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Informatics, Agriculture,
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Organized by King Mongkut's Institute of Technology Ladkrabang,
Prince of Chumphon Campus, Chumphon, Thailand



The 11th National Conference and The 7th International Conference
on Informatics, Agriculture, Management, Business Administration, Engineering,
Sciences and Technology (IAMBEST 2026)

Organized by:

King Mongkut's Institute of Technology Ladkrabang, Prince of Chumphon Campus and University
Network

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MESSAGE FROM THE EXECUTIVE VICE PRESIDENT KMITL-CHUMPHON

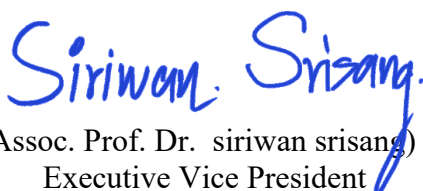
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The 7th International Conference on Informatics, Agriculture, Management, Business Administration, Engineering, Science, and Technology (IAMBEST 2026), along with the 11th National Conference on Informatics, Agriculture, Management, Business Administration, Engineering, Science, Technology, Social Sciences, and Humanities, are well-known conferences for academics, experts, and many researchers. The conferences are hosted by the King Mongkut's Institute of Technology Ladkrabang Prince of Chumphon Campus (KMITL-Chumphon), in collaboration with various university networks and professional associations in Thailand. During 8-9 May 2026 at Royal Phuket City hotel, Phuket, Thailand. The aim of these conferences is to provide an exchange stage for ideas, knowledge, and research among researchers from various fields. The continued development of research fosters the exchange of knowledge, connections, collaboration, and integration among one another. All of the above contributes to the development of the community, society, and country.

As the chairman of IAMBEST 2026 by KMITL-Chumphon, I sincerely thank all committee members for your time and determination in organizing this wonderful conference. I thank all attendees and guests for sharing your research, innovation and technology. This conference is grateful and successful. I wish that the exchange of ideas and sharing of knowledge from this conference will provide you with another step to advance your knowledge and technology and to benefit your communities and countries.

Best wishes to all of you.



(Assoc. Prof. Dr. Siriwan Srisang)
Executive Vice President
KMITL, Prince of Chumphon Campus
Chairman of the Organizing Committee



IAMBEST
KMITL PRINCE OF CHUMPHON
8-9 MAY 2026



Dr. David L. Hyten Jr.
University of Nebraska-Lincoln (USA)

Keynote Speaker

Topic: Genomic Tools to Accelerate Soybean Improvement in an Era of Climate Volatility

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PROGRAM CONFERENCE

Program IAMBEST2026: Oral Presentation

Day 1: Registration Day		Fri 8 May 2026	
15:30-17:00	Registration and installation of poster and soft file presentation (ppt or pdf format)		
16:00-18:30	Organizing committee and session chair meeting		
Day 2: Opening Day		Sat 9 May 2026	
International Session		National Session	
(2) Sci. and Agri.		(1) Management and B.A.	(3) Tech.
Sessions		(2) Management and B.A.	
Chairperson	K. Eawsakul P. Nittayacharn	A. Jansuri R. Taweephol	C. Boonnual P. Chatchawanchanchanakij
Room	Hay Room	Nakha Room	Bon Room
08:30-10:30	S52, S54, S61, S68, S78, A41, A42, A83	MB21, MB27, MB37, MB96, MB117, MB143	IS01, T7, T28, T55, T87, T89, T113, T70
10:30-10:50	Coffee Break		
10:50-11:10	Opening Ceremony (Ballroom2)		
11:10-12:00	Keynote: Genomic Tools to Accelerate Soybean Improvement in an Era of Climate Volatility, Dr. David L. Hyten Jr. University of Nebraska-Lincoln (USA)		
12:00-13:00	Lunch Break (Ballroom1)		
Sessions		(4) Sci. and Agri.	(6) Eng.
Chairperson	N. Chanthap R. T. Palayon	P. Youryon C. Sriket	M. Subongkod R. Dangwilailux
Room	Hay Room	Nakha Room	Bon Room
13:00-15:30	SS4, SS13, SS56, SS80, SS111, SS112	I95, T20, T64, E10, E24, E29, E39, E63, E69	SS34, SS36, SS128, SS129, SS130, SS131, SS132, SS134
15:30-15:45	Coffee Break		
Sessions		(7) Management and B.A.	(9) Eng.
Chairperson	N. Chanthap R. T. Palayon	C. Lalaeng B. Hongsakul	T. Archevapanich N. Ruttanadech
Room	Hay Room	Nakha Room	Bon Room
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18:30-21:00	Banquet & Awarding (Ballroom1)		

Note: Each oral presentation is allocated for 15 min.

PROGRAM CONFERENCE

Program IAMBEST2026: Poster Presentation

Day 1: Registration Day		Fri 8 May 2026	
15:30-17:00	Registration and installation of poster and soft file presentation (ppt or pdf format)		
16:00-18:30	Organizing committee and session chair meeting		
Day 2: Opening Day		Sat 9 May 2026	
10:30-10:50	Coffee Break		
10:50-11:10	Opening Ceremony (Ballroom2)		
11:10-12:00	Keynote: Genomic Tools to Accelerate Soybean Improvement in an Era of Climate Volatility, Dr. David L. Hyten Jr. University of Nebraska-Lincoln (USA)		
12:00-13:00	Lunch Break (Ballroom1)		
International Session		National Session	
(1) Agri.		(3) Info., Eng. and Tech.	
Sessions	(2) Sci.	(4) Sci. and Agri.	(5) Soc. Sci. & Humanities
Chairperson	P. Phansak N. Suwannarat	R. Sombutkaew N. Kanjak	K. Bunya-atichart P. Narkthewan
Room	Foyer	Foyer	Foyer
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15:30-15:45	Coffee Break		
18:30-21:00	Banquet & Awarding (Ballroom1)		

Note: Each poster presentation is allocated for 10 min.

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Zamboanga Peninsula Polytechnic State University, Philippines

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| 47. Asst. Prof. Dr. Pijug Summpunn | Walailak University, Thailand |

Oral Presentation

Regional Analysis of Wet Atmospheric Temperature Variability in Thailand using Machine Learning and COSMIC Satellite Measurements

Daniel Okoh^{1,b)}, Anongnat Intasam^{2,c)}, Punyawit Jamjareegulgarn^{2,d)}
and Sarawut Paspanthong^{2,a)}

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Abstract. Wet atmospheric temperature is a key variable governing tropical thermodynamics and convection, yet its regional variability remains challenging to quantify. This study presents a grid-based machine-learning framework for estimating three-dimensional wet atmospheric temperature over Thailand using COSMIC-1 and COSMIC-2 radio occultation data from 2006 to 2025. Feedforward neural networks were trained on satellite profiles and independently validated using radiosonde observations. The training dataset comprised 83,081,049 COSMIC temperature data points from 182,472 radio occultation profiles, ensuring robust sampling across the study region. The model accurately reproduces vertical temperature structure from the lower troposphere to the stratosphere, achieving mean absolute errors of 1.6 to 2.1 degree Celsius and correlation coefficients above 0.995. Spatial fields capture realistic diurnal and seasonal variability associated with monsoonal processes. Trend analysis reveals persistent tropospheric warming of approximately 0.03 to 0.10 degree Celsius per year, with the strongest warming during the rainy season. In contrast, the stratosphere exhibits systematic cooling of about -0.02 to -0.10 degree Celsius per year across all seasons.

Index Terms— Atmospheric temperature, machine learning, neural networks, radio occultation, wet atmospheric temperature.

I. INTRODUCTION

Wet atmospheric temperature, defined as the moisture-adjusted temperature of an air parcel, is a key variable for characterizing atmospheric thermodynamics, convective processes, and regional weather patterns in the tropics. Over Thailand and the surrounding Southeast Asian region, atmospheric variability is strongly shaped by monsoonal circulation, seasonal moisture transport, and large-scale convective systems [1]. These features produce complex nonlinear interactions between temperature, humidity, and atmospheric dynamics, making it difficult for traditional empirical or climatological models to represent wet temperature accurately across space, altitude, and time [2]. The emergence of high-quality satellite-based radio occultation (RO) observations provides an opportunity to overcome many of these limitations. COSMIC-1 and COSMIC-2 RO profiles offer globally consistent, high-vertical-resolution measurements of temperature and other atmospheric parameters from the surface through the stratosphere [3]. For the Thailand region, RO measurements

capture the full vertical structure of the atmosphere (from the near-surface boundary layer through the moist convective troposphere to the dry, radiatively driven stratosphere), making them an excellent foundation for regional atmospheric modeling.

In recent years, several studies (e.g. [4], [5]) have summarized advances in satellite-based atmospheric observations and machine-learning applications in climate and weather research. For example, [6] and [7] have highlighted the growing role of radio occultation measurements in atmospheric profiling and climate monitoring, and [8] has demonstrated the increasing use of machine-learning techniques for modeling complex atmospheric variables. These studies emphasize the importance of combining high-quality observational datasets with data-driven approaches to improve the representation of atmospheric thermodynamics, particularly in data-sparse tropical regions.

Machine-learning techniques, particularly neural networks, offer powerful tools for capturing the nonlinear relationships inherent in tropical atmospheric behavior [9].

Unlike deterministic parameterizations, neural networks can learn complex mappings between atmospheric variables and can operate seamlessly in three dimensions (longitude, latitude, and altitude) [10]. Recent studies (e.g., [11] and [12]) have demonstrated the capability of machine learning for temperature and humidity retrievals, yet there remains limited research focused specifically on wet atmospheric temperature over Southeast Asia, especially with long-term satellite records extending nearly two decades. In particular, recent review papers (e.g., [8], [13], [14]) have emphasized the success of machine-learning approaches in atmospheric parameter retrieval, forecasting, and climate variability analysis, highlighting their advantages over traditional empirical and physics-based models in handling high-dimensional and nonlinear datasets.

In this study, we develop a regional machine-learning model to estimate wet atmospheric temperature over the Thailand region using COSMIC satellite measurements as the primary input dataset. Radiosonde observations from stations around the Thailand region are used exclusively for independent validation, ensuring an unbiased performance assessment. The analysis spans 2006-2025, allowing investigation of not only seasonal and spatial patterns but also long-term temperature trends in both the troposphere and stratosphere, where changes are driven by different physical processes such as monsoon variability, deep convection, and large-scale radiative forcing. A major contribution of this paper is the characterization of atmospheric variability and trends, including spatial gradients, seasonal cycles, and multi-year temperature trends in the troposphere and stratosphere.

II. DATA AND METHODS

A. COSMIC Radio Occultation Data

Wet atmospheric temperature profiles were obtained from COSMIC-1 (2006-2020) and COSMIC-2 (2019-2025) Level-2 wetPrf/wetPf2 products. All NetCDF profiles within 5-25°N and 90-110°E were extracted, covering the Thailand region. Quality control followed the CDAAC “badness flag” criterion, and only profiles with badness = 0 were retained [15], [16]. The final dataset provides high-vertical-resolution coverage of the regional troposphere and stratosphere for the 2006-2025 period. In total, the dataset used for model training consisted of 83,081,049 atmospheric temperature measurements derived from 182,472 COSMIC radio occultation profiles.

B. Radiosonde Data for Independent Testing

Independent evaluation was performed using radiosonde temperature profiles from the Integrated Global Radiosonde Archive (IGRA). Four stations located within the selected Thailand region were used (see Fig. 1), ensuring region-specific validation. Measurements were screened using the IGRA temperature and geopotential height quality flags (TFLAG = B, ZFLAG = B) [17]. Radiosonde profiles were not used in model training and served exclusively as an external testing dataset.

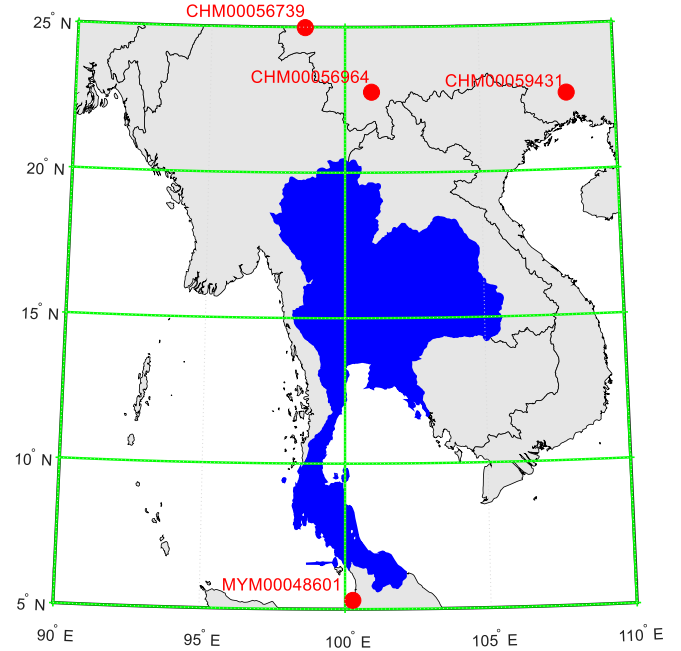


Fig. 1. Map of Thailand region showing sites of radiosonde experiment (red dots) used for testing, and the 10°×5° grid overlaid to show how the ANN sub-models are organized. Thailand country is shown in blue color.

C. Regional Spatial Binning

To account for spatial heterogeneity across Thailand’s land-ocean-topographic environment, and for feasibility of the neural network training, the model domain was divided into 10° × 5° (longitude × latitude) grid cells, resulting in a total of eight grid cells as shown in Fig. 1. This grid resolution was selected as a balance between spatial representativeness and data availability, ensuring sufficient sampling density within each cell for stable neural network training while still resolving regional-scale atmospheric variability across Thailand. The COSMIC measurements were assigned to respective grid cells using the longitudes and latitudes of the RO, and separate machine-learning sub-models were trained for each cell. Each of the bins contained sufficient sampling density (greater than 9,700,000 temperature measurements) to support stable ANN training.

D. Neural Network Modeling Framework

A feedforward artificial neural network was developed for each grid cell. The input features included latitude, longitude, altitude, year, day of year (DOY), and local solar time (LST), with DOY and LST represented using sine/cosine pairs to preserve temporal periodicity. The output variable was wet atmospheric temperature.

For each sub-model, the COSMIC dataset was partitioned into 70% training, 15% validation, and 15% cross-validation. After spatial binning, and after removing validation and cross-validation datasets, the number of temperature measurements per grid cell ranged from approximately 8.5 million to 14.2 million, corresponding to about 20,000 to 29,000 COSMIC profiles per cell, ensuring adequate

sampling density for stable neural network training across all regions. The Levenberg-Marquardt optimization algorithm [18] was used for training due to its rapid convergence on nonlinear regression problems. Based on preliminary optimization, hidden-layer sizes of 9-11 neurons were tested, and the lowest-error configuration was selected for each of the sub-models. The hyperbolic tangent activation function was used for both the hidden and output layers, as this configuration is well suited for bounded target variables such as wet atmospheric temperature, avoiding extrapolation beyond physically meaningful limits, and has been widely applied in similar geophysical modeling contexts (e.g., [19], [20]). Model performance was quantified using root-mean-square errors (RMSEs) computed for the validation and cross-validation datasets.

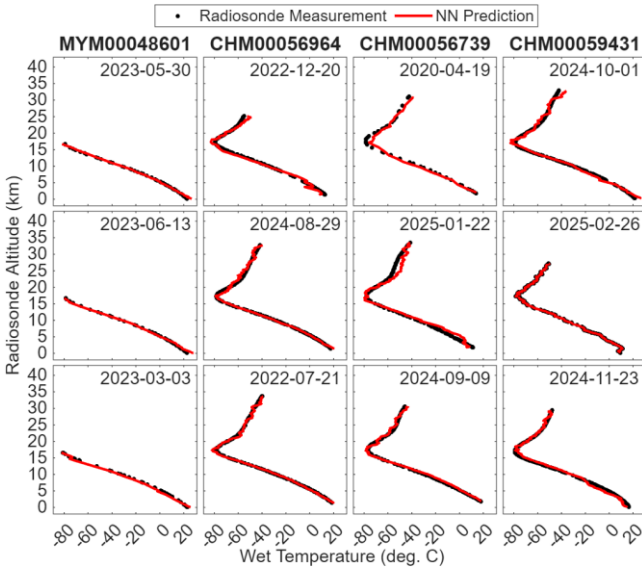


Fig. 2. Comparison between neural-network-predicted wet atmospheric temperature profiles and independent radiosonde measurements at four radiosonde experiment stations.

III. RESULTS AND DISCUSSION

A. Validation Against Independent Radiosonde Measurements

Fig. 2 presents a comprehensive comparison between the neural network (NN) predictions and radiosonde measurements for experiments selected across different months/seasons and years at the four validation stations. Across all 12 cases shown, the NN model successfully reproduces the full vertical structure of the 3-D atmospheric temperatures from the lower troposphere to the stratosphere.

At all stations, the predicted temperature profiles lie nearly on top of the radiosonde measurements, with only minor deviations occurring in regions of strong vertical gradients (typically near the tropopause or within moist boundary-layer layers). These deviations are small and remain consistent with expected differences arising from differences in sampling techniques between RO-derived training data and in-situ radiosondes. To assess the performance

quantitatively across all radiosonde experiments, the mean absolute errors (MAEs) and correlation coefficients were computed by aggregating all of the radiosonde experiments at each station. The resulting MAEs and correlation coefficients are as follows: MYM00048601 (1.64 °C, 0.998), CHM00056964 (1.84 °C, 0.997), CHM00056739 (2.14 °C, 0.995), and CHM00059431 (1.88 °C, 0.997).

These low MAE values (each on the order of 1.6-2.1 °C) are well within expected error ranges for tropical troposphere-stratosphere temperature retrievals (for example, [21] and [22]) and further confirm that the model generalizes effectively beyond its training dataset. All correlation values exceed 0.995, indicating near-perfect agreement between the predicted and observed vertical temperature structures. These extremely high correlations confirm that the NN not only matches temperature magnitudes but also captures the correct vertical gradients, inflection points, and structural transitions across all seasons. These results generally demonstrate that the neural network approach provides a robust and accurate representation of vertical wet-temperature structure across the Thailand region, giving confidence in its application to broader spatial and temporal analyses in subsequent sections.

B. Sample Spatial Distribution of the Wet Temperature

Fig. 3 illustrates a sample of the spatial pattern of wet atmospheric temperature at 10 km altitude on day 200 of 2025, corresponding to early morning local time (00 UT $\approx$ 07 LT). To generate the full continuous map, the individual NN sub-model outputs were first interpolated across grid boundaries using a linear gridded interpolation scheme. A two-dimensional median filtering technique was then applied to the interpolated field to reduce local inconsistencies and enhance spatial smoothness, particularly near grid boundaries. This ensures a smooth and physically consistent temperature field over the entire region. The map provides a snapshot of mid-tropospheric conditions during the peak southwest monsoon season across the Thailand region.

The northern portions of the domain (located farther inland and more strongly influenced by continental heating) exhibit the highest temperatures overall, consistent with reduced maritime moderation and enhanced convective uplift. These spatial characteristics primarily reflect monsoon-driven atmospheric structure, with warmer temperatures over land and cooler values offshore. This warming over land reflects typical Southwest Monsoon conditions, where persistent deep convection and accumulated daytime heating elevate the mid-tropospheric temperatures above surrounding oceanic environment [23].

The smooth and coherent spatial structure of the temperature field indicates that the grid-based neural-network framework is able to preserve realistic horizontal gradients without introducing discontinuities at grid boundaries. This result also highlights one of the strengths of the model: although trained independently within spatial sub-domains, the outputs collectively form a physically

consistent regional field. This ability is critical for applications such as regional weather modeling.

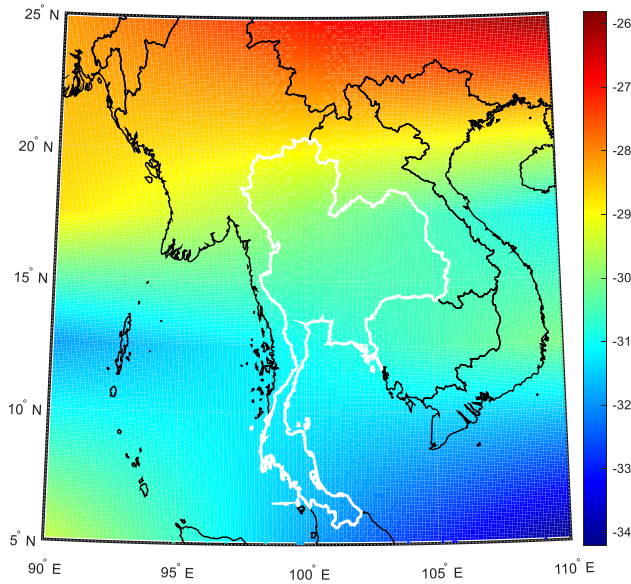


Fig. 3. Spatial distribution of wet atmospheric temperature at 10 km altitude over the Thailand region on day 200 of 2025 at 00:00 UT (≈07:00 LT).

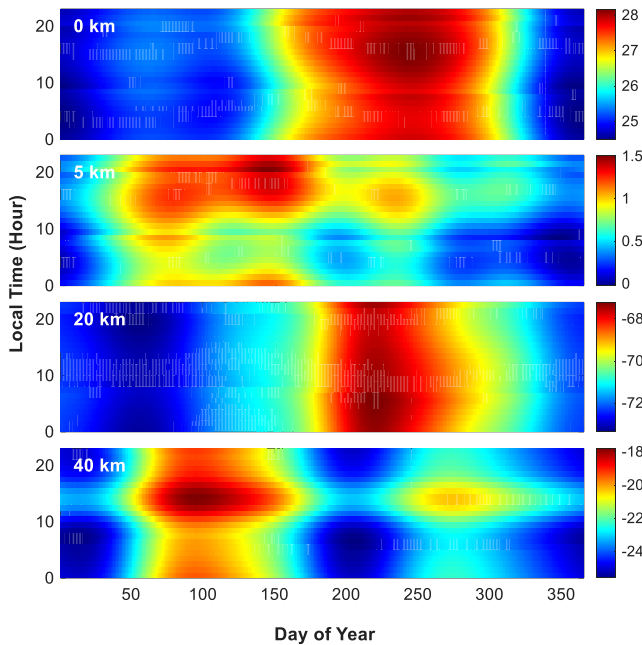


Fig. 4. Diurnal and seasonal variability of wet atmospheric temperature at Bangkok (100.5°E, 13.8°N) at altitudes of 0, 5, 20, and 40 km. Distinct color scales are purposefully used for each altitude to emphasize temporal variability rather than absolute magnitude, this is because the emphasis is on identifying spatiotemporal patterning rather than comparing absolute magnitudes.

C. Local Time and Seasonal Variability at Selected Altitudes

Fig. 4 summarizes the diurnal and seasonal evolution of wet atmospheric temperature at Bangkok (100.5°E, 13.8°N) for altitudes of 0, 5, 20, and 40 km. Because each panel uses a distinct color scale, the emphasis is on identifying spatial-

temporal patterning rather than comparing absolute magnitudes. This is why we have intentionally used different color bar limits for the different heat maps. The surface-level temperature field (top panel) exhibits a strong annual cycle, with peak temperatures centered around mid-year and thereafter (DOY 150-300), characteristic of the monsoon season and enhanced convective activity. A clear diurnal cycle is also present, with daytime warming and nighttime cooling.

At 5 km, the diurnal cycle becomes weaker but remains visible during the pre-monsoon and monsoon periods. Enhanced warming signals appear around DOY 60-180 (corresponding to the hot season in Bangkok), likely linked to deep convection lofting moisture and heat to mid-tropospheric altitudes. Cooler temperatures dominate outside the monsoon season, during the months of December and January which is the cool season in Bangkok.

Meanwhile, a strong annual cycle emerges with minimal diurnal structure at 20 km. The warmest temperatures occur in northern summer (July-August), while the coldest appear in northern winter (December-February), reflecting seasonal modulation of the tropical tropopause layer and variations in deep convective outflow. This pattern is consistent with known large-scale dynamical influences such as the Brewer-Dobson circulation [24] and seasonal expansion/contraction of the Hadley cell [25].

Moreover, the temperature variability is distinctly stratospheric in character at 40 km: a semiannual oscillation is clearly evident, driven by radiative-dynamical interactions and the phase-locking of the semiannual oscillation (SAO) [26] that dominates the equatorial lower stratosphere. There is also a weak diurnal variation which indicates strong radiative control at these altitudes.

D. Multi-Year Mean Temperature Change as a Function of Altitude and Latitude

To examine long-term atmospheric temperature tendencies captured by the neural network model, Fig. 5 presents the mean year-to-year temperature change along longitude 100.5°E (passing through Bangkok) over the 2006-2025 period. For each altitude-latitude pair, the neural network was used to predict the wet atmospheric temperature for the same local time and day of year (DOY) across all years in the record. Differences between successive years were then computed and averaged to produce an estimate of the mean temperature trend per year, expressed in °C/year. The three panels correspond to 05 UT (~12 LT) on DOY 1, DOY 100, and DOY 210, chosen to represent the Cool, Hot, and Rainy seasons, respectively.

Across all three seasons, the most prominent feature is the consistent warming in the troposphere (0-17 km), visible as positive trends of approximately 0.03-0.10 °C/year. The strongest warming occurs during the Rainy season (DOY 210). In this season, widespread positive trends exceeding 0.07-0.10 °C/year appear across most latitudes, likely reflecting the combined effects of persistent deep convection, enhanced moisture transport, and sustained

latent heat release associated with monsoonal rainfall. The Hot season (DOY 100) exhibits warming as well, but with slightly lower magnitude and more spatial variability, while the Cool season (DOY 1) shows the weakest tropospheric warming, particularly at northern latitudes where surface heating is reduced. These seasonal differences support the conclusion that monsoon-related convective processes play a dominant role in regulating long-term tropospheric temperature tendencies over Thailand.

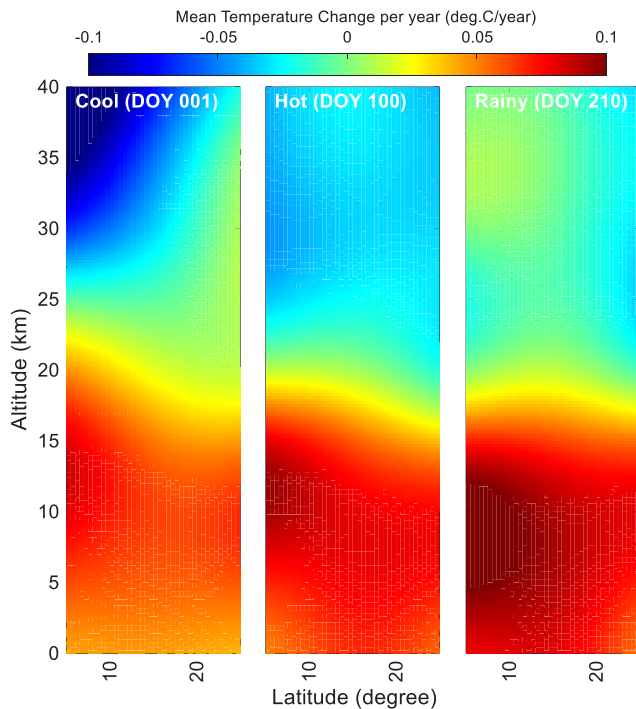


Fig. 5. Mean year-to-year wet atmospheric temperature change ($^{\circ}\text{C yr}^{-1}$) as a function of altitude and latitude along 100.5°E for the period 2006-2025, shown for the Cool (DOY 1), Hot (DOY 100), and Rainy (DOY 210) seasons.

Above ~ 20 km, the trend structure changes sharply, with cooling tendencies in the stratosphere. This band of negative trends, ranging from ~ -0.02 to -0.10 $^{\circ}\text{C}/\text{year}$, appears in all three seasons but is most pronounced in the Cool-season. The presence of long-term cooling in the stratosphere is consistent with known tropical stratospheric trends associated with changes in ozone, radiative forcing, and the strengthening of large-scale overturning circulations [27].

Fig. 5 demonstrates that the neural-network-derived temperature fields reproduce physically plausible vertical and meridional structures of long-term regional temperature change. In general, the results reveal a warming troposphere and cooling stratosphere over Thailand. Importantly, the seasonal differences highlight how monsoonal processes modulate both the magnitude and spatial structure of these trends.

IV. CONCLUSION

This work demonstrates that neural networks trained on long-term COSMIC radio occultation measurements can reliably resolve wet atmospheric temperature variability over Thailand across altitude, season, and time. Independent radiosonde validation confirms strong agreement throughout the troposphere and stratosphere, with typical errors below 2.1 $^{\circ}\text{C}$ and correlations exceeding 0.995 , indicating that the model captures both temperature magnitudes and vertical gradients.

The reconstructed temperature fields reveal robust long-term tendencies with clear vertical structure. Tropospheric warming is consistently observed, with mean rates ranging from about 0.03 to 0.10 $^{\circ}\text{C yr}^{-1}$, and reaches its maximum during the rainy season, indicating the role of monsoon-driven convection and latent heat release. Above approximately 20 km, the trend reverses sign, with stratospheric cooling of roughly -0.02 to -0.10 $^{\circ}\text{C yr}^{-1}$ evident in all seasons, most strongly during the cool season. These contrasting trends are physically consistent with enhanced tropical convection and large-scale radiative-dynamical processes influencing the upper atmosphere. The approach provides a robust framework for diagnosing regional climate variability and long-term atmospheric change in the tropics. The resulting high-resolution, three-dimensional atmospheric temperature fields provide a robust framework that could be applied to future studies in regional climate monitoring, seasonal forecasting, and improved weather prediction over Thailand and similar tropical regions.

Despite the strong performance of the proposed approach, the following limitations are acknowledged. First, the model relies entirely on COSMIC radio occultation-derived temperature products for training, and any inherent uncertainties or biases in these datasets may propagate into the neural network predictions. Also, the model is purely data-driven and does not explicitly incorporate physical constraints, which may affect its extrapolation capability under conditions outside the range of the training data. Future work could address these limitations by integrating multi-source datasets, and incorporating physics-informed machine-learning approaches.

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Waterfall as Biodiversity Hotspots for Aquatic Insects (Trichoptera) and Freshwater Fish in Tropical Streams

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Abstract. Waterfall ecosystems are important components of tropical freshwater systems but remain relatively understudied. This study examined the diversity of Trichoptera and freshwater fish, together with selected water quality parameters, at Kaeng Khoi Waterfall, Chumphon Province, southern Thailand. Sampling was conducted during the hot (February 2025) and rainy (August 2025) seasons. A total of 2,695 Trichoptera individuals representing nine species were recorded, with assemblages dominated by *Chimarra* spp. in both seasons. Diversity indices were similar between seasons (Shannon index, $H' = 1.666$ in the hot season and 1.654 in the rainy season), indicating a relatively stable community structure. Freshwater fish assemblages consisted of 12 species representing multiple trophic guilds, including insectivores, omnivores, and predators, reflecting a functionally structured food web. Water quality parameters were within suitable ranges for tropical freshwater ecosystems, with relatively high dissolved oxygen and low nutrient concentrations. These conditions are consistent with those reported for headwater streams and are known to support flow-adapted taxa. The species composition observed in this study differs from that typically reported in lowland river systems, suggesting that waterfall habitats support distinct assemblages adapted to high-flow conditions.

Overall, the findings indicate that Kaeng Khoi Waterfall provides suitable habitat for aquatic biodiversity and plays an important role in maintaining ecological integrity in tropical freshwater systems.

Index Terms— Aquatic insects, Freshwater fish, Waterfall ecosystems, Trophic guilds, Biodiversity, Bioindicators, Tropical freshwater streams

I. INTRODUCTION

Freshwater ecosystems are among the most biologically diverse environments on Earth, supporting a wide range of aquatic organisms and providing essential ecosystem services [2]. Tropical streams and waterfalls create unique environmental conditions characterized by fast-flowing water, high dissolved oxygen concentrations, and heterogeneous substrates such as rocks, gravel, and leaf litter. These habitat features support diverse assemblages of aquatic organisms, including aquatic insects and freshwater fishes, which play important roles in maintaining ecosystem structure and function [9].

Aquatic insects are widely recognized as effective bioindicators for assessing the ecological condition of freshwater ecosystems. Among them, caddisflies (order Trichoptera) are particularly important because many species are sensitive to environmental changes and water quality

conditions [7]. The larvae of many caddisfly species inhabit riffle habitats with strong water currents and high oxygen levels, where they construct cases or silk nets to capture organic particles from flowing water. Due to their ecological specificity and sensitivity to environmental disturbance, caddisflies are frequently used in biodiversity assessments and biomonitoring programs in freshwater environments [8].

Freshwater fishes also play a crucial role in stream ecosystems and are commonly used as ecological indicators of habitat quality and ecosystem integrity. Fish communities often reflect environmental conditions through their trophic structure, species composition, and habitat preferences. Different species occupy various trophic guilds, including insectivores, herbivores, omnivores, and piscivores, forming complex food webs that regulate energy flow and nutrient cycling within aquatic ecosystems [10]. Waterfall ecosystems represent ecologically important habitats within tropical stream systems. The combination of steep gradients,

turbulent flow, and well-oxygenated water creates favorable conditions for many specialized aquatic organisms [1]. However, Southeast Asia, including southern Thailand. Kaeng Khoi Waterfall, located in Pathio District, Chumphon Province, southern Thailand, is characterized by clear, fast-flowing water and rocky substrates typical of tropical waterfall streams [6]. These environmental conditions provide suitable habitats for various aquatic organisms, including caddisflies and freshwater fishes. However, information regarding the biodiversity and ecological structure of aquatic communities in this area remains limited. Therefore, the present study aimed to (1) assess the diversity of caddisflies and freshwater fishes at Kaeng Khoi Waterfall and (2) evaluate selected water quality parameters in relation to the ecological condition of the waterfall ecosystem. The findings of this study provide baseline information on aquatic biodiversity and contribute to a better understanding of the ecological characteristics of tropical freshwater waterfall habitats. Despite their ecological significance, waterfall habitats remain poorly studied in tropical freshwater systems, particularly in Southeast Asia. As transitional zones linking headwaters to downstream reaches, they often harbor assemblages adapted to high-flow environments. Examining biodiversity patterns in these systems is therefore important for understanding their role within tropical river ecosystems.

II. METHODOLOGY

a). Study Area

The study was conducted at Kaeng Khoi Waterfall (10°54'N, 99°16'E) in Pathio District, Chumphon Province, southern Thailand. The stream is characterized by clear, fast-flowing water, rocky and gravel substrates, and high dissolved oxygen conditions typical of tropical headwater systems (Fig. 1).

b). Sampling Design

Field sampling was conducted during two seasons: the hot season (February 2025) and the rainy season (August 2025) to capture seasonal variation in aquatic communities. Sampling sites were selected along the waterfall stream to represent typical riffle habitats with flowing water and rocky substrates.

c). Collection of Caddisflies

Adult caddisflies were collected using ultraviolet (UV) pan light traps placed near the stream during the evening. The traps were operated for several hours after sunset to attract adult insects. Collected specimens were preserved in 80% ethanol and transported to the laboratory for identification. Species identification was conducted based on morphological characteristics using standard taxonomic keys for Trichoptera.

d). Fish Sampling

Freshwater fish were collected using hand nets in shallow stream sections. Collected specimens were identified to species level using standard taxonomic references for freshwater fishes of Southeast Asia. All collected fish were identified to species level and released back into their natural habitat immediately after sampling to minimize ecological disturbance.

e). Water Quality Measurement

Selected water quality parameters were measured in situ at each sampling site. These included water temperature, dissolved oxygen (DO), and pH. Measurements were taken using portable water quality meters during the sampling period.

f). Data Analysis

The collected specimens were counted and identified to determine species composition and diversity. The ecological roles of fish species were categorized into trophic guilds (e.g., insectivores, omnivores, herbivores, and piscivores) based on published ecological information. The results were used to evaluate the ecological condition and biodiversity of the waterfall ecosystem. Species diversity was further quantified using the Shannon–Wiener diversity index (H'), which is commonly used to evaluate biodiversity in freshwater ecosystems.



Fig. 1. Stream habitat at the study site in Kaeng Khoi Waterfall, Chumphon Province, Thailand.

III. RESULTS AND DISCUSSION

a) Trichoptera Diversity

The composition of Trichoptera species during the hot season is shown in Fig. 2. The assemblage was mainly dominated by species of the genus *Chimarra*. Among these, *Chimarra Chiangmaiensis* was the most abundant species (34%), followed by *C. okuihorum* (30%) and *C. bimbeltona* (17%). Other species were present in lower proportions, including *Macrostemum floridum* (7%) and *Dipseudopsis*

robustior (6%). Minor species included *Cheumatopsyche chrysothemis* (4%), *C. dhanikari* (3%), and *Leptocerus dirghachuka* (3%), while *Macrostemum hestia* was the least abundant (1%). In the rainy season (Fig. 3), the assemblage showed a similar pattern. *Chimarra okuihorum* was the most abundant species (36%), followed by *C. changmaiensis* (31%) and *C. bimbeltona* (17%). Other species occurred in smaller proportions, including *Macrostemum floridum* (5%), *Dipseudopsis robustior* (6%), and *Cheumatopsyche dhanikari* (3%). Minor contributions were observed for *C. chrysothemis* (4%) and *Leptocerus dirghachuka* (2%), while *Macrostemum hestia* remained the least abundant species (1%).

Overall, the results suggest that the Trichoptera community remained relatively stable between seasons. However, there was a slight shift in dominant species, with *C. changmaiensis* more abundant in the hot season and *C. okuihorum* more dominant in the rainy season.

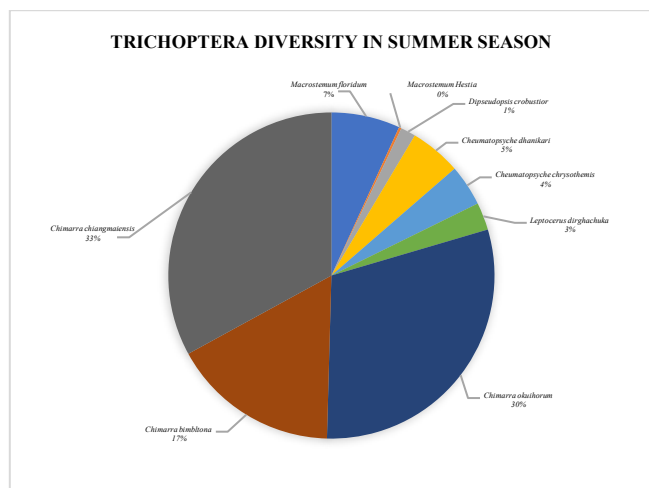


Fig. 2. Relative abundance (%) of Trichoptera species during the hot season

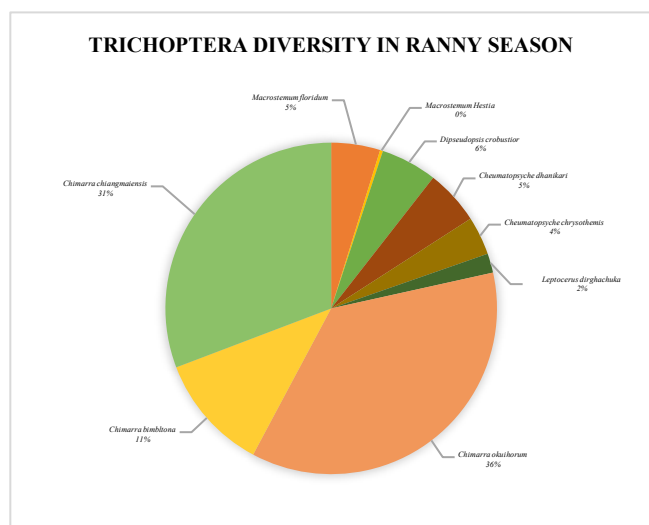


Fig. 3. Relative abundance (%) of Trichoptera species during the rainy season.

The diversity indices of Trichoptera were similar between seasons (Table 1). Species richness remained constant at nine species in both periods. The Shannon index (H') was slightly higher in the hot season (1.666) than in the rainy season (1.654), while evenness values were comparable (0.758 and 0.753, respectively). These results indicate a relatively stable community structure, with a moderate distribution of individuals among species and consistent dominance of *Chimarra* spp. across seasons.

Table 1. Diversity indices of Trichoptera assemblages in Kaeng Khoi Waterfall across seasons

Season	N(Individuals)	Species richness (S)	Shannon index (H')	Evenness (J')
Hot season	807	9	1.666	0.758
Rainy season	692	9	1.654	0.753

b) Freshwater fish Diversity

A total of 12 freshwater fish species representing several trophic guilds were recorded in the study area. Insectivorous species included *Rasbora paviana*, *Devario regina*, and *Dermogenys siamensis*, while omnivores comprised *Barbodes rhombeus*, *Systemus rubripinnis*, and *Striuntius lateristriga*. Detritivorous and herbivorous feeding habits were observed in *Cyclocheilichthys apogon* and *Osteochilus vittatus*. Predatory species were represented by *Hampala macrolepidota*, *Silurus phaiosoma*, *Channa lucius*, and *Channa limbata*, indicating a multi-trophic structure within the stream ecosystem (Table 2).

Table 2. freshwater fish species recorded at the study site

Number	Scientific name	Feeding guild (Ecological role)	Relative abundance (%)
1	<i>Rasbora paviana</i>	Insectivore / zooplanktivore	Dominant
2	<i>Devario regina</i>	Insectivore	Subdominant
3	<i>Barbodes rhombeus</i>	Omnivore	Dominant
4	<i>Cyclocheilichthys apogon</i>	Omnivore / detritivore	Dominant
5	<i>Osteochilus vittatus</i>	Herbivore / detritivore	Dominant
6	<i>Systemus rubripinnis</i>	Omnivore	Rare
7	<i>Striuntius lateristriga</i>	Omnivore	Subdominant
8	<i>Hampala macrolepidota</i>	Carnivore / piscivore	Frequent
9	<i>Dermogenys siamensis</i>	Insectivore (surface feeder)	Subdominant
10	<i>Silurus phaiosoma</i>	Carnivore	Rare
11	<i>Channa lucius</i>	Carnivore / piscivore	Frequent
12	<i>Channa limbata</i>	Carnivore	Frequent

Species dominance categories were defined based on relative abundance: dominant (>20%), subdominant (10–20%), frequent (5–10%), and rare (<5%)

c) Water Quality and Ecological Condition

Water quality parameters at the study site were within suitable ranges for tropical freshwater ecosystems (Table 3). Dissolved oxygen levels were relatively high (6.8 ± 0.6 mg/L), reflecting well-aerated conditions associated with fast-flowing water. The pH remained near neutral (7.3 ± 0.3), and water temperature (25.4 ± 1.2 °C) was within the typical range for tropical streams. Nutrient concentrations were low, with ammonia (0.02 ± 0.01 mg/L) and nitrate (0.8 ± 0.2 mg/L) remaining below levels associated with pollution. Electrical conductivity was also relatively low (112 ± 15 μ S/cm), indicating limited dissolved solids and minimal anthropogenic impact. These environmental conditions are typical of headwater and waterfall ecosystems and are known to support diverse aquatic communities. High dissolved oxygen, stable substrates, and low nutrient levels provide suitable habitat for sensitive taxa such as Trichoptera. The dominance of flow-adapted genera, particularly *Chimarra*,

further supports the interpretation that the study site is in good ecological condition.

Table 3. Water quality parameters at the study site (Mean $\pm$ SD)

Parameter	Unit	Mean $\pm$ SD	Suitable range	Status
Temperature	°C	25.4 $\pm$ 1.2	22–28	Suitable
Dissolved Oxygen (DO)	mg/L	6.8 $\pm$ 0.6	> 5	Good
pH	-	7.3 $\pm$ 0.3	6.5–8.5	Suitable
Electrical Conductivity (EC)	μ S/cm	112 $\pm$ 15	50–300	Low
Ammonia (NH ₃)	mg/L	0.02 $\pm$ 0.01	< 0.05	Safe
Nitrate (NO ₃ ⁻)	mg/L	0.8 $\pm$ 0.2	< 1–5	Low

d) Ecological Implications

The results suggest that Kaeng Khoi Waterfall functions as a stable and suitable habitat for aquatic organisms. The combination of relatively consistent species composition, moderate diversity, and good water quality indicates that the ecosystem is not heavily disturbed. Compared with lowland or slow-flowing rivers, which are often dominated by more tolerant species, the assemblage observed in this study reflects adaptation to high-flow environments. Waterfall ecosystems therefore play an important role in maintaining biodiversity in tropical freshwater systems. They may act as refuges for species adapted to fast-flowing and well-oxygenated conditions and contribute to the overall ecological integrity of river.

IV. CONCLUSION

This study provides the first integrated assessment of caddisfly and freshwater fish diversity in Kaeng Khoi Waterfall, southern Thailand. The results demonstrate that the waterfall ecosystem supports a diverse and functionally structured aquatic community, characterized by multiple trophic guilds and dominance of flow-adapted taxa such as *Chimarra*. The species composition observed in this study differs from that typically found in lowland river systems, indicating that waterfall habitats support distinct ecological assemblages adapted to high-flow and well-oxygenated conditions. [3] [4]. These ecosystems function as important transitional zones within river continua and play a key role in maintaining regional biodiversity. Therefore, waterfall ecosystems should be recognized as biodiversity hotspots in tropical freshwater environments and considered in future conservation and biomonitoring programs. [11] [10]. The observed water quality conditions are consistent with those reported for relatively undisturbed tropical headwater streams. High dissolved oxygen levels and low nutrient concentrations are particularly important for sustaining sensitive taxa such as Trichoptera. The presence of dominant flow-adapted genera, such as *Chimarra*, further supports the interpretation that the study site maintains good ecological condition. These findings suggest that Kaeng Khoi Waterfall provides a suitable habitat for aquatic biodiversity and functions as an important refuge for species adapted to high-flow environments.

V. ACKNOWLEDGMENT

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Effect of *Gymnema* leaves (*Gymnema Inodorum* (Lour.) Decne.) Supplement on Physical Quality of Northern Thai noodle.

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Abstract. Noodle is a great source of carbohydrate. Even though, wheat-based noodle frequently lacks ingredients that may enhance the value of the new product. To overcome this nutritional shortage and provide health advantages for consumers, a feasible strategy was to integrate gymnema leaves (*Gymnema Inodorum* (Lour.) Decne.) in Northern Thai noodles. The study aimed to produce Northern Thai noodles supplemented gymnema leaves at the ratio of 1:1 (w/w), 1:2 (w/w), and 2:1 (w/w) between leaves and limewater. The assessment of color, cooking quality, and texture profile within the noodles were evaluated. The results revealed that incorporating gymnema leaves in Northern Thai noodles significantly decreased yellowness and lightness compared with the control. The noodles supplemented gymnema leaves also showed a reduction of cooking time; the cooking yield was increased compared to the control. In addition, the texture profiles showed that the incorporated gymnema leaves increased the hardness of noodle. The findings indicate that incorporating gymnema leaves in Northern Thai noodles can be considered as a functional food ingredient.

Index Terms—*Gymnema* leaves, Northern Thai noodle (Khao Soi), Texture

V. INTRODUCTION

Food serves as a critical instrument for representing local identity and culture, significantly influencing the reputation of a tourist destination. Furthermore, the consumption of local cuisine by tourists contributes to the growth of regional economies. According to a survey on cultural identity in Northern Thai cuisine, Khao Soi Kai (Chicken Curry Noodles) exhibited a high frequency of recognition at 82% [1]. Khao Soi is a noodle-based dish originating from a cultural fusion between the Yunnanese (Haw) Chinese and Lanna people, which has evolved over time. It is a highly popular local delicacy in Northern Thailand, particularly in Chiang Mai, a city renowned internationally for its tourism [2]. The primary ingredients of Khao Soi, such as chicken or beef, are locally sourced and reflect the Lanna way of life and culinary traditions. The traditional production process involves kneading a mixture of wheat flour, eggs, salt, and water until smooth, pressing it into sheets, and then slicing (soi) them into strips, which gives the dish its name, "Khao Soi". This noodle-making technique and the dissemination of noodle culture were significantly influenced by Chinese and Burmese culinary traditions [3]. High-quality Khao Soi noodles share characteristics with fresh egg noodles, consistent with what J. Luecha et al. [4] mentioned, a staple

consumed extensively across Asian countries, including Japan, China, Korea, Malaysia, and Thailand. Generally produced from wheat flour, water, and salt, sometimes with additional ingredients like eggs, modified starch, or spices to enhance quality and variety, the production involves mixing the ingredients into a dough, sheeting, and cutting into desired sizes before blanching or boiling.

Gymnema leaves (*Gymnema Inodorum* (Lour.) Decne) contains various phytochemicals, including polyphenols, flavonoids, and a key active compound known as gymnemic acid, which possesses hypoglycemic properties [5] *gymnema* leaves, as a health-promoting herb, has gained increasing popularity due to its efficacy in regulating blood sugar levels in diabetic patients, balancing body lipids, and alleviating symptoms of allergies, asthma, and gout A. Sanguanwaew et al. [6] In Thailand, *gymnema* leaves is typically found in the cooler climates of the Northern region. Botanically, it is a perennial climber with green stems that produce a white milky latex. Its elliptical or ovate leaves are arranged in opposite pairs, featuring pointed tips and smooth or slightly wavy margins. According to traditional medicine records, the plant is used to treat diabetes, reduce fever (for external use on the forehead), relieve coughs, and act as an expectorant. In culinary terms, young shoots and leaves are preferred for consumption, either as a side dish for chili pastes (Nam Phrik) or as an ingredient in stir-fries and curries. These young

leaves are exceptionally high in antioxidants [7]. The growing awareness of its medicinal properties has established gymnema leaves as a popular indigenous vegetable in Northern households and a promising economic crop.

Consequently, the researcher aims to develop a Khao Soi noodle formula enriched with gymnema leaves. This initiative seeks to provide consumers with a functional food alternative that offers nutritional benefits while utilizing local raw materials from Northern Thailand, specifically gymnema leaves. This study focuses on identifying the optimal production process and the effect of gymnema leaves extract on the physical characteristics and quality of the Northern Thai noodle to produce a flat-shaped noodle similar to "Jade Noodles" (green noodles).

VI. METHODOLOGY

I. Materials

Gymnema leaves was collected from Tha Pla District in Uttaradit, Thailand. The wheat flour (UFM), salt (prung tip), egg (CP Selection Egg), limewater and soybean oil (A-Ngoon) were purchased from fresh market in Uttaradit province.

II. Methods

II.I. Preparation of stock solution of gymnema leaves

gymnema leaves were washed with tap water and then removed the excess water the leaves were cut into the dimension of N. D. Qumbisa et al. [8] and they were blanched in hot water at 100 °C for 2 minutes. After that they were immediately soaked in an ice-water bath to rapid cool room temperature. The washed gymnema leaves were split into three batches For the first batch, the leaves were homogenized in a blender (Mallory, Brazil) For 3 s with the limewater in the ratio of 1 to 1 (w/w) between gymnema leaves and limewater. Another batch, the leaves were also homogenized in the limewater with the ratio of 1 to 2 (w/w). The last batch of leaves they were homogenized with the limewater in the ratio of 2 to 1 (w/w).

II.II. Fresh northern Thai noodle preparation

As control, fresh noodle was prepared with the following formulation: wheat flour (60 g), salt (0.29 g), egg (5 g), limewater (26 g), and soybean oil (12 g). For the test noodle samples, at the ratio of 1:2, 1:1 and 2:1 (w/w) of gymnema leaves and limewater in the control formulation was replaced with limewater. The dough was rolled to a thickness of 2 mm, and laminated into a tagliatelle form with a Model 4100 iPasta Classic noodle roller machine (Imperia, Italy) The fresh noodles was cooked in boiling water for 7 minutes and then soaked into cold water for 2 mins. They were physically analyzed characterized 1 hour after production.

II.III. Cooking quality of northern Thai noodle

II.III.I. Optimum Cooking time

The optimum cooking time for each noodle was assessed as previously described by S. Klamklomjit et al. [9] Briefly, 25 g of sample was placed into 300 ml of boiled water at 100 °C. The sample was then randomised and squeezed between two glass plates every 30 seconds. The optimum cooking time was determined as the time point at which the white core of noodle is no longer visible.

II.III.II. Cooking yield

The cooking yield was analyzed using the method of S. Klamklomjit et al. [9]. Briefly, 25 g of noodle was soaked in 300 ml of boiling water and cooked in accordance with the predetermined optimum cooking time. The water was then drained and the sample was set aside and weighed and the cooking yield was calculated using the equation 1.

$$\text{cooking yield} = \frac{W1}{W2} \times 100\% \quad (1)$$

W1 = weight of cooked noodle

W2 = weight of uncooked noodle

II.III.III. Cooking loss

The cooking loss of noodle was evaluated by the method of S. Klamklomjit et al. [9]. Briefly, 25 g of noodle was places into 300 ml of boiling water in a beaker and cooked for a present cooking time. remove noodle, evaporate the remaining cooking water to dryness at 105±2 °C to constant weight. weigh the dried residue. The cooking loss was calculated as equation 2.

$$\text{cooking loss} = \frac{W(S)}{W2} \times 100\% \quad (2)$$

W(S) = weight of dried residue from cooking water

W2 = weight of uncooked noodle

II.IV. Physical properties of Northern Thai noodle

Measurement of Water Activity, pH and color.

The water activity was measured at 25 °C using Water Activity (LabSwift-aw, Novasina, Switzerland). For the pH measurement, the sample (10 g) was suspended in 90 ml of water and soaked for 20 mins. The solution was subsequently homogenised using homogenizer (UltimateTaste 300, Electrolux, Thailand) . The homogenised solution was measured pH with five replication using Benchtop (pH700, EUTECH, Singapore). The color of noodle was performed using L*, a*, b* system. Where L* refers to the lightness, a* (intensity of red (+) and green (-) and b* (intensity of yellow (+) and blue (-). The noodles were analyzed in tens of the referred parameter using Hunter lab (Colorflex EZ, Hunter Lab, USA).

II.V. Texture analysis

The adhesive, hardness, adhesive, cohesiveness, gumminess, springiness, and resilience of both uncooked and cooked noodles were carried out using a texture analyzer (TA Hdplus, stable micro systems, England) as described by The test setting include a probe P/35, load cell 5 kg, pre-test speed of 2 mm/s, post-test speed of 10 mm/s and initial probe distance of 90% of sample. Five replicates were performed for each treatment.

II.VI. Statistical analysis

The data were presented as mean±standard deviation (CRD) was performed to analysed the data. An analysis of variance (ANOVA) was performed using Program spss version 30. Whenever a significant F value

($p < 0.05$) . was found, the Duncan's Multiple Range Test (DMRT) was used to identify significant differences among means.

III. RESULTS AND DISCUSSION

I. Cooking quality of Northern noodle with gymnema leaves

The cooking quality of Northern Thai noodle added gymnema leaves including cooking time, cooking yield, and cooking loss are presented in Table I

TABLE I
COOKING QUALITIES OF NORTHERN THAI NOODLES SUPPLEMENTED WITH GYMNEMA LEAVES

Cooking qualities	Northern Thai noodles supplemented with gymnema leaves			
	Control	1:2 (w/w) (gymnema leaves: limewater)	1:1(w/w) (gymnema leaves: limewater)	2:1(w/w) (gymnema leaves: limewater)
Cooking time (min)	7.38	7.18	7.04	7.22
Cooking yield ^{ns} %	178.62±18.66	189.94±8.02	190.54±6.62	182.20±7.95
Cooking loss %	6.97±2.13 ^b	5.94±1.21 ^{ab}	4.34±0.99 ^a	7.15±1.65 ^b

Different superscripts in same row indicate significant differences ($p < 0.05$)

^{ns} means non significant ($p > 0.05$)

According to Table I, the cooking time of the developed Northern Thai noodles ranged from 7.04 to 7.22 minutes. The cooking yield (%) of the Northern Thai noodles fortified with varying contents of gymnema leaves (*Gymnema Inodorum* (Lour.) Decne.) showed no statistically significant difference ($P > 0.05$) compared to the control. However, the formulation with a 2:1 ratio of gymnema leaves to limewater exhibited the highest percentage of cooking loss. This finding is consistent with S. Khemthong et al.[10], who reported that increasing the concentration of water meal (*Wolffia globosa*) powder led to higher cooking loss in noodles. This phenomenon occurs because the fiber content in the additive interferes with the formation of disulfide bonds between proteins and starch in the noodle structure. Consequently, this interference causes starch granules to rupture during the

blanching process, leading to increased amylose leaching into the water and a higher loss of total solids. Furthermore, these results align with S. Chooklin et al. [11] observed that cooking loss increased relative to the control as the concentration of Champignon mushroom flour increased. In this development of gymnema leaves for Northern Thai noodles, the total solid loss remained below 10%, which is in agreement with the research conducted by S. Klamklomjit et al. [9]. The 2:1 ratio of gymnema leaves to limewater was the highest concentration that used in this experiment, was found to result in an increased percentage of weight loss after cooking.

II. Physical properties of Northern Thai noodle supplemented with gymnema leaves

TABLE II
PHYSICAL PROPERTIES OF UNCOOKED NORTHERN THAIL NOODLE SUPPLEMENTED WITH GYMNEMA LEAVES

uncooked Northern Thai noodle				
Physical properties	Control	1:2 (w/w) (gymnema leaves: limewater)	1:1(w/w) (gymnema leaves: limewater)	2:1(w/w) (gymnema leaves: limewater)
Color				
L^*	71.62±0.00 ^c	57.28±1.31 ^b	53.72±0.95 ^a	53.31±0.70 ^a
a^*	2.83±0.23 ^b	-4.50±0.53 ^a	-4.72±0.73 ^a	-4.98±0.65 ^a
b^*	11.63±0.47 ^a	18.86±1.79 ^d	17.28±1.62 ^c	15.61±0.86 ^b
uncooked Northern Thai noodle				
Physical properties	Control	1:2 (w/w) (gymnema leaves: limewater)	1:1(w/w) (gymnema leaves: limewater)	2:1(w/w) (gymnema leaves: limewater)
a_w	0.92±0.00 ^b	0.92±0.01 ^b	0.917±0.01 ^a	0.93±0.00 ^b
pH	6.57±0.02 ^c	6.54±0.05 ^c	6.29±0.08 ^a	6.45±0.01 ^b
Adhesiveness (g)	-81.34±19.12 ^a	-44.54±24.90 ^b	-45.90±17.23 ^{ab}	-53.27±16.58 ^{ab}
Hardness (N)	143.29±43.85 ^a	536.19±19.36 ^c	508.40±46.93 ^c	439.93±23.09 ^b
Cohesiveness (g) ^{ns}	0.22±0.06	1.67±0.87	0.33±0.06	0.31±0.05
Chewiness (g)	18.42±11.35 ^a	146.81±32.98 ^c	98.57±13.76 ^b	82.69±34.99 ^b
Gumminess (g)	32.30±11.45 ^a	238.21±76.32 ^c	169.17±24.41 ^{bc}	138.60±23.58 ^b
Springiness(g) ^{ns}	0.53±0.17	0.64±0.19	1.76±0.66	0.57±0.18
Resilience (g)	0.17±0.04 ^a	0.49±0.195 ^c	0.33±0.06 ^b	0.29±0.05 ^{ab}

Different superscripts in same row indicate significant differences ($p < 0.05$)

^{ns} means non significant ($p > 0.05$)

From Table II illustrates the properties of the gymnema leaves for Northern Thai noodle before thermal processing. As the concentration of gymnema leaves increased, lightness (L^*), b^* (yellowness), and a^* (redness/greenness) all showed a downward trend due to increased chlorophyll content. Textural profile analysis such as Cohesiveness and springiness did not show statistically significant differences ($P > 0.05$), while higher concentrations increased Adhesiveness and Gumminess. Water activity (a_w) was approximately 0.9, indicating high moisture, and pH ranged

from 6.29 to 6.57. The developed noodles were then subjected to blanching for further quality analysis, as detailed in Table III springiness did not show statistically significant differences ($P > 0.05$), while higher concentrations increased Adhesiveness and Gumminess. Water activity (a_w) was approximately 0.9, indicating high moisture, and pH ranged from 6.29 to 6.57. The developed noodles were then subjected to blanching for further quality analysis, as detailed in Table III

TABLE III
PHYSICAL PROPERTIES OF COOKED NORTHERN THAIL Noodle SUPPLEMENTED WITH GYMNEMA LEAVES

Physical properties	cooked Northern Thai noodle			
	Control	1:2 (w/w) (gymnema leaves: limewater)	1:1(w/w) (gymnema leaves: limewater)	2:1(w/w) (gymnema leaves: limewater)
color				
L^*	67.96±18.74 ^b	56.45±1.35 ^a	56.73±1.57 ^a	53.52±1.83 ^a
a^*	0.65±0.33 ^c	-2.85±0.89 ^b	3.32±0.92 ^{ab}	-3.87±1.65 ^a
b^*	5.99±0.58 ^a	10.98±2.07 ^{ab}	15.83±19.58 ^b	12.01±2.68 ^{ab}
a_w^{ns}	0.96±0.01	0.96±0.01	0.89±0.14	0.96±0.01
pH	7.03±0.02 ^d	6.79±0.01 ^c	6.51±0.06 ^a	6.73±0.02 ^b
Adhesiveness(g)	-290.81±98.62 ^c	-340.41±63.85 ^{bc}	-412.08±59.05 ^{ab}	-526.19±110.22 ^a
Hardness (N)	221.73±10.48 ^a	220.31±6.20 ^a	248.70±24.30 ^b	278.64±8.34 ^c
Cohesiveness (g) ^{ns}	0.30±0.05	0.35±0.02	0.30±0.02	0.42±0.27
Chewiness (g) ^{ns}	53.80±6.97	61.29±9.42	57.42±12.19	68.84±13.02
Gumminess (g)	67.57±12.40 ^a	78.04±6.50 ^{ab}	77.01±10.31 ^{ab}	84.37±11.86 ^b
Springiness (g) ^{ns}	0.80±0.07	2.00±2.67	0.71±0.09	0.49±0.28
Resilience (g)	0.11±0.0 ^a	0.12±0.02 ^b	0.11±0.01 ^{ab}	0.12±0.01 ^{ab}

Different superscripts in same row indicate significant differences ($p < 0.05$)

^{ns} means non significant ($p > 0.05$)

As shown in Table III, the cooked properties of the noodles were evaluated. Regarding color, the L^* value decreased as the concentration of gymnema leaves increased. Similarly, b^* and a^* values decreased due to the presence of chlorophyll; these values were lower than those of the raw noodles after exposure to heat, though the differences were not statistically significant ($P > 0.05$). These findings have similar trend to S. Plalasuwan et al. [12] which observed that adding Bai Liang or Malindjo leaves (*Gnetum gnemon* L. var. *tenerum*) to fresh noodles led to a reduction in L^* and b^* values as concentration increased. The a^* values, representing greenness (-), became more intensely green following the blanching of noodles with higher vegetable content. Regarding the textural properties of the cooked Northern Thai noodle, no statistically significant differences ($P > 0.05$) were found in cohesiveness, chewiness, or springiness. However, the increased gymnema leaves content after heating led to higher values for adhesiveness, hardness, gumminess, and resilience. This aligns with the research by S. Tempiam et al. [13], which noted that calcium ions from limewater (calcium hydroxide) exhibit high ionic activity, forming strong bonds with starch. This results in high viscosity and elasticity, similar to cooked Singapore Sweet Dumplings (Lod Chong). S. Sirikhan. [14] also noted that blanching noodles until cooked enhances their aesthetic green color and provides a soft, elastic texture. Additionally, related research on Yanang leaf *Tiliacora triandra* (Colebr.)

Diels) water at a 50:50 (w/w) ratio found high consumer acceptance. The a_w values after cooking ranged from 0.8 to 0.9, indicating high moisture and a slight increase compared to the raw noodles. The pH of the fortified noodles ranged from 6.51 to 7.03, which is comparable to the study by N. Dangkhaw. [15] on Milkfish (*Chanos chanos* Forskal) noodles fortified with sea grapes (*Caulerpa lentillifera* J. Agardh), which reported a pH of 6.11 in Fig. 1.

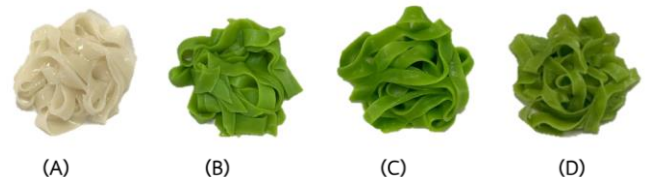


Fig. 1. Appearance of cooked Northern Thai noodle supplemented with gymnema leaves (A) control (B) 1:2 (w/w) (C) 1:1 (w/w) (D) 2:1 (w/w).

IV. CONCLUSION

In determining the prototype formula for gymnema leaves for Northern Thai noodle, it was found that the control formula's water content could be replaced with gymnema leaves extract at three ratios: 1:2, 1:1, and 2:1. The noodles achieved optimal color and textural quality at the 2:1 ratio (1,000g of gymnema leaves to 500g of water). At this maximum concentration, gumminess, cohesiveness, and springiness remained no statistically significant difference,

while adhesiveness, hardness, chewiness, and resilience increased. These findings provide a viable pathway for value-adding to indigenous Northern Thai vegetables and developing new functional health-food products.

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Air Pollution Management in Welding: PM_{2.5} Removal by Water Trapping and HEPA Filter

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Abstract. A lot of pollutants from welding fumes have an effect on the serious risks to human health, leading to respiratory and cardiovascular diseases, increased mortality rates. This study investigated the PM_{2.5} removal efficiency at varying distances from welding fume to hood in the welding area at There Engineering & Consulting Co., LTD in Bangkok. It focused on Tungsten Inert Gas (TIG) and Stick Welding processes. The welding fume extractor was designed using Autodesk Inventor and Computational Fluid Dynamics (CFD) was simulated with Solidworks. The experimental purification system used dual-stage process (water-trapping system and PM_{2.5} HEPA filter) was implemented. The performances were monitored in real-time by IoT-based air and water quality sensors. The findings highlight PM_{2.5} reduction efficiency around 80.76 to 90.57% at a distance of 20 to 200 mm from the source. The AQI color changed from red (Very unhealthy) and purple (Unhealthy) to yellow (Moderate). The PM_{2.5}/PM₁₀ ratio were in the range of 0.69 to 0.98. The study directly demonstrates a tangible improvement in the workplace environment for welders.

Index Terms— Air Pollution, PM_{2.5}, Welding, Water-trapping, HEPA filter

VII. INTRODUCTION

A lot of pollutants (PM_{2.5}, SO₂, NO₂, and CO) have an effect on the serious risks to human health, leading to respiratory symptoms, decreased pulmonary functions, cardiovascular diseases, increased mortality rates [1][2]. Welding fumes have been classified as carcinogenic to humans by the International Agency for Research on Cancer (IARC) linked to cancers such as trachea, bronchus and lung cancer [3]. Welding fumes are composed of fine and ultrafine particles, which may reach the distal airways and represent a risk factor for respiratory diseases. Dose, exposure duration, and composition of welding fumes affect lung injury. The toxicity of complex metal-containing particulates can be associated with soluble forms of transition metals. Fumes generated during manual metal arc welding were found to be highly water soluble, whereas gas metal arc welding fumes were relatively insoluble [4]. Thus, understanding the air quality of welding area is vital for designing air pollution removal. This study seeks to provide an efficient air pollution removal system to improve air quality.

Welding workshops contribute significant amounts of air pollutants and pose health risks to welders and residents alike [5]. Carbon monoxide (CO) and formaldehyde (HCHO) levels are frequently higher than recommended limits in certain locations. CO is particularly dangerous due to its

toxicity and the frequent lack of, or improper use of, safety devices in welding workplaces. Exposure to these emissions can lead to both short-term and long-term health effects for workers and others in the vicinity [6]. The air quality index (AQI) for Fine Particle Pollution (PM_{2.5}) of Environmental Protection Agency (EPA) updated in 2024 was shown in Table 1 [7].

TABLE I
AIR QUALITY INDEX (AQI) FOR FINE PARTICLE
POLLUTION

Fine Particle Pollution AQI, µg/m ³	AQI Value	AQI Category	AQI Color
0.0 to 9.0	0 – 50	Good	Green
9.1 to 35.4	51 – 100	Moderate	Yellow
35.5 to 55.4	101 – 150	Unhealthy for sensitive groups	Orange
55.5 to 125.4	151 – 200	Unhealthy	Red
125.5 to 225.4	201 – 300	Very unhealthy	Purple
225.5+	301+	Hazardous	Maroon

Due to weak regulations and poor work ethics regarding workshop safety, both welders and non-welders are highly susceptible to the risks emanating from welding processes. The use of personal protective equipment (PPE)—such as welding masks, face shields, and goggles—remains very low, despite continuous exposure to harmful ultraviolet (UV) radiation [8]. Implementing these necessary precautions and enforcing stricter environmental standards are vital for worker safety [9]. Furthermore, preventive measures such as improved ventilation systems can effectively reduce indoor air pollutant concentrations [10].

Low Volume High Velocity (LVHV) ventilation systems were tested at a distance of 15 cm from the torch tip, following the National Institute of Occupational Safety and Health (NIOSH) Method No. 7302. The removal efficiency for manganese in a bell-shaped hood averaged 84.92%, while the lowest efficiency (13.39%) was recorded for chromium in a slot hood. Factors such as hood shape, the distance from the electric arc, and the exhaust flow rate at the hood opening are critical to the design and performance of an LVHV system [11]. Additionally, a water spray system operating at two bars of pressure is most compatible with the dimensions of a V6 nozzle. This configuration is likely to succeed in significantly reducing welding emissions before they are discharged into the environment [12].

VIII. METHODOLOGY

The sampling was conducted at the workshops of Therec Engineering & Consulting Co., Ltd., located on Kanchanaphisek Road, Bang Bon Nuea, Bangkok, Thailand. The facility's processes involve the manual fabrication of various metal products using mechanical equipment such as cyclones, hoppers, enclosures, strainers, rotary valves, and diverter valves. Two welding processes were Tungsten Inert Gas (TIG) and Shielded Metal Arc Welding (Stick). TIG welding was performed on stainless steel, while Stick welding was used for iron. Welding activities are performed without separation barriers, with all work occurring in the same open area. Regarding the spatial arrangement, there is no specific layout design or proper air circulation system, as shown in Fig.1. Consequently, various forms of dust are generated, ranging from invisible fine particles to coarse dust visible to the naked eye. This lack of effective ventilation increases health risks for workers who are exposed to these conditions over 8-hour shifts.

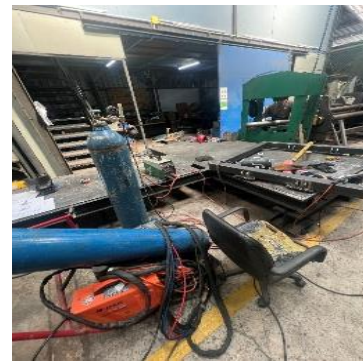


Fig.1. Inadequate air circulation during welding operations.

Air quality data were collected using sensors placed near the welding area to monitor the dust exposure levels of the workers. The instruments used in this study consisted of various air quality sensors. The measured environmental parameters included temperature, relative humidity, and concentrations of CO₂, PM_{2.5}, PM_{1.0}, PM₁₀. The sensors collected air quality data hourly during 8-hour work shifts. Baseline data were recorded throughout April and May prior to the installation of the welding fume extraction machine. An experimental dual-stage purification system, comprising a water-trapping system and a high-efficiency HEPA PM_{2.5} grade filter, was implemented. Data were collected during November and December.

IX. RESULTS AND DISCUSSION

Humidity levels near the welding area ranged from 60% to 70%, with an average of $65.41 \pm 5.52\%$. The temperature fluctuated between 30°C and 33.4°C, averaging 31.60 ± 1.44 °C. CO₂ levels ranged from 408 ppm to 432 ppm (412.77 ± 16.91 ppm), while HCHO concentrations remained low, between 0.002 mg/m³ to 0.003 mg/m³. Finally, TVOC levels were recorded at a constant 2.445 ppm.

PM₁₀ concentrations near the welding area ranged from 57 µg/m³ to 145 µg/m³, with an average of 101.94 ± 45.10 µg/m³. PM_{1.0} levels fluctuated between 35 µg/m³ to 100 µg/m³ (average: 65.10 ± 31.08 µg/m³). Similarly, PM_{2.5} values were between 50 µg/m³ to 119 µg/m³, averaging 91.41 ± 38.45 µg/m³. These findings highlight that PM_{2.5} levels exceed standard limits and are "Unhealthy". Since welders work 8 hours a day, 5 days a week in this environment, this duration and concentration pose significant health risks. Common health issues related to PM_{2.5} exposure include eye, nose, and throat irritation, skin infections, respiratory allergies, difficulty breathing, and asthma attacks.

Fine particle pollution AQI can be related to the detected AQI (Ambient Air Quality). The AQI sensor can be easily set for small factory. The Air Quality Index (AQI) is used to communicate daily air pollution levels and health risks. It measures five primary air pollutants: PM_{2.5}, PM₁₀, ozone, nitrogen dioxide, sulfur dioxide, and carbon monoxide [13]. while the ACGIH was detected in the 8 hours [14].

The PM_{2.5} to PM₁₀ ratios in this study is in the range of 0.69-0.98 and is similar to the previous reports as shown in Table 2. The factors of the spatial distribution of PM_{2.5} and PM₁₀ are the result of the combined effect of natural factors and human. The natural factors of PM_{2.5} and PM₁₀ spatial distribution are mainly illustrated as low sand dust and weak high-pressure system in spring while high sand dust caused by the elevated thermal inversion in winter. The PM_{2.5}/PM₁₀ ratio is lower in spring, ranging from 0.3 to 0.6 while these ratios exceeding 0.85 in winter [17,18].

TABLE 2
THE PM_{2.5} TO PM₁₀ RATIOS IN THAILAND

City	PM _{2.5} to PM ₁₀ Ratio	Area	Reference
Chiang Mai	0.30–1.14	Urban	[15]
Nan	0.27–1.57	Rural	
Bangkok	0.53–0.73	Urban	[16]
Bangkok	0.69–0.98	Urban	This study

The welding fume extraction machine was designed using Autodesk Inventor, and the Computational Fluid Dynamics (CFD) simulations were performed in SolidWorks. The fume flow direction within this process was simulated to utilize a cyclonic effect. The accuracy of the simulation was validated by testing the velocity range in front of the filter, which measured between 0.3 and 1 m/s as shown in Fig. 2. The boundary conditions as static pressure for CFD section were 101325 Pa. The air flow type of laminar and turbulent within the extraction machine were used to design for the interaction maximum between fumes and water as shown in Fig. 3. The particle fume size of the mild steel and stainless steel were 0.59 and 0.46 μm , respectively [19]. Due to the use of a small flexible pipe at the hood face, the velocity of welding fume extractor machine is 15 m/s was simulated. The velocity ventilation design through the open area of the perforated plate was 10.2 m/s [20].

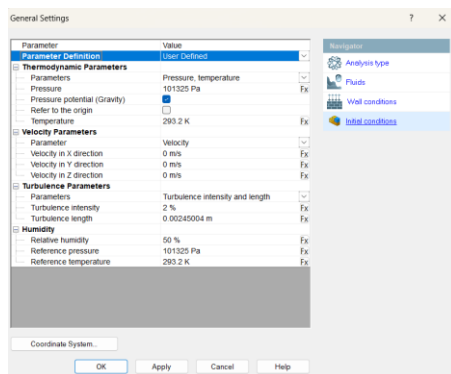


Fig. 2. CFD design for boundary condition.

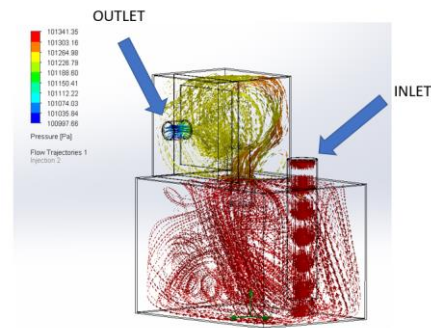


Fig.3. CFD simulation of fume extraction.

Additionally, the welding fumes were extracted through water to prevent the risk of explosion. The PM_{2.5} removal efficiency of the fume extraction machine—composed of a

water-trapping system and a PM_{2.5} filter (HEPA grade)—was tested, as shown in Fig. 4. The fume extraction system consists of two air quality sensors, a water quality sensor, a PM_{2.5} filter, a blower, and a water tank. Detailed specifications of the fume extraction machine are provided in Table 3. The total cost of the machine is 650 USD.

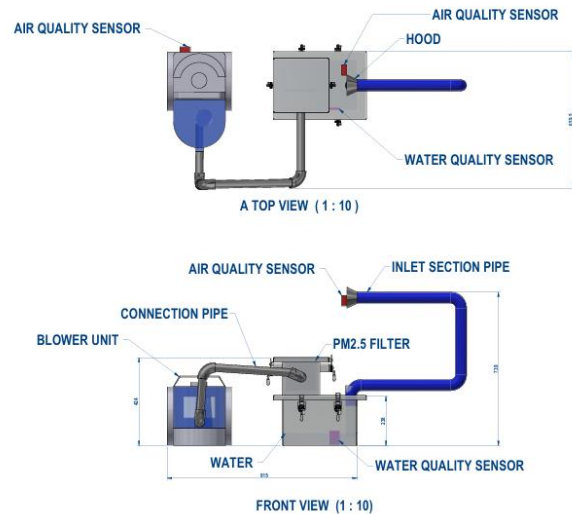


Fig.4. Diagram of fume extraction machine

TABLE 3
DETAILS OF WELDING FUME EXTRACTION MACHINE (WFEM)

Equipment	Specification of WFEM	Cost of WFEM (USD)
WFEM	Carbon Steel (SS 400) 2mm thickness	220
Blower	Electrolux EFC 52512 with 1800 Watts	95

Air quality sensor	2C08 with (WIFI) measured the Temperature, humidity, CO ₂ , HCHO, TVOC, PM _{2.5} , PM ₁₀ , PM _{1.0}	115
PM _{2.5} filter	High Efficiency Particulate Air filter (HEPA) Hysure POLO Q9 with mesh size 2.5-micron meter	30
Water quality sensor	Temperature, pH, TDS, EC	80
Piping system	Flexible pipe and pipe fittings	110
Total		650

The research utilizes a welding fume extraction system equipped with both water-trapping and air-filtration components. The air velocity was measured by using a UNIT UT363 digital anemometer. A water quality sensor continuously monitors the temperature and pH of the water used to trap pollutants. An increase in these values indicated that the water has successfully captured contaminants from the welding fumes. The system integrates two air quality sensors and one water quality sensor, all of which were IoT-enabled for continuous monitoring and display via a mobile application. This application employed digital twin technology to visualize real-time water quality and provide data on the volume of toxic fumes removed.

The efficiency analysis compares air quality parameters recorded before installation—where high PM_{2.5} levels were observed—with post-installation data to determine the percentage reduction in pollution. Routing the fumes through water prior to the PM_{2.5} filter is an effective method for pre-treating the air, which prevents premature filter clogging and increases overall system efficiency. At a capture velocity of 15 m/s at the hood, the system achieves PM_{2.5} removal efficiency of 80.76 to 90.57% at a distance of 20 to 200 mm from the welding area as shown in Table 4. This is consistent with previous studies [21], which utilized a radial impeller outlet velocity of 10.85 m/s to effectively extract welding fumes. The PM₁₀ removal efficiency of 80.00 to 87.14% at a distance of 20 to 200 mm from the welding area as shown in Table 5 while the exhaust air cleaned by passing it through a highly efficient filter MERV 16 (HEPA filtration efficiency test to MIL Std. 282 was achieved 99.97% on 0.3 micrometer thermally particles [22]. The peripheral leakage can be occurred by using a manual clamping.

TABLE 4
THE EFFICIENCY OF PM_{2.5} REMOVAL VARIED
ACCORDING TO THE DISTANCES FROM
THE FUME-EMITTING AREA.

Distance (mm)	Velocity (m/s)	Inlet side (µg/m ³)	Outlet side (µg/m ³)	Efficiency, %
20	14±1	138	13	90.57
40	12±1	110	14	87.27
60	8±2	100	13	87.00
80	7±2	88	15	82.95
100	5±2	84	13	84.52
150	2±0.5	78	15	80.76
200	1±0.5	73	14	80.82

TABLE 5
THE EFFICIENCY OF PM₁₀ REMOVAL VARIED
ACCORDING TO THE DISTANCES FROM
THE FUME-EMITTING AREA.

Distance (mm)	Velocity (m/s)	Inlet side (µg/m ³)	Outlet side (µg/m ³)	Efficiency (%)
20	14±1	140	18	87.14
40	12±1	126	20	84.12
60	8±2	126	18	85.71
80	7±2	126	21	83.33
100	5±2	104	18	82.69
150	2±0.5	104	21	79.80
200	1±0.5	100	20	80.00

The sodium or alkaline elements act as stabilizers allows for improved arc control and more reliable performance during high-current metal-cored arc welding (MCAW)

Operations [23]. These cause alkaline particle in dust and can increase the initial water pH level from 7.4 to 8.0 for 6 hours testing times. However, the optimum of wet scrubber was pH level 5.5 – 8.5 [24]. The maintenance was established by refilling water once the pH level exceeds 8.5. The extended working times can increase from 7.4 to 11.2 within 6 months. This increase in pH indicates that the welding fumes successfully mixed with and were trapped by the water, suggesting that the water requires periodic monitoring or replacement.

While standard portable fume extractors, which use dry filters and operate with reverse pulse cleaning, require additional air supply pipe line, air compressor, pressure tank and electronic pulse controller to manage the cleaning cycles [25]. The portable fume extractor without reverse pulse cleaning price with the efficiency around 95.00% was \$2,427.00 as in Table 6 [26]. However, the cost per % of efficiency was \$24.28, which was 3.17 times higher than this research. Therefore, the economic motivation for a water-based pre-filter was interested. Furthermore, the advantages of the welding fume extraction machine were safety and longevity. Welding fumes often contain hot solids (sparks). A water-trap neutralizes these high-temperature particles, eliminating explosion and burning. Furthermore, the water trap removes approximately 70% of pollutants and prevents the clogging of the HEPA media.

TABLE 6
WELDING FUME EXTRACTION SYSTEM
AND INDUSTRIAL UNIT

Category	welding fume extraction system	Industrial Unit
Capital Cost	\$650	\$2,427
Measured Efficiency	85%	95.00%
Cost per % of Efficiency	\$7.64	\$24.28

X. CONCLUSION

Initial findings revealed a significant environmental hazard, as PM_{2.5} levels near the welding area consistently exceeded safety standards. These elevated concentrations represent a serious state of air pollution and a high health risk for welders. To address this, a vacuum-type welding fume extraction machine was developed utilizing a dual-stage purification system. The first stage employed a water-trapping system, which not only captures pollutants but also extended the life of the secondary filter by quenching high-temperature sparks and preventing potential explosions. The second stage cleaned the remaining fumes using a high-efficiency PM_{2.5} filter (air purifier grade). Tests confirmed a PM_{2.5} removal efficiency of 80.76 to 90.57% at a distance of 20 to 200 mm from the welding area. The AQI color changed from red and purple to yellow. The PM_{2.5}/PM₁₀ ratio were in the range of 0.69 to 0.98.

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Study of CO₂ Nanoparticle Flooding using Micromodel Experiment: Pore-scale Visualization and Quantitative Image Analysis

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Abstract. Carbon dioxide (CO₂) flooding is a well-established Enhanced Oil Recovery (EOR) technique; however, its sweep efficiency is often limited by unfavorable mobility ratio, viscous fingering, and early gas breakthrough. Recent studies suggest that the incorporation of nanoparticles may improve CO₂ flooding performance, yet the underlying pore-scale mechanisms remain poorly understood. In this study, CO₂ nanoparticle flooding was experimentally investigated using transparent micromodels, with a focus on pore-scale visualization and quantitative image analysis. A series of micromodel flooding experiments were conducted to directly observe fluid flow behavior, phase interactions, and displacement patterns during CO₂ injection with and without nanoparticles. High-resolution image sequences were acquired throughout the experiments and processed using ImageJ™ software to segment grains and fluid phases, enabling quantitative evaluation of oil and water-gas saturations as well as sweep efficiency. Color-filling and image overlay techniques were employed to clearly visualize phase distributions and displacement evolution. The results indicate that nanoparticle-assisted CO₂ flooding improves pore-scale sweep efficiency by stabilizing displacement fronts, reducing viscous fingering, and enhancing oil mobilization compared with conventional CO₂ flooding. The CO₂ nanoparticle flooding achieved 85% oil recovery, whereas CO₂ injection without nanoparticles resulted in 80% recovery. The findings provide valuable pore-scale insights into the mechanisms governing CO₂ nanoparticle flooding and support its potential application as an effective EOR strategy.

Index Terms—CO₂ flooding, nanoparticle, image analysis, micromodel

XI. INTRODUCTION

Carbon dioxide (CO₂) flooding is one of the most widely used Enhanced Oil Recovery (EOR) techniques due to its ability to increase oil recovery due to several oil recovery mechanisms including reduction of oil viscosity through miscibility, oil swelling, and lowering of interfacial tension between oil and the injected gas phase. These mechanisms contribute to improving microscopic displacement efficiency and increased oil recovery from mature reservoirs. In addition to its role in EOR, CO₂ injection has gained increasing attention as it can simultaneously support Carbon Capture and Storage (CCS) applications. However, conventional CO₂ flooding often faces operational challenges such as unfavorable mobility ratio leading to viscous fingering, and early gas breakthrough, which reduce sweep efficiency and leave a significant amount of residual

oil trapped in the porous media. Previous studies discussed the displacement mechanisms and limitations associated with CO₂ flooding processes (Holm and Josendal 1974; Alvarado and Manrique 2010).

In recent years, nanoparticles have been investigated as promising additives to enhance oil recovery performance. Due to their extremely small size and large surface area, nanoparticles can significantly influence rock-fluid interactions within porous media. When introduced into the reservoir, nanoparticles can adsorb onto rock surfaces and alter wettability from oil-wet to more water-wet conditions, facilitating the detachment and mobilization of trapped oil. In addition, nanoparticles can accumulate at the oil–water interface and reduce interfacial tension, thereby lowering capillary forces that trap oil within pore throats. Another important mechanism is the generation of structural disjoining pressure, where nanoparticles form a wedge-shaped film between the oil phase and rock surface that

promotes the detachment of thin oil films. Furthermore, nanoparticles can improve displacement efficiency by partially blocking high-permeability channels (log-jamming effect) and modifying the mobility ratio of the displacing fluid, which leads to improved sweep efficiency in porous media. These combined mechanisms contribute to enhanced microscopic displacement efficiency and reduced residual oil saturation in porous media. The main mechanisms of nanoparticle enhanced oil recovery are illustrated schematically in Fig. 1 (Ju and Fan 2009; Hendraningrat et al. 2013; Li et al. 2014; Fu et al. 2025). Despite these promising mechanisms, a detailed understanding of the pore-scale displacement behavior during CO₂ nanoparticle flooding remains limited, particularly in terms of fluid distribution, displacement patterns, and residual oil trapping within porous structures. As a result, the integration of nanoparticles with CO₂ flooding has emerged as a promising strategy for improving the effectiveness of EOR operations.

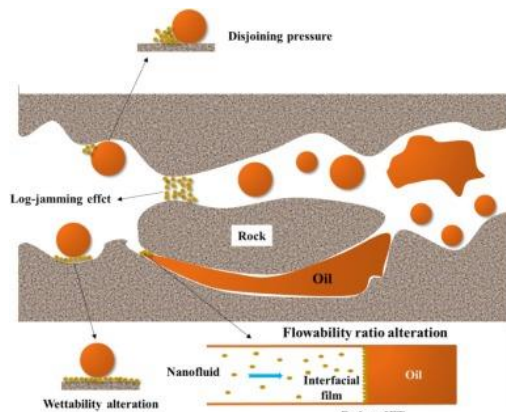


Fig. 1. Schematic mechanism of enhance oil recovery by nanoparticles in porous media. (Fu et al. 2025)

A detailed understanding of the displacement mechanisms of CO₂ nanoparticle flooding requires investigation at the pore scale. Conventional core flooding experiments provide microscopic recovery data but do not allow direct observation of fluid flow behavior within the pore structure. Micromodel experiments provide a useful platform for visualizing multiphase flow in porous media under controlled conditions. Previous studies have demonstrated that micromodel visualization can provide valuable insights into displacement patterns, residual oil trapping, and pore-scale flow mechanisms during EOR processes (Sohrabi et al. 2004; Karadimitriou and Hassanizadeh 2012). Therefore, investigation of CO₂ nanoparticle flooding using micromodel experiments combined with pore-scale visualization and quantitative image analysis is performed in this study to evaluate its effectiveness in improving oil displacement efficiency and its potential application for field-scale CO₂ EOR operations.

Whether the study aims to compare displacement behaviors, sweep efficiency, and oil recovery between baseline and CO₂ flooding nanoparticle-assisted.

XII. METHODOLOGY

Micromodel Fabrication and Experimental Setup

The pore-scale flooding experiments were conducted using a transparent glass micromodel designed to represent a two-dimensional porous medium. The micromodel was fabricated using photolithography and chemical patterning techniques to create a network of interconnected pores and throats with heterogeneous geometry. The characteristic pore diameters ranged from approximately 50 to 300 μm , while throat widths varied between 10 and 80 μm . The pore area fraction of the micromodel was approximately 0.38, representing typical porosity conditions observed in sandstone reservoirs. The patterned glass plate was bonded with a transparent cover plate to form a sealed microfluidic channel, enabling controlled fluid injection through the pore network. Stainless-steel tubing was connected to the inlet and outlet ports to allow fluid delivery and production during flooding experiments.

The experimental setup consisted of a syringe pump used to maintain a constant injection rate, a CO₂ supply cylinder equipped with a pressure regulator, and an optical microscope coupled with a high-resolution digital camera, as illustrated in Fig. 2. The micromodel was positioned on the microscope stage to allow real-time visualization of fluid flow within the pore network during the flooding experiments.

To simulate reservoir conditions, the experiments were conducted at a temperature of 114°C and a pressure of 3000 psi to simulate representative reservoir conditions. Fluids were injected at a constant flow rate of 5 $\mu\text{L}/\text{min}$, corresponding to a stable displacement condition suitable for pore-scale visualization. Images were captured at a resolution of 2048×2048 pixels, corresponding to a spatial resolution of approximately 3-5 μm per pixel, with a frame capture interval of 5 seconds throughout the flooding experiments.

Fluid Preparation and Micromodel Saturation

Prior to the flooding experiments, the micromodel was thoroughly cleaned using ethanol and deionized water to remove contaminants and ensure the consistent of wettability conditions. The micromodel was then dried and vacuumed to remove trapped air within the pore network.

A live oil sample obtained from reservoir S1 field was used in this study to represent realistic reservoir fluid conditions. The live oil contained dissolved gas components and exhibited properties similar to those of in-situ reservoir oil. Prior to the experiments, the oil sample was filtered to remove any impurities that could potentially obstruct the pore channels within the micromodel. The live oil was subsequently injected into the micromodel at a low flow rate until the entire pore network was fully saturated, thereby establishing the initial oil saturation condition required for the flooding experiments.

Synthetic formation water was prepared to represent reservoir brine conditions. The brine solution had a total salinity of 80,000 ppm, which is typical of high-salinity

formation waters found in many oil reservoirs. The brine was prepared by dissolving appropriate inorganic salts in deionized water to achieve the desired salinity. This formation water was used as the aqueous phase in the flooding experiments and as the base fluid for nanofluid preparation.

For the nanoparticle flooding experiments, silica (SiO_2) nanoparticles were used due to their chemical stability, high surface area, and proven effectiveness in enhanced oil recovery applications. The silica nanoparticles had an average particle diameter in the range of 20-50 nm and a high specific surface area, which promotes strong interaction with rock and fluid interfaces.

The nanoparticles were dispersed in the prepared formation water at a concentration of 500 ppm to produce the nanofluid solution. The dispersion process involved magnetic stirring for 30 minutes, followed by ultrasonic treatment for 60 minutes to ensure uniform dispersion and prevent nanoparticle aggregation. The prepared nanofluid served as the aqueous phase for the nanoparticle flooding experiments.

Flooding Procedure

Two displacement experiments were conducted to evaluate the performance of nanoparticle-assisted CO_2 flooding. In the first experiment, brine CO_2 flooding was performed as the baseline case. After establishing the initial oil saturation within the micromodel, CO_2 was injected at a constant flow rate of 5 $\mu\text{L}/\text{min}$ under the experimental conditions of 114 $^\circ\text{C}$ and 3000 psi. The displacement process continued until no significant additional oil production was observed. In the second experiment, CO_2 nanoparticle flooding was carried out using a co-injection method. CO_2 and nanofluid were injected simultaneously into the micromodel at a volumetric ratio of 1:1. The nanofluid consisted of 500 ppm silica nanoparticles dispersed in formation water with 80,000 ppm salinity. The CO_2 and nanofluid streams were combined through a mixing junction before entering the micromodel inlet to ensure uniform mixing of the injected phases.

The combined injection rate was maintained at 5 $\mu\text{L}/\text{min}$, allowing a direct comparison between the conventional CO_2 flooding and the CO_2 nanoparticle flooding experiments. The displacement process was continuously monitored using the optical microscope system to observe the evolution of the displacement front and the mobilization of trapped oil within the pore network.

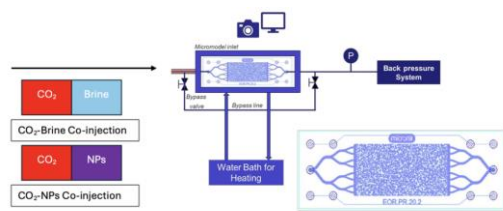


Fig. 2. Schematic diagram of the micromodel experimental setup used for CO_2 brine and CO_2 nanoparticle co-injection flooding experiments.

Image Acquisition and Quantitative Analysis

The flooding experiments were recorded using a high-resolution digital camera connected to the optical

microscope. Images were captured at regular time intervals of 5 seconds throughout the flooding experiments to monitor changes in fluid distribution within the micromodel. The captured images were subsequently analyzed using digital image processing techniques to quantify oil recovery and evaluate displacement efficiency.

Image processing involved converting the captured images to grayscale followed by threshold segmentation to distinguish between oil and injected fluids. The processed images were then converted into binary images representing the two fluid phases. The oil saturation at each stage of the flooding process was calculated based on the pixel area occupied by oil within the pore network. The oil recovery factor was determined by comparing the initial oil saturation with the remaining oil saturation after flooding. This quantitative analysis enabled comparison of displacement performance between brine CO_2 flooding and CO_2 nanoparticle flooding while providing insights into pore-scale displacement mechanisms.

XIII. RESULTS AND DISCUSSION

The micromodel visualization results indicate that CO_2 nanoparticle flooding produced a more uniform displacement front and lower residual oil saturation compared with conventional CO_2 brine flooding. Quantitative analysis of segmented images further shows improved sweep efficiency and reduced formation of disconnected residual oil clusters during nanoparticle assisted displacement. These observations suggest enhanced displacement performance at the pore scale in the presence of silica nanoparticles. The improved displacement behavior observed in this study is consistent with mechanisms commonly reported in previous nanoparticle assisted EOR studies, including possible interfacial tension reduction and wettability alteration, which may contribute to improved mobility control and reduced oil trapping. However, because interfacial tension and contact angle measurements were not conducted in the present experiments, these mechanisms cannot be confirmed directly and should therefore be interpreted as plausible explanations rather than definitive mechanisms.

Visualization analysis.

The pore-scale displacement behavior during CO_2 brine and CO_2 nanoparticle in co-injection was evaluated using micromodel visualization at different injected pore volumes (PV), as shown in Fig. 3. The red regions represent the remaining oil phase, while the dark regions indicate areas swept by the injected fluids.

At 0.1 PV, both flooding cases show similar displacement behavior, with most of the pore network still occupied by oil. The estimated residual oil-area fractions are approximately 9.4% for CO_2 brine flooding and 12.0% for CO_2 nanoparticle flooding. This indicates that the displacement front had not yet propagated significantly through the pore network at this stage, and the influence of nanoparticles on sweep efficiency remained limited during the initial injection period.

At 0.5 PV, differences between the two flooding cases become more apparent. The residual oil-area fraction decreases to approximately 6.0% in the CO_2 brine flooding

case, whereas the CO₂ nanoparticle flooding case shows a lower residual oil-area fraction of approximately 4.3%, corresponding to an improvement of about 28% in sweep-area efficiency. The nanoparticle-assisted flooding case also exhibits a more uniform displacement front with reduced evidence of preferential flow channel formation compared with the baseline flooding case.

At 1 PV, the improvement in displacement performance becomes more pronounced. The residual oil-area fraction further decreases to approximately 3.3% in the CO₂ brine flooding case and 1.35% in the CO₂ nanoparticle flooding case, representing nearly 60% reduction in remaining oil area after nanoparticle-assisted flooding. In addition, the baseline flooding case shows numerous disconnected residual oil clusters distributed throughout the pore network, whereas the nanoparticle-assisted flooding case results in fewer isolated oil clusters and improved displacement uniformity.

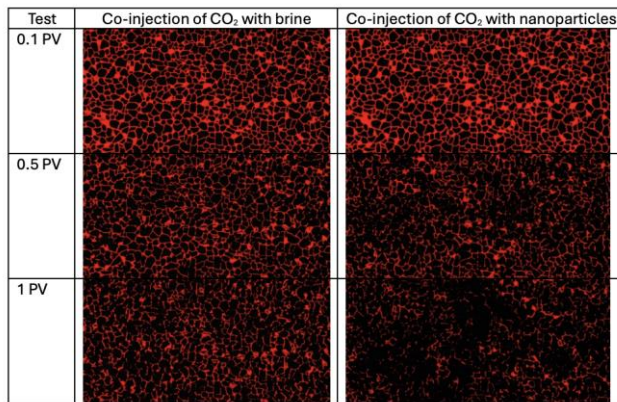


Fig. 3. Visuals of co-injection into oil-saturated micromodel at different type of CO₂ injection and injected pore volume. The fluid was injected from the left to the right side of images, oil and mixed CO₂ are presented in red and black respectively, in the processed images.

Breakthrough behavior was evaluated by monitoring displacement-front propagation across the micromodel snapshots. The CO₂ brine flooding case shows earlier development of preferential flow paths compared with the nanoparticle-assisted flooding case, indicating reduced displacement stability during conventional flooding. In contrast, the nanoparticle-assisted flooding case maintains a more stable displacement front and improved areal sweep distribution across the pore network.

The quantitative image analysis confirms that nanoparticle-assisted CO₂ flooding improves microscopic sweep efficiency and reduces residual oil trapping compared with conventional CO₂ brine flooding. Although previous studies suggest that silica nanoparticles may reduce interfacial tension and alter wettability conditions during enhanced oil recovery processes, these mechanisms were not directly measured in the present study and are therefore interpreted as possible contributing factors rather than confirmed mechanisms.

Oil recovery.

The oil recovery as a function of cumulative fluid injection in pore volume (PV) is presented in Fig. 4 for both CO₂ brine and CO₂ nanoparticle in co-injection experiments. In both cases, oil recovery increases rapidly during the early stage of injection as the injected fluids displace oil from the larger pore channels. This stage corresponds to the dominant displacement of mobile oil within the porous network.

As the injected pore volume increases to approximately 0.5–1.0 PV, the rate of oil recovery gradually decreases. This behavior indicates that most of the easily movable oil has already been displaced, and the remaining oil is trapped within smaller pores, pore throats, and isolated clusters due to capillary forces. Consequently, the recovery curves approach a plateau as the flooding progresses.

A clear difference between the two flooding scenarios is observed throughout the injection process. The CO₂ nanoparticle co-injection consistently demonstrates higher oil recovery compared with the CO₂ brine flooding. The presence of nanoparticles enhances microscopic displacement efficiency by improving oil mobilization and reducing residual oil trapping within the pore structure. At approximately 1 PV of injection, the CO₂ nanoparticle flooding achieves a final recovery of about 85%, while the CO₂ brine flooding reaches approximately 80%.

The improved recovery performance can be attributed to the ability of nanoparticles to enhance microscopic displacement efficiency. The presence of nanoparticles may reduce interfacial tension and alter wettability toward more water-wet conditions, facilitating the detachment and mobilization of trapped oil droplets within the pore network. Consequently, less oil remains trapped in the porous structure, resulting in higher oil recovery. These results demonstrate that incorporating nanoparticles into CO₂ flooding can improve pore-scale displacement efficiency and enhance overall oil recovery performance.

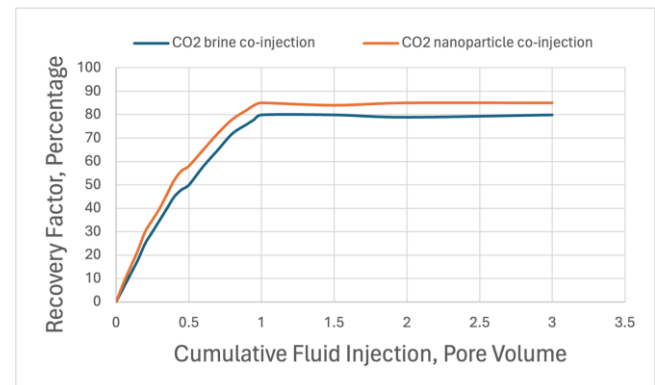


Fig. 4. Recovery factor as a function of cumulative fluid injection during CO₂ injection for co-injection with brine/nanoparticles.

Quantitative analysis of the captured images confirmed the improvement in oil recovery performance. The CO₂ nanoparticle co-injection experiment achieved an oil recovery factor of approximately 85%, representing a 5% increase in recovery compared with the conventional CO₂ flooding case. The additional recovery was primarily

associated with improved displacement of oil trapped within smaller pores and pore throats.

These results demonstrate that the incorporation of nanoparticles in CO₂ flooding can enhance pore-scale displacement efficiency and reduce residual oil saturation within the porous medium. The improved recovery observed in the micromodel experiments indicates the potential of CO₂ nanoparticle flooding as an effective strategy for enhancing oil recovery in subsurface reservoir systems.

XIV. CONCLUSION

This study investigated the pore-scale displacement performance of CO₂ flooding and CO₂ nanoparticle co-injection flooding using a glass micromodel under reservoir-representative conditions. The experiments were conducted at 114 °C and 3000 psi with a constant injection rate of 5 µL/min, enabling direct visualization and quantitative analysis of fluid displacement behavior within the porous structure.

The experimental results show that both flooding methods effectively reduced oil saturation during the early stage of injection. A rapid decline in oil saturation was observed within the first 0.5 pore volume (PV), indicating efficient displacement of mobile oil located in larger pore channels. As the injected pore volume increased, the rate of oil saturation reduction gradually decreased due to the presence of oil trapped within smaller pores and pore throats by capillary forces.

A noticeable improvement in displacement efficiency was observed for the CO₂ nanoparticle co-injection case compared with conventional CO₂ flooding. The residual oil saturation after approximately 1 PV of injection decreased from about 0.20 for CO₂ brine flooding to approximately 0.15 for CO₂ nanoparticle flooding. This reduction in residual oil saturation resulted in an increase in oil recovery.

Quantitative image analysis indicated that CO₂ nanoparticle co-injection achieved a final oil recovery of approximately 85%, representing a 5% improvement compared with the 80% recovery obtained from conventional CO₂ flooding. The enhanced performance is attributed to the ability of silica nanoparticles to improve microscopic displacement efficiency through mechanisms among which interfacial tension reduction, wettability alteration toward more water-wet conditions, and improved mobilization of trapped oil droplets within the pore network.

Overall, the results demonstrate that the incorporation of

nanoparticles into CO₂ flooding can significantly enhance pore-scale displacement efficiency and reduce residual oil saturation. To further validate and better understand the mechanisms responsible for the improved recovery performance, additional experimental studies are recommended. These investigations should include scanning electron microscopy and energy-dispersive spectroscopy (SEM/EDS) to evaluate nanoparticle distribution and surface interactions, core flooding experiments to assess displacement behavior at the core scale, and interfacial tension (IFT) and contact angle measurements to quantify the effects of nanoparticles on fluid–fluid and fluid–rock interactions. Such complementary analyses would provide a more comprehensive understanding of nanoparticle-assisted CO₂ flooding and support its potential application in field-scale enhanced oil recovery operations.

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Visual Greenery and Students Emotional Responses in Campus Environment: Examining Green View Index and Spatial Typologies

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Abstract. The psychological benefits of campus greenery have been widely acknowledged. However, limited research has examined how visible greenery intensity and spatial typology influence students' emotional responses in tropical university contexts. This study investigates the relationship between the Green View Index (GVI) and emotional responses across three campus greenery typologies: open green space, under-canopy vista line, and built-environment greenery integration. A controlled photo-simulation experiment was conducted using nine systematically manipulated campus images with varying GVI levels. A total of 45 university students evaluated the images using a 9-point emotional scale measuring pleasure, arousal, and perceived stress. Quantitative analysis included descriptive statistics, one-way ANOVA, and simple linear regression, complemented by thematic analysis of open-ended responses. The results indicate that higher GVI levels are significantly associated with increased pleasure and reduced perceived stress. However, no statistically significant differences were found across greenery typologies. The regression results indicate that visible greenery explains a moderate effect on pleasure and smaller, but significant, changes in arousal and stress. These findings suggest that perceptual green visibility may play a more substantial role in shaping student's emotional responses than spatial configuration alone. The study contributes empirical evidence to campus landscape design by emphasizing the importance of visible greenery exposure in supporting student well-being within dense tropical urban environments.

Index Terms— Biophilic design, Emotional response, Green View Index (GVI), Student wellness, Tropical university campus, Visual perception

XV. INTRODUCTION

University students experience increasing psychological pressure heightened from academic competition, social expectations, and post-pandemic mental health challenges. As students spend a substantial amount of their daily lives within campus environments, the spatial and visual qualities of these environments play a crucial role in shaping their psychological experiences and overall well-being [1, 2]. University campuses not only serve as a physical setting for educational functions, but also as environmental contexts that can either intensify stress or promote emotional restoration. This is a crucial factor to consider when designing modern campuses or renovating existing campuses, by identifying spatial characteristics that support psychological resilience.

Environmental psychology has conducted extensive research on exposure to natural environments as a mechanism for psychological restoration and emotional regulation. Theoretical frameworks such as Attention Restoration Theory (ART) focus on the cognitive benefits of nature, while Stress Recovery Theory (SRT) emphasizes the

emotional and physiological benefits of exposure to natural settings [3]. Empirical studies have consistently demonstrated that exposure to green environments improves mood, reduces anxiety, and enhances individual's well-being [1, 4]. However, the majority of these studies have been conducted in Western temperate climates, where climatic variations and landscape typologies differ substantially from tropical urban contexts.

In tropical metropolitan contexts such as Bangkok, vegetation is often spatially discontinuous and visually obstructed by high-density built environments. Conventional green space metrics, such as total green area coverage, may not accurately reflect an individual's perceptual exposure to greenery [5]. The Green View Index (GVI) quantifies visible greenery by measuring the proportion of vegetation within visual fields captured at pedestrian eye level [6]. Although GVI has been widely applied in urban environmental studies, there are limited systematic examinations of the relationship between visible greenery and emotional responses which are measured through the Pleasure-Arousal framework, particularly within tropical university campuses using the controlled visual simulation methods. This gap limits the development of evidence-based biophilic design strategies

specifically for dense tropical institutional environments.

Chulalongkorn University, located in central Bangkok, represents a dense tropical urban campus characterized by diverse spatial typologies and various integrations in vegetation. It is essential to understand how variations in eye-level visible greenery influence students' emotional states within this context.

Therefore, this study aims to examine the relationship between the Green View Index and student's emotional responses, specifically evaluating whether higher levels of visible greenery are associated with increased pleasure and arousal and reduced stress. It is hypothesized that greater eye-level greenery exposure will positively correlate with pleasure and arousal while negatively correlating with stress indicators. The study integrates semi-automated image segmentation by combining AI-assisted classification, manual refinement in Adobe Photoshop, and pixel-based quantification using ImageJ to calculate GVI values, alongside subjective emotional assessments collected through the survey instrument. By integrating objective environmental metrics with perceptual emotional responses, this research provides empirical evidence to inform biophilic campus design strategies in tropical urban contexts.

XVI. CONCEPT THEORY FRAMEWORK

2.1 Green View Index and Perceptual Green Exposure

Urban greenery has traditionally been quantified using area-based indices such as the Normalized Difference Vegetation Index (NDVI) or Green Cover Percentage (GCP) [7-9]. While these methods are suitable for macro-scale analysis, these measures do not reflect how individuals visually experience greenery at eye level [10].

The Green View Index (GVI) addresses this limitation by quantifying the proportion of visible vegetative elements within a pedestrian's visible field through image-based pixel analysis [11, 12]. In contrast to measurements derived from aerial or satellite-based imagery, GVI captures the experiential dimensions of greenery through the individual's perception within urban environments. In recent studies, GVI has become increasingly applied to examine associations with walkability, thermal comfort, and subjective environmental perception. However, its direct relationship with emotional responses within educational environments, specifically in tropical urban campuses, remains underexplored [13, 14].

In dense tropical cities such as Bangkok, where vegetation may be spatially dispersed or partially disrupted by built structures, eye-level visual greenery becomes crucial. In this study, GVI is conceptualized as the objective independent variable, representing the intensity of visible greenery within the selected campus environments. This implementation enables direct comparison between environmental conditions and emotional responses.

2.2 Emotional Responses and the Pleasure-Arousal Framework

Emotional responses to environmental stimuli are commonly assessed using the Pleasure-Arousal-Dominance (PAD) model, which conceptualizes affective states based on three

dimensions: pleasure, arousal, and dominance [15, 16]. Pleasure refers to the degree of positive or negative affect experienced by an individual, arousal indicates the level of stimulation or activation, and dominance reflects the sense of control in a particular emotional state.

In architectural and environmental psychology research, the pleasure and arousal dimensions are often employed to evaluate affective responses to spatial environments because they capture the direct experiential qualities of environmental evaluation [17]. However, the dominance dimension is generally considered less directly relevant to visual environmental perception, thus, it is not emphasized in the present study. In existing restorative environmental research, exposure to natural settings is associated with increased pleasure and moderate arousal, while contributing to emotional regulation and stress reduction [15].

In this study, emotional responses primarily focus on the two dimensions of the PAD model; pleasure and arousal dimensions to evaluate student's affective responses to variations in visible greenery. Emotional responses are measured using a 9-point Likert-type scale, in which participants rate their perceived levels of pleasure, arousal, and stress after viewing each simulated campus image. The 9-point scale ranged from low to high level, enabling participants to distinguish variations across the manipulated GVI conditions. The inclusion of stress as an additional evaluation dimensions enables further examination of the potential restorative effects of visible greenery.

The resulting numerical ratings of pleasure, arousal, and stress constitute the subjective dependent variables used in the statistical analysis of the relationship between the Green View Index (GVI) and student's emotional responses.

2.3 Conceptual Framework

According to environmental psychology, this study conceptualizes visible greenery as an environmental stimulus that influences affective evaluation. The proposed framework provides a direct relationship between perceptual green exposure which quantified by using the Green View Index (GVI) and students' emotional responses measured through pleasure, arousal, and stress ratings.

In this framework, GVI functions as the objective independent variable, representing the intensity of eye-level visible greenery within campus environments. Emotional responses serve as subjective dependent variables, reflecting student's emotional evaluations of the simulated greenery conditions.

By integrating objective spatial metrics with subjective psychological assessment, this framework provides an empirical structure for examining how visual environmental

characteristics contribute to students' well-being within a tropical urban university context.

2.4 Research Hypotheses

Based on the proposed conceptual framework, the following hypotheses are formulated:

H1: Higher GVI levels are positively associated with pleasure.

H2: Higher GVI levels are positively associated with arousal.

H3: Higher GVI levels are negatively associated with perceived stress.

XVII. METHODOLOGY

3.1 Research Design

This study employed a mixed-methods design integrating a controlled photo-simulation experiment with thematic analysis of open-ended responses. The quantitative component examined the statistical relationship between the Green View Index (GVI) and students' emotional responses, while the qualitative component provided contextual insights into students' experiential perceptions of campus greenery at Chulalongkorn University.

A controlled image-based simulation approach was adopted to systematically vary the level of visible greenery across the selected campus environments. This design enabled the isolation of Green View Index (GVI) as the primary measurable environmental variable while maintaining consistency in spatial, composition, perspective, and built context in the image stimuli.

The study examined two environmental dimensions:

- (1) greenery typology, representing spatial configuration (categorical variable), and
- (2) GVI, representing perceptual green intensity (continuous variable).

Emotional responses are measured through pleasure, arousal, and perceived stress which served as dependent variables. This design enabled investigation of how students' emotional responses are shaped by both spatial configuration and perceptual greenery level.

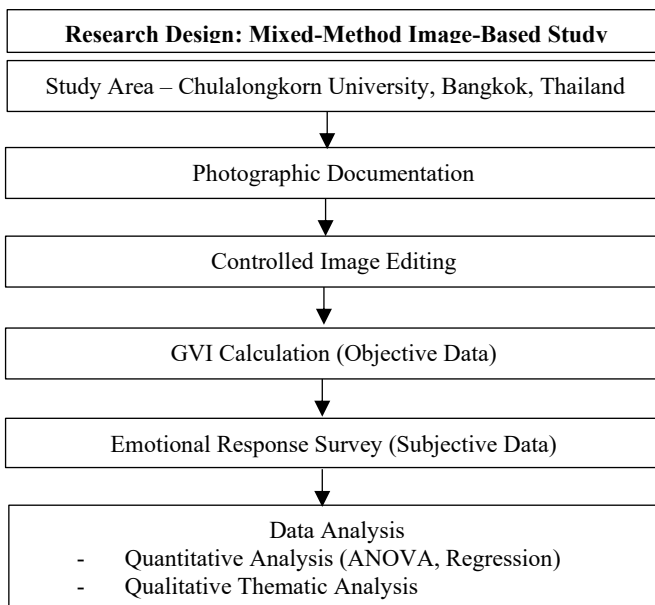


Fig. 1: Flowchart of Methodology

3.2 Study Area and Visual Stimulation Development

The study was conducted at Chulalongkorn University, Bangkok, Thailand. The campus represents a dense tropical urban environment characterized by diverse spatial typologies and varying levels of vegetation integration, making it an ideal context for examining perceptual green exposure.

Three greenery typologies were selected to represent distinct spatial configurations commonly found within dense tropical university settings:

- (1) Open green space
- (2) Under-canopy vista line
- (3) Built environment integrated with vegetation

These typologies were selected to capture variations in spatial enclosures, vegetation distribution, and visual continuity.



Fig. 2: Selected Three Greenery Typologies

3.3 Image Processing and GVI Quantification

Photographic documentation was conducted at a standardized pedestrian eye-level height (approximately 1.5 meters) to simulate pedestrian visual experience. To ensure comparability, lighting, framing, and perspective were controlled during the photographic documentation.

Photographic images manipulation proceeded in two stages. Preliminary vegetation adjustments were conducted using AI-assisted editing to modify greenery elements while preserving overall spatial composition. Subsequently, detailed refinements were completed manually using Adobe Photoshop to maintain visual realism and consistency across image stimulus conditions.

GVI values were calculated using ImageJ software. Each image was converted into binary format to differentiate vegetation pixels from non-green vegetation elements. GVI was computed as the percentage of green pixels relative to total image pixels:

$$GVI (\%) = \left(\frac{\text{Green Pixel Area}}{\text{Total Image Pixel Area}} \right) \times 100\% \quad (1)$$

Based on the calculated GVI values, images were categorized into low, medium, and high visibility conditions for comparative analysis.



Fig. 3: Representative under-canopy vista typology edited images to display low, medium, and high Green View Index (GVI) levels for the photo-simulation experiment

3.4 Participants and Data Collection Procedure

A total of 45 university students voluntarily participated in the study. Participants were recruited through convenience sampling within the Chulalongkorn University campus.

Each participant evaluated on nine edited images of three typologies in three different GVI levels, assessing through a structured questionnaire consisting of three sections:

1. Demographic and green space usage information
2. Emotional responses ratings using a 9-point Likert-type scale
3. Open-ended qualitative responses

Emotional responses were measured using two primary dimensions of the PAD model – pleasure and arousal along with perceived stress with an additional evaluation dimension. Participants rated each image independently, resulting in 405 total observations.

The study adhered to standard ethical guidelines for human subject research. Participants were voluntary, and informed consent was obtained from all respondents, and anonymity and confidentiality were ensured.

3.5 Quantitative Data Analysis

Descriptive statistics were first calculated to summarize the distribution of Green View Index (GVI) values and emotional responses ratings of all the 9 visual stimuli. Means and standard deviations were computed to provide an overview of average and variability.

To examine differences in emotional responses across three greenery typologies, one-way analysis of variance (ANOVA) was conducted for each emotional dimension (pleasure, arousal, and stress). ANOVA was selected to compare mean differences across the three different spatial categories.

To assess the relationship between Green View Index (GVI) and emotional responses, simple linear regression analyses were performed. Separate regression models were constructed for each dimension, with GVI as the independent variable and emotional ratings as dependent variables. Although measured using a 9-point Likert scale, emotional responses were carried out as a continuous variable for statistical analysis.

3.6 Qualitative Data Analysis

Open-ended responses were analyzed using an inductive thematic analysis approach. All responses were reviewed and manually coded using Microsoft Excel.

Initial codes were identified based on recurring words, phrases, and expressed perceptions. These codes were then

systematically grouped into subthemes and then thematic categories through iterative comparison and refinement.

The coding process focused on identifying patterns related to emotional reactions, perceived environmental qualities, and restorative experiences associated with varying levels of visible greenery within the campus. Representative participant quotations were selected to illustrate key themes and to maintain transparency in interpretation.

The qualitative findings were used to support and contextualize the quantitative results, providing deeper insights into how perceptual green exposure influences student's emotional experiences within the campus environment.

XVIII. RESULTS AND DISCUSSION

4.1 Emotional Responses Across Greenery Typologies

Descriptive statistics suggest minor variation in emotional responses across the three selected greenery typologies: open green space, under-canopy vista line, and built environment integrated with vegetation. Open green spaces exhibited slightly higher mean pleasure scores and slightly lower perceived stress levels compared to the other typologies. Under-canopy vista lines demonstrated comparable emotional responses, while built-integrated environments showed similar average values across the three affective dimensions. [Table 1] presents the mean and standard deviation values for pleasure, arousal, and stress across the typologies.

To determine whether these observed differences were statistically significant, one-way ANOVA analyses were conducted for each emotional dimension. The results indicated that typology did not have a statistically significant effect on pleasure, $F(2,402) = 0.94$, $p = 0.393$, arousal, $F(2, 402) = 0.67$, $p = 0.513$, or stress, $F(2, 402) = 0.24$, $p = 0.790$.

Although insignificant variations in mean scores are observable in the descriptive results and illustrated in [Figure 4], these differences were not statistically significant. The distribution of emotional responses across typologies demonstrates considerable overlap, indicating that spatial configuration alone does not substantially influence students' emotional evaluations.

Overall, while open green spaces appear descriptively favorable, the absence of statistically significant differences suggests that typological form may be less influential than other environmental factors, such as the overall intensity of visible greenery.

Table 1: MEAN (SD) EMOTIONAL SCORES BY IMAGE STIMULI
AND GVI LEVELS

Image	GVI Values	Mean		
		Pleasure (M ± SD)	Arousal (M ± SD)	Stress (M ± SD)
	13	4.40 (1.88)	3.82 (1.89)	4.91 (2.08)
	34	5.58 (1.47)	4.69 (1.52)	4.11 (1.63)
	77	6.10 (1.44)	5.16 (1.51)	3.49 (1.41)
	8	3.50 (1.55)	3.18 (1.51)	5.49 (2.06)
	32	4.93 (1.23)	4.31 (1.40)	4.51 (1.65)
	56	6.76 (1.26)	5.51 (1.85)	2.93 (1.37)
	2	3.53 (1.80)	3.31 (1.76)	5.47 (2.04)
	27	5.16 (1.62)	4.24 (1.61)	4.13 (1.60)
	52	6.84 (1.43)	5.42 (1.98)	2.91 (1.50)

4.2 Relationship between GVI and Emotional Responses

Although spatial typology did not demonstrate statistically significant differences in emotional responses, the Green View Index (GVI) was examined as a numerical variable to determine whether variations in visible greenery levels were associated with emotional responses. GVI quantifies the proportion of visible greenery within the visual fields. To examine whether variations in visible greenery were associated with emotional responses, simpler linear regression analyses were conducted using participant-level data (n = 405).

As shown in [Table 2], GVI significantly predicted pleasure ratings, $F(1, 403) = 172.17$, $p < 0.001$, with an R^2 value of 0.299. This indicates that approximately 29.9% of the variance in pleasure scores can be explained by differences in visible greenery intensity. The positive regression coefficient ($B = 0.045$) suggests that higher levels of eye-level greenery are associated with increased pleasure.

Similarly, GVI significantly predicted arousal, $F(1, 403) = 77.62$, $p < 0.001$, with R^2 value of 0.161. This reflects a moderate positive association between greenery visibility and activation levels. Despite a lower R^2 value relative to pleasure, the regression model indicates a statistically significant relation between GVI and arousal.

In contrast, GVI showed a significant negative association with perceived stress, $F(1, 403) = 87.02$, $p < 0.001$, with an R^2 value of 0.178. The negative regression coefficient ($B = -0.035$) indicates that higher levels of visible greenery correspond to lower stress ratings.

Linear relationships are presented in [Figure 5], which demonstrates the regression trends between GVI and each emotional dimension. The relatively stronger effect observed for pleasure suggests that positive effects may be more influenced by variations in greenery visibility than arousal or stress.

Although all regression models reached statistical significance, the R^2 values indicate that GVI explains a moderate proportion of variance in pleasure and a comparatively smaller proportion in arousal and stress. These findings suggest that while visible greenery contributes meaningfully to emotional responses, additional environmental, contextual, and individual factors may also influence students' emotional experiences. Factors such as previous stress levels, familiarity with specific campus settings, microclimatic conditions, and social context may further shape emotional perception and should be considered in future research.

Overall, the findings indicate that the intensity of visible greenery plays a more substantial role in shaping emotional responses than only spatial typology, emphasizing the importance of perceptual green exposure in campus design.

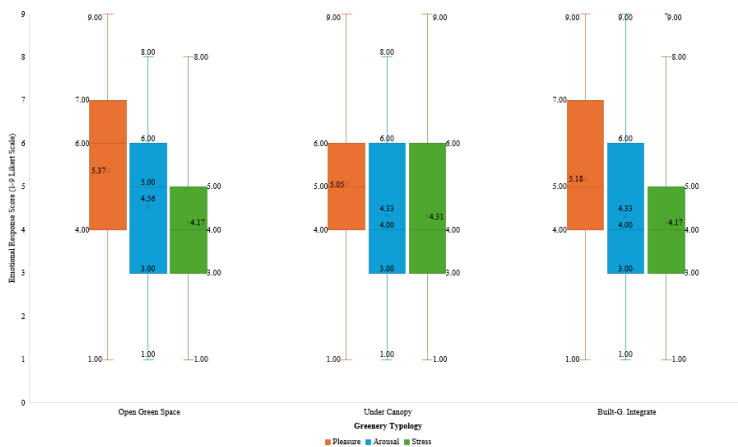


Fig. 4: Distribution of emotional responses across greenery typologies

TABLE 2: LINEAR REGRESSION RESULTS - EFFECT OF GVI ON EMOTIONAL RESPONSES (n = 405)

Dependent Variable	B (slope)	SE	β	R ²	F	p
Pleasure	0.045	0.003	0.547	0.299	172.17	< .001
Arousal	0.032	0.004	0.402	0.161	77.62	< .001
Stress	-0.035	0.004	-0.421	0.178	87.02	< .001

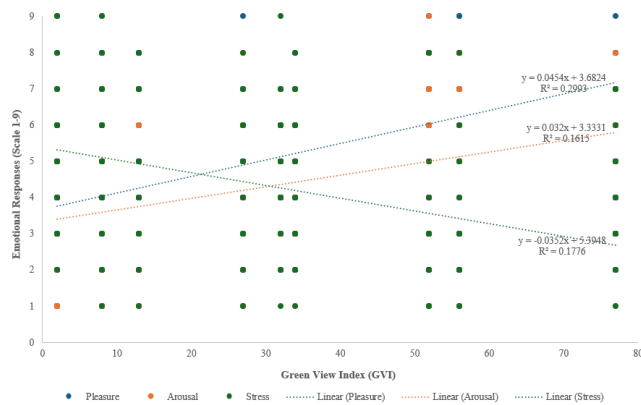


Fig. 5: Relationship between GVI and Emotional Dimensions

4.3 Thematic Analysis of Open-Ended Responses

Thematic analysis of the participant's open-ended responses was conducted to complement the qualitative findings. This analysis aimed to explore how students subjectively interpret and emotionally frame different campus environments.

As summarized in [Table 3], the analysis identified four main themes: (1) Psychological Restoration, (2) Positive Emotional Enhancement, (3) Nature Connection & Environmental Aesthetics, and (4) Environmental Comfort & Engagement.

The first three themes align closely with established environmental psychology literature. Participants frequently described green environments as calming, refreshing, and mentally restorative, reflecting principles consistent with restorative environments theories. Subthemes such as emotional recovery, cognitive clarity, and stress reduction reinforce previous findings that exposure to visible greenery enhances affective well-being and psychological restoration.

In addition to these established themes, an emergent theme of "Environmental Comfort & Engagement" was identified. Participants indicated that greenery environments not only reduce stress but also enhanced social interaction, spatial liveliness, and a sense of belonging. This suggests that visible greenery contributes not only to emotional restoration but also to experiential activation within campus spaces.

The qualitative findings therefore strengthen and extend the quantitative results. Thematic responses show how students cognitively perceive these environments as restorative, relaxing, and stimulating, while statistical analysis shows quantifiable correlations between exposure to greenery and emotional dimensions.

TABLE 3: SUMMARY OF IDENTIFIED THEMES FROM OPEN-ENDED RESPONSES

Themes	Representative Initials	Participant's Comments
Psychological Restoration	stress reduction, anxiety relief, calming, refreshment	"It helps me relieve my stress and makes me feel relax"
Positive Emotional Enhancement	mood improve, mood uplift, mood refresh	"My mood is uplifted from tiring day after walking pass greenery"
Nature Connection & Environmental Aesthetics	visual preference, aesthetic enjoyment, natural connection, natural break	"It is beautiful to look at and also felt near with nature while walking pass."
Environmental Comfort & Engagement	thermal comfort, need for privacy, outdoor engagement, physical activity, socialization	"Provided me shades from sun and enjoy physical activity" "Greenery provides a good level of privacy"

4.4 Discussion

The findings of this study indicate that the level of visible greenery plays a more substantial role in shaping student's emotional responses than spatial typology alone. While descriptive statistics indicated minor variations across greenery typologies, the one-way ANOVA results did not reveal statistically significant differences in pleasure, arousal, or stress among open green space, under-canopy environment, and built-integrated greenery settings. This suggests that spatial configuration independently may not be a determining factor in emotional evaluation under controlled visual conditions.

In contrast, the regression analyses demonstrated that variations in the Green View Index (GVI) were significantly associated with the differences in emotional responses. Higher levels of visible greenery were significantly related to increased pleasure and reduced perceived stress. The regression models indicated that GVI explained a moderate proportion of variance in pleasure, and smaller but statistically significant, proportions in arousal and stress. These findings suggest that the proportion of greenery visible at eye level may exert a more influential role in shaping emotional responses than the categorical classification of spatial typology.

These findings are consistent with established theories in environmental psychology, including Attention Restoration Theory and Stress Reduction Theory, which emphasize the importance of visual engagement with natural elements in promoting psychological restoration and reducing stress. The results of this study extend existing literature by demonstrating that measurable variations in visible greenery levels are associated with differences in emotional response within a campus context.

Although the regression models were statistically significant, the R² values indicate that visible greenery explains only one component of the variation in emotional responses. This suggests that, while GVI is an important factor, other environmental and individual variables may also contribute to student's emotional experiences. Factors

such as spatial characteristics, environmental comfort, social context, and individual psychological states may further influence emotional responses. Future research could incorporate these variables to provide a more comprehensive understanding of emotional experiences in campus environments.

Overall, the findings suggest that enhancing the perceptual visibility of greenery may be an important consideration in campus landscape design. In contrast to approaches that rely only on spatial typology, the results indicate that eye-level exposure to greenery within everyday campus environments may be more closely associated with students' emotional responses. The visual conditions examined in this study, including open green spaces, under-canopy vista environments, and built-integrated greenery, indicate that increased visible green exposure may be associated with more positive emotional responses. However, these findings may be limited due to the use of simulated images and a relatively small sample size.

From the design perspective, these findings suggest important implications for campus landscape strategies. This suggests that design strategies should primarily focus on increasing visible greenery at pedestrian eye level, rather than relying only on spatial typology. This can be achieved through interventions such as incorporating street trees, shaded walkways, vertical greenery, and layered planting along the circulation spaces which are frequently used. Thus, these approaches can have more effective contributions to improve students' emotional well-being in tropical urban campus settings.

4.5 Limitations and Future Study

There are several limitations that should be acknowledged. First, the sample size was relatively limited, which may restrict the generalizability of the findings beyond the study context. Future research should include larger and more diverse participant groups to enhance the strength and external validity of the results.

Second, the study relied on photo-simulated images to represent campus environments. Although this approach allows for controlled visual comparison, it may not fully replicate real environmental immersion. Future research may incorporate virtual reality simulations or on-site field experiments to enhance environmental validity.

Third, although Green View Index significantly predicted emotional responses, the relatively moderate and low R^2 values indicate that additional variables such as individual stress levels, familiarity with campus spaces, microclimatic conditions, and social context may also influence emotional perception. Future research should integrate multiple environmental and psychological factors into more comprehensive predictive models.

Finally, further investigation is needed to clarify whether spatial typology independently influences emotional responses beyond greenery visibility intensity.

XIX. CONCLUSION

This study examined the relationship between visible greenery and a students' emotional responses within a

tropical university campus. The findings indicate that higher Green View Index (GVI) levels are significantly associated with increased pleasure and reduced perceived stress. Visible greenery also showed a positive relationship with arousal, although the effect was smaller.

In contrast, no statistically significant differences were found across the three spatial typologies. This suggests that the intensity of visible greenery may play a more important role in shaping emotional responses than spatial configuration alone.

By integrating objective GVI measurement with subjective emotional evaluation, this study provides empirical validation for the importance of perceptual greenery exposure in campus design. The findings highlight that enhancing visible greenery at eye level may contribute to enhance students' emotional well-being, specifically in dense tropical urban environments.

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Indoor Air Quality and Thermal Comfort Improvement of Naturally Ventilated Deep Plan Sport Facility: A Case Study of Combat Sport Facility in Chulalongkorn University Sports Complex

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Abstract. With concern for Indoor Air Quality (IAQ), harmful impacts of naturally ventilated indoor environments on high intensity sports demand close attention. To improve the conditions, the relations between airflow, air velocity and IAQ need to be investigated. This study examines these relations and parameter standards of air movement, air change rate (ACH), thermal sensation, and comfort and experiments in mechanical fan configurations as independent variables within selected case study, a Combat Sports Facility in CU Sports Complex, where excessive plan depth restricts cross ventilation. Its ventilation is assessed, evaluated and find a gap for improvement. Computational Fluid Dynamics (CFD) simulations are conducted integrating KRUGER induced jet fans in 6 different configurations. Findings in this study suggest ways to determine required parameters of the indoor airflow characteristics like indoor airflow pattern, air velocity, and ACH for comfort. Results show that adding fans generally improve airflow distribution and indoor air velocity throughout the space but does not impact inlet velocities and therefore, does not improve ACH, needing further tests with opening variations as independent variables. Working with CFD simulation, this study also contributes to the study structure of IAQ and ventilation via CFD simulation.

Index Terms—Deep Plan Cross Ventilation, Indoor Sports Facility, Indoor Air Movement, Air Flow, Thermal Comfort.

XX. INTRODUCTION

With the increasing interest in Indoor Air Quality (IAQ) due to almost 90% of the day being spent in indoor environments, low air exchange between indoor and outdoor gained more awareness since 1970s [1]. Although sport and active lifestyles are important, indoor environmental conditions have influences on sport practices, with complaints ranging from poor ventilation causing stuffiness to overheating in summer and cold draughts in winter [2]. One impact of indoor environment upon sports is that, as physical exercises cause rapid respiratory rate, the metabolic response exposes the body to 4-8 times greater inhalation of air and particles compared to resting body as well as to higher body temperature [3].

Study by Zhang, Ortiz & Bluysen (2023), mentions how requirements for air velocity, air temperature and relative humidity in the playing area in arena are usually stricter than other areas due to influence on athletes' performance. Thus, it is noticeable that the indoor air quality, air movement and

outdoor air exchange are important for indoor physical exercises and sport practices both for practitioners' health and performance. That is why the ventilation and air recirculation of the indoor fitness environments, especially with high intensity trainings, should be investigated.

Indoor Combat Sports room on 3rd floor of CU sports complex, Figure 1, is the case study to investigate for this research. This room is used by Chula students for group practices like Boxing, Muay Thai, JiuJitsu, Aikido, Fencing and Thai Martial Arts. While joining the JiuJitsu club's practice in busy Wednesday evenings with all different practices, the room air felt thick, stuffy and stale despite the direct elevated air from portable fans around the practice areas. From preliminary survey, this room with a deep plan seems to lack adequate air flow around the room and thermal comfort during practices.

The room currently is relying mainly on openings for natural ventilation and since it is not performing well enough due to the long distance between the opposite windows, portable fans are used when all practice areas are busy. Thus, what type of fans could be used and how could they be placed

in the cross ventilated deep-plan room to distribute the air flow better into the desired area where the air might be stale for the comfort benefit of occupants with intense practices? Can fans be rearranged and used to manage airflow velocity in and out of the room? The IAQ of the room is aimed to be improved through air movement.



Fig. 1: Interior of Combat Sports Facility on 3rd Floor of CU Sports Complex

Previous studies done on this issue focused on monitoring, experimenting, investigating, evaluating, comparing thermal comfort, IAQ, air movement and speed during exercises and creating assessments and prediction models. Moreover, studies were conducted in air-conditioned rooms, climate-controlled chambers, rooms with shallow depth or without natural ventilation as main ventilation system. These settings are different from the room examined in this research.

Furthermore, previous studies mention sports halls or gymnasiums without specifically describing what kind of sports or activities they hold, resulting in either different or uncertain range of considered metabolic rates compared to combat sports considered in this research. There was no research focusing on intervention to improve IAQ through air movement and speed, of a naturally ventilated indoor combat sports hall with openings on distant opposite walls. The objective of this study is to investigate and study relationship between air movement and indoor air quality parameters like airflow and thermal comfort in the deep-plan natural ventilated room and to understand how a semi mechanical ventilation impact air movement, improving IAQ in indoor high intensity sports facility.

XXI. LITERATURE REVIEW

As indoor air quality and thermal comfort have impact on both health and performance of the practitioners, indoor comfort in high intensity sports facilities is crucial. According to Fantozzi and Lamberti (2019), the indoor comfort can be ensured through environmental control like Indoor Air Quality (IAQ), Thermal Comfort, Lighting, and Acoustical Quality. Thus, IAQ, Air Movement, Air Velocity, Thermal Sensation and Air Change Rate (ACH) will be the basic terminologies of ventilation requirements in sport facilities to be studied together with their standards.

2.1. Indoor Air Quality (IAQ)

Indoor air quality has impact on health of the athletes, stated by Fantozzi and Lamberti (2019), due to the particles in air and its breathability. High concentration of particulate matters in fitness centers and gyms occurs during classes which suggests the pollutants concentration being partly influenced by the number of occupants and the intensity of performed exercises. In conclusion, physical activities

contribute to constant particle resuspension, further leading to stuffy and polluted indoor environment indicating inadequate air exchange rates, potentially signaling the concurrent buildup of pollutants. [1]

2.2. Air Movement & Air Speed

Adequate air movement at higher temperatures results in equal or better comfort and perceived air quality (PAQ) compared to lower temperatures with less air movement. Investigation by Toftum and Nielsen (1996) and investigation by Griefahn et al. (2001) on thermal comfort, thermal sensation, and air movement perception for various metabolic rates both depict that during higher intensity activities, elevated air movement is perceived as pleasant when the body is warm.

The positive impact of air movement for subject's thermal comfort comes due to the convective and evaporative heat loss through elevated air speeds, offsetting the heat gain caused by increased MET. Without air movement, PAQ also decreases significantly once MET increases due to higher bio contaminants generation and resuspension. [4] Moreover, as mentioned by ASHRAE (2021), air quality can indeed be improved by fan flows above 0.3 m/s disrupting the body's thermal plume that carries pollutants upward to the breathing zone. Therefore, air movement is important both for cooling occupants and dispersing polluted air. [5] [6]

As increased air movement increases the comfortable temperature for people, elevated air speed acts as a major tool in comfort for most naturally conditioned spaces. [7] According to the study by Jones et al. (1986), at same MET level, for subjects with different clothing, the elevated air speed improves thermal sensation at higher temperatures for all subjects. Again, from the research by Zhai et al. (2015), subjects at 6 MET and 20-26 °C preferred air speeds up to 2.3 m/s, indicating people at higher MET level accept and prefer higher air speeds than sedentary people at lower temperatures according to ASHRAE Standard 55. This is supported even more by the note by Khedari et al. (1999) on how increased air velocity reduces thermal response time.

2.3. Thermal Sensation & Comfort

With a combat sports facility as a study setting, evaluation of the thermal environment must be considered. Warm and humid environments are critical for athletes, disturbing their thermoregulatory system from proper operating. High room temperature prevents heat transfer to the environment and high humidity restricts sweat evaporation [8].

Cooler thermal sensation during the high metabolic rate activities is necessary for comfort than during low metabolic rate activities as stated in the study by Mihara et al. (2019). The relation between MET Level and thermal sensation can be seen in the study by Zhai et al. (2015) where subjects cycle at three different MET levels. Thermal sensation result tells that when the intensity of activity and environment temperature rise, air movement helps reduce the sensation of rising heat. For aforementioned relations, just like air

movement, air speeds above 0.3 m/s also increase acceptable operative temperature limits if the indoor operative temperature is over 25°C as seen in Table 1. [9]

TABLE I

INCREASES IN ACCEPTABLE OPERATIVE TEMPERATURE
LIMITS IN OCCUPANT-CONTROLLED NATURALLY
CONDITIONED SPACES WITH INCREASING AIR SPEED [9]

Average Air Speed V_a		
0.6 m/s	0.9 m/s	1.2 m/s
1.2 °C	1.8 °C	2.2 °C

2.4. Air Change per Hour (ACH) or Air Change Rate

The most used indicator for assessing and affecting IAQ in ventilation is the exchange of indoor air with outdoor fresh air. [10] In halls and enclosed public zones, room air needs to be sufficiently exchanged with fresh air. This is more important in sports spaces, with rapid respiratory process happening. Although fresh outdoor air can be taken in through windows, calm wind conditions isn't enough for improving thermal sensation and IAQ. According to the paper by Bayoumi (2017), to achieve comfortable indoor environment, saturated air must be removed and if wind is calm, active air circulation system like fans must be applied. Room air exchange is measured by Air Change per Hour or ACH which is the value of inlet opening area, multiplied by inlet air velocity, multiplied by 3600s and divided by room volume as in (1).

$$ACH = \frac{(Inlet\ Opening\ Area\ m^2 * Inlet\ Air\ Velocity\ m/s) * 3600s}{Room\ Volume\ m^3} \quad (1)$$

2.5. Standard ACH and Metabolic Rates

According to the list by Robertson (2024) of ACH requirements for different spaces adapted from ASHRAE 62.1. (2022), and the list of dose-related IAQ indicators for sports facilities including their required values gathered by Zhang, Ortiz & Bluysen (2023), ACH value of 10, suggested for most intense activities, will be taken as target minimum value for this research's case study space.

During sport activities, a metabolic heat production takes place due to muscular strain and it's assessed as metabolic rate (MET). This is about 58 W/m² and is called 1 MET. [11] Therefore, as high metabolic rate produces heat, and releases heat outward, once the room temperature catches up with the body temperature, the body just stays heated up with nowhere to release the body heat [8]. This issue is more severe in combat sports considering the MET values listed by Compendium of Physical Activities (Table 2) selected from study by Ainsworth (2024).

TABLE II

METABOLIC RATES IN MET FOR DIFFERENT INTENSITY OF
COMBAT SPORT PRACTICES ACCORDING TO COMPETITION
OF PHYSICAL ACTIVITIES (CPA) ADAPTED FROM [12]

Adult Compendium of Physical Activities (CPA)	
Metabolic Rate in MET	Combat Sport Practices of Different Intensity
12.3	Boxing, in ring, general
7.0	Boxing, punching bag, 60b/ min
9.8	Fencing, competitive
6.0	Fencing, general
10.3	Martial Arts, different types, moderate pace (eg., Judo, Jujitsu, Karate)
5.3	Martial Arts, different types, slow pace, novice performers, practice

2.6. Standard Environmental Parameters for Sports

The international comfort standards, such as ASHRAE 55 and ISO 7730, are mainly for sedentary activities as in offices. In the absence of standards for sports facilities, Sport Federations provide more diverse standards of environmental parameters, such as maximum air velocity, air temperature and relative humidity, to be maintained in active areas for more specific sports but certain combat sports like boxing, fencing, etc. still lack the values, as observed in Table 3. [8]

TABLE III

ENVIRONMENTAL PARAMETERS TO BE MAINTAINED IN
THE ACTIVE AREA OF DIFFERENT SPORTS GIVEN BY
INTERNATIONAL OLYMPIC COMMITTEE (IOC) [8]

Federation	Air temperature (°C)	Relative Humidity (%)	Maximum Air Velocity (m/s)
Aquatics (FINA) [74]	2 °C higher than water temperature (water temperature 25–28 °C)	-	-
Badminton (BWF) [75]	18–30	-	<0.2
Basketball (FIBA) [76]	16–20	<50	-
Boxing (AIBA)	-	-	-
Curling (WCF) [77]	6–7	controlled	No constant air movement
Fencing (FIE)	-	-	-
Gymnastics (IFG) [78]	Humidex = 22–38	-	-
Handball (IHF) [79]	15–22 (heated halls) 18–24 (cooled halls)	-	<1
Ice Hockey (IIHF) [80]	6	<70	-
Ice skating (ISU) [80]	6–12	<70	-
Judo (IJF) [81]	17–26	30–40	-
Table tennis (ITTF) [82]	12–25	-	<0.1
Taekwondo (WT)	-	-	-
Tennis (ITF) [83]	13–17 (winter) 6–8 below the external temperature (summer) >10	55–60	-
Volleyball (FIVB) [84]	16–15 (for official competitions)	-	-
Weightlifting (IWF)	-	-	-
Wrestling (UWW) [85]	18–22	-	-

Defined maximum air velocities to be maintained in some sports are due to the concern on the disturbance to the way one sport is performed, like high air velocity disturbing the flight of shuttlecock in badminton. This concern does not apply on combat sports since they normally don't involve any form of equipment or performance that could be visibly and physically disturbed immediately by the speedy air movement. This, on the other hand, relieves the concern about potential disturbance of elevated air movement and velocity upon combat sport performances.

Another study by Roghanchi et al. (2017), about air flow velocity's effect on thermal comfort of underground mine workers, found that for metabolic rates from 3.45 to 5.86 MET at under 80% relative humidity, air velocity of 1 m/s to 2 m/s provides thermal comfort. Beyond 2 m/s, an air velocity increase negatively affects thermal comfort with respect to the sweat rate of the worker. For example, at relative humidity of 80% and based on maximum skin wetness, a 1.5 m/s air flow provides comfort for air temperature up to 30.88 °C, but the maximum allowable temperature drops to 29.82 °C at 4 m/s. However, this velocity range of 1-2 m/s cannot be taken as maximum allowable values for the case in this research as the discomfort comes from impact of dust particles moved by air flow in underground mine and the upper metabolic rate of 5.8 MET is low compared to the highest MET activity listed in Table 2. Therefore, it can be assumed that the air velocity for combat sports should be at least 1 m/s for comfort and could be over 2 m/s without no disturbance or discomfort as long as the relative humidity is kept under 80%

While some of these reviewed papers conducted literature overviews, equation solving and developing, some investigated the theories and hypotheses through climate chamber experiments, questionnaire surveys, field studies plus measurements and performed CFD simulations and wind tunnel experiments to observe and verify the results on the issue of interest. The gaps here are the lacking of experiments on a high intensity sports building with the length longer than five times the ceiling height, and lacking of intervention to improve IAQ and thermal comfort through wind-induced cross ventilation in such buildings with high intensity activities inside.

XXII. METHODOLOGY

The method starts with the literature review, studying and determining the interested variables to learn the standards and relations between indoor physical exercise spaces, natural ventilation and thermal comfort and to experiment. Another step is to document and evaluate the selected case setting with determined variables and required standards so that problems can be identified that further help determine the independent variables to test. Then the base model of selected case setting for CFD simulation is created, with existing boundary conditions, and tested to validate the software. Next step is to test experimental models and to assess whether the conditions satisfy the desirable standards for parameters like required ACH value, target indoor air velocity at measured points and airflow distribution with least stagnant zones within occupiable area on breathing planes. If the simulation results do not satisfy, there comes problem identification and solution determination to further define gap and suggestion for future research. Based on the simulation outcomes and suggestions defined, the discussion and conclusion will be composed.

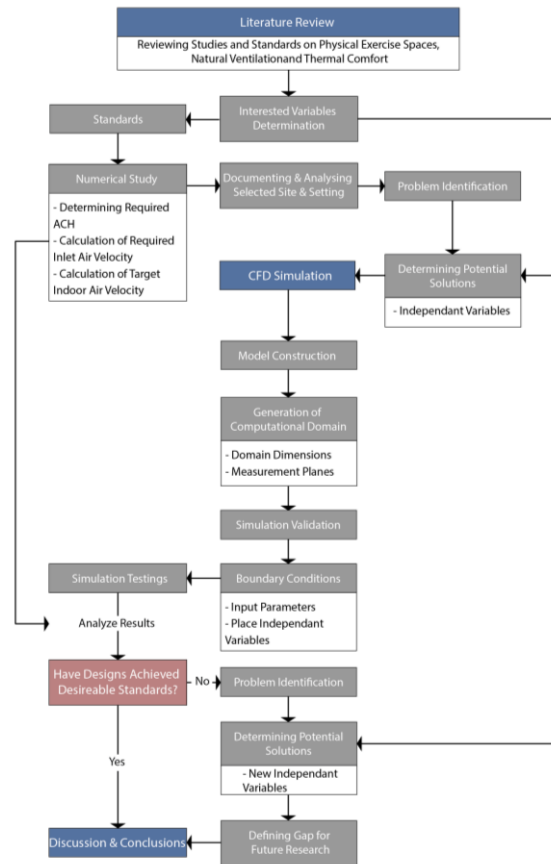


Fig. 2: Flowchart of the Study

3.1. Site & Setting

The Combat Sports room, Figure 3, of the CU sports complex is a case study for this research. The room is 36 m wide, 36 m long and 3.6 m high with 1 m high Boxing ring, 0.3 m elevated pitch for Jiu-jitsu and Aikido, Fencing mat strips and one not-assigned foam pitch. It operates with cross ventilation through windows on East-Southeast and West-Northwest walls with standing fans near practice areas.

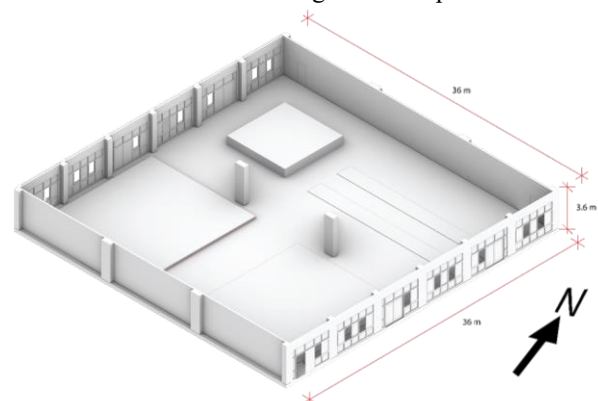


Fig. 3: Isometric Model of Selected Combat Sports Room on CU Sports Complex 3rd Floor

As observed from statement by Chu and Chiang (2014) and ASHRAE (2022) of how the building length should be less than five times the ceiling height for effective wind-

driven cross ventilation, for a room with 3.6 m ceiling height and 36 m distance between opposite windows, the natural ventilation of the room is preliminarily not adequate in terms of indoor air movement and air exchange with potential low velocity sluggish zones. The layout and dimensions of each opening types are as seen in Figure 5.

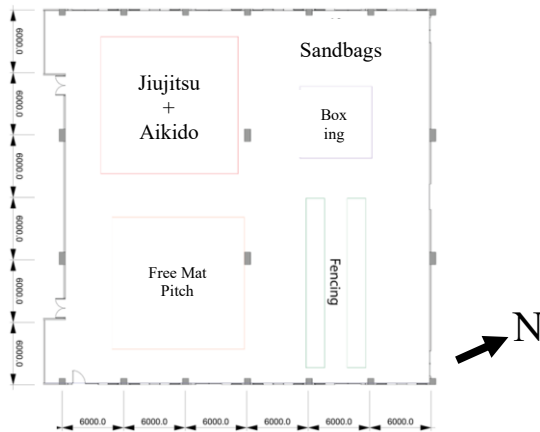


Fig. 4: Plan of the Selected Room to Test

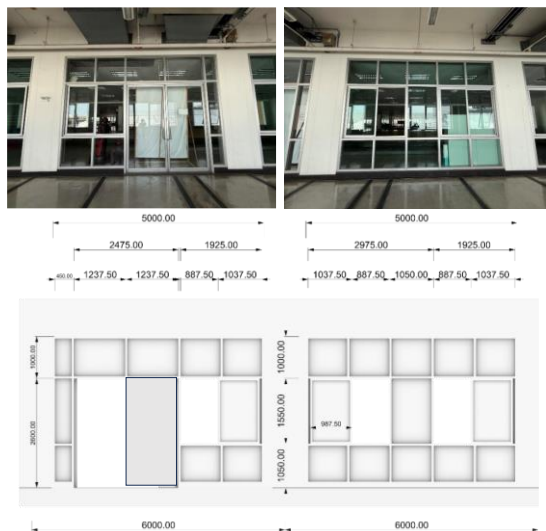


Fig. 5: Photos and Dimensions of Existing Openings on Two Types of Panels Between Columns

3.2. Dependent & Independent Variables

In 1962, Macpherson defined six factors affecting thermal comfort: four environmental variables (air temperature, air velocity, relative humidity, mean radiant temperature), and two personal variables (clothing insulation and activity level, or MET) [13] [14]. Among them, Air Temperature, in this study, is harder to maintain or adapt than Air Velocity due to changing MET of occupants and uncontrollable temperature of natural wind. Since the selected room is not a controlled climate chamber, thermal sensation can be more suitable to manipulate through air velocity.

This research focuses on Indoor Air Distribution/ Airflow Pattern, Air Exchange and Indoor Air Velocity for Thermal Sensation mainly. The personal variables will be considered average constants, if needed, since the design solutions will

be configurations of mechanical fans. Thus, the indexes to be examined are characteristics of air flow such as indoor air movement and distribution for improving stale air areas, ACH for contaminant dispersion and air velocity for better thermal sensation and comfort. How can these factors be controlled in a cross ventilated, non-conditioned room?

The design approach for this research will be a mixed mode in which mechanical fans will be used in selected setting to observe data from simulations. The independent variable to test whether it can improve the existing ventilation in any of dependent variables will be different configurations of fans with an intention to direct high velocity air efficiently into sluggish areas and benefit thermal sensation while drawing more air through the inlet openings which in turn increases the ACH.

TABLE V
DEPENDENT AND INDEPENDENT VARIABLES TO TEST AND ANALYZE

Dependent Variables	Independent Variables
Airflow Pattern (Distribution) mainly over the practice areas	6 Configurations of Mechanical Fans (Table 6)
Air Change Per Hour (ACH)	
- Inlet Air Velocity	
Indoor Air Velocity at Measure Grid Points within Used Areas (Figure 8)	- Positions
	- Amount

3.3. Required Inlet Air Velocity from Air Change Rate

Having the volume of 36 m x 36 m x 3.6 m, with the target ACH value of 10 according to the ASHRAE 62.1 as one of the indexes from (2), required inlet air velocity (V_{ia}) for the selected room is derived as follows:

$$Q = V_{ia} * A \quad (2)$$

$$ACH = \frac{3600s}{hr} * \frac{Q}{Room Volume} \quad (3)$$

$$\frac{10}{hr} = \frac{3600s}{hr} * \frac{V_{ia} * A}{(36 * 36 * 3.6)m^3}$$

$$\frac{10}{hr} * \frac{hr}{3600s} * \frac{4665.6 m^3}{A} = V_{ia}$$

$$\frac{12.96 m^3}{17.17 m^2s} = V_{ia}$$

$$V_{ia} = 0.76 m/s$$

Therefore, to achieve ACH of 10 with the existing openings, average inlet velocity needs to be at least 0.76 m/s.

3.4. Target Air Velocity from Required Outdoor Airflow in Breathing Zone

The outdoor airflow required in the breathing zone of the occupiable space (V_{bz}) shall not be less than the value determined by Eq (4) in accordance with Table 6 as follows:

$$V_{bz} = R_p \times P_z + R_a \times A_z \quad (4)$$

where,

A_z = net occupiable floor area of the ventilation zone (m^2),

P_z = number of people in the ventilation zone during use

TABLE VI
MINIMUM VENTILATION RATES IN BREATHING ZONE [15]

Occupancy Category	People Outdoor Air Rate R_p		Area Outdoor Air Rate R_a		Default Values Occupant Density		Air Class	OS (6.2.6.1.4)
	cfm/person	L/s/person	cfm/ft ²	L/s/m ²	#/1000 ft ² or #/100 m ²			
Sports and Entertainment								
Bowling alley (seating)	10	5	0.12	0.6	40	1		
Disco/dance floors	20	10	0.06	0.3	100	2		✓
Gambling casinos	7.5	3.8	0.18	0.9	120	1		
Game arcades	7.5	3.8	0.18	0.9	20	1		
Gym, sports arena (play area)	20	10	0.18	0.9	7	2		
Health club/aerobics room	20	10	0.06	0.3	40	2		
Health club/weight rooms	20	10	0.06	0.3	10	2		
Spectator areas	7.5	3.8	0.06	0.3	150	1		✓
Stages, studios	10	5	0.06	0.3	70	1		✓
Swimming (pool and deck)	—	—	0.48	2.4	—	2		

Inserting values for gym (sports arena) from Table 6 and field survey value of 36 x 36 m² (13950 ft²) for A_z , 100 person for P_z , 20 cfm/person for R_p and 0.18 cfm/ft² for R_a , the required outdoor airflow (V_{bz}) resulted as 4511 cfm (2.13 m³/s). The area for one person in the zone which is derived from occupant density value from Table 6 with room height of 3.6 m (Figure 6). The airflow area for one occupant of 13.7 m² is then inserted to (5) to divide the V_{bz} value of 2.13 m³/s and get the required air velocity per airflow area for one person in occupied zone. Thus, the minimum target air velocity to be achieved at points in the occupied zone is achieved as 0.16 m/s.

$$V_a = \frac{V_{bz}}{13.7 \text{ m}^2} \quad (5)$$

$$V_a = \frac{2.13 \text{ m}^3}{13.7 \text{ m}^2 \text{ s}}$$

$$V_a = 0.16 \text{ m/s}$$

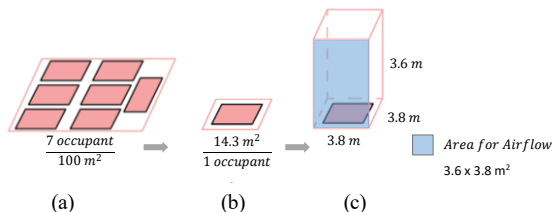


Fig. 6. Deriving Area for Airflow per Person (a) Occupants per 100 sqm for Sports Arena, (b) Area for 1 Occupant and (c) Dimensions of Volume for 1 Occupant with 3.6 m Room Height [15]

To achieve targets of inlet air velocity and indoor air

velocity, positions and numbers of mechanical fans are to be varied and tested to learn the results of air distribution and air exchange between indoor and outdoor.

3.5. Simulation Conditions

SolidWorks software is chosen for simulating the existing condition and experimental cases due to its use of Reynolds-Averaged-Navier-Stokes (RANS) models. According to Evola and Popov (2006) study, although RANS models lack some precisions, they require less computing time while they are considered satisfying in describing the natural ventilation flow inside the building. Lastly, it is said that the RANS and LES (Large Eddy Simulation) results do not show outstanding differences, at least in describing indoor flow.

Furthermore, SolidWorks has been used for CFD simulation by Widiastuti et al. (2020) in the process to develop building thermal performance through natural ventilation by observing air velocities, air pressure and indoor air temperature. The study by Ang et al. (2024) also conducted CFD simulations in SolidWorks for wind tunnel analysis, indoor air velocity and temperature distribution to test natural ventilation and IAQ improvement strategies for a classroom. [16]

3.6. Software Validation

American Institute of Aeronautics and Astronautics (AIAA) (1998) defines validation as “the process of determining the degree to which a (CFD) model is an accurate representation of the real world from the perspective of the intended uses of the model.” A simplified base model, Figure 7, for the CFD simulation is created in SolidWorks with focus on wall openings and training pitches.

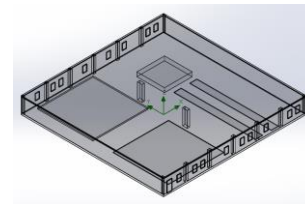


Fig. 7: Isometric Simplified Model of the Selected Existing Room for Simulation

A handheld Testo 405i smart probe thermal anemometer is used for measuring the actual data of base model air flow like inlet air velocity and indoor air velocity on a 6m x 6m grid and inlet and outlet points as seen in Figure 8. In warm conditions, the comfort of the head and hands dictates a person's overall discomfort. [11] Therefore, the measuring heights are set at 1.5 m and 1 m above the foot levels as these are also the average breathing zone heights of adults standing and sitting positions respectively (Figure 9). [17] [18] [19] This measured data is used to validate the simulation data.

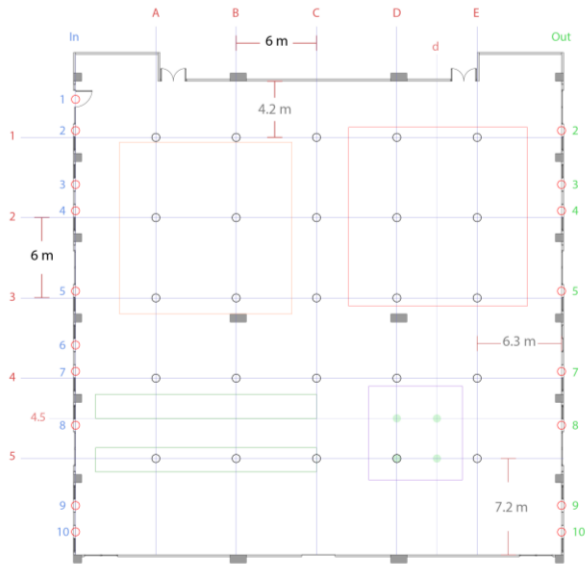


Fig. 8: Grid of Measuring Points in Selected Room



Fig. 9: Vertical Measuring Heights for Different Sport Areas

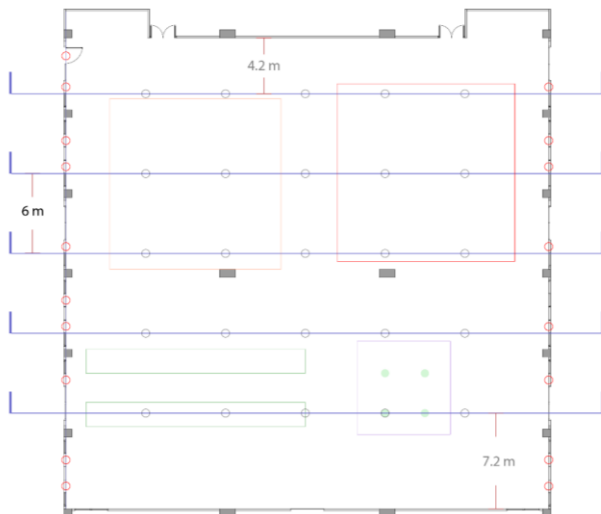


Fig. 10: Plan Showing Vertical Cut Plot Positions

The measured data were collected on four different days, 6th and 10th in January and 20th and 21st in February 2026, with one-hour intervals from 9 am till 6 pm. Each time has 25 indoor measuring points for 2 different heights (1.0m and 1.5m) and 10 inlet points. All measured values at each point for all four days are averaged to get overall average values for 10 inlet points, 25 points at 1.0m and 25 points at 1.5m as in Table 7.

Having 0.76 m/s as the target minimum inlet air velocity, the measured average values show that 8 out of 10 openings do not reach the target for adequate ACH. With 0.16 m/s as a target indoor air velocity, the measured values for A1 to E5 at 1.5m do not show many differences (0.05 m/s max) but the ones at 1.0m show larger differences (0.08 m/s max). Therefore, it

could be concluded that there is room for improvement to be made or even increase beyond the target minimum values.

TABLE VII
OVERALL AVERAGE VALUES OF MEASURED VELOCITIES
AND TEMPERATURES AT (A) INLET OPENINGS, (B) INDOOR
POINTS AT 1.5M HEIGHT AND (C) INDOOR POINTS AT 1.0M
HEIGHT

(A)

	In1	In2	In3	In4	In5	In6	In7	In8	In9	In10
m/s	0.89	0.57	0.85	0.35	0.52	0.62	0.53	0.60	0.41	0.45
°C	30.2	30.3	30.3	30.3	30.3	30.3	30.3	30.3	30.3	30.3

(B)

	m/s	°C
A1	0.23	30.2
A2	0.11	30.2
A3	0.15	30.2
A4	0.15	30.2
A5	0.13	30.2
B1	0.29	30.1
B2	0.13	30.1
B3	0.15	30.1
B4	0.14	30
B5	0.09	30
C1	0.21	30
C2	0.12	30
C3	0.12	30
C4	0.14	30
C5	0.12	30
D1	0.22	30
D2	0.16	30
D3	0.12	30
D4	0.12	29.9
D5	0.13	29.9
E1	0.19	29.9
E2	0.19	29.9
E3	0.15	29.9
E4	0.16	29.9
E5	0.12	29.9

(C)

	m/s	°C
A1	0.25	28.5
A2	0.16	28.5
A3	0.18	28.5
A4	0.13	28.5
A5	0.09	28.5
B1	0.23	28.5
B2	0.15	28.5
B3	0.21	28.5
B4	0.18	28.5
B5	0.09	28.5
C1	0.22	28.5
C2	0.11	28.5
C3	0.12	28.6
C4	0.13	28.6
C5	0.09	28.6
D1	0.24	28.5
D2	0.13	28.5
D3	0.14	28.5
D4	0.16	28.6
D5	0.09	28.6
E1	0.17	28.6
E2	0.11	28.6
E3	0.14	28.6
E4	0.1	28.6
E5	0.08	28.6

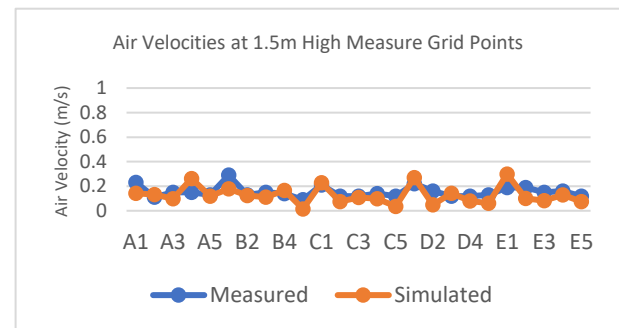


Fig. 11: Indoor Air Velocity Values at 1.5m Height

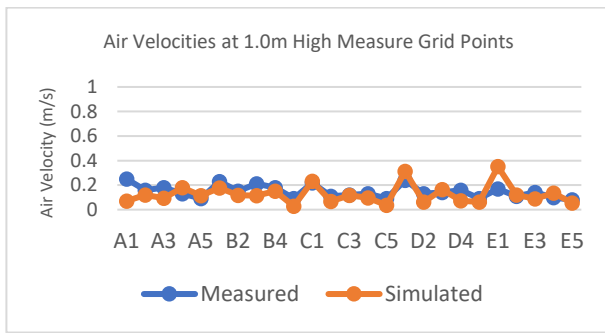


Fig. 12: Indoor Air Velocity Values at 1.0m Height

When the simulation was run with the measured inlet averages as in Table 7(A), the simulated values for grid points at 1.5m and 1.0m show average error percentage of 18.5% and 15.1% respectively. Some points show large error percentage, due to the very small values of parameters as a small difference in a decimal value shows large error percentage (Figure 11 and 12). High errors in row 1 arise from unmodeled openings to an adjacent room, which are present in the real setting. Error percentages at 1.0m and 1.5m differ due to differences between the incoming flow and initial condition in the space which is not set with fluctuating conditions in reality. Thus, SolidWorks software's flow simulation is regarded as reliable for this research.

From the base model simulation, airflow pattern over the space is evaluated (Figure 15). As observed in the result, areas with very low air velocity as well as swirling flow patterns can be seen covering practice areas on both vertical and horizontal planes. Thus, it can be concluded that there is room for airflow pattern improvement with higher velocity over stale air areas.

3.6. Simulation Procedure

Air velocity and air distribution are observed in both the vertical (Figure 10) and horizontal planes at 1.5m and 1.0m heights within the breathing zone as defined by ASHRAE (2022) and DeKay and Brown (2014); not closer than 0.6 m to the walls and not higher than 1.8 m above the floor. While the air flow pattern will be observed on overall planes, the indoor air velocity will be observed at same measuring points as in Figure 8 and the inlet velocities are evaluated for ACH.

3.7 Simulation Approach

Base model simulation was first run for finding the travel value where the convergence is achieved to mark as standard to determine sufficient runs for further models. To achieve better Airflow Pattern, Indoor Air Velocities, and ACH Values, 4 to 6 tunnel fans attached to the ceiling are tested. The IJB series induced jet fan (Figure 13) by Kruger Ventilation Industries Asia Co. Ltd. is chosen to test and observe changes in selected dependent variables. [20]

Two different numbers of fans are tested, four fans normal to the columns and six fans in between each columns. Placing each amount at three positions (6m, 18m and 30m from windward wall) and at 2.85 m height between fan's center and

floor, 6 fan configurations (Table 8) are tested.

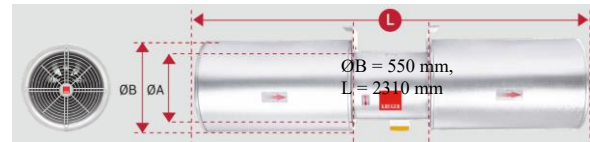
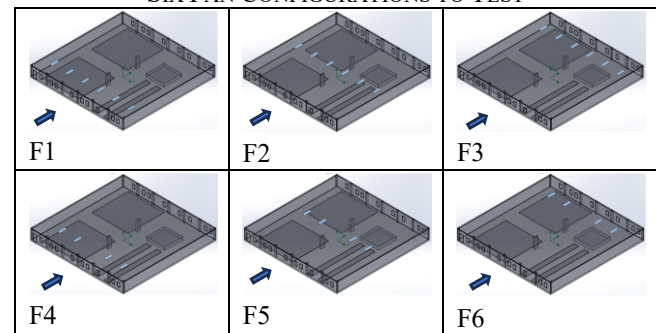


Fig. 13: Dimension of IJB Induced Jet Fan Selected to Test

The locations of fans are decided with an intention to extend the inlet flow further and stronger into the space, to bridge the inlet airflow to the leeward wall and to draw the indoor airflow towards the outlet windows instead of losing strength and airflow dropping to the floor. 6 fans aligned with openings are intended to extend the inlet flow while 4 fans normal to columns are intended to distribute airflow to areas between inlet flow paths. All are experimented to control airflow distribution, Indoor Air Velocity in occupied spaces and ACH minimizing stale air zones on the used areas of the space.

TABLE VIII
SIX FAN CONFIGURATIONS TO TEST



New simulation results from the six fan configurations and fan output velocity of 9 m/s as provided by Kruger (2023) show increases in indoor air velocities as well as changes in airflow pattern on horizontal and vertical planes (Figure 16).

XXIII. RESULT AND DISCUSSION

4.1 Airflow Distribution and Stale Area Results

Comparing horizontal planes at 1.5m of Figure 15 to ones in Table 9, all configurations show improvement in terms of air distribution around the room except F3 and F6 with fans closest to outlet openings. This is because the fans are too far at the other end to draw inlet flows. Therefore, F3 and F6 both result in very similar horizontal and vertical planes with the whole room, other than near the walls, having low air velocities. Among the other four configurations, F1 and F4 with fans at windward end result in better airflow pattern on vertical planes with air reaching more areas of the whole space than F2 and F5 that show stronger airflow only on the leeward side of the room.

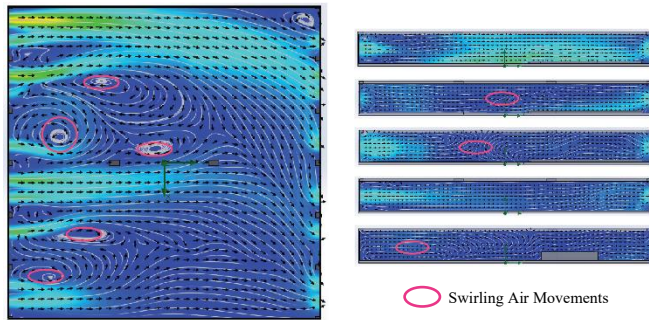
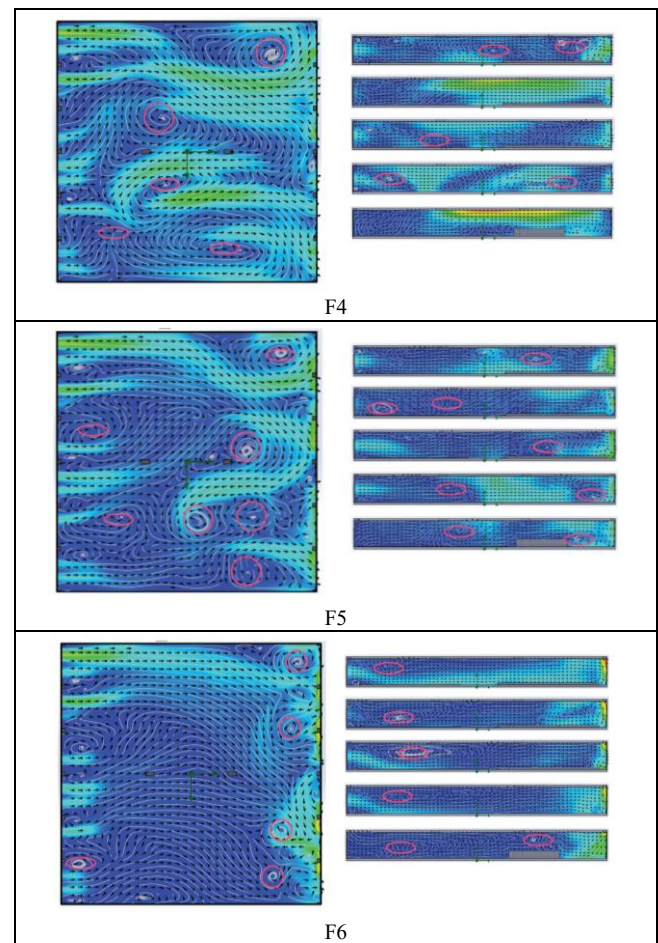
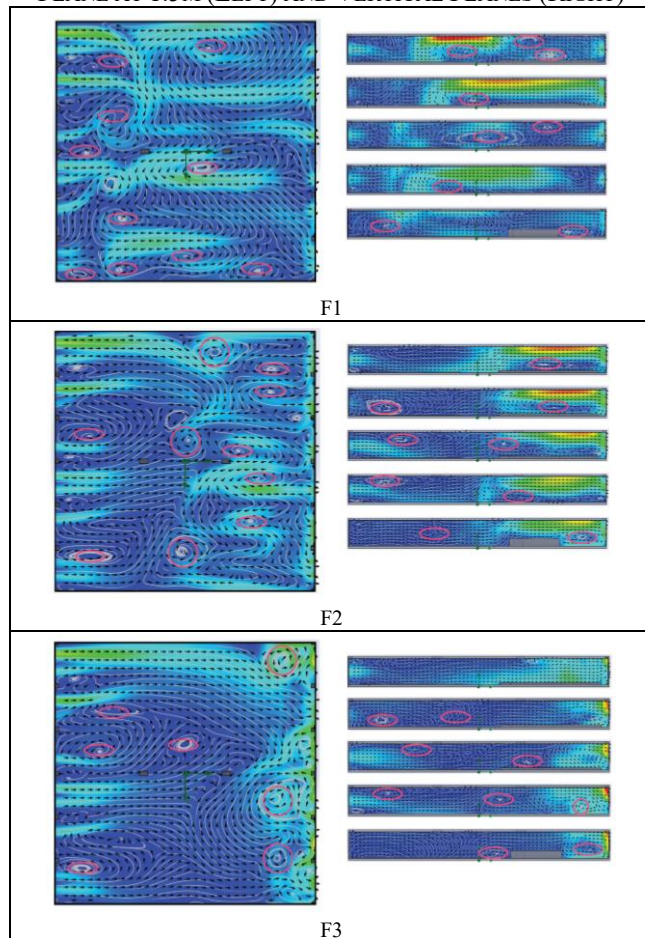


Fig. 15: Cut Plots with Streamlines and Velocity Contour on Horizontal Plane at 1.5m (left) and Vertical Planes (right) of Base Model Simulation

F1 provides stronger but narrow airflows due to air directly drawn from inlet flow paths while F4 can bring more spread out distribution with smoother flows because it's fans hang in between the inlet flow paths and diffuse the flows into further areas. In vertical planes, F1 depicts stronger and straighter airflows, and F4 displays more smoothly spread out flows. With same or more amount of stale air areas than the base model, F4 has fewer stale air areas over the used regions. Therefore, in terms of airflow pattern, configuration F4 with four fans on windward side gives the best result.

TABLE IX

CUT PLOTS OF 6 FAN CONFIGURATIONS ON HORIZONTAL PLANE AT 1.5M (LEFT) AND VERTICAL PLANES (RIGHT)



4.2 Indoor Air Velocity Results

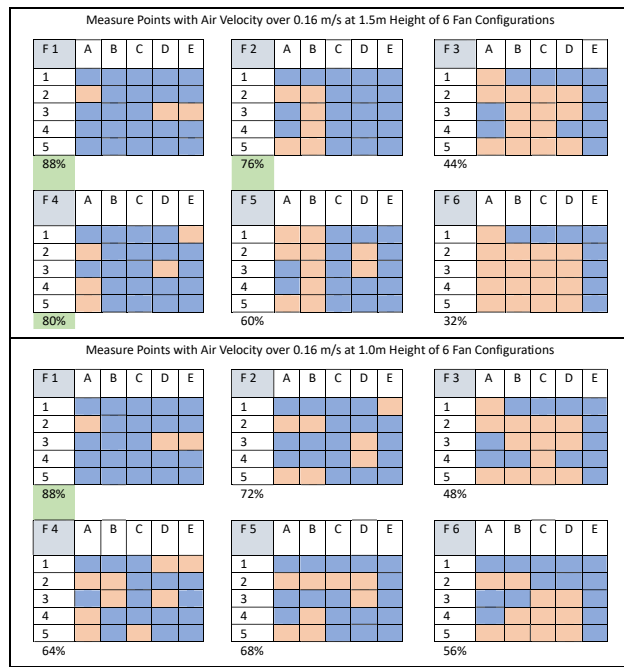
With 0.16 m/s as target indoor air velocity at measure points, the results of all models are assessed as in Table 10. Although all 6 configurations increase in percentage of points with over 0.16 m/s velocity, only three (F1, F2 and F4) hold more than 75% of points on 1.5 m plane with velocity over target value. On 1.0 m plane, only F1 configuration achieves over 75% of points with over-target value. Analyzing overall results, the fan placements near inlet openings spread air velocity more evenly than others (F2, F3, F5, F6) which mostly have low velocities on the windward side of the room.

TABLE X

PERCENTAGES OF MEASURE POINTS WITH AIR VELOCITY OVER 0.16 M/S OF EACH MODEL

Measure Points with Air Velocity over 0.16 m/s of Base Model											
Base 1.5m	A	B	C	D	E	Base 1.0m	A	B	C	D	E
1						1					
2						2					
3						3					
4						4					
5						5					
24%						24%					

< 0.16 m/s
> 0.16 m/s



4.3 Air Change Rate (ACH) Results

Although adding fans improved inlet velocities and airflow distribution, it did not satisfy the intended ACH improvement. Fans do not have impact on the inlet velocity. The reasons fans do not affect inlet velocity can be the large distance between inlets and fans or fans hanging outside the window frames or both. Therefore, the initial ACH value of base model stays unchanged (Figure 16), below the required ACH of 10 and more variables are to be tested for ACH improvement.

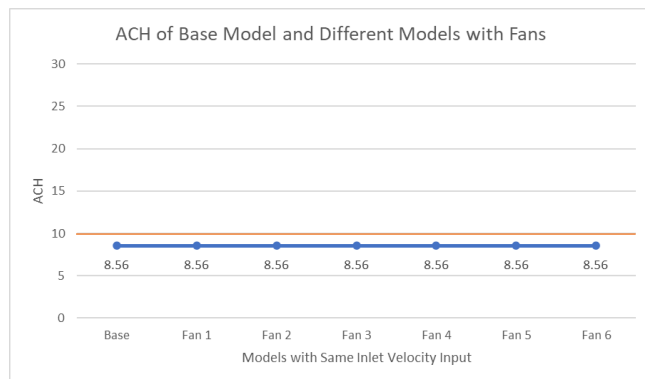


Fig. 16: Air Change Rate (ACH) of Base Model and Experimental Models with Different Fan Configurations

Overall, the configuration F1, satisfies the air distribution and indoor velocity target while the F4 serves the best air distribution. However, there is still a gap to test more variables for ACH improvement and one of which can be the openings.

The existing sliding windows of the room are not fully using the openable area, opening only one panel at a time. There are also several doors being closed currently due to the access control suggesting potentials for more or larger openings. These potential components can be reconfigured

while more fan configurations like combination of F1 and F4 in this research are tested to improve the ventilation in future research.

XXIV. CONCLUSION

This study explores the interest in relation among air movement, indoor air quality and thermal comfort with particular emphasis on cross-ventilated deep-plan spaces accommodating high intensity activities highlighting the need for careful consideration of key environmental parameters for maintaining good indoor conditions.

The literature review identifies critical parameters to be tested for IAQ improvement through air movement and air velocity. With comparison between measured and simulated data sets, this study also validated the software's ability to support the flow simulation studies.

The results of the experimental simulations also reveals how the placement of mechanical fans can improve airflow pattern, and indoor air velocity. However, the proposed configurations are insufficient to meet the ACH requirement, due to limited influence on inlet air velocity. While the study points out a feasible fan configuration, findings also bring up the gap for future research to test opening variations for improved ACH.

Overall, this study gives a frame of thinking for further experiments and simulations in pursuing IAQ and comfort improvement through airflow in deep-plan cross-ventilated combat sports facilities.

ACKNOWLEDGMENT

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Optimizing Canteen Layout for Improved Circulation Efficiency and Seating Capacity: A Visibility Graph Analysis of the Chula Chakrabongse Building Canteen, Chulalongkorn University, Thailand

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Abstract. University canteens often encounter spatial inefficiencies during peak hours due to inadequately organized seating layouts and circulation paths. These conditions often lead to overcrowding, circulation congestion, and difficulty locating available seating. This study investigates spatial design strategies to improve seating capacity and circulation efficiency in the Chula Chakrabongse Building Canteen at Chulalongkorn University, Thailand. The research adopts a mixed-method approach combining field observation, spatial documentation, and computational spatial analysis. The existing canteen layout was modeled using AutoCAD and analyzed through Visibility Graph Analysis (VGA) implemented in DepthmapX within the framework of Space Syntax. Key performance indicators include seating capacity, circulation area, and Average Visual Integration (HH). A redesigned layout was developed using circulation standards from Neufert Architects' Data to reorganize table alignment, establish a clear hierarchy of aisles, and introduce designated queuing spaces. Simulation results indicate that the proposed layout increases seating capacity from 672 to 720 seats, representing a 7.14% increase, while improving the Average Visual Integration value from 11.80 to 13.88, corresponding to a 17.63% improvement. These findings suggest that strategic furniture arrangement and circulation organization can significantly enhance spatial efficiency in high-density dining environments without requiring additional physical space. This study offers practical design ideas for optimizing university canteen layouts and illustrates the relevance of spatial analysis techniques in interior space planning.

Index Terms—Circulation design, space syntax, spatial optimization, university canteen, visibility graph analysis

XXV. INTRODUCTION

University dining facilities are essential communal spaces that support daily campus activities and social interaction. However, many existing canteens experience operational inefficiencies during peak hours due to poorly organized seating layouts and circulation patterns [1, 2]. Congestion frequently occurs when large numbers of users arrive simultaneously, leading to conflicts between seated users, walking paths, and queuing areas. Spatial configuration plays a crucial role in shaping how users perceive and navigate interior environments. According to research in Space Syntax by Bill Hillier and Julienne Hanson, spatial visibility and connectivity significantly influence human movement patterns and spatial accessibility. Environments with clearer visual connections typically support smoother circulation and more intuitive wayfinding [3] [4].

Environmental design research also suggests that spatial

cues can influence user behavior by clarifying possible actions within a space. This idea relates to the concepts of affordances and signifiers discussed by Don Norman in *The Design of Everyday Things*, which explain how design elements communicate how users should interact with an environment [5]. Similarly, wayfinding theory highlights that clear visual cues and well-organized spatial layouts can improve navigation and reduce congestion in complex environments, as discussed by Romedi Passini and Paul Arthur in *Wayfinding: People, Signs, and Architecture* [6]. Earlier studies on environmental legibility also emphasize the importance of visual clarity and spatial organization in supporting user orientation. For example, Kevin Lynch's seminal book *The Image of the City* examines how legible spatial structures enable users to form mental maps of their surroundings and navigate urban environments effectively [7]. Similarly, John Weisman's study *Evaluating Architectural Legibility: Way-Finding in the Built Environment* explains that architectural legibility and clear

visual access can significantly improve wayfinding performance in complex buildings [8].

In dining environments, furniture arrangement and aisle configuration strongly affect both seating efficiency and circulation performance. Architectural design references such as Neufert Architects' Data recommend specific spatial standards for circulation widths and seating arrangements to ensure functional movement within dining spaces [9].

Despite these guidelines, many existing canteens evolve through incremental furniture placement rather than systematic spatial planning. As a result, aisle widths often become inconsistent, seating clusters may obstruct circulation routes, and queuing behavior may spill into circulation paths. At Chulalongkorn University, the Chula Chakrabongse Building Canteen (Fig. 1) was selected as the case study due to its significant role as a high-density dining facility serving a large number of students, staff, and visitors during peak hours, while also exhibiting several spatial inefficiencies.



Fig. 1. Inside view of the canteen during peak hours

First, the canteen experiences high user density during lunchtime, resulting in frequent overcrowding and circulation congestion. Preliminary field observations during peak hours show that many users arrive simultaneously, creating conflicts between seated users, pedestrians, and individuals waiting in queues. These conditions make the canteen an appropriate environment for investigating spatial efficiency in dining spaces.

Second, the existing seating layout lacks systematic spatial organization. Tables are arranged in a relatively random manner, producing inconsistent aisle widths and inefficient circulation paths. Some aisles are excessively wide while others are too narrow to accommodate comfortable pedestrian movement. This uneven distribution of circulation space reduces the overall efficiency of the seating layout.

Third, queuing behavior in front of food stalls often interferes with circulation paths. Due to the absence of

clearly defined queue areas or visual guidance, users tend to stand in the main aisles while waiting for food, which obstructs pedestrian flow. Informal feedback from frequent users suggests that clearer environmental cues, such as floor markings or queue barriers, could help organize waiting behavior and reduce circulation conflicts.

These spatial issues make the canteen an appropriate environment for investigating how layout optimization can improve seating capacity and circulation efficiency. This issue is significant because the canteen serves students from multiple faculties, while physical expansion of the canteen is not possible, improving spatial efficiency through layout optimization becomes a practical design strategy. Therefore, this study investigates how reorganizing seating layouts and circulation paths can enhance both seating capacity and circulation efficiency within the existing canteen area. Although previous studies have examined aspects such as queue management, ergonomics, and user satisfaction in dining facilities [10-12], relatively limited research has focused on spatial design interventions for optimizing seating layouts in existing university canteens. This study addresses this gap by investigating spatial design strategies that improve seating capacity and circulation efficiency through layout simulation and spatial analysis.

The objective of this research is to evaluate the spatial performance of existing and proposed canteen layouts using Visibility Graph Analysis and to identify design strategies that improve seating capacity and circulation efficiency. Improving seating capacity refers to increasing the number of seats available to users within the existing canteen space, while circulation efficiency refers to improving the clarity, continuity, and usability of movement routes within the canteen, particularly during peak hours. This includes reducing congestion and movement conflicts, minimizing overlap between seating areas, queuing zones, and circulation paths. By improving circulation efficiency, the study aims to ensure that available space is used more effectively, thereby supporting both user comfort and the functional performance of the seating area.

XXVI. METHODOLOGY

A. Research Design

This study adopts a mixed-method approach combining qualitative field observation and quantitative spatial analysis to investigate and evaluate architectural design strategies for improving seating capacity and circulation efficiency in the Chula Chakrabongse Building Canteen. The qualitative component involves on-site observation and spatial mapping to document user behavior, movement patterns, and congestion during peak hours, following ethnographic principles in architectural research by Galen Cranz [13]. The quantitative component focuses on analyzing the relationship between spatial configuration and user movement using Space Syntax methodology developed by Bill Hillier and Julienne Hanson [3].

A comparative analysis was conducted between the

existing layout and a proposed layout to examine how spatial reconfiguration could enhance seating capacity, visual accessibility and circulation performance. The evaluation of both layouts was conducted using three key spatial performance indicators: seating capacity, circulation area, and Average Visual Integration (HH). Seating capacity reflects the total number of users that the space can accommodate, while circulation area represents the spatial allocation for movement within the canteen. Average Visual Integration (HH), calculated through Visibility Graph Analysis (VGA), indicates the level of visual connectivity and spatial accessibility within the layout.

The research process consists of three main stages (Fig. 2). The first stage is Fieldwork Data Collection, which includes on-site observation, user behavior documentation, and spatial measurement of the existing layout. The second stage is Layout Redesign, where the seating arrangement and circulation structure are reorganized based on architectural design standards and spatial planning principles to improve spatial efficiency. The third stage is Data Analysis, which involves conducting Visibility Graph Analysis (VGA) simulations using the software DepthmapX to evaluate the spatial performance of both the existing and the proposed layouts. The results of the analysis are then compared using the selected performance indicators to assess the effectiveness of the proposed spatial design strategies [14].



Fig. 2. Methodology diagram

B. Fieldwork Data Collection

Fieldwork was conducted at the Chula Chakrabongse Building Canteen to document the existing spatial configuration and user behavior within the space. The data collection process involved direct on-site observation, spatial measurement, and photographic documentation of the canteen layout. Observations were carried out during peak usage periods in order to capture typical user movement patterns, seating behavior, and areas of congestion.

The spatial layout of the canteen was recorded by measuring the dimensions of the dining area, table arrangements, circulation paths, and service areas. The existing seating configuration and circulation routes were documented and converted into a digital floor plan for further analysis. In addition, field observations were used to identify spatial issues such as overcrowding, inefficient circulation paths, and underutilized areas within the canteen. The collected data provided the basis for evaluating the current spatial performance and for developing a redesigned layout aimed at improving spatial efficiency.

C. Layout Redesign

Based on the findings from the fieldwork observations, a redesigned canteen layout was developed to improve seating

capacity and circulation efficiency. The redesign process focused on reorganizing the seating arrangement and circulation pathways while maintaining functional accessibility to the food service counters and entrances.

The layout redesign was guided by architectural planning principles and spatial standards related to dining facilities and pedestrian movement [6, 9]. Key considerations included maintaining adequate circulation widths between tables, improving visibility across the seating area, and maximizing the use of available floor space without creating congestion. The arrangement of tables was adjusted to increase seating capacity while ensuring that movement paths remained clear and accessible.

Several iterations of seating configurations were explored to balance spatial density and circulation efficiency. The final proposed layout was selected based on its ability to increase the number of seats while maintaining sufficient circulation space for users. The redesigned layout was then prepared as a digital floor plan to be used for spatial simulation and comparative analysis.

D. Data Analysis

To evaluate the spatial performance of the existing and proposed layouts, spatial simulation was conducted using the software DepthmapX [14] [15]. Visibility Graph Analysis (VGA), a method within the Space Syntax framework [3], was used to analyze the visual accessibility and spatial connectivity within the canteen environment.

Both the existing and redesigned layouts were converted into visibility graph models within the DepthmapX software. A grid-based analysis was applied to the floor plan to calculate spatial relationships between visible points within the space. The primary indicator used in this analysis was Average Visual Integration (HH), which measures the degree to which a location is visually connected to other areas in the spatial system.

In addition to visual integration analysis, two additional performance indicators were evaluated: seating capacity and circulation area. Seating capacity was calculated based on the total number of available seats in each layout configuration, while circulation area was measured as the total space allocated for movement within the dining area. By comparing these indicators between the existing and proposed layouts, the study evaluates whether the redesigned configuration improves spatial efficiency and user accessibility within the canteen.

XXVII. RESULTS AND DISCUSSION

A. Existing Canteen Layout Performance

Field observations revealed several spatial issues affecting the functionality of the Chula Chakrabongse Building Canteen, particularly during peak lunch hours. The most significant problems include overcrowding, circulation congestion, and inefficient table arrangements. The lack of clearly defined queuing areas in front of food stalls causes

users to form informal queues within the primary circulation aisles. As a result, students frequently stand in the main circulation paths, obstructing pedestrian flow and creating conflicts between moving and stationary users.

Informal user feedback further supported this observation. A frequent canteen user reported that the absence of marked queue lines or barriers makes it unclear where to stand while waiting for food, leading users to occupy circulation paths and block the main aisles (first-year student from the Faculty of Science, personal communication, Oct. 10, 2025). This indicates that the circulation problem is not solely due to

spatial limitations but also due to insufficient spatial guidance and visual organization.

The existing layout (Fig. 3) contains a total of 66 tables with eight seats and 36 tables with four seats, providing a total seating capacity of 672 seats. The total circulation area within the layout is approximately 548.05 m² out of the 853.45 m² total canteen area (Table I). However, the arrangement of tables is irregular, resulting in inconsistent aisle widths. Some aisles are excessively narrow, restricting movement, while others are wider than necessary, leading to inefficient use of space.

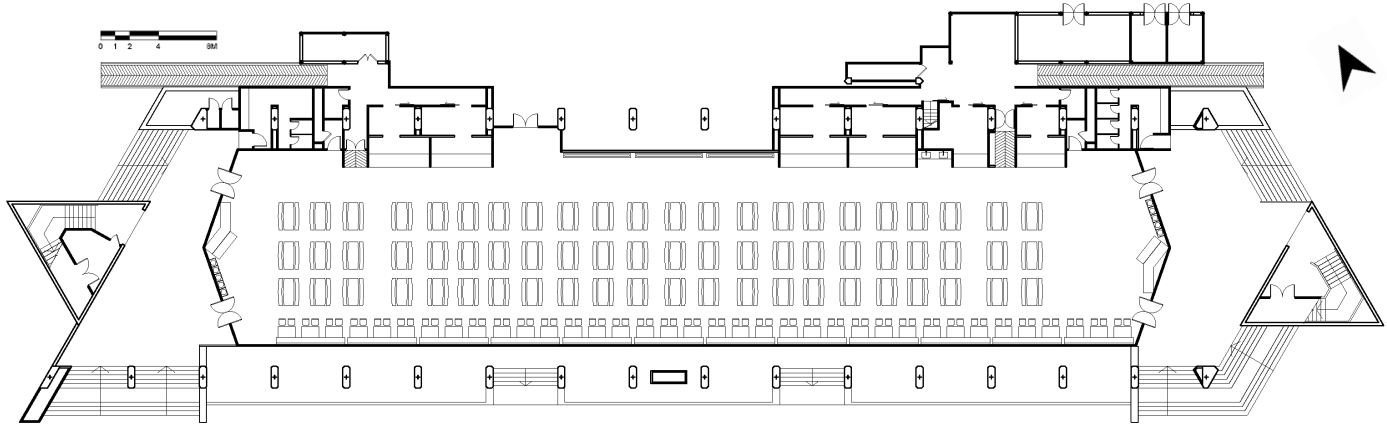


Fig. 3. Existing first-floor plan of the Chula Chakrabongse Building canteen

TABLE I
SPATIAL CHARACTERISTICS OF THE EXISTING
CANTEEN LAYOUT

Indicator	Value
Total canteen area	853.45 m ²
Total number of tables	102 tables
Seating capacity	672 seats
Circulation area	548.05 m ²

Spatial analysis using Visibility Graph Analysis (VGA) in DepthmapX (Fig. 4) indicates that the existing layout has an Average Visual Integration (HH) value of 11.80, with a minimum value of 4.62 and a maximum value of 18.63 (Table II). In the VGA visualization, spatial integration is represented through a color spectrum ranging from blue to red, where blue indicates visually segregated spaces with low levels of visual connectivity and red represents highly integrated locations that are visually connected to a large portion of the spatial system.

In the existing canteen layout, the highest integration values represented by red tones appear primarily near the entrance located on the right side of the canteen. This entrance area functions as a visually dominant access point where users entering the space can perceive multiple directions within the canteen. Within the interior of the

canteen, the main circulation paths between seating clusters appear mostly in yellow and orange tones, indicating relatively high but not maximum levels of visual integration. Since these routes function as the primary movement corridors where users frequently walk while searching for seats or moving toward food stalls, higher integration values would typically be expected along these paths. However, the concentration of the highest integration values mainly at the entrance suggests that visual connectivity within the interior circulation network is not evenly distributed.

In contrast, the smaller aisles located between tables appear predominantly in cyan tones, indicating lower levels of visual integration. These aisles primarily function as local access routes where users approach or leave their seats rather than move through the space. While lower integration values are appropriate for these localized seating areas, the overall distribution of integration values in the existing layout suggests that the spatial hierarchy between primary circulation routes and secondary seating aisles is not clearly defined.

B. Proposed Layout Performance

Based on the spatial problems identified during field observations, a redesigned layout was developed to improve seating capacity, circulation efficiency, and spatial organization. The redesign strategy focused on reorganizing table arrangements to create continuous and clearly defined circulation aisles while maximizing the use of available floor space.

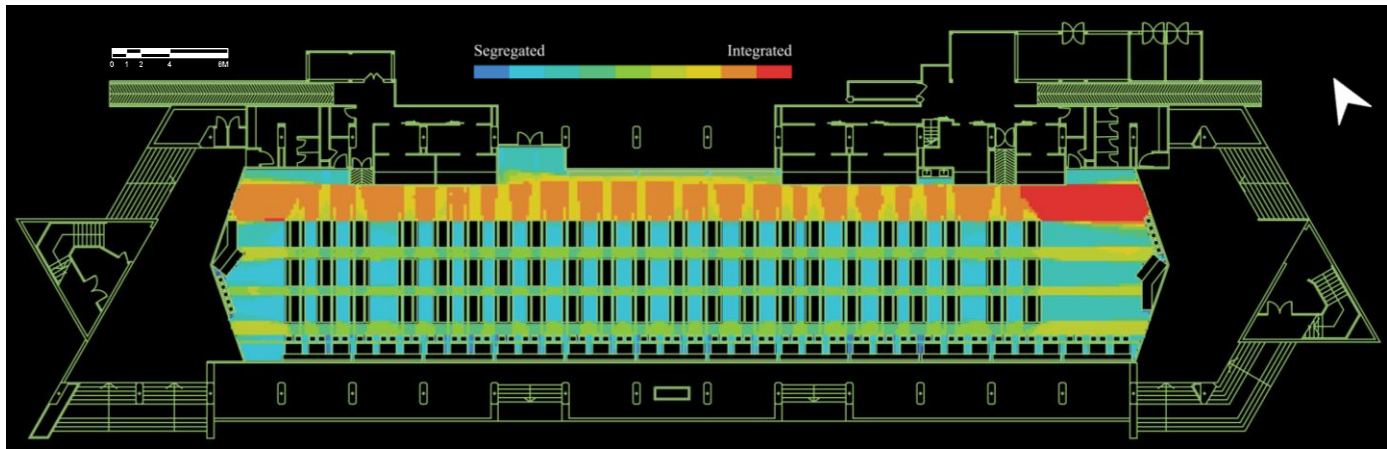


Fig. 4. Visibility graph analysis of the existing first-floor plan of the Chula Chakrabongse Building canteen using DepthmapX

TABLE II
VISIBILITY GRAPH ANALYSIS OF THE EXISTING
CANTEEN LAYOUT

Indicator	Value
Average Visual Integration	11.80
Minimum Visual Integration	4.62
Maximum Visual Integration	18.63

The redesign follows spatial design strategies for space optimization proposed by Merrell [16], which emphasize both functional and visual criteria in furniture layout planning. Functional criteria include adequate clearance around furniture, smooth circulation routes, appropriate spatial relationships between tables, and seating arrangements that support comfortable face-to-face interaction. Visual criteria focus on balance, alignment, and emphasis within the spatial composition. In the proposed layout, tables were aligned systematically to create continuous circulation aisles and to maintain consistent spacing between seating areas. This organized arrangement improves both the functional efficiency of movement and the visual clarity of the dining space, helping users navigate the canteen more easily while locating available seating.

The proposed layout also aligns tables in a more structured configuration to reduce irregular gaps and minimize unnecessary small aisles. Circulation paths were designed according to architectural planning standards provided in Neufert Architects' Data (4th edition). The main circulation aisle was maintained at a width of 2500 mm to accommodate high pedestrian flow. Secondary aisles were designed with a width of 1200 mm, while the intermediate spacing between tables was maintained at a minimum width of 900 mm to ensure adequate user movement and accessibility [9].

In addition, designated queuing spaces were introduced in front of food stalls. These spaces provide a minimum standing width of 625 mm per person, based on ergonomic standards from Neufert Architects' Data. Clear visual cues, such as floor markings or barriers, were proposed to guide queue formation and prevent users from occupying circulation aisles. This design approach follows wayfinding principles suggesting that environmental cues can influence user behavior by clarifying appropriate actions within a space. As discussed by Norman [5], design elements can function as signifiers that communicate how users should interact with an environment, while wayfinding research by Arthur and Passini [6] highlights that clear spatial cues and organized layouts can improve navigation and reduce congestion in complex environments. The provision of defined queue zones helps separate stationary activities from movement paths, which can reduce circulation conflicts.

As a result of these spatial adjustments, the proposed layout (Fig. 5) increases the number of tables with eight seats from 66 to 72 while maintaining 36 tables with four seats. This change increases the overall seating capacity from 672 seats to 720 seats. At the same time, the circulation area is slightly reduced to 531.55 m², indicating a more efficient use of floor space without compromising pedestrian accessibility (Table III).

TABLE III
SPATIAL CHARACTERISTICS OF THE PROPOSED
CANTEEN LAYOUT

Indicator	Value
Total canteen area	853.45 m ²
Total number of tables	108 tables
Seating capacity	720 seats
Circulation area	531.55 m ²

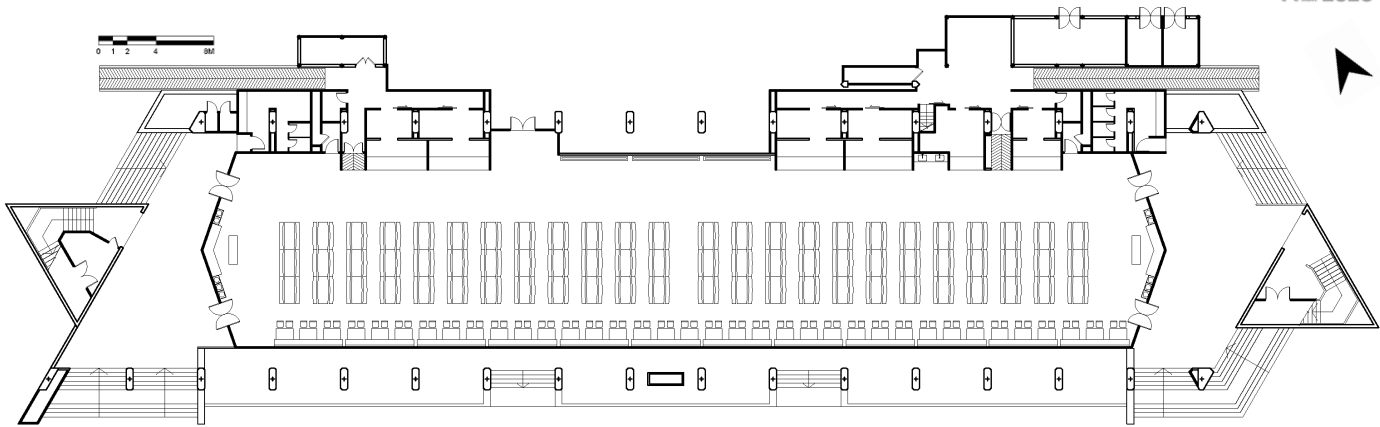


Fig. 5. Proposed first-floor plan of the Chula Chakrabongse Building canteen

Spatial simulation using Visibility Graph Analysis (VGA) in DepthmapX (Fig. 6) shows that the proposed layout achieves an Average Visual Integration (HH) value of 13.88, with a minimum value of 5.85 and a maximum value of 21.40 (Table IV). In the VGA visualization, spatial integration is represented through a color spectrum ranging from blue to red, where blue indicates visually segregated spaces with low levels of visual connectivity and red represents highly integrated locations that are visually connected to a large portion of the spatial system.

In the proposed layout, the highest integration values represented by red tones appear primarily along the main circulation aisle that runs through the center of the canteen. This main circulation route is the area where users most frequently move when entering the canteen, searching for seating, or accessing food stalls. The presence of highly integrated zones along this path indicates that the spatial configuration allows users within the main aisle to visually access many surrounding areas, supporting orientation and continuous pedestrian movement.

In contrast, smaller aisles located between seating clusters appear mostly in blue to green tones. These areas function primarily as local access paths for users approaching or leaving their seats rather than as major movement corridors. Because these spaces are used less frequently for through-movement, lower levels of visual integration are spatially appropriate. The distribution of integration values therefore reflects the functional hierarchy of movement within the canteen, with highly integrated areas supporting primary circulation and more segregated zones accommodating localized seating activities.

TABLE IV
VISIBILITY GRAPH ANALYSIS OF THE PROPOSED
CANTEEN LAYOUT

Indicator	Value
Average Visual Integration	13.88
Minimum Visual Integration	5.85
Maximum Visual Integration	21.40

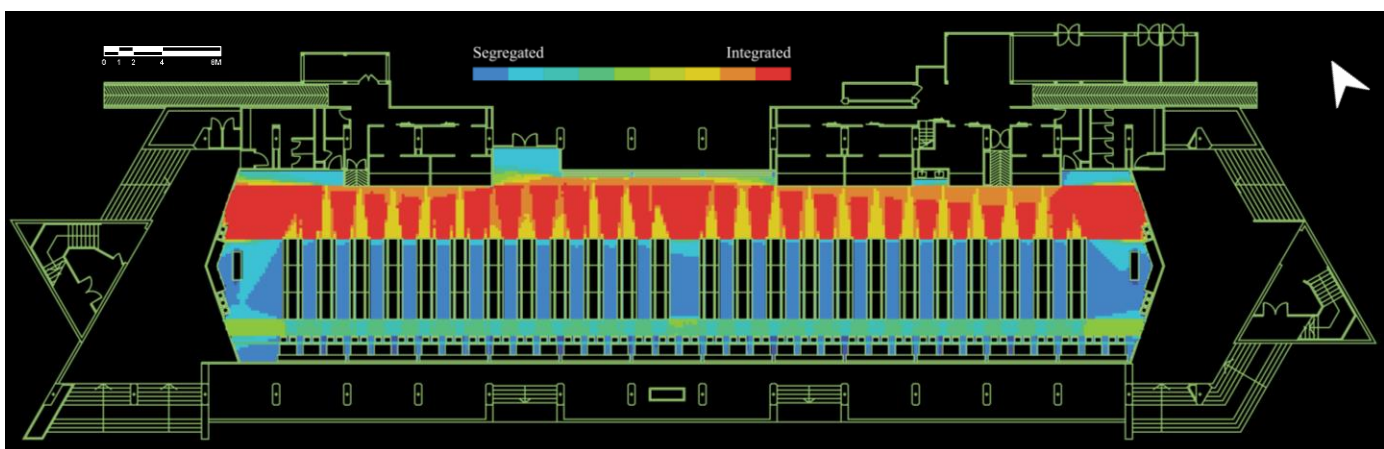


Fig. 6. Visibility graph analysis of the proposed first-floor plan of the Chula Chakrabongse Building canteen using DepthmapX

C. Comparative Spatial Performance Analysis

A comparison between the existing and proposed layout demonstrates that the redesigned configuration improves multiple aspects of spatial performance within the canteen environment.

First, the seating capacity increases from 672 seats in the existing layout to 720 seats in the proposed layout, representing an increase of 48 seats. This improvement is achieved through a more systematic arrangement of tables that reduces irregular gaps between seating clusters and allows the available floor area to be used more efficiently. The redesign therefore increases seating capacity without expanding the total canteen area.

Second, the circulation area decreases slightly from 548.05 m² to 531.55 m² while maintaining circulation widths that comply with architectural planning standards. This indicates that the redesigned layout allocates circulation space more efficiently by organizing tables in a clearer structure and eliminating unnecessary or inconsistent aisle widths.

Third, the Visibility Graph Analysis results show an improvement in visual connectivity within the spatial system. The Average Visual Integration (HH) value increases from 11.80 in the existing layout to 13.88 in the proposed layout. This increase suggests that the proposed spatial configuration provides stronger visual connections across the canteen environment (Table V).

TABLE V
COMPARISON OF SPATIAL PERFORMANCE
BETWEEN EXISTING AND PROPOSED LAYOUT

Indicator	Existing Layout	Proposed Layout	Change
Seating capacity	672 seats	720 seats	+48 seats
Circulation area	548.05 m ²	531.55 m ²	Optimized
Average Visual Integration	11.80	13.88	Increased
Minimum Visual Integration	4.62	5.85	Increased
Maximum Visual Integration	18.63	21.40	Increased

The VGA visualization further reveals a clearer spatial hierarchy between primary circulation routes and secondary seating aisles in the proposed layout. In the existing layout, the highest integration values are concentrated mainly near the entrance, while the interior circulation paths show mostly moderate integration values. In contrast, the proposed layout distributes highly integrated areas along the main central aisle, which functions as the primary movement corridor of the canteen. This spatial arrangement allows users moving through the main circulation path to visually access a larger portion of the seating area.

At the same time, smaller aisles located between tables remain less integrated, reflecting their function as localized access paths rather than major movement routes. This clearer differentiation between highly integrated circulation corridors and more segregated seating aisles indicates that the redesigned layout establishes a more organized spatial structure.

Overall, the comparison demonstrates that the proposed layout improves spatial efficiency by increasing seating capacity, optimizing circulation space, and enhancing visual connectivity. The findings demonstrate that systematic table alignment, standardized circulation widths, and the creation of a clear circulation hierarchy can significantly improve the usability and spatial performance of high-density dining environments.

Beyond the case study context of the Chula Chakrabongse Building Canteen, the findings of this research demonstrate broader applicability to other high-density interior environments where spatial efficiency is critical and physical expansion is constrained. These include university dining halls, food courts, office cafeterias, and other shared dining facilities that experience similar challenges related to congestion, circulation conflicts, and inefficient space utilization.

The study shows that systematic furniture arrangement, a clear circulation hierarchy, and the integration of spatial analysis tools such as Visibility Graph Analysis (VGA) can support more efficient space utilization and improved user movement patterns. By combining design strategies with quantitative spatial evaluation, the research highlights the role of analytical methods in informing evidence-based layout decisions.

Therefore, the design strategies identified in this research may serve as practical guidelines for optimizing spatial performance in comparable environments with similar functional requirements. At the same time, the methodological approach adopted in this study offers a transferable framework for evaluating and improving interior spatial configurations in other high-density settings.

IV. CONCLUSION

This study investigated spatial design strategies to improve seating capacity and circulation efficiency in the Chula Chakrabongse Building Canteen. Field observations identified several spatial issues, including overcrowding during peak hours, circulation congestion, and unclear queuing areas that interfere with pedestrian movement. To address these issues, a redesigned layout was developed based on architectural planning standards and spatial design strategies focusing on organized furniture alignment, adequate circulation widths, and clearly defined queuing areas.

The redesigned layout increased seating capacity from 672 to 720 seats while maintaining appropriate circulation space within the canteen. Spatial simulation using Visibility Graph Analysis (VGA) further demonstrated an improvement in

spatial connectivity, with the Average Visual Integration (HH) value increasing from 11.80 in the existing layout to 13.88 in the proposed layout. These results indicate that systematic table alignment and standardized circulation design can significantly improve spatial efficiency and visual accessibility in high-density dining environments. The findings highlight the importance of integrating spatial analysis tools with architectural design strategies to enhance the functionality and usability of communal dining spaces.

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Environment-Behavior Study of Campus Coworking Space Design and Student Proxemic Experience at Chulalongkorn University, Thailand

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Abstract. This study investigated how spatial configurations in the Central Library at Chulalongkorn University shape students' proxemic experiences and utilization patterns in campus coworking and informal learning spaces. Drawing on proxemics theory and environment-behavior research, it examines how layout variety, visibility, privacy gradients, furniture flexibility, and service amenities influence interpersonal distance, work modes, and patterns of space appropriation. A qualitative single-case design was employed, combining systematic behavior mapping across regular teaching weekdays with semi-structured interviews with eleven frequent student users; behavior mapping documented occupancy patterns, movement flows, and proxemic distances across analytically defined zones, while interview data were thematically coded to capture spatial preferences, perceived comfort, interaction patterns, and adaptive strategies. Findings show that wall-side and window-edge zones attract the highest and most sustained occupancy and are used to negotiate "privacy without isolation," whereas focus and isolated zones remain dedicated to individual work even at peak crowding; students manipulate semi-fixed furniture, use personal objects to manage territory, and tolerate higher densities when natural light, acoustic conditions, and technological amenities remain satisfactory. While international design principles for coworking and learning spaces are largely reflected in student behavior at Chulalongkorn University, several patterns, including students accepting crowding, staying in the same seat for long periods, and using personal items to mark territory, point to the influence of local social norms on shared-space behavior. The study offers design recommendations for library coworking spaces that may also inform comparable Southeast Asian universities.

Index Terms— campus coworking spaces, environment-behavior, proxemics, spatial configuration, student learning environment

XXVIII. INTRODUCTION

Campus coworking environments and learning commons have become important infrastructures for supporting students' individual study, collaboration, and social interaction in higher education [1, 2]. University libraries have shifted from book-centered repositories to multifunctional spaces that integrate individual study areas, group rooms, informal zones, and technology-rich services to accommodate diverse learning activities [3-5]. Case studies from Asia and Europe show that campus coworking spaces and innovation-oriented libraries can strengthen student engagement, entrepreneurial activity, and collaboration when spatial design is aligned with user needs [5-7]. Despite these developments, there remains limited empirical evidence on how specific spatial configurations within university libraries in Thailand shape students' experiences and patterns of space utilization.

A growing body of work links spatial configuration to user behavior in coworking spaces and educational buildings.

Studies of commercial and campus coworking spaces indicate that layout variety, visual connectivity, and furniture flexibility influence users' choice of work settings, collaboration intensity, and satisfaction [2, 8]. Research on educational complexes and informal learning spaces further demonstrates that connectivity, clustering, and privacy gradients shape students' social interaction and informal learning activities [9-11]. However, much of this literature focuses on Western or commercial contexts, and comparatively few studies apply proxemics-based analysis to university library coworking spaces in Southeast Asia settings, where cultural norms may shape spatial behavior differently. This gap limits understanding of where international design principles hold and where local adaptation is needed [11, 12].

Environment-behavior research and proxemics theory offer a useful lens for understanding these issues. Hall (1966) distinguishes between fixed-feature space (permanent architectural elements), semi-fixed-feature space (movable furniture and artifacts), and informal space defined by

interpersonal distance zones, highlighting how spatial arrangements mediate social interaction and perceptions of privacy. Subsequent environment–behavior studies emphasize that users actively manipulate semi-fixed elements and territories to negotiate comfort, control, and social engagement in shared spaces [13]. In campus coworking settings, these ideas suggest that walls, partitions, furniture layouts, and seating patterns may significantly influence how students manage interpersonal distance, balance privacy and interaction, and appropriate space for different work modes [8, 10, 11].

This study focuses on coworking and informal learning zones within the Central Library at Chulalongkorn University to address these gaps. It investigates how spatial configurations, particularly layout variety, visibility, proximity to communal areas and services, privacy gradients, and furniture flexibility, influence student experiences, proxemic distances, and utilization patterns across different times of day during a regular teaching week [8-11]. By doing so, the study contributes empirical evidence on which global design principles apply in a Thai university context and which behaviors appear shaped by local social norms, informing design for Chulalongkorn University and comparable Southeast Asian institutions [3, 6, 14].

XXIX. METHODOLOGY

This study employs a qualitative single-case design to examine how spatial configurations in the Central Library at Chulalongkorn University shape students' experiences in coworking and informal learning zones. The Central Library was selected because it integrates open study areas, group rooms, and coworking floors, offers extended operating hours, and is widely used by students, making it a key campus node for examining the effects of spatial design. Focusing on a single, information-rich case allows detailed analysis of spatial features and user behavior within a specific cultural and institutional context.

The research is grounded in environment–behavior theory and proxemics. Hall's (1966) [15] distinction between fixed-feature space (permanent architectural elements), semi-fixed-feature space (movable furniture and artifacts), and informal space (interpersonal distance zones) provides a framework for analyzing how the library's walls, partitions, furniture layouts, and seating configurations mediate social interaction and perceptions of privacy. Environment–behavior studies further emphasize that users actively manipulate semi-fixed elements and territories to manage comfort, control, and social engagement in shared spaces [8, 13]. Building on this work, the present study examines how spatial configurations in the Central Library enable or constrain students' proxemic behavior, including interpersonal distance management, transitions between individual and group work, and patterns of space appropriation [8, 10, 11].

Two research questions guide the study: (1) How do spatial configurations within the Central Library shape student

experiences and utilization patterns in coworking and informal learning zones? and (2) Which specific spatial elements, such as layout variety, visibility, proximity to communal areas and services, privacy gradients, and furniture flexibility, most strongly influence student behaviors and proxemic preferences? The scope is limited to coworking and informal learning areas within the Central Library on the Pathumwan campus, and it focuses on undergraduate and graduate students who regularly use these spaces for individual study, group work, and project-based collaboration.

Data were collected through semi-structured interviews and systematic behavior mapping. Semi-structured interviews were conducted with students who use the Central Library's coworking and informal learning zones at least twice per week, selected via purposive sampling to capture variation in academic level, discipline, and activity types [16]. A total of 11 interviews (15–30 minutes each) explored usage patterns, perceptions of fixed and movable spatial elements, interpersonal distance and comfort, circulation and interaction, and adaptive strategies and design recommendations. Behavior mapping was conducted across regular teaching weekdays between 11:00 and 17:00 using annotated floor plans, time-based observation records, field notes, and photographs to capture occupancy, work modes, movement flows, and proxemic distances [8, 10, 13]. Seven observation rounds were conducted at one-hour intervals (i.e., hourly scans) to document use patterns within each analytically defined zone. Interpersonal distances were estimated visually by comparing observed spacing between seated users against the physical dimensions of furniture units derived from floor plans; these distances were then classified according to Hall's proxemic distance zones (intimate: 0-0.45m; personal: 0.45-1.2m; social: 1.2-3.6m; public: >3.6m)

Interview recordings were transcribed and analyzed using thematic coding, with codes organized into categories such as permanent spatial features, flexible design elements, distance preferences, spatial appropriation strategies, social norms, and design recommendations [16]. Observational data were analyzed through occupancy analysis, comparison of observed distances with Hall's proxemic zones, movement-pattern analysis, and spatial clustering to identify how different layouts and zones are used under varying conditions [8, 10, 15]. Triangulation of interview and behavior-mapping data was used to compare stated preferences with observed behaviors, enhancing the credibility of findings [17]. Additional strategies for ensuring research quality include re-coding a subset of transcripts to check consistency, conducting member checks with participants where appropriate, and adhering to institutional ethical guidelines, including anonymization of all participant data [17, 18]. The coworking floor was divided into five analytical zones: Central, Wall, Focus, Entrance, Isolated, and Window, based on furniture type and spatial position (Fig. 1).

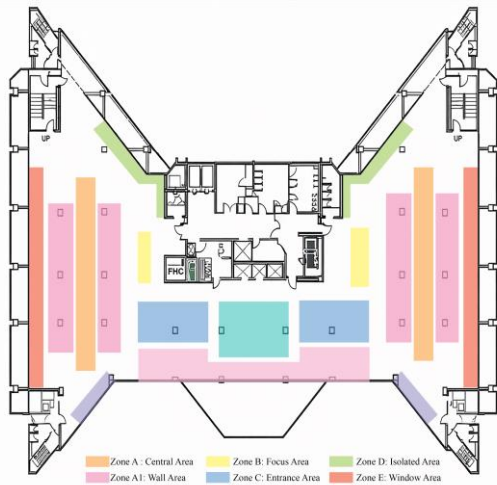


Fig. 2. Spatial Zoning of the Central Library Coworking Floor. Six analytical zones were defined based on furniture type, enclosure level, and spatial position: Zone A (Central) – open-plan tables and chairs in the middle of the floor, accommodating mixed individual and group activity; Zone A1 (Wall-Side) – linear seating along perimeter walls, primarily individual fixed desks with back-to-wall orientation, associated with focused individual work; Zone B (Focus) – semi-enclosed individual workstation separated by partitions, designed for concentrated individual tasks; Zone C (Entrance) – seating near the floor entry and main circulation corridor, most exposed to foot traffic; Zone D (Isolated) – semi-private carrels away from main circulation, intended for deep-focus private online meeting; Zone E (Window) – seating along the glazed perimeter with exterior views, used for both individual focus and small-group collaboration.

XXX. RESULTS AND DISCUSSION

This section presents the findings from behavior mapping and semi-structured interviews conducted in the Central Library at Chulalongkorn University. Results are organized into three subsections: overall usage and occupancy patterns, proxemic experiences and the privacy–interaction balance, and spatial appropriation strategies and design implications. Interview transcripts were thematically coded using a framework derived from proxemics and environment–behavior theory (Table I).

Notes: the six code groups represent higher-order themes. Individual codes (e.g., F1, S2) represent specific behavioral patterns identified in the data. Participant identifiers P1-P11 indicate the source of each excerpt.

TABLE I INTERVIEW CODEBOOK

Code group	Code	Short definition	Example from interviews
Fixed-feature space	F1 Wall-side preference	Preference for seats next to walls/partitions/columns to support focus or privacy	“If I sit next to the wall, I can focus better on my work, but in the middle of the space I can’t focus.” (P4)
	F2 Window & view preference	Importance of windows, natural light, and outside view for	“I prefer to sit next to the window... I can concentrate better and look at the view when I am stressed.” (P2)

Code group	Code	Short definition	Example from interviews
		comfort and concentration	
	F3 Avoiding circulation/entrances	Avoiding central zones, corridors, or entrances because of busy foot traffic or being too visible	“I avoid the central space because it is next to the corridor and full of busy circulation.” (P3)
	F4 Layout variety & zoning	Perceiving the floor as offering multiple layouts/zones for different activities	“They provide variety of layouts for every function, individual work, meeting room, group collaboration.” (P8)
Semi-fixed space	S1 Preferred furniture type	Likes/dislikes for specific furniture types (individual desks, focus booths, standing tables)	“Individual desk is one of my fav.” (P1) / “Standing table is one of my favorite things.” (P6)
	S2 Furniture adjustment	Changing fixed chairs to movable chairs or rearranging furniture for comfort or group work	“Change the fixed chair to the movable chair... It’s more convenient and comfortable.” (P6)
	S3 Territorial marking (self)	Using bags, books, or bottles to reserve a seat or define a personal area	“I put something down to secure a seat... during midterm or final period.” (P7)
	S4 Territorial marking (others)	Noticing others reserving seats or using objects as boundaries/privacy	“Yes, I used to see someone do that.” (P5)
Proxemics	P1 Preferred distance solo	Desired distance from others when working alone and reasons	“I need a bit of distance... to keep my stuff or talk without bothering anyone.” (P1)
	P2 Distance in group work	Different distance tolerance with group members vs. strangers	“For group work I’m fine to close with my group member but far from other group.” (P9)
	P3 Comfort near strangers	(Dis)comfort sitting beside unknown people and conditions that make it acceptable	“I’m okay to sit next to the stranger who comes alone.” (P10)
	P4 Crowding	Feeling crowded (time/period)	“Around 4:30–5:00 pm it’s too crowded...”

Code group	Code	Short definition	Example from interviews
	discomfort & response	and responses such as leaving or using headphones	normally I leave when it's time." (P1) / "I always use my own earphone with noise cancellation." (P9)
Privacy–interaction balance	B1 Privacy without isolation	Wanting privacy and control while still feeling part of the shared space	"I need privacy but not isolation." (P3) / "Partition helps me focus more without feeling isolated." (P6)
	B2 Focus locations	Zones chosen for deep focus or online meetings (isolated zone, focus zone, wall seats)	"If I need to be focused... I prefer to sit in the Isolated zone; for less focus, the Focus Zone." (P11)
	B3 Social/collaborative locations	Places selected for being social or doing group work (window zone, open area, meeting room)	"For collaborative work, I normally book a meeting room." (P5) / "I love to sit in the window zone for discussion." (P8)
Amenities & comfort	A1 Power & Wi-Fi importance	Importance of power outlets, charging, and Wi-Fi for seat choice and staying put	"They provide everything I need like Wi-Fi, power outlet... the Internet is really stable so I never move." (P11)
	A2 Lighting satisfaction / issues	Positive or negative comments about natural or artificial lighting	"Lighting... excellent/good, especially with the white table." (P5) / "Lighting is really bad for me; I want to change the light color." (P8)
	A3 Thermal & acoustic comfort	Comments on temperature, noise, and acoustic separation	"The temperature... sometimes too cool, sometimes too hot." (P10) / "I love they provide the partition and the acoustic of this space." (P6)
	A4 Food & duration of stay	Role of food/beverage access in choosing the space and length of stay	"Access to food is somewhat/not important for me." (P7)
Design evaluation	D1 Perceived strengths	Features that work well (layout variety, partitions, atmosphere, standing tables, amenities)	"The variety of layouts that they provide... and the partitions and acoustic are what I love." (P8)

Code group	Code	Short definition	Example from interviews
	D2 Perceived weaknesses	Frustrating aspects (lighting, temperature, tables too close, noisy mix of zones)	"Lighting is really bad... the tables are too close to each other... temperature sometimes too cool, sometimes too hot." (P4)
	D3 Desired design changes	Concrete suggestions for redesign or management	"They should separate the noise area from the focus zone... switch the chair and change the light color." (P3)

A. Usage and Occupancy Patterns

Behavior mapping over seven observation rounds (11:00–17:00) showed that the coworking floor shifted from light use in the morning to heavy use in the afternoon. Total occupancy rose from 52 users at 11:00 to 70 at 12:00 and 81 at 13:00, then jumped to between 152 and 175 users from 14:00 to 15:00, and stayed high at 171 and 160 users at 16:00 and 17:00. This pattern indicates that the coworking floor operates as a major afternoon hub, particularly after regular classes finish, mirroring reports that learning commons spaces become focal points during peak study periods [1, 3].

The spatial distribution of users showed a clear preference for perimeter and edge areas. The wall-side zone (Zone A1) consistently attracted the most students, increasing from 10 users at 11:00 to 58 at 14:00–15:00, and remaining above 44 users for the rest of the afternoon. In contrast, the entrance zone (Zone C) was empty in the late morning and only gradually increased to about 10–18 users after 13:00. The isolated focus zone (Zone D) and the window edge (Zone E) grew more steadily over the day, from 8–18 users at 11:00 to peak values of 22 and 45 users, respectively. This concentration around the wall-side and window-edge seats comes from several factors working together. Proxemic comfort is one factor: seats along the edge provide support, reducing the feeling of being watched from behind, and create clearer personal boundaries, which aligns with earlier findings that more enclosed seats are preferred when the space is crowded. However, seat availability, natural light, and access to power outlets also influence these choices. As one participant said, "I prefer to sit next to the window... I can concentrate better and look at the view when I am stressed" (P2), while another noted, "I avoid the central space because it is next to the corridor and full of busy circulation" (P3). Overall, these accounts show that the preference for perimeter zones comes from a mix of spatial environmental and practical reasons, not proxemic factors alone [8, 10].

In contrast, the wall and window zones (A1 and E) were used more for pair and small-group work in the afternoon,

often by groups of two to four students working on projects or discussing ideas. The central and entrance areas (A and C) changed from mainly social use in the late morning to a mix of group and individual activities later in the day. Interviewees said they chose isolated and wall-side seats when they needed deep concentration or had online meetings: “If I need to be focused... I prefer to sit in the isolated zone” (P11), and preferred open or window-edge areas when working with others: “For collaborative work, I normally book a meeting room” (P5)/ “I love to sit in the window zone for discussion. Together, these patterns echo studies of learning spaces that identify a gradient from open, highly visible settings that support social and group activity to more enclosed, peripheral zones dedicated to focused individual work [10, 11].

B. Proxemic Experiences and Privacy–Interaction Balance

Proxemic observations showed that the Focus Zone (B) consistently had the smallest interpersonal distances across the day. At every interval from 11:00 to 17:00, Zone B fell within Hall’s personal distance range, indicating dense clustering of students along the focus tables. In contrast, the zones with the largest distances alternated between the wall-side zone A1, the more open central area (A), and the window edge (E), suggesting that these areas allowed students to maintain social or public-distance spacing when there were enough free seats. This distribution aligns with proxemics theory, which holds that fixed features such as walls and edges structure the range of interpersonal distances that feel acceptable in shared spaces [15].

Interview findings help explain how students understand these spatial and proxemic conditions. Across the eleven participants, window and wall-side locations were repeatedly described as offering a balance between privacy and visibility. Students characterized window-edge seats as providing “good light” and an outside view that reduced stress, while also being away from busy circulation routes and central visibility, which made it easier to concentrate than in the middle of the floor. Others stressed that sitting next to a wall or within the Focus Zone partitions allowed them to focus without feeling completely isolated, reflecting a recurring desire for “privacy but not isolation”. This combination of back support, side shielding, and visual connection echoes environment–behavior studies of learning spaces and coworking environments that highlight walls and partitions as key devices for negotiating territorial control and perceived privacy [8, 13].

Participants also differentiated between acceptable distances for strangers and for collaborators. Some preferred to keep several meters from unknown users during individual study, mainly to avoid overheard conversations or accidental eye contact, but were comfortable with close-personal distances when working with teammates. Others accepted sitting within approximately 0.5-2m of other solo users – corresponding to Hall’s personal-to-social distance range – as long as they had sufficient desk space and could use

headphones, particularly during busy afternoon periods. These accounts resonate with Zhang’s typology of quiet-private versus quiet-public settings in informal learning spaces, but in the Central Library at Chulalongkorn University case, such distinctions are managed less through fully enclosed rooms and more through selective occupation of wall-sides, focus, and window zones [11].

Crowding was most frequently mentioned in relation to the late afternoon. Participants reported that the space felt ‘too crowded’ around 16:00-17:00, yet they typically remained until their planned departure time, relying on noise-cancellation earphones rather than relocating: “Around 4:30-5:00 pm it’s too crowded... normally I leave when it’s time” (P1), while others noted “I always use my own earphone with noise cancellation” (P9). Several participants also reported, based on prior general experience rather than the present observation period, that territorial marking with personal objects became more common during the midterm and final examination periods; these accounts reflect participants’ perceptions and should not be read as directly observed patterns. This relatively high tolerance for crowding, together with the good amenities, suggests that students see the Central Library coworking floor as one of their preferred places to study on campus, similar to other coworking spaces that remain popular even when they are crowded [2, 12].

Several patterns in this study show how local social norms influence student behavior and add nuance to findings from Western learning-space research. First, students often stay in their seats even when the space feels crowded, which may relate to Thai values of maintaining harmony and avoiding actions that disturb or inconvenience others. Second, instead of moving away from other people, many students manage proximity by using headphones, placing objects on the table, or adjusting their chairs, which fits with a collectivist way of sharing space where people quietly protect their boundaries without open conflict. Third, almost all participants cared more about strong Wi-Fi and enough power outlets than about having high spatial privacy, suggesting that technology is a key source of comfort in this setting, similar to patterns reported in some Chinese university studies and less emphasized in Western coworking research. Together, these differences support the idea that the same physical layout can elicit different proxemic behaviors, depending on users’ cultural backgrounds.

C. Spatial Appropriation and Design Implications

Although the behavior-mapping sheet recorded only a few explicit instances of appropriation, the interviews showed that students actively adjusted semi-fixed elements and used personal objects to negotiate territory. Several participants described switching from fixed to movable chairs to achieve a more comfortable posture or to reorient themselves relative to nearby users, and occasionally rearranging chairs when working in groups. Many also reported, based on general usage experience rather than the present observation period, placing bags, books, or water bottles on table to ‘secure a

seat' during busy period: "I put something down to secure a seat.... During midterm or final period" (P07), and had observed others doing the same: "Yes, I used to see someone do that" (P5). These statements describe how participants say they usually use the space and should be understood as self-reported behavior rather than patterns directly observed by the researchers. These practices align with arguments that semi-fixed furniture and personal artifacts act as tools for territorial negotiation and social regulation in shared workspaces [8, 13].

Amenities and environmental comfort influenced seat choice and length of stay across three interrelated dimensions. In terms of technological infrastructure, all interviewees rated power outlets, charging points, and Wi-Fi quality as very important, and most noted that a stable connection removed any motivation to relocate once seated: "They provide everything I need like Wi-Fi, power outlet.... The internet is really stable, so I never move" (P11), pointing to the need for high outlet density and consistent wireless coverage across all zones. Regarding daylighting and views, window zones were consistently described as desirable because natural light and external views supported focus and reduced fatigue, underscoring the value of maximizing daylight access and keeping glazed perimeter seating unobstructed. Finally, in terms of acoustic and thermal comfort, areas near entrances, elevators, and corridors were avoided due to noise and foot traffic: "I avoid the central space because it is next to the corridor and full of busy circulation" (P3), and air conditioning was noted as inconsistent: "The temperature... sometime too cool, sometime too hot"(P10), calling for clearer acoustic zoning between high-traffic and study areas and more responsive thermal controls [1, 2].

Despite generally high satisfaction, students reported recurring problems with artificial lighting, temperature, furniture spacing, and noise between zones. One participant praised the standing tables but criticized the "really bad" lighting color and the tight spacing between tables, which made the area feel visually and physically cramped. Others noted that the air conditioning was sometimes too cold and sometimes too warm, and that the overall atmosphere grew noisy in the late afternoon as student numbers increased. Several suggested a clearer separation between noisy and focus zones, adjustments to lighting color and brightness, more ergonomic movable chairs, and slightly greater distances between tables. These criticisms support the idea that soundproofing, ergonomic furniture, and finely tuned environmental controls are important for successful learning commons and coworking designs. They also suggest that small changes to lighting, zoning, and furniture layout could make people much more comfortable in the Central Library at Chulalongkorn University [1, 3, 5, 14]. Taken together, the results indicate that fixed-feature configuration, semi-fixed elements, and informal proxemic practices collectively shape how students use and appropriate the Central Library coworking floor. Wall-side and window-edge layouts, combined with partitions and reliable technological

infrastructure, help students achieve privacy without isolation and tolerate high densities, whereas shortcomings in lighting, thermal comfort, and acoustic zoning sometimes disrupt this balance. The case thus extends existing environment-behavior and coworking literature into a Southeast Asian university context, illustrating how global design principles intersect with local proxemic preferences [8, 11, 12, 15].

Several methodological limitations should be considered when interpreting these findings. First, behavior mapping was conducted on a single regular teaching weekday between 11:00 and 17:00. This window captures afternoon peak-use patterns well but likely misses morning behaviors – the 11:00 occupancy count of only 52 users suggests qualitatively different dynamics may operate earlier in the day. Patterns on the weekend, during examination period, or across different points in the academic semester are entirely absent from the observational data; accordingly, claims about exam-period crowding and territorial making rest solely on participants' recall rather than direct observation. Second, the purposive sample of 11 participants is appropriate for a qualitative exploratory study but may not represent the full range of student experiences across disciplines, academic levels, or user types. Third, interpersonal distance estimates were based on visual approximations referenced against scaled floor plans, which introduces measurement imprecision, particularly in crowded afternoon conditions. Fourth, since observations were limited to the coworking floor, the study does not support direct comparison with other campus spaces; any such comparisons in the discussion should be understood as contextual framing rather than empirically grounded claims. Future research should expand observations across multiple days and academic periods, increase the interview sample size, and, where feasible, supplement visual distance estimation with a calibrated measurement tool.

XXXI. CONCLUSION

This study examined how spatial configurations in the Central Library at Chulalongkorn University shape students' experiences and utilization patterns in coworking and informal learning zones, using a qualitative single-case approach. Evidence was drawn from eleven semi-structured interviews ($n = 11$) and from systematic behavior mapping conducted on one regular teaching weekday between 11:00 and 17:00, across seven hourly observation rounds. Beyond showing that well-known international spatial design principles also apply here, the study adds a contextual contribution by identifying behavioral patterns, such as students accepting high levels of crowding, staying attached to a chosen seat because of technology access, and using personal objects to mark territory, that seem to reflect local social norms and indicate that proxemic behavior in this Thai university setting cannot be fully explained by Western-based frameworks. Together, the findings indicate that students do not use the coworking floor randomly; instead, they adapt their seating choices and interpersonal distance

based on privacy needs, task type, and the spatial cues provided by the environment.

In response to research question 1, observed utilization patterns differed across zones. Central and open areas supported mixed activities and occasional interaction, while edge conditions and semi-enclosed settings were repeatedly associated with more focused work and longer stays. Seating near walls, window edges, and partition-adjacent positions was frequently preferred, suggesting that boundary conditions and reduced exposure to circulation improve perceived control and comfort in shared environments. Movement-flow observations also indicate that areas with higher pass-through activity were less suitable for sustained concentration, reinforcing the importance of separating circulation from work settings.

In response to research question 2, the spatial elements most strongly influencing student behaviors and proxemic preferences were (1) privacy gradients created by transitions from open zones to more secluded or shielded areas, (2) fixed and semi-fixed boundary features such as walls, partitions, and window edges that support territoriality and reduce unwanted visual contact, and (3) furniture layout and flexibility, which enable users to adjust orientation, manage interpersonal distance, and appropriate space for either individual work or small-group collaboration. Interview accounts further suggest that environmental comfort factors—particularly acoustic conditions and perceived crowding—interact with these spatial elements to shape whether students remain in a zone or relocate to another setting.

Overall, the study suggests that campus coworking and informal learning environments can better support diverse student work modes by providing a clear range of spatial options rather than a single open-plan configuration. Design implications include: organizing layouts to create legible privacy gradients; offering boundary-supported seating for focused work alongside open areas for collaboration; minimizing conflict between circulation paths and study zones; and using semi-fixed elements (movable furniture, partitions, and varied seating types) to enable flexible appropriation while maintaining proxemic comfort. Future research could strengthen inference by expanding observations across multiple days and time periods and increasing the interview sample, enabling comparison across varying occupancy conditions.

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Demonstration of Solar Reforming of Methane for Syngas Production

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Abstract. The development of solar thermochemical technologies has attracted increasing attention as a promising approach for converting methane into value-added products using renewable energy. In solar thermochemical processes, the solar chemical reactor plays a critical role in enabling high-temperature reactions driven by concentrated solar energy. In this study, an indirect tubular solar reactor was developed and experimentally tested for methane reforming. Experiments were conducted at 600 °C under two operating conditions: (i) methane pyrolysis and (ii) methane reforming with CO₂. The evolution of gaseous products was monitored to analyze the reaction behavior in the reactor. The results show that methane reforming reactions can be initiated in the developed reactor. For methane pyrolysis, methane cracking was expected to be the dominant reaction thermodynamically. However, side reactions were observed due to air leakage into the reaction zone. The addition of CO₂ did not significantly enhance dry reforming of methane under the present experimental conditions, and a considerable amount of unreacted CO₂ remained in the system. Overall, the results provided a preliminary experimental demonstration of methane reforming using the developed indirect tubular solar reactor; nonetheless, Improving the sealing mechanisms and achieving tighter system control are key next steps for future development.

Index Terms—Solar reactor, Solar thermochemical conversion, Methane reformin

XXXII. INTRODUCTION

The global shift toward renewable energy is driven by the urgent need to reduce reliance on fossil fuels and mitigate the environmental impacts of greenhouse gas emissions [1]. Solar energy, while abundant and environmentally friendly, faces significant integration barriers due to its inherent intermittency [2]. To circumvent this, solar energy can be harnessed to power endothermic reactions, effectively upgrading it into storable chemical fuels [3]. Within this framework, the dry reforming of methane (DRM) is particularly noteworthy, as it facilitates the simultaneous conversion of two major greenhouse gases into valuable syngas [4].

In solar-driven methane reforming processes, concentrated solar radiation is used to supply the thermal energy required to drive endothermic chemical reactions [5, 6]. In such systems, a solar chemical reactor facilitates the interaction between the reactants and the concentrated solar energy [7]. In packed-bed solar reactors, the reactant gases pass through a bed of solid particles that are heated by the absorbed solar radiation [8]. As the reactants flow through the heated bed, methane reforming reactions occur,

converting methane into valuable products such as hydrogen and synthesis gas.

Various solar reactor configurations have been investigated for thermochemical applications, including directly and indirectly irradiated reactor designs [9, 10]. Among these configurations, indirectly irradiated tubular reactors provide several advantages, such as improved temperature control, protection of reactive materials from direct solar irradiation, and enhanced reactor durability [10]. Nevertheless, the development and experimental validation of solar chemical reactors for methane reforming remain challenging due to issues such as heat losses, reactor stability, and operational control under fluctuating solar conditions.

Therefore, the development and demonstration of practical solar reactor systems are essential for advancing solar thermochemical methane reforming technologies. In this study, an indirect tubular solar reactor was tested for methane reforming and pyrolysis, as described in Eqs. (1) and (2), respectively. Both reactions are highly endothermic and require substantial heat input, particularly suitable for solar-driven thermochemical processes.



The reactor performance was evaluated under two experimental conditions: methane pyrolysis and methane reforming with CO₂ (DRM), which were conducted at 600 °C to assess the preliminary thermal performance and chemical behavior of the developed reactor under non-catalytic conditions. The evolution of gaseous products was monitored to analyze the reaction behavior occurring inside the reactor. The objective of this work is to provide a preliminary experimental demonstration of methane reforming using the developed solar reactor and to provide insights for further reactor optimization and system improvement.

XXXIII. SETUP AND METHODS

Fig. 1 schematically illustrates the experimental setup of the indirect tubular solar reactor for methane reforming. Inside the cavity, a vertical alumina tube with a 20 mm inner diameter is installed as the reaction zone. The cavity is insulated with ISOWOOL ceramic fiber blanket insulation (0.12 W/m·K). An electric heater with a capacity of 2 kW_e is positioned alongside the tube to provide indirect heating, with a heating zone length of 80 mm. A K-type thermocouple was positioned inside the alumina tube to monitor and control the reaction temperature.

Prior to the experiment, the reactor was heated to its operating temperature of 600°C. Once reached, N₂ gas was injected at 200 NmL/min for 10 min to remove residual air. Subsequently, the inlet gas flow rates of N₂ carrier gas with 99.98% purity, CH₄ with 99.999% purity, and CO₂ with 99.98% purity were regulated using digital mass flow controllers (Aalborg DPC17, Aalborg Instruments, USA) range of 0–1.0 NL/min (±0.2% of full scale). The total inlet gas flow rate was 300 NmL/min, and the gas residence time within the heated zone is approximately 1.57 seconds. Each experimental run was maintained for a duration of 10–15 min.

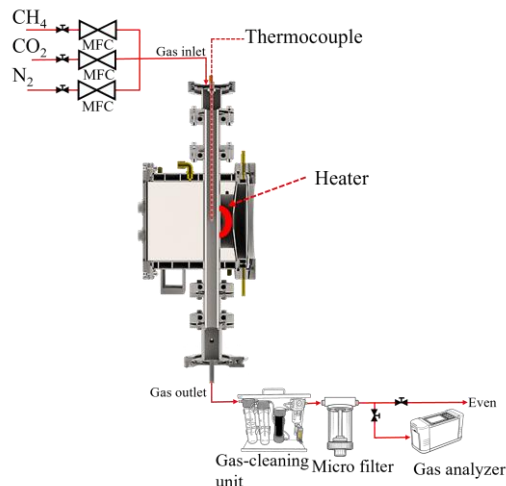


Fig. 1. Schematic diagram of the experimental setup.

At the reactor outlet, the product gas mixture passes through a gas-cleaning unit followed by a micro gas filter to remove excess water and solid carbon. The composition of the dried gas was analyzed using a portable gas analyzer (Gasboard-3100P, Cubic Instruments, uncertainty <2%).

The amounts of byproducts generated (water and solid carbon) were calculated from the atomic mass balance of the outlet gases. The generated H₂O and solid carbon were calculated by hydrogen atom balance (Eq. 3) and carbon atom balance (Eq. 4), respectively.

$$H_{\text{balance}} = \frac{4\dot{Q}_{\text{CH}_4}^{\text{out}} + 2\dot{Q}_{\text{H}_2}^{\text{out}} + 2\dot{Q}_{\text{H}_2\text{O}}}{4\dot{Q}_{\text{CH}_4}^{\text{in}} + 2\dot{Q}_{\text{H}_2\text{O}}^{\text{in}}} \quad (3)$$

$$C_{\text{balance}} = \frac{\dot{Q}_{\text{CH}_4}^{\text{out}} + \dot{Q}_{\text{CO}_2}^{\text{out}} + \dot{Q}_{\text{CO}}^{\text{out}} + \dot{Q}_{\text{C}_s}}{\dot{Q}_{\text{CH}_4}^{\text{in}} + \dot{Q}_{\text{CO}_2}^{\text{in}}} \quad (4)$$

XXXIV. RESULTS AND DISCUSSION

Fig. 2 shows the gas production rates and reaction temperature for methane pyrolysis at 600 °C. Thermodynamically, methane cracking (Eq. 2) is expected to be the dominant reaction under the present conditions. However, O₂ was detected at the reactor outlet, indicating air leakage into the reactor system. As expected, once CH₄ was introduced into the reactor, O₂ decreased and remained constant between 2 and 12 min, indicating that the leaked O₂ reacted with CH₄ via methane combustion or partial oxidation, both of which are exothermic reactions, thereby contributing to the observed temperature rise in the reactor. Under oxygen-rich conditions, complete combustion of methane (CH₄ + 2O₂ → CO₂ + 2H₂O) occurred, leading to increased CO₂ and water formation. In contrast, under oxygen-limited conditions, partial oxidation (CH₄ + 1/2 O₂ → CO + 2H₂) may occur, producing CO and H₂. As a result, minor amounts of H₂ and CO were observed, suggesting that partial oxidation and methane cracking may occur simultaneously. Meanwhile, the presence of CO₂ and the temperature rise indicate that methane reaction with O₂ contributes to the overall reaction pathway. In addition, byproducts such as water vapor and solid carbon could be calculated using an atomic mass balance in Eqs. 3 and 4, yielding approximately 150 mL/min of H₂O and 70 mL/min of solid carbon. These results suggest that methane combustion reactions contributed to water formation, while methane cracking likely led to carbon deposition in the reactor.

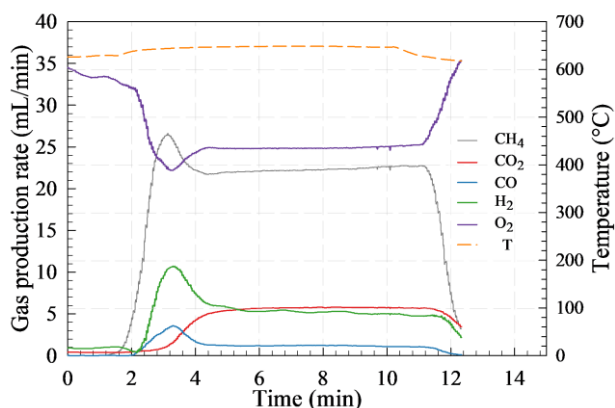


Fig. 2. Gas production rates and reaction temperature during methane reforming without oxidant at 600 °C and 1 bar ($\text{CH}_4 = 100 \text{ mL/min}$, $\text{N}_2 = 200 \text{ mL/min}$).

Fig. 3 shows the syngas production rates and reaction temperature for methane reforming with CO_2 at 600 °C. Similar to the previous experiment, O_2 was detected in the reactor system and reacted with methane after the reactants were introduced. The addition of CO_2 to the reactant stream did not significantly promote the dry reforming of methane ($\text{CH}_4 + \text{CO}_2 \rightarrow 2\text{CO} + 2\text{H}_2$) under the present experimental conditions, as no substantial increase in syngas (H_2 and CO) production was observed. Instead, a considerable amount of unreacted CO_2 remained in the system. Meanwhile, the calculated water vapor and solid carbon were approximately

165 mL/min. This limited conversion is attributed to the relatively low operating temperature for DRM (600 °C)[11]. Furthermore, O_2 from air leakage likely diverted CH_4 toward oxidation pathways rather than the intended DRM reactions.

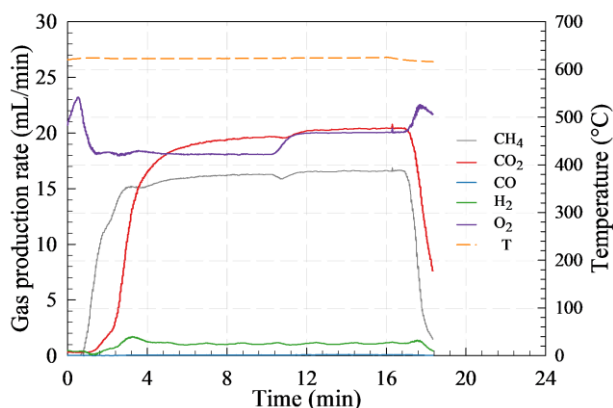


Fig. 3. Gas production rates and reaction temperature during methane reforming with CO_2 at 600 °C and 1 bar ($\text{CH}_4 = 100 \text{ mL/min}$, $\text{CO}_2 = 100 \text{ mL/min}$, $\text{N}_2 = 100 \text{ mL/min}$).

XXXV. CONCLUSION

This study demonstrates the feasibility of methane reforming using an indirect tubular solar reactor. Two experimental conditions were investigated, including

methane reforming without an oxidant and methane reforming with CO_2 at 600 °C. The results show that methane reforming reactions can be successfully carried out in the developed reactor and the evolution of gaseous products can be monitored during the experiments. However, oxygen leakage into the reactor system was observed in both cases, which promoted oxidation reactions involving methane and consequently decreased syngas production.

In the case without CO_2 , methane cracking was expected to be the dominant reaction thermodynamically. Nevertheless, air leakage led to oxidation reactions such as methane combustion and partial oxidation, resulting in only minor syngas formation. Meanwhile, byproducts, including water vapor and solid carbon, were estimated at approximately 150 and 70 mL min⁻¹, respectively. The addition of CO_2 did not significantly enhance dry reforming of methane under the present conditions, and a considerable amount of unreacted CO_2 remained in the system. In addition, the calculated formation rates of water vapor and solid carbon increased to approximately 165 mL min⁻¹, indicating that side reactions remained important in the system.

Overall, the results indicate that air leakage strongly influences the reaction behavior and reduction efficiency of the current reactor system. Consequently, improving the sealing mechanisms is a critical step for future development to ensure a purely reductive environment for optimal thermochemical conversion. Despite this limitation, the present work demonstrates the potential of the developed solar reactor for methane reforming and represents an important step toward further reactor development and system improvement in future studies.

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For RGBD Sensor Calibration by Underdetermined Solutions and Iterative Overdetermined Solutions

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Abstract. This paper presents a method for solving a system of equations to find the parameters of a homogeneous transformation matrix from a Kinect camera to the base of a manipulator. This transformation is applied to coordinate calibration between the camera and the manipulator's end-effector, where the homogeneous matrix consists of rotation and translation parameters. A square system requires at least four manipulator poses to obtain an overdetermined system and solve it using the Iterative Least-Squares (ILS) solution. The objective of this paper is to reduce the number of poses to one, to obtain an underdetermined system and solve it using the Least-Norm solution to check whether the values can still be accurately obtained. As a result, the equations can be solved, but the obtained rotation and translation values are not accurate. Therefore, the Iterative Least-Squares solution was applied again, and noise was added gradually from low to high levels (up to 50 mm) at the end-effector along the x, y, and z axes to test whether this method is robust to noise. The results show that increasing noise levels lead to larger errors as well, reaching larger values in rotation and translation, and increasing the number of poses helps to reduce errors. In addition, The study found that different joint angles of the manipulator also influence errors. In this paper, the joint angles were randomly selected within the specified range.

Index Terms—homogeneous transformation matrix, image noise, iterative least-squares solutions, least-norm solutions, robot position calibration

XXXVI. INTRODUCTION

Currently, in the industrial sector, robotic manipulators are widely used in manufacturing processes, such as robotic arms for welding car bodies or for pick-and-place operations. Within the same workspace as the manipulator, there may be other things operating simultaneously such as conveyor belts, workpieces, human operators, or even other robotic arms. Therefore, the manipulator must be capable of avoiding obstacles and preventing collisions to ensure safety.

There may be different methods to avoid obstacles. This paper will focus on a technique of obstacle detection based on a computer vision approach for real-time tracking of the manipulator's position using a camera. Accurate detection of the manipulator's pose through vision requires precise camera calibration. Therefore, we will emphasize the importance of the camera calibration process in order to accurately identify the position of the manipulator.

Vision-based obstacle detection can be categorized into several data analysis approaches, namely 2D, 2½D and 3D. In 2D [1]-[3], collision detection identifies obstacles by separating them from the background using color information. However, errors may occur if the robot and the

obstacles have similar colors.

In 2½D approaches, [4]-[7] generate surfaces inside a scene using disparity information. On the other hand, [8] reconstruct objects using epipolar parameterization instead of using the disparity images. However, the results are less detailed compared to those obtained from full surface reconstruction methods. In any case, 2½D approaches still rely on clear differences in color or texture between the robot and its surrounding environment.

In 3D approaches, various algorithms have been proposed for surface reconstruction. For example, corner detection can be used to reconstruct a 3D object model [9]. Another algorithm is the shape-from-silhouette algorithm [10]-[12], which constructs 3D surfaces by computing volume intersections from multiple viewpoints. These techniques typically rely on stereo or multiple cameras. However, these reconstructed 3D surfaces usually contain noise, especially when the robot and the background share similar colors or textures.

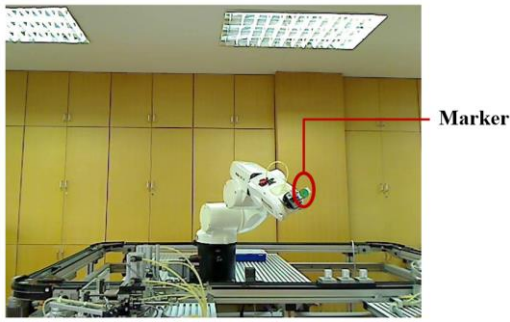


Fig. 1. A workspace consists of obstacle (such as a pneumatic cylinder) and green marker is used to indicate the position of the end-effector.

To solve the aforementioned problem, a depth-sensing device can be directly employed to reconstruct 2½D or 3D surfaces. Currently, there is a new device available that generates less noise compared to stereo camera systems. In this paper, a Kinect sensor is utilized to acquire depth images, including the x and y coordinates.

In this paper, we focus on a method for solving a system of equations to find the parameters of a homogeneous transformation matrix from a Kinect camera to the base of a manipulator. The differences from conventional hand-eye calibration is as follows. The first difference is to reduce the calibration time by taking images only once with one robot pose and solving the equation using the least-norm solution. The second is to compare the least-norm solution and the iterative least-squares solution and how the accuracy differs. The third is that if one solution is more accurate, noise will be simulated to test its robustness to noise. The fourth is to study whether the robot pose has an effect on the accuracy of calibration.

XXXVII. METHODOLOGY

The target is to find the rotation and translation values contained in the homogeneous transformation matrix from the Kinect camera to the base of a manipulator (${}^{cam}_{base}H$). The robot arm used in this study is the Bosch Rexroth AR6 (see Fig. 2.).

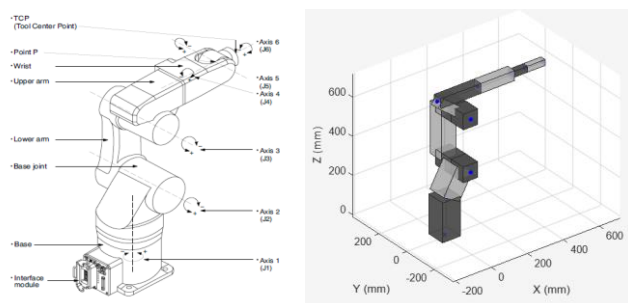


Fig. 2. The configuration of Bosch Rexroth AR6 articulated robot arm [13].

Instead of using a tape measure to measure the distances along the x, y, and z axes and using an inclinometer to measure the tilt angle resulting from the camera installation, which may result in measurement errors, we chose the Kinect camera itself to capture images instead. Capturing images

directly at the base of the manipulator, which has quite a large diameter (approximately 200 mm), may make the position less accurate. Therefore, we chose to capture images at the end-effector where a green marker is attached (see Fig. 1.) because it provides a smaller focus point, resulting in lower position error.

In this paper, MATLAB is used to construct model equations and perform all calculations, beginning with the construction of the homogeneous transformation equations from the camera to the base and from the base to the end-effector. These two parts are combined to obtain the homogeneous transformation relationship from the camera to the end-effector.

Then a simulation is performed by defining the prototype homogeneous transformation from the camera to the base.

The rotation coordinate axes are defined as shown in Fig. 5, and to better reflect real-world implementations, this study follows [14], which suggests using a depth range of 1–3 m. At greater distances, the accuracy of depth measurements decreases due to noise and reduced depth resolution. Therefore, so the example translation distances along x, y, and z are set to (-0.5, 1.0, 3.0) meters. The end-effector coordinate is set to (0,0,0) as a reference position, and the example rotation angles of each joint of the manipulator are randomly generated within the range of ± 75 degrees. The angles are randomly generated four times, corresponding to four image poses. Then MATLAB code is written to find a homogeneous transformation matrix from the camera to the base and it is obtained as the prototype value. Then the experiment is changed to capture one pose and solve the equations using the least-norm solution. Then experiment is then modified to capture 50 poses and solve the equations using the iterative least-squares solution.

The rotation and translation errors are compared to determine the difference between them. If one method is more accurate, noise will be added at the end-effector position (noise that may occur during image capture) by increasing the noise and To better reflect real-world implementations too, noise is applied up to a maximum of 25 mm at depth, follow [14]. A graph will be plotted with the horizontal axis showing the standard deviation of noise and the vertical axis showing the rotation and translation errors in order to test robustness to noise.

XXXVIII. FEATURES AND CALIBRATION OF DEPTH CAMERA

The Microsoft Kinect was originally developed as a motion-sensing device and has been utilized for gaming purposes. It is capable of capturing both color and depth images at the same time (see Fig. 3.). Therefore, we applied it to obstacle detection in a scene due to its high performance and cost-effectiveness. The Kinect is composed of an RGB camera, a depth sensor, and a multi-array microphone (see Fig. 4.). The depth sensor consists of an infrared laser projector and a monochrome CMOS sensor.



Fig. 3. Color and depth images captured from Kinect device.

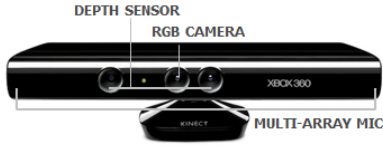


Fig. 4. The configuration and component of Kinect device.

In order to use Kinect for depth measurement, one must first calibrate it using the calibration method described in [15]. Note that the Kinect has an image resolution of 24 bits and returns a raw depth image which contains integer values between 0 and 2047 (11 bits).

XXXIX. TRANSFORMATION BETWEEN THE DEPTH CAMERA AND THE MANIPULATOR

This section describes how to derive a homogeneous transformation between the depth camera and the robot's base (${}^{cam}_{base}H$). This calibration requires a green marker to be attached to the robot's end-effector as shown in Fig. 1.



Fig. 5. Coordinate system of depth camera and manipulator.

First, we define an equation to derive for ${}^{cam}_{base}H$ in world coordinate system in (1).

$$P_{cam} = {}^{cam}_{base}H {}^{base}_{end-eff}H P_{end-eff} \quad (1)$$

Where P_{cam} is a position of the marker in the depth camera coordinate.

$$P_{cam} = [X_c \ Y_c \ Z_c \ 1]^T$$

$P_{end-eff}$ is a position of the marker in the end-effector coordinate. For convenience, we attach the marker at the center of the gripper.

$$P_{end-eff} = [X_{end-eff} \ Y_{end-eff} \ Z_{end-eff} \ 1]^T = [0 \ 0 \ 0 \ 1]^T$$

${}^{base}_{end-eff}H$ is a homogeneous matrix from base of the robot to its end-effector. This matrix is known as we can acquire the robot's forward kinematic relationship. And unknown ${}^{cam}_{base}H$ contains 12 parameters.

$${}^{base}_{end-eff}H = \begin{bmatrix} a_{11} & a_{12} & a_{13} & a_{14} \\ a_{21} & a_{22} & a_{23} & a_{24} \\ a_{31} & a_{32} & a_{33} & a_{34} \\ 0 & 0 & 0 & 1 \end{bmatrix}, \quad {}^{cam}_{base}H = \begin{bmatrix} r_{11} & r_{12} & r_{13} & t_{14} \\ r_{21} & r_{22} & r_{23} & t_{24} \\ r_{31} & r_{32} & r_{33} & t_{34} \\ 0 & 0 & 0 & 1 \end{bmatrix}$$

Here, equation (1) then be represented by equation (2).

$$\begin{bmatrix} X_c \\ Y_c \\ Z_c \\ 1 \end{bmatrix}_{cam} = \begin{bmatrix} r_{11} & r_{12} & r_{13} & t_{14} \\ r_{21} & r_{22} & r_{23} & t_{24} \\ r_{31} & r_{32} & r_{33} & t_{34} \\ 0 & 0 & 0 & 1 \end{bmatrix} \begin{bmatrix} a_{11} & a_{12} & a_{13} & a_{14} \\ a_{21} & a_{22} & a_{23} & a_{24} \\ a_{31} & a_{32} & a_{33} & a_{34} \\ 0 & 0 & 0 & 1 \end{bmatrix} \begin{bmatrix} 0 \\ 0 \\ 0 \\ 1 \end{bmatrix}_{end-eff} \quad (2)$$

To derive the unknown, the first 3 rows of (2) can be rewritten in a different matrix form.

$$Ax = B \quad (3)$$

Where A is a known coefficient matrix.

$$A = \begin{bmatrix} a_{14} & a_{24} & a_{34} & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 \\ 0 & 0 & 0 & a_{14} & a_{24} & a_{34} & 0 & 0 & 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & a_{14} & a_{24} & a_{34} & 0 & 0 & 1 \end{bmatrix}$$

And B contains the position of the marker in the depth camera coordinate.

$$B = [X_c \ Y_c \ Z_c]^T$$

For i , it is the number of data frame that and is indexed with $i = 1, 2, 3, 4, 5, \dots, n$. Our unknown is matrix x that contain 12 parameters of matrix ${}^{cam}_{base}H$.

$$x = [r_{11} \ r_{12} \ r_{13} \ r_{21} \ r_{22} \ r_{23} \ r_{31} \ r_{32} \ r_{33} \ t_{14} \ t_{24} \ t_{34}]^T$$

In order to solve the 12 unknown parameters, 9 rotations and 3 translations, multiple numbers of robot configurations are required.

XL. LEAST-NORM AND ITERATIVE LEAST-SQUARES SOLUTIONS

The least-norm solution is used to obtain a unique solution for an underdetermined linear system [16]-[18]. Among all possible solutions of $Ax = B$, the least-norm solution selects the vector with the minimum Euclidean norm. This solution can be computed using the Moore-Penrose pseudoinverse A^+ , given by $x = A^+B$. If the matrix A has full row rank, the pseudoinverse can be written as

$$A^+ = A^T(AA^T)^{-1} \quad (4)$$

Therefore,

$$x = A^T(AA^T)^{-1}B \quad (5)$$

The iterative least-squares method is an extension of the classical least-squares technique used to estimate unknown parameters in an over-determined system of linear equations [19]-[21]. The objective of the least-squares method is to determine the parameter vector that minimizes the sum of squared residual errors between the observed measurements and the predicted model. From equation $Ax = B$, as A is not a square matrix, x matrix can be derived using pseudo inverse as $x = A_i^+ B_i$.

Where A_i^+ denotes the left matrix pseudo inverse given by

$$A_i^+ = (A_i^T A_i)^{-1} A_i^T \quad (6)$$

Summation matrix A_i^+ and B_i from i to N the equation of iterative least-square method can be written as (7).

$$x = \left(\sum_{i=1}^N A_i^T A_i \right)^{-1} \left(\sum_{i=1}^N A_i^T B_i \right) \quad (7)$$

The error mainly comes from the $^{cam}_{base}H$. This occurs because the camera placement is different each time. In contrast, the $^{base}_{end-eff}H$ is more accurate because it is a constant value, as it is determined by the joint coordinates and the link lengths defined in the configuration of that specific robotic arm. Applying this method in the calculation means that many robot poses will help reduce the error, resulting in a more accurate value of the transformation matrix as well.

XLI. RESULTS AND DISCUSSION

The results for all graphs were obtained from MATLAB calculations, beginning with the least-norm solution, which shows the rotation error in Fig. 6. and the translation error in Fig. 7.

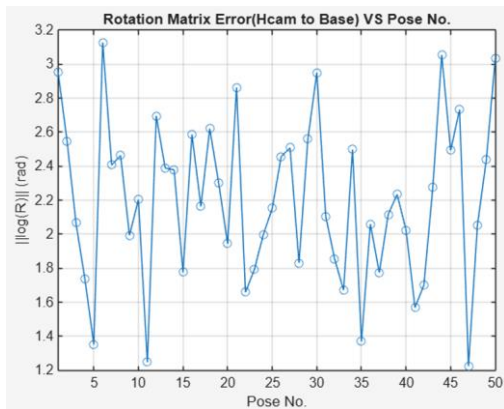


Fig. 6. Rotation error using least-norm solution.

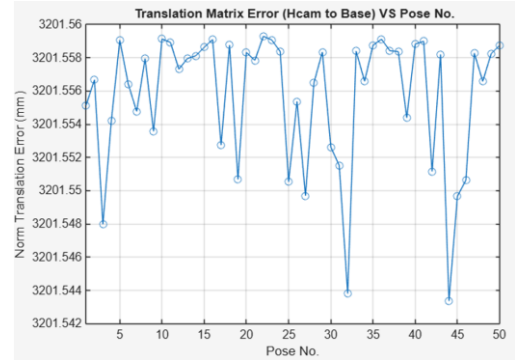


Fig. 7. Translation error using least-norm solution.

The results obtained from calculations using the iterative least-squares solution with noise added up to ± 25 mm show the rotation error in Fig. 8. and the translation error in Fig. 9.

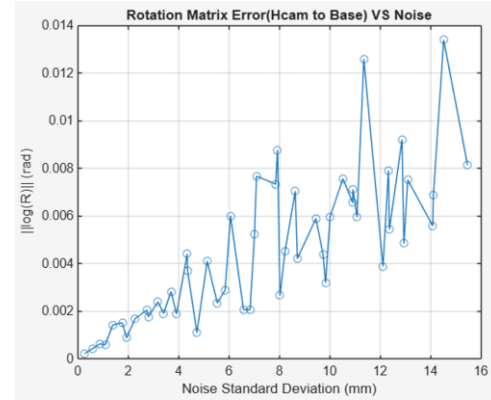


Fig. 8. Rotation error using iterative least-squares solution with increasing of noise.

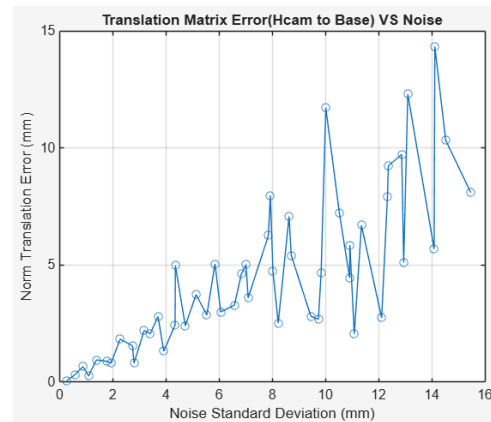


Fig. 9. Translation error using iterative least-squares solution with increasing of noise.

The results obtained from calculations using the iterative least-squares solution with the number of poses ranging from 5 to 50 and random noise within ± 25 mm show the rotation error in Fig. 10. and the translation error in Fig. 11.

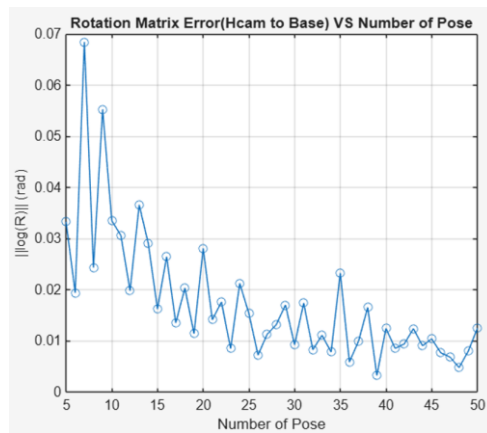


Fig. 10. Rotation error using iterative least-squares solution with increasing number of pose.

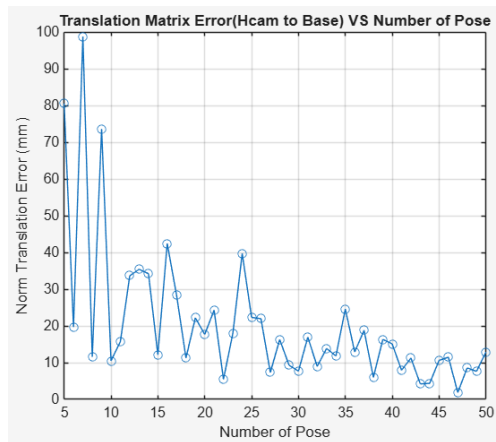


Fig. 11. Translation error using iterative least-squares solution with increasing number of pose.

The results show that using the least-norm solution to solve the equations leads to relatively large rotation and translation errors. In contrast, the iterative least-squares solution produces smaller errors and is more robust to noise. In addition, increasing the number of poses helps reduce the estimation errors.

XLII. CONCLUSION

This paper proposes a calibration method to obtain the transformation matrix between a Kinect camera and the base of a manipulator. The Kinect camera, which has a depth sensor, is used to measure distances in the x, y, and depth directions. The experimental results show that the least-norm solution can solve the system of equations, but the obtained rotation and translation values have large errors. In contrast, the iterative least-squares solution provides more accurate results.

Furthermore, the results show that increasing the number of robot poses can reduce the errors caused by noise. In this study, noise up to ± 25 mm was considered. Therefore, it is recommended to use at least 25–30 poses for the calculation. In addition, each pose should have sufficiently different joint angles (not similar poses) so that the transformation matrix can clearly separate the effects of rotation and translation.

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Mechanical Evaluation of an Adjustable End-Effector for Wood Ear Mushroom (*Auricularia polytricha*) Harvesting

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Abstract. Harvesting Wood Ear mushrooms (*Auricularia polytricha*) in bag cultivation is labor-intensive and highly dependent on skill. In this study, a new end-effector for an automated harvesting system has been designed and tested. The influences of the loading rate and the clearance between the end-effector and the mushroom bag on the detachment behavior of bag-grown wood ear mushrooms were investigated. A 3D-printed mechanical end-effector was tested using a Universal Testing Machine (UTM) under three loading rates, i.e., 200, 250, and 300 mm/min, and three clearance levels, i.e., 5.0, 7.5, and 10.0 mm, with five replications for each treatment. Force–displacement responses were recorded to determine maximum force and total energy required. Results showed that smaller clearance significantly increased the maximum force and energy requirement, while loading rate had no significant effect for the tested range. Analysis of variance associated with Duncan multiple range test indicated that clearance was the dominant factor, and showed that 10-mm clearance required significantly lower force and energy than 5 mm and 7.5 mm. A strong linear relationship between the peak force and total energy was observed with an R^2 of 0.8829, suggesting that peak force can serve as a practical indicator of energy requirement. On the other hand, the smaller clearance of 5 mm resulted in 100% harvesting success rate with no damage, while the clearances of 7.5 mm and 10 mm resulted in lower harvest success rates with some damage. The present study suggested that an end-effector clearance of 5 mm with a loading rate of 300 mm/min can be used for automated harvesting.

Index Terms- Wood Ear Mushroom, Automatic Harvesting, End-effector, Universal Testing Machine.

XLIII. INTRODUCTION

Wood ear mushrooms (*Auricularia polytricha*) are conventionally produced in polypropylene bag systems, although picking remains a manual, time-consuming task. In the production facilities, which contain thousands of cultivation bags, the mushrooms must be harvested within a short time to preserve quality and market price. This results in a labor bottleneck and quality variations. Several research studies have been conducted on developing automatic mushroom cultivation systems covering different tasks, ranging from growing to harvesting. Those systems, however, are highly specific to the mushroom species and local cultivation practice. To the best of our knowledge, a fully automatic system that supports Wood Ear mushroom cultivation in Thailand and surrounding regions has not been developed. In harvesting, the design of an end effector for soft-tissue manipulation is crucial. However, a few studies have been reported regarding the handling concept and the end-effector design for the mechanical harvesting of the bagged Wood Ear mushroom.

Mechanical and physical characterization establishes the design boundaries required for the gentle harvesting of soft biological materials. In representative studies, experiments

using some standardized instruments, such as a universal testing machine, could quantify the effects of geometry and moisture content on mechanical properties such as modulus, failure stress, detachment force, and fracture energy. These parameters define acceptable contact pressures and operating speeds for end-effectors [1], [2], [3], [4]. For bag-cultivated fungi, in-season measurements indicate that failure commonly occurs at the base, specifically at the stipe-substrate interface, and that detachment loads vary with loading rate and pulling angle, directly informing decisions on clearance settings and the selection between shear and twist-lift trajectories [1], [3], [4]. Cross-commodity findings show consistent engineering patterns: with increasing moisture content, bulk density increases, porosity decreases, and static friction coefficients on typical surfaces rise. Such conditions require more conservative gripping and transport parameters to prevent bruising or tearing during wet handling [2]. These established benchmarks underpin the force, pressure, and motion constraints incorporated into the present harvester design.

In fruit and vegetable harvesting, overall system performance depends on three main elements: dependable perception in dense or cluttered canopies, crop-friendly end-

effectors, and properly adjusted motion settings. Reviews and field implementations indicate that selective harvesting improves when 3D geometry is used to locate stems and estimate pose under occlusion, when grippers are compliant or incorporate twist-pluck actions to minimize tearing, and when variables such as approach speed, gripping force, and tool spacing or operating frequency are systematically optimized rather than kept constant [5], [6], [7], [8], [9]. Soft or astringent mechanisms further enhance manipulability by spreading contact stress and conforming to uneven surfaces, thereby reducing bruising risk and increasing tolerance to positional errors [10]. Studies involving greenhouse cucumber, tomato, and asparagus systems consistently report challenges such as occlusion, clustered produce, and the balance between speed and damage; however, satisfactory harvesting success and throughput are achieved once sensing strategies and end-effector parameters are appropriately tuned to the specific crop and environmental conditions [5], [6], [7], [8], [9].

Edible mushrooms pose additional harvesting challenges due to their soft caps, short stems, dense clustering, and limited access when grown on shelves or bag supports. Field-deployed systems for oyster mushrooms demonstrate that vision-guided, crop-specific end-effectors can operate effectively in production settings; however, harvesting success and product integrity remain highly dependent on approach orientation, cluster arrangement, and gentle detachment mechanics [11]. Dynamic investigations on *Agaricus bisporus* have measured loads during twist-lift actions and revealed that detachment forces at the stipe-substrate interface vary with loading rate and pulling angle, guiding controller set-points that minimize bruising and tearing [3]. In the case of *Auricularia*, recent engineering developments follow two main directions: (i) the design of integrated automatic harvesters adapted to bag cultivation and (ii) the optimization of operational parameters such as clearance or spacing, knife or stroke frequency, and feed or tray speed in relation to qualified pick rate, breakage, and throughput. These studies indicated that effective performance depends on defined operating windows rather than fixed parameter values [12], [13]. Together, these findings support our emphasis on end-effector clearance and motion speed as primary design variables, and on bench-scale validation based on measurements of harvesting success rate, damage percentage, and cycle time.

The objectives of the present study were: (i) to design an end effector which is capable of adjusting the clearance between its internal ring and the cylindrical surface of the mushroom bag, and (ii) to investigate the effects of end-effector clearance and loading rate on force and energy requirement, and on the detachment capability of the wood ear mushrooms from substrate bags by using a Universal Testing Machine.

XLIV. METHODOLOGY

The wood ear mushroom samples used in the present study were collected from a local mushroom farm in Kamphaeng Saen district of Nakhon Pathom province, Thailand. In the mushroom house, the mushroom bags were tied into bundles

of 6–7 bags using polypropylene raffia string and suspended from the growing racks (Fig. 1). A mushroom bag is approximately cylindrical, with an average diameter of 120 mm and a height of about 150 mm. The bags were prepared by making eight diagonal slits of about 5 cm in length around the cylindrical surface and one slit on the bottom surface to allow primordia development. The mushroom age at harvesting ranged from 15 to 20 days after fructification induction. The average size of the mushrooms at the harvest stage in this study was 31.15 ± 6.46 mm. The average moisture content of the mushroom samples used in the experiments, as determined by the oven-dry method at a temperature of 60°C for 72 h, was $72.93 \pm 13.74\%$ (w.b.).



Fig 1. Wood ear mushroom growing house at a local farm in Kamphaeng Saen, Nakhon Pathom, Thailand.

A harvesting end-effector has been designed and prototyped as a part of an automatic wood ear mushroom cultivation platform. The end-effector is a fully mechanical module mounted on a circular carrier and fabricated from 3D-printed components to achieve a lightweight yet sufficiently stiff structure for repeatable operation (Fig 2). The structural frame maintains coaxial alignment with the mushroom bag during actuation. The detachment mechanism employs a compression-shear principle, where downward compressive load induces localized shear stress at the stem-substrate interface, resulting in controlled mechanical separation.

To accommodate variations in bag diameter and fruiting body thickness, the radial gap between the contact of the end-effector and the bag surface is adjustable. Interchangeable spacers are used to set three nominal clearances, i.e., 5.0, 7.5, and 10.0 mm. These selected clearances were fixed before testing to ensure dimensional stability during repeated cycles. The mounting interface incorporates a concentric locating feature and a multi-bolt pattern to maintain consistent alignment. All 3D-printed components were designed with dimensional allowances to compensate for fabrication tolerances, and critical holes were post-processed to ensure accurate fitting of mechanical elements.

The harvesting tests have been carried out using a Universal Testing Machine (Instron 5569) set up to simulate the compressive shear motion of the actual harvesting mechanism. The end-effector was fixed to the upper moving fixture machine, while the wood ear mushroom bag was placed on the base, as shown in Fig 3. The end-effector which was mounted to the upper fixture was then triggered to move downward at a specified loading rate, to strip the mushroom

clusters off the bag. The upper fixture of the UTM's moving stroke was set at 150 mm to cover the bag height. When this maximum displacement limit had been reached, the fixture was commanded to stop.



Fig 2. A 3D-printed prototype of the developed adjustable end-effector.



Fig 3. Arrangement of the mushroom bag sample and the end effector with the universal testing machine in mechanical tests.

During the loading test, the force and displacement were concurrently recorded by the UTM's associated software. For each test, the maximum force and total energy were determined from the force-displacement curve. The maximum force refers to the highest force among several peaks appeared in the force-displacement curve in the 150-mm range of loading. The total energy could be obtained by integrating the differential rectangular areas under the force-displacement curve.

The mechanical loading experiments consisted of two independent variables, i.e., the clearance between the end-effector and the outer surface of the mushroom bag, which was varied in three levels: 5, 7.5, and 10 mm, and the loading rate, which included 200, 250, and 300 mm/min. A full factorial experimental design was used with five replications for each treatment. The analysis of variance (ANOVA) associated with Duncan's multiple range test was performed. The p-value of less than 0.05 was considered significant.

The harvesting success rate (*HSR*) and the damage percentage (*D_p*) of the mushroom were assessed according to Eq. (1) and Eq. (2) as follows:

$$HSR = \frac{N_{harvested}}{N_{target}} \times 100\% \quad (1)$$

and,

$$D_p = \frac{N_{damaged}}{N_{harvested}} \times 100\% \quad (2)$$

where *N_{harvested}* is the number of mushroom clusters detached by the end-effector, *N_{target}* denotes the total number of mushroom clusters on the bag, and *N_{damaged}* represents the number of mushroom damaged by the end-effector.

XLV. RESULTS AND DISCUSSION

Representative force-displacement curves for the three clearance levels are shown in Fig 4. The force rose and fell when the end effector started contacting and passed over a mushroom cluster. The first peak in each curve represents the initial contact between the end-effector and the mushroom surface, marking the onset of compressive-shear interaction. For some samples, the force increased more or less continuously if the mushroom clusters were accumulated on the end effector. For the 5-mm clearance, the initial contact peak was approximately 60 N, followed by a higher maximum detachment force of over 100 N. The 7.5-mm clearance showed a similar force pattern with a lower maximum force of about 30 N. With a greater clearance of 10 mm, the force curve did not show obvious peaks like those in the 5- and 7.5-mm clearances, but remained almost constant at approximately 10 N. From Fig. 4, it is quite obvious that the smaller end-effector clearance increased the maximum force required to detach the mushroom.

When considering the averaged peak force values across all loading rates (Table III), the mean peak forces were 70.58 N (5 mm), 61.99 N (7.5 mm), and 25.94 N (10 mm). This confirms that smaller clearance increases mechanical demand during detachment.

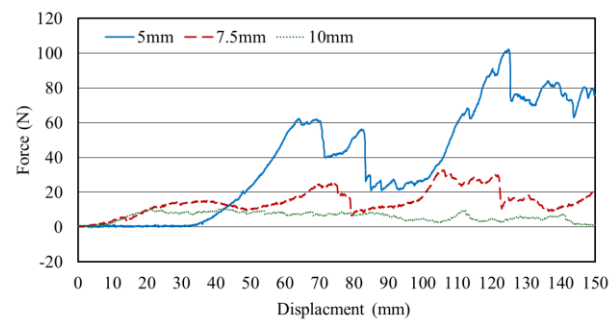


Fig 4. Example of force-displacement curves of different end-effector to bag clearances of 5, 7.5, and 10 mm.

Statistical analysis has been performed to clarify the effects of end-effector clearance and the loading rate on the maximum force and total energy required for mushroom detachment. Analysis of variance associated with Duncan's multiple range test showed that the end-effector clearance was the dominant factor influencing the force and energy

requirement. As shown in Table 1, the greater clearance of 10 mm significantly ($p < 0.05$) required a lower value of force than 5 and 7.5 mm. The force requirements for the clearance of 5 and 7.5 mm were not significantly different. The same pattern was found for the total energy (Table 2).

The effect of loading rate on force and energy was not systematic. Statistical analysis indicated that the loading rate had no significant effect on maximum force and total energy. The ANOVA further suggested that the interaction between the end-effector clearance and the loading rate had no significant effect on both maximum force and total energy.

TABLE I
MAXIMUM FORCE (N) REQUIRED FOR MUSHROOM
DETACHMENT

Loading rate (mm/min)	End-effector clearance (mm)		
	5	7.5	10
200	64.76±28.27 ^{aA}	74.78±35.91 ^{aA}	38.84±17.78 ^{bA}
250	59.12±32.55 ^{aA}	60.02±35.00 ^{aA}	10.50±5.50 ^{bA}
300	87.86±43.43 ^{aA}	51.16±30.79 ^{aA}	28.48±24.03 ^{bA}

*Values in the table are the mean±standard deviation. Values in a row followed by the same lowercase alphabet, and values in a column followed by the same uppercase alphabet are not significantly different at 0.05 level.

TABLE II
TOTAL ENERGY (J) REQUIRED FOR MUSHROOM
DETACHMENT

Loading rate (mm/min)	End-effector clearance (mm)		
	5	7.5	10
200	2.89±1.8 ^{aA}	3.9±1.4 ^{aA}	2.46±1.07 ^{bA}
250	2.64±1.3 ^{aA}	3.16±1.97 ^{aA}	0.45±0.35 ^{bA}
300	4.58±2.23 ^{aA}	2.31±1.98 ^{aA}	1.73±1.56 ^{bA}

*Values in the table are mean±standard deviation. Values in a row followed by the same lowercase alphabet, and values in a column followed by the same uppercase alphabet are not significantly different at 0.05 level.

A relatively wide range of variability in force and energy was observed. This is likely associated with biological differences among individual mushrooms, including variation in moisture content, stem thickness, and attachment strength at the substrate interface, which are inherent to agricultural products.

The relationship between the maximum force and the total energy was observed. Fig. 5 showed a strong positive linear correlation between maximum force and the total energy ($p < 0.001$). This suggests that the maximum force can be a valid descriptor of total energy consumption in mushroom harvesting for the specific end-effector used in the present study, which supports real-time monitoring of harvesting performance.

The harvesting performance quantified by *HSR* and D_p has been observed. The end-effect clearance of 5 mm was significantly more effective in detaching the mushroom from its bag. At this clearance, the *HSR* attained 100% (Table 3). The larger clearances of 7.5 and 10 mm resulted in lower

HSR values. These two sizes of clearance were not significantly different. For the experimental range, the loading rate did not affect the *HSR*.

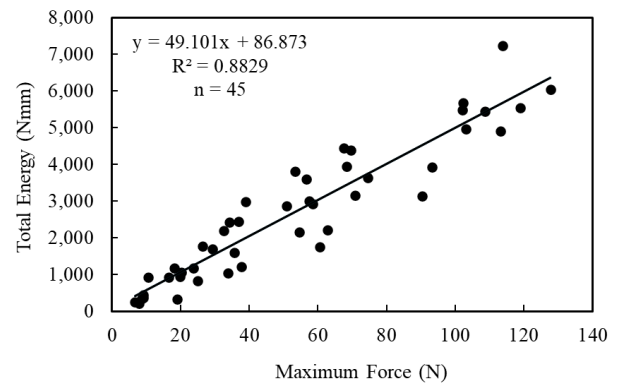


Fig 5. Correlation between maximum force and total energy.

In Table 4, the 5-mm clearance provided zero damage to the mushroom, while the 7.5- and 10-mm clearances caused some damage. This phenomenon is the opposite of our expectation in that the smaller clearance should cause greater damage. During the experiment, it was noticed that the 5-mm clearance provided more complete contact between the end effector and the mushroom. Furthermore, the smaller clearance caused the end effector to contact the mushroom at its basal part, which is a stronger portion, while the greater clearances of 7.5 and 10 mm caused the end effector to contact the mushroom at its edge. This, therefore, caused the mushroom ears to be torn. However, statistical analysis showed that the difference in damage percentage was not significant. Likewise, the loading rate had no significant effect on the mushroom damage.

TABLE III
HATRVESTING SUCCESS RATE

Loading rate (mm/min)	End-effector clearance (mm)		
	5	7.5	10
200	100±0.00 ^{aA}	78.18±12.89 ^{bA}	87.22±12.04 ^{bA}
250	100±0.00 ^{aA}	88.00±17.89 ^{bA}	70.00±44.72 ^{bA}
300	100±0.00 ^{aA}	76.67±32.49 ^{bA}	80.00±20.92 ^{bA}

*Values in the table are the mean±standard deviation. Values in a row followed by the same lowercase alphabet, and values in a column followed by the same uppercase alphabet are not significantly different at 0.05 level.

TABLE IV
DAMAGE PERCENTAGE

Loading rate (mm/min)	End-effector clearance (mm)		
	5	7.5	10
200	0.00±0.00 ^{aA}	4.00±8.94 ^{aA}	7.33±10.11 ^{aA}
250	0.00±0.00 ^{aA}	0.00±0.00 ^{aA}	0.00±0.00 ^{aA}
300	0.00±0.00 ^{aA}	0.00±0.00 ^{aA}	20.00±44.72 ^{aA}

*Values in the table are the mean±standard deviation. Values in a row followed by the same lowercase alphabet, and values in a column followed by the same uppercase alphabet are not significantly different at 0.05 level.

The present study was conducted under laboratory conditions and focused on two primary factors, i.e., end-effector-to-bag clearance and loading rate. However, there might be other factors, such as pulling angle, mushroom maturity variation, cluster density, and ambient temperature and humidity during harvest, that affect the harvesting performance of the device. In addition, further evaluation of the end effector when operated with its completed automated system in real cultivation conditions is necessary. Furthermore, the cycle time and cost benefit of the developed system should be analyzed in addition to the harvest success rate and the damage percentage.

XLVI. CONCLUSION

A new end-effector for wood ear mushroom harvesting has been designed and prototyped to support further development of an automatic mushroom cultivation and harvesting system. The prototype was tested using a universal testing machine to evaluate maximum force and total energy required to detach the mushroom clusters from their substrate bags, as affected by the end-effector and bag clearance, and the loading rate. The results suggested that a clearance of 5 mm with a loading rate of 300 mm/min can be used for automated harvesting. With this condition, the best harvest success rate of 100% and no damage could be achieved. Further development of an automatic harvesting platform for the wood ear mushroom can be carried out using the end-effector developed in this study.

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Retrofitting Strategies with High-Performance Glasses and Shading Devices to Optimize Building Energy Performance in Tropical Climate: A Case Study of a Hotel in Tachileik, Myanmar

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Abstract. This study evaluates retrofit strategies focusing on high-performance glazing and external shading devices to reduce energy consumption and improve cost effectiveness in an existing hotel building in Tachileik. While previous building envelope studies have primarily focused on office, residential, and educational buildings, this research addresses a gap in the hospitality industry, which operates 24/7 despite the country's significant energy constraints. Building energy simulations were conducted using OpenStudio, and calibrated against the actual electricity data, achieving a 3.75% error rate. The study examines the influence of glazing properties, including Solar Heat Gain Coefficient (SHGC), U-value, and Visible Light Transmittance (VLT), together with various shading configurations applied to the south and north guestroom façades, which were identified as the main contributors to cooling demand. The economic feasibility was evaluated using a simple payback period based on material costs and auxiliary expenses. The result shows that the integrated retrofit strategy reflective glazing (SHGC 0.28) combined with 1 m egg-crate shading on the south façade and vertical fins on the north façade achieves the optimal performance among the evaluated scenarios, reducing annual energy consumption by 30% with a payback period of 2.9 years. These findings demonstrate a cost-effective approach to improving energy efficiency in hotel buildings and provide guidance for retrofit strategies in tropical climates.

Index Terms—Retrofitting Strategies, High Performance Glasses, Shading Devices, Tropical Hotel, Building Energy Simulation

I. INTRODUCTION

Buildings represent one of the largest energy-consuming sectors globally, accounting for approximately 36% of global final energy consumption and 37% of energy-related carbon emissions [1]. The hospitality sector is among the most energy-intensive building types due to its continuous 24/7 operation and the need to provide comfortable indoor environments for guests [2]. This demand is particularly significant in tropical climates, where cooling loads account for a large proportion of total building energy consumption [3]. In the context of Myanmar, this demand is significant due to insufficient and unreliable electricity supply [4]. Although Tachileik receives electricity through cross-border towns with Thailand and Laos, energy shortages and power interruptions still occur due to regional energy constraints [5]. These frequent power outages force hotels to rely on expensive fuel generators, making passive envelope upgrades an essential strategy to reduce the cooling loads while minimizing dependence on energy-intensive cooling systems [6].

Retrofitting buildings has become an important approach

to improving existing building performance. Over time, building energy standards have become increasingly stringent, evolving from modest energy reduction targets of around 10% to more ambitious goals of 30–50% improvements in energy efficiency in many national energy policies and building regulations [1]. However, the development of energy-efficient buildings in Myanmar is currently hindered by a lack of mandatory building energy regulations. Consequently, a large proportion of the 74 registered hotels in Tachileik were constructed without energy-efficient design strategies, creating a critical need to evaluate the efficiency of existing buildings. Previous research indicates that building envelope retrofit strategies can significantly reduce cooling loads and improve overall building energy performance in tropical climates [7] [8]. In tropical climates, solar heat gain through glazing systems can significantly increase cooling demand [9]. Therefore, glazing systems with lower Solar Heat Gain Coefficient (SHGC) values are generally preferred in warm climates because they reduce the amount of solar radiation entering the building [10]. Shading devices also plays an important role on building envelope energy performance [11] [12].

A study conducted on a high-rise office building with a window-to-wall ratio of 1 in Malaysia suggested that a 600 mm shading depth could effectively balance solar shading and daylight penetration [13]. However, such studies often focus on office and educational buildings, with daytime occupancy primarily focused on daylighting, and do not fully represent the 24/7 operational profiles of hotels in tropical climates. Furthermore, existing research often overlooks the critical link between energy performance and practical economic feasibility. To address these research gaps, this study investigates façade retrofit strategies for a representative hotel building in Tachileik using calibrated energy simulation. The objective of this research is to identify retrofit solutions that achieve at least 30% reduction in energy consumption while maintaining a payback period of less than five years, providing a practical guide for hotel owners to balance energy savings with investment costs.

II. METHODOLOGY

The study employs a quantitative research approach, following the workflow illustrated in Figure 1. Building energy performance was evaluated using OpenStudio, which was selected as the primary building energy simulation tool due to its suitability for analyzing and optimizing building energy performance [14]. The building model was developed in SketchUp using the OpenStudio plug-in, and simulate building energy in the OpenStudio Program. Due to the lack of EPW weather file for Tachileik, the nearest available climate file from Chiang Rai was utilized as a proxy, given their similar geographic and climatic characteristics. A baseline simulation model was developed and validated using the actual electricity consumption data of the case study hotel. The model was calibrated until acceptable agreement was achieved and subsequently used to evaluate various retrofit scenarios, including whole-building glazing replacement, shading devices applied to the south façade, north façade, and combined south-north façades, as well as integrated strategies combining both glazing and shading improvements. The objective of these simulations was to identify retrofit configurations that achieve higher energy savings while maintaining shorter payback periods. To assess the broader applicability of the proposed retrofit strategy, the validated building model was further simulated in different tropical climate conditions while maintaining identical building parameters.

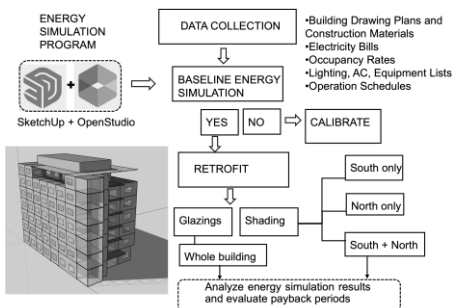


Fig. 1. Workflow diagram

III. SITE AND SETTING

The case study building is located in Tachileik, at latitude 20°27'11.2" N and longitude 99°53'19.9" E (Figure 2). The region is characterized by a hot and humid tropical climate, classified as ASHRAE Climate Zone 1A. To ensure the generalizability of the findings, the building was selected as a representative model for the 74 registered hotels currently operating in Tachileik. Due to the nature of urban density of this border town, most hospitality infrastructures follow a similar elongated, mid-rise architectural typology and window-to-wall ratio design as shown in Figure 2. The building specifications used for the baseline energy simulation are summarized in Table 1. The typical floor layout, illustrated in Figure 3, shows that the building has an elongated plan configuration, with guest rooms arranged along both the north and south façades. This geometry is a dominant feature of the local existing building, making façade design and solar exposure the primary factors influencing cooling energy demand. To maintain a scalable baseline, the study specifically focuses on guestroom-dominant energy loads, excluding specialized facilities such as event spaces and fitness center, which may result in higher energy consumption. Consequently, the building serves as a representative case for evaluating retrofit strategies applicable to the standard hospitality typology in a tropical climate.



Fig. 2. Exterior View of Case Study Hotel

TABLE 1. BUILDING SPECIFICATION OF CASE STUDY HOTEL

Building Parameters	Specification
Service Life	13 years
Orientation	East-West elongated
Floor Area	4094 m ²
Number of floors	7 floors +service floor
Floor-to-floor height	3.51m
Building Layout	34.56m x 19.04m
Total guestrooms	70
Wall Type	14cm brick (k= 0.473 W/m.K) 1.5cm plaster at both sides
Window Type	Single Clear 6mm (U-value = 5.74 W/m ² .K, SHGC = 0.82, VLT = 0.88)

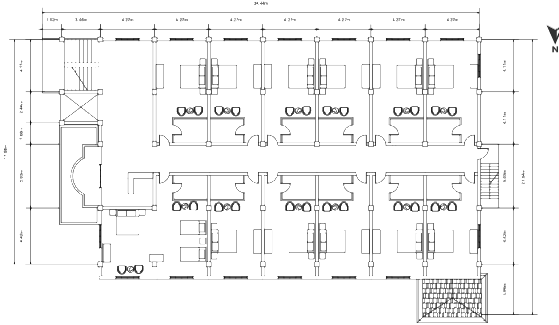


Fig. 3. Typical Floor Plan

VI. BASELINE SIMULATION

The baseline energy model was calibrated using operational data from the case study hotel to ensure reliability. Operational schedules were derived from staff interviews and on-site observations, establishing an average 16-hour guestroom occupancy profile which linked to the hotel's keycard systems, while cooling setpoints were maintained at 25 °C during occupied hours. Lighting in common areas was scheduled from 6:00 PM to 6:00 AM. Following ENERGY STAR guidelines, which allow for a 12-month average for fluctuating occupancy, an average 50% annual occupancy rate was applied to reflect the current operational reality in Tachileik. The guestroom spaces were categorized into 'occupied' and 'vacant' space types to align with actual energy usage. The resulting annual consumption of 357,042 kWh showed a minimal 3.75% variance from the actual billing data of 344,149 kWh, validating the reliability of these operational assumptions.

V. WHOLE BUILDING GLASS RETROFIT

The glazing specifications presented in Table 2 were obtained from a Thai glass manufacturer that complies with the standards of the National Fenestration Rating Council (NFRC)[15]. The dataset provides key thermal and optical properties of glazing systems, including U-value, Solar Heat Gain Coefficient (SHGC), and Visible Light Transmittance (VLT). A total of ten glazing types were selected for evaluation, including 6 mm single-pane tinted and reflective glass as well as 24 mm double-glazing systems (e.g., G7: 6 mm + 12 mm air gap + 6 mm) and double low-E glazing. In the glazing notation, #2 indicates the low-emissivity coating applied on the inner surface of the exterior glass pane, which helps reflect heat back toward the exterior and reduce solar heat gain entering the building. These glazing options were chosen to investigate how variations in thermal and solar properties influence the energy consumption.

The case study hotel has an overall window-to-wall ratio (WWR) of 23.64%, with façade distributions of 16.63% on the north façade, 36.05% on the east façade, 29.97% on the south façade, and 12.56% on the west façade. To evaluate the impact of glazing retrofit strategies, energy simulations were conducted by replacing the existing glazing with the selected alternatives while keeping all other building parameters unchanged.

TABLE 2. PROPOSED GLASS SPECIFICATIONS

Code	Glass Type	Thickness (mm)	U-value (W/m ² .K)	SHGC	VLT
Tinted					
G1	Ocean Green	6	5.25	0.6	0.73
G2	Energy Green	6	5.25	0.5	0.6
G3	Euro Grey	6	5.25	0.61	0.45
Reflective					
G4	Dark Grey	6	4.25	0.35	0.22
G5	Dark Green	6	3.99	0.28	0.13
G6	Dark Blue	6	3.93	0.28	0.1
Double Glazing					
G7	G1+ Air + Clear	24	2.84	0.48	0.65
G8	G3+ Air + Clear	24	2.84	0.49	0.4
Double Low-E					
G9	Vision50 #2+ Air + Clear	24	1.6	0.29	0.49
G10	Smart30 #2+ Air + Clear	24	1.6	0.21	0.29

Among the evaluated single glazing types, reflective glass G6 with SHGC of 0.28 achieved the highest energy saving of 21%. slightly outperforming G5, which has a comparable SHGC value but marginally higher U-value and VLT. Reflective glazing types demonstrated better energy performance than double-glazing systems because their lower SHGC effectively reduced solar heat gain entering the building.

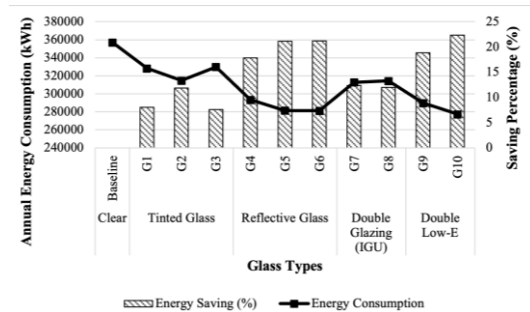


Fig. 4. Comparison of Annual Energy Consumption and Saving Percentages of Different Glass Types

In the case of the double-glazing alternatives, low-E glazing G10 demonstrated the best thermal performance due to its improved solar control and enhanced insulation properties. Although all glazing alternatives contributed to some degree of energy reduction, their economic feasibility varies depending on the material costs and resulting payback periods.

To evaluate the economic performance of the glazing retrofit scenarios, glass prices shown in Table 3 were obtained from a local supplier in Chiang Rai inquired by the author in February 2026, as material costs may vary by region. The simple payback period was determined by

dividing the total initial cost by the annual energy cost savings. Annual savings were calculated using the current electricity price of 7.2 Thai Baht per kWh in Tachileik. The total initial investment cost was estimated based on the total glazing area of 636 m², multiplied by the unit price of each glazing type. An auxiliary 40% cost factor was included to account for regional logistics and skilled labor costs, acknowledging that additional site-specific construction overheads may apply.

TABLE 3. PAYBACK PERIOD FOR GLASSES

Code	Glass Price (THB/m ²)	Total Initial Cost (THB)	Annual Saving (THB)	Payback Year
G1	229	203902	207079	1
G2	323	287421	305266	0.9
G3	506	450293	195559	2.3
G4	915	814360	458626	1.8
G5	915	814360	544205	1.5
G6	915	814360	545119	1.5
G7	4304	3832282	318586	12
G8	4842	4311317	307721	14
G9	6133	5461001	484805	11.3
G10	6133	5461001	574366	9.5

VI. ANALYSIS OF SHADING DESIGN

Table 4 presents the solar position data for the case study location in Tachileik, Myanmar (20°27'11.2" N, 99°53'19.9" E). The solar altitude and solar azimuth angles were obtained using the Andrew Marsh Sun Path tool [16] for three representative dates: the spring/autumn equinox (March 21/September 21), the summer solstice (June 21), and the winter solstice (December 21). For each date, solar positions were evaluated at 8:00 AM, 12:00 PM, and 4:00 PM.

TABLE 4. SOLAR ALTITUDE AND AZIMUTH ANGLES ON THREE REFERENCE DAYS

Study Time	Mar 21 and Sept 21		June 21		December 21	
	Alt	Azi	Alt	Azi	Alt	Azi
8:00am	28.53	101.4	36.44	75.38	19.22	125.4
12:00pm	69.8	181.5	86.51	329.3	46.04	183.9
4:00pm	27.59	259.1	32.94	285.3	14.67	237.6

Solar geometry analysis was conducted using solar altitude and azimuth angles to determine the Horizontal Shadow Angle (HSA) (1) and Vertical Shadow Angle (VSA) (2). Based on these angles, the required shading dimensions were calculated using geometric relationships for overhang depth and vertical fin depth, considering the window height and window width of the guestroom façade [17]. Equations (3) and (4) were used to determine the depths of the overhang and vertical fins.

$$\text{HSA} = \text{Solar Azimuth} - \text{Wall Azimuth} \quad (1)$$

$$\text{VSA} = \tan^{-1}(\tan(\text{Solar altitude}) / \cos(\text{HSA})) \quad (2)$$

$$\text{Overhang Depth} = \text{Window Height} / \tan(\text{VSA}) \quad (3)$$

$$\text{Vertical Fin Depth} = \text{Spacing} / \tan(\text{HSA}) \quad (4)$$

During the summer solstice, the sun path shifts toward the northern side of the building; therefore, shading calculations focused primarily on the north façade. During the equinox and winter solstice, solar exposure is more significant on the south façade, and shading requirements were analyzed accordingly. The calculated angles and corresponding shading depths are summarized in Table 5. Some calculated vertical fin depths resulted in negative values, indicating that the sun is positioned behind the façade and does not produce direct solar exposure. The results indicate that a minimum overhang depth of approximately 0.5 m is required for the south façade during most daytime periods, while greater depths may be required under certain conditions, such as winter noon and afternoon. For the north façade, smaller overhang depths (0.5-0.6 m) and vertical fin depths around 0.6m were identified. However, these geometric calculations only consider direct solar obstruction and do not account for diffuse radiation, reflected heat, or thermal interactions within the building envelope. Therefore, a range of overhang and vertical fin depths was further evaluated through energy simulation.

TABLE 5. CALCULATION OF HSA, VSA, SHADING DEPTH FOR SOUTH AND NORTH FACADES

Study Time/ Facade	Wall Azi	HSA	VS A	Overhang Depth	Vertical Depth
Mar 21 & Sept 21/ South					
8:00 AM	180	79	70	0.5	-0.5
12:00 PM	180	1	70	0.5	92.9
4:00 PM	180	79	70	0.5	0.5
Dec 21/ South					
8:00 AM	180	55	31	2.3	-1.7
12:00 PM	180	4	46	1.3	35.4
4:00 PM	180	58	26	2.8	1.5
Jun 21/ North					
8:00 AM	0	75	71	0.5	0.6
12:00 PM	0	329	87	0.1	-4.0
4:00 PM	0	285	68	0.6	-0.7

VII. ENERGY SIMULATION OF SOUTH FAÇADE SHADING

Based on the solar geometry analysis, shading depths ranging from 0.5m to 1.2m were selected for further evaluation, as these dimensions represent feasible structural

depths that can be implemented without additional structural supports. While the solar analysis considered only three representative dates, the energy performance of the proposed shading configurations was assessed using an annual building energy simulation. The configurations of the proposed shading systems are illustrated in Figure 5. The sub-surface shading, which covers only the window area, and integrated shading, which extends across the entire façade, also shade portions of the wall surface. In addition, egg-crate shading configurations combining horizontal and vertical elements were evaluated for both sub-surface and integrated applications. For the integrated shading configurations, vertical fins were arranged according to the room-to-room spacing rather than the window width. This approach was adopted to better accommodate passive design strategies, including daylight penetration and natural ventilation through operable windows in the guestrooms.

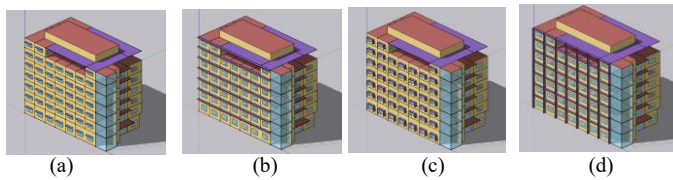


Fig. 5. (a) Overhang Sub-Surface (b) Overhang Integrated (c) Eggcrate Sub-Surface (d) Eggcrate Integrated

The results indicate that increasing shading depth gradually reduces the annual energy consumption of the building. In general, every 0.1 m increase in shading depth results in a small but consistent reduction in cooling energy demand, demonstrating the effectiveness of external shading devices in limiting solar heat gain on the south façade. Among the four shading configurations, the eggcrate integrated shading achieves the highest savings, while overhang sub-surface design performs the least as shown in Figure 6. Notably, the eggcrate sub-surface configuration performs less efficiently than the overhang integrated design, indicating that shading applied only to window areas is less effective than full façade coverage. This suggests that solar heat gain through both glazing and wall surfaces contributes significantly to overall cooling load, highlighting the importance of integrated façade shading in tropical climates.

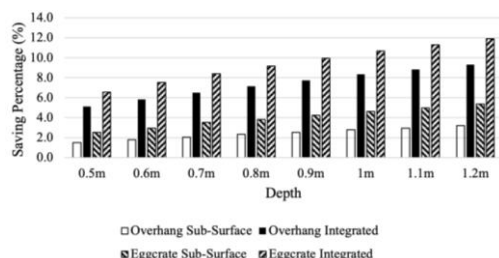


Fig. 6. Comparison of Saving Percentages for South Shading Designs

The shading devices evaluated in this study are assumed to be constructed using Aluminum Composite Panel (ACP) with a thickness of 0.5 mm, A2 fire-resistant core, and double-layer coating. A base material cost of 1,800 Baht/m² was adjusted by a 40% multiplier to incorporate logistics and labor overheads. For the sub-surface shading configuration, the retrofit is applied to 45 windows, each with a width of 2.4 m. The integrated overhang design spans across the façade with seven continuous overhang elements covering a total length of 30.1 m. In the eggcrate sub-surface configuration, each window requires one horizontal overhang and two vertical fins, resulting in 135 shading elements in total for each depth scenario. In contrast, the integrated eggcrate configuration requires only seven horizontal overhangs and eight vertical fins, extending across the façade height of 24.84 m, which reduces the number of shading components despite the larger façade coverage. The results in Table 6 indicate that the integrated overhang configuration achieves the shortest payback period, ranging from 2.0 to 2.7 years, due to its relatively lower material costs and effective energy savings.

TABLE 6. PAYBACK YEARS OF SOUTH SHADING

Depth	Overhang		Eggcrate	
	Sub-Surface	Integrated	Sub-Surface	Integrated
0.5m	3.5	2.0	4.6	3.1
0.6m	3.5	2.1	4.7	3.2
0.7m	3.6	2.2	4.6	3.3
0.8m	3.6	2.3	4.9	3.5
0.9m	3.7	2.4	4.9	3.6
1m	3.8	2.5	5.0	3.8
1.1m	3.9	2.6	5.1	3.9
1.2m	4.0	2.7	5.2	4.0

VIII. NORTH FAÇADE SHADING

Although solar geometry analysis provides an initial estimate of shading dimensions, energy simulation is required to evaluate their thermal performance under real climatic conditions. Therefore, shading depths of 0.6 m, 0.8 m, 1 m, and 1.2 m with integrated design were tested through energy simulation to determine the optimal configuration.

The simulation results indicate that increasing the shading depth improves building energy performance on the north façade; however, the improvement is limited due to lower solar exposure. Although deeper shading devices can reduce diffuse solar gains and enhance façade shading effectiveness, the overall impact remains relatively small. Table 7. shows that increasing shading depth from 0.6m to 1.2m leads to a gradual improvement in energy savings for all shading type. Among the three shading types, eggcrate achieves the highest energy savings but results in higher investment cost and longer payback periods, while vertical fins provide slightly higher

savings than overhangs with shorter payback periods. These results suggest that shading on the north façade has limited impact and is less economically effective when applied alone. Therefore, simpler and cost-effective shading solutions are more appropriate, particularly when used in combination with other façade retrofit strategies.

TABLE 7. SAVING PERCENTAGE AND PAYBACK YEARS OF NORTH SHADING

Type	Depth	Saving Percentage (%)	Payback Year
Overhang	0.6	1.0	11.0
	0.8	1.2	12.1
	1	1.4	12.6
	1.2	1.6	13.6
Vertical Fin	0.6	1.1	9.1
	0.8	1.4	9.0
	1	1.8	9.2
	1.2	2.0	9.7
Eggcrate	0.6	1.6	12.8
	0.8	2.0	13.6
	1	2.3	14.5
	1.2	2.6	15.5

XLVII. COMBINATION OF SOUTH AND NORTH SHADING

Based on the results of the individual façade shading analysis, a combined shading strategy was developed by integrating the most effective configurations from the south and north façades. To streamline the analysis while maintaining representative cases, the 0.6 m depth was excluded due to its consistently low energy savings in both façade studies. As shown in Table 8, a matrix of nine scenarios (S1–S9) was established by combining three selected shading depths (0.8 m, 1.0 m, and 1.2 m) for both façades, incorporating variations of horizontal overhangs and vertical fins. This approach enables the study to systematically evaluate the combined effects of shading devices on overall building energy performance.

The results in Table 9 indicate that increasing shading depth enhances energy performance by improving solar control on both façades. However, this improvement also leads to higher initial costs, resulting in longer payback periods. Rather than relying solely on deeper shading, the results suggest that performance can be optimized through an orientation-based approach. More effective shading configurations, such as eggcrate systems, are better suited to the south façade where solar exposure is higher, on the other

TABLE 8. SHADING DESIGNS MATRIX OF SOUTH AND NORTH

Code	South		North	
	Overhang (m)	Vertical Fin (m)	Overhang (m)	Vertical Fin (m)
S1	0.8	0	0	0.8
S2	1	0	0	1
S3	1.2	0	0	1.2
S4	0.8	0.8	0	0.8
S5	1	1	0	1
S6	1.2	1.2	0	1.2
S7	0.8	0.8	0.8	0.8
S8	1	1	1	1
S9	1.2	1.2	1.2	1.2

hand, simpler elements such as vertical fins are appropriate for the north façade due to their lower cost and sufficient performance under lower solar heat gain. This combination provides a balanced trade-off between energy savings and investment cost, making it appropriate for further integration with glazing retrofit strategies.

TABLE 9. SAVING PERCENTAGE AND PAYBACK YEARS OF SOUTH AND NORTH SHADING RETROFIT

Case	Saving Percentage (%)	Payback Year
S1	8.6	3.4
S2	10.1	3.7
S3	11.3	3.9
S4	10.6	4.3
S5	12.4	4.5
S6	13.9	4.9
S7	11.1	5.3
S8	13.0	5.7
S9	14.5	6.1

XLVIII. INTEGRATION OF GLASS AND SHADING RETROFIT

The glazing and shading options were selected based on their energy-saving performance and payback period from previous analyses. The three best-performing glazing types—G2 (tinted glass), G6 (reflective glass), and G10 (low-E glass)—were combined with the selected shading configurations (S2, S3, S5, and S6) that achieved payback periods of less than five years. By integrating the three glazing types with the four selected shading configurations, a matrix of twelve integrated retrofit scenarios (C1–C12) was established in Table 10.

TABLE 10. INTEGRATED RETROFIT MATRIX OF
GLAZING AND SHADING

Glazing Type	S2	S3	S5	S6
G2 (Tinted)	C1	C2	C3	C4
G6(Reflective)	C5	C6	C7	C8
G10 (Low-E)	C9	C10	C11	C12

The integrated retrofit cases demonstrate that combining glazing and shading strategies achieves significantly energy reductions of up to 31.8%. The optimal configuration (Case C7), combining reflective glazing with eggcrate shading on the south facade and vertical fins on the north façade, achieved a balanced performance of 30.3% energy savings with a payback period of 2.9 years, meeting the study objective of achieving at least 30% energy reduction with a payback period below five years. This result highlights the synergistic effect of reducing solar heat gain through both glazing and shading design. Compared to previous studies in Thailand, where combined building envelope and system retrofits achieved up to 49% energy savings [18], the present study focuses on façade interventions; therefore, further energy savings could be achieved by incorporating building system upgrades.

TABLE 11. SAVING PERCENTAGE AND PAYBACK
YEARS OF GLAZING AND SHADING

Case	Saving Percentage (%)	Payback Year
C1	20.8	2.3
C2	21.8	2.5
C3	23.0	2.9
C4	24.2	3.2
C5	28.2	2.4
C6	29.1	2.6
C7	30.3	2.9
C8	31.3	3.2
C9	28.7	8.69
C10	29.6	8.68
C11	30.7	8.7
C12	31.8	8.82

XLIX. RESULTS AND DISCUSSION

The simulation results indicate that building envelope retrofits can significantly improve the energy performance of the case study hotel. Among the evaluated single glazing types, reflective glass with an SHGC of 0.28 demonstrated the most significant energy reduction, achieving approximately 21% with a payback period of 1.5 years. While a previous study on an office building in India with 66.98% glazing percentage achieved 62.23% with a payback period of 5.41 years [19], highlighting that variations in window-to-wall ratio also influence overall energy performance. An optimized 1 m egg-crate shading on the south façade achieved approximately 12% energy savings,

consistent with a study in Malaysia [13], showed that the egg-crate shading devices outperform vertical and horizontal shading in hot-humid climates. However, shadings on the north façade contributed only 3% of energy saving due to lower solar exposure. When shading devices were applied to both façades, energy savings increased further. In integrated scenarios combining glass and shading on both façades, energy savings ranged from 20.8% to 31.8%. Within the scope of this study, the combination of reflective glazing with 1m egg-crate shading on the south façade and vertical fins on the north façade yielded the optimal balance between energy efficiency and cost-effectiveness. This configuration achieved a 30.3% reduction with a payback period of 2.9 years. The relatively rapid payback period obtained in this study is mainly due to high electricity price in Tachileik, which amplifies the financial impact of energy savings. While these estimates are based on regional averages, actual costs may vary depending on construction complexities and market fluctuations.

To evaluate the applicability of the optimal retrofit strategy beyond the case study, the validated building model was applied to multiple tropical cities, including Bangkok, Chiang Mai, Yangon and Mandalay. In this analysis, the same building geometry, construction properties, and operational schedules were maintained, while only the weather data were varied. The results in Table-12 indicate that baseline energy consumption differs significantly across climates. the proposed retrofit strategy consistently achieves energy reductions from 21% to 30%. The highest saving is observed in the case study location, while Bangkok is found to have relatively lower performance. These findings demonstrate that the proposed façade retrofit strategy is adaptable across tropical regions. However, the economic analysis was conducted only for the case study location, and payback periods may vary in other regions due to differences in electricity tariffs, material and construction costs. Compared to previous studies of residential envelope retrofits in Thailand have demonstrated energy reduction of 49.36% with 4.36 payback years [18]. Another study in Ghana found passive design strategies reduced 48-50% within 6-10 years [20]. As this study focused exclusively on two envelope strategies, future research should explore building system upgrades, such as HVAC and lighting optimization to improve the energy performance.

TABLE 12. MULTI-CITY COMPARISON OF
BASELINE AND RETROFIT PERFORMANCE

Region	Baseline (kWh)	Retrofit (kWh)	Energy Reduction (%)
Tachileik	357042	249033	30.25
Bangkok	470000	370472	21.18
Chiang Mai	366711	264647	27.83
Yangon	427631	323508	24.35
Mandalay	448581	334853	25.35

L. CONCLUSION

This study investigated the effectiveness of façade retrofit strategies focusing on glazing replacement and exterior shading devices to improve the energy performance of an existing hotel in a tropical climate. The findings show that reducing solar heat gain through the building envelope improvements is a key strategy to lowering cooling energy demand in hot-humid regions. Reflective glazing significantly improves energy performance due to its lower SHGC value, while shading devices further enhance façade performance by reducing solar radiation. The integrated solution, combining reflective glazing with optimized shading devices, achieved approximately 30% energy reduction with a payback period of less than three years, demonstrating a balance between energy efficiency and economic feasibility. This is particularly relevant in the context of Myanmar, where unreliable electricity supply and high operational costs increase the importance of passive design strategies. Furthermore, the findings demonstrate that the proposed retrofit strategies are applicable to similar mid-rise hotel buildings in tropical climates, providing consistent energy savings under different climatic conditions. Across the evaluated cities, the strategy achieved energy reduction ranging from approximately 21% to 30%, despite differences in baseline energy consumption. These results highlight the potential of integrated façade retrofit strategies as a practical and cost-effective solution for enhancing energy efficiency in existing hotel buildings, and supporting informed investment decision-making.

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RSSI-based Indoor Positioning System Using ESP32 with Trilateration Algorithm

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Abstract. As location-based services become increasingly integral to modern infrastructure, positioning systems have become a fundamental component across various sectors, including logistics, healthcare, and security. However, the Global Positioning System (GPS), which serves as the international standard for outdoor positioning, faces severe performance limitations when deployed indoors due to signal attenuation caused by building structures and obstacles. This paper presents the development and study of an Indoor Positioning System (IPS) by applying the ESP32 microcontroller, a high-performance and cost-effective device, in conjunction with WiFi technology to measure the Received Signal Strength Indicator (RSSI). The proposed system utilizes the Trilateration Algorithm to calculate the coordinates of a tracking device (Tag Node) based on distance data estimated from at least three reference points (Anchor Nodes) via the Log-Normal Shadowing Path Loss Model. The calculated coordinates are transmitted via the MQTT (Message Queuing Telemetry Transport) protocol to a graphical visualization system developed in Python for real-time positioning display. Experimental results from a real-world environment demonstrate the potential of using RSSI for positioning. Despite challenges related to signal fluctuation caused by environmental factors, the implementation of signal filtering techniques and the appropriate calibration of parameters A (the received signal strength at a 1-meter reference distance) and n (the environmental path loss exponent) can enhance accuracy, significantly improving the overall system performance and practicality for general applications.

Index Terms— ESP32, Indoor Positioning System, Kaman Filter, RSSI, Trilateration Algorithm.

I. INTRODUCTION

Navigation and positioning technologies have influenced to daily life and critical infrastructure during the digital and AI ages, for example, smartphone navigation maps, ride-hailing services, and logistics management. It is well-known that Global Positioning System (GPS) serves as the standard technology for precise outdoor positioning, its application in indoor environments—such as shopping malls, hospitals, office buildings, or large warehouses—encounters significant limitations. Satellite signals cannot effectively penetrate walls, concrete ceilings, or building structures, and even when its signals are received, the multipath effect often causes high discrepancies, rendering the data unusable for practical applications.

Consequently, the demand for developing Indoor Positioning System (IPS) has arisen to address the needs in GPS-denied areas. The primary challenge in developing the IPS lies in balancing "Accuracy," "Complexity," and "Cost." One popular and economically viable approach is to leverage

existing wireless infrastructure within the buildings, such as WiFi networks, in which RSSI (Received Signal Strength Indicator) measurement is the simplest method to act as the IPS. The RSSI method does not require specialized hardware and the standard wireless devices can measure the signal strength, estimate the distance, and determine the position. Although its accuracy may not match advanced technologies like Ultra-Wideband (UWB), its significantly lower cost makes it an attractive option for developing the applications that do not require millimeter-level precision.

The main objectives of this study are to investigate the IPS principles and develop RSSI-based IPS. The research focuses on developing a prototype using the ESP32 board to function as both a transmitter (tag) and a reference receiver (anchor), emphasizing cost-effectiveness and efficiency. Furthermore, trilateration algorithm and linear equation solving technique are employed to calculate position coordinates (x, y), as well as both server communication and a real-time 2D visualization system are also developed based on Message Queuing Telemetry Transport (MQTT) protocol

to facilitate convenient and rapid object tracking. Finally, the experimental results from a real-world environment are analyzed to evaluate system performance and propose guidelines for future improvements.

II. METHODOLOGY

Currently, various technologies have been developed for the IPS, each offering distinct advantages and limitations. WiFi Fingerprinting, for instance, relies on creating a database of signal maps (radio Map) at reference points; while it offers high accuracy and resistance to multipath effects. It requires significant time and resources for offline data collection and must be updated whenever the environment changes. As for prior researches with higher equipment costs, Ultra-Wideband (UWB) technology along with Time of Flight (ToF) technique are often employed in some applications requiring centimeter-level accuracy, despite its. Also, Inertial Navigation System (INS) using IMU sensors are utilized in conjunction with other systems to calculate the positions relative to a starting point. On the other hand, the RSSI-based trilateration approach is proposed in this present work as it gives the lowest cost, easy installation, flexible relocation, and no pre-collected fingerprint data requirement.

A. Distance Estimation Model and RSSI

The fundamental principle of using RSSI for distance determination involves considering the received signal power. Naturally, the radio signals attenuate as they travel over longer distances. The relationship between RSSI and distance (d) is described by the mathematical model (log-normal shadowing path loss model) as shown in Eq. (1).

$$RSSI = A - 10n \log_{10}(d) \quad (1)$$

where RSSI represents the measured signal strength at a receiver (unit: dBm), and d denotes the distance between a transmitter and a receiver (unit: meters). The variable “ A ” corresponds to the received signal strength measured at a reference distance of 1 meter (unit: dBm), while the variable “ n ” represents the path loss exponent depending on environmental conditions ($n > 2$ for indoor environments with obstacles). To achieve maximum accuracy, it is essential to determine the appropriate values for two studied variables (A and n) for our actual indoor environment. The calibration process was conducted by placing the Tag node at specific reference distances from the Anchor nodes (e.g., 1m, 2m, 3m, and 4m). At each distance, continuous RSSI measurements were collected for a predefined duration to obtain a statistically significant dataset. Subsequently, a logarithmic curve fitting technique based on linear regression analysis was applied to these empirical datasets. This allowed us to extract the most accurate baseline signal strength (A) and path loss exponent (n) that specifically represent the characteristics of the test environment.

B. Trilateration Algorithm

Trilateration is a geometric method used to determine the coordinates of an unknown point (tag node) using distances from at least three reference points with known positions (Anchor Nodes). It involves finding the intersection of three

circles centered at the Anchor coordinates (x, y) with radii equal to the measured distances d . We assume that the Tag is located at coordinate (x, y) and the Anchors are at known positions (x_1, y_1) , (x_2, y_2) , and (x_3, y_3) , the relationship can be defined by the circle equation for each anchor. The system of equations is given by:

$$\begin{cases} (x - x_1)^2 + (y - y_1)^2 = d_1^2 \\ (x - x_2)^2 + (y - y_2)^2 = d_2^2 \\ (x - x_3)^2 + (y - y_3)^2 = d_3^2 \end{cases} \quad (2)$$

To solve for (x, y) , these non-linear equations are typically linearized by expanding the squared terms and subtracting the equations from one another. This process eliminates the squared terms $(x^2 + y^2)$, resulting in a system of linear equations in the form $Ax = B$. This system can then be solved using matrix operations to determine the precise coordinates of the Tag.

C. Signal Filtering Techniques

Due to the high fluctuation of RSSI signals, using raw data to calculate distance directly causes the position to jump erratically. Therefore, signal filtering techniques are necessary. The basic technique commonly used is the Moving Average Filter, which calculates the average of the past N samples to smooth the signal. Additionally, in order to tackle the significant fluctuations in RSSI caused by multipath fading and external interference in indoor environments, this study employed the Kalman Filter. In contrast to the Moving Average filter, which may cause delays and treats all past samples with equal importance, the Kalman Filter is notably effective in evaluating the actual state of a system that experiences dynamic noise. It utilizes mathematical modeling and forecasting, along with adjustments determined by noise covariance, resulting in distance estimations that are more stable and precise than those produced by straightforward averaging.

For smoothing the RSSI in a static indoor environment, the one-dimensional Kalman Filter can be simplified into a recursive update process. The core equations for the Kalman gain (K_t), the current state estimate (x_t), and the error covariance (P_t) at time step t are formulated as follows:

$$K_t = \frac{P_{t-1}Q}{P_{t-1}Q + R} \quad (3)$$

$$x_t = x_{t-1} + K_t(z_t + x_{t-1}) \quad (4)$$

$$P_t = (1 - K_t)(P_{t-1} + Q) \quad (5)$$

where z_t is the newly measured RSSI, Q is the process noise covariance, and R is the measurement noise covariance. This iterative approach effectively minimizes variance, providing a significantly more stable RSSI value (x_t) for the subsequent distance calculation.

III. SYSTEM DESIGN AND METHODOLOGY

D. System Architecture

The proposed system is designed based on a three-layer architecture to improve modularity, scalability, and ease of

development. The system communication concept diagram of this work is depicted in Fig. 1. The first layer is the Sensing Layer (Physical Layer), which consists of ESP32 hardware devices deployed as three Anchor Nodes and one Tag Node. The Anchor Nodes are installed at fixed locations around the target area and are responsible for transmitting or receiving wireless signals, while the Tag Node is a mobile device whose position is to be estimated.

The second layer is the Network Layer (Communication Layer), which handles data transmission between nodes. Wi-Fi or BLE is used to measure the Received Signal Strength Indicator (RSSI) between the Tag and Anchors, while the MQTT protocol is employed to transmit measured signal data or preliminary distance information to the server over a wireless network.

The final layer is the Application Layer (Processing and Visualization Layer). This layer performs high-level data processing and visualization using Python scripts executed on a computer. The application subscribes to the MQTT broker, computes the Tag position using the trilateration algorithm, and displays the estimated position through a graphical user interface (GUI) in real time.

E. Hardware Implementation

The hardware implementation of the proposed system (Fig. 2) consists of three LILYGO T-SIM7600 boards configured as Anchor nodes and one ESP32-WROOM module configured as the mobile Tag node.

The T-SIM7600 board integrates an ESP32 microcontroller with extended communication capabilities, making it suitable for reliable signal reception and RSSI measurement. Each Anchor node is deployed at a fixed position within the indoor test area and powered independently using 18650 lithium-ion batteries.

The Tag node is implemented using a standalone ESP32-WROOM module configured to transmit Wi-Fi signals at predefined intervals. The Anchor nodes receive the transmitted signals and extract RSSI values for distance estimation.

A Wi-Fi Access Point is used to provide network connectivity, while a laptop computer runs the Python-based data processing and visualization system. During the experimental setup, a measuring tape is used to accurately determine the ground-truth positions of the Tag for performance evaluation. All devices operate on the same Wi-Fi channel to ensure stable communication and consistent RSSI measurements throughout the experiments.

F. Software Implementation

The software implementation is divided into two primary components: embedded firmware and data processing software. The embedded firmware is developed using the Arduino framework and deployed on the ESP32-based Anchor and Tag nodes. The firmware manages Wi-Fi communication, periodic signal transmission from the Tag node, RSSI acquisition at the Anchor nodes, data formatting, and publishing of collected data via the MQTT protocol to a central broker.

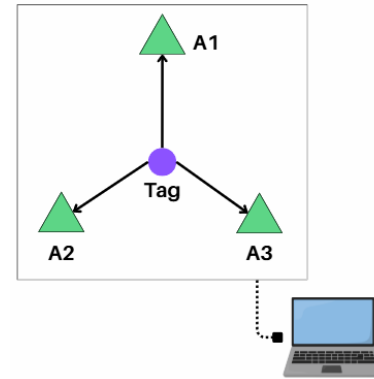


Fig. 1. System Communication Concept Diagram.



Fig. 2. Hardware Implementation.

On the host computer, the data processing software is implemented in Python. The application subscribes to the MQTT broker and continuously receives RSSI measurements from multiple Anchor nodes. To mitigate signal fluctuation caused by multipath and environmental interference, a signal filtering technique is applied prior to distance estimation.

The filtered RSSI values are converted into distance estimates using the Log-Normal Shadowing Path Loss Model with calibrated parameters A and n obtained from experimental regression analysis. The estimated distances are then used in a trilateration algorithm, where the nonlinear circle equations are linearized and solved in matrix form to compute the two-dimensional coordinates (x, y) of the Tag node.

Finally, the calculated positions are visualized on a real-time two-dimensional map corresponding to the indoor test environment, enabling both live monitoring and post-experimental performance analysis.

IV. RESULTS AND DISCUSSION

G. System Testing

The indoor positioning system was evaluated using one ESP32 board as a Tag and three ESP32 boards as Anchors. The system estimates the two-dimensional position (X, Y) of the Tag based on Wi-Fi RSSI measurements and a trilateration algorithm. The experiments were conducted in an indoor environment with an approximate area of 20×20 meters as illustrated in Fig. 3.

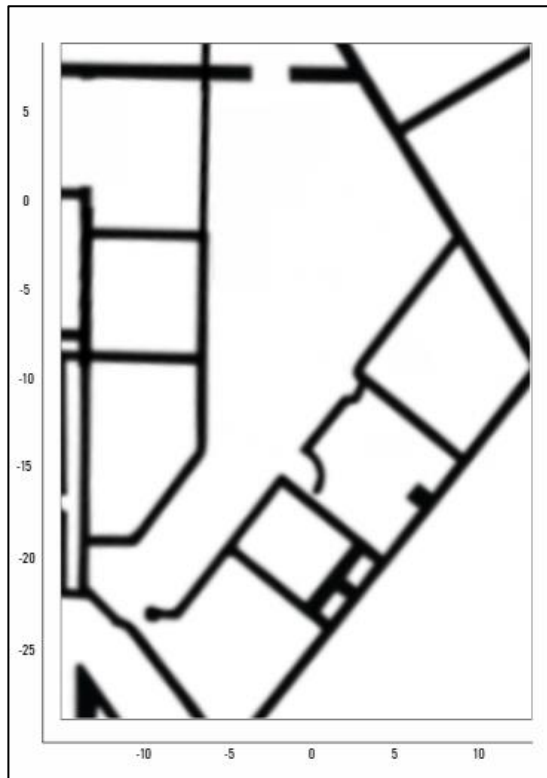


Fig. 3. Indoor experimental layout (approximately 20 × 20 meters) indicating anchor positions and tag measurement points for positioning evaluation.

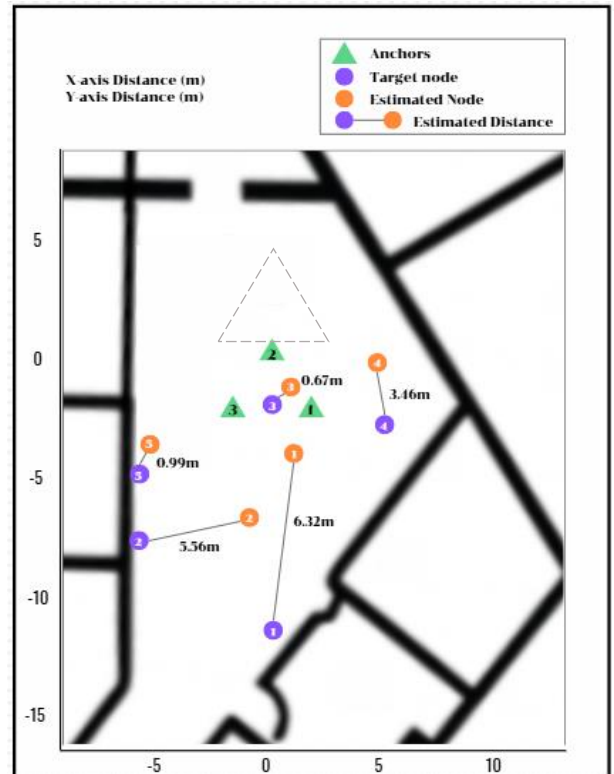


Fig. 5. Equilateral Triangle anchor placement configuration.

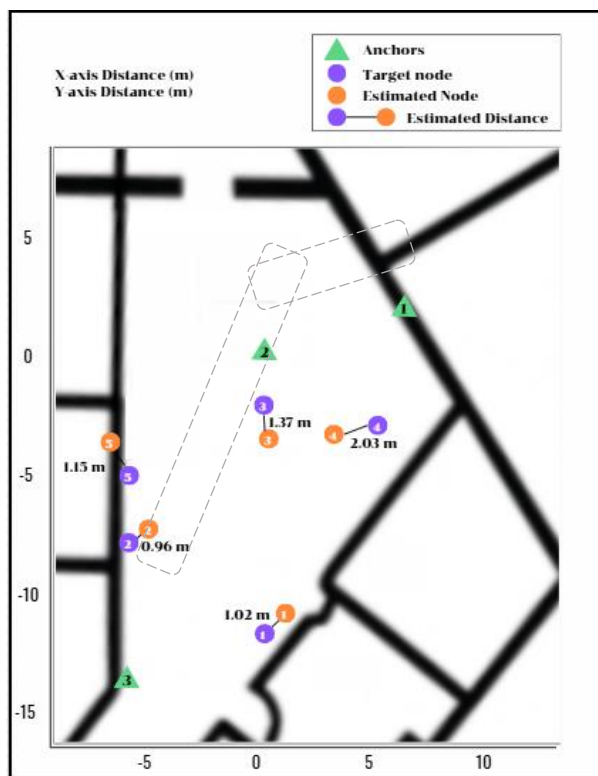


Fig. 4. Line of Sight (LOS) anchor placement configuration.

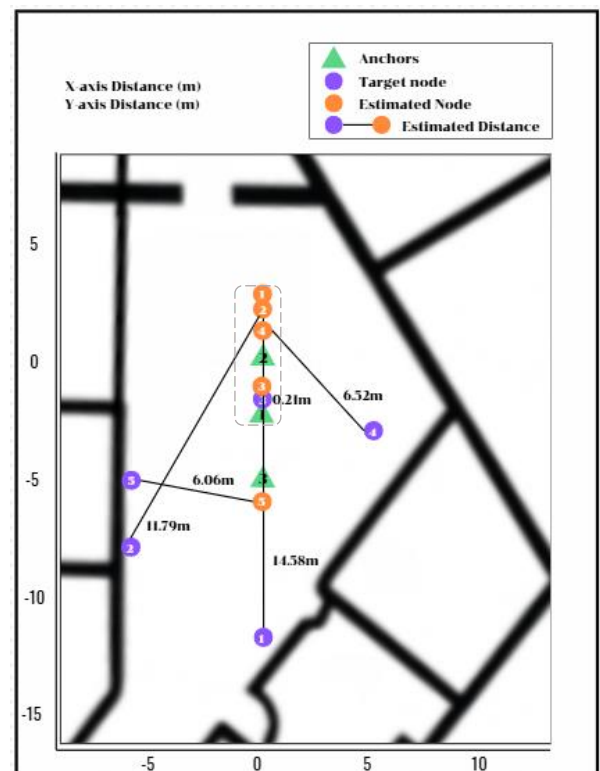


Fig. 6. Linear Alignment anchor placement configuration.

To investigate the effect of anchor placement on positioning accuracy, three different anchor configurations were tested: Line of Sight or LOS (Fig. 4), equilateral triangle (Fig. 5), and linear alignment (Fig. 6). Note that the small triangles denote the anchor positions and the dashed rectangles in Figs. 4-6 represent the anchor placements. Each configuration was evaluated at multiple Tag positions. To ensure statistical reliability and account for signal fluctuations, the RSSI data was recorded continuously for 100 samples at a rate of 1 sample per second (totaling 100 seconds) at each specific test point. The final positioning error was then calculated by comparing the average estimated position with the actual ground-truth coordinates.

H. Positioning Accuracy Results

The experimental results indicate that anchor placement has a significant impact on positioning accuracy as shown in Table I. The LOS configuration provides the lowest positioning error due to stable signal propagation and minimal multipath interference. The equilateral triangle configuration shows increased error when the Tag is located outside the anchor coverage area, mainly due to Geometric Dilution of Precision (GDOP). The linear alignment configuration produces the largest error, as the anchors lack sufficient geometric diversity for accurate trilateration.

TABLE I: THE EXPERIMENTAL RESULTS OF
DIFFERENT ANCHOR PLACEMENTS.

Case	Anchor Placement Configuration (Figs. 4-6)	Mean Error (m)	Mean Error (%)
1	Line of Sight (LOS)	1.31	16.6%
2	Equilateral Triangle	3.40	44.3%
3	Linear Alignment	7.83	102%

Although the system can estimate indoor positions with reasonable accuracy under favorable conditions, several limitations were observed. RSSI measurements are highly sensitive to environmental factors such as multipath propagation, signal reflection, and obstruction by walls or objects. In addition, improper anchor placement leads to high GDOP, which significantly degrades positioning performance.

Therefore, careful anchor deployment is essential to ensure system stability and reliable positioning accuracy. Future improvements may include RSSI filtering techniques, adaptive anchor placement strategies, or the integration of additional sensors to enhance robustness in complex indoor environments.

V. CONCLUSION

This research presents the design and implementation of a Wi-Fi RSSI-based indoor positioning system using ESP32 devices as Anchors and Tag. The system estimates the two-dimensional position (X, Y) of the Tag through RSSI-based distance estimation and a trilateration algorithm. The experiments were conducted in a 20 × 20 meters indoor

environment to evaluate system performance under different anchor placement configurations.

The experimental results demonstrate that anchor placement significantly affects positioning accuracy. Among the three tested configurations, the Line of Sight (LOS) placement achieved the best performance with a mean error of 1.31 meters. The equilateral triangle configuration resulted in moderate accuracy with a mean error of 3.40 meters, mainly due to increased GDOP when measurement points were located outside the anchor coverage area. The linear alignment configuration produced the highest positioning error at 7.83 meters, as the lack of geometric diversity led to severe degradation in trilateration accuracy.

The experimental results clearly demonstrate that system accuracy is directly correlated with the Anchor placement strategy. Configuration 1 (LOS) achieves the highest accuracy due to its balanced area coverage and optimal multi-directional signal reception. Conversely, Configuration 2 (Equilateral Triangle) exhibits increased errors because some test points fall outside the enclosed coverage area, leading to incomplete signal reception and unbalanced signal angles. Furthermore, Configuration 3 (Linear Alignment) yields the highest error, as this setup fails to support adequate multi-directional signal distribution, severely limiting the spatial data required for precise coordinate calculation.

Overall, the results confirm that balanced anchor geometry and proper deployment strategy are critical factors for improving the accuracy of RSSI-based indoor positioning systems.

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IoT-Based Sugar Quality Measurement System Using Electrochemical Sensing and ESP32

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Abstract Sugar quality evaluation is an important process in the food industry to ensure product standards and consistency. Conventional methods for analyzing sugar properties often rely on laboratory instruments that are expensive and unsuitable for rapid field measurements. This study proposes an IoT-based sensing system for evaluating sugar solutions using a Hall Effect sensor and an ESP32 microcontroller. The sensing system detects variations in electrical conductivity of sugar solutions through an electrochemical sensing unit and a magnetic detection mechanism. White sugar and brown sugar samples with concentrations ranging from 1 to 4 teaspoons dissolved in 250 mL of water were prepared and analyzed. Experimental results show that brown sugar solutions exhibit higher conductivity and Hall sensor output voltage compared to white sugar solutions due to the presence of molasses and mineral ions. The measured data were transmitted to a Telegram-based monitoring platform for real-time visualization and remote observation. The proposed system demonstrates a low-cost and portable approach for preliminary sugar quality evaluation and IoT-based monitoring.

Index Terms—Sugar quality measurement, Hall Effect sensor, Electrical conductivity, IoT sensing system, ESP32 microcontroller, Telegram monitoring.

I. INTRODUCTION

Sugar is one of the most widely used ingredients in the global food industry, and its quality directly affects product standards, processing efficiency, and the economic value of food products [1]. In general, sugar can be classified into several types, such as white granulated sugar and brown sugar, which differ in processing methods, color, impurity levels, and physical properties [2]. Therefore, the evaluation of sugar quality plays an important role in quality control processes within the food industry.

Conventional methods for evaluating sugar quality typically rely on laboratory instruments such as polarimeters,

refractometers, and spectrophotometers to measure sucrose content, solution concentration, and color characteristics of sugar samples [3]. Although these techniques can provide accurate analytical results, the instruments are relatively expensive and usually require specialized laboratory environments and trained personnel for operation. As a result, these conventional techniques are not suitable for rapid, portable, or on-site measurements, particularly for small-scale producers or field applications.

In recent years, the rapid development of embedded systems and Internet of Things (IoT) technologies has enabled the development of low-cost and portable monitoring systems. Microcontrollers such as the ESP32 have become widely used in IoT-based sensing applications because they provide sufficient processing capability

together with built-in wireless communication modules, including Wi-Fi and Bluetooth [4]. These capabilities allow sensor data to be collected and transmitted in real time for remote monitoring and analysis [5].

The Hall Effect Sensor is widely used for detecting magnetic fields based on the Hall effect phenomenon, where a voltage is generated when an electric current flows through a conductor in the presence of a perpendicular magnetic field [6]. Hall sensors have been applied in various applications, including position detection, current measurement, and magnetic field sensing [7]. When combined with an inductive coil, variations in the physical properties of nearby materials can influence the magnetic field distribution and result in measurable changes in the sensor output signal [8].

Therefore, this study proposes the development of an IoT-based sugar quality measurement system using a Hall Effect Sensor and an ESP32 microcontroller. The proposed system analyzes variations in the sensor response caused by different sugar samples and transmits measurement data in real time via the internet. Similar sensing-based approaches have also been explored for food quality detection and monitoring systems [9].

II. METHODOLOGY

To prepare the sugar samples for the experiment, both white sugar and brown sugar were tested separately. Each type of sugar was divided into four measuring cups to obtain different concentration levels. A standard measuring spoon set was used in the experiment, where one teaspoon was used as the basic unit for measuring sugar. Fig. 1 shows the preparation of sugar solutions used in the experiment.



Fig. 1 Experimental preparation of sugar solutions with different concentrations: (a) white sugar and (b) brown sugar samples, each dissolved in 250 mL of water using 1–4 teaspoons of sugar.

For each sugar type, four samples were prepared with different sugar quantities ranging from 1 to 4 teaspoons, and each sample was dissolved in 250 mL of distilled water. The solutions were stirred until the sugar was completely dissolved to ensure a homogeneous mixture, resulting in four concentration levels for each sugar type. This preparation method allows the sensing system to observe variations in electrical responses corresponding to different sugar concentrations.

Electrical conductivity describes the ability of a solution to conduct electric current through the movement of charged ions in the medium [10]. In aqueous solutions, the conductivity mainly depends on the concentration and

mobility of ions present in the solution, which directly influences the electrical behavior of the sensing system.

Sucrose, which is the main component of refined sugar, is classified as a non-electrolyte compound and does not significantly dissociate into ions when dissolved in water, resulting in very low electrical conductivity [11]. However, brown sugar contains molasses and mineral impurities such as potassium, calcium, and magnesium, which increase the ionic concentration and therefore enhance the conductivity of the solution [12-13].

As a result, brown sugar solutions generally show higher electrical conductivity compared to refined white sugar solutions. Each experiment was repeated three times for each concentration level to ensure the reliability and reproducibility of the results. The reported values represent the average of the measured data.

The Hall voltage is related to sugar concentration through the electrical conductivity of the sugar solution. When the sugar concentration increases, the number of dissolved particles in the solution also increases, causing the conductivity to rise. This relationship can be expressed as

$$\sigma \propto C \quad (1)$$

where σ is the electrical conductivity and C is the sugar concentration.

The electrical conductivity of the solution can be expressed as :

$$\sigma = \frac{L}{RA} \quad (2)$$

where σ is the electrical conductivity (S/m), R is the electrical resistance of the solution (Ω), L is the distance between the electrodes (m), and A is the cross-sectional area of the electrode surface (m^2).

After preparation, the sugar solutions were transferred into the electrochemical sensing cell for measurement. The sensing system detected the electrical current generated from the solution, and the resulting signal was subsequently processed by the microcontroller for further analysis.

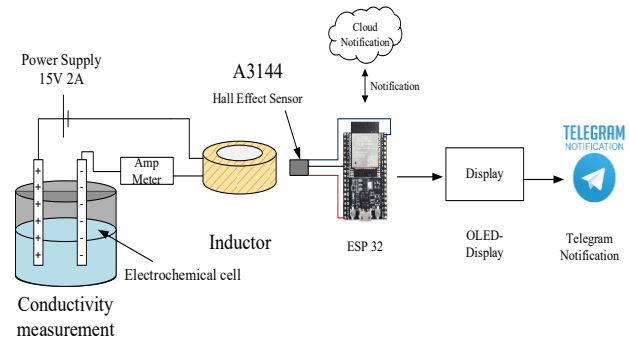


Fig. 2 Block diagram of the proposed IoT-based sugar measurement system using an electrochemical sensing unit, ESP32 microcontroller, and cloud notification.

The proposed system consists of an electrochemical sensing unit, a signal conditioning circuit, an ESP32 microcontroller, a display module, and a cloud-based notification system. As illustrated in Fig. 2, the sugar sample is placed inside an electrochemical cell where two electrodes are immersed in the solution. When a voltage is applied across the electrodes, an electrochemical interaction occurs within the solution, generating an electrical current that is related to the concentration and ionic properties of the sugar solution.

In this study, the sensing coil is constructed using a toroidal core with a diameter of 27 mm. A copper wire with a diameter of 31AWG is wound around the toroid for approximately 700 turns. The resulting current flowing through the coil generates a magnetic field, which can be

detected by a Hall Effect sensor such as the A3314 Hall Effect Sensor.

The electrochemical current generated in the sensing cell can be described by

$$I = \frac{V}{R} \quad (3)$$

where I is the current flowing through the solution, V is the applied voltage, and R is the electrical resistance of the sugar solution. The resistance depends on the concentration and conductivity of the dissolved sugar.

The magnetic field generated by the current flowing through the coil can be approximated by

$$B = \frac{\mu N I}{2\pi r} \quad (4)$$

where B is the magnetic flux density (Tesla), μ is the magnetic permeability of the core material, N is the number of turns of the coil, I is the current flowing through the coil, and r is the radius of the toroidal core. The Hall Effect sensor detects the magnetic field produced by the current and generates an output voltage proportional to the magnetic field strength.

The Hall voltage is expressed as :

$$V_H = K_H B \quad (5)$$

Where V_H is the Hall voltage, K_H is the sensitivity constant of the Hall sensor, and B is the magnetic flux density. The detected Hall voltage signal is amplified through a signal conditioning circuit to obtain a suitable signal level for the microcontroller. The conditioned signal is then processed by the ESP32, which performs analog-to-digital conversion, data acquisition, and signal processing.

Therefore, the Hall voltage increases with increasing sugar concentration, and the overall relationship can be summarized as

$$V_H \propto I \propto C \quad (6)$$

This shows that changes in sugar concentration directly affect conductivity, which in turn influences the Hall voltage output of the sensing system.

After processing, the measured values are displayed on a local display module for real-time monitoring. In addition, the ESP32 transmits the measurement data through a wireless network to a cloud-based notification platform such as Telegram, enabling users to remotely monitor the measurement results and system status.

III. RESULTS AND DISCUSSION

The experimental results obtained from the proposed sensing system are summarized in Table I, which presents the measured electrical conductivity and Hall sensor output voltage for both white sugar and brown sugar solutions at different concentration levels ranging from 1 to 4 teaspoons dissolved in 250 mL of water.

As shown in Table I, both the electrical conductivity and Hall voltage increase as the sugar concentration increases for both types of sugar. However, the brown sugar solutions exhibit significantly higher conductivity and Hall voltage compared to the white sugar solutions. This behavior can be attributed to the presence of molasses and mineral ions in brown sugar, which increases the ionic conductivity of the solution.

TABLE I.
CONDUCTIVITY AND HALL VOLTAGE OF WHITE
AND BROWN SUGAR SOLUTIONS WITH
PERCENTAGE DIFFERENCE.

Sample	Sugar Type	Sugar Amount (Teaspoon)	Conductivity (μS/cm)	Hall Voltage (mV)
S1	White Sugar	1	5.27	12.25
S2	White Sugar	2	5.84	14.12
S3	White Sugar	3	6.32	16.55
S4	White Sugar	4	6.94	18.78
S5	Brown Sugar	1	11.25	28.92
S6	Brown Sugar	2	14.84	32.11
S7	Brown Sugar	3	17.25	36.64
S8	Brown Sugar	4	19.67	41.38

Based on the results presented in Table I, both conductivity and Hall voltage increase with increasing sugar concentration from 1 to 4 teaspoons. For the white sugar samples, the conductivity ranges from 5.27 μS/cm to 6.94 μS/cm, while the Hall voltage increases from 12.25 mV to 18.78 mV. This relatively small increase is expected because sucrose is a non-electrolyte, meaning that it does not significantly dissociate into ions when dissolved in water.

In contrast, the brown sugar samples exhibit significantly higher conductivity values, ranging from 11.25 μS/cm to

19.67 $\mu\text{S}/\text{cm}$, with Hall voltage values increasing from 28.92 mV to 41.38 mV. The higher conductivity observed in brown sugar solutions is mainly caused by the presence of molasses and mineral impurities, which introduce additional ions into the solution and enhance electrical conduction.

Overall, the results demonstrate a clear relationship between sugar concentration and Hall sensor output voltage. As the conductivity of the solution increases, a larger electrical current flows through the sensing circuit, generating a stronger magnetic field in the sensing coil. As a result, the Hall effect sensor produces a higher output voltage, confirming the effectiveness of the proposed sensing system for detecting variations in solution conductivity.

As illustrated in Fig. 3, to further evaluate the sensing system, measurements were conducted for 1 minute for each sample, with voltage data recorded at 5-second intervals. During this period, the sensor continuously monitored the output voltage corresponding to the conductivity of the sugar and the Hall voltage exhibits time-dependent behavior due to ongoing electrochemical reactions within the sensing cell. These variations arise from ion dissociation, ionic migration, electrode polarization, and gradual corrosion of the electrode surfaces, all of which influence solution conductivity. As a result, the current through the sensing coil changes over time, producing corresponding fluctuations in the measured Hall voltage. solution, producing multiple data samples for analysis of signal stability and variation.

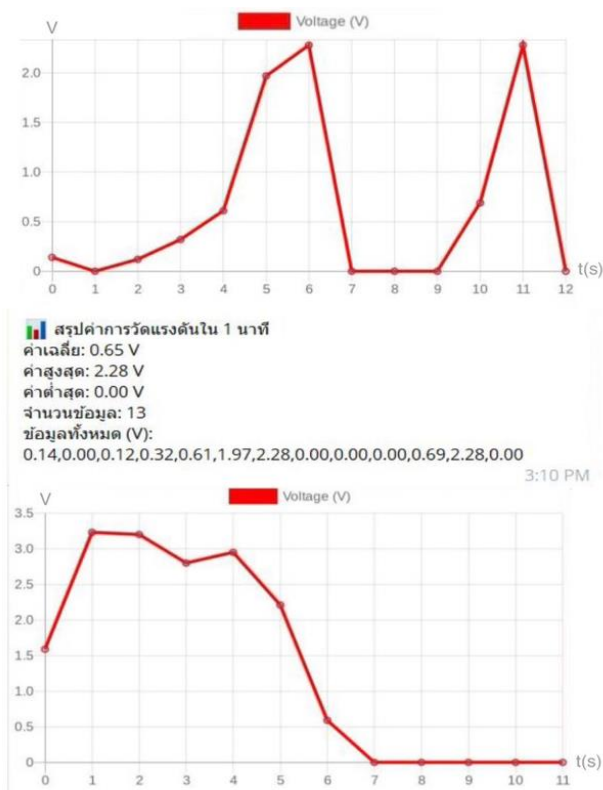


Fig. 3 Telegram monitoring interface

The collected data were transmitted to a Telegram notification system through the ESP32 microcontroller. The Telegram bot automatically plotted the measured voltage values as a time-based graph, allowing users to visually observe the signal behavior. In addition, the system summarized the measurement results by reporting the average, maximum, and minimum voltage values. This integration enables real-time monitoring and remote observation of the sensing system through a mobile application.

IV. CONCLUSION

This study proposed an IoT-based sensing system for evaluating sugar solutions using a Hall Effect sensor and an ESP32 microcontroller. The system detects variations in electrical conductivity through an electrochemical sensing unit and converts the resulting current into a measurable Hall voltage. Experimental results show that both conductivity and Hall voltage increase with increasing sugar concentration from 1 to 4 teaspoons dissolved in 250 mL of water. Brown sugar solutions exhibited significantly higher conductivity and Hall voltage than white sugar solutions due to the presence of molasses and mineral ions. Hall voltage (V_H) is directly proportional to the electric current (I) and the sugar concentration (C). The integration of the ESP32 with a Telegram notification platform enables real-time monitoring, graphical visualization, and remote access to measurement data. Overall, the proposed system provides a low-cost, portable, and IoT-enabled approach for preliminary sugar quality evaluation and demonstrates the feasibility of using Hall sensor-based detection for solution conductivity analysis.

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A Customized NGS Panel for The Detection Of MDS/AML-Associated Gene Mutations and Predisposing Genes; A Preliminary Finding in 20q Deletion Cases

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Abstract. Myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) are heterogeneous hematologic malignancies driven by diverse genetic abnormalities that influence disease initiation, progression, and clonal evolution. Accurate molecular characterization is critical for diagnosis, risk stratification, and therapeutic decision-making. This study aimed to investigate driver mutations and genetic predisposition markers in patients with MDS/AML harboring a 20q deletion, and to develop a customized mutation panel tailored to specific cytogenetic subgroups. 9 patient samples with 20q deletion were analyzed using an Illumina-based next-generation sequencing (NGS) platform and a comprehensive targeted panel of 92 genes, encompassing both recurrently mutated and newly reported genes relevant to MDS/AML pathogenesis. Mutations were identified in genes associated with epigenetic regulation (*ASXL1*, *TET2*, *KMT2D*, and *BCOR*), signaling and transcription factors (*TP53*, *KRAS*, *KIT*, *CBL*, *PHF6*, and *IKZF1*), RNA splicing (*SF3B1* and *U2AF1*), the cohesin complex (*CTCF*), and pharmacogenetics (*NUDT15**2, *3, and *TPMT**3C). These findings support the use of a customized mutation panel for MDS/AML with 20q deletion and contribute to establishing a national reference database, providing a valuable resource for precision medicine in MDS/AML patients in Thailand.

Index Terms—Acute Myeloid Leukemia, Myelodysplastic Syndrome, Next-generation sequencing, 20q deletion

I. INTRODUCTION

Myeloid malignancies, such as myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML), are caused by abnormalities in hematopoietic stem and progenitor cells at the myeloid lineage, leading to clonal proliferation driven by acquired genetic alterations [1]. The progression of MDS to AML occurs in up to 20% of patients within five years and is influenced by multiple factors, particularly the accumulation of somatic gene mutations. These gene mutations play a critical role in disease initiation, progression, and clinical heterogeneity [2].

Cytogenetic abnormalities remain a cornerstone in the diagnosis and prognostic stratification of MDS/AML. For instance, the deletion of the long arm of chromosome 20 (20q deletion) is found in approximately 4–7% of MDS/AML patients. This abnormality can be further categorized into isolated 20q deletion, which is generally associated with a good prognostic subgroup, and 20q deletion accompanied by

additional chromosomal aberrations or a complex karyotype, which is associated with more aggressive disease and a poorer prognosis [3]. Despite their clinical relevance, conventional karyotyping alone is insufficient to fully characterize the underlying molecular mechanisms and gene mutations driving these diseases.

Since MDS/AML exhibits high genetic heterogeneity, identifying distinct genetic mutations in individual patients is essential for precise diagnosis, risk assessment, prognosis, and therapy decision-making [4]. Single-gene analyses are inadequate to capture the full spectrum of clinically important mutations and may yield incomplete information, limiting patients' opportunities to receive appropriate treatment [5]. As a result, international clinical practice guidelines increasingly emphasize the application of molecular genetic testing into standard diagnostic and management algorithms for MDS/AML, including the use of genetic biomarkers to monitor treatment response [1, 6–8].

Next-generation sequencing (NGS) has emerged as a powerful and widely used technology for detecting genetic abnormalities, including somatic and germline mutations. Compared with conventional sequencing methods, NGS offers superior sensitivity, specificity, accuracy, and cost-effectiveness, enabling simultaneous analysis of many mutations associated with disease pathogenesis and inherited predisposition [9]. To satisfy these clinical and research requirements, a variety of new NGS platforms and customized gene panels have been developed to date [10-12].

In this context, the present study aims to characterize the mutational landscape of patients with MDS/AML harboring 20q deletion using a targeted NGS approach. In parallel, the study aims to provide recommendations on which clinically significant genetic mutations should be included in the gene panel-based assay used for this patient subgroup. The ultimate goal is to establish a cost-effective, high-accuracy diagnostic tool that supports precise diagnosis, improved prognostic evaluation, and informed therapeutic planning. It is anticipated that the findings from this study will contribute to the development of a valuable genetic database and provide a critical foundation for advancing precision medicine strategies in the management of MDS/AML.

II. METHODOLOGY

Ethical approval

This study was reviewed and approved by Human Research Ethics Committee, Faculty of Medicine Ramathibodi Hospital, Mahidol University (COA. No. MURA2025/507).

Study Design and Sample Collection

This study was conducted as a cross-sectional observational investigation. Nine leftover specimens preserving cells in fixative, with an estimated volume of 1 mL per sample, were retrospectively collected from patients diagnosed with MDS or AML harboring 20q deletion. All specimens were obtained and authorized for research use from the Human Genetics Laboratory, Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University.

The inclusion criteria included leftover samples from routine clinical diagnostics of patients with confirmed MDS or AML who demonstrated a primary cytogenetic abnormality of 20q deletion, as identified by chromosomal analysis. Specimens were excluded if the patient had a diagnosis other than MDS/AML, the extracted DNA yield was <10 ng, as quantified by Qubit fluorometric measurement, or the quantitative PCR (qPCR) quality control (QC) score was > 12.

DNA Sample Preparation

Genomic DNA was extracted from leftover specimens containing cells preserved in fixative using the QIAamp Blood Mini Kit (Qiagen, Germany). Briefly, samples were centrifuged at 3,000 rpm for 5 minutes, and the cell pellet

was resuspended in 200 μ L of $1\times$ PBS.

Protease (20 μ L) and Buffer AL (200 μ L) were added, followed by incubation at 56 °C. After lysis, 200 μ L of 100% ethanol was added, and the lysate was transferred to a spin column. The column was sequentially washed with Buffer AW1 and Buffer AW2, then centrifuged according to the manufacturer's instructions. DNA was eluted in 40 μ L of 10 mM Tris-HCl (pH 8.0) and stored at 4 °C until further analysis.

Quantification and Quality Assessment

Genomic DNA concentration was quantified using the Qubit™ 1x dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, 1 μ L of each DNA sample was mixed with Qubit working solution and measured against the provided standards.

Genomic DNA quality was assessed using the Preseq™ DNA QC Assay (Integrated DNA Technologies, Inc.) according to the manufacturer's protocol, with minor modifications. DNA samples were diluted to 1 ng/ μ L, loaded into a 0.1-mL 96-well block, analyzed using the QuantStudio™ 5 Real-Time PCR system (Thermo Fisher Scientific), and calculated as shown below in (1).

$$\text{QC score} = C_T \text{ of sample} - C_T \text{ of standard} \quad (1)$$

Where C_T is the quantification cycle or the precise number of cycles at which the fluorescent signal surpasses background levels and crosses a threshold.

NGS Library preparation

Library preparation was performed using the Archer VARIANT Plex HS/HGC protocol for Illumina platforms. The workflow uses Anchored Multiplex PCR (AMP) technology to generate target-enriched libraries from genomic DNA (Fig. 1). Briefly, purified genomic DNA (10–250 ng) was adjusted to 50 μ L and enzymatically fragmented, followed by end repair to generate blunt-ended fragments. After purification with AMPure XP beads, adapters containing unique molecular barcodes (MBCs) and P5 indices were ligated in two sequential steps. Libraries were purified using bead-based cleanup and eluted according to the manufacturer's protocol. Target enrichment was performed by two rounds of PCR using gene-specific primers (GSP1 and GSP2). The second PCR incorporated P7 indices to complete library construction. Final libraries were purified with AMPure XP beads and eluted in 10 mM Tris-HCl for downstream sequencing.

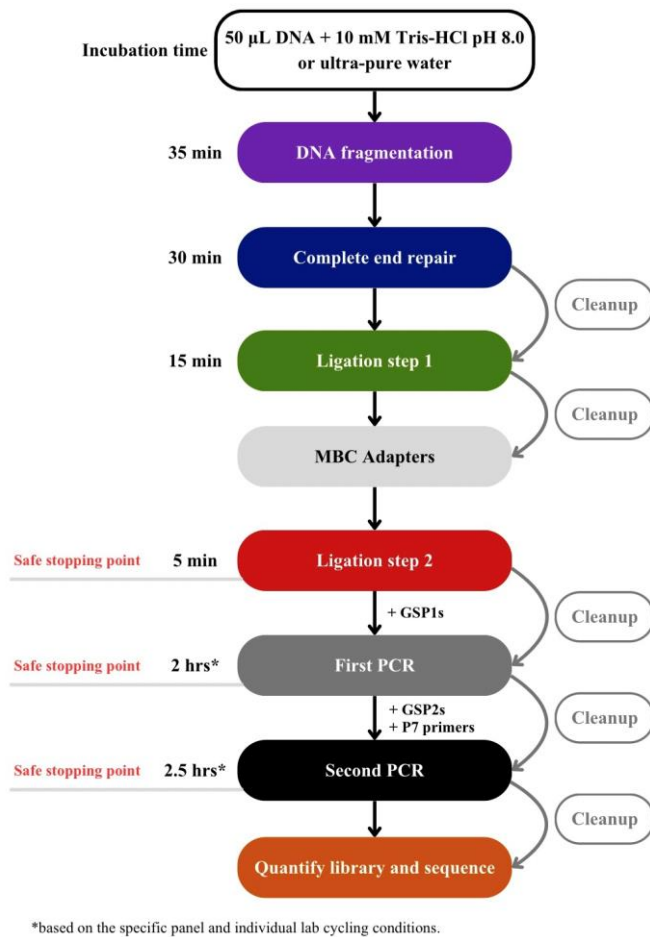


Fig. 1. Library preparation workflow

Archer™ VARIANTPlex™ Myeloid v2 Panel

In response to new findings, a growing need for biomarker detection, and updated guidelines from esteemed organizations such as the WHO, NCCN, and ESMO, the VARIANTPlex Myeloid v2 Panel has been developed to address these requirements. This panel, as shown in Table I, will incorporate 92 target genes, including those newly reported and commonly observed in association with MDS/AML [12]. These genes include both driver mutations, which play a direct role in disease pathogenesis, and germline mutations, which are associated with an increased predisposition to developing MDS/AML.

Sequencing and data analysis

The concentration must be determined using the Qubit™ 1x dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific), and the sample quality must be assessed using a Fragment Analyzer (Novogene, Ltd.) in accordance with the manufacturer's instructions prior to sequencing.

NGS libraries were quantified using KAPA SYBR FAST qPCR kits (KAPA Biosystems, Wilmington, WA), normalized, and then sequenced on the Novaseq X plus, 2x150 base pair reads (Illumina, San Diego, CA). Archer Analysis software, version 7.5.1 (Integrated DNA Technologies), was utilized for sequence processing, alignment, and variant calling, employing molecular barcode

information to remove duplicate reads (deduplication) and enables accurate and reliable variant calling. Analysis required at least 1.5 million reads and variant calls with a minimum variant allelic fraction (VAF) of 2.7%.

TABLE I
VARIANTPlex MYELOID V2 PANEL [12].

VARIANTPlex Myeloid v2 panel				
<i>ABL1</i>	<i>CUX1</i>	<i>IL7R</i>	<i>PDGFRA</i>	<i>STAT3</i>
<i>ANKRD26</i>	<i>CXCR4</i>	<i>JAK1</i>	<i>PHF6</i>	<i>STAT5B</i>
<i>ASXL1</i>	<i>DCK</i>	<i>JAK2</i>	<i>PPM1D</i>	<i>TET2</i>
<i>ATRX</i>	<i>DDX41</i>	<i>JAK3</i>	<i>PRPF8</i>	<i>TP53</i>
<i>BCOR</i>	<i>DHX15</i>	<i>KDM6A</i>	<i>PTEN</i>	<i>TPMT</i>
<i>BCORL1</i>	<i>DNMT3A</i>	<i>KIT</i>	<i>PTPN11</i>	<i>U2AF1</i>
<i>BRAF</i>	<i>EED</i>	<i>KMT2A</i>	<i>RAD21</i>	<i>U2AF2</i>
<i>BRCC3</i>	<i>ETNK1</i>	<i>KMT2D</i>	<i>RBBP6</i>	<i>UBA1</i>
<i>BTK</i>	<i>ETV6</i>	<i>KRAS</i>	<i>RPS14</i>	<i>UBTF</i>
<i>CLAR</i>	<i>EZH2</i>	<i>LUC7L2</i>	<i>RUNX1</i>	<i>WT1</i>
<i>CBL</i>	<i>FBXW7</i>	<i>MAP2K1</i>	<i>SAMD9</i>	<i>XPO1</i>
<i>CBLB</i>	<i>FLT3</i>	<i>MPL</i>	<i>SAMD9L</i>	<i>ZRSR2</i>
<i>CBLC</i>	<i>GATA1</i>	<i>MYC</i>	<i>SETBP1</i>	
<i>CCND2</i>	<i>GATA2</i>	<i>MYD88</i>	<i>SF3B1</i>	
<i>CDC25C</i>	<i>GNAS</i>	<i>NF1</i>	<i>SH2B3</i>	
<i>CDKN2A</i>	<i>GNB1</i>	<i>NOTCH1</i>	<i>SLC29A1</i>	
<i>CEBPA</i>	<i>HRAS</i>	<i>NPM1</i>	<i>SMC1A</i>	
<i>CREBBP</i>	<i>IDH1</i>	<i>NRAS</i>	<i>SMC3</i>	
<i>CSF3R</i>	<i>IDH2</i>	<i>NUDT15</i>	<i>SRSF2</i>	
<i>CTCF</i>	<i>IKZF1</i>	<i>PAX5</i>	<i>STAG2</i>	

III. RESULTS AND DISCUSSION

Patient characteristics

The median age of patients was 77 years (range 62-92 years), with a greater number of males (7). Cell samples in fixative were obtained during the diagnosis and treatment of MDS (n=7) and AML (n=2) (Table II).

Chromosomal analysis results

Among the 9 patients, as shown in Table II, 4 (44.44%) had an isolated 20q deletion; the remaining samples (55.56%) exhibited more than one aberration.

TABLE II
CHARACTERISTICS OF MDS/AML PATIENTS WITH 20Q DELETION.

Chromosome abnormality [MDS (n=7), AML (n=2)]	MDS n (%)	AML n (%)
Isolated 20q deletion	4 (44.4)	0
Female	1 (11.1)	0
80-89 years old	1 (11.1)	0
Male	3 (33.3)	0
60-69 years old	1 (11.1)	0
70-79 years old	1 (11.1)	0
80-89 years old	1 (11.1)	0
20q deletion with complex karyotype	3 (33.3)	2 (22.2)
Female	1 (11.1)	0
60-69 years old	1 (11.1)	0
Male	2 (22.2)	2 (22.2)
60-69 years old	0	1 (11.1)
70-79 years old	1 (11.1)	0
80-89 years old	1 (11.1)	0
90-99 years old	0	1 (11.1)

Quantity and Quality Assessment results

The concentration and quality requirements established in the manufacturer's instructions were satisfied by all nine samples. Figure 2 showed that the average concentration after preparing the Library was 22.03 ng/μL (range 11.6-32.6 ng/μL), and the quality was between 7.435 and 8.36, remaining below the specified threshold of 12. These results indicate that all samples had appropriate quality and concentration, suggesting a good probability of producing adequate libraries for sequencing.

In addition to Figure 2, the average insert size across all samples was 160.6 bp, within the expected range (100-300 bp). All samples exhibited a single peak with a stable shape and showed no abnormal features, such as impurity peaks, adapter contamination, or primer dimers.

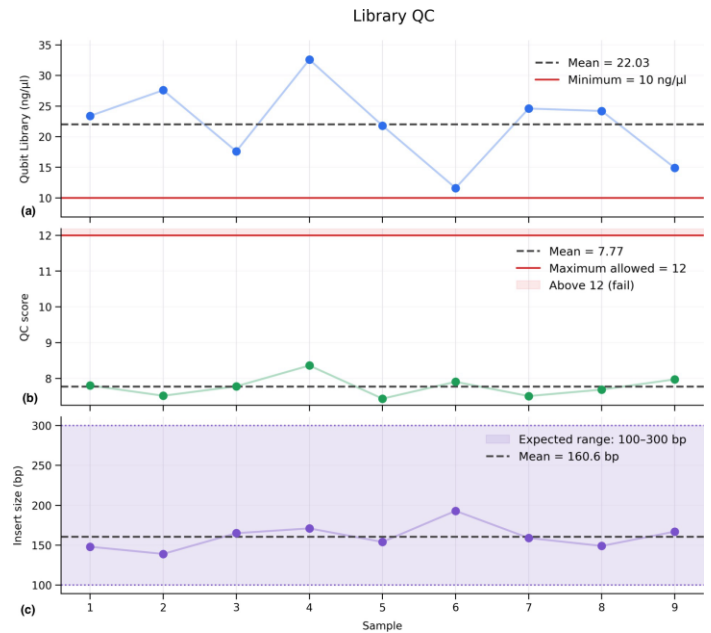


Fig. 2. Quantity (a), Quality (b), and Insert size (c) before the sequence of all nine samples.

Results and discussion of sequencing on the Illumina NGS platform.

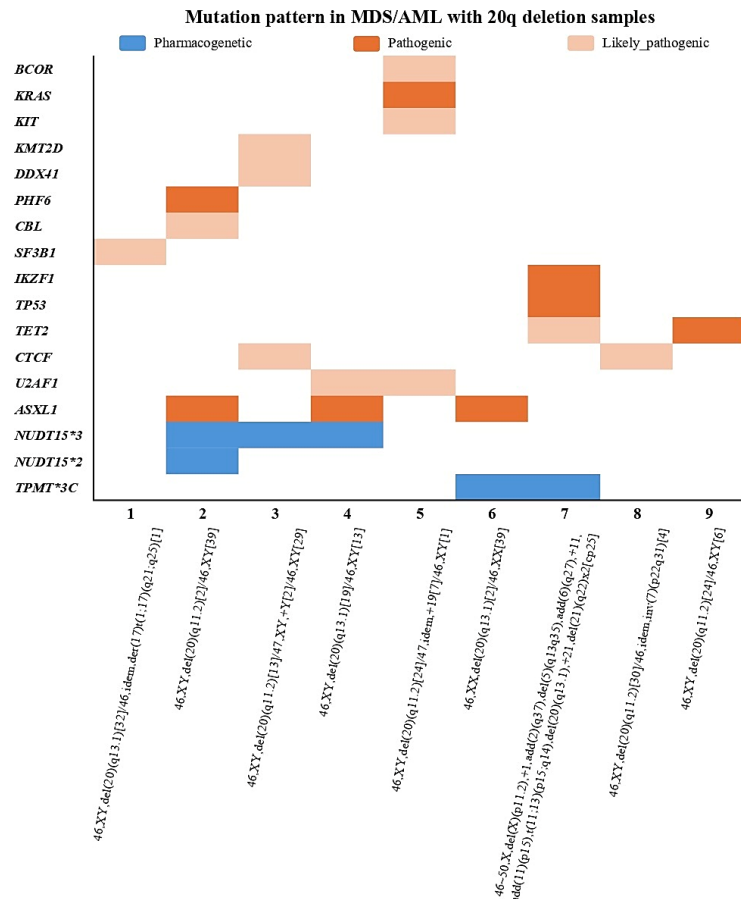


Fig. 3. Mutation pattern in MDS/AML with 20q deletion samples

TABLE III
DETAILS OF THE MUTATED GENES FOUND IN MDS/AML WITH 20Q DELETION SAMPLES

No.	Karyotyping	Symbol	HGVSp	HGVSc	AF	ACMG Grading And Significant	DBSNPID
1	46,XY,del(20)(q13.1)[32]/46, idem,der(17)t(1;17)(q21;q25)[1]	<i>SF3B1</i>	NP_036565.2:p.Lys700Glu	NM_012433.4:c.2098A>G	0.456467	Likely_pathogenic	rs559063155
2	46,XY,del(20)(q11.2)[2]/ 46,XY[39]	<i>ASXL1</i>	NP_056153.2:p.Glu635ArgfsTer15	NM_015338.6:c.1900_1922del	0.520273	Pathogenic	rs766433101
		<i>CBL</i>	NP_005179.2:p.Cys381Arg	NM_005188.4:c.1141T>C	0.603744	Likely_pathogenic	rs757874631
		<i>NUDT15*2</i>	NP_060753.1:p.Gly17_Val18dup	NM_018283.4:c.50_55dup	0.470111	Pharmacogenetic	rs746071566
		<i>NUDT15*3</i>	NP_060753.1:p.Arg139Cys	NM_018283.4:c.415C>T	0.544351	Pharmacogenetic	rs116855232
3	46,XY,del(20)(q11.2)[13]/ 47,XY,+Y[2]/46,XY[29]	<i>PHF6</i>	NP_115711.2:p.Arg275Ter	NM_032335.3:c.823C>T	0.148342	Pathogenic	rs1556019107
		<i>CTCF</i>	NP_006556.1:p.His373Arg	NM_006565.4:c.1118A>G	0.042857	Likely_pathogenic	COSV50461937
		<i>DDX41</i>	NP_057306.2:p.Ser21ThrfsTer7	NM_016222.4:c.62_63del	0.497166	Likely_pathogenic	rs756004929
		<i>KMT2D</i>	NP_003473.3:p.Leu797Val	NM_003482.4:c.2389C>G	0.027972	Likely_pathogenic	rs1057518680
4	46,XY,del(20)(q13.1)[19]/ 46,XY[13]	<i>NUDT15*3</i>	NP_060753.1:p.Arg139Cys	NM_018283.4:c.415C>T	0.490251	Pharmacogenetic	rs116855232
		<i>ASXL1</i>	NP_056153.2:p.Gly646TrpfsTer12	NM_015338.6:c.1934dup	0.035117	Pathogenic	rs750318549
		<i>NUDT15*3</i>	NP_060753.1:p.Arg139Cys	NM_018283.4:c.415C>T	0.995885	Pharmacogenetic	rs116855232
		<i>U2AF1</i>	NP_006749.1:p.Ser34Phe	NM_006758.3:c.101C>T	0.443645	Likely_pathogenic	rs371769427
5	46,XY,del(20)(q11.2)[24]/ 47,idem,+19[7]/46,XY[1]	<i>BCOR</i>	NP_001116857.1:p.Ser336LeufsTer45	NM_001123385.2:c.1005dup	0.78481	Likely_pathogenic	-
		<i>KIT</i>	NP_000213.1:p.Asp816Val	NM_000222.3:c.2447A>T	0.28139	Likely_pathogenic	rs121913507
		<i>KRAS</i>	NP_004976.2:p.Gly13Asp	NM_004985.5:c.38G>A	0.075055	Pathogenic	rs112445441
		<i>U2AF1</i>	NP_006749.1:p.Ser34Phe	NM_006758.3:c.101C>T	0.432367	Likely_pathogenic	rs371769427
6	46,XX,del(20)(q13.1)[2]/ 46,XX[39]	<i>ASXL1</i>	NP_056153.2:p.Gly646TrpfsTer12	NM_015338.6:c.1934dup	0.040953	Pathogenic	rs750318549
		<i>TPMT*3C</i>	NP_000358.1:p.Tyr240Cys	NM_000367.5:c.719A>G	0.518519	Pharmacogenetic	rs1142345
7	46-50,X,del(X)(p11.2),+1, add(2)(q37),del(5)(q13q35), add(6)(q27),+11,-add(11)(p15), t(11;13)(p15;q14),del(20)(q13.1), +21,del(21)(q22)x2[cp25]	<i>IKZF1</i>	NP_006051.1:p.Asn159Ser	NM_006060.6:c.476A>G	0.031123	Pathogenic	rs374333820
		<i>TET2</i>	NP_001120680.1:p.Arg1440ThrfsTer38	NM_001127208.3:c.4317dup	0.470785	Likely_pathogenic	rs777908833
		<i>TP53</i>	NP_000537.3:p.Arg248Gln	NM_000546.6:c.743G>A	0.945744	Pathogenic	rs11540652
		<i>TPMT*3C</i>	NP_000358.1:p.Tyr240Cys	NM_000367.5:c.719A>G	0.475248	Pharmacogenetic	rs1142345
8	46,XY,del(20)(q11.2)[30]/ 46,idem,inv(7)(p22q31)[4]	<i>CTCF</i>	NP_006556.1:p.Thr317ArgfsTer91	NM_006565.4:c.950_951del	0.032751	Likely_pathogenic	rs1470210640
9	46,XY,del(20)(q11.2)[24]/ 46,XY[6]	<i>TET2</i>	NP_001120680.1:p.Gln1030Ter	NM_001127208.3:c.3088C>T	0.106535	Pathogenic	rs780043982

According to Figure 3 and Table III, a total of 16 genes were identified with pathogenic, likely pathogenic, or pharmacogenetic variants. The most frequently mutated gene was *ASXL1*, occurring in 33% of the cohort and previously linked to disease progression in MDS with 20q deletion [13]. A complex karyotype was also observed in many samples in this study. For example, Sample 7 showed a particularly aggressive molecular profile, characterized by the co-occurrence of *TP53*, *IKZF1*, and *TET2* mutations. Notably, *TP53* is widely acknowledged and strongly associated with poor prognosis due to its extreme genomic instability, and the presence of *IKZF1* and *TET2* suggests a multi-hit model of leukemogenesis, in which the combination of epigenetic dysregulation and DNA damage response failure drives disease progression [14, 15].

The similarity between Samples 3 and 5 from AML patients and other MDS samples indicates the presence of driver mutations involving *U2AF1* and *CTCF*. These discovered mutations are consistent with those previously reported. *U2AF1* mutations are significantly associated with poorer overall survival and a higher risk of transformation to AML compared to patients without these mutations [16]. Moreover, it has been demonstrated that *CTCF* overexpression or incorrect binding is a crucial factor in

maintaining myeloid cells in an immature, malignant state [17].

In addition to mutations found in Figure 3 and Table III, pharmacogenetic markers are crucial for the metabolism of thiopurine, 6-mercaptopurine (6-MP), and azathioprine (AZA), and are utilized in ongoing therapy, especially acute lymphoblastic leukemia (ALL), and as immunosuppressants for autoimmune or inflammatory diseases, especially inflammatory bowel disease (IBD) [18]. Patients with *NUDT15*2*, **3*, or *TPMT*3C* variants have reduced ability to metabolize active thiopurine metabolites, increasing the risk of myelosuppression. In MDS/AML cases, standard-dose thiopurines may lead to severe myelosuppression and neutropenia in slow metabolizers [18, 19]. CPIC guidelines recommend considering significant dose reductions (30–90%) or alternative therapies for these patients to prevent the risk of hematologic toxicity [20].

Due to limitations in accessing sample data, the sample size in this study was small (n = 9), which may reduce the reliability and applicability of the findings when compared to studies that included a larger cohort of patients with MDS/AML with 20q deletion. Therefore, future studies should aim to include a larger sample size to strengthen the validity of the results.

Although the sample size was small, the results provided valuable information about the importance of choosing gene targets specific to the disease for this patient group. This approach could eliminate the need for large gene panels, thereby reducing turnaround time, technical complexity and operating cost.

All in all, Epigenetic Regulators (*ASXL1*, *TET2*, *KMT2D*, and *BCOR*), Signaling & Transcription Factors (*TP53*, *KRAS*, *KIT*, *CBL*, *PHF6*, and *IKZF1*), RNA Splicing Factors (*SF3B1* and *U2AF1*), Cohesin Complex (*CTCF*), and Pharmacogenetic (*NUDT15**2, *3, and *TPMT**3C) were detected in the study. These mutations should be introduced for application in the development of a customized panel for MDS/AML with 20q deletion.

VI. CONCLUSION

This study identified several genes in MDS/AML with 20q deletion samples, which are recommended as significant genetic mutations for a new gene panel-based assay. In the future, we intend to perform similar investigations on other populations, such as patients with 5q or 7q deletions. We expect that the resulting data will support the refinement of diagnostic workflows at the Hospital and contribute to a national reference database for molecular testing in hematologic cancers. Ultimately, this research can improve diagnostic accuracy, guide personalized treatment, and enhance clinical outcomes for MDS/AML patients across Thailand.

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Development and Validation of Real-Time PCR Application for Screening of *DDX41* Mutations on MDS/AML

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Abstract. Myelodysplastic Syndrome (MDS) and Acute Myeloid Leukemia (AML) are hematological malignancies frequently associated with genetic mutations, including those in the *DDX41* gene. *DDX41* mutations have increasingly been recognized as important in the pathogenesis, prognosis, and familial predisposition of these diseases. Current methods for mutation detection, such as Sanger sequencing and next-generation sequencing (NGS), are accurate but time-consuming and costly, limiting their use for rapid clinical screening. This study developed and validated a real-time PCR-based assay for the rapid and sensitive screening of *DDX41* mutations with three key hotspots: *R525H*, *M1L* and *D140fs*2* in patients with MDS/AML. The assay was optimized for primer and probe specificity, reaction efficiency, and detection sensitivity. Analytical validation was conducted using synthetic plasmid controls containing wild-type and mutant sequences, followed by clinical validation on DNA samples from confirmed MDS/AML cases. The performance of the Real-time PCR assay was compared with conventional sequencing to evaluate sensitivity, specificity, and turnaround time. The developed assay demonstrated a rapid turnaround time of 4-6 hours, significantly faster than the 2-4 weeks required for Next-Generation Sequencing and results demonstrated 100% concordance with NGS and Sanger sequencing. These results provide a validated Real-time PCR application for *DDX41* mutation screening to facilitate earlier diagnosis and genetic counseling in MDS/AML patients, enabling timely clinical decision-making and potentially improving patient outcomes.

Index Terms—Acute Myeloid Leukemia, *DDX41*, Genotyping, Myelodysplastic Syndrome, Real-Time PCR.

I. INTRODUCTION

Myelodysplastic Syndrome (MDS) and Acute Myeloid Leukemia (AML) are hematological malignancies characterized by clonal abnormalities of hematopoietic stem cells and progenitor cells, with a high propensity for progression to aggressive leukemia [1],[2]. Over the past decade, advances in molecular genetics have highlighted the pivotal role of recurrent gene mutations in the pathogenesis, diagnosis, prognosis, and therapeutic stratification of MDS/AML. Among these, mutations in the DEAD-box helicase 41 (*DDX41*) gene have emerged as clinically significant [3], [3].

DDX41 is a member of the DEAD-box family of RNA helicases and plays a critical role in RNA splicing, ribosome biogenesis, and innate immune signaling. Germline and somatic mutations in *DDX41* have been increasingly recognized as predisposing factors for familial myeloid neoplasms. Patients with *DDX41* mutations often present with late-onset MDS or AML, and these alterations are associated with distinct clinical and pathological features.

Identifying such mutations is crucial not only for diagnostic and prognostic purposes but also for guiding family screening and genetic counseling [3],[4].

Despite their importance, detecting *DDX41* mutations in routine clinical practice remains challenging due to limitations in accessibility and cost of next-generation sequencing (NGS), the current gold standard. Real-time polymerase chain reaction (RT-qPCR) offers a rapid, sensitive, and cost-effective alternative for mutation screening, making it highly suitable for clinical laboratories, particularly in resource-limited settings. The development of a reliable real-time PCR-based genotyping for *DDX41* mutation screening could therefore significantly improve early detection, risk stratification, and personalized management of MDS and AML patients [4],[5]. The selection of *R525H*, *M1L* and *D140fs*2* was based on clinical significance. *R525H* is recognized as the most frequent somatic second-hit mutation in *DDX41*-mutated patient globally and have been specifically identified in Thai cohorts[6]. *M1L* and *D140fs*2* were included as they present the most prevalence germline mutations in globally[7].

The purpose of this research is to develop and validate a real-time PCR-based application for the rapid and specific screening of common pathogenic *DDX41* mutations in patients with MDS/AML. By establishing a robust, efficient screening tool, this study aims to streamline the diagnostic workflow and facilitate the early identification of patients who may benefit from tailored surveillance and treatment strategies. The findings will provide a foundation for integrating targeted molecular screening into routine clinical practice, thereby improving patient outcomes and family management in the context of *DDX41*-associated myeloid malignancies.

II. METHODOLOGY

A. Clinical Sample and Ethics

A total of 91 (n=91) remnant bone marrow samples were collected from patients diagnosed with Myelodysplastic Syndrome (MDS, n=35) and Acute Myeloid Leukemia (AML, n=56) at the Department of Pathology, Ramathibodi Hospital, Mahidol University, between January 2021 and December 2024. Ethical approval was granted by the Institutional Review Board of Mahidol University (COA.NO. MURA2025/606). This study utilized de-identified archived samples (2021-2024); the 2025 COA covers the analysis of this cohort. All samples were archived and de-identified to maintain patient confidentiality in accordance with the Declaration of Helsinki[8].

B. DNA Extraction and Qualification

Genomic DNA was isolated using the QIAamp DNA Blood Mini Kit. Sample lysis was performed at 56°C with AL buffer and protease. DNA purification followed a standard spin-column protocol, with washing steps using AW1 and AW2 buffers. Quantification was performed using a NanoDrop™ 2000 Spectrophotometer, targeting an A260/A280 ratio of 1.8-2.0.

C. real-time PCR reaction setup

Assays utilized TaqMan™ Genotyping Master Mix containing 2xAmpliTaQ Gold™ DNA Polymerase 12.5µl. Each 25 µl reaction included 10 ng /5µl of DNA template and 1.25 µl of 20x TaqMan Assay Mix. Specific primer and probe sequences were obtained as pre-designed TaqMan™ genotyping assays (Thermo Fisher Scientific, USA). The ***R525H*** variant was analyzed using a custom assay (ID: ANAAXTK), while ***D140fs*2***(rs762890562) and ***MIL*** (rs141601766) were analyzed using MTO (Made-To-Order) assays (IDs: C_392253550_10 and C_171186806_10, respectively). Details of target variants, protein changes, and assay ID are shown in Table I.

TABLE. I. DETAIL TARGET VARIANTS OF *DDX41* GENE

Target variant	Protein Changes	dbSNP ID	Thermofisher Assay ID
<i>R525H</i> (Somatic)	p.Arg 525His	-	ANAAXTK (Custom)
<i>MIL</i> (Germline)	p.Met1Ile	rs141601766	C_171186806_10 (MTO)
<i>D140fs*2</i> (Germline)	p.Asp140 delinsGly Ter	rs762890562	C_392253550_10 (MTO)

TABLE. II. REACTION MIX COMPONENTS

Components	Volume per reaction (µl)	Final Concentration
TaqMan™ Genotyping Master Mix (2x)	12.5	1x
TaqMan™ Genotyping Assay Mix (20x)	1.25	1x
Genomic DNA template (10ng)	5	
Nuclease-free water	6.25	
Total Volume	25	

D. Thermal cycling condition

The thermal profile was optimized on the QuantStudio™ 5 system, as shown in Table III.

TABLE. III. PCR THERMAL CYCLING CONDITION

Step	Temp (°C)	Duration	Cycles
AmpliTaQ Gold, UP Enzyme Activation	95	10 min	Hold
Denature	95	15 sec	40
Anneal/Extend	60	1 min	

E. Data Analysis

Data acquisition and allelic discrimination analysis were performed using Diomni design and analysis software v3.0. Genotype calls were determined using an automated clustering algorithm, with manual inspection of the multicomponent plots to ensure clear separation of fluorescent clusters.

III. RESULTS AND DISCUSSION

A. Demographic Profile of the Patient Cohort

The demographic characteristics of the 91 patients diagnosed with MDS and AML were analyzed to establish a baseline for this study. The participants' ages ranged across several decades, with a notable concentration in the elderly population. Figure 1 illustrates the age distribution, showing that the highest frequency of cases occurred in the 70-79 age group. Following the age analysis, gender composition was evaluated. Figure 3 shows the sex distribution of the patients, with female patients (n=53) accounting for 58% of the total cohort, compared with male patients (n=38).

Age Distribution of Patients with MDS and AML

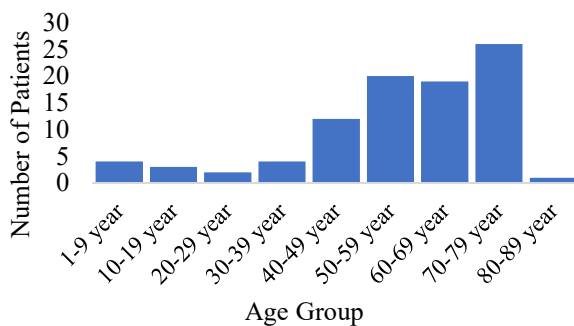


Figure. 1. Age Distribution of Patients with MDS and AML

Sex Distribution of Patients with MDS and AML

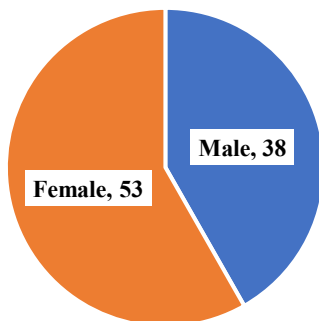


Figure. 2. Sex Distribution of Patients with MDS and AML

Numbers of Patients with MDS and AML

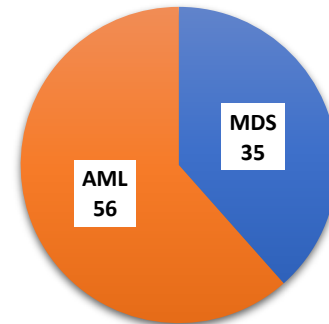


Figure. 3. Numbers of Patients with MDS and AML

B. Cytogenetic Characteristics

Cytogenetic abnormalities are critical prognostic factors in myeloid neoplasms. The chromosome distribution among 91 samples was categorized to identify recurring patterns associated with MDS and AML. Figure 4 displays the variety of chromosomal alterations detected. Normal karyotype was the most prevalent finding, followed by complex karyotypes and specific abnormalities, such as trisomy 8, t(9;11), and so on.

Chromosome Distribution of Patients with MDS and AML

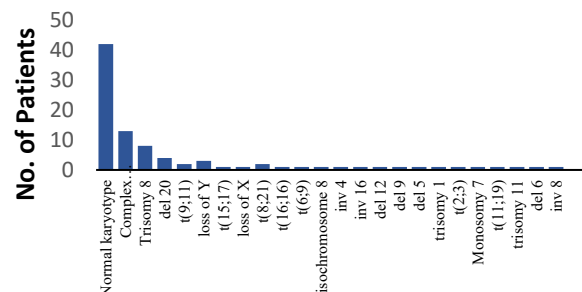


Figure. 4. Chromosome Distribution of Patients with MDS and AML

C. Real-Time PCR Assay Development and Allelic Discrimination

The optimized Real-time PCR assays for the *R525H*, *MIL* and *DI40fs*2* variants underwent rigorous analytical validation. The success of the allelic discrimination strategy is best visualized in the scatter plots. These plots demonstrate clear, tight clustering of samples into distinct populations. The clear separation of these clusters confirms the high analytical specificity and robustness of the developed qPCR assays for accurate genotype calling. The interpretation of genotypes is summarized in table IV.

TABLE. IV. INTERPRETATION CRITERIA FOR
ALLELIC DISCRIMINATION PROBES

	VIC dye	FAM dye
Wild-type homozygous	Positive	Negative
Mutant homozygous	Negative	Positive
Heterozygous	Positive	Positive
No-template control	No amplification	

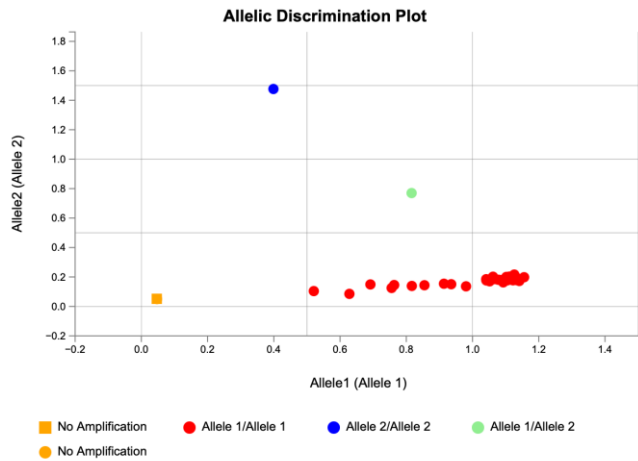


Figure. 5. Allelic discrimination plot for *DDX41 R525H* variant.

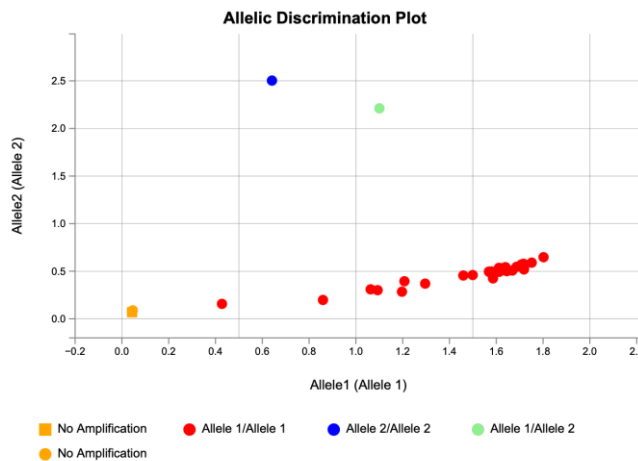


Figure. 6. Allelic discrimination plot for *DDX41 MIL* variant.

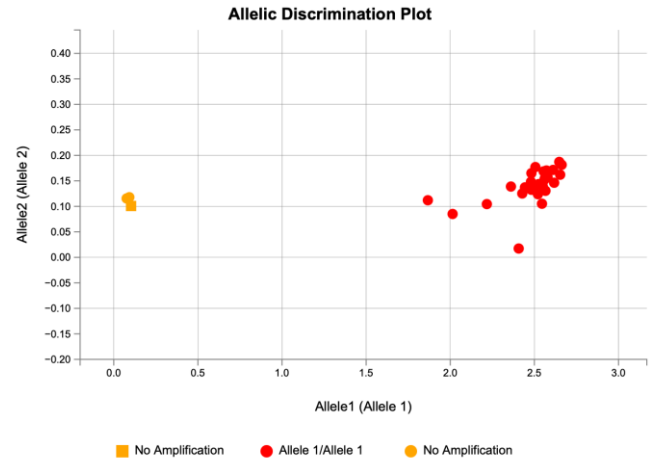


Figure. 7. Allelic discrimination plot for *DDX41 D140fs*2* variant.

During the assay development phase, an optimization challenges was encountered regarding master mix compatibility. Successful genotyping results (n=91), summarized in Table V, were obtained using a complete Thermo Fisher Scientific reagent system, including the TaqMan Genotyping Master Mix, Assay Mix, GeneBlock 10x6 concentration for control. While the allelic discrimination for *R525H* and *MIL* show clear cluster separation, the *D140fs*2* variant (Figure 7) exhibited a tighter cluster distribution. This variation is likely due to frameshift mutation, which can influence the hybridization kinetics and thermal stability of the TaqMan probes. Despite the reduced separation, the assay remains capable of distinguishing the wild-type population from the non-template controls, and further optimization of the thermal cycling conditions is currently underway to enhance the discrimination power for this specific variant.

TABLE. V. SUMMARY OF CLINICAL AND
MUTATIONAL PROFILES (COHORT OF n=91)

Variable	Category	Frequency(n)	Percentage (%)
Diagnosis	AML	56	61.5%
	MDS	35	38.5%
Gender	Male	49	53.8%
	Female	42	46.2%
Hematological finding	Bicytopenia	21	23.1%
	Pancytopenia	24	26.4%
	Leucocytosis	10	11.0%
	Others	36	39.5%
	Normal	43	47.3%
Cytogenetics	Complex	13	14.3%
	Karyotype		
	Trisomy 8	8	8.8%
	Deletions	8	8.8%
	Others	19	20.8%
Genotype	Homozygous	87	95.6%
	Wild type		
	Undetermined	4	4.4%

D. Reagent Optimization and Troubleshooting Analysis

As summarized in Table V, genotyping was performed on a cohort (n=91) to evaluate the assay's performance across Master Mix formulations.

Challenges with Hybrid System: Preliminary experiments using the PCRBIO Master Mix (PCR Biosystems, UK) in combination with TaqMan assays yielded suboptimal allelic discrimination. As illustrated in Figure 8 and 9, the genotyping clusters for *DDX41 R525H* and *MIL* variants exhibited an elongated distribution along the diagonal axis, suggesting inconsistent probe hybridization or competitive binding when using non-proprietary master mixes. And the *DDX41 D140fs*2* variant remained relatively low, as seen in the cluster positional near the origin in Figure 10.

To maintain methodological consistency and ensure the highest diagnostic accuracy, the study reverted to the validated Thermo Fisher Scientific reagent system. Consequently, all samples were processed using the standardized Thermo Fisher protocol to achieve reliable and reproducible allelic discrimination across the entire cohort of 91 samples.

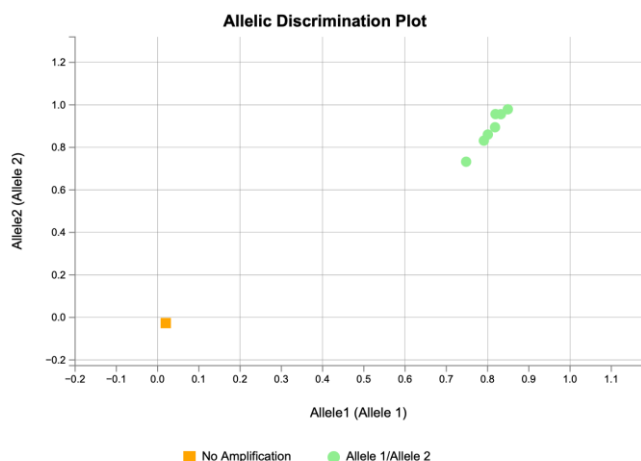


Figure. 8. Allelic discrimination plot for *DDX41 R525H* variant.

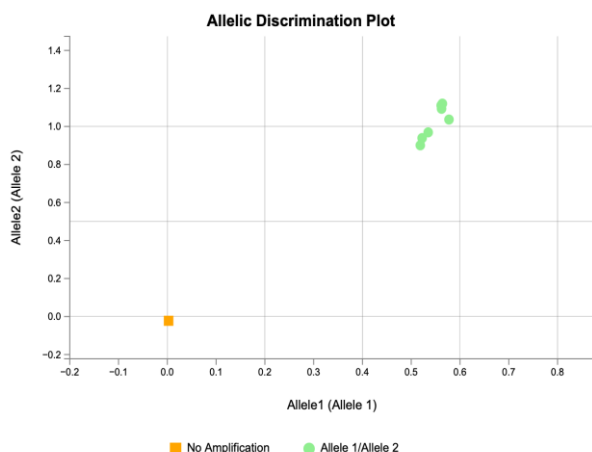


Figure. 9. Allelic discrimination plot for *DDX41 MIL* variant.

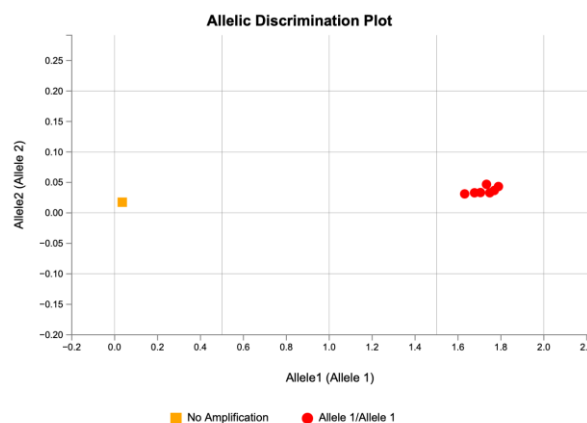


Figure. 10. Allelic discrimination plot for *DDX41 D140fs*2* variant.

E. Analytical Sensitivity and Positive Control Validation

The analytical performance of the RT-qPCR assay was evaluated using synthetic **GeneBlock** standards (Genscript, USA) to establish a baseline for genotype calibration and sensitivity.

Standard Curve and Sensitivity: As illustrated in Figure 11, a serial dilution of the wild-type (WT) GeneBlock ranging from 10^2 to 10^6 copies was performed to assess the assay's detection limit. The Allelic Discrimination Plot demonstrates a high degree of linearity and precise clustering of the standards.

Control Reliability: The tight diagonal distribution of the standards confirms that *DDX41* variants provide consistent signal amplification across the wide dynamic range. No amplification on No-template control and Negative control, ensuring absence of contamination or primer-dimer artifacts.

Clinical Application: This high sensitivity allows the assay to reliably detect *DDX41* alleles even in clinical samples with relatively low DNA yields, facilitating early screening for MDS/AML predisposition.

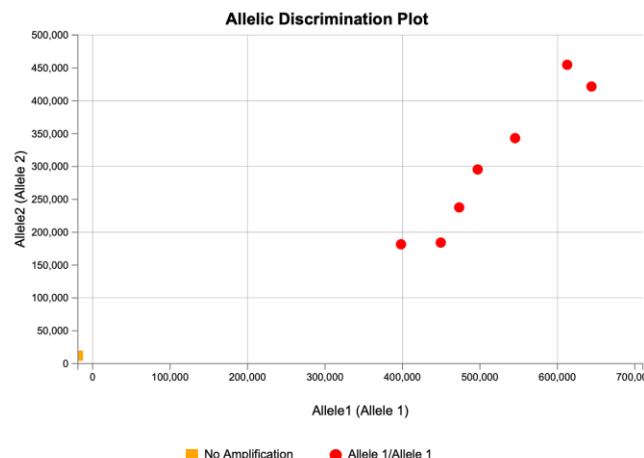


Figure. 11. Allelic discrimination plot of wild-type GeneBlock standards showing a serial dilution range from

10^2 to 10^6 copies, the sample, the negative control and the NTC.

F. Turnaround Time and Cost-Effective

The clinical utility of the developed RT-qPCR assay was evaluated by comparing its Turnaround Time (TAT) and operational costs with those of the current gold standard, Next-Generation Sequencing (NGS). While NGS typically requires 2-4 weeks to provide results due to complex library preparation and bioinformatic analysis, the developed RT-qPCR assay can complete the entire workflow within 4-6 hours. And the operational cost per sample is significantly lower, making it a suitable tool for large-scale population screening in resource-limited settings.

Table. VI. COMPARISON OF PERFORMANCE AND COST-EFFECTIVENESS: RT-qPCR, SANGER SEQUENCING AND NEXT-GENERATION SEQUENCING

	RT-qPCR	Sanger sequencing	Next-Generation Sequencing
Mutation scope	Targeted (3variants)	Targeted (Single fragment)	Whole gene
Turnaround time	4-6 Hours	2-3 Days	2-3 Weeks
Sensitivity	High	Low	Very high
Cost per Sample	Low (300-500 THB)	Moderate (800-1,200 THB)	High (>10,000 THB)
Suitability	Rapid screening	Single variant confirmation	Comprehensive profiling

Although our current cohort of 91 patients provides a valuable preliminary screening of *DDX41* hotspots in the Thai population, the absence of mutations may be influenced by limited sample size. To further validate these findings and improve the sensitivity of our prevalence data, recruitment is ongoing, with plans to expand the cohort future.

IV. CONCLUSION

This study successfully developed and validated a rapid, cost-effective Real-Time PCR-based screening for *DDX41* mutations (*R525H*, *MIL*, and *D140fs*2*) in patients with MDS and AML. Our findings demonstrate that the optimized TaqMan genotyping assay provides high analytical sensitivity, capable of detecting as few as 10^6 copies of mutant DNA, with clear allelic discrimination as shown in the distinct clustering of clinical and synthetic standards.

The investigation into reagent optimization revealed that Master Mix compatibility is a critical factor for achieving diagnostic-grade results. While Next-Generation Sequencing (NGS) remains the gold standard for comprehensive profiling, this RT-qPCR method offers a cost-effective, rapid alternative, reducing the diagnostic turnaround time to a single working day.

In conclusion, the developed assay facilitates earlier identification of *DDX41* variants, which is essential for timely clinical decision-making, risk stratification, and genetic counseling for familial myeloid neoplasms. Future work will focus on expanding the mutation panel and increasing the current cohort of 91 patients to further validate these findings and improve the sensitivity of regional prevalence data. This will involve intergrading this tool into routine hematological molecular diagnosis.

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Comparative Flow Cytometric Evaluation of Differential T Helper Cell Activation Phenotypes in Inflammatory Autoimmune Diseases

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Abstract. Autoimmune diseases, including axial spondyloarthritis (axSpA), psoriatic arthritis (PsA), rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE), share overlapping inflammatory pathways driven by T helper 1 (Th1) and Th17 cells. While total Th cell frequencies reflect the overall composition of the adaptive immune compartment, they do not distinguish between resting and actively engaged cells. This exploratory cross-sectional study aimed to compare total and activated Th1 and Th17 profiles across these diseases to determine whether activation-defined phenotypes improve discrimination among disease groups. Peripheral blood mononuclear cells from 48 patients (21 axSpA, 11 PsA, 12 RA, 4 SLE) were stimulated with PMA, ionomycin, and monensin, then stained for surface and activation markers and intracellular cytokines. Flow cytometry was used to evaluate Th1 and Th17 subsets, defining activated Th1 and Th17 cells as IFN- γ +HLA-DR+CD38+ and IL-17A+HLA-DR+CD38+ within CD45+CD3+CD4+ T cells, respectively. Activated Th17 cells showed the most distinct cross-disease distribution, with the highest median in axSpA and the lowest in PsA. Post hoc analysis revealed that activated Th17 frequencies were significantly higher in axSpA than in PsA ($p = 0.015$). Overall, activated Th1 frequencies differed significantly across groups ($p = 0.046$), although no pairwise comparisons remained significant after Holm correction. These findings suggest that Th17 activation phenotypes may offer additional immunophenotypic information beyond total subset frequencies, with the most notable difference observed between axSpA and PsA. The SLE cohort was small ($n = 4$), which limits interpretation for that group, and all findings should be considered preliminary pending validation in larger, independent cohorts.

Index Terms— T helper 1 cell, T helper 17 cell, activation marker, autoimmune disease, flow cytometry

I. INTRODUCTION

Autoimmune diseases are chronic inflammatory conditions caused by immune dysregulation and loss of self-tolerance. Clinical manifestations vary considerably, ranging from articular and musculoskeletal involvement to multisystem disease. Among diseases affecting the axial skeleton and peripheral joints, axial spondyloarthritis (axSpA) and psoriatic arthritis (PsA) share overlapping features of spondyloarthritis, including inflammatory back pain and enthesitis [1, 2]. AxSpA mainly affects the spine and sacroiliac joints, whereas PsA is characterized by psoriatic skin or nail involvement and may also present with dactylitis [3]. Rheumatoid arthritis (RA) presents with synovitis and symmetric small-joint polyarthritis. RA may also be associated with erosive disease [4, 5]. Systemic lupus erythematosus (SLE) is an autoimmune disease involving multisystem inflammation [6, 7].

Adaptive immune cells, T helper cells (Th), play an important role in inflammatory responses through cytokine production and immune regulation [8]. Two key cell types

drive pro-inflammatory responses. T helper 1 (Th1) cells produce interferon-gamma (IFN- γ), which activates macrophages, promotes inflammatory signaling, and amplifies local immune activation [9]. T helper 17 (Th17) cells produce interleukin-17A (IL-17A), which induces pro-inflammatory mediators and chemokines, including interleukin-6 (IL-6), granulocyte colony-stimulating factor (G-CSF), and neutrophil-recruiting chemokines, thereby amplifying the inflammatory response and promoting neutrophil-driven inflammation [10, 11]. Previous studies have shown that IL-17-producing CD4+ T cells are increased in inflammatory arthritis, supporting an important role for Th17-related immunity in inflammatory disease [12, 13]. Together, these observations suggest that comparative evaluation of Th phenotypes may provide insight into disease-associated immune heterogeneity across autoimmune rheumatic diseases.

While total Th cell frequencies reflect the overall composition of the adaptive immune compartment, they do not capture the degree of cellular activation, potentially

limiting pathophysiological insight. In flow cytometric studies, co-expression of HLA-DR and CD38 has been used to identify activated T-cell states and to quantify immune activation in inflammatory and immune dysregulation settings [14, 15]. Despite increasing evidence for the role of Th17-related immunity in inflammatory rheumatic diseases, comparative evaluation of Th1, activated Th1, Th17, and activated Th17 across clinically overlapping immune-mediated inflammatory diseases using a standardized flow cytometric protocol remains limited [16, 17]. Therefore, this study aimed to compare Th1, activated Th1, Th17, and activated Th17 among patients with axSpA, PsA, RA, and SLE for mechanistic immune profiling and to determine whether activation-defined phenotypes provide additional immunophenotypic information for disease characterization compared with total Th1 and Th17 frequencies.

II. METHODOLOGY

Study participants

Inclusion criteria required participants to be aged 18 years or older and to have been diagnosed by a specialist physician with axSpA according to the ASAS classification criteria [18], PsA according to the CASPAR criteria [19], RA according to the 2010 ACR/EULAR criteria [20], or SLE according to the 2019 EULAR/ACR criteria [21]. The study was approved by the Ethics Committee of the Department of Medicine, Faculty of Medicine Ramathibodi Hospital, Mahidol University (COA No. MURA2025/470, approved June 5, 2025), prior to sample collection, and written informed consent was obtained from all participants. Sample sizes across groups reflect the consecutive enrollment of eligible patients within the study period; the small SLE cohort (n = 4) is acknowledged as a limitation. Individuals with autoimmune diseases outside the study-defined groups, recent or chronic infection, active or recent malignancy, pregnancy, organ transplantation, or current biologic disease-modifying anti-rheumatic drug (bDMARD) or targeted synthetic DMARD (tsDMARD) treatment were excluded.

Flow cytometry analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by density gradient centrifugation. Surface staining was performed using BD Tritest™ CD3 FITC/CD4 PE/CD45 PerCP (clone SK7/SK3/2D1; Cat. No. 342413; BD Biosciences, USA). Activation status was assessed using anti-human HLA-DR APC (clone L243; Cat. No. 307610; BioLegend, USA) and anti-human CD38 APC-Cy7 (clone HIT2; Cat. No. 356616; BioLegend, USA), with fluorescence minus one (FMO) controls used for gating of activation markers. For intracellular cytokine detection, cells were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate and 1 µg/mL ionomycin in the presence of monensin (BD GolgiStop™; BD Biosciences, USA), with unstimulated cells serving as negative controls. After fixation and permeabilization using BD Fixation/Permeabilization solution (BD Biosciences, USA), intracellular staining was performed using anti-human IFN-γ PE-Cy7 (clone B27; Cat. No. 557643; BD Biosciences, USA) and anti-human IL-17A BV421 (clone BL168; Cat. No. 512321; BioLegend, USA), with corresponding Mouse IgG1κ isotype controls (PE-Cy7, clone MOPC-21, Cat. No. 557646; BV421, clone X40, Cat. No. 562438; both from BD Biosciences, USA). Co-expression of HLA-DR and CD38 was used to identify activated T-cell states, as both markers are upregulated upon T-cell activation, with HLA-DR reflecting antigen-presenting capacity and CD38 reflecting metabolic activation, and their co-expression has been applied to quantify activated T-cell populations in inflammatory settings [15, 16]. Surface staining for HLA-DR and CD38 was performed prior to cell stimulation to reflect the in vivo activation state of circulating T cells at the time of blood collection. The gating strategy was applied hierarchically. Lymphocytes were identified by FSC/SSC characteristics, followed by CD45⁺ selection, then CD3⁺CD4⁺ T cells. Th1 and Th17 cells were defined as IFN-γ⁺ and IL-17A⁺ populations, respectively, within CD3⁺CD4⁺ T cells. Activated subsets were further identified by co-expression of HLA-DR and CD38 (Fig. 1). Samples were acquired on a BD FACSLytic™ flow cytometer with a 3-laser configuration (2 violet, 4 blue, and 2 red detectors; BD Biosciences, USA) and analyzed using FlowJo™ software (BD Biosciences, USA).

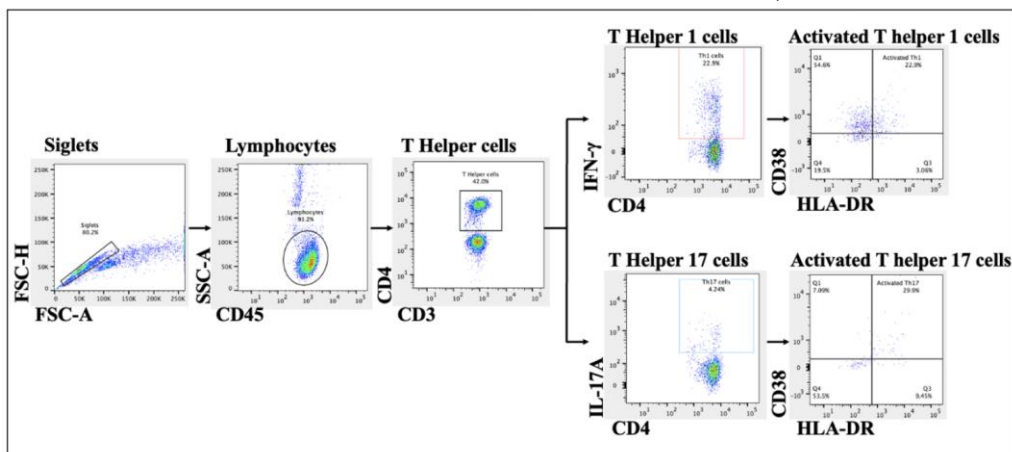


Fig. 1. Gating strategy for identification of Th1, Th17, activated Th1, and activated Th17 cells by multiparameter flow cytometry

Statistical analysis

Categorical variables were compared using the chi-square test or Fisher's exact test, as appropriate. Continuous variables were expressed as median (interquartile range, IQR) and compared across disease groups using the Kruskal–Wallis test. Dunn's multiple comparisons test was applied for post hoc pairwise analysis when appropriate, with Holm correction applied within each parameter. A p -value less than 0.05 was considered statistically significant. Values in tables are presented as median [IQR] for continuous variables and n (%) for categorical variables. Statistical analyses were performed using GraphPad Prism version 10 (GraphPad Software, San Diego, CA, USA)

III. RESULTS AND DISCUSSION

A total of 48 participants were included in the analysis, comprising 21 patients with axSpA, 11 with PsA, 12 with RA, and 4 with SLE. All participants were enrolled consecutively during the study period, and none were receiving bDMARDs or tsDMARDs at the time of enrollment. The proportion of female participants differed significantly across disease groups ($p = 0.015$), with the highest proportions observed in the PsA (90.9%) and SLE (100%) groups, whereas axSpA was predominantly male (57.1%) (Table I). This sex distribution reflects the composition of the consecutively enrolled patient population during the study period and is consistent with the known sex predilection of these diseases. Age and body mass index (BMI) did not differ significantly among groups. No patients were receiving bDMARDs or tsDMARDs at enrollment.

Total Th1 and Th17 frequencies did not differ significantly ($p = 0.676$ and $p = 0.096$, respectively), indicating that activation state rather than total subset frequency provides greater immunophenotypic discrimination across these diseases (Fig. 2). Within the

CD45+CD3+CD4+ T cell population, Th1 and Th17 cells were defined as IFN- γ + and IL-17A+ cells, respectively, while activated Th1 and activated Th17 were further identified by co-expression of HLA-DR and CD38. Post hoc Dunn's multiple comparisons test with Holm correction revealed that the frequency of activated Th17 cells was significantly higher in axSpA than in PsA ($p = 0.015$) (Fig. 2D). This was the only pairwise comparison to reach statistical significance after correction. These data are presented as individual points with the median and interquartile range. Activated Th17 cells showed a distinct distribution across the groups, with the highest median level in axSpA and the lowest in PsA. Although overall activated Th1 frequencies differed significantly, no pairwise comparison remained significant after adjustment.

These findings suggest that activated Th phenotypes, particularly activated Th17, may provide additional immunophenotypic information beyond total Th subset frequencies. Surface activation markers were stained prior to stimulation to capture the basal immune activation state at the time of sampling. Although Th immunophenotyping may reflect distinct patterns of immune activation among these diseases, it captures only one component of the broader immune landscape. Autoimmune diseases involve a complex network of innate immune cells, regulatory T cells, B cells, cytokines, and inflammatory proteins that collectively influence disease activity and treatment response [22–24].

Among the pairwise comparisons reaching significance after Holm correction, activated Th17 was significantly higher in axSpA than in PsA. Although these diseases share overlapping inflammatory and clinical features, including enthesitis and inflammatory back pain, this finding suggests a distinct Th17 activation profile in axSpA that is not captured by total Th17 frequency alone. This is biologically plausible, as the IL-23/IL-17 axis is particularly implicated in enthesal and axial inflammation in axSpA [10, 11]

TABLE I
BASELINE DEMOGRAPHIC CHARACTERISTICS ACROSS DISEASE GROUPS

Variable	AxSpA (n=21)	PsA (n=11)	RA (n=12)	SLE (n=4)	p -value
Sex, female, n (%)	9 (42.9)	10 (90.9)	6 (50.0)	4 (100.0)	0.015
Age (years)	51.00 [43.00, 59.00]	41.00 [32.50, 53.50]	59.00 [31.50, 65.25]	49.00 [42.25, 52.25]	0.301
BMI (kg/m ²)	24.94 [21.11, 28.26]	26.60 [22.10, 28.71]	22.69 [21.95, 26.90]	23.96 [21.63, 27.22]	0.986

Data presented as median [IQR]. IQR, interquartile range.

TABLE II
FLOW CYTOMETRIC ANALYSIS OF TH1, ACTIVATED TH1, TH17, AND ACTIVATED TH17 ACROSS DISEASE GROUPS

Variable	AxSpA (n=21)	PsA (n=11)	RA (n=12)	SLE (n=4)	p -value
Th1 (%)	6.98 [5.29, 13.90]	6.52 [3.97, 8.46]	8.11 [6.98, 9.24]	6.15 [4.82, 8.87]	0.676
Th17 (%)	2.50 [1.70, 4.01]	1.75 [1.32, 2.49]	1.87 [1.17, 2.38]	2.10 [1.71, 2.53]	0.096
Activated Th1 (%)	0.65 [0.30, 1.23]	0.34 [0.21, 0.55]	1.03 [0.68, 1.51]	1.86 [1.18, 2.33]	0.046
Activated Th17 (%)	0.79 [0.37, 1.14]	0.24 [0.18, 0.50]	0.40 [0.31, 0.53]	0.67 [0.49, 0.81]	0.013

Data presented as median [IQR]. IQR, interquartile range.

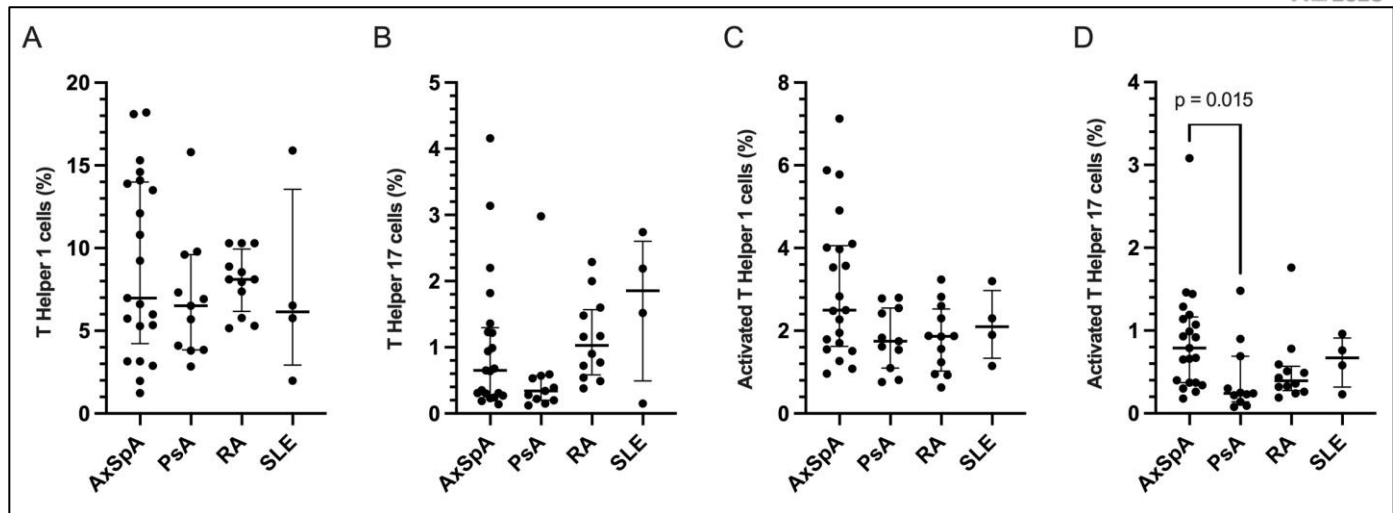


Fig. 2. Comparison of Th cell parameters across disease groups (A) Th1 (%), (B) activated Th1 (%), (C) Th17 (%), (D) activated Th17 (%)

The difference in activated Th17 between axSpA and PsA may reflect differences in pathway dominance, as axSpA is thought to be driven more prominently by axial Th17-related inflammation, while PsA involves a more heterogeneous immune landscape including Th1, Th17, and innate immune components. Regarding activated Th1, while the overall Kruskal–Wallis test was significant, no individual pairwise comparison survived Holm correction, underscoring the need for larger cohorts to detect specific between-group differences reliably.

Compared with a previous PsA study, both activated Th1 and activated Th17 levels in the present cohort were lower. Mean activated Th1 and activated Th17 values were 1.32% and 1.32%, respectively, whereas the corresponding median values in the present study were 0.34% and 0.24%. However, the activated Th1 and activated Th17 cells were defined using different markers and analytical strategies. Th subsets were identified as activated Th1 and activated Th17 using CXCR3 and CCR6 along with HLA-DR and CD38, whereas the present study defined these subsets using intracellular IFN- γ and IL-17A together with HLA-DR and CD38 [16]. Previous studies have also reported increased Th17 frequencies in RA, as well as increased Th17 frequencies in RA and axSpA, relative to control groups. In our cohort, Th17 levels were consistent with what was reported in axSpA, whereas Th17 levels appeared lower than previously reported [25]. In this study, the RA Th1 level was broadly comparable to prior reports, whereas the RA Th17 level appeared lower than that reported in some earlier studies [26]. These differences may reflect variation in cohort characteristics, stimulation conditions, comparator groups, and flow cytometric methodology.

Several limitations of this study warrant consideration. First, the overall sample size was modest, and the SLE cohort was particularly small ($n = 4$), precluding reliable post hoc analysis for that group. Second, no healthy control group was included. Therefore, all findings represent relative differences among disease groups rather than evidence of abnormal elevation above normal immune status. Third, the significant sex imbalance across groups, with PsA and SLE

being predominantly female and axSpA predominantly male, may represent a potential confounder in between-group comparisons. Fourth, the cross-sectional design limits causal inference and precludes assessment of how Th activation phenotypes evolve with disease activity or treatment. Finally, the definition of activated Th subsets using intracellular cytokines combined with HLA-DR and CD38 co-expression differs from chemokine receptor-based approaches used in some prior studies, which may contribute to quantitative differences in reported frequencies.

Despite these limitations, this exploratory cross-sectional study provides comparative flow cytometric data across four inflammatory autoimmune diseases using a consistent immunophenotyping protocol at a single institution. Future studies should address these limitations by recruiting larger and more balanced cohorts, including a healthy control group, and adopting a longitudinal design to assess whether activated Th17 and activated Th1 phenotypes track with disease activity indices, structural progression, and treatment response over time. Integration of broader immune profiling, including serum cytokines, innate immune cell characterization, and regulatory T cell assessment, would further clarify the immunological landscape across these diseases. Exploration of whether Th activation phenotyping can guide bDMARD selection represents a clinically meaningful direction, pending validation in adequately powered prospective cohorts.

IV. CONCLUSION

In this exploratory comparative flow cytometric study, activation-related Th phenotypes appeared to provide additional immunophenotypic information beyond total Th1 or Th17 frequencies across the four inflammatory autoimmune diseases examined. Among the evaluated parameters, activated Th17 showed the most distinct between-group variation, with the only statistically significant pairwise difference observed between axSpA and PsA after post hoc correction. These findings should be interpreted cautiously given the small overall sample size,

the particularly limited SLE cohort (n = 4), the absence of a healthy control group, and the cross-sectional study design. Nevertheless, they support the potential value of incorporating activation-defined Th phenotypes into comparative immune profiling across clinically overlapping autoimmune rheumatic diseases. Longitudinal studies in larger, independently validated cohorts, integrating Th activation phenotyping with clinical disease activity indices, serum cytokine measurements, and treatment outcomes, are warranted to confirm these preliminary observations and to clarify their clinical applicability.

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Whole Genome Sequencing-Based Pharmacogenomic Analysis of 6-Mercaptopurine Response in Pediatric Acute Lymphoblastic Leukemia

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Abstract. Thiopurine drugs, particularly 6-mercaptopurine (6-MP), are essential components of maintenance therapy for pediatric acute lymphoblastic leukemia (ALL) but are frequently associated with hematologic toxicity, especially neutropenia. Although pharmacogenetic variants in *NUDT15* and *TPMT* are established predictors of thiopurine intolerance, some patients with normal metabolizer phenotypes still develop neutropenia. This study aimed to identify additional genetic variants associated with thiopurine-induced neutropenia using a genome-wide association study (GWAS). Pediatric ALL patients receiving 6-MP during maintenance therapy were prospectively recruited from multiple centers in Thailand. Whole-genome sequencing was performed, and variants were analyzed using logistic regression to compare patients with and without neutropenia among individuals with normal metabolizer phenotypes for *NUDT15* and *TPMT*. A total of 62 patients were included. Baseline characteristics did not differ significantly between groups. Notably, a considerable proportion of patients with normal metabolizer phenotypes still developed neutropenia. Although several variants showed suggestive associations, none reached the genome-wide significance threshold ($p < 5 \times 10^{-8}$). These findings suggest that *NUDT15* and *TPMT* alone may not fully explain susceptibility to thiopurine-induced neutropenia. Larger studies are needed to identify additional genetic and clinical factors that may improve prediction of thiopurine toxicity.

Index Terms— Acute lymphoblastic leukemia, Genome-wide association study, 6-mercaptopurine, Neutropenia, Pharmacogenetics, Whole genome sequencing

I. INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common pediatric malignancy, accounting for approximately 25% of all childhood cancers. In recent decades, survival outcomes for children with ALL have improved substantially, with cure rates reaching approximately 85% in developed countries. These improvements are largely attributable to advances in risk-adapted therapeutic strategies and the implementation of multi-agent chemotherapy protocols (1).

Treatment of ALL generally consists of three sequential phases: induction, consolidation, and maintenance therapy, each designed to achieve specific therapeutic goals. Among these, the maintenance phase represents the final and longest stage of treatment, typically lasting approximately 2–3 years in pediatric patients. This phase plays a crucial role in

sustaining remission and preventing relapse while minimizing treatment-related toxicities. The two backbone agents used during maintenance therapy are 6-mercaptopurine (6-MP), administered daily, and methotrexate (MTX), administered weekly. Both agents have been used for decades and are supported by extensive clinical evidence (2) (3).

Ideally, medications administered over prolonged periods should cause minimal toxicity or only manageable adverse effects. However, in clinical practice, both 6-MP and MTX may cause severe toxicities and potentially life-threatening adverse events in susceptible patients. This has led to increasing interest in pharmacogenetics (PGx) as an approach to identify patients at higher risk of toxicity and to enable personalized therapeutic strategies that balance

treatment efficacy with safety (2).

6-MP, a thiopurine analogue, exerts its cytotoxic effects primarily through incorporation into DNA and RNA, leading to impaired leukemic cell proliferation. Despite its effectiveness, 6-MP has a narrow therapeutic index and is associated with a high risk of hematological toxicities, particularly myelosuppression. These adverse effects may necessitate dose reduction or temporary treatment interruption, thereby compromising treatment continuity and potentially increasing the risk of relapse. Importantly, the severity of 6-MP-related toxicity varies substantially among individuals. Pharmacogenetic studies have provided strong evidence that genetic variation is a major contributor to this interindividual variability (4).

Two genes, *thiopurine S-methyltransferase (TPMT)* and *nudix hydrolase 15 (NUDT15)*, play key roles in thiopurine metabolism. Loss-of-function variants in these genes impair drug metabolism, leading to excessive accumulation of cytotoxic metabolites and an increased risk of severe adverse effects. Consequently, the Clinical Pharmacogenetics Implementation Consortium (CPIC) recommends genotyping for *TPMT* and *NUDT15* prior to initiating thiopurine therapy in order to guide dose adjustments and improve treatment safety (5).

Nevertheless, clinical observations from 70 patients with inflammatory diseases who discontinued azathioprine therapy due to myelotoxicity at Vanderbilt University Medical Center in the United States found that the majority of affected individuals (57/70; 81.4%) were classified as normal or indeterminate metabolizers (6). This highlights the insufficiency of relying solely on *TPMT* and *NUDT15* for predicting adverse drug reactions (ADRs).

To address this gap, several studies have sought to identify additional pharmacogenetic determinants of thiopurine toxicity. Recent findings suggest that specific single nucleotide polymorphisms (SNPs) may play an important role. For example, rs3821169 in the *CRIM1* gene and rs13306435 in *IL6* have been associated with increased hematologic toxicity from 6-MP, particularly among Asian populations (7), (8) Moreover, variants in *CYP2A7* have been linked to susceptibility to leukopenia, while polymorphisms in *COMT* have been implicated in increased risk of hepatotoxicity during 6-MP treatment (9).

Genome-wide association studies (GWAS) are an unbiased way to examine the whole genome to find links between common genetic variants (SNPs) and specific traits. This method helps identify new genetic regions that may influence differences in drug response and improve our understanding of disease biology. GWAS are especially useful for finding variants with small effects that are often missed by candidate gene studies (10).

Therefore, this study aimed to perform a genome-wide association study (GWAS) to identify novel genetic variants associated with 6-mercaptopurine-induced neutropenia in pediatric patients with acute lymphoblastic leukemia.

II. METHODOLOGY

Study population

The study population comprised pediatric patients with acute lymphoblastic leukemia (ALL) who received 6-mercaptopurine (6-MP) treatment at multiple care centers in Thailand, including the Faculty of Medicine Ramathibodi Hospital, Mahidol University; the Faculty of Medicine, Chiang Mai University; the Faculty of Medicine, Chulalongkorn University; and the Department of Pediatrics, Phramongkutklao Hospital. Between 2021 and 2025, 103 pediatric patients with acute lymphoblastic leukemia (ALL) were prospectively enrolled and followed during maintenance therapy. Eligible patients were those diagnosed before 15 years of age. Patients were excluded if they met any of the following criteria: (1) classified as poor or intermediate metabolizers based on *TPMT* and *NUDT15* genotypes; (2) lost to follow-up or deceased during the study period; (3) had a history of treatments or medications associated with neutropenia, such as immunosuppressive agents; (4) had undergone prior hematopoietic stem cell transplantation; (5) had chronic conditions that could affect drug metabolism, including severe liver disease or chronic renal failure; or (6) were receiving concomitant medications known to interfere with 6-mercaptopurine (6-MP) metabolism, such as allopurinol. (11). In this study, GWAS was restricted to patients with normal metabolizer status for both *TPMT* and *NUDT15*, comprising a total of 62 patients in the study cohort.

The sample size was determined pragmatically rather than based on a formal statistical power calculation. This approach was adopted due to practical considerations, including the limited number of eligible patients, the rarity of the condition, and the predefined study period. As such, all patients who met the inclusion criteria during the study timeframe were included in the analysis.

Neutropenia was defined as an absolute neutrophil count (ANC) <1,000 cells/ μ L occurring within 9 weeks after initiation of maintenance therapy. The severity of neutropenia was classified and graded according to the National Cancer Institute Common Toxicity Criteria (NCI-CTC) (12).

This study was conducted in accordance with ethical standards, and written informed consent was obtained from the parents or legal guardians of all participating patients. The study protocol was approved by the Human Research Ethics Committee of the Faculty of Medicine Ramathibodi Hospital, Mahidol University (COA MURA2025/682).

Whole-Genome Sequencing and Variant Calling

Whole-genome sequencing (WGS) was performed on 101 samples using the BGISEQ-500 and NovaSeq sequencing platforms. All experimental procedures were carried out in accordance with applicable guidelines and regulations. Raw sequencing reads in FASTQ format underwent initial quality assessment prior to downstream analysis. Variant discovery was conducted using the NVIDIA Parabricks (version 3.8.1) HUMAN PAR pipeline on an NVIDIA A-100 GPU platform. The sequencing reads were aligned to the GRCh38 human reference genome using BWA-MEM (version 0.7.17), generating binary alignment map (BAM) files. Subsequently, the BAM files were processed using the Genome Analysis Toolkit (GATK) workflow for germline variant detection. Variant calling was performed with GATK HaplotypeCaller, which generated individual genomic variant call format (gVCF) files for each sample. These gVCF files were then combined and jointly genotyped to produce a consolidated variant call format (VCF) file for subsequent analyses (13).

Predicted phenotypes

TPMT and *NUDT15* star-allele genotyping was performed using Aldy (version 4.8.2), which infers pharmacogenetic haplotypes from sequencing data. The identified diplotypes were subsequently translated into predicted metabolizer phenotypes according to the Clinical Pharmacogenetics Implementation Consortium (CPIC) guidelines (5).

Variant Quality Control and Population Stratification

The merged VCF file was processed using bcftools (version 1.13) and converted to PLINK binary format (pgen, pvar, and psam files) for downstream analyses using PLINK version 2.0.

Quality control filtering was applied prior to association analysis. Sex concordance was evaluated based on X-chromosome heterozygosity, where samples with an inbreeding coefficient (F) greater than 0.8 were classified as male and those with values lower than 0.2 as female. Variants were retained if they met the following criteria: minor allele frequency (MAF) > 0.05 and Hardy-Weinberg equilibrium (HWE) *p*-value > 1×10^{-10} . Samples showing evidence of relatedness ($F > 0.2$) were excluded. Individuals with abnormal heterozygosity rates, defined as values outside mean ± 2 standard deviations, were also removed to reduce potential genotyping artifacts (14).

Population structure was evaluated using principal component analysis (PCA) implemented in PLINK version 2.0. Principal components derived from autosomal variants passing quality control were included as covariates in the association analysis to account for population stratification (14).

Statistical analysis

Demographic and clinical characteristics between the case and control groups were analyzed using Pearson's chi-squared test or Fisher's exact test for categorical variables,

with Fisher's exact test applied when expected or observed cell counts were fewer than five. Continuous variables were compared using the Wilcoxon rank-sum test. Categorical variables are presented as frequencies and percentages, while continuous variables are summarized as medians with interquartile ranges (IQR). A *p*-value < 0.05 was considered statistically significant.

Genome-wide association analysis was performed using logistic regression, with sex included as a covariate in the model. The threshold for genome-wide significance was set at $p < 5 \times 10^{-8}$, corresponding to the conventional Bonferroni-corrected significance level used in GWAS (15).

III. RESULTS AND DISCUSSION

Baseline demographic, clinical, and pharmacogenetic characteristics of the study population stratified by neutropenia status are presented in Table 1. The median age of participants was 4 years, and 62.71% were male. There were no significant differences between the neutropenia and non-neutropenia groups with respect to age, sex, risk group, or initial 6-mercaptopurine (6-MP) dose (all $p > 0.05$).

When stratified by metabolizer phenotype, a substantial proportion of patients with the normal metabolizer (NM) phenotype for *TPMT* and *NUDT15* also developed neutropenia, accounting for 58.95% and 60.64%, respectively. These findings suggest that neutropenia may occur even in individuals without reduced-function variants in these genes.

Over time, patients who developed neutropenia exhibited significantly lower absolute neutrophil counts (ANC) during both the early and late phases of treatment compared with those without neutropenia.

Together, these results indicate that genetic variation in *TPMT* and *NUDT15* alone may not fully explain susceptibility to thiopurine-induced hematologic toxicity, particularly neutropenia, suggesting that additional genetic or clinical factors may contribute to this adverse drug reaction (7) (16).

Table 2 summarizes the clinical and demographic characteristics of patients with normal *TPMT* and *NUDT15* metabolizer phenotypes ($n = 62$), stratified into control ($n = 25$) and case ($n = 37$) groups. The median age was comparable between groups (5.5 vs. 4 years, $p = 0.6552$). There was no significant difference in gender distribution, with males comprising 41.17% in the control group and 58.83% in the case group ($p = 0.85$).

Risk group classification (standard, high, very high, and Philadelphia chromosome-positive) did not differ significantly between groups ($p = 0.141$). The initial 6-mercaptopurine (6MP) dose was also similar between control and case groups (39.24 vs. 37.5 mg/m²/day, $p = 0.8796$), indicating comparable treatment initiation.

However, significant differences were observed in absolute neutrophil count (ANC). Early ANC (weeks 1–9) and late ANC (weeks 10–24) were significantly lower in the case group compared to controls ($p = 0.0002$ and $p < 0.0001$, respectively), suggesting increased hematologic toxicity in the case group.

In contrast, liver enzyme levels, including aspartate aminotransferase (AST) and alanine aminotransferase (ALT), both in early (weeks 1–9) and late phases (weeks 10–24), were not significantly different between groups (all $p > 0.05$), indicating no marked difference in hepatotoxicity.

Overall, while baseline characteristics and treatment dosing were comparable, the case group exhibited significantly greater neutropenia, as reflected by lower ANC levels, without significant differences in liver toxicity markers.

A genome-wide association analysis was performed to identify genetic variants associated with thiopurine-induced neutropenia among patients with normal metabolizer phenotypes for *TPMT* and *NUDT15*. As shown in Table 3, the top 10 variants with the lowest p -values were identified from the analysis. However, none of the variants reached the conventional genome-wide significance threshold ($p < 5 \times 10^{-8}$).

The most strongly associated variants demonstrated modest effect sizes but did not achieve statistical significance at the genome-wide level. . These findings suggest that no common variant with a large effect on neutropenia risk was identified in this cohort.

SCUBE2 (Signal peptide–CUB–EGF domain-containing protein 2) is a member of the SCUBE family of proteins, characterized as a membrane-bound and secreted glycoprotein. Structurally, it contains an NH₂-terminal signal peptide, multiple epidermal growth factor (EGF)-like repeats, and a CUB domain at the COOH terminus. *SCUBE2* is highly expressed in vascular endothelial cells and in highly vascularized tissues, and is also detected in certain epithelial cell types. Functionally, *SCUBE2* plays a critical role in cell–cell signaling, particularly through its involvement in the Hedgehog (Hh), bone morphogenetic protein (BMP), and vascular endothelial growth factor (VEGF) signaling pathways, which regulate cell proliferation, survival, and angiogenesis (17) (18).

Although direct evidence of *SCUBE2* in hematopoiesis remains limited, its molecular functions suggest an important indirect role. *SCUBE2* is known to modulate Hedgehog signaling, a key pathway in maintaining hematopoietic stem cells (HSCs), including their self-renewal, proliferation, and differentiation. By facilitating the release and activity of Hedgehog ligands such as sonic hedgehog (Shh), *SCUBE2*

may contribute to the regulation of HSC homeostasis and progenitor cell expansion. In addition, *SCUBE2* acts as an antagonist of BMP signaling, thereby influencing the balance between Hedgehog and BMP pathways, which is essential for lineage commitment, particularly within the myeloid lineage (17) (19) (18).

Within the bone marrow microenvironment, or vascular niche, *SCUBE2* may also play a regulatory role through its effects on endothelial function and angiogenesis. Through VEGF/VEGFR2 and downstream AKT/MAPK signaling pathways, *SCUBE2* may influence the structural and functional integrity of the vascular niche that supports HSC maintenance and hematopoietic activity. Dysregulation of *SCUBE2* could therefore impair the bone marrow microenvironment and subsequently disrupt normal hematopoiesis. Furthermore, *SCUBE2* expression is modulated by proinflammatory cytokines such as IL-1 β and TNF- α , which are key regulators of stress hematopoiesis and immune-mediated hematopoietic responses (17) (18).

In the context of neutropenia, a condition characterized by reduced neutrophil counts, *SCUBE2* may be involved through several potential mechanisms. Reduced *SCUBE2* expression could impair Hedgehog signaling, leading to decreased proliferation and differentiation of myeloid progenitor cells, and consequently diminished neutrophil production (granulopoiesis). Disruption of the balance between Hedgehog and BMP signaling may further compromise myeloid lineage differentiation. Additionally, alterations in *SCUBE2* within the bone marrow vascular niche may impair endothelial support and the secretion of critical growth factors required for neutrophil development (18).

Moreover, *SCUBE2* is linked to inflammatory signaling pathways and may influence emergency granulopoiesis during infection or systemic inflammation. Dysregulation of *SCUBE2* could result in an inadequate hematopoietic response to increased demand for neutrophils. Its involvement in VEGF/AKT/MAPK signaling also suggests a role in cell survival, where reduced *SCUBE2* activity may increase apoptosis of neutrophil precursors (18).

One possible explanation for the lack of significant associations is the relatively small sample size, which may have limited the statistical power to detect variants with modest effect sizes. In complex traits such as drug-induced hematologic toxicity, the genetic architecture is likely to involve multiple variants with small effects rather than a single strong genetic determinant.

Additionally, neutropenia during thiopurine therapy may be influenced by a combination of genetic and non-genetic factors, including treatment intensity, drug exposure, and

individual clinical characteristics. Therefore, larger cohorts and collaborative studies may be necessary to identify additional genetic determinants contributing to susceptibility to thiopurine-induced neutropenia.

IV. CONCLUSION

In summary, this study evaluated genetic factors associated with thiopurine-induced neutropenia in pediatric patients receiving thiopurine therapy. Although variants in *NUDT15* and *TPMT* are well-established predictors of thiopurine toxicity, a substantial proportion of patients with normal metabolizer phenotypes for these genes still developed neutropenia. Genome-wide association analysis restricted to this subgroup did not identify variants reaching genome-wide significance ($p < 5 \times 10^{-8}$), suggesting that *TPMT* and *NUDT15* alone do not fully explain susceptibility to thiopurine-induced neutropenia. These findings highlight the likely contribution of additional genetic and clinical factors. However, the lack of significant associations may be

partly due to limited statistical power. Given the expected small to moderate effect sizes of relevant variants, substantially larger cohorts are required to detect associations at genome-wide significance thresholds. For variants with smaller effect sizes (OR ~1.3), the required sample size may exceed 4,000–5,000 individuals.

Future studies should therefore incorporate larger sample sizes and collaborative, multi-center designs to enhance statistical power and facilitate the identification of clinically relevant genetic determinants, ultimately improving risk prediction and personalized thiopurine therapy.

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TABLE I
CHARACTERISTICS OF ALL RECRUITED PATIENTS AND COMPARISON OF BASELINE VARIABLES BETWEEN PATIENTS WITH NEUTROPENIA AND THOSE WITHOUT NEUTROPENIA

All participants (N=103)	Non-Neutropenia (n=40)	Neutropenia (n=63)	<i>p-value</i>
Age, year	5	4	0.2633
Gender, n (%)			
Male	22 (37.29%)	37 (62.71%)	0.709
Female	18 (40.91%)	26 (59.09%)	
Risk groups, n (%)			
Standard	18 (38.30%)	29 (61.71%)	0.141
High	13 (32.50%)	20 (62.50%)	
Very High	8 (72.73%)	3 (27.27%)	
Philadelphia	0 (0%)	1 (100%)	
6MP initial dose (mg/m ² /day)	37.32	34.52	0.5534
<i>TPMT</i> phenotype			
Normal Metabolizer (NM)	39 (41.05%)	56 (58.95%)	0.112
Intermediate Metabolizer (IM)	1 (12.50%)	7 (87.50%)	
Poor Metabolizer (PM)	0	0	
<i>NUDT15</i> phenotype			
Normal Metabolizer (NM)	31 (39.24%)	48 (60.76%)	0.92
Intermediate Metabolizer (IM)	8 (36.36%)	14 (63.64%)	
Poor Metabolizer (PM)	1 (50%)	1 (50%)	
Early ANC (weeks 1-9)	2414	1490	<0.0001
Late ANC (weeks 10-24)	2436	1489.42	<0.0001
Early AST (weeks 1-9)	35.08	40	0.0844
Late AST (weeks 10-24)	36.33	39.33	0.288
Early ALT (weeks 1-9)	32.33	53	0.0724
Late ALT (weeks 10-24)	35.67	50.33	0.1066

TABLE II
CHARACTERISTICS OF PATIENTS WITH NORMAL *NUDT15* AND *TPMT* METABOLIZER PHENOTYPES.

Study cohort (n=62)	Control (n=25)	Case (n=37)	P-value
Age, year	5.5	4	0.6552
Gender, n (%)			
Male	14 (41.17%)	20 (58.83%)	0.85
Female	11 (39.28%)	17 (60.72%)	
Risk groups, n (%)			
Standard	9 (34.62%)	17 (65.38%)	0.141
High	10 (37.04%)	17 (62.96%)	
Very High	6 (75%)	2 (25%)	
Philadelphia	0 (0%)	1 (100%)	
6MP initial dose (mg/m ² /day)	39.24	37.5	0.8796
Early ANC (weeks 1-9)	2414	140	0.0002
Late ANC (weeks 10-24)	2400	1511	<0.0001
Early AST (weeks 1-9)	35.08	40.66	0.1679
Late AST (weeks 10-24)	34.67	52.03	0.3017
Early ALT (weeks 1-9)	25.27	60	0.2723
Late ALT (weeks 10-24)	37.5	53.33	0.245

TABLE III
TOP 10 GENETIC VARIANTS IDENTIFIED FROM A GENOME-WIDE ASSOCIATION ANALYSIS OF THIOPURINE-INDUCED NEUTROPENIA AMONG PATIENTS WITH NORMAL *NUDT15* AND *TPMT* METABOLIZER PHENOTYPES.

CHR:POS	RSID	Gene	REF	ALT	OR	p
3:190443155	rs6791090	<i>TMEM207</i> : Intron Variant	T	G	0.071687	8.49E-05
11:9052988	rs3751051	<i>SCUBE2</i> : Intron Variant	C	T	12.5586	9.58E-05
11:9058134	rs72632953	<i>NRIP3-DT</i> : Intron Variant, <i>SCUBE2</i> : Intron Variant	T	C	12.5586	9.58E-05
1:115641290	rs1553185390	<i>VANGL1</i> : 2KB Upstream Variant	GGTGT	GGT	0.049268	9.59E-05
3:190373241	rs10659925	<i>CLDN16</i> : Intron Variant	G	GAAGC	0.088037	9.79E-05
2:47568133	rs1065650	<i>MSH2</i> : Intron Variant, <i>KCNK12</i> : Intron Variant	G	A	0.106008	9.97E-05
11:72819955	rs58682117	<i>ATG16L2</i> : Intron Variant	T	C	0.075434	0.000105
11:72843362	rs3217361	<i>FCHSD2</i> : Intron Variant, <i>ATG16L2</i> : 3 Prime UTR Variant	A	AGGG	0.075434	0.000105
11:122203336	rs10892819	<i>MIR100HG</i> : Intron Variant	T	G	10.1604	0.000119
10:13914618	rs2698110	<i>FRMD4A</i> : Intron Variant	C	T	0.087891	0.000133

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Basophil Activation Test as a Diagnostic Tool for Immediate Reaction to Chlorhexidine and Ceftriaxone

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Abstract. Anaphylaxis is a rapid-onset, potentially life-threatening systemic hypersensitivity reaction and its reported incidence has increased over the past decade. The basophil activation test (BAT) may offer a safe and accurate alternative diagnostic method for patients with suspected drug-induced hypersensitivity. This study addresses a critical diagnostic gap by evaluating the clinical utility of BAT in identifying immediate hypersensitivity to chlorhexidine and ceftriaxone which are two common yet high-risk agents in clinical practice. Chlorhexidine and ceftriaxone were used as test allergens. Patients with a history of drug-induced immediate hypersensitivity were included in the study. The skin prick test (SPT) was performed to confirm the diagnosis, while individuals with negative SPT results were included as controls. Flow cytometry with specific CD markers was used to identify activated basophils. The diagnostic performance of BAT was assessed in comparison with SPT in 11 patients and 10 controls. Our findings reveal a striking discrepancy in marker sensitivity. Notably, CD203c achieved 100% sensitivity, vastly outperforming CD63, which reached only 36.4%. Both markers maintained 100% specificity. CD203c demonstrated perfect agreement with SPT results ($\kappa=1.00$, $p<0.001$), identifying it as a superior indicator in this clinical context. The results indicate that CD203c is a more accurate and reliable marker than CD63 for diagnosing chlorhexidine and ceftriaxone allergies. Integrating BAT with CD203c improves diagnostic safety and accuracy, reduces reliance on high-risk skin tests, and highlights BAT's potential as a new standard for evaluating drug-induced anaphylaxis.

Index Terms—Anaphylaxis, Basophil activation test, Drug-induced immediate hypersensitivity, Skin prick test.

I. INTRODUCTION

Anaphylaxis is an acute systemic allergic reaction that can progress rapidly and become fatal without prompt treatment and its reported incidence has increased over the past decade. Antibiotic agents are frequently implicated in drug-induced hypersensitivity reactions, although a wide range of medications including antiseptics, contrast media, and biologic products have also been associated with such events. In its most critical presentation, immediate hypersensitivity involves an IgE-dependent immune mechanism that can progress to a rapid, systemic reaction clinically recognized as anaphylaxis [1-3].

The basophil activation test (BAT) is an in vitro assay that measures the upregulation of activation markers on the surface of peripheral blood basophils and is used to investigate drug-induced allergies. BAT evaluates functional basophil activation in vitro by quantifying activation-associated surface markers such as CD63 and CD203c after drug stimulation [4-6]. Growing data indicate that the BAT

can serve as a valuable adjunct in the evaluation of suspected immediate drug hypersensitivity particularly in situations where skin prick tests (SPT) yield equivocal findings or pose safety concerns. Evidence from clinical investigations suggests that measurement of activation markers most notably CD203c enhances the diagnostic performance of BAT, demonstrating favorable sensitivity alongside strong specificity in reactions attributed to antibiotics, neuromuscular blocking agents and other frequently reported culprit medications [7, 8]. For example, in patients with confirmed chlorhexidine-induced anaphylaxis, increased CD63 and CD203c expression was observed after chlorhexidine stimulation and inhibition testing suggested an IgE-mediated mechanism [9].

Despite growing evidence supporting its utility, further evaluation of BAT in real clinical settings is still needed. In this study, we aim to validate BAT for the clinical diagnosis of drug-induced immediate hypersensitivity. chlorhexidine and ceftriaxone were used as test allergens.

II. METHODOLOGY

This study is a cross-sectional study conducted over a one-year period in patients diagnosed with chlorhexidine and ceftriaxone allergy. All patients and controls provided us with written informed consent for study participation. The study included patients with confirmed drug-induced allergy and control subjects without the condition, all of whom had negative skin test results. Both patient and control groups were recruited from the Division of Allergy, Immunology, and Rheumatology, Department of Internal Medicine, Ramathibodi Hospital, Mahidol University. Ethical approval was obtained from the Human Research Ethics Committee, Faculty of Medicine, Ramathibodi Hospital, Mahidol University. Blood will be drawn from both patients and control individuals in a single 6 mL K-EDTA venipuncture tube.

Peripheral whole blood samples were collected from both patients and healthy controls. BAT was performed using flow cytometry (BD FACSLyric™, Biosciences) with the BÜHLMANN Flow CAST® to identify activated basophils. Samples were incubated at 37°C with a fluorochrome-conjugated CD marker together with serial dilutions of ceftriaxone (n=2) and chlorhexidine (n=9). The kit-provided positive controls were included in each run, and stimulation buffer served as the negative control. After incubation, red blood cells were lysed according to the manufacturer's instructions, followed by centrifugation and washing steps. The cell pellet was resuspended in wash buffer, and data acquisition was subsequently carried out using a flow cytometer.

For the gating strategy, basophils were identified by CCR3^{pos} and SSC^{low} gating (Figure 1). Basophils incubated with stimulation buffer alone served as the negative control (Figure 2a and 2b). As shown in Q2 (Figure 2a) and Q6 (Figure 2b), basophils were negative for CD63 and CD203c while remaining positive for CCR3 and the threshold used for determining positive basophil activation. Basophils stimulated with anti-FcεRI antibody served as the positive control (figure 2c and 2d) showing marked upregulation of CD63 and CD203c with maintained CCR3 positivity (Figure 2c; Q2 and figure 2d; Q6). This control verifies basophil viability and the functional responsiveness of the assay (Figures 2c and 2d). However, an additional positive control was included in this study but the data are not shown.

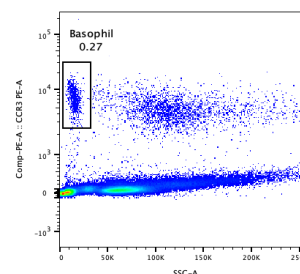


Figure 1 selection of basophilic cells (CCR3^{pos} and SSC^{low}).

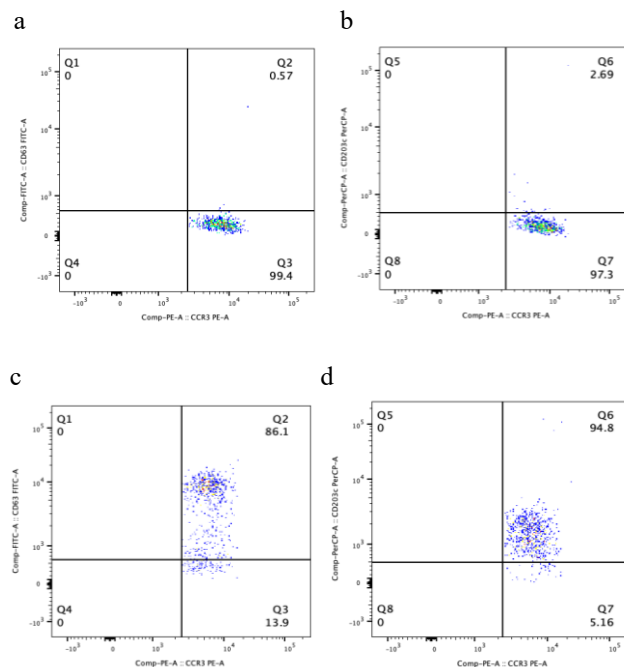


Figure 2 Negative control for CD63 and CD203c (a, b) and stimulation control (Positive control with anti-FcεRI antibody) for CD63 and CD203c (c, d).

Statistical analyses were conducted using SPSS (version 31.0.1.0). Diagnostic accuracy of BAT was determined by calculating sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) using SPT as the reference standard. Agreement between tests was evaluated with Cohen's kappa coefficient. A p-value <0.05 was considered statistically significant, and 95% confidence intervals were reported.

III. RESULTS AND DISCUSSION

A total of 11 patients who met the study inclusion criteria were enrolled. The majority were female with ages ranging from 50 to 77 years. Chlorhexidine was identified as the culprit drug in 9 patients whereas ceftriaxone was implicated in 2 patients. All participants exhibited positive results on both the SPT and BAT (Table 1).

TABLE I

DEMOGRAPHIC CHARACTERISTICS AND BLOOD AND SKIN
TEST RESULTS OF 11 PATIENTS

Patient No.	Drug culprit	Age	Sex	SPT result	BAT result
1	Chlorhexidine	77	Female	Positive	Positive
2	Chlorhexidine	73	Female	Positive	Positive
3	Chlorhexidine	74	Female	Positive	Positive
4	Chlorhexidine	71	Male	Positive	Positive
5	Chlorhexidine	63	Male	Positive	Positive
6	Chlorhexidine	50	Male	Positive	Positive
7	Chlorhexidine	66	Female	Positive	Positive
8	Chlorhexidine	59	Female	Positive	Positive
9	Chlorhexidine	65	Male	Positive	Positive
10	Ceftriaxone	61	Female	Positive	Positive
11	Ceftriaxone	50	Female	Positive	Positive

Illustration shows the distribution of SPT and BAT results among the 21 participants. Of the total study population, 11 individuals demonstrated positive SPT results, whereas 10 had negative findings. All participants subsequently underwent BAT analysis. For CD63 expression, 4 cases were classified as positive and 17 as negative. In contrast, CD203c expression was positive in 11 cases and negative in 10 cases. Concurrent positivity of both CD63 and CD203c was observed in 3 participants (Figure 3).

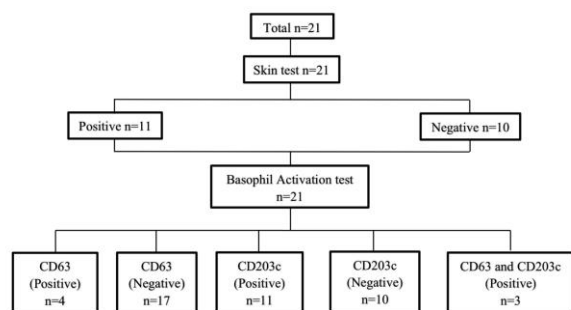


Figure 3 Results of skin test and basophil activation test.

Representative BAT dot plots are presented. In the negative control (Figure 4a, 4b, 4c), basophils showed minimal baseline activation, with CD63 and CD203c expression remaining below the established cut-off as shown in Q2 (Figure 4a) and Q6 (Figure 4b), respectively. When both markers were considered, the percentage of activation also remained low. Stimulation with anti-FcεRI antibody (Figures 4d, 4e, 4f) and fMLP (Figures 4g, 4h, 4i) was used

as a positive control and resulted in marked upregulation of activation markers, confirming basophil viability and functional responsiveness as observed in Q2, Q6 and Q10 of Figure 4d, 4e, 4f for anti-FcεRI and Figure 4g, 4h and 4i for fMLP. Figures 4j-4l, 4m-4o, and 4p-4r represent BAT results at high, medium, and low concentrations of

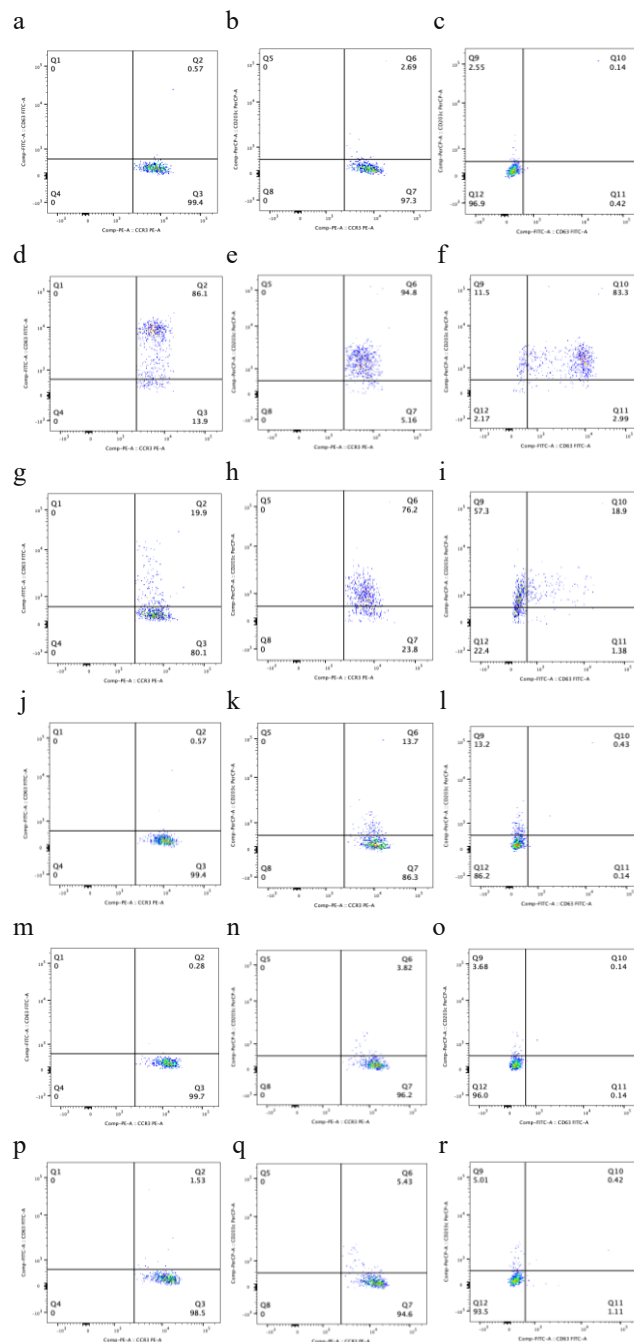


Figure 4 Representative flow cytometry plots showing CD63 expression, CD203c expression, and co-expression of both markers in a patient with a drug-induced immediate hypersensitivity reaction after exposure to the chlorhexidine. Increased expression of activation markers was observed following stimulation compared with the negative control, indicating a positive basophil activation response.

chlorhexidine, respectively. Increased CD203c expression was also observed at two concentrations (Figures 4k, 4q) of the chlorhexidine indicating a positive activation result.

The diagnostic performance of the BAT was evaluated in comparison with the SPT. Sensitivity, specificity, PPV, NPV and Cohen's kappa were calculated for CD63, CD203c, and their combined expression in 11 patients and 10 healthy controls.

CD203c demonstrated greater sensitivity than CD63 alone, while specificity was 100% in all analyses. Agreement between BAT and SPT based on CD203c expression was good (Cohen's kappa = 1.00, $p < 0.001$). In contrast, CD63 showed lower sensitivity and correspondingly weaker agreement. When positivity was defined as the expression of either marker, overall diagnostic performance improved and strong concordance with SPT was maintained (Table 2).

TABLE 2

DIAGNOSTIC ACCURACY AND AGREEMENT ANALYSIS
BETWEEN SPT AND BAT CD MARKERS (N = 21 PATIENTS)

	Sen (%)	Spec (%)	PPV (%)	NPV (%)	κ	p^*
SPT and CD63	36.4	100	100	58.8	0.35	0.034
SPT and CD203c	100	100	100	100	1.00	<0.001
SPT and CD63 and 203c	27.3	100	100	55.6	0.26	0.074
SPT and CD63 or CD203c	100	100	100	100	1.00	<0.001

An asterisk (*) denotes the p-value of Cohen's kappa and bold numbers indicate statistical significance. κ , Cohen's Kappa; p , p-value; Sen, sensitivity; Spec, specificity; PPV, positive predictive value; NPV, negative predictive value.

Overall, CD203c appears to be a more reliable marker than CD63 for detecting drug-induced immediate hypersensitivity, given its superior sensitivity and strong concordance with SPT. These findings suggest that CD203c may serve as a more robust indicator of basophil activation in this clinical setting (Table 2).

In this study, we validated BAT in patients with drug-induced immediate hypersensitivity. CD203c showed higher sensitivity than CD63 while specificity remained high and CD203c also demonstrated good agreement with SPT.

Drug-induced immediate hypersensitivity reactions are typically IgE-mediated and may present with rapid systemic symptoms including anaphylaxis [10]. SPT remains a routine diagnostic approach in clinical settings. However, the procedure is not entirely without risk as systemic reactions may occasionally occur [7]. Therefore, in vitro tests such as

BAT have been increasingly explored as supportive diagnostic tools.

The BAT evaluates functional basophil activation in vitro by quantifying activation-associated surface markers such as CD63 and CD203c after drug stimulation [2, 5]. In this study, CD63 appeared to represent degranulation after basophil activation whereas CD203c seemed to increase at an earlier stage of the response. This temporal difference might explain why CD203c showed higher sensitivity in our results. Consistent with previous literature, BAT generally demonstrates high specificity. However, the sensitivity can differ depending on the type of drug tested and the marker chosen for analysis [2, 7]. In the context of antibiotic- and disinfectant-related immediate hypersensitivity, CD203c appears to respond more readily compared with other basophil activation markers [7, 9]. However, this study has some limitations. The drugs included in the analysis may not fully represent the wide range of potential allergens. Further studies involving a broader variety of drugs would help improve the accuracy and robustness of the findings.

Overall, our findings highlight the potential of BAT particularly CD203c as a useful complementary tool in the diagnosis of drug-induced immediate hypersensitivity. In addition, this approach may offer a safer alternative as patients do not need to be directly exposed to the suspected allergen.

IV. CONCLUSION

Our findings suggest that the BAT may be useful and relatively safe for the evaluation of drug-induced hypersensitivity. By assessing basophil activation in vitro, BAT provides additional information that can support the diagnosis in patients with suspected drug allergy particularly CD203c which showed great results in the study. Although, it should be interpreted alongside clinical history and other test results.

ACKNOWLEDGMENT

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Animation for Promoting Knowledge of Triptans for Acute Migraine in Community Pharmacists in Sai Mai, Bangkok

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Abstract. This quasi-experimental study aimed to develop an educational animation on Triptans for acute migraine treatment, to assess the knowledge of community pharmacists before and after watching the animation, and to identify factors associated with differences in knowledge scores. Forty-one community pharmacists in Sai Mai, Bangkok participated in the study, with data collected from January 30 to February 11, 2026. The 8-minute animation and a 10-item knowledge test were validated by clinical experts prior to use, with Index of Item Objective Congruence (IOC) values of 0.66 to 1.00 for all items. Post-test scores (6.90 ± 1.71) were significantly higher than pre-test scores, with a mean score difference of 2.61 ± 1.24 points out of 10 ($p < 0.001$). The most substantial gains were observed in understanding the mechanism of action, contraindications, and correct dosage and administration of Triptans. Among all participant characteristics examined, the type of community pharmacy was the only factor significantly associated with the degree of score improvement, with pharmacists working at chain pharmacies showing larger gains than those at independent pharmacies. Satisfaction assessment revealed consistently high ratings exceeding 95% across all dimensions evaluated, including design, content quality, titivation, and usability. These results demonstrate that educational animation is an effective and accessible tool for improving community pharmacist knowledge of Triptans for migraine management.

Index Terms—Animation, Community pharmacist, Knowledge, Migraine, Triptans.

I. INTRODUCTION

Migraine headache is one of the most common primary headache disorders, and it substantially impairs both working capacity and daily functioning. A prevalence survey conducted in the Klong Toey district of Bangkok found that migraine occurred in 10.4% of men and 35.3% of women, with the highest frequency in the 30 to 39 age group [1]. The economic and functional burden of migraines makes appropriate recognition and treatment at every point of care critically important. The 2022 clinical practice guideline for migraine diagnosis and treatment, issued by the Headache Study Group under the Neurological Society of Thailand, recommends Ergotamine or Triptans for patients experiencing moderate-to-severe migraine headache or for those with mild-to-moderate symptoms who do not respond to, or cannot tolerate, general analgesics. Within this framework, Triptans receive a Grade I recommendation supported by Level A evidence, while Ergotamine carries Level B evidence. Both drugs are assigned the same strength of recommendation under the guideline, though Ergotamine and caffeine, as a listed drug on the National Drug List of

Thailand, is recommended for initial use before proceeding to Triptans. Importantly, Triptans and Ergotamine should never be used together because of additive vasoconstrictive effects, and if switching between the two drugs is required, a gap of at least 24 hours must be observed [2].

Triptans are selective serotonin 1B/1D (5-HT_{1B/1D}) receptor agonists that exert their antimigraine effects through three main mechanisms. First, they cause vasoconstriction by activating 5-HT_{1B} receptors on meningeal, dural, cerebral, and pial blood vessels. Second, they inhibit neurogenic inflammation by activating presynaptic 5-HT_{1D} receptors on trigeminal C-fibers to suppress the release of substance P and neurokinin A, and on trigeminal A-fibers to suppress the release of calcitonin gene-related peptide, thereby reducing dural vasodilation. Third, they modulate pain transmission by inhibiting trigeminal nucleus cell activation and stimulating 5-HT_{1B/1D} receptors in the brainstem [3]. The two oral Triptans currently available in Thailand are Sumatriptan and Eletriptan. Sumatriptan is taken as a 50 mg tablet when symptoms begin and may be repeated every two hours up to a maximum of 200 mg per day if the headache

does not resolve [2]. Eletriptan is taken as a 40 mg tablet, which may be repeated after two hours for a maximum of 80 mg per day [2]. A head-to-head randomized controlled trial in 2,113 migraine patients found that Eletriptan 40 mg produced a two-hour pain response rate of 67% compared to 59% for Sumatriptan 100 mg, and Eletriptan also outperformed Sumatriptan on secondary endpoints including one-hour response and pain-free rates [4]. These clinical properties make both agents important options for community pharmacists to understand and counsel on appropriately.

Community pharmacists serve as a frontline healthcare contact for the public in Thailand, and they are often the first professional that migraine patients consult when seeking treatment. Because Thai community pharmacists are also responsible for initial symptom assessment under national law, their knowledge of migraine management directly affects patient outcomes. A study of pharmacy practice patterns across 142 community pharmacies in southern Thailand [5], conducted using simulated patients, found that pharmacy personnel were significantly more likely to engage in history taking and appropriate drug selection when the presenting migraine severity was moderate rather than mild (mean history-taking score 2.6 ± 1.5 versus 1.8 ± 1.6 out of 12). Despite this, pharmacists still dispensed ergotamine inappropriately for mild migraine in 42.3% of cases, and preventive migraine treatment was recommended by fewer than 6% of pharmacists across both severity groups. A simulated patient study in 57 community pharmacies in Phayao province [6] focusing on severe migraine found even more pronounced gaps. In that study, no pharmacy dispensed Triptans at all, despite Triptans being the most appropriate treatment for the case scenario. NSAIDs were the most commonly dispensed drug (24.56%), followed by Ergotamine (14.04%). A study from the UK [7] similarly showed that community pharmacists failed to ask the most relevant clinical history questions, with only half of pharmacists referring to patients appropriately when clinical red flags were present. A cross-sectional survey on analgesic use in southern Thailand [8] found that community pharmacies were the most common source of analgesics, with 46.1% of 1,982 respondents obtaining their pain medications from a community pharmacist. The study also found that respondents reported using paracetamol above the recommended daily dose, and that inappropriate NSAIDs administration was associated with obtaining the drug from a community pharmacist (OR = 1.27, 95% CI 1.01 to 1.61). These findings reinforce the importance of accurate pharmacist knowledge not only for migraine-specific treatment but also for general analgesic counseling. A randomized controlled trial in Denmark [9] evaluated a one-day pharmacist education intervention on Triptan use across 22 community pharmacies and found no statistically significant change in Triptan consumption among patients following the training at three, six, or nine months of follow-

up. The authors noted that pharmacist time constraints, patient reluctance to change behavior, and inappropriate prescribing by physicians were all contributing factors. While the intervention effect was not significant, the authors emphasized that the concept of pharmaceutical care for migraine could not be dismissed, and that education design and delivery require careful consideration. Given these practice gaps, educational interventions targeting community pharmacists are important and warranted. A meta-analysis of 26 studies [10] comparing instructional animation to static images across diverse subject areas found that animation was significantly more effective for knowledge building (effect size $d = 0.37$, 95% CI 0.25 to 0.49), particularly when the animation showed the actual lesson's content rather than decorative motion ($d = 0.40$ versus $d = -0.05$). A video-based education intervention in 92 patients with co-occurring migraine and depression [11] demonstrated that online migraine education video significantly reduced headache frequency ($b = -1.32$), pain intensity ($b = -0.20$), and depression scores ($b = -0.28$) over a 12-week follow-up period.

The Sai Mai district of Bangkok was selected for this study because it has one of the highest population densities in the city, with 91 registered community pharmacies [12], and no comparable study had previously been conducted in this area. The study is therefore designed to extend the existing evidence on animation-based pharmacist education into a community pharmacy context, in a new geographic area, and with a more heterogeneous pharmacist sample than a single hospital setting. The three objectives of this study are to develop an educational animation on Triptans for acute migraine treatment, to assess pharmacist knowledge before and after watching the animation, and to identify factors associated with differences in knowledge scores.

II. METHODOLOGY

A. Sample Size and Data Collection

The trial was approved by Srinakharinwirot ethics committee (Protocol code: SWUEC-681056). This study uses a quasi-experimental design with a one-group pretest-posttest approach, without a control group. Pair t-test is used for statistical analysis to compare dependent sample as the average of the same group before and after (as itself control) to see if there is a significant difference. The study was conducted at community pharmacies registered with the Pharmacy Council of Thailand in the Sai Mai district of Bangkok, Thailand. The target population consisted of pharmacists working at 91 registered community pharmacies in Sai Mai, Bangkok, with one pharmacist assumed per pharmacy. Sample size was calculated using the Cochran formula at 95% confidence and 5% margin of error, then corrected for the finite population of 91. The formula applied was:

$$n_0 = \frac{Z^2 pq}{e^2} \quad (1)$$

$$n = \frac{n_0}{\left(1 + \frac{n_0 - 1}{N}\right)} \quad (2)$$

where $Z = 1.96$ for 95% confidence level, $p = 0.5$ (maximum variability), $q = 1 - p = 0.5$, $e = 0.05$ (margin of error), $N = 91$ (population size), n_0 = initial sample size, and n = adjusted sample size for finite population. The calculation yielded $n_0 = 384.16$, which was rounded to 385, and after finite population correction, $n = 73.76$, rounded to 74 pharmacists.

The target sample was 74 pharmacists. Data collection was conducted from January 30 to February 11, 2026, through in-person visits to all 91 pharmacies. Inclusion criteria required participants to be licensed pharmacists working at a registered community pharmacy in Sai Mai during the data collection period, aged 20 to 80 years, with access to a device to complete the Google Form questionnaire, and willing to participate voluntarily. Exclusion criteria included pharmacists who had already participated from a different pharmacy during the same period, those whose license had expired, and those who did not complete the full study protocol.

Each data collection session lasted approximately 30 minutes. Participants completed a demographic questionnaire and the pre-test through a Google Form (9 minutes), then watched the animation (approximately 8 minutes), completed the post-test (7 minutes), and filled out a satisfaction questionnaire (7 minutes). Each pharmacy was visited on one occasion, and if more than one pharmacist was present, only one was randomly invited to participate. The trial was approved by the Srinakharinwirot University Ethics Committee (SWUEC-681056).

B. Educational Animation and Knowledge Test

The animation was produced by an educational media specialist at Srinakharinwirot University based on content developed collaboratively by the research team. The animation covered six topic areas referenced to the 2022 Thai clinical practice guideline: migraine diagnostic criteria (International Classification of Headache Disorders-3; ICHD-3), classification of migraine, pharmacy-based history taking, red flag symptoms requiring hospital referral, the treatment selection framework for acute migraine, and Triptan-specific information for Sumatriptan and Eletriptan. Regarding to diagnostic criteria according to ICHD-3, migraine should be diagnosed as follows: at least 5 headache attacks characterized as unilateral, pulsatile, moderate or severe pain intensity, lasting for 4-72 hours or aggravation by routine physical activity. Other associated symptoms included nausea and/or vomiting, photophobia, or phonophobia.

The knowledge test consists of 10 five-choice closed-ended questions worth one point each, for a maximum score of 10 points. Questions cover migraine history taking,

migraine diagnostic criteria, appropriate community pharmacy management, Triptan indications per the 2022 Thai guideline, mechanism of action, dosage and administration, adverse effects, drug interactions, contraindications, and dose adjustment in hepatic impairment. Question 7 is a case-based scenario designed to test application of Triptan drug interaction knowledge in a real dispensing situation. A satisfaction questionnaire was also administered to assess participants' satisfaction with the animation. The questionnaire contained 20 items covering four aspects: design (5 items), content (5 items), titivation (5 items), and usability (5 items).

Content validity of the animation and the knowledge test was assessed using the Index of Item Objective Congruence (IOC), calculated using the formula:

$$IOC = \frac{\sum R}{N} \quad (3)$$

where R is the score assigned by each expert (+1 if the item clearly aligns with the objective, 0 if uncertain, -1 if clearly misaligned) and N is the total number of experts. Items with IOC values of 0.50 to 1.00 are considered valid. The content quality of the animation and knowledge test was evaluated by three clinical pharmacy experts, while the quality of the animation's design components was assessed by four experts (three clinical pharmacy experts and one media specialist). After revisions, all items achieved IOC values of 0.66 to 1.00, indicating acceptable validity across all domains.

Content reliability was assessed through pilot testing with 10 pharmacists who had similar characteristics to the target study population but were not part of the main sample. Internal consistency of the knowledge test was measured using the Kuder-Richardson formula 20 (KR-20), while the satisfaction questionnaire was assessed using Cronbach's alpha coefficient. A reliability coefficient of 0.70 or above was considered acceptable for both instruments.

C. Statistical Analysis

Descriptive statistics including frequencies, percentages, means, and standard deviations were used to characterize participant demographics. Because the sample size was 41, fewer than 50, the Shapiro-Wilk test was used to assess normality of the score differences, with the Kolmogorov-Smirnov test applied as a supplementary check. The score differences were found not to follow a normal distribution (Shapiro-Wilk $p = 0.014$; Kolmogorov-Smirnov $p = 0.006$). The Wilcoxon signed rank test was therefore used to compare pre- and post-test scores, because the variables are related and not independent of each other. The McNemar test was applied to evaluate changes in the proportion of correct and incorrect responses for each individual knowledge question, since the data for each question consisted of paired nominal observations. To identify factors associated with the magnitude of score improvement, subgroup correlation analyses were conducted using Spearman correlation for ordinal variables (age group, program duration, educational level, and years of

experience), the Kruskal-Wallis H test for nominal variables with more than two categories (pharmacy degree program), and point-biserial correlation for binary nominal variables (employment type, pharmacy type, prior Triptan knowledge, Triptan availability, dispensing history, and drug of choice). A p-value below 0.05 was considered statistically significant.

III. RESULTS AND DISCUSSION

A. Development of Educational Animation

The educational animation on Triptans for acute migraine treatment was successfully developed to fulfill the first objective of this study. The final version was 8 minutes and 37 seconds in length, which aligns with the recommendations by Brame [13] on learner engagement, who indicated that videos with optimal length that maintain learner attention should be approximately 6 to 9 minutes. As video length increases, learner engagement and completion rates tend to decrease accordingly. Fig. 1. Showed the example contents in the educational animation for this study.



Fig. 1. Example of educational animation.

Content validation demonstrated that the animation and knowledge test items were valid and consistent with the study objectives, with IOC values ranging from 0.66 to 1.00 for all items. Content reliability testing of the pre-test and post-test yielded a KR-20 value of 0.807, which exceeds the acceptable threshold of 0.70, indicating that each test item is correlated with other items in the same instrument. The satisfaction questionnaire demonstrated a Cronbach's alpha coefficient of 0.972, also exceeding the acceptable threshold of 0.70. These reliability values confirm that the educational animation is reliable, valid, measures what it is intended to measure, and provides accurate assessment. Regarding participant satisfaction with the animation, participants reported very high satisfaction across all four evaluated aspects, as shown in Table I. The design aspect received a mean score of 4.89 ± 0.32 (97.76%), content scored 4.82 ± 0.41 (96.39%), titivation scored 4.86 ± 0.37 (97.27%), and usability scored 4.77 ± 0.48 (95.70%). All four aspects achieved very high satisfaction levels, demonstrating that participants were highly satisfied with the animation's quality across design, content delivery, engaging appeal, and ease of use.

B. Participant Characteristics

The overall target population consisted of 91 pharmacists working at registered community pharmacies in Sai Mai, Bangkok. The calculated sample size was 74 pharmacists with confidence interval as $95.0 \pm 5.0\%$. Among 91 visited pharmacists, fourteen pharmacies (15.39%) declined to

participate, 14 pharmacies (15.39%) had no pharmacist present at the time of the visit, and 12 pharmacies (13.19%) were found to be permanently or temporarily closed. Thereby, the final sample was 41 participants (45.05% of 91 participants). Although less than the initial target of 74, the final sample of 41 pharmacists remains statistically acceptable. Under the Taro Yamane formula, this sample size aligns with a $\pm 10.0\%$ margin of error at a 95% confidence level (calculated requirement: 41.36). Thus, the 41 participants were deemed sufficient for all subsequent analyses.

TABLE I

PARTICIPANT SATISFACTION SCORES WITH THE ANIMATION
(N = 41)

Section	Mean	%	S.D.	Satisfaction Level
Design	4.89	97.76	0.32	Very high
Content	4.82	96.39	0.41	Very high
Titivation	4.86	97.27	0.37	Very high
Usability	4.77	95.70	0.48	Very high

Note: Satisfaction was measured on a 5-point Likert scale.

Table II presents the demographic characteristics of the 41 participants. The largest age group was 21 to 40 years (58.4%), reflecting a relatively young pharmacist workforce in this district. The most common degree background was a non-specialized pharmacy program (43.9%), which reflects the older pre-2007 Thai pharmacy curriculum that did not require students to specialize in a particular track. The majority of participants completed a 6-year program (51.2%), held a Bachelor's degree (92.7%), and worked full time (73.2%). The most common experience range was 6 to 10 years (31.7%), and most participants worked at independent pharmacies (80.5%), which is consistent with the known distribution of pharmacy types in Sai Mai. In terms of migraine-related practice, 43.9% reported seeing one to five migraine patients per week. While 85.4% had some prior awareness of Triptans, 68.3% had Triptans in stock and 70.7% had previously dispensed them. NSAIDs (65.9%) and Ergotamine (61.0%) were the most common chosen medications for moderate-to-severe migraine, while only 34.1% selected Triptans. The high selection rate for Ergotamine can be attributed to its long history of use and lower cost, which is an important consideration in community pharmacy practice, despite having more drug interactions and adverse effects compared to Triptans. This finding highlights a gap between guideline-recommended practice and actual dispensing behavior, consistent with previous studies in Thai community pharmacies, and underscores the need for enhanced education on Triptans [10, 11].

TABLE II
DEMOGRAPHIC OF PARTICIPANTS (N = 41)

Characteristics	n	%
Age group		
21-40 years	24	58.4
41-60 years	13	31.7
61-80 years	4	9.8
Pharmacy degree program		
Pharmaceutical Care	11	26.8
Industrial Pharmacy	10	24.4
Pharmaceutical and Health Consumer Protection	2	4.9
Non-specialized program	18	43.9
Pharmacy program duration		
5-year program	20	48.8
6-year program	21	51.2
Highest pharmacy educational level		
Bachelor's degree	38	92.7
Master's degree	3	7.3
Employment type at community pharmacy		
Full time	30	73.2
Part time	11	26.8
Years of experience at community pharmacy		
<1 year	5	12.2
1-5 years	12	29.3
6-10 years	13	31.7
>10 years	11	26.8
Type of community pharmacy		
Independent pharmacy	33	80.5
Chain pharmacy	8	19.5
Number of migraine patients seen per week		
1-5 patients	18	43.9
6-10 patients	11	26.8
>10 patients	12	29.3
Prior knowledge of Triptans		
Previously aware	35	85.4
Never aware	6	14.6
Triptans available at community pharmacy		
Yes	28	68.3
No	13	31.7
Previously dispensed Triptans to patients		
Yes	29	70.7
No	12	29.3
Drug of choice for moderate-to-severe migraine attacks*		
Paracetamol	2	4.9
NSAIDs	27	65.9
Ergotamine	25	61.0
Triptans	14	34.1
NSAIDs + paracetamol	12	29.3
Amitriptyline	2	4.9
Cinnarizine	1	2.4
Muscle relaxant	1	2.4

Note: *Percentages exceed 100% as respondents could select more than one option.

C. Knowledge Test: Pre- and Post-test Comparison

The mean pre-test score was 4.29 ± 1.57 out of 10 (median 4, score range 1 to 8), indicating that pharmacists' baseline knowledge of Triptans was moderate at best, with the majority of participants answering fewer than half the questions correctly. The three knowledge areas with the highest pre-test error rates were migraine diagnostic criteria

(Q2, 92.7% wrong), mechanism of action of Triptans (Q5, 75.6% wrong), and both hospital referral criteria (Q3) and contraindications for Eletriptan and Sumatriptan (Q8), both with 70.7% wrong. These findings are consistent with previous studies documenting inadequate pharmacist knowledge of migraine pathophysiology and Triptan pharmacology in community settings [10, 11]. After watching the animation, the mean post-test score increased to 6.90 ± 1.71 (median 7, score range 3 to 10). The Wilcoxon signed rank test confirmed that post-test scores were significantly higher than pre-test scores, with a mean difference of 2.61 ± 1.24 points ($p < 0.001$), indicating that watching the animation produced a statistically significant improvement in Triptan-related knowledge among community pharmacists. This finding is consistent with the study by Thakur et al. [14] demonstrating that animated educational media can effectively promote learning. In their study examining the benefits of an online migraine education video for patients with co-occurring migraine and depression, participants who watched the animation showed significant improvements in multiple outcome measures. Specifically, scores measuring headache frequency, pain intensity, and depression symptoms all showed significant differences after viewing the animation compared to baseline scores, and the animation enabled participants to better manage their migraine condition and reduce headache symptoms.

Table III presents the item-level analysis using the McNemar test. Three questions showed statistically significant improvements at $p < 0.001$, namely Q5 on mechanism of action (61.0% increase in correct answers), Q8 on which patients can use both Eletriptan and Sumatriptan (51.2% increase), and Q10 on correct dosage and administration of Triptans (51.2% increase). These were precisely the items where baseline knowledge was lowest, confirming that the animation effectively targeted and addressed real knowledge gaps. For Q2 on migraine diagnostic criteria, 68.3% of participants answered incorrectly both before and after watching, which the highest rate of persistent error across all questions. This suggests that the ICHD-3 diagnostic criteria content in the animation may not have been presented in a way that was memorable or easy to apply and may benefit from a more structured or repeated format in future iterations. For Q7, a clinical case scenario on the drug interaction between Eletriptan and Itraconazole, 17.1% of participants regressed, meaning they answered correctly before watching but incorrectly after, likely because of the complexity of the pharmacokinetic reasoning required in that scenario. Only four participants (9.8%) achieved a perfect score on the post-test, and Q1 on migraine history taking was the only item where all participants answered correctly after watching.

TABLE III
PRE-TEST AND POST-TEST KNOWLEDGE SCORES BY
QUESTION (N = 41)

Question	Pre-test		Post-test		Increased correct answers (%)
	Correct (%)	Incorrect (%)	Correct (%)	Incorrect (%)	
Q1	40 (97.6)	1 (2.4)	41 (100.0)	0 (0.0)	1 (2.4)
Q2	3 (7.3)	38 (92.7)	11 (26.8)	30 (73.2)	8 (19.5)
Q3	12 (29.3)	29 (70.7)	22 (53.7)	19 (46.3)	10 (24.4)
Q4	34 (82.9)	7 (17.1)	36 (87.8)	5 (12.2)	2 (4.9)
Q5	10 (24.4)	31 (75.6)	35 (85.4)	6 (14.6)	25* (61.0)
Q6	18 (43.9)	23 (56.1)	28 (68.3)	13 (31.7)	10 (24.4)
Q7	14 (34.1)	27 (65.9)	16 (39.0)	25 (61.0)	2 (4.9)
Q8	12 (29.3)	29 (70.7)	33 (80.5)	8 (19.5)	21* (51.2)
Q9	19 (46.3)	22 (53.7)	26 (63.4)	15 (36.6)	7 (17.1)
Q10	14 (34.1)	27 (65.9)	35 (85.4)	6 (14.6)	21* (51.2)

Note: * $p < 0.05$ (McNema test), Q1. Key questions that should be asked when counseling a migraine patient, Q2. Which statement is correct regarding migraine diagnosis, Q3. Which patient scenario with headache can be managed at a community pharmacy, Q4. Which is the indication for Triptans according to the 2022 Thai migraine clinical practice guideline, Q5. What is the mechanism of action of Triptans, Q6. Which is a rare but severe adverse effect of Triptans, Q7. Case study: A female patient regularly uses Eletriptan 40 mg for migraine and requests to purchase oral Itraconazole because she previously used it to treat a fungal infection of the nails (Tinea unguium). Can the patient use Itraconazole together with her migraine medication, Q8. Which migraine patient can use both Eletriptan and Sumatriptan, Q9. Which statement is correct regarding Sumatriptan dose adjustment in patients with mild-to-moderate hepatic impairment, Q10. What is the correct dosage and administration of Triptans

D. Factors Associated with Knowledge Score Difference

Table IV presents the results of the subgroup analyses examining which participant characteristics were associated with the magnitude of knowledge score improvement. Of all tested, only the type of community pharmacy was significantly associated with the pre-post score difference ($r = 0.357$, $p = 0.022$). Pharmacists working at chain pharmacies showed larger score improvement compared to those at independent pharmacies. This positive association suggests that chain pharmacies may provide more structured continuing education, more frequent exposure to updated clinical guidelines, or more standardized dispensing protocols that create a foundation for learning new pharmacotherapy content. We suggest that independent pharmacists should update clinical guidelines via online continuing pharmacy education (CPE) or attend

pharmacotherapy academic meetings at least once a year. All other factors, including age group, pharmacy degree program, program duration, educational level, employment type, years of experience, migraine patient volume, prior Triptan awareness, Triptan availability, previous dispensing history, and drug of choice, did not show statistically significant associations with the score difference. Pharmacists who had never dispensed Triptans and those with little prior familiarity showed comparable improvements to those with more experience, indicating that the animation was effective across a wide range of baseline knowledge and clinical exposure levels.

TABLE IV
FACTORS ASSOCIATED WITH KNOWLEDGE SCORE
IMPROVEMENT (N = 41)

Factor	Correlation (r)	p-value
Age group	- 0.104 ¹	0.517
Pharmacy degree program	Df = 3 H = 0.627 ²	0.890
Pharmacy program duration (5-year / 6-year program)	0.232 ¹	0.144
Highest pharmacy educational level (Bachelor's / Master's degree)	0.090 ¹	0.576
Employment type at community pharmacy (Full time / Part time)	0.058 ³	0.719
Years of experience at community pharmacy	- 0.003 ¹	0.987
Type of community pharmacy (Independent / Chain)	0.357 ³	0.022*
Number of migraine patients seen per week	0.119 ¹	0.458
Prior knowledge of Triptans (Not aware / Aware)	0.149 ³	0.351
Triptans available at community pharmacy (Yes / No)	- 0.890 ³	0.582
Previously dispensed Triptans to patients (Never / Previously dispensed)	- 0.169 ³	0.292
Drug of choice for moderate-to-severe migraine attacks		
Paracetamol	- 0.297 ³	0.059
NSAIDs	0.022 ³	0.889
Ergotamine	- 0.254 ³	0.109
Triptans	- 0.232 ³	0.144
NSAIDs + paracetamol	0.217 ³	0.174
Amitriptyline	- 0.020 ³	0.900
Cinnarizine	0.050 ³	0.755
Muscle relaxant	0.050 ³	0.755

Note: * $p < 0.05$, ¹Spearman correlation, ²Kruskal-Wallis H test, ³Point-biserial correlation.

The present study shows that a similar approach works in community pharmacy settings across a larger and more heterogeneous sample. The substantially larger score difference observed in the hospital pharmacist study (5.05 ± 1.80 points) [15] compared to this study (2.61 ± 1.24 points) may reflect higher baseline knowledge among hospital

pharmacists, a ceiling effect, or differences in the test instruments between the two studies.

Although the results of this study may not be directly comparable to the hospital pharmacist study due to differences in the practice contexts between community pharmacy and hospital settings, particularly in terms of the history-taking process and assessment of headache red flags, the findings demonstrate that animation can significantly enhance learner knowledge in both settings. Future work should address the persistent gap in migraine diagnostic criteria knowledge, consider redesigning the drug interaction scenario question, and expand the study to a multi-center sample in Bangkok or Thailand to improve generalizability. In addition, we plan to assess pharmacists' knowledge at 1 year later to confirm knowledge retention in long term period.

IV. CONCLUSION

Our 8-minute educational animation on Triptans for acute migraine treatment significantly improved knowledge and comprehension among community pharmacists in Sai Mai, Bangkok. The mean post-test score is 2.61 points, significantly higher than the pre-test score, with the most substantial gains in understanding the mechanism of action, contraindications, and correct dosage and administration of Triptans. Item-level analysis confirms that the animation effectively addressed knowledge areas where pharmacists had the greatest difficulty at baseline. The type of community pharmacy is the only pharmacist characteristic significantly associated with the degree of improvement, with chain pharmacists showing greater gains. The animation is broadly effective regardless of pharmacist age, educational background, years of experience, or prior Triptan exposure, supporting its potential as a scalable continuing education tool for community pharmacists. Participant satisfaction assessment revealed very high satisfaction across all four evaluated aspects: design (97.76%), content (96.39%), titivation (97.27%), and usability (95.70%), with an overall Cronbach's alpha coefficient of 0.972, indicating excellent internal consistency and participant acceptance of the educational intervention.

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Design Optimization of Perforated Solar Screens for Enhanced Daylighting and Outdoor Views in Tropical Gymnasiums with Bilateral Open Façades

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Abstract. This study optimizes non-uniform perforated solar screens (NUPSS) to achieve uniform useful daylight, visual comfort, and outdoor environment visibility (OEV) in tropical gymnasiums with bilateral open façades. Parametric NUPSS models varying maximum and minimum perforation diameters were evaluated via annual climate-based daylight simulations and image-based OEV calculations across Opening-to-Wall Ratios (OWR) in three levels, i.e. 50, 70, and 100%, and four orientations. Single and multi-objective optimizations were employed, evaluating a total of 1,800 iterations, to maximize daylight performance and balance outdoor views. Open façade facing North and South is found to be optimal. For maximum daylight performance, a fully open façade (100% OWR) with minimal perforations (<10% Perforated Percentage) achieved the highest performance. This configuration is overly optimized for unobstructed, shallow play area resulting in low outdoor visibility. Conversely, a 70% OWR façade with a moderate 15-27% Perforated Percentage delivered the optimal balance, yielding 96.4% useful daylight illumination (UDI_a), 67.9% uniformity variance autonomy (UVA), 2.2% spatial disturbing glare (sDG), and 36.1% OEV, making it highly recommended for dense urban contexts. This balanced configuration ensures sufficient daylight and minimal glare across varying tropical latitudes and opening heights, successfully accommodating the clear-height requirements of different indoor sports.

Index Terms— Daylighting, Design Optimization, Gymnasium, Outdoor Environment View, Perforated Solar Screen, Visual Comfort

I. INTRODUCTION

Natural lighting is a highly effective passive design strategy that significantly improves energy efficiency, reduces emissions, and supports sustainable building development [1, 2]. Sports centers and gymnasiums are typically characterized by wide-span spaces and high ceilings, making it difficult to provide daylight deep into the building's interior. To overcome this spatial challenge, it is technically possible to rely entirely on artificial luminaires to meet illuminance and uniformity thresholds. Doing so in a tropical climate drives up lighting and ancillary costs [3]. Beyond reducing reliance on artificial lighting, the reasonable use of natural light enhances visual comfort and user efficiency while positively influencing physical and mental health [4, 5]. Research in tropical regions consistently finds that occupants value natural daylight over electric lighting reporting higher satisfaction when spaces allow generous access to daylight and views even when adequate electric lighting is available [6-8]. Moreover, natural daylight is linked to greater physical activity, better mood and sleep, and higher perceived leisure and social benefits [9, 10]. Most gymnasiums lack daylight integration, as they are designed for stable lighting conditions to support

competitive sports. For gymnasiums with recreational purpose, they need large openings to allow daylight, causing visual comfort problems such as direct sunlight, glare, and indoor overheating [11]. As modern architectural development shifts toward sustainable quality improvement, the use of passive strategies has become a priority in the early stages of building design [12]. Among them, solar shading is considered an effective approach to improve the thermal and daylighting environment, especially in hot climates [13].

Application of solar shading on gymnasiums requires addressing challenges unique to their expansive spatial volumes. Shi et al. [14] established that relying on large side-façade windows or skylights to distribute natural light to the building's core often introduces severe glare. Yue et al. [15] noted that envelope optimization must carefully mitigate harsh perimeter sunlight without over-darkening the interior, which would otherwise increase artificial lighting loads. Furthermore, inadequate shading causes visual discomfort that disrupts athletes' visual-motor responses, while excessive shading blocks essential daylight [16]. Fan et al. [17] found that facilities in tropical region with unshaded, multi-sided open façades experience peak daylight intensities exceeding 2500 lux, whereas facilities with façade louvers successfully reduce illuminance but yield the lowest illumination satisfaction among the users. The expansive

spatial volumes of sports halls cultivate psychological expectations for daylight levels well above standard baselines for indoor artificial lighting (300-750 lux).

The application of non-uniform shading, where the façade is split into multiple sections, is a well-established concept. Chan and Tzempelikos [18] explored the concept of three-section façade with a top daylighting section to distribute daylight evenly throughout the space, a middle viewing section to balance view with glare protection, and a bottom spandrel section that is typically opaque. Building on this, Brzezicki [19] developed a bi-sectional shading system regulated by a façade closure scheme closing lower fins to block glare, and finally to an all-closed state if excessive illuminance persists. However, the operational success of adaptive façades is often compromised by the steep learning curve required for users and the use of fully automated system can deprive occupants of their personal environmental control [20].

Recent studies on uniform perforated solar screens (UPSS) indicate that the optimal perforated percentage (PP), having largest influence on daylighting performance, varies depending on climate and orientation. In a hot-arid Iranian climate (33.98°N), utilizing 60% PP provided the most optimal balance, successfully generating a highly effective daylit floor area of up to 95% at noon while maintaining imperceptible glare levels [21]. In temperate climate, Srisamranrungruang and Hiyaama [22] recommended a much lower PP of 40% for South façades, and a strict reduction to 10% for West-facing façades to prevent intolerable glare. Similar orientation-specific variances are observed in subtropical climates (28.7°N), where Wang et al. [23] favored a low PP of 30% for East and West façades to block intense solar radiation, while requiring a higher PP of up to 70% on North-facing windows to maximize daylight sufficiency. In a Mediterranean climate, Pool [24] found that lowering PP (<50%) using fewer, larger holes improved the fully daylit area while minimizing solar gain.

While reducing PP effectively lowers Energy Use Intensity and mitigates overlit perimeters, it inherently creates non-daylit areas at the rear of deep spaces, resulting in sharp contrast [25, 26]. Attempts to resolve this contrast in the Tropics (7.6°S) have combined uniform PSS with light shelves [27]. However, the uniformity provided by light shelves decreases as ceiling height increases, making them unsuitable for the geometries of gymnasiums [28, 29]. Furthermore, these studies focus mainly on offices or classrooms with a single open façade in isolation. Effective daylighting in wide-span buildings with openings on multiple façades requires a more comprehensive framework. When Lavin and Fiorito [30] applied UPSS to adjacent North and West façades in Sydney (33.9°S), the asymmetric solar load demanded different optimal PP (45% and 7%).

The limitation of UPSS in distributing daylight evenly [26, 31], has motivated research into non-uniform perforated solar screens (NUPSS), which aim to provide uniform, useful daylight and visual comfort [31-33]. Abdelhamid et al. [31] implemented a vertical gradient strategy with perforation sizes decreasing from top to bottom, minimizing glare at the perimeters, while allowing daylight into the core. Huang et

al. [32] found that NUPSS with perforations that gradually decrease in size from top to bottom enhance uniformity, outperforming UPSS with the same PP. Previous studies have largely confined their analysis to small rooms with a single exposed façade. The application of NUPSS to large-volume spaces like gymnasiums, where providing uniform useful daylight across multiple open façades as a primary design objective, has not yet been investigated. To address these gaps, this study introduces parameters that control maximum and minimum perforation size, expanding the optimization design space by enabling continuous transitions between fully uniform and non-uniform gradient pattern.

II. METHODOLOGY

The systematic workflow employed in this study and the tools used is presented in **Fig. 1**. The study includes the parametric generation of NUPSS applied to the openings of gymnasium, and usage of single and multi-objective optimization to identify the optimal design with highest daylight performance and find a balance between daylight performance and outdoor visibility. A robustness analysis is further conducted after selecting the NUPSS designs to find out the limitation of the results found in this study.

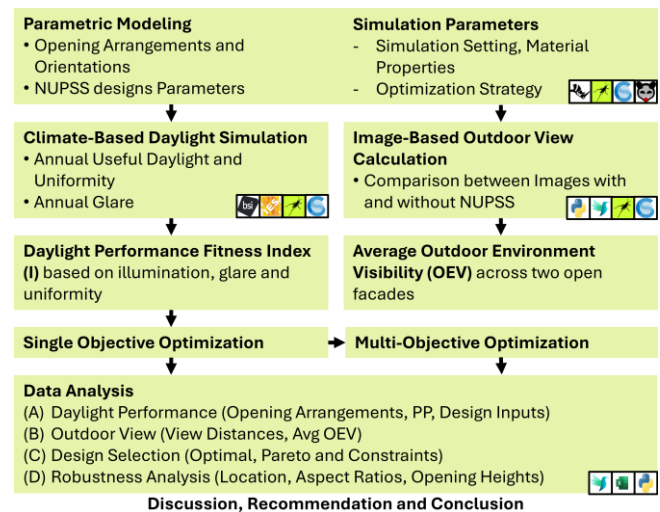


Fig. 1. Methodology and tools used in this study.

A. Base Cases

The spatial dimensions of the base case models follow international architectural standards [34]. The play area consists of a standard basketball court (28 m × 14 m) surrounded by a mandatory 2 m perimeter clearance zone for safety run-out and circulation, resulting in a gross play area of 32 m × 18 m and a recommended clear height of 7 m. The base cases are modelled in Rhino 8. The analyzed facility is assumed to be in Bangkok (13.75° N, 100.50° E), to represent typical tropical daylighting conditions.

In rectangular sports facilities such as basketball courts, the primary visual focus of the players is directed toward the short ends of the playing area. To prevent disabling glare in this direct line of sight, these short sides are devoid of fenestration,

making the gymnasium reliant on bilateral open façades along its long sides for natural light. To evaluate the performance of the solar screens under different opening arrangements, three Opening-to-Wall Ratios (OWR) are assessed in this study visualized in Fig. 2. For the 0.5 (50%) and 0.7 (70%) OWR configurations, the openings and opaque sections alternate along the façade to ensure an even distribution of daylight. Moreover, these configurations are highly characteristic of the adaptive reuse of industrial warehouses, where existing solid shear walls and column grids dictate the fenestration pattern. The orientation of the gymnasium is defined by North Offset, representing the clockwise rotation angle of the gymnasium relative to True North. The model is evaluated at 0°, 45°, 90°, and 135° to capture a range of standard solar exposures.

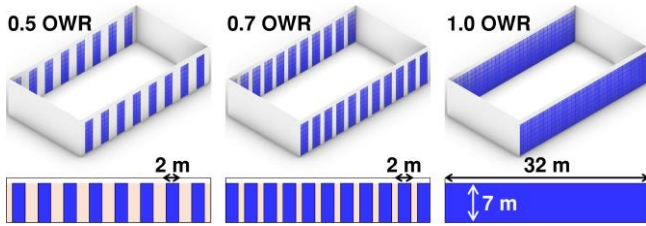


Fig. 2. Base Cases with Different Opening Arrangements.

B. Design Parameters for NUPSS

A vertical gradient NUPSS with perforations decreasing in size from top to bottom was selected for this study, as this configuration optimizes daylight distribution and lowers glare, while fulfilling the specific need for higher side openings to illuminate the core of deep-plan gymnasiums [31, 32]. Unlike adaptive louvers, utilizing fixed shading system avoids operational complexities and maintenance burdens [20] that are impractical for the transient occupancy of public sports facilities. To generate NUPSS in grasshopper (GH), perforations are arranged on a uniform square grid with a pitch (p) of 100mm. The varying diameters of these perforations follow a linear gradient pattern, which is strictly governed by two primary parameters: the perforation sizing factor (F) and the gradient contrast (G). Expressed in equation (1), the perforation sizing factor ($0.5 \leq F \leq 0.9$) sets maximum perforation diameter (d_{max}) at the top of the panel as a direct fraction of the grid pitch p .

$$d_{max} = F \times p \quad (1)$$

The gradient contrast ($0.0 \leq G \leq 1.0$) controls the transition from a fully uniform pattern ($G = 0.0$) to a full linear gradient ($G = 1.0$). Expressed in equation (2), this factor determines minimum perforation diameter (d_{min}) at the bottom of the panel by scaling it against maximum diameter.

$$d_{min} = (1 - G) \times d_{max} \quad (2)$$

Together, F and G establish the absolute boundaries for

perforation size, with all intermediate perforations scaling linearly between d_{max} on top and d_{min} on bottom of the panel as shown in Fig 3.

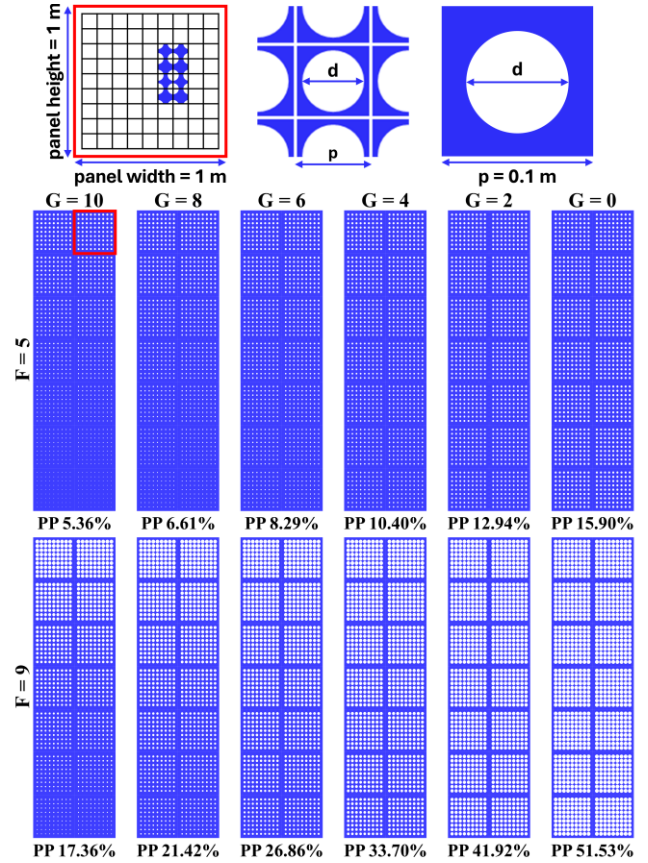


Fig. 3. Generated PSS designs based on Parameters G and F.

C. Simulation Parameters

Annual daylight and glare simulations are carried out in ClimateStudio 2.0.9 using the Bangkok Metropolis energy-plus weather file. The analyzed plane is set up at 1 m for daylight simulation recommended in ANSI/IES RP-6-24 [35]. Annual glare is calculated across eight view directions per sensor, with the analysis plane positioned at 1.5 m above the play area in accordance with BS EN 12193 [36]. The sensor spacing of 1 m and the simulation period between 8:00 and 18:00 are used. For outdoor view analysis, high-fidelity Radiance fisheye renderings are generated to simulate realistic daylight conditions and visually capture the impact of the perforated screens.

For design optimization, Radial Basis Function Optimization (RBFOpt) from Opossum interface (GH plugin) is selected over a broad range of traditional metaheuristics and direct search methods, due to the severe computational constraints of annual daylight and glare simulations. While population-based and stochastic methods like GA or PSO typically require thousands of time-intensive function evaluations to converge, RBFOpt dynamically constructs a mathematical surrogate model to rapidly locate optimal solutions within hundreds of iterations [37, 38].

Simulation parameters and material properties used in this study are shown in **Table I**.

TABLE I
SIMULATION PARAMETERS

		Reflectance
Simulation Settings	Sample rays per sensor	16,38
	Ambient bounce	4
	Weight limit	0.01
Material Properties	Wall	50%
	Floor	20%
	Ceiling	70%
	PSS	67.6%

D. Assessment Criteria for Optimization

For daylight performance, this study evaluates functional illumination, visual comfort, and daylight uniformity, which are important for recreational gymnasiums. UDIa measures the percentage of occupied time where daylight levels fall within the autonomous and acceptable range of 300 to 3000 lx [39]. This range provides adequate illumination for recreational sports while avoiding excessive solar gains that increase cooling loads [40, 41]. Standard Daylight Glare Probability (DGP) is limited to point-in-time assessments. To capture annual performance, this study uses sDG. It calculates the percentage of the analyzed area that experiences disturbing or intolerable glare ($DGP > 38\%$) across eight viewing directions for at least 5% of occupied hours [42]. Illuminance uniformity is assessed using the Coefficient of Variation (CV), the ratio of standard deviation to average illuminance [43]. Uniformity Variance Autonomy (UVA) calculates the percentage of occupied hours where CV remains at or below 0.30, adhering to IESNA recommendations for indoor basketball [40].

Previous research on tropical community gymnasiums indicates that illuminance uniformity is the primary determinant of integrated daylight and thermal comfort [17]. As the main objective, the fitness index (I) aims to maximize UDI, minimize sDG and maximize UVA and can be expressed in equation (3).

$$I = UDIa \times 0.25 + (1 - sDG) \times 0.25 + UVA \times 0.5 \quad (3)$$

For view connection, this study evaluates Outdoor Environment Visibility (OEV) using a color image recognition approach, which effectively quantifies the visual differences between a judged image with PSS and an original base image without PSS, done similarly in previous study [44]. To ensure the metric strictly evaluates the façade's performance, a spatial mask is applied to extract only the part of the visual field containing the outdoor environment, effectively excluding static interior surfaces from the calculation as shown in **Fig 4**. The pixel count across the degrees of the red, blue, and green (RGB) color bands for both masked images are extracted and

OEV is calculated by determining the similarity between the two images based on the differences in these RGB pixel numbers as expressed in equations (4) and (5).

$$OEV = \frac{1}{3} \times \left(\frac{\sum_{i=0}^{255} D_i(R) + \sum_{i=0}^{255} D_i(G) + \sum_{i=0}^{255} D_i(B)}{256} \right) \quad (4)$$

$$D_i = \begin{cases} 1, a_i = b_i \\ 1 - \frac{|a_i - b_i|}{\max(a_i, b_i)}, a_i \neq b_i \end{cases} \quad (5)$$

Here, D_i is the calculated similarity score for a specific brightness level, i represents the specific color brightness value on a scale from 0 to 255, a_i denotes the total number of pixels at brightness level i in the screened image, b_i denotes the total number of pixels at brightness level i in the unobstructed image, and R, G, B represent the red, green, and blue color bands, respectively. Because the view through PSS is highly dependent on the observer's proximity [45], the arithmetic mean of OEV values calculated at view distances VD (1 m and 9 m) across two open façades is used as secondary objective.

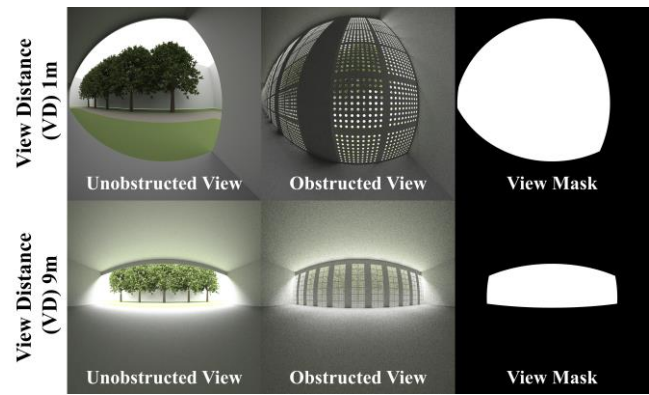


Fig. 4. Image processing setup for calculating OEV.

The design variables for each façade consist of the Gradient Contrast (G) and the Sizing Factor (F). The gradient contrast ranges from 0.0 to 1.0 in 0.1 increments, while the sizing factor ranges from 0.5 to 0.9 in 0.1 increments. This yields 55 unique pattern combinations per façade. Because the two opposing façades are optimized independently, the resulting search space expands to 3,025 possible combinations for any orientation. Additionally, the building orientation (N Offset) is evaluated as a discrete variable ranging from 0° to 135° in 45° intervals. To identify the optimal solution that yields the highest daylight performance (I), single-objective optimization is performed for 300 iterations on each OWR configurations (0.5, 0.7, and 1.0). To generate pareto frontiers and identify balanced solutions with high daylight fitness (I) and average OEV, multi-objective optimization is performed for additional 300 solutions. Overall, this study analyzed a total of 1,800 PSS design iterations. After the selection of optimal and pareto solutions, robustness analysis is conducted for selected designs on different locations in the tropical region (Singapore 1.35°N, Bangkok 13.75°N).

Kolkata 22.5°N), street aspect ratios (SAR) of 0.5, 1.0, 1.5 and 2.0, building aspect ratios (BAR) with a fixed length of 32 m and widths ranging from 18 to 36 m, and opening heights from 7m to 13m for accommodating different clear height requirements for various sports.

III. RESULT AND DISCUSSION

A. Daylight Performance

The impact of OWR on daylight performance across different rotations (N Offsets) is analyzed in **Fig. 5**. For gymnasium with open façades on the North and South, OWR of 0.7 yields the optimal balance, maximizing useful daylight (UDIa 91.8%) and daylight uniformity (UVA 54.2%) while maintaining sDG below 10%. Having fully opened façades (OWR 1.0) causes annual glare to more than double (sDG 21.9%), demonstrating the requirements of alternating solid sections to break up visual discomfort. This reliance on restricted openings is especially critical for highly exposed East-West and diagonal orientations, where utilizing a fully open façade (1.0 OWR) actively degrades useful daylight and causes excess glare. Overall, while UDIa demonstrates remarkable stability across partial opening configurations (2.0% variance between OWR 0.5 and OWR 0.7), UVA reveals high sensitivity to building rotation (31.8% variance), alongside moderate improvement (up to 12.3%) as OWR increases from 0.5 to 1.0.

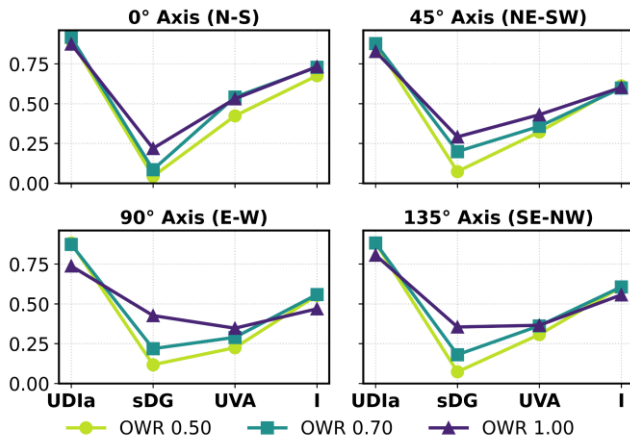


Fig. 5. Mean Performance Analysis based on OWR.

Fig. 6 demonstrates that daylighting quality and visual comfort are highly dependent on the average PP of the solar screens, revealing that useful daylight does not scale linearly with larger perforations. A gymnasium with open façades on the North and South achieves maximum useful daylight when the PP is between 20-30% (UDIa 93.2%), whereas high PP (>50%) causes UDIa to collapse (63.5%) due to excess illumination. On the highly exposed East and West façades, maintaining 10-20% PP provides low annual glare (sDG 8.7%), but pushing the PP to 40-50% increases sDG to 60.7%. Furthermore, smaller PP consistently distributes light more evenly across the deep-plan interior (<10% PP yields UVA 79.1% on North and South façades), concentrating the highest overall Fitness Index scores in the lowest perforation

ranges (I 0.86). As shown in **Fig. 7**, applying a steep gradient pattern (High G) prevents the severe performance drop seen in a more uniform PSS with high PP (>30%), allowing daylight to the core area while maintaining strict perimeter glare control. Overall, combining high G inputs with small perforations (Low F) maximizes daylight uniformity hours (UVA 61.9%) and minimizes glare (sDG 2.9%), whereas using large perforations (High F) boosts UDIa (92.6%) at the slight expense of UVA (52.9%).

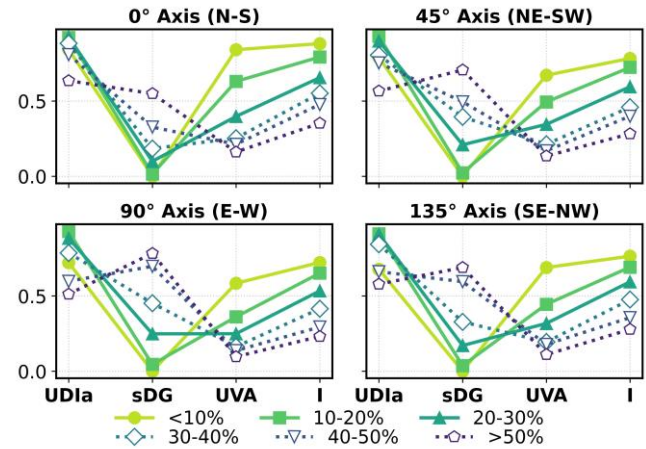


Fig. 6. Mean Performance Analysis based on PP ranges.

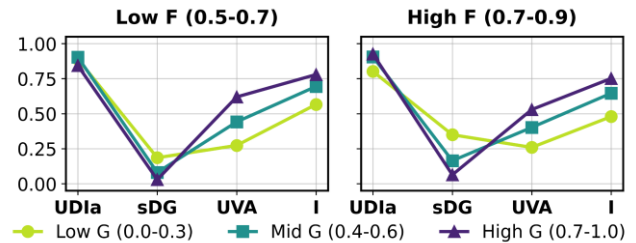


Fig. 7. Mean Performance Analysis based on Design Input (G/F).

B. Outdoor Environment Visibility

The variation of OEV changes significantly depending on the viewing distance. Deeper inside the gymnasium (VD = 9m), the outdoor view is mostly controlled by the maximum perforation size (F), maintaining a large 20.0-23.9% variance regardless of the gradient contrast (G). Conversely, near-façade views (VD = 1m) are heavily dictated by the minimum perforation size at the bottom of the screen. Because a steep gradient (high G) creates very small perforations right at eye level, it severely blocks the observer's line of sight. As a result, changing the size of the perforations at the top of the wall barely affects the view from 1m distance, causing the variance of OEV to drop from 15.5% (at G=0.0) down to just 5.5% (at G=1.0). Overall, the Average Outdoor Environment Visibility (Avg OEV) scales proportionally with larger maximum perforation sizes and inversely with steep gradient contrasts as depicted in **Fig. 8**.

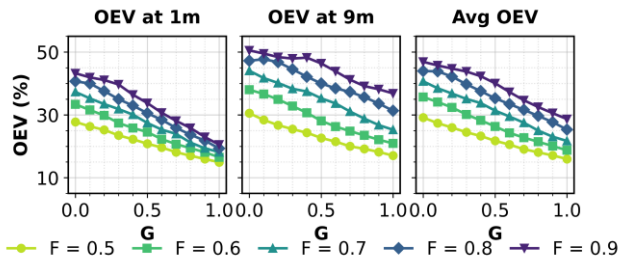


Fig. 8. Impact of Design Inputs for Perforation Sizes on OEV.

C. NUPSS Design Selection

To evaluate the trade-offs between indoor daylighting and outdoor views, three distinct solution tiers were selected for the Pareto frontiers illustrated in Fig. 9. The optimal solutions strictly maximize pure daylight fitness; to eliminate glare and maximize daylight uniformity, low levels of PP are applied ($PP < 15\%$), resulting in lowest OEV. In contrast, pareto solutions without high daylight performance criteria are selected based on the distance from the utopia point (point with maximum fitness and average OEV). These solutions resulted in high PP up to 27-34% to maximize views but inevitably fail in visual comfort (sDG) and uniformity (UVA) thresholds as a result. Thus, strict criteria ($UDI_a \geq 0.90$, $sDG \leq 0.10$, $UVA \geq 0.50$) are applied before finding the closest point to utopia point. The resulting solutions utilize a moderate PP (15-27%) to secure high outdoor views without high annual glare and low uniformity.

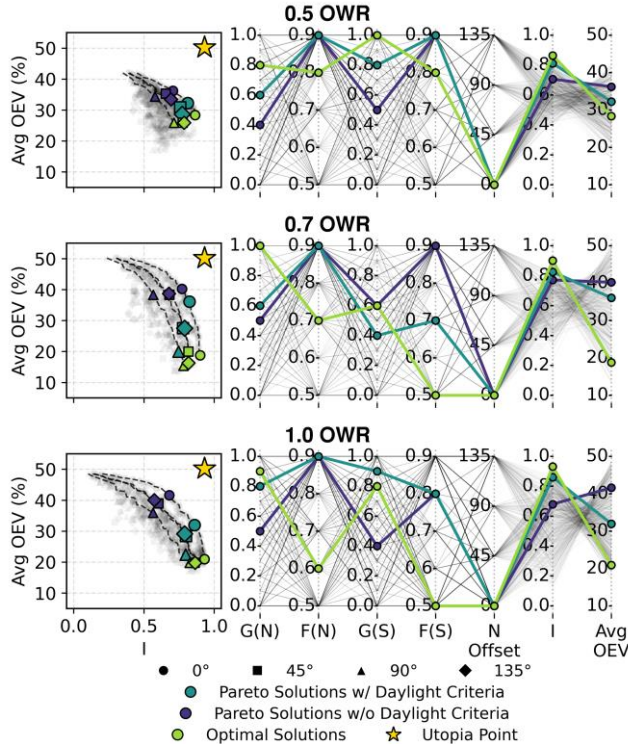


Fig. 9. Pareto Front for different Building Rotations (N Offsets) and Parallel Coordinate Plot with Selected Solutions highlighted.

Gymnasiums with open façades facing North and South (N Offset 0°) perform the best overall, allowing the façade to sustain significantly larger perforations before direct solar

glare becomes uncontrollable. When attempting to maximize the outdoor view while meeting the optimal daylight criteria, OWR of 0.7 emerges as the sweet spot with high 28.8% PP, 40.7% average OEV, 6.5% sDG (0.065) and 55.6% UVA (0.556). For each OWR arrangement, three specific NUPSS configurations are selected: the optimal solutions for maximum daylight fitness, pareto solutions for balanced performance, and pareto solutions meeting high daylight criteria and high outdoor environment visibility.

D. Robustness Analysis of Selected Solutions

The robustness analysis in Fig. 10 reveals that the optimized façade configurations are highly resilient to geographical changes but sensitive to urban and architectural changes. Across varying tropical latitudes, all performance metrics remain stable, indicating that differences in solar altitude across these regions do not disrupt the façade's fundamental shading logic. However, as the urban canyon becomes denser (H:W increasing from unobstructed 0.0 to deep 2.0), UVA and UDI_a drop significantly across all configuration types. The most critical point of failure occurs with the building aspect ratio (L:W). Transitioning from a rectangular shallow-plan (L:W = 32/18) to a deep square plan (L:W = 32/36) decreases UVA to near-zero, and overall Fitness drops by more than 50%.

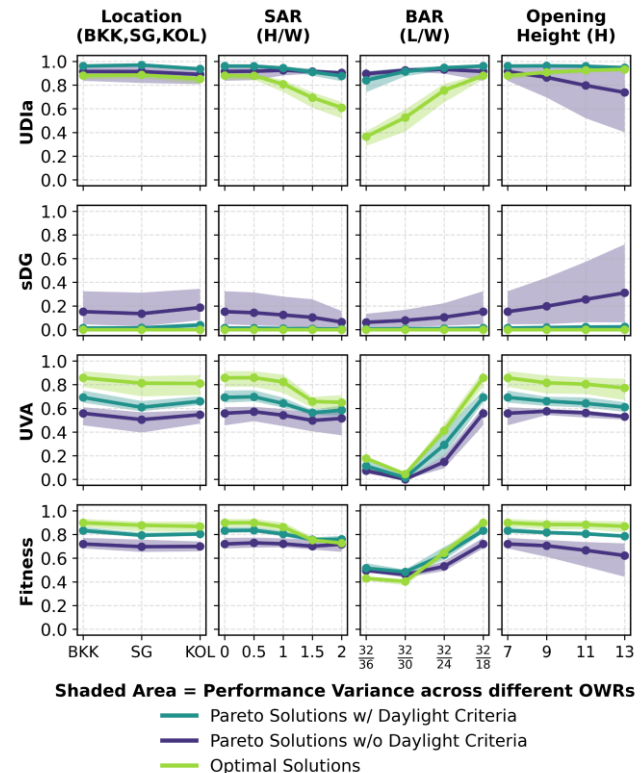


Fig. 10. Robustness Analysis of Selected Solutions.

The optimal solutions suffer from severe overfitting to the boundary conditions (BKK, H:W = 0.0, L:W = 32/18, H = 7m). While they achieve the highest possible daylight uniformity in an unobstructed environment, due to minimal PP, their performance drops significantly in denser urban

contexts or deeper plans. The pareto solutions meeting high OEV and daylight criteria demonstrate exceptional robustness. While they sacrifice a marginal amount of uniformity, they maintain high functional daylight ($UDI_a > 0.85$) and glare control ($sDG < 0.1$) even with H:W of 1.5. Both the optimal and constrained Pareto solutions maintain highly stable useful daylight ($UDI_a \geq 88.0\%$) and strict glare control ($sDG \leq 2.3\%$) even when the opening height is increased from 7 m to 13 m. However, increasing the façade height slightly reduces daylight uniformity, causing UVA to decrease by approximately 8%.

E. Discussion

This study advances the design of passive daylighting in wide-span buildings by introducing a quantitative method to precisely coordinate perforation sizing (F) and gradient contrast (G). While previous literature largely analyzed non-uniform patterns at a fixed perforated percentage (PP)[32] or used randomized perforation sizes [31, 33], the mathematical relationship between F and G used in this study enables a continuous parametric transition between fully uniform and steep gradient screens. This approach unlocks a significantly larger design space, offering architects greater flexibility in optimizing daylight distribution. Furthermore, these designs are evaluated against an image-based OEV metric. Unlike traditional analyses that rely on ray-traced view angles, the OEV approach calculates direct variations in view pixels, offering a significantly more accurate assessment of visual connectivity through complex perforated obstructions.

Single-objective optimization favors a low perforated percentage ($<15\%$ PP) to maximize useful and uniform daylight without glare. This is notably lower than recommendations for classrooms (30-70% PP [23]), residential buildings (60% PP [21]) and offices (30-50% PP [26, 27]) attributed to the multi-façade configuration and non-uniform PSS with larger perforations at the top, which reduces the need for high transparency. In contrast, previous studies primarily evaluated uniform screens for single-sided apertures in higher-latitude locations. In these scenarios, daylight can only reach further into space from single direction and with fewer sun hours in higher latitude locations, the need for larger PP is further amplified. Moreover, the optimal result found in this study is severely overfitted to unobstructed environments. As illustrated in **Fig. 11**, the optimal case applied to a fully open façade (100% OWR) delivers peak daylight uniformity, but its reliance on minimal perforations makes it highly vulnerable to external obstructions. Thus, architects should deploy these optimal solutions exclusively for unobstructed, shallow-plan gymnasiums.

To the contrary, multi-objective optimization proves that balancing daylight with OEV yields a moderate PP (15-27%) that is superior for practical application. For urban gymnasiums embedded in dense city fabrics, natural canopies or featuring deeper floor plans, the Pareto solution found for gymnasium with 70% OWR stands out as the most viable choice, meeting strict daylight criteria ($UDI_a \geq 0.90$, $sDG \leq 0.10$, $UVA \geq 0.50$) and maintaining daylight sufficiency and glare control across tropical latitudes and

dense urban obstructions. Additionally, both configurations possess the flexibility to be utilized with higher opening heights, successfully accommodating the elevated clear-height requirements of diverse indoor sports without sacrificing visual comfort. These findings are particularly valuable in the context of adaptive reuse. When converting former industrial warehouses into multipurpose spaces, the structural necessity of retaining existing shear walls and column grids often results in alternating solid and open façades. Since this perfectly aligns with the 70% OWR configuration analyzed in this study, these lightweight perforated screens act as highly practical retrofit solutions. In these applications, the screens can also function as expressive design elements that enhance the aesthetic appeal while establishing visual connection to the outdoor environment.

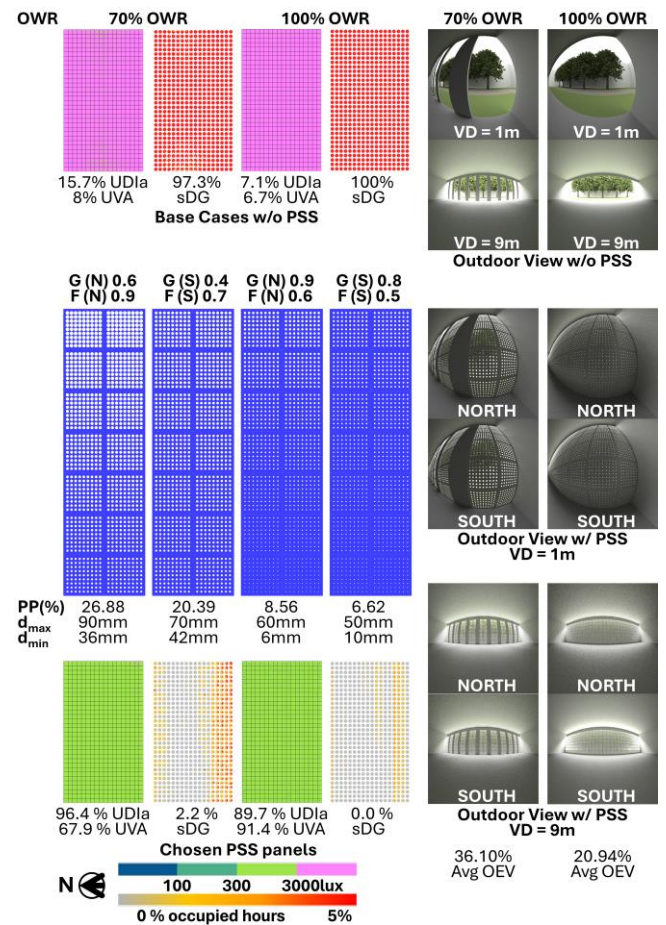


Fig. 11. Recommended NUPSS panels and Comparison with Base Cases.

IV. CONCLUSION

While this study successfully establishes a framework for enhancing daylight using perforated solar screens with outdoor views as secondary objective, certain limitations remain. The potential contribution of supplementary artificial lighting or skylights, which would influence illuminance requirements at the deep core, remained outside the scope of this research. Although the pareto analysis

demonstrates that NUPSS with moderate PP range (15-27%) allows higher outdoor visibility while maintaining daylight criteria, the corresponding impact on Thermal Comfort was not evaluated. Since increasing the Opening-to-Wall Ratio (OWR) and Perforation Percentage (PP) directly impacts solar heat gain, future studies should incorporate an analysis of Thermal Comfort and total Building Energy Consumption alongside daylighting performance to ensure a truly holistic design approach. Furthermore, subsequent research should expand this methodology to gymnasiums with asymmetrical daylighting conditions, such as those with two adjacent open façades, or three or more highly exposed open façades.

Use of Generative Artificial Intelligence (AI) and AI-Assisted Technologies

During the preparation of this manuscript, Google Gemini AI is used to improve readability and language of some passages. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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Poster Presentation

The Effect of Pre-Harvest Irrigation on the Severity of Chilling Injury in Queen Group Pineapples

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Abstract. Pineapples are tropical fruits that are susceptible to chilling injury when stored at low temperatures. This injury typically manifests as brown core, which negatively affects fruit quality and consumer acceptance. This study aimed to investigate the effect of pre-harvest water management on the severity of chilling injury in Queen pineapple varieties. Pineapples were grown under greenhouse conditions and subjected to two irrigation treatments. Regular irrigation throughout fruit development with water withheld before harvest, and Regular irrigation combined with heavy watering before harvest. The experimental parameters included brown core score, flesh color, total soluble solids content, total organic acid content, malondialdehyde content, and electrolyte leakage. The results showed that excessive pre-harvest water application significantly increased the severity of brown core, particularly with longer storage durations. The increase in brown core score was associated with a decrease in L* value and changes in a* and b* values, indicating internal tissue deterioration. Furthermore, pineapples subjected to high pre-harvest water application exhibited significantly higher malondialdehyde content and electrolyte leakage, especially on day 14 of storage, suggesting cell membrane damage caused by lipid peroxidation. In addition, changes in total soluble solids and organic acid content reflected an imbalance in fruit metabolic processes under chilling injury conditions. In conclusion, pre-harvest water management is a key factor influencing the severity of chilling injury in 'Sawi' pineapple. Excessive water application close to harvest increases the risk of cellular damage and postharvest internal browning during low-temperature storage.

Index Terms—Irrigation, Membrane lipid peroxidation, Pineapple

I. INTRODUCTION

Pineapple (*Ananas comosus* L.) is an economically important tropical fruit widely traded in both fresh and processed forms. Thailand is one of the world's leading producers and exporters, contributing approximately 45–50% of the global processed pineapple market. Processed products, including canned pineapple, juice, and jam, generate more than 25 billion baht annually [1]. Despite its economic importance, the export of fresh pineapple is constrained by postharvest physiological disorders, particularly internal browning. Internal browning is a form of chilling injury that develops when pineapple fruit are exposed to low storage temperatures for extended periods. Symptoms are characterized by brown discoloration of the flesh, especially in tissues adjacent to the core, commonly referred to as brown core [2]. Chilling injury in pineapple has

been associated with membrane deterioration, oxidative stress, and metabolic imbalance during cold storage. Preharvest environmental conditions, particularly water availability during fruit development, have been reported to influence fruit susceptibility to postharvest disorders. Elevated water content in developing fruit has been linked to a higher incidence of chilling injury and brown core after cold storage. This effect may be associated with alterations in cellular water status, membrane stability, and stress-response mechanisms that influence tissue sensitivity to low temperatures [3]. However, the relationship between water status during fruit development and subsequent chilling injury under controlled production conditions remains insufficiently understood. Therefore, this study aimed to evaluate the effect of water content during fruit development under greenhouse conditions on the incidence and severity of

chilling injury in Queen pineapple following low-temperature storage.

II. METHODOLOGY

Plant Material, Treatment, and Storage Conditions

Pineapple plants were cultivated under greenhouse conditions. Two irrigation treatments were applied during fruit development Treatment1 Watering irrigation throughout fruit development 1.2 liters per day followed by the suspension of watering one week prior to harvest and Treatment 2 Watering irrigation throughout fruit development 1.2 liters per day with intensified watering during the final week before harvest. Ten fruits of uniform size and maturity were selected from each treatment. The harvested fruits were stored at 13 °C for 7 or 14 days and subsequently transferred to 25 °C for 2 days to simulate shelf-life conditions. After storage, fruits were longitudinally cut, and the severity of brown core symptoms was evaluated [4]. The proportion of affected flesh area relative to the total fruit area was determined, and severity was scored on a scale of 0–5. The brown core severity index was then expressed as a percentage. Fruit physical quality was assessed using a chromameter to measure flesh lightness (L*) values in tissues adjacent to the core. Measurements were performed at least three times per fruit.

Chemical Qualitative Analysis

Total soluble solids (TSS)

TSS was determined using a hand-held refractometer. The data were expressed as Brix.

Titrateable acidity (TA)

TA was determined using the method described. A 20 g of sample was homogenized and filtrated through filter cloth. Two mL of the extract was mixed with 20 mL of distilled water and 1–2 drops of 1% phenolphthalein. The mixture was titrated with 0.1 N NaOH until the solution turned to pink at least 30 sec. The volume of used 0.1 N NaOH solution was recorded. The data were presented as g citric acid per 100 g fresh weight (g 100 g⁻¹).

$$TA (\%) = \frac{V \times N \times MEq}{[\text{solution of sample (2 mL)}]} \times 100 \quad (1)$$

V: Volume of used for titration (mL)

N: Normality of 0.1 N

MEq: Milliequivalent weight of 0.064 for citric

Malondialdehyde (MDA) Quantification

Malondialdehyde (MDA) content was measured to assess the level of membrane damage caused by lipid peroxidation. Five grams of pineapple pulp Weigh and fill into a 50ml tube. (from the inner part between the core and the flesh) were mixed with 20 mL of 5% trichloroacetic acid (TCA) solution, homogenized, and centrifuged at 10,000 rpm for 20 minutes. The resulting supernatant was then collected for

MDA analysis. For the assay, 1 mL of the sample was transferred into a test tube, and 2 mL of 15% TCA solution containing 0.5% thiobarbituric acid (TBA) was added. The mixture was incubated in a water bath at 60°C for 30 minutes and then immediately cooled. The MDA content was determined using a UV–visible spectrophotometer at wavelengths of 532 and 600 nm. described by Wang et al. [5].

Electrolyte Leakage (EL) Analysis

Electrolyte leakage was measured to evaluate changes in membrane integrity in pineapple pulp. Ten cylindrical pieces of pineapple pulp (from the area between the core and the flesh) were obtained using a ¼-inch cork borer. The samples were rinsed with deionized (DI) water, blotted dry with filter paper, weighed, and immersed in 30 mL of DI water for 30 minutes. Electrical conductivity was measured using a conductivity meter. The samples were then autoclaved at 121°C for 15 minutes to completely disrupt the tissues. After cooling to room temperature, electrical conductivity was measured again. The percentage of electrolyte leakage was calculated described by Promyou et al [6].

$$EL (\%) = \frac{(\text{initial conductivity})}{(\text{final conductivity})} \times 100 \quad (2)$$

Statistical Analysis

The experiment was conducted using a completely randomized design (CRD). Mean comparisons were performed using Duncan's Multiple Range Test (DMRT). All statistical analyses were conducted using SPSS for Microsoft Windows, version 29.

III. RESULTS AND DISCUSSION

Pre-harvest water management markedly influenced brown core development in 'Sawi' pineapple. Fruits receiving high water input prior to harvest exhibited significantly greater brown core severity compared with water-deprived fruits. Symptom intensity further increased during storage at 13 °C, [7] indicating that excessive water availability during fruit development enhances susceptibility to chilling injury under suboptimal storage temperatures. Given that pineapple is a tropical crop inherently sensitive to temperatures below 15 °C [8] these findings suggest that pre-harvest water status modulates physiological resilience to postharvest cold stress. Mechanistically, chilling-induced membrane destabilization and lipid peroxidation likely facilitated the interaction between phenolic substrates and polyphenol oxidase, accelerating oxidative browning [9]. The progressive increase in brown core scores (Fig. 1) was closely associated with CIELAB color shifts, particularly a decline in L* values (Fig. 2A), reflecting reduced flesh brightness. Fruits exposed to high pre-harvest water levels exhibited a more rapid decrease in L* during storage, indicating enhanced internal tissue disintegration and oxidative deterioration. In contrast, water-deficit fruits maintained higher L* values, suggesting improved membrane integrity and structural stability. Concomitantly, the increase in a* values (Fig. 2B) in high-water fruits,

especially at later storage stages, indicates enhanced chlorophyll degradation and pigment oxidation, whereas the decline in b^* values (Fig. 2C) suggests loss of yellowness associated with tissue breakdown. The inverse relationship between L^* and internal browning severity observed here corroborates previous findings in pineapple [10], reinforcing the reliability of instrumental color parameters as indicators of chilling-induced internal disorders. Collectively, these results demonstrate that excessive pre-harvest water supply predisposes ‘Sawi’ pineapple to chilling injury by amplifying membrane vulnerability and oxidative processes during cold storage. Thus, strategic water management prior to harvest may represent a critical intervention to enhance postharvest tolerance and mitigate internal browning in tropical fruit systems.

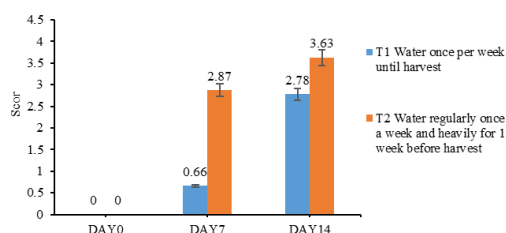


Fig. 1. Internal browning score of pineapple treated with pre-harvest irrigation storage at 13 °C for 0, 7 and 14 day

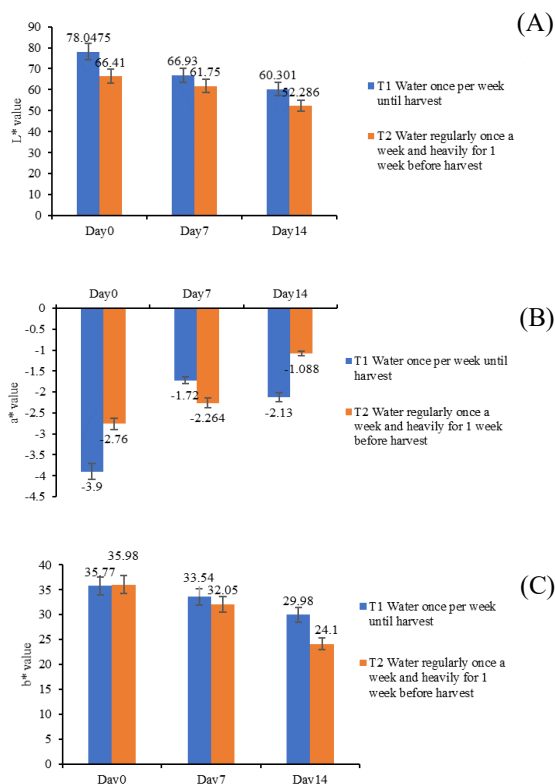


Fig. 2. Color attributes, L^* (A), a^* (B), and b^* (C), of tissue adjacent to the core of ‘Queen’ pineapple subjected to different irrigation levels during storage at 13 °C for 0, 7 and 14 days

Pre-harvest water management also significantly influenced chemical quality during storage. A clear

association was observed between total soluble solids (TSS), titratable acidity (TA), and brown core severity. At the onset of storage, TSS and TA values did not differ among treatments, and no internal browning was detected, indicating comparable metabolic status and structural integrity at harvest. However, after 14 days (Fig. 3A) at 13 °C, fruits subjected to high pre-harvest water supply exhibited a significant increase in TSS and a pronounced decline in TA (Fig. 3B), concomitant with severe brown core development and late-stage increase in TSS is unlikely to represent normal ripening progression, as pineapple is a non-climacteric fruit. Rather, it likely reflects stress-induced metabolic disruption under chilling injury conditions. Membrane destabilization and lipid peroxidation may impair compartmentalization, leading to leakage of intracellular solutes and altered carbohydrate distribution [9]. Similarly, chilling stress in tropical commodities has been shown to induce metabolic imbalance, intracellular leakage, and water redistribution [8], which may collectively contribute to the apparent elevation in soluble solids. The marked reduction in TA, particularly in high-water fruits, suggests enhanced utilization of organic acids as respiratory substrates under stress conditions. Increased respiratory demand during chilling injury can accelerate organic acid depletion, while loss of membrane integrity further disrupts acid compartmentalization. Such alterations in sugar–acid balance have been closely associated with tissue deterioration and internal browning in pineapple [7]. These findings support the current understanding of postharvest physiology [11], emphasizing that pre-harvest environmental factors, including water availability, critically determine fruit tolerance to subsequent storage stress. Collectively, the data indicate that excessive pre-harvest water supply predisposes ‘Sawi’ pineapple to chilling-induced metabolic dysregulation. Enhanced membrane vulnerability likely accelerates oxidative processes, organic acid depletion, and solute redistribution, culminating in severe internal browning. This reinforces the concept that water management prior to harvest is not merely an agronomic factor but a determinant of postharvest biochemical stability and storage resilience in tropical fruit systems.

Changes in malondialdehyde (MDA) content and electrolyte leakage (EL), two established indicators of membrane integrity, further elucidated the physiological basis of internal browning development. Both parameters increased progressively during storage and were positively associated with brown core severity. At harvest, MDA concentrations (Fig. 4A) were low and did not differ among treatments, indicating minimal lipid peroxidation and preserved membrane structure. Correspondingly, EL values (Fig. 4B) were also low, reflecting intact membrane selectivity and effective maintenance of intracellular ion homeostasis. These findings are consistent with the absence of visible browning symptoms at the initial storage stage. By day 7 of storage at 13 °C, MDA levels increased, particularly in fruits exposed to high pre-harvest water supply, indicating the onset of lipid peroxidation under chilling stress.

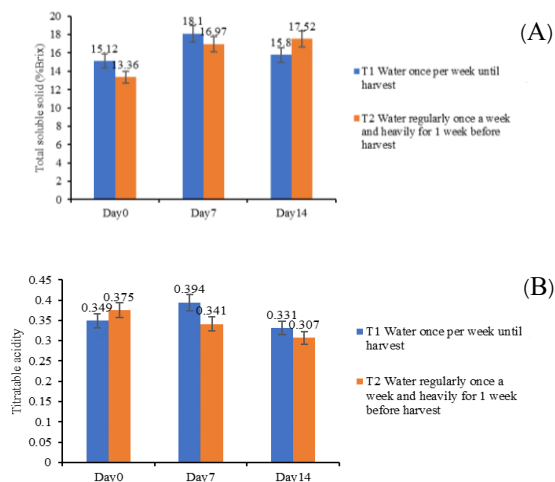


Fig. 3. TSS (A), TA (B) of tissue adjacent to the core of 'Queen' pineapple subjected to different irrigation levels during storage at 13 °C for 0, 7 and 14 days

Pineapple, as a tropical fruit, is highly susceptible to chilling injury, and internal browning has been closely linked to the accumulation of lipid oxidation products [9]. Low-temperature stress is known to induce metabolic imbalance and excessive generation of reactive oxygen species (ROS), which attack membrane lipids and initiate peroxidative chain reactions [8]. As membrane lipid structure deteriorates, permeability increases, leading to reduced regulation of ion transport and a concomitant rise in EL. Membrane damage became most pronounced after 14 days of storage, when MDA levels significantly increased across all treatments, with the highest accumulation observed in fruits receiving excessive pre-harvest water. This indicates severe oxidative degradation of membrane lipids and sustained loss of structural integrity. The concurrent increase in EL supports this interpretation, as elevated leakage reflects compromised membrane semi-permeability and impaired retention of intracellular electrolytes. Previous studies have demonstrated that internal browning in pineapple is closely associated with membrane disintegration and intracellular leakage [7], consistent with the present findings. Notably, the temporal sequence observed in this study an initial rise in MDA followed by increased EL suggests that lipid peroxidation precedes and drives membrane permeability loss. This mechanistic progression supports the hypothesis that oxidative damage is a primary trigger of membrane dysfunction, ultimately facilitating phenolic oxidation and brown core development. Within the framework of postharvest physiology, pre-harvest factors such as irrigation regime may modulate membrane composition, antioxidant capacity, and tolerance to oxidative stress during storage [11]. These results demonstrate that excessive pre-harvest water supply enhances susceptibility to chilling-induced oxidative stress in 'Sawi' pineapple. Elevated MDA accumulation reflects intensified lipid peroxidation, which subsequently increases membrane permeability (EL) and accelerates internal browning. Thus, membrane stability represents a critical physiological link between pre-harvest

water management and the development of postharvest disorders.

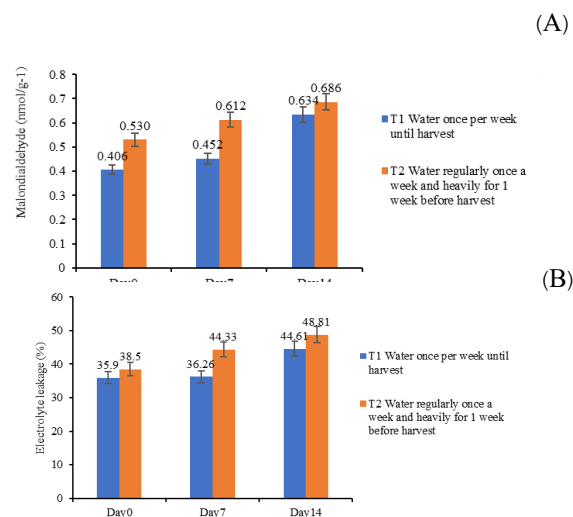


Fig. 4. MDA (A) and electrolyte leakage (B) of tissue adjacent to the core of 'Queen' pineapple subjected to different irrigation levels during storage at 13 °C for 0, 7 and 14 days

IV. CONCLUSION

The present findings demonstrate that pre-harvest water management significantly modulates chilling injury susceptibility in 'Queen' pineapple during low-temperature storage. Excessive irrigation near harvest markedly increased brown core severity after 14 days at 13 °C. This disorder was closely associated with coordinated alterations in chromatic, biochemical, and membrane integrity parameters. Specifically, increased browning severity corresponded with declining L* values, elevated a* values, and reduced b* values, indicating progressive pigment oxidation and tissue discoloration. These visual changes were accompanied by increases in total soluble solids (TSS) and declines in titratable acidity (TA), reflecting disruption of intragrain metabolic balance under chilling stress. Concomitantly, significant increases in malondialdehyde (MDA) and electrolyte leakage (EL) in fruits receiving excessive pre-harvest water indicate enhanced lipid peroxidation and loss of membrane semi-permeability. The accumulation of MDA suggests intensified oxidative degradation of membrane lipids, while elevated EL confirms impaired ion homeostasis resulting from structural membrane damage. These processes collectively facilitate phenolic oxidation and the development of brown core symptoms. However, the results indicate that excessive pre-harvest water supply predisposes pineapple fruit to chilling-induced oxidative stress, metabolic dysregulation, and membrane destabilization during storage. Therefore, strategic irrigation management particularly the avoidance of excessive watering close to harvest represents a critical agronomic intervention to enhance membrane stability, mitigate internal browning, and maintain postharvest quality under low-temperature storage conditions.

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Internal Browning Alleviation of ‘Sawi’ Pineapples During Cold Storage Using Methyl Jasmonate Spray

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Abstract. This study aimed to investigate the effectiveness of methyl jasmonate (MeJA) in alleviating internal browning in ‘Queen’ pineapple cv. Sawi under low-temperature storage at 13 °C. Pineapple fruits were sprayed with MeJA solutions at concentrations of 0.1 and 1 mM and compared with an untreated control. The fruits were stored for 0, 7, and 14 days, followed by an additional 2 days at room temperature. The results demonstrated that treatment with 1 mM MeJA resulted in the lowest incidence and severity of internal browning, followed by 0.1 mM MeJA, whereas the control exhibited the highest browning severity. Particularly on day 14, the internal browning score and browning index were significantly correlated with the decrease in lightness (L*) values of the flesh adjacent to the core. MeJA treatment significantly suppressed polyphenol oxidase (PPO) activity, reduced malondialdehyde (MDA) accumulation, controlled electrolyte leakage, and maintained antioxidant capacity as determined by DPPH radical scavenging activity and FRAP assay. In addition, MeJA treatment helped maintain total soluble solids (TSS) and titratable acidity (TA) compared with the control. Therefore, the application of methyl jasmonate at 1 mM can be considered an effective postharvest strategy to delay internal browning, maintain fruit quality, and extend the storage life of ‘Queen’ pineapple cv. Sawi under low-temperature storage conditions.

Index Terms— Queen pineapple, Plant growth regulators, Chilling injury, Low temperature.

I. INTRODUCTION

Pineapple (*Ananas comosus* L.) is an economically important tropical fruit in Thailand and is classified as a non-climacteric fruit [1]. More than 70% of the total production is utilized for processing and export, while the remaining portion is consumed as fresh fruit domestically especially ‘Queen’ pineapples [2]. However, low-temperature storage and prolonged transportation are major factors inducing chilling injury [3], which leads to a physiological disorder known as internal browning in ‘Queen’ pineapple [4]. This disorder is commonly observed when fruit are stored at commercial refrigeration, approximately 10–13 °C [5], resulting in deterioration of internal quality and reduced marketability. Internal browning in pineapple is associated with physiological alterations at the cellular level, particularly membrane lipid peroxidation, as indicated by increased ion leakage, and the accumulation of malondialdehyde (MDA), a by-product of lipid peroxidation, ultimately leading to flesh

discoloration [6]. Previous studies have investigated the application of plant growth regulators to delay internal browning, especially methyl jasmonate (MeJA), a signaling molecule that regulates physiological processes and stress responses in plants. MeJA has been recognized as an effective alternative for reducing chilling injury and other oxidative stresses due to its role in regulating plant growth, wound responses, development, and tolerance to environmental stress. As a derivative of jasmonic acid, MeJA induces biochemical changes that enhance plant defense mechanisms [7]. MeJA treatment helps maintain phenolic compounds within the vacuole, thereby reducing their contact with oxidative enzymes in the cytosol. This is a key mechanism in delaying postharvest tissue breakdown. [8]. Furthermore, postharvest application of MeJA contributes to maintaining cellular redox balance by preserving antioxidant compounds, such as phenolics, leading to reduced reactive oxygen species (ROS) accumulation and decreased phenolic oxidation. Consequently, quinone formation and tissue browning are

alleviated during low-temperature storage [9]. In addition, MeJA reduces the activity of browning-related enzymes, including polyphenol oxidase (PPO), thereby mitigating the severity of chilling injury compared with untreated fruit [10]. Therefore, this study aimed to evaluate the effects of postharvest MeJA application at various concentrations of 0.1 and 1 mM on the alleviation of chilling injury in Queen pineapple during low-temperature storage conditions at 13 °C followed by an additional 2 days at 25 °C.

II. METHODOLOGY

Plant Material, Treatment, and Storage Conditions

Queen pineapple fruits cv. Sawi were harvested at 135 days after flower induction. Fruits of uniform size and free from defects were selected for the experiment. The experimental design was a completely randomized design (CRD) consisting of three treatments with six replications, two fruits per replication. Prior to storage, fruits were sprayed with MeJA solutions at concentrations of 0.1 and 1 mM, applied to both the crown and fruit surface. The treated fruits were then stored at 13 °C for 7 and 14 days, followed by an additional 2 days at 25 °C. At the end of the storage period, fruits were longitudinally cut to evaluate internal browning severity described by Teisson [11]. The affected area of flesh adjacent to the core exhibiting internal browning symptoms was assessed relative to the total flesh area. Browning severity was scored on a scale of 0–5, and the obtained scores were subsequently converted into percentage of internal browning severity. Physical quality was further evaluated using a chromameter to determine flesh lightness (L^*) at the area adjacent to the core. Measurements were taken at least three points per fruit.

Chemical Qualitative Analysis

Browning index (BI) Quantification

The browning index was determined using spectrophotometric method. A 5 g sample of flesh tissue adjacent to the core was homogenized with 20 mL of 65% (v/v) ethanol using a homogenizer. The homogenate was allowed to stand for 90 min and subsequently filtered through Whatman No. 1 filter paper. The clear filtrate was collected and analyzed for browning intensity using a UV–visible spectrophotometer at a wavelength of 420 nm, following the method described by Supapvanich [12].

Polyphenol Oxidase (PPO)

Polyphenol oxidase (PPO) activity was analyzed as an indicator of phenolic oxidation associated with flesh browning in pineapple. PPO activity was determined using a UV–Visible spectrophotometer at a wavelength of 470 nm according to the method described by Galeazzi et al. [13]. A 5 g sample of flesh tissue adjacent to the core was placed in a conical tube and homogenized with 0.3 g polyvinylpyrrolidone (PVPP) and 30 mL of 100 mM sodium phosphate buffer (pH 7.8) using a

homogenizer. The homogenate was then centrifuged at $10,000 \times g$ for 15 min, and the supernatant was collected as the

crude enzyme extract. The reaction mixture was prepared in a test tube containing 3.9 mL of 100 mM phosphate buffer (pH 7.8) and 0.1 mL of catechol. Subsequently, 1.0 mL of enzyme extract was added to initiate the reaction. PPO activity was expressed as Units g^{-1} FW, where one unit (U) was defined as a change in absorbance of 0.001 min^{-1} .

Malondialdehyde (MDA)

Malondialdehyde (MDA) content was determined to evaluate membrane damage resulting from lipid peroxidation. A 5 g sample of pineapple flesh tissue located between the core and the outer flesh, which was selected due to its higher sensitivity to physiological stress and susceptibility to internal browning, was homogenized with 20 mL of 5% (w/v) trichloroacetic acid (TCA) using a homogenizer. The homogenate was centrifuged at 10,000 rpm for 20 min, and the supernatant was collected for MDA analysis. An aliquot of 1 mL of the supernatant was transferred into a test tube, followed by the addition of 2 mL of a solution containing 15% (w/v) trichloroacetic acid (TCA) and 0.5% (w/v) thiobarbituric acid (TBA). The mixture was incubated in a water bath at 60 °C for 30 min and then immediately cooled in an ice bath. The absorbance was measured using a UV–visible spectrophotometer at 532 and 600 nm to determine MDA content, following the method described by Wang et al. [14].

Electrolyte leakage (EL)

EL was measured to indicate changes in membrane integrity of pineapple flesh tissue. Ten flesh discs were excised from the region between the core and the outer flesh using a ¼-inch cork borer. The tissue discs were rinsed with deionized (DI) water and gently blotted dry with filter paper. The samples were weighed and then immersed in 30 mL of DI water for 30 min. Initial electrical conductivity was measured using a conductivity meter. Subsequently, the samples were autoclaved at 121 °C for 15 min to completely disrupt the tissues, and the final conductivity was measured again. The percentage of EL was calculated according to the method described by Promyou et al [15].

$$EL (\%) = \frac{(\text{initial conductivity})}{(\text{final conductivity})} \times 100 \quad (1)$$

Lipoxygenase (LOX)

Lipoxygenase (LOX) activity was analyzed to determine the level of unsaturated fatty acid oxidation and membrane damage in pineapple tissues, which contribute to the development of internal browning during storage. A 10 g sample of flesh tissue adjacent to the core was placed in a conical tube and homogenized with 0.5 g polyvinylpyrrolidone (PVP) and Tris–HCl buffer (pH 8.4) using a homogenizer. The homogenate was then centrifuged at $10,000 \times g$ for 20 min, and the supernatant was collected for enzyme activity

analysis. The reaction mixture was prepared in a test tube containing 2.85 mL of 100 mM sodium phosphate buffer (pH 6.0) and 50 μ L of substrate solution. Subsequently, 100 μ L of the enzyme extract was added to initiate the reaction. LOX activity was determined by measuring the increase in absorbance at 234 nm using a UV–Visible spectrophotometer, following the method described by Wang et al [14].

DPPH free radical scavenging activity

Radical scavenging activity was determined using methanol extracts of pineapple flesh adjacent to the core. A 5 g sample was homogenized with 50 mL of methanol using a homogenizer and then centrifuged to obtain the supernatant. An aliquot of 5 mL of the extract was used to evaluate free radical scavenging activity by the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay according to the method described by Brand Williams et al. [16]. Briefly, 50 μ L of the extract was mixed with 2.45 mL of distilled water and 0.25 mL of DPPH reagent. The absorbance was immediately measured at 517 nm using a UV–visible spectrophotometer to obtain the initial absorbance (A_0). The mixture was then incubated in the dark at room temperature for 30 min, and the absorbance was measured again to obtain (A_{30}). The percentage of DPPH inhibition was subsequently calculated.

$$\text{DPPH (\%)} = [(A_0 - A_{30}) / A_0] \times 100 \quad (2)$$

A_0 = absorbance of the sample at 0 min
 A_{30} = absorbance of the sample at 30 min

Antioxidant capacity

Antioxidant capacity was determined using the ferric reducing antioxidant power (FRAP) assay. A 5 g sample of pineapple flesh adjacent to the core was homogenized with 50 mL of distilled water using a homogenizer and then centrifuged to obtain the supernatant. An aliquot of 0.3 mL of the extract was reacted with 3 mL of FRAP reagent and incubated in the dark at room temperature for 30 min. The absorbance was measured at 630 nm using a UV–Visible spectrophotometer. Antioxidant capacity was expressed as μ mol Trolox equivalents per 100 g fresh weight (μ mol TE/100 g FW).

Statistical Analysis

The experiment was arranged in a Completely Randomized Design (CRD) with six replications, two fruits per replication. Statistical analysis was performed using analysis of variance (ANOVA). Mean comparisons were conducted using Duncan's New Multiple Range Test (DMRT). All statistical analyses were carried out using IBM SPSS Statistics Version 29.

III. RESULTS AND DISCUSSION

The severity of internal browning in pineapple fruit sprayed with MeJA postharvest and stored at 13 °C increased progressively with storage duration. The control fruit exhibited the highest internal browning score, followed by fruit treated with 0.1 mM MeJA, whereas treatment with 1 mM MeJA most effectively delayed the development of

internal browning (Figure 1A). The increase in internal browning score was consistent with changes in the browning index (BI). BI values gradually increased in all treatments as storage time progressed; however, the control showed the highest BI, while fruit treated with 1 mM MeJA consistently exhibited the lowest BI throughout storage, particularly on day 14 (Figure 1B). MeJA is known to stimulate the production of antioxidant compounds, such as ascorbic acid and phenolic compounds, as well as to enhance the activity of antioxidant enzymes, which play crucial roles in reducing oxidative stress [17]. Therefore, the application of methyl jasmonate at a concentration of 1 mM was the most effective in delaying the development of internal browning during low-temperature storage.

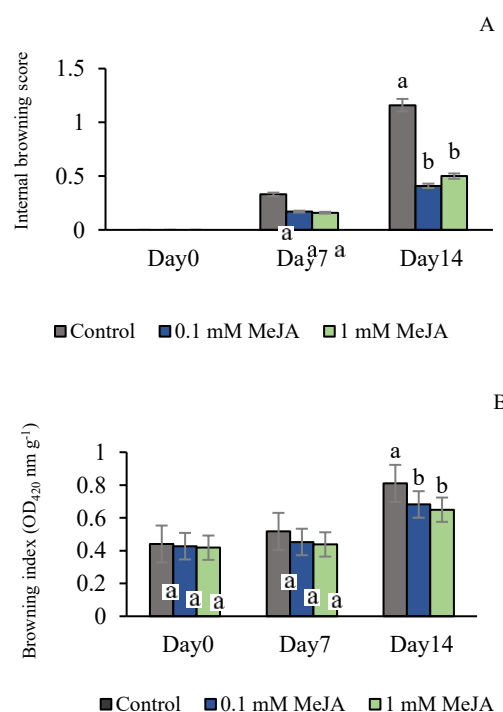


Figure 1. Internal browning (score) (A), Browning index and (B) in pineapple treated with methyl jasmonate sprayed at various concentrations 14 days postharvest during storage at 13 °C for 0, 7, and 14 days.

The L* value (lightness) of the flesh adjacent to the core of pineapple fruit sprayed with methyl jasmonate after harvest and stored at 13 °C decreased with increasing storage duration. After 7 days of storage, the control fruit exhibited the lowest L* value, followed by fruit treated with 0.1 mM MeJA, whereas fruit treated with 1 mM MeJA showed the highest L* value (Figure 2A). By day 14 of storage, L* values decreased due to the deterioration of cell membrane structure, which led to increased oxidation of pigments within the fruit. However, fruit treated with 1 mM MeJA still maintained the highest L* value. The reduction in L* was closely associated with the activity of polyphenol oxidase

(PPO), the key enzyme involved in enzymatic browning in fruit tissues. PPO catalyzes the oxidation of phenolic compounds into quinones, which subsequently polymerize to form brown pigments [18], resulting in a marked decrease in tissue lightness. This was consistent with the observed increase in PPO activity during storage. At the initial stage, no significant differences in PPO activity were found among treatments; however, after 7 and 14 days of storage, PPO activity increased markedly. The control fruit showed the highest PPO activity, followed by fruit treated with 0.1 mM MeJA, while fruit treated with 1 mM MeJA exhibited the lowest PPO activity (Figure 2B). These results indicate that MeJA application effectively suppressed the increase in browning-related enzyme activity during storage.

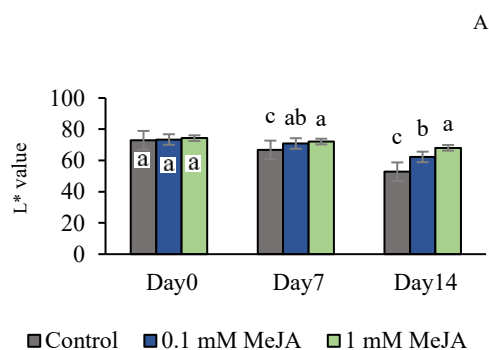
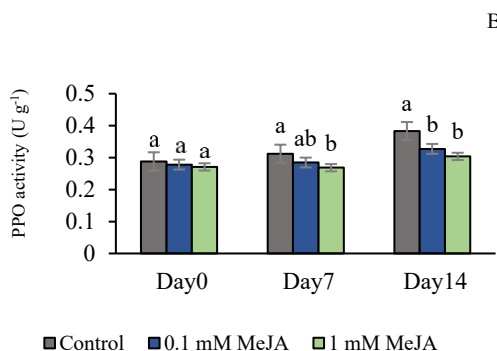


Figure 2. L* (lightness) values (A) and PPO activity (B) in pineapple treated with methyl jasmonate sprayed at various concentrations 14 days postharvest during storage at 13 °C for 0, 7, and 14 days.

Changes in the chemical quality of pineapple fruit sprayed with MeJA after harvest and stored at 13 °C were associated with storage duration and MeJA concentration. TSS showed no significant differences among treatments at the initial stage of storage, indicating that MeJA application did not immediately affect sugar levels. However, after 7 days of storage, TSS values tended to increase. Fruit treated with 1 mM MeJA exhibited higher TSS than those treated with 0.1 mM, while the control showed the lowest TSS (Figure 3A).



This increase may be attributed to changes in carbohydrate metabolism and shifts in the balance of major sugars, including sucrose, glucose, and fructose, as well as water loss

during storage [19], resulting in increased total soluble solids. After 14 days of storage, TSS values in all treatments tended to decrease, and no significant differences were observed among treatments. This decline may be due to the utilization of internal substrates for respiration and tissue senescence during prolonged storage. Meanwhile, TA tended to increase during the mid-storage period in all treatments, with the control exhibiting the highest TA, followed by fruit treated with 0.1 and 1 mM MeJA, respectively (Figure 3B), although no significant differences were detected at that stage. By day 14, TA values increased markedly, and the control fruit showed significantly higher TA than fruit treated with 1 mM MeJA (Figure 3B). The increase in TA during storage may be associated with membrane damage and leakage of organic acids from the vacuole under low-temperature conditions. In contrast, MeJA treatment helped maintain membrane integrity, reduce lipid peroxidation, and delay tissue senescence, thereby slowing changes in organic acid levels within the fruit [20].

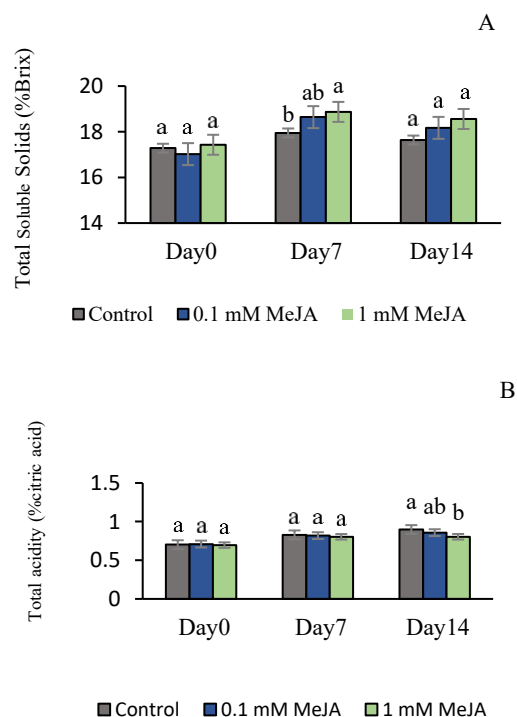


Figure 3. Total soluble solids (A) and Total acidity (B) of pineapple treated with methyl jasmonate sprayed at various concentrations 14 days postharvest during storage at 13 °C for 0, 7, and 14 days.

MDA content was closely associated with the severity of internal browning, reflecting the extent of membrane structural damage. MDA levels increased progressively with storage duration. At the initial stage of storage, no significant differences in MDA content were observed among treatments, indicating that lipid peroxidation had not yet occurred. After 7 days of storage, MDA content increased in all treatments, with the control fruit showing higher MDA

levels than fruit treated with 0.1 and 1 mM MeJA. By day 14, MDA accumulation became more pronounced, with the control exhibiting the highest MDA content, whereas fruit treated with 1 mM MeJA showed the lowest MDA levels (Figure 4A) ($P < 0.05$). These results indicate that lipid peroxidation in the cell membrane intensified as storage duration increased. This trend was consistent with changes in EL, which also increased over time. On day 7, EL values increased markedly, with the control showing the highest EL, while MeJA-treated fruit exhibited significantly lower EL values. After 14 days of storage, EL continued to rise, with the control fruit maintaining the highest EL and fruit treated with 1 mM MeJA showing distinctly lower EL (Figure 4B). These findings demonstrate the ability of MeJA to maintain membrane structural stability. When considering MDA content together with EL values, the increase in MDA reflects the level of lipid peroxidation, a key factor leading to membrane integrity loss and increased EL, ultimately resulting in greater internal browning severity [21]. In contrast, postharvest application of 1 mM MeJA effectively delayed MDA accumulation, reduced membrane deterioration, and alleviated the severity of internal browning during storage.

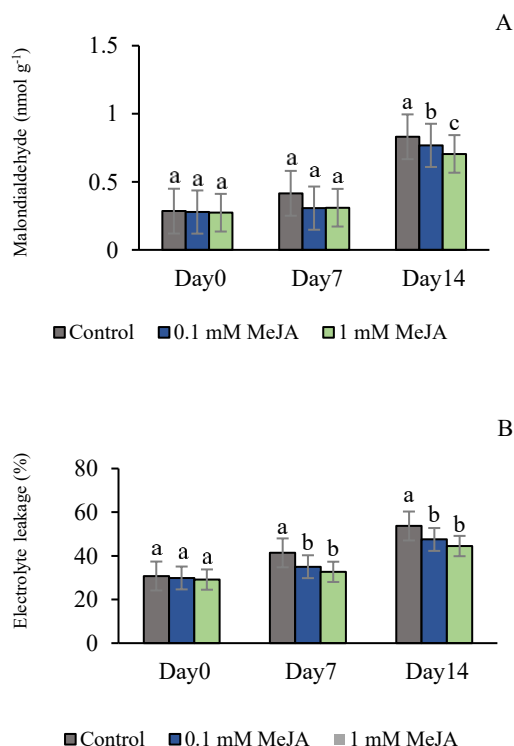


Figure 4. Malondialdehyde (A) and Electrolyte leakage (B) of pineapple treated with methyl jasmonate sprayed at various concentrations 14 days postharvest during storage at 13 °C for 0, 7, and 14 days.

Postharvest application of MeJA influenced the activity of enzymes associated with oxidative processes and internal browning in 'Queen' pineapple cv. Sawi, particularly LOX. Enzyme activity changed markedly with storage duration, reflecting tissue responses to postharvest stress. LOX activity, which is involved in the oxidation of unsaturated fatty acids and represents an initial step in lipid peroxidation, showed an increasing trend during storage. After 14 days of storage, the control fruit exhibited the highest LOX activity (Figure 5A), whereas treatment with 1 mM MeJA significantly suppressed the increase in LOX activity. When LOX activity was considered in relation to internal browning development, the increase in this parameter reflected elevated oxidative stress within the tissues [22], leading to membrane structural damage, tissue deterioration, and the development of internal browning in pineapple fruit. These results demonstrate that postharvest application of MeJA, particularly at 1 mM, effectively reduced oxidative stress, delayed tissue senescence, and alleviated the severity of internal browning during storage.

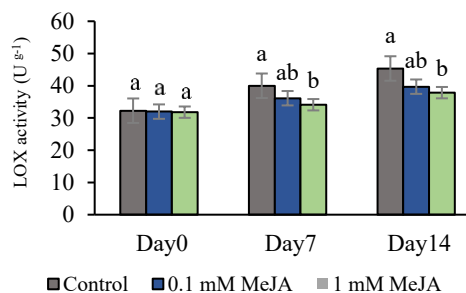


Figure 5. LOX activity of tissue adjacent to the core of 'Sawi' pineapple treated with methyl jasmonate sprayed at various concentrations 14 days postharvest during storage at 13 °C for 0, 7, and 14 days.

Changes in antioxidant compounds in 'Queen' pineapple cv. Sawi following postharvest application of methyl jasmonate (MeJA) were evaluated during storage. Antioxidant capacity varied with storage duration. At the initial stage of storage, no significant differences in DPPH radical scavenging activity were observed among treatments, indicating that antioxidant compounds in the tissues remained intact. However, after 7 days of storage, DPPH values decreased in all treatments. The control fruit exhibited the lowest DPPH activity, whereas MeJA-treated fruit showed a delayed decline, with 1 mM MeJA maintaining the highest DPPH value, followed by 0.1 mM. After 14 days of storage, DPPH activity decreased markedly; nevertheless, fruit treated with 1 mM MeJA retained significantly higher DPPH activity compared with the control (Figure 6A). The changes in DPPH were consistent with FRAP values, which also showed a decreasing trend with prolonged storage. After 7 days, FRAP values declined in all treatments; however, fruit treated with 0.1 and 1 mM MeJA exhibited higher FRAP values than the control (Figure 6B). By day 14, MeJA treatment effectively delayed the reduction in FRAP, with 1 mM MeJA maintaining the highest FRAP value, followed by

0.1 mM, while the control showed the lowest FRAP value, with significant differences among treatments. Prolonged storage resulted in membrane damage, leakage of phenolic compounds, and increased oxidative enzyme activity, leading to continuous oxidative reactions that consumed antioxidant compounds in the tissues, thereby reducing DPPH and FRAP values. Therefore, this study demonstrates that postharvest application of MeJA, particularly at 1 mM, plays an important role in maintaining antioxidant capacity in pineapple during storage, which is a key mechanism in reducing oxidative stress and delaying postharvest quality deterioration.

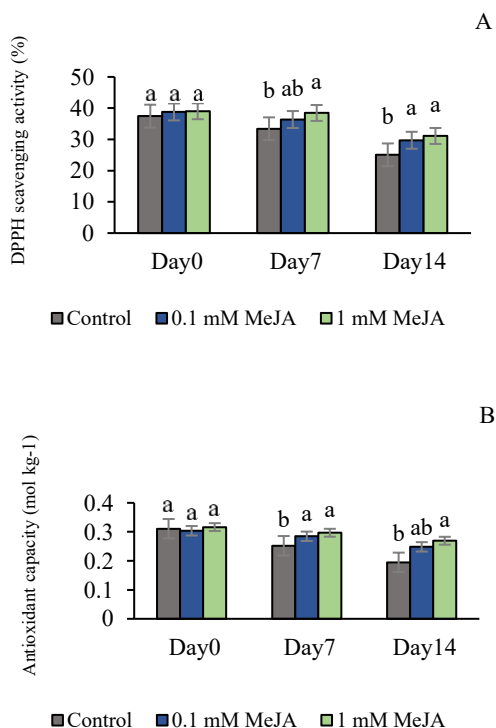


Figure 6. DPPH scavenging activity (A) and Antioxidant capacity value (B) of pineapple treated with methyl jasmonate sprayed at various concentrations 14 days postharvest during storage at 13 °C for 0, 7, and 14 days.

IV. CONCLUSION

Postharvest spraying with 1 mM MeJA effectively reduced the severity score of internal browning and the browning index, while maintaining higher lightness (L^*) values in the flesh adjacent to the core. In addition, MeJA inhibited the activity of PPO, a key enzyme involved in oxidative reactions leading to browning. MeJA treatment also helped preserve chemical quality by delaying changes in TSS and TA. Furthermore, it alleviated membrane damage by reducing MDA accumulation and EL, suppressed the activity of LOX, and better maintained

antioxidant capacity as determined by DPPH and FRAP assays compared with the control. These effects were observed during storage of pineapple fruit at 13 °C.

ACKNOWLEDGMENT

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Wavelength-Dependent Variability in Optical Density (OD) Measurements for Growth Assessment of Marine Microalgae

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Abstract. Optical density (OD) measurement is one of the most commonly used approaches for monitoring microalgal growth due to its simplicity and rapidity. However, the accuracy of OD-based growth assessment may vary depending on the wavelength used and the taxonomic characteristics of microalgae. This study investigated wavelength-dependent variability in growth estimation across six marine microalgal genera (*Chaetoceros*, *Thalassiosira*, *Isochrysis*, *Tetraselmis*, *Chlorella*, and *Nannochloropsis*) using OD measurements at multiple wavelengths (420, 550, 680, and 750 nm). Microalgae representing different taxonomic and physiological groups were cultivated under controlled laboratory conditions (25±1 °C, 28 psu, 12L:12D, 3,000 Lux light intensity, Conway medium). OD was measured throughout the exponential growth phase. Growth performance was evaluated using growth rate (μ), division rate (K), and doubling time (D) derived from OD data. The results revealed clear species-specific responses to measurement wavelength. Growth estimates for *Isochrysis*, *Tetraselmis*, and *Chlorella* differed significantly among wavelengths ($p<0.05$), indicating strong wavelength dependence of OD-derived parameters. In contrast, growth parameters for *Chaetoceros*, *Thalassiosira*, and *Nannochloropsis* showed no significant differences across wavelengths ($p>0.05$), suggesting wavelength-insensitive behavior. Among the evaluated parameters, D was generally less sensitive to wavelength variation, although significant differences ($p<0.05$) were observed in selected taxa. These findings demonstrate that OD does not universally represent microalgal growth across species and wavelengths. For practical application, OD measurement at longer wavelengths (e.g., 750 nm) is recommended for general biomass estimation due to reduced pigment interference, whereas species-specific or multi-wavelength approaches are required for accurate growth assessment in wavelength-sensitive taxa. The study highlights the necessity of species-aware and multi-wavelength approaches for accurate growth assessment and provides practical guidance for interpreting OD-based growth data in comparative microalgal studies and cultivation systems.

Index Terms—Growth kinetics, Marine microalgae culture, Optical properties, Species-specific response

I. INTRODUCTION

Microalgae are photosynthetic microorganisms with high taxonomic and physiological diversity and are widely utilized in aquaculture, biotechnology, and bioresource production. The success of microalgal production depends on various factors, primarily those influencing microalgal growth, such as temperature, salinity, light, nutrients, and pH [1,2]. Accurate assessment of microalgal growth was essential for both fundamental research and applied aquaculture systems [3,4]. Among the available techniques, spectrophotometric optical density (OD) measurement was widely used due to its rapid, non-destructive, and practical nature for routine monitoring [5-7]. In practice, OD measurements were typically conducted at a single wavelength, most commonly in the red region of the visible spectrum due to its association with chlorophyll absorption. However, microalgae exhibited substantial variation in

pigment composition, cell size, morphology, and internal structure, all of which influenced light absorption and scattering [8].

Microalgal growth performance was generally characterized using kinetic parameters, including specific growth rate, cell division rate, doubling time, etc. [1-3]. Systematic comparisons of growth kinetics across multiple wavelengths were feasible; however, despite the widespread use of OD measurements, a critical limitation remains: the lack of mechanistic understanding of how wavelength-dependent optical properties (absorption and scattering) influence growth estimation across different microalgal taxa. Previous studies have reported variability in OD–biomass relationships; however, systematic evaluation across multiple wavelengths and taxonomic groups remains limited. This gap restricts the development of standardized protocols for cross-species comparison and applied aquaculture

systems.

Accurate growth monitoring of microalgae is essential; therefore, this study aimed (1) to evaluate growth kinetic parameters of microalgae derived from OD measurements at different wavelengths, (2) to compare the consistency of growth estimates among microalgal genera, and (3) to identify groups of microalgal genera exhibiting wavelength-sensitive and wavelength-insensitive growth responses. The findings may contribute to the development of standardized protocols for OD-based growth measurement in microalgal research and applied in live feed cultivation.

II. METHODOLOGY

Experimental design and set-up

A multi-species experimental design was employed to evaluate wavelength-dependent optical density measurements in marine microalgae. The experiment was conducted using ten replicates ($n = 10$) in a completely randomized design (CRD).

The experiment was conducted at the Phytoplankton Laboratory of the Klongwan Fisheries Research Station (PL-KFRS), Prachuap Khiri Khan Province, Thailand. Original inoculum samples of six marine microalgal species—diatoms (*Chaetoceros calcitrans* and *Thalassiosira weissflogii*), green microalgae (*Chlorella* spp. and *Nannochloropsis oculata*), and flagellates (*Tetraselmis suecica* and *Isochrysis galbana*)—were obtained from the Prachuap Khiri Khan Coastal Fisheries Research and Development Center, Department of Fisheries, Thailand.

Microalgal cultures were raised in 250 mL Erlenmeyer flasks containing sterilized seawater adjusted to a salinity of 28 psu and enriched genus-specific growth media (Conway medium), and silicate was added only for the diatoms. The cultures were inoculated at 10% (v/v) and maintained in a temperature-controlled room at PL-KFRS at $25 \pm 1^\circ\text{C}$ under a 12-h light to 12-h darkness photoperiod (12L:12D) using cool white fluorescent lamps at a light intensity of approximately 3,000 Lux. The microalgae were cultured in the Erlenmeyer flasks until the cell density reached approximately 10^6 cells/mL. Each culture was then scaled up to 1 L glass bottles and used in the experiment (Fig. 1).

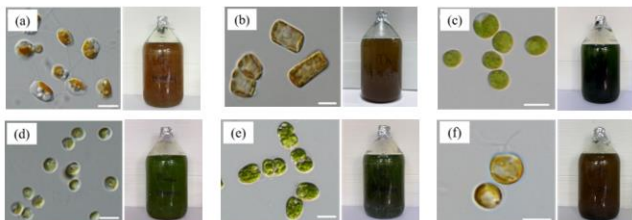


Fig. 1. The characteristics of microalgal cells examined in this study (modified from Ma and Hu, 2024; scale bars = 5 μm), with corresponding culture samples shown to illustrate coloration during the exponential growth phase. Diatoms: (a) *Chaetoceros calcitrans* and (b) *Thalassiosira weissflogii*; green microalgae: (c) *Chlorella* spp. and (d) *Nannochloropsis oculata*; and flagellates: (e) *Tetraselmis suecica* and (f) *Isochrysis galbana*.

Optical density measurements

Microalgae were cultivated in 1 L glass bottles in a temperature-controlled room ($25 \pm 1^\circ\text{C}$, 28 psu, 12L:12D, 3,000 Lux light intensity, Conway medium) for 15 days. All cultures were started with equal amounts of inoculum (approximately $1-2 \times 10^5$ cells/mL), and aeration was applied to prevent cell sedimentation and to ensure homogeneous culture conditions.

Growth was monitored by measuring optical density (OD) using a spectrophotometer (i5 UV-Vis Hanon spectrophotometer) at four wavelengths (420, 550, 680, and 750 nm). OD measurements were recorded daily during the exponential growth phase. Prior to measurement, cultures were gently mixed to ensure uniform cell distribution. Cell-free culture media were used as blanks for each wavelength.

Growth kinetics calculation

During the experiment, algal cell samples were collected daily to estimate cell density and maximum cell density, expressed as cells/mL. Cells were fixed with 5% formalin and counted using a hemocytometer under a compound microscope at $40\times$ magnification. To support the interpretation of OD as a proxy for microalgal growth, cell density (cells/mL) was independently measured using hemocytometer counts. Although direct calibration between OD and biomass was not the primary objective of this study, the use of cell density data provided an independent reference for evaluating growth trends across wavelengths. The exponential phase was identified as the period showing a linear increase in $\ln(\text{cell density})$ over time, and only data within this phase were used for growth rate calculation.

Growth parameters were calculated separately for each wavelength. The specific growth rate (μ), division rate (K), and doubling time (D) of each culture were calculated using Equations 1, 2, and 3, respectively [3]:

$$\mu = \ln N_2 - \ln N_1 / t_2 - t_1 \quad (1)$$

$$K = \mu / \ln(2) \quad (2)$$

$$D = 1 / K \quad (3)$$

where μ = the specific growth rate (day⁻¹), K = division rate (day), D = doubling time (day), N_1 = the cell count at time t_1 (cells/mL), N_2 = the cell count at time t_2 (cells/mL), t_1 = the first sampling time (day), t_2 = the second sampling time (day), and $\ln$ indicates the Napierian logarithm.

Statistical Analysis

One-way ANOVA was applied separately to each microalgal genus to evaluate the effect of measurement wavelength on growth parameters, allowing assessment of wavelength-dependent responses within each taxonomic group. Significant differences were further analyzed using post hoc multiple comparison tests ($p < 0.05$). All analyses

were performed using IBM SPSS Statistics (version 26.0; IBM Corp., Armonk, NY, USA).

Wavelength sensitivity was determined based on the consistency of growth parameters across wavelengths within each genus; genera showing significant variation were classified as wavelength-sensitive, whereas those with no significant differences were considered wavelength-insensitive. Data are presented as mean $\pm$ standard deviation.

III. RESULTS AND DISCUSSION

In this experiment, the growth dynamics of six marine microalgal species, expressed as cell density and maximum cell density ($\times 10^5$ cells/mL), were evaluated under controlled laboratory conditions. As illustrated in Fig. 2, all species exhibited distinct growth trajectories during the cultivation period, reflecting interspecific variation in growth capacity and biomass accumulation. The observed differences in growth patterns highlight species-specific growth responses under identical environmental conditions, providing a basis for comparing growth performance among the studied microalgae.

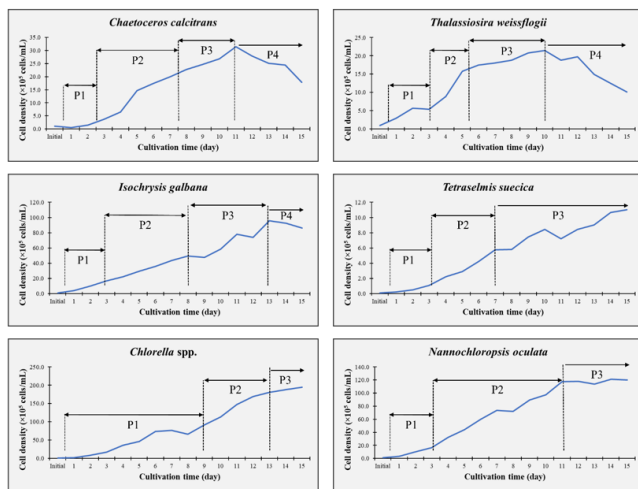


Fig. 2. Growth dynamics of six marine microalgal species cultured under controlled laboratory conditions (25 $\pm$ 1 $^{\circ}$ C, 28 psu, 12L:12D photoperiod, light intensity of 3,000 lux, Conway medium; n = 10). Growth phases were classified as P1 (lag), P2 (exponential), P3 (stationary), and P4 (death).

The growth kinetic parameters (μ , K, and D) of six microalgal genera derived from OD measurements at 420, 550, 680, and 750 nm are presented in Table 1. Statistical analyses indicated that the effect of measurement wavelength on growth estimates was strongly species-specific. Analysis of variance revealed a significant effect of wavelength ($p < 0.05$) on at least one growth parameter in genera *Isochrysis*, *Tetraselmis*, and *Chlorella*, whereas no significant differences ($p > 0.05$) among wavelengths were observed for genera *Chaetoceros*, *Thalassiosira*, and *Nannochloropsis*.

Genera exhibiting significant wavelength effects—*Isochrysis*, *Tetraselmis*, and *Chlorella*—showed wavelength-dependent differences in growth kinetics. In *Isochrysis*, both μ and K differed significantly among wavelengths ($p < 0.05$), whereas D did not ($p > 0.05$),

indicating that OD-based growth estimates were strongly influenced by wavelength and were not directly interchangeable. In *Tetraselmis*, μ and K did not vary significantly among wavelengths ($p > 0.05$); however, D differed significantly ($p < 0.05$), suggesting wavelength-dependent effects on D estimation. *Chlorella* exhibited moderate wavelength sensitivity, with significant differences in D ($p < 0.05$) but no significant variation in μ or K ($p > 0.05$), indicating that wavelength influenced the interpretation of cell proliferation dynamics more than overall growth rate.

In contrast, genera that did not exhibit significant wavelength effects—*Chaetoceros*, *Thalassiosira*, and *Nannochloropsis*—showed no statistically significant differences in growth kinetic parameters across wavelengths ($p > 0.05$). For these microalgae, μ , K, and D derived from OD measurements at 420, 550, 680, and 750 nm did not differ significantly, indicating low sensitivity to wavelength selection. Consequently, OD measurements at multiple wavelengths yielded consistent growth estimates, demonstrating that growth assessment for these species was stable and largely unbiased with respect to wavelength choice.

The results underscore the importance of evaluating wavelength-dependent differences in optical density (OD)–based microalgae growth measurements. Accordingly, selecting an appropriate, genus-specific wavelength is essential to improve the accuracy and efficiency of OD-based growth assessment.

TABLE 1
SPECIFIC GROWTH RATE (μ ; day⁻¹), DIVISION RATE (K; day), AND DOUBLING TIME (D; day) OF SIX MARINE MICROALGAE DETERMINED FROM OPTICAL DENSITY (OD) MEASUREMENTS AT FOUR WAVELENGTHS (n = 10)

Microalgal species	Item	OD measurements				p-value
		420 nm	550 nm	680 nm	750 nm	
<i>Chaetoceros</i>	μ	0.32 $\pm$ 0.06	0.35 $\pm$ 0.04	0.36 $\pm$ 0.04	0.35 $\pm$ 0.04	0.318
	K	0.46 $\pm$ 0.08	0.51 $\pm$ 0.06	0.53 $\pm$ 0.08	0.51 $\pm$ 0.07	0.321
	D	2.30 $\pm$ 0.48	2.00 $\pm$ 0.00	2.00 $\pm$ 0.00	2.10 $\pm$ 0.31	0.084
<i>Thalassiosira</i>	μ	0.20 $\pm$ 0.03	0.21 $\pm$ 0.03	0.22 $\pm$ 0.02	0.21 $\pm$ 0.02	0.555
	K	0.29 $\pm$ 0.04	0.31 $\pm$ 0.04	0.32 $\pm$ 0.03	0.31 $\pm$ 0.03	0.560
	D	3.60 $\pm$ 0.69	3.40 $\pm$ 0.69	3.00 $\pm$ 0.00	3.30 $\pm$ 0.48	0.122
<i>Isochrysis</i>	μ	0.17 $\pm$ 0.00 ^b	0.18 $\pm$ 0.01 ^a	0.18 $\pm$ 0.00 ^a	0.18 $\pm$ 0.00 ^a	0.000
	K	0.25 $\pm$ 0.00 ^a	0.27 $\pm$ 0.00 ^c	0.27 $\pm$ 0.00 ^c	0.26 $\pm$ 0.00 ^b	0.000
	D	4.00 $\pm$ 0.00	3.90 $\pm$ 0.31	4.00 $\pm$ 0.00	4.00 $\pm$ 0.00	0.404
<i>Tetraselmis</i>	μ	0.29 $\pm$ 0.05	0.30 $\pm$ 0.04	0.31 $\pm$ 0.03	0.31 $\pm$ 0.03	0.532
	K	0.42 $\pm$ 0.08	0.44 $\pm$ 0.06	0.46 $\pm$ 0.04	0.46 $\pm$ 0.04	0.529
	D	2.50 $\pm$ 0.52 ^c	2.40 $\pm$ 0.51 ^{bc}	2.00 $\pm$ 0.00 ^a	2.10 $\pm$ 0.31 ^{ab}	0.025
<i>Chlorella</i>	μ	0.21 $\pm$ 0.02	0.21 $\pm$ 0.01	0.22 $\pm$ 0.00	0.22 $\pm$ 0.01	0.076
	K	0.30 $\pm$ 0.03	0.31 $\pm$ 0.01	0.32 $\pm$ 0.01	0.33 $\pm$ 0.01	0.069
	D	3.30 $\pm$ 0.48 ^b	3.00 $\pm$ 0.00 ^a	3.00 $\pm$ 0.00 ^a	3.00 $\pm$ 0.00 ^a	0.017
<i>Nannochloropsis</i>	μ	0.24 $\pm$ 0.03	0.23 $\pm$ 0.02	0.24 $\pm$ 0.02	0.23 $\pm$ 0.01	0.944
	K	0.35 $\pm$ 0.05	0.34 $\pm$ 0.03	0.35 $\pm$ 0.03	0.34 $\pm$ 0.02	0.945
	D	2.90 $\pm$ 0.56	2.90 $\pm$ 0.31	2.90 $\pm$ 0.31	2.90 $\pm$ 0.31	1.000

Note: Different lowercase superscripts within the same row indicate significant differences ($p < 0.05$; gray bars).

These results demonstrate that microalgal growth estimates based on optical density (OD) measurements are strongly species-dependent and influenced by measurement wavelength. While some genera exhibited consistent OD-derived growth parameters across wavelengths, others showed significant variability or inconsistent trends, indicating that both optical properties and species-specific

biological characteristics play critical roles in determining the reliability of OD-based growth estimation.

Previous studies have similarly reported that OD is a practical and non-destructive proxy for microalgal biomass; however, its accuracy depends on wavelength selection and species-specific calibration. Nielsen and Hansen [9] showed that the OD–biomass relationship is strongly influenced by growth stage and physiological conditions, including variations in pigment composition and metabolic activity. This supports the present findings that OD measurements are not directly comparable across species or wavelengths, particularly when physiological characteristics differ among taxonomic groups.

The influence of wavelength selection on OD measurements was substantial. Optical analyses indicate that near-infrared wavelengths (e.g., ~750 nm) minimize pigment interference and more accurately reflect cell concentration, whereas visible wavelengths are more sensitive to chlorophyll and accessory pigments [10]. In the present study, *Chaetoceros*, *Thalassiosira*, and *Nannochloropsis* exhibited consistent OD responses across wavelengths, suggesting relatively stable optical properties associated with uniform pigment composition or cell morphology. In contrast, *Isochrysis*, *Tetraselmis*, and *Chlorella* showed significant variability, indicating wavelength-dependent optical responses likely driven by differences in pigment composition, cell size, cellular structure, and motility. Such variability has been reported to introduce substantial discrepancies in OD–biomass relationships, resulting in biomass estimation errors of up to 25–52% [10–12].

This wavelength-dependent variability can be mechanistically explained by differences in pigment composition and cellular structure among microalgal taxa. Chlorophyll-*a* strongly absorbs light in the red region (~680 nm), whereas accessory pigments absorb shorter wavelengths. Consequently, OD measurements at visible wavelengths reflect a combination of light absorption and scattering, while near-infrared wavelengths (e.g., 750 nm) primarily capture light scattering associated with biomass [13]. Furthermore, structural differences among taxa contribute to these patterns. Diatoms such as *Chaetoceros* and *Thalassiosira* possess silica frustules that enhance light scattering, leading to relatively wavelength-insensitive OD responses. In contrast, flagellated microalgae such as *Isochrysis* and *Tetraselmis* exhibit more variable pigment composition and cellular organization, resulting in greater wavelength sensitivity.

Santos-Ballardo et al. [14] reported that OD measurements at a single wavelength (e.g., 680 nm) were insufficient to distinguish biomass differences among *Chaetoceros gracilis*, *Isochrysis galbana*, *Nannochloropsis gaditana*, and *Phaeodactylum tricornutum* under identical culture conditions, particularly in the absence of species-specific calibration. This limitation was attributed to interspecific differences in pigment composition. Similarly, Chirivella-Martorell et al. [15] demonstrated that multi-wavelength OD approaches provide improved growth assessment across multiple genera, including *Chaetoceros*, *Isochrysis*, *Nannochloropsis*, *Dunaliella*, and *Tetraselmis*. These

findings highlight that variability in optical properties among taxa leads to distinct OD response patterns, reinforcing the importance of wavelength-dependent analyses in microalgal studies [16].

Overall, the findings of this study, together with previous research, indicate that OD measurements should be interpreted with caution when applied to microalgal growth assessment, particularly in comparative or multi-species systems. For practical applications, OD measurements at longer wavelengths (e.g., 750 nm) are recommended for general biomass estimation due to reduced pigment interference, whereas species-specific calibration or multi-wavelength approaches are necessary for wavelength-sensitive taxa [17]. The integration of OD measurements with complementary methods, such as direct cell enumeration or biomass quantification, is therefore essential to improve the accuracy and robustness of growth estimation, and future studies should further develop species-specific calibration models to strengthen OD-based approaches [18].

IV. CONCLUSION

This study demonstrates that microalgal growth estimates based on optical density (OD) measurements are strongly species-dependent and influenced by measurement wavelength. While some genera (*Chaetoceros*, *Thalassiosira*, and *Nannochloropsis*) produced consistent growth estimates across wavelengths, others (*Isochrysis*, *Tetraselmis*, and *Chlorella*) exhibited marked wavelength sensitivity, potentially leading to biased growth interpretation. These findings highlight the importance of selecting appropriate, genus-specific wavelengths when applying OD measurements for microalgal growth assessment. For practical applications, OD measurement at longer wavelengths (e.g., 750 nm) is recommended for general biomass estimation due to reduced pigment interference. However, for wavelength-sensitive taxa, species-specific calibration or multi-wavelength approaches are necessary to improve accuracy and comparability.

Overall, this study provides practical guidance for improving the reliability of OD-based growth monitoring and supports the development of more standardized protocols for microalgal cultivation and comparative studies. Future studies should incorporate direct biomass validation (e.g., dry weight or chlorophyll content) and develop species-specific calibration models to further enhance the robustness of OD-based growth estimation.

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Effects of Light Spectrum Ratios on the Yield and Bioactive Compound Contents of Lemon Basil Microgreens

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Abstract. Lemon basil (*Ocimum x africanum* Lour.) microgreens are recognized as nutrient-dense functional foods; however, the influence of light spectral composition on their growth and phytochemical quality under controlled environments remains insufficiently characterized. This study investigated the effects of varying red–blue ratios (1R:1B, 3R:7B, 7R:3B) with and without ultraviolet (UV) supplementation, alongside natural light as a control, on biomass accumulation, pigment synthesis, total sugar, phenolic compounds, flavonoids, and antioxidant activity in lemon basil microgreens cultivated in a plant factory with artificial lighting (PFAL) system. All LED treatments outperformed natural light across all parameters. The blue-enriched 3R:7B produced the highest biomass, while 1R:1B and 3R:7B+UV generated the highest yield efficiency (10.05% and 9.78%, respectively). The β -carotene (0.48 mg/100 g FW), and total sugar (10.59 mg glucose/g FW) were maximized under UV-supplemented non-balanced spectra, indicating UV effectively enhances carotenoid biosynthesis and carbon assimilation under specific spectral conditions. The balanced 1R:1B simultaneously maximized total phenolic content (48.00 mg GAE/g FW), total flavonoid content (0.66 mg QE/g FW), and antioxidant activity (40.00%), attributed to synergistic activation of phenylpropanoid metabolism. These findings recommend 1R:1B for phytochemical quality, blue-enriched spectra for biomass, and strategic UV supplementation for pigment and carbohydrate enhancement in PFAL-based lemon basil microgreen production.

Index Terms— lemon basil, microgreen, PFAL system, red-blue ratio, ultraviolet supplementation.

I. INTRODUCTION

Microgreens are young seedlings harvested within 7–14 days after germination that have gained increasing popularity in the food and nutrition industries due to their high nutritional value, diverse flavors, and suitability for controlled-environment agriculture addressing global food security and limited arable land [1-2]. They are rich sources of bioactive compounds, including ascorbic acid, carotenoids, phenolics, flavonoids, and glucosinolates, particularly in species belonging to the Brassicaceae and Lamiaceae families, exhibiting strong antioxidant activity and potential role in preventing chronic diseases. Pre- and postharvest factors, including light quality and intensity, nutrient management, temperature, and cultivation systems, significantly influence bioactive compound accumulation and overall microgreen quality [3].

Plant Factory with Artificial Lighting (PFAL) represents a promising indoor production system in which crops are cultivated on multilayer vertical shelves within a closed, thermally insulated facility under precisely controlled environmental conditions, including temperature, humidity, carbon dioxide concentration, and light [4]. This precise environmental control enables stable, high-yield production

independent of external climatic variability. Although operational costs remain relatively high, PFAL systems are increasingly recognized as an important agricultural innovation supporting sustainable food production, reduced dependence on natural resources, and contributions to environmental conservation and economic development [5-6]. Crops with short production cycles, such as microgreens, are therefore particularly well-suited for PFAL cultivation to maximize economic returns.

Among artificial lighting technologies, light-emitting diodes (LEDs) have become the preferred option in controlled-environment agriculture due to their high energy efficiency, low heat emission, spectral tunability, and long operational lifespan [7-8]. Light quality plays a critical role in regulating plant growth and metabolism. Red light is primarily associated with photosynthetic efficiency and biomass production, whereas blue light regulates stomatal function, chloroplast development, and secondary metabolism. Additionally, ultraviolet (UV) radiation acts as an abiotic elicitor, stimulating the accumulation of phenolic compounds and enhancing antioxidant capacity through activation of phenylalanine ammonia-lyase (PAL) and related pathways [9-10].

Species within the Lamiaceae family, including basil (*Ocimum* spp.), are well known for their high concentrations of bioactive compounds such as phenolics, flavonoids, and essential oils. Previous studies have demonstrated that light spectral composition significantly influences growth and phytochemical accumulation in basil. For instance, Chutimanukul et al. [11] reported that biomass production and physiological responses in holy basil varied markedly under different red–blue light ratios, highlighting the importance of spectral optimization in PFAL systems.

Lemon basil (*Ocimum x africanum* Lour.), a member of the Lamiaceae family, is widely recognized for its aromatic leaves and high nutritional and functional value, characterized by abundant phenolic and flavonoid compounds that contribute to strong antioxidant activity. Its rapid growth and adaptability make it a promising candidate for microgreen production under controlled environments. However, information regarding optimal artificial light spectrum conditions for simultaneously improving yield and phytochemical quality in lemon basil microgreens remains limited. Therefore, this study aimed to investigate the effects of red–blue light ratio and UV supplementation on yield, pigment biosynthesis, carbohydrate metabolism, and bioactive compound accumulation in lemon basil microgreens cultivated in a PFAL system.

II. METHODOLOGY

Microgreen cultivation

Lemon basil seeds (1 g per box; 7 × 11.5 × 5 cm) were sown in plastic boxes pre-filled with peat moss to one-third of their volume. The seeds were evenly distributed over the substrate surface, thoroughly watered, and kept in darkness for 5 days to allow germination. Thereafter, the germinated seedlings were transferred to growth conditions under artificial lighting. The experiment was arranged in a Completely Randomized Design (CRD) with seven treatments and five replicates: natural light; red and blue light (R:B) ratios of 1R:1B, 3R:7B, and 7R:3B; and the same R:B ratios supplemented with UV-A radiation (10 μmol m⁻² s⁻¹). All treatments were exposed to a light intensity of 200 μmol m⁻² s⁻¹ PPFD with a 12-h photoperiod for 13 days, while the temperature was maintained at 22°C. Measurements were taken at the end of the experimental period.

Biomass and yield percentage of lemon basil microgreens

Microgreen samples were weighed in grams to determine fresh biomass yield per box. The samples were then dried in a hot-air oven at a constant temperature of 45°C until a constant dry weight (DW) was obtained. The resulting data were used to calculate yield percentage using the following equation:

$$\text{Yield (\%)} = (\text{DW}/\text{FW}) \times 100$$

Phytochemical analysis of lemon basil microgreens

Chlorophyll Content Analysis: Lemon basil microgreen (0.5 g) was finely chopped and extracted with acetone: hexane (4:6, v/v) at a volume of 10 mL, then left to stand overnight. The extract was analyzed using a UV–VIS spectrophotometer at wavelengths of 453, 505, 645 and 663 nm [12]. Chlorophyll

a and chlorophyll b contents were calculated using the following equations:

$$\text{Chlorophyll a (mg/100 g FW)} = 0.999A_{663} - 0.0989A_{645}$$

$$\text{Chlorophyll b (mg/100 g FW)} = -0.328A_{663} + 1.77A_{645}$$

$$\beta\text{-carotene (mg/100 g FW)} = 0.216A_{663} - 1.22A_{645} - 0.304A_{505} + 0.452A_{453}$$

Microgreen extraction: Sample extraction was conducted by homogenizing 1 g of fresh lemon basil microgreens with 15 mL of 60% ethanol, followed by continuous agitation on a mechanical shaker overnight to ensure thorough extraction of soluble compounds. The resulting mixture was subsequently filtered through filter paper to remove plant debris and clarify the extract. The obtained filtrate was then transferred into amber-colored vials and stored at –20 °C until further analysis to minimize degradation of bioactive compounds.

Total Sugar Content Analysis: Total soluble sugar content was determined following the phenol-sulfuric acid colorimetric method described by Dubois et al. [13]. An aliquot of 1 mL of lemon basil microgreen extract was transferred into a test tube, followed by the addition of 1 mL of 5% phenol solution and 5 mL of concentrated sulfuric acid. The mixture was left to stand for 10 minutes, vortexed for 30 seconds, and incubated in a temperature-controlled water bath at 30 °C for 20 minutes. The absorbance was measured using a spectrophotometer at 490 nm.

A standard calibration curve was prepared using glucose solutions. A stock solution was prepared by dissolving 1 mg of glucose in 10 mL of distilled water, then diluted to obtain standard concentrations of 0, 20, 40, 60, 80, and 100 mg/L. Total sugar content was calculated from the standard curve using the linear regression equation ($y = 0.0217x + 0.0334$; $R^2 = 0.9906$) and expressed as milligrams of glucose equivalent per gram of fresh weight (mg glucose/g FW).

Total Phenolic Content (TPC) Analysis: Total phenolic content was determined using the Folin–Ciocalteu colorimetric method as described by Slinkard and Singleton [14]. An aliquot of 0.3 mL of the filtrate was transferred into a test tube, followed by the addition of 1.5 mL of 10% Folin–Ciocalteu reagent and 1.2 mL of 7.5% sodium carbonate solution. The mixture was incubated at room temperature for 30 minutes for color development, after which the absorbance was measured using a spectrophotometer at 765 nm.

A standard calibration curve was prepared using gallic acid solutions. A stock solution was prepared by dissolving 1 mg of gallic acid in 10 mL of distilled water, then diluted to obtain standard concentrations of 0, 20, 40, 60, and 80 mg/L. Total phenolic content was calculated from the gallic acid standard curve ($y = 0.0013x - 0.0124$; $R^2 = 0.9973$) and expressed as milligrams of gallic acid equivalent per gram of fresh weight (mg GAE/g FW).

Total Flavonoid Content (TFC) Analysis: Total flavonoid content was determined using the aluminum chloride colorimetric method as described by Zhishen et al. [15]. An aliquot of 0.25 mL of lemon basil microgreen extract was transferred into a test tube, followed by the addition of 1.25 mL

of distilled water and 75 μ L of 0.5% sodium nitrite solution. The mixture was left at room temperature for 6 minutes, after which 150 μ L of 10% aluminum chloride solution was added and allowed to stand for 5 minutes. Then, 0.5 mL of 1 M sodium hydroxide was added. The absorbance was measured using a spectrophotometer at 517 nm.

A standard calibration curve was prepared using quercetin solutions. A stock solution was prepared by dissolving 1 mg of quercetin in 10 mL of distilled water, then diluted to obtain standard concentrations of 0, 20, 40, 60, 80, and 100 mg/L. Total flavonoid content was calculated from the linear regression equation ($y = 0.0185x - 0.0035$; $R^2 = 0.9980$) and expressed as milligrams of quercetin equivalent per gram of fresh weight (mg QE/g FW).

Antioxidant Activity Analysis: Antioxidant activity was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay following the method of Blois [16]. An aliquot of 1 mL of lemon basil microgreen extract was transferred into a test tube, followed by the addition of 3 mL of 60 μ M DPPH solution per tube. The mixture was kept in the dark for 30 minutes, after which the absorbance was measured using a spectrophotometer at 517 nm. The percentage of DPPH radical inhibition was calculated using the following equation.

$$\text{Inhibition (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

where A_{control} represents the absorbance of the DPPH solution without sample extract, and A_{sample} represents the absorbance of the reaction mixture containing the extract.

Statistical Data Analysis

All data were statistically analyzed using Statistix software version 8.0. Mean comparisons were performed using the Least Significant Difference (LSD) test at a significant level of 0.05.

III. RESULTS AND DISCUSSION

Biomass accumulation and Yield

The results showed that light spectral composition significantly affected biomass accumulation and yield of lemon basil microgreens (Fig. 1 and Table I). Plants grown under natural light exhibited the lowest fresh and dry weights, likely due to fluctuating irradiance conditions that limit photosynthetic efficiency [4, 17]. All LED treatments produced greater biomass than natural light, confirming the advantage of controlled spectral environments in PFAL systems [18], although this did not consistently translate into improved yield efficiency.

Among LED treatments, the highest fresh and dry weights were observed under 3R:7B, indicating that a higher proportion of blue light contributed to enhanced biomass accumulation. This response is likely associated with improved stomatal regulation and photosynthetic performance [19-20]. However, biomass responses were not strictly proportional to blue light content, as 1R:1B produced lower fresh weight than 3R:7B, and 7R:3B+UV exceeded 1R:1B, suggesting that biomass accumulation is regulated by

interactions among spectral components rather than individual wavelengths. Increasing red light proportion (7R:3B) resulted in intermediate fresh weight but relatively low dry weight, indicating that red-dominant spectra may promote elongation growth rather than biomass accumulation [21].

Regarding yield efficiency, 1R:1B and 3R:7B+UV produced the highest values and were significantly greater than natural light, whereas red-dominant treatments resulted in lower yield efficiency. This supports the importance of balanced red-blue spectra for optimizing biomass partitioning [11]. UV supplementation exerted a spectrum-dependent effect on yield. For example, 3R:7B+UV showed reduced fresh weight but increased yield compared to 3R:7B, suggesting a possible shift in dry matter allocation or biomass composition. Such responses are consistent with reports that UV radiation can alter growth patterns through stress-mediated physiological adjustments [22].

Accordingly, blue-enriched spectra favor biomass accumulation, balanced red-blue ratios optimize yield efficiency, and UV supplementation modulates biomass allocation in a spectrum-dependent manner in lemon basil microgreens under PFAL conditions.

Pigment accumulation and total sugar

Light spectral composition significantly influenced pigment accumulation and carbohydrate metabolism in lemon basil microgreens (Table II). Not surprisingly, microgreens grown under natural light still exhibited the lowest chlorophyll, β -carotene, and total sugar contents.

Chlorophyll accumulation was enhanced under both balanced and UV-supplemented spectra. Chlorophyll a was highest under 7R:3B+UV, 1R:1B+UV, and 1R:1B, with comparable value under 3R:7B+UV. A similar trend was observed for chlorophyll b, which was maximized under 7R:3B+UV and remained relatively high under balanced spectra. These results indicate that both red-blue interactions and UV supplementation contribute to chlorophyll biosynthesis, likely through coordinated regulation of photochemical activity and chloroplast development [20, 23].



Fig. 1. Comparison of lemon basil microgreens grown under different light treatments.

β -carotene was significantly highest under 7R:3B+UV and 3R:7B+UV (0.48 and 0.47 mg/100g FW), while 1R:1B+UV (0.43 mg/100g FW) was significantly lower despite UV supplementation, indicating that UV most effectively induces carotenoid biosynthesis when combined with non-balanced spectral ratios [24-26].

TABLE I
BIOMASS ACCUMULATION AND YIELD OF LEMON
BASIL MICROGREENS UNDER DIFFERENT LIGHT
SPECTRAL RATIOS

Treatment	Fresh Weight (g/box)	Dry Weight (g/box)	Yield (%)
Natural light	6.26±0.83e	0.52±0.11d	8.23±0.85b
1R:1B	9.39±0.60cd	0.95±0.13ab	10.05±0.78a
3R:7B	13.97±0.64a	1.05±0.10a	7.55±0.53b
7R:3B	11.65±1.15b	0.71±0.13c	6.07±0.58c
1R:1B+UV	11.18±1.40b	0.83±0.13bc	7.45±0.58b
3R:7B+UV	8.06±1.60d	0.78±0.14bc	9.78±0.97a
7R:3B+UV	10.31±1.40bc	0.84±0.20bc	8.10±1.15b
Mean	10.20	0.81	8.07
CV (%)	11.12	16.05	9.91
P-value	< 0.01	< 0.01	< 0.01

*Different letters in column within each variable indicate differences among light intensity treatments according to LSD test (n=5).

β -carotene was significantly highest under 7R:3B+UV and 3R:7B+UV (0.48 and 0.47 mg/100g FW), while 1R:1B+UV (0.43 mg/100g FW) was significantly lower despite UV supplementation, indicating that UV most effectively induces carotenoid biosynthesis when combined with non-balanced spectral ratios [24-26].

Total sugar was highest under 3R:7B+UV (10.59 mg glucose/g FW), followed by 1R:1B (9.09, labeled b), an intermediate group of 7R:3B+UV and 1R:1B+UV (8.63 and 8.55, both labeled c), and the lowest values under 7R:3B and 3R:7B (6.57 and 6.39, both labeled d). The highest sugar content under 3R:7B+UV suggests that blue-enriched spectra combined with UV supplementation most effectively enhance carbon assimilation through improved photosynthetic efficiency and UV-A induced activation of carbohydrate-metabolizing enzymes, while the second highest value under 1R:1B without UV confirms that balanced red-blue light alone also effectively supports carbohydrate metabolism [27-28]. Overall, tailored integration of red, blue, and UV light spectra offers an effective strategy for simultaneously enhancing pigment biosynthesis and carbohydrate accumulation in lemon basil microgreens under controlled environments.

Phytochemical contents

Light spectral composition significantly influenced TPC, TFC, and antioxidant activity (DPPH inhibition) in lemon basil microgreens (Table III), with TPC ranging from 22.18 to 48.00 mg GAE g⁻¹ FW, TFC from 0.13 to 0.66 mg QE g⁻¹ FW, and DPPH inhibition from 26.69% to 40.00%. In contrast to pigment accumulation patterns, the highest TPC, TFC, and antioxidant activity were observed under the balanced 1R:1B treatment, suggesting that phytochemical accumulation does not necessarily follow the same response pattern as pigment biosynthesis. This superior performance highlights the synergistic role of red and blue wavelengths in enhancing carbon assimilation and activating phenylalanine

ammonia-lyase (PAL), a key enzyme in the phenylpropanoid pathway [20, 23, 29].

Plants grown under natural light exhibited the lowest TPC and TFC, indicating that uncontrolled spectral conditions are less effective in sustaining secondary metabolism [4, 17]. UV supplementation exerted a non-uniform, spectrum-dependent effect on phytochemical accumulation. TPC remained relatively high under 1R:1B+UV and 3R:7B+UV but was reduced under 7R:3B+UV, indicating that UV responses depend on interactions with the underlying red-blue spectral ratio rather than acting as a uniform elicitor. Among UV-supplemented treatments for TFC, only 7R:3B+UV was statistically comparable to 1R:1B, while 1R:1B+UV and 3R:7B+UV were significantly lower, confirming that UV supplementation did not consistently enhance flavonoid accumulation beyond that achieved by the balanced red-blue spectrum alone. These responses are consistent with the role of UV as an abiotic signal activating UVR8-mediated pathway and phenylpropanoid [30-32].

TABLE II
PIGMENT ACCUMULATION AND TOTAL SUGAR
CONTENTS OF LEMON BASIL MICROGREENS

Treatment	chlorophyll a (mg/100g FW)	chlorophyll b (mg/100g FW)	β -carotene (mg/100 g FW)	Total Sugar (mg Glucose/ g FW)
Natural light	0.92±0.08d	0.34±0.03e	0.23±0.01d	3.15±0.07e
1R:1B	1.91±0.02a	1.01±0.04ab	0.40±0.01bc	9.09±0.15b
3R:7B	1.74±0.08b	0.75±0.08c	0.39±0.02bc	6.39±0.29d
7R:3B	1.48±0.10c	0.53±0.06d	0.38±0.03c	6.57±0.19d
1R:1B+UV	1.91±0.10a	0.97±0.15ab	0.43±0.03b	8.55±0.25c
3R:7B+UV	1.84±0.07ab	0.89±0.12bc	0.47±0.03a	10.59±0.54a
7R:3B+UV	1.92±0.11a	1.10±0.27a	0.48±0.07a	8.63±0.20c
Mean	1.67	0.80	0.40	7.57
CV (%)	5.08	16.29	8.57	3.69
P-value	< 0.01	< 0.01	< 0.01	< 0.01

*Different letters in column within each variable indicate differences among light intensity treatments according to LSD test (n=5).

TABLE III
ANTIOXIDANT ACTIVITY, TOTAL PHENOLIC AND
FLAVONOID CONTENTS OF LEMON BASIL
MICROGREENS

Treatment	TPC (mg GAE/g FW)	TFC (mg QE/g FW)	Inhibition (%)
Natural light	22.18±2.86f	0.13±0.06e	28.42d
1R:1B	48.00±2.68a	0.66±0.10a	40.00a
3R:7B	33.21±0.56d	0.49±0.08c	32.97c
7R:3B	25.75±0.59e	0.23±0.08d	38.59a
1R:1B+UV	41.61±3.16b	0.51±0.06c	35.90b
3R:7B+UV	42.90±1.10b	0.54±0.05bc	34.00bc
7R:3B+UV	37.87±2.07c	0.61±0.07ab	26.69d
Mean	35.93	0.45	33.99
CV (%)	5.91	16.36	4.71
P-value	< 0.01	< 0.01	< 0.01

*Different letters in column within each variable indicate differences among light intensity treatments according to LSD test (n=5).

Antioxidant activity did not strictly correlate with TPC and TFC. The 7R:3B treatment exhibited high DPPH

inhibition despite relatively low phenolic and flavonoid contents, indicating a clear dissociation between metabolite concentration and antioxidant capacity. Conversely, 7R:3B+UV showed reduced antioxidant activity despite relatively high flavonoid content. These patterns suggest that antioxidant capacity is governed not only by total metabolite levels but also by the composition and structural properties of individual compounds [33-35]. Therefore, a balanced red–blue spectrum (1R:1B) is the most effective for maximizing phytochemical accumulation and antioxidant capacity, while UV supplementation acts as a secondary, spectrum-dependent modulator in PFAL systems.

IV. CONCLUSION

This study demonstrates that light spectral composition is a critical determinant of growth performance, pigment biosynthesis, and phytochemical quality in lemon basil microgreens cultivated under PFAL conditions. Natural light consistently produced the lowest values across all measured parameters, confirming that spectral inconsistency and irradiance fluctuation are limiting factors for both primary and secondary metabolite accumulation. Among all LED treatments, distinct spectral responses were observed depending on the red–blue ratio and UV supplementation. The balanced red–blue spectrum (1R:1B) proved most effective for simultaneously maximizing total phenolic content, total flavonoid content, and antioxidant activity. The 3R:7B treatment produced the greatest fresh weight and the highest dry weight. Meanwhile, 3R:7B+UV promoted the highest total sugar content among all treatments, significantly exceeding natural light. Chlorophyll synthesis was most effectively enhanced under UV-supplemented treatments combined with balanced or red-dominant spectra, particularly 7R:3B+UV and 1R:1B+UV, as well as under the balanced 1R:1B treatment without UV, indicating that both spectral balance and UV supplementation independently contribute to chlorophyll biosynthesis. Carotenoid accumulation was most pronounced under blue-enriched and red-dominant UV-supplemented treatments, particularly 3R:7B+UV and 7R:3B+UV, consistent with UV radiation activating photoprotective carotenogenic pathways. Overall, UV supplementation functioned primarily as a complementary modulator of pigment biosynthesis and secondary metabolite accumulation rather than a direct promoter of vegetative growth. These findings collectively highlight that the red–blue light ratio is the dominant regulatory factor governing both growth and phytochemical quality, with UV radiation exerting a targeted modulatory influence on specific metabolic pathways. Consequently, tailored optimization of light spectra in PFAL systems represents a scientifically grounded and practically viable strategy for simultaneously enhancing yield and nutritional quality of lemon basil microgreens. Future research should investigate the underlying molecular and enzymatic mechanisms governing light-mediated metabolic responses, assess the economic feasibility of implementing customized LED light regimes at a commercial scale, and explore the

potential of dynamic light scheduling to further maximize both productivity and functional quality in controlled-environment microgreen production.

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Effects of Coconut Cream Residue and *Houttuynia cordata* Extract on the Growth and Survival of Giant Freshwater Prawn

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Abstract This study evaluated the effects of coconut cream residue enriched with *Houttuynia cordata* extract (CRP) on the growth performance and survival of giant freshwater prawns (*Macrobrachium rosenbergii*). A 16-week feeding trial utilized four diets: T1 (0% CRP - Control), T2 (5% CRP), T3 (10% CRP), and T4 (15% CRP). Chemical analysis confirmed that CRP contained a total phenolic content of 20.64 ± 0.38 mg GAE/g with distinct antioxidant activity. The most significant finding of this study is that the 10% CRP inclusion level (T3) represents the physiological optimum for *M. rosenbergii*. At this level, prawns achieved the highest final weight (2.070 ± 0.037 g), the maximum specific growth rate (SGR), and the highest survival rate (92.33%) ($P < 0.05$). Furthermore, T3 exhibited the most efficient feed conversion ratio (FCR) among all treatments. Notably, although the 15% CRP group (T4) provided the highest crude protein (41.07%), it did not surpass the growth performance of the 10% group, confirming that 10% CRP is the definitive threshold for maximizing nutrient utilization. In conclusion, incorporating 10% CRP is the most effective strategy for enhancing production metrics and survival in sustainable prawn aquaculture.

Index Terms— Coconut Cream Residue, Growth performance, *Houttuynia cordata*, *Macrobrachium rosenbergii*

I. INTRODUCTION

The global aquaculture industry is a vital economic pillar, with the Giant Freshwater Prawn (*Macrobrachium rosenbergii*) serving as a high-value commodity in international markets. However, the transition toward intensive farming systems has led to a rise in environmental stressors and a higher susceptibility to pathogenic infections, particularly from *Vibrio* species. These bacterial outbreaks often result in mass mortality and significant financial instability for farmers [1]. While antibiotics were historically used to mitigate these risks, their over-application has triggered global concerns regarding antibiotic-resistant bacteria and chemical residues in food products, prompting a strategic shift toward sustainable, plant-based functional feed additives [2]. Phytogetic feed additives (PFAs) have gained prominence for their ability to enhance growth and immunity without ecological harm. Among these, *Houttuynia cordata* (Plu-kaow) stands out due to its diverse profile of bioactive compounds, including polyphenols, flavonoids (such as quercetin and rutin), and essential oils. These metabolites possess potent antimicrobial, anti-inflammatory, and antioxidant properties that can bolster the non-specific immune system of crustaceans [3,4]. Nevertheless, the efficacy of *H. cordata* is often

compromised by traditional extraction methods. The use of synthetic organic solvents not only poses environmental hazards but can also lead to the thermal degradation of

sensitive bioactives, thereby reducing their functional potency [5]. To overcome these limitations, this study explores a green extraction paradigm using coconut milk as a natural, biodegradable bio-solvent. Coconut milk is a unique oil-in-water emulsion rich in medium-chain triglycerides (MCTs), which provide a superior hydrophobic environment for the partitioning and stabilization of lipophilic bioactives from *H. cordata* [6,7]. The primary novelty of this research lies in its alignment with the Bio-Circular-Green (BCG) Economy framework through the upcycling of the extraction byproduct. Instead of discarding the solid waste, this study utilizes cold-fractionation to develop a Bioactive-Rich Matrix (Coconut Cream Residue with *Houttuynia cordata* extract; CRP). This CRP matrix acts as a functional "phyto-lipid scaffold," where key metabolites are adsorbed onto the protein-lipid structure of the residue. This integration is hypothesized to protect antioxidants from premature oxidation and enhance their bioavailability within the prawn's digestive tract by mimicking natural lipid absorption pathways [10]. Despite

the recognized potential of *H. cordata*, the application of such a solid, integrated phyto-lipid matrix in aquafeed—especially one derived from zero-waste processing—remains largely unexplored. Therefore, this study aims to evaluate the efficacy of CRP in optimizing the growth performance and survival of *M. rosenbergii*, seeking to establish the optimal inclusion level for commercial application. This research provides a scalable, sustainable strategy for "Blue Transformation," ensuring both environmental responsibility and economic viability for the aquaculture sector [11].

II. METHODOLOGY

2.1 Materials

Mature coconuts (*Cocos nucifera* L., 10–12 months old) were obtained from local markets in Thungsong District, Nakhon Si Thammarat, Thailand. Fresh *Houttuynia cordata* Thunb. (Plu-kaow) was sourced from Kawtong Garden, Chiang Rai, Thailand. The CRP was prepared using a green cold-fractionation process, wherein the *H. cordata* extract was integrated and partitioned into the coconut milk emulsion. The resulting solid residue was collected, dried in a hot-air oven at (60°C) until a constant weight was achieved, and pulverized into a fine powder. The CRP powder was subsequently stored in airtight containers at 4°C for further dietary formulation.

2.2 Antioxidant Activity

The antioxidant capacity of the CRP was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, adapted from the method described by Brand-Williams et al. (1995) [12] with minor modifications to suit the solid matrix. Briefly, 1.0 g of the dried and pulverized CRP was extracted with 10 mL of ethyl acetate and filtered to obtain a clear extract. Subsequently, 1.0 mL of the filtrate was transferred into a 10 mL volumetric flask containing a freshly prepared 10⁻⁴ M DPPH radical solution in ethyl acetate. The reaction mixture was thoroughly agitated using a vortex mixer for 10 s and then incubated in the dark at room temperature for 30 min to allow for complete reaction. The absorbance was measured at 517 nm using a spectrophotometer (Biochrom S22) with a 1 cm quartz cell against an ethyl acetate blank. To ensure precision and reproducibility, all measurements were performed in triplicate. The radical scavenging activity, expressed as the percentage of inhibition, was calculated according to the following equation (1) [13]:

$$\% \text{ Inhibition} = \frac{(A_{\text{control}} - A_{\text{sample}}) \times 100}{A_{\text{control}}} \quad (1)$$

Where A_{control} is the absorbance of the DPPH solution without the sample, and A_{sample} is the absorbance of the reaction mixture containing the coconut residue extract.

2.3 Determination of Total Phenolic Content (TPC)

The total phenolic content (TPC) of the raw coconut cream residue and CRP was determined using the Folin-Ciocalteu colorimetric method, adapted from Singleton et al. (1999) [14]. Briefly, 1.0 g of each sample was extracted with 10 mL of an 80:20 (v/v) methanol-water mixture and filtered to

obtain a clear supernatant. Subsequently, 1.0 mL of the extract was mixed with 1.0 mL of Folin-Ciocalteu reagent (diluted 1:10 v/v with distilled water). After a 5-min initial reaction, 8.0 mL of a 7.5% (w/v) sodium carbonate solution was added to neutralize the mixture. The reaction mixture was then incubated in the dark at room temperature for 30 min to ensure complete chromophore development. The absorbance was measured at 725 nm using a spectrophotometer (Biochrom S22). Gallic acid was used as the standard, with a calibration curve constructed over a concentration range of 0–500 µg/mL. All measurements were performed in triplicate, and results were expressed as milligrams of gallic acid equivalents per gram of dry sample (mg GAE/g).

2.4 Preparation of Coconut Cream Residue (CCR) and CRP

2.4.1 Preparation of Coconut Cream Residue (CCR)

CCR, serving as the control matrix, was prepared using a cold-extraction method adapted from Raghavendra and Raghavarao (2010) [15]. Briefly, 5 kg of mature coconut endosperm was homogenized with distilled water at a 1:1 (w/v) ratio. The resulting emulsion was chilled at 4–5 °C for 12 hours to induce gravitational creaming and fat solidification. Subsequently, the mixture was centrifuged at 3,500 ×g for 7 min to facilitate phase separation into an aqueous phase, coconut oil, and the CCR. The collected CCR was dehydrated in a hot-air oven at 60 °C for 24 hours until a constant weight was achieved. The dried matrix was pulverized using a laboratory grinder, passed through a 60-mesh sieve to ensure uniform particle size, and stored in hermetically sealed plastic bags at 4 °C [6].

2.4.2 Preparation of Coconut Residue with *H. cordata* Extract (CRP)

The CRP was prepared by integrating the bioactive constituents of *H. cordata* into the coconut milk matrix. Fresh *H. cordata* leaves (1 kg) were cleaned, comminuted, and homogenized with deionized water (1:1 w/v). The homogenate was filtered through a double-layered cheesecloth to obtain the crude aqueous extract. This extract was then incorporated into freshly prepared coconut milk (derived from 5 kg of endosperm and 5 L of water). To facilitate the partitioning and adsorption of *H. cordata* metabolites into the lipid-protein scaffold, the mixture was chilled at 4–5 °C for 12 hours. The stabilized emulsion was then centrifuged at 3,500 ×g for 7 min to isolate the CRP fraction. The resulting CRP was dried in a hot-air oven at 60 °C for 24 hours, pulverized, sieved (60-mesh), and stored in light-protected containers at 4 °C [10, 15].

2.5 Experimental Diet Preparation and Nutritional Analysis

Four experimental diets were formulated by incorporating the pulverized and sieved CRP powder into a high-quality commercial basal diet at levels of 0% (T1/Control), 5% (T2), 10% (T3), and 15% (w/w). To ensure maximum homogeneity, the dried CRP was first finely ground and passed through a 60-mesh sieve to achieve a uniform particle size before being thoroughly blended with the basal diet. The mixtures were then processed through a laboratory-scale

pellet mill to form the experimental feed. During this process, distilled water was added to the blend as a binder to reach the appropriate consistency for pelleting. The resulting pellets were dried in a hot-air oven at a low temperature (< 60°C) for 12 hours to preserve the heat-sensitive bioactive compounds of *H. cordata*. Once a constant weight was achieved, the diets were stored in airtight containers at 4°C until use. The proximate composition of the diets was determined following AOAC (2019) protocols [16]. As shown in Table II, the inclusion of CRP resulted in a progressive, non-isonitrogenous, and non-isocaloric series, with crude protein ranging from 36.84% to 41.07% and gross energy from 342.04 to 372.37 Kcal/100 g. These variations reflect the intrinsic nutritional contribution of the CRP matrix under a practical supplementation model [1, 8].

2.6 Experimental Design, Animal Acquisition, and Rearing Management

A total of 1,200 prawns were sourced from a reputable private hatchery in Thung Song District, Nakhon Si Thammarat, Thailand. Before the trial, the prawns were acclimated for 30 days in concrete tanks and fed a standard commercial diet to ensure health uniformity and physiological stability. The feeding trial was conducted over a 16-week period using a Completely Randomized Design (CRD). Prawns with an initial mean weight of 0.135 ± 0.007 g were randomly distributed into 12 circular indoor concrete ponds (diameter: 1.5 m; effective water volume: 883.13 L). A stocking density of 100 individuals per pond was maintained. The four experimental treatments, each performed in triplicate, were categorized based on the CRP supplementation levels:

T1 (Control): 100% basal commercial diet (0% CRP)

T2: Basal diet + 5% CRP (w/w)

T3: Basal diet + 10% CRP (w/w)

T4: Basal diet + 15% CRP (w/w)

It is important to note that the dietary formulations were non-isonitrogenous and non-isocaloric due to the practical supplementation (top-dressing/incorporation) model used. This approach was intentionally selected to evaluate the CRP matrix as a complete functional package, allowing its intrinsic nutritional and bioactive components to contribute naturally to the prawn's diet without the interference of synthetic fillers or protein adjustments.

Rearing was managed in an indoor system with continuous aeration to maintain dissolved oxygen (DO) levels above 5 mg/L. Water exchange was performed every 15 days to ensure optimal hygiene. Prawns were fed to apparent satiety three times daily (08:00, 12:00, and 17:00 h). Water temperature and pH were monitored regularly and maintained within the optimal range for *M. rosenbergii*.

Growth performance and feed utilization parameters, including weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), and survival rate (SR), were calculated using (2) – (5). In these equations, W_i and W_f represent the initial and final body weights (g), respectively; T denotes the total feeding duration (days); and N_i and N_f are the initial and final number of prawns [1, 2].

$$WG = W_f - W_i$$

(2)

$$SGR(\%/day) = \frac{(\ln W_f - \ln W_i) \times 100}{T} \quad (3)$$

$$FCR = \frac{\text{Total feed intake (g)}}{\text{Wet weight gain (g)}} \quad (4)$$

$$SR = (N_f / N_i) \times 100 \quad (5)$$

2.7 Water Quality Monitoring

To ensure optimal rearing conditions, critical water quality parameters in all experimental ponds (T1–T4) were monitored weekly throughout the 16-week study period. Parameters including temperature, dissolved oxygen (DO), and pH were measured *in situ*. Additionally, ammonia-nitrogen, nitrite-nitrogen, total alkalinity, calcium (Ca^{2+}), and magnesium (Mg^{2+}) were analyzed using a commercial colorimetric water test kit (V-unique, v-color 9750, Thailand). All measurements were performed consistently at 09:00 h to minimize diurnal fluctuations. Throughout the experiment, water quality parameters were maintained within the recommended ranges for the optimal growth and physiological health of *M. rosenbergii* [1].

2.8 Statistical data analysis

All experimental data were expressed as mean $\pm$ standard deviation (SD). Statistical analyses were conducted using **standardized statistical software**. Data were tested for normality and homogeneity of variance using Shapiro-Wilk and Levene's tests, respectively. Significant differences among treatments were determined using one-way ANOVA, followed by Scheffe's test for post-hoc multiple comparisons at a significance level of $P < 0.05$.

III. RESULTS AND DISCUSSION

3.1 Antioxidant Activity and Total Phenolic Content

The total phenolic content (TPC) and DPPH radical scavenging activity of the plain CCR and CRP are summarized in Table 1. The integration of *H. cordata* extract into the coconut residue matrix resulted in a significant twofold increase in TPC, rising from 8.70 ± 0.06 mg GAE/g in CCR to 20.64 ± 0.38 mg GAE/g in CRP ($P < 0.05$). This chemical enrichment was closely mirrored by the radical scavenging activity, where CRP exhibited a significantly higher DPPH capacity (40.82 ± 0.00 %) compared to CCR (18.68 ± 0.41 %). The strong correlation between TPC and DPPH activity indicates that these phenolic constituents are the primary drivers of the enhanced antioxidant capacity observed in the CRP matrix. When compared to other agricultural by-products utilized in aquaculture, the TPC of CRP is remarkably high. For instance, fermented rice bran or distillers dried grains with solubles (DDGS) typically yield TPC values in the range of 2.0 to 5.5 mg GAE/g [20, 21]. Furthermore, a study by Chakraborty et al. [31] on herbal-enriched fish feed reported that simple inclusion of dried herbal powders often results in TPC values below 12 mg GAE/g due to the dilution effect of the basal diet. The superior phenolic retention in CRP (> 20 mg GAE/g) suggests that the cold-extraction and partitioning process

used in this study is highly efficient at capturing and concentrating the polyphenolic fraction of *H. cordata*. The stability and high retention of these phenolics are likely facilitated by the physicochemical architecture of the coconut residue. Coconut lipids, specifically those rich in medium-chain triglycerides (MCTs) like lauric acid, function as a natural lipophilic carrier system [22, 25]. This lipid-protein scaffold potentially encapsulates and stabilizes the hydrophobic flavonoids of *H. cordata*—specifically quercetin and rutin [3, 4]. According to Upadhyaya et al. [32], lipid-based matrices can protect the sensitive free hydroxyl groups of phenolics from thermal degradation and premature oxidation during feed processing (< 60 °C) and subsequent storage [10, 19]. While these *in vitro* results provide a robust chemical baseline, it is important to distinguish between chemical antioxidant capacity and biological immune response. Although this study did not directly measure immunological markers (e.g., phenoloxidase or superoxide dismutase), the high concentration of stabilized bioactives in the CRP suggests a potential for enhanced bioavailability. Unlike simple dry-mixing, the partitioning of bioactives into the lipid-rich CRP ensures a more uniform delivery system. This interaction potentially offers protection against feed-borne oxidative stress, such as lipid peroxidation within the prawn's digestive tract. By safeguarding the integrity of these compounds, a higher concentration of intact bioactives may remain available for intestinal absorption, thereby supporting the overall physiological stability and growth performance observed in the subsequent 16-week trial [8, 18, 27].

TABLE I

%INHIBITION AND TOTAL PHENOLIC CONTENT OF CCR AND CRP

Sample	TPC (mg GAE/1g)	% Inhibition
CCR	8.70±0.06 ^a	18.68±0.41 ^a
CRP	20.64±0.38 ^b	40.82±0.00 ^b

b)

Values are expressed as mean ± standard deviation (n = 3). Superscript letters (a, b) within the same column indicate significant differences (p < 0.05).

3.2 Nutritional Composition and Bioactive Potency of CPR

The nutritional profiles of CCR and CRP are presented in Table 2. Proximate analysis revealed that CRP is a highly nutrient-dense ingredient, containing 44.37% crude protein and 38.94% crude lipid, which contributes to a substantial gross energy of 556.30 kcal/100 g. The integration of *H. cordata* extract significantly enhanced the bioactive potency of the matrix, with CRP exhibiting a TPC of 20.64 ± 0.38 mg GAE/g. This twofold enrichment compared to CCR directly correlated with superior DPPH radical scavenging activity (40.82 ± 0.00 %). This enhanced activity is attributed to the synergistic interaction between *H. cordata* flavonoids—

specifically quercetin and afzelin—and the lipid-rich scaffold of the coconut residue [3, 4]. The green extraction approach facilitated the partitioning of these hydrophobic metabolites into the medium-chain triglycerides (MCTs) within the CCR, which act as a natural stabilizer, protecting polyphenols from oxidative degradation during feed processing and storage [7, 10, 32].

3.2.1 Protein-Sparing Effect and Energy Optimization

All experimental diets exceeded the minimum protein requirement of 35% recommended for *Macrobrachium rosenbergii* [20, 21, 24]. However, the incremental nutrient density provided by CRP (T2–T4) introduced a significant metabolic advantage. According to Babu et al. [25], the inclusion of MCTs in crustacean diets enhances energy availability without the typical oxidative stress associated with long-chain fatty acids (LCFAs). In this study, the peak growth performance observed in T3 (9.80% lipid) aligns perfectly with the optimal dietary lipid threshold (8–10%) reported for juvenile *M. rosenbergii* [21, 24]. Lipid levels exceeding 12% (near the T4 level) have been shown to potentially cause growth retardation due to satiety feedback mechanisms, which explains why growth slightly declined in the T4 group compared to T3. The medium-chain fatty acids (MCFAs) prevalent in CRP serve as an immediate metabolic energy source that bypasses the complex lymphatic transport system required by LCFAs [22, 26]. Unlike the soybean oil-based control (T1), these lipids are directly transported to the hepatopancreas for rapid oxidation [25, 26]. This allows the prawns to effectively utilize CRP-derived lipids for high-energy demanding processes, such as ecdysis (molting), thereby "sparing" dietary protein for muscle synthesis and tissue growth. This Protein-Sparing Effect is a primary driver for the improved Feed Conversion Ratio (FCR) and Specific Growth Rate (SGR) observed in the T3 group.

3.2.2 Comparative Insights on Matrix-Mediated Stability

The superiority of CRP provides a unique case for circular economy in aquaculture [8, 9]. When compared to other plant-based additives, several key distinctions emerge:

1) **Lipid-Mediated Bioactive Stability:** The TPC of CRP (20.64 mg GAE/g) is significantly higher than fermented agricultural residues or herbal powders, which often report TPC values below 12 mg GAE/g due to high leaching and oxidative degradation during pelleting [29, 31]. The coconut-lipid matrix acts as a "lipid-shield", protecting sensitive flavonoids through the digestive transit [32].

2) **MCFA-Driven Energy Efficiency:** Unlike LCFAs from vegetable oils which can lead to excessive lipid deposition in the hepatopancreas [28, 30], the MCFAs in CRP are oxidized rapidly for energy. This explains why the nutritional shift toward higher lipid levels in T3 optimized performance rather than hindering metabolic health.

3) **Bioavailability Baseline:** Compared to the simple dry-mixing of herbal extracts [27], our results indicate that the coconut-lipid scaffold enhances the retention and delivery of *H. cordata* metabolites. This provides a consistent chemical baseline that supports the physiological resilience observed in the prawns, ensuring bioactives remain effective until they reach the midgut.

3.2.3 Ash Content and Molting Success

The ash content showed a decreasing trend from T1 (15.06%) to T4 (11.69%). While T1 contained higher mineral concentrations from fishmeal, the balanced ash level in T3 (14.03%) was sufficient to support the mineral requirements for successful molting. The high survival rate in T3 (92.33%) suggests that the organic-rich nutrients in CRP, combined with adequate mineral levels, provided a superior balance for the physiological demands of *M. rosenbergii* compared to the high-ash, low-energy control diet [23, 24].

TABLE II
CHEMICAL COMPOSITION OF THE FOUR DIETARY FORMULATIONS

Chemical Composition	Treatment			
	T1	T2	T3	T4
Ash (%)	15.06	14.83	14.03	11.69
carbohydrate (%)	32.20	33.47	30.26	27.52
Energy Kcal/100 g	342.04	355.43	365.36	372.37
Total fat (%)	7.32	7.47	9.80	10.89
Humidity (%)	8.58	5.65	6.88	8.83
Protein (%)	36.84	38.58	39.03	41.07

3.3 Growth Performance, Survival, and Feed Utilization

The growth trajectories, survival rates, and feed efficiency of *M. rosenbergii* over the 16-week feeding trial are summarized in Table 3. The results demonstrate that the incorporation of CRP significantly enhances all growth metrics compared to the control group ($P < 0.05$). Among the formulations, Treatment 3 (T3), containing 10% CRP, emerged as the physiological optimum, yielding the highest production performance across all measured parameters.

3.3.1 Synergistic Nutrient Utilization and the Protein-Sparing Effect

A key observation in this study was the non-linear response to dietary protein levels. Although the T4 group (15% CRP) contained the highest crude protein (41.07%), it did not surpass T3 (39.03% protein) in weight gain (1.943 ± 0.043 g) or SGR (2.492 ± 0.063 %). This indicates that the enhanced growth is not solely a function of increased protein intake, but rather the result of an optimal Energy-to-Protein (E:P) ratio and the bioactive concentration within the CRP matrix. The superior growth in T3 highlights a powerful synergy between the medium-chain triglycerides (MCTs) from the coconut residue and the protein fraction. Unlike the long-chain fatty acids (LCFAs) in traditional diets, the MCFAs in coconut are absorbed and oxidized rapidly, serving as an immediate metabolic fuel [25, 26]. This rapid energy availability triggers a "Protein-Sparing Effect", where lipids are prioritized for energy production, thereby sparing high-quality dietary protein for muscle biosynthesis and tissue growth [24, 25]. This metabolic efficiency is confirmed by the significant improvement in Feed Conversion Ratio (FCR) in the CRP-supplemented groups.

3.3.2 Enhanced Survival and the "Lipid-Shielding" Mechanism

The inclusion of CRP significantly bolstered survival rates, with T3 achieving a peak of 92.33 ± 1.15 %, compared to 85.00 ± 1.00 % in the control ($P < 0.05$). While immunological markers were not directly measured, this enhanced resilience is likely linked to the antioxidant-mediated robustness provided by the *H. cordata* extract.

The flavonoids within the CRP matrix, such as quercetin, are protected by a Lipid-Shielding Effect. The coconut-lipid scaffold encapsulates these bioactives, protecting them from aqueous leaching in the water and enzymatic degradation in the gut [32]. This ensures a consistent delivery of intact polyphenols to the midgut, mitigating oxidative stress and maintaining homeostatic stability throughout the culture period [28]. This mechanism explains the higher survival rates observed here compared to studies using simple dry herbal powders [27, 29].

3.3.3 Dose-Response and the Physiological Threshold

The growth data reflects a quadratic-like dose-response, where performance peaked at 10% inclusion (T3) before experiencing a decline at the 15% level (T4). Despite T4 having a lower FCR (2.389 ± 0.051), its reduced SGR suggests a Nutritional Satiety Threshold or a metabolic plateau. According to Ng and Koh [22], excessive MCFAs can trigger satiety feedback mechanisms in decapods, potentially reducing voluntary feed intake. Additionally, higher inclusion levels might introduce minor anti-nutritional effects or affect diet palatability. Therefore, the 10% inclusion of CRP represents the ideal balance—maximizing nutrient assimilation and physiological resilience without exceeding the metabolic capacity of *M. rosenbergii* [24, 30].

TABLE III
GROWTH PERFORMANCE AND SURVIVAL OF PRAWNS FED CRP-SUPPLEMENTED DIETS OVER A 16-WEEK PERIOD

Parameter	Treatment			
	T1	T2	T3	T4
Initial weight (g)	0.141±0.008 ^a	0.129±0.001 ^a	0.127±0.007 ^a	0.134±0.013 ^a
Final weight (g)	1.228±0.014 ^a	1.529±0.038 ^b	2.070±0.037 ^d	1.734±0.028 ^c
Weight gain (g)	1.087±0.005 ^a	1.400±0.038 ^b	1.943±0.043 ^d	1.600±0.036 ^c
DWG	0.010±0.001 ^a	0.013±0.001 ^b	0.017±0.001 ^d	0.014±0.001 ^c
SGR (% BW day ⁻¹)	1.936±0.046 ^a	2.208±0.019 ^b	2.492±0.063 ^c	2.288±0.092 ^b
Survival (%)	85.000±1.000 ^a	85.667±1.528 ^{ab}	92.333±1.155 ^c	88.667±1.154 ^b
FCR (g feed g gain ⁻¹)	2.870±0.101 ^a	2.536±0.038 ^{bc}	2.437±0.063 ^c	2.389±0.051 ^c

Values are expressed as mean ± standard deviation (n = 3). Superscript letters (a, b) within the same column indicate significant differences ($p < 0.05$).

3.4 Water Quality Dynamics

Throughout the 16-week trial, water quality parameters across all treatments (T1–T4) remained within optimal ranges for *M. rosenbergii* (Table 4). Key parameters, including water temperature (25–30 °C), pH (7.0–8.5), and dissolved oxygen (7.0–8.5 mg/L), were strictly maintained. Nitrogenous metabolites, specifically total ammonia (NH₃: 0.1–0.2 mg/L) and nitrite (0.1–0.3 mg/L), were kept at non-toxic levels via the 15-day water exchange protocol. Additionally, essential minerals for crustacean molting—alkalinity (102–119 mg/L), calcium (100–120 mg/L), and magnesium (338–386 mg/L)—were successfully stabilized within the indoor concrete pond system. The uniformity of these parameters across all groups is crucial as it confirms that the rearing environment did not impose physiological stress or mineral deficiencies that could confound the growth and survival data [1, 11]. High DO levels and adequate mineral concentrations supported efficient metabolism and regular molting cycles, ensuring that water quality was not a limiting factor in this closed indoor system.

TABLE IV

WATER QUALITY PARAMETERS MONITORED
THROUGHOUT THE EXPERIMENTAL PERIOD

Parameters	Measured Values (Range)
pH	7.0-8.5
Temperature (°C)	25-30
Total Ammonia (mg/L)	0.1-0.2
Nitrite (mg/L)	0.1 -0.3
Calcium (mg/L)	100-120
Alkalinity (mg/L)	102-119
Dissolved Oxygen (mg/L)	7.0-8.5
Magnesium(mg/L)	338-386

IV. CONCLUSION

This study identifies a 10% inclusion level of CRP (T3) as the optimal formulation for enhancing the growth and survival of *M. rosenbergii*. The superior performance of T3 over the higher-protein T4 group underscores that the bioactive-lipid synergy is the key driver of production, rather than protein quantity alone. This approach supports the BCG economy model by upcycling agricultural by-products into high-value functional feed. As direct immunological parameters were not measured, future research should focus on immune-related gene expression and disease-challenge trials to fully validate the practical efficacy of CRP in intensive aquaculture systems.

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Effect of Extraction Solvents and Maceration Temperature on Antioxidant Activity of *Limnophila aromatica* Crude Extract

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Abstract. *Limnophila aromatica* is a traditional herb widely used in Southeast Asian cuisine and medicine and has gained scientific attention for its antioxidant potential. This study investigated the effects of solvent type and maceration temperature on the antioxidant activity of *L. aromatica* crude extracts to determine optimal extraction conditions. Aqueous and organic solvents (ethanol and acetone at 50% and 100%) were examined, and antioxidant activity was evaluated using the DPPH radical scavenging assay. The results indicated that 100% ethanol was the most effective solvent, producing the highest antioxidant activity ($93.47 \pm 0.59\%$), significantly greater than other solvent systems. Extracts obtained with 100% ethanol at maceration temperatures of 4 °C and 25 °C showed comparable DPPH scavenging activities (93.40% and 93.47%, respectively), suggesting that moderate temperatures are sufficient to preserve thermolabile antioxidant compounds. In contrast, extraction at temperatures above 50 °C resulted in a marked decline in activity, likely due to thermal degradation of phenolic and flavonoid constituents. Compared with the standard antioxidant ascorbic acid (99.29% inhibition), the optimized extract exhibited nearly equivalent activity. From a practical perspective, extraction at 25 °C offers advantages for industrial scalability due to lower energy requirements than cold extraction. In conclusion, extraction using 100% ethanol at ambient temperature is an effective and practical approach for maximizing the antioxidant potential of *L. aromatica*, supporting its application in functional foods, cosmetics, and nutraceutical products as a natural alternative to synthetic antioxidants.

Index Terms—Antioxidant activity, DPPH assay, *Limnophila aromatica*, Maceration temperature, Solvent extraction

I. INTRODUCTION

In recent years, considerable attention has been given to medicinal plants due to their potent antioxidant properties. Numerous studies have indicated that consuming foods naturally high in antioxidants may reduce the risk of degenerative diseases, especially cardiovascular ailments and various forms of cancer [1]. Bioactive compounds with antioxidant capacity, particularly those isolated from aromatic, spicy, and medicinal plants, have been actively investigated as promising natural ingredients for antioxidant formulations in food, cosmetic, and health products [2]. Among the principal classes of plant secondary metabolites—terpenoids, phenolic metabolites, and alkaloids [3], phenolic compounds are recognized as the most relevant to dietary applications and are among the most thoroughly studied for their health-promoting functions [4].

The search for safe and effective antioxidants from natural sources is a priority, especially for applications in functional foods, nutraceuticals, and cosmetic products. In this context, *Limnophila aromatica*, commonly known as rice paddy herb, has attracted growing attention due to its traditional use and potential health benefits. Native to Southeast Asia, *L.*

aromatica thrives in tropical, water-rich environments such as flooded rice fields [5]. Traditionally used as a culinary herb and a medicinal plant, it is prominently featured in Thai cuisine for its aromatic qualities and traditional Thai medicine [6, 7]. In Thailand, *L. aromatica* is a widely utilized species valued for its distinctive flavor and traditional medicinal applications. This plant has been reported to contain bioactive flavonoids that exhibit antioxidant potential [6].

Several studies have investigated the antioxidant activities of extracts obtained from *L. aromatica* [5, 7, 8]. Much of this research has focused on examining the effects of different extraction solvents and drying temperatures on antioxidant efficiency. Although valuable, these studies often assessed each factor independently and did not systematically evaluate how these parameters might interact.

In this study, we aim to compare the antioxidant activities of crude extracts obtained using various solvents to identify the most effective solvent system. Once the optimal solvent is determined, we further investigate how varying the maceration temperature influences antioxidant activity when combined with this selected solvent. This stepwise approach is essential because solvent polarity and temperature are

interdependent factors—temperature affects antioxidant compounds' solubility and extraction kinetics. It may also influence the stability or degradation of thermosensitive phenolics and flavonoids. By studying these factors sequentially, our work fills a knowledge gap and provides a more comprehensive understanding of optimizing antioxidant extraction from *L. aromatica*. Insights gained from this research will help maximize extraction protocols and serve as a foundation for future investigations into other extraction parameters. Ultimately, this will support the development of high-efficiency, plant-based antioxidant formulations suitable for use in the food and cosmetic industries, where natural and functional ingredients are increasingly in demand.

II. METHODOLOGY

Chemicals Used

Ethanol and acetone were purchased from QR&C (New Zealand). 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ascorbic acid were purchased from Sigma-Aldrich GmbH (Sternheim, Germany).

Sample Preparation

The *L. aromatica* samples used in this study were purchased from local markets in Bangkok, Thailand, in March 2024. The plant was identified by Department of botany, Faculty of science, Chulalongkorn University, and the voucher specimen was deposited at the Herbarium of Botany dept., Chulalongkorn University [H.B. No. A17868 (BCU)]. After removing the roots, the aerial parts were thoroughly washed, sliced, and left at room temperature to drain excess moisture. The plant material was then dried in a hot air oven at 50 °C, which has been reported as the optimal drying temperature for this method [8]. The drying duration was adjusted to ensure that the final moisture content did not exceed 10%. Moisture content was determined using azeotropic distillation with water-saturated toluene, following the procedure outlined in the Pharmacopoeia (1998). Once dried, the *L. aromatica* was ground into a fine powder using a kitchen milling machine and stored at -20 °C until further use in extraction procedures [9].

Extraction of Crude Extracts

The dried ground aerial parts of *L. aromatica* (1 g) were extracted with 10 mL of solvent under constant stirring for 48 h. Two sets of experiments were conducted.

Effect of Extraction Solvent

To determine the influence of solvent polarity, extractions were performed at 25 °C using five different solvents: distilled water, 50% ethanol, 100% ethanol, 50% acetone, and 100% acetone.

Effect of Extraction Temperature

Based on solvent screening, 100% ethanol was selected for subsequent temperature variation experiments. Extractions were conducted at 4, 25, 40, 50, and 60 °C for 48 h to

investigate the effect of temperature on antioxidant activity. After extraction, the mixtures were filtered through Whatman No. 1 filter paper, and the filtrates were measured for antioxidant activity by DPPH assay.

Antioxidant Activity by DPPH Assay

The antioxidant activity of the extracts was assessed using the DPPH radical scavenging method, following the procedure outlined by Wanyo et al. (2018). In brief, 0.1 mL of the extract was added to 3 mL of a 0.1 mmol·L⁻¹ DPPH solution prepared in ethanol. The mixture was vortexed for 1 minute and then incubated in the dark at room temperature for 30 minutes. After incubation, the absorbance was measured at 517 nm. The percentage of radical scavenging activity was calculated using the formula: $[(A_0 - A_e) / A_0] \times 100$, where A_0 represents the absorbance of the control (without extract) and A_e represents the absorbance in the presence of the extract.

III. RESULTS AND DISCUSSION

Effect of Solvent on Antioxidant Activity

DPPH is a stable organic free radical with a characteristic absorption peak at 517 nm. This absorption diminishes upon receiving an electron or a hydrogen atom from an antioxidant compound, leading to a visible color change from deep purple to yellow. The method allows for the rapid screening of multiple samples and is sufficiently sensitive to detect bioactive compounds even at low concentrations [10].

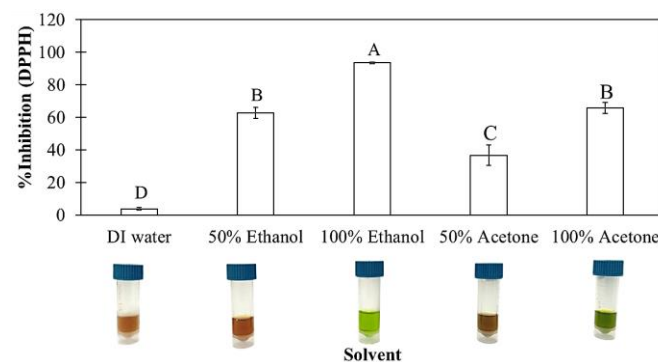


Fig. 1 Effect of different solvents on the antioxidant activity of *L. aromatica* crude extracts evaluated by DPPH radical scavenging assay. The representative visual appearance of each extract is shown below the X-axis. (Data are expressed as mean $\pm$ SD, n = 3. Different letters indicate significant differences at $p < 0.05$.)

As illustrated in Fig. 1, the antioxidant activity of *L. aromatica* crude extracts varied significantly with the type of solvent used. The extract obtained with 100% ethanol exhibited the highest percentage of DPPH radical scavenging activity with 93.47 ± 0.59 , followed by 50% Ethanol, 100% acetone, 50% Acetone, and distilled (DI) water. This result highlights the importance of solvent polarity in influencing the solubility and extractability of antioxidant compounds, particularly phenolics and flavonoids, which are known to contribute significantly to free radical scavenging activity.

These findings are consistent with previous studies [5], which reported that 100% ethanol was the most effective solvent for extracting antioxidant constituents from *L. aromatica*, yielding the highest total phenolic and flavonoid contents along with the lowest IC₅₀ value in the DPPH assay. While their approach involved the use of defatted freeze-dried samples and a broad range of aqueous ethanol mixtures, our findings demonstrate that extraction efficiency can be further enhanced under practical conditions by employing a moderate maceration temperature (45 °C) without the need for pre-defatting or complex purification procedures. This improvement may be attributed to the combined effect of temperature-enhanced diffusion and ethanol's ability to solubilize mid-polarity bioactive compounds—such as caffeic acid, chlorogenic acid, and 8-oxygenated flavonoids—previously identified in *L. aromatica* [5, 11]

Ethanol, a solvent of moderate polarity, is capable of effectively dissolving a wide range of both polar and non-polar phytochemicals. In contrast, although water is highly polar, it is generally less efficient in extracting certain bioactive constituents, particularly those with low polarity, due to their limited solubility in aqueous media. As illustrated below the X-axis in Fig. 1, the visual differences among the extracts further indicate extract composition and concentration variations.

Interestingly, although aqueous acetone appeared to yield the highest amount of extract based on the visual intensity of extract color presented in their study, it did not correspond to higher antioxidant activity. This observation suggests that estimated extraction yield—particularly when inferred from qualitative visual indicators such as color intensity—may not accurately reflect the actual bioactive compound content. These findings underscore the importance of not only selecting an appropriate solvent system but also optimizing thermal conditions to ensure efficient recovery of functional phytochemicals with high antioxidant potential.

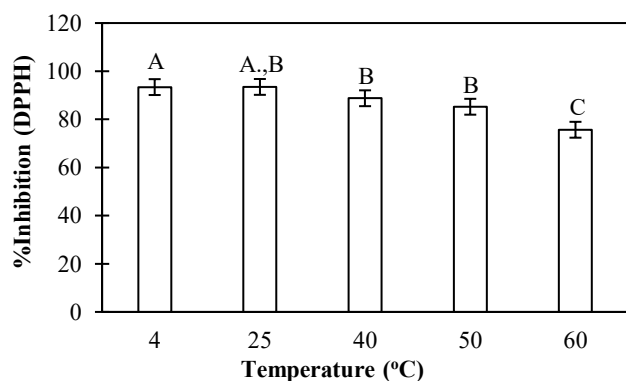


Fig. 2 Effect of maceration temperature on the antioxidant activity of *L. aromatica* extracts using 100% ethanol as the solvent. (Data are expressed as mean $\pm$ SD, n = 3. Statistically different letters indicate significance at $p < 0.05$.)

Effect of Maceration Temperature on Antioxidant Activity

The maceration temperature also significantly affected the antioxidant activity of the extracts (Fig. 2). When 100%

ethanol was used as a solvent, extraction at 4 °C and 25 °C produced the highest antioxidant activity with no significant difference. This optimal temperature likely enhances cell wall permeability and increases the diffusion of active constituents without degrading thermolabile compounds.

A decline in activity was observed at temperatures above 50 °C, suggesting thermal degradation of sensitive compounds such as phenolic acids and flavonoids. Conversely, lower temperatures may lead to incomplete extraction. These results support findings in similar studies that have demonstrated a bell-shaped relationship between temperature and antioxidant yield [12]. This may be due to conjugated hydroxyl groups in polyphenolic compounds, which are more stable at moderate temperatures. At higher temperatures (>50 °C), oxidative degradation or polymerization may reduce their radical scavenging ability.

Although both maceration conditions of 4 °C and 25 °C resulted in similarly high antioxidant activity, consideration of practical applications suggests that 25 °C may be more suitable for industrial or large-scale extraction. Maintaining low temperatures such as 4 °C typically requires additional energy input and specialized cooling systems, which may increase operational costs. In contrast, extraction at ambient temperature (25 °C) is more energy-efficient and economically viable, particularly in resource-limited or scalable production settings. Therefore, 25 °C offers a more feasible option for optimizing antioxidant extraction from *L. aromatica* for potential use in developing natural antioxidant ingredients in the food and cosmetic industries.

Comparison with Ascorbic Acid Standard

To assess the antioxidant potential of the optimized extract under practical conditions, its DPPH radical scavenging activity was compared with that of ascorbic acid, a well-known standard antioxidant (Fig. 3). Ascorbic acid exhibited the highest activity, with a percent inhibition of $99.29 \pm 0.15\%$. Notably, extracts obtained using 100% ethanol at 4 °C and 25 °C demonstrated remarkably similar antioxidant activities, with percent inhibition values of $93.40 \pm 0.17\%$ and $93.47 \pm 0.59\%$, respectively. Although slightly lower than that of the pure standard, the activity of these extracts is remarkably high, especially considering they represent crude plant extracts without further purification.

These findings are particularly significant as they indicate that *L. aromatica* extracts prepared under optimized conditions possess antioxidant activity that closely approaches that of ascorbic acid. The minimal difference—less than 6%—in radical scavenging capacity underscores the extract's strong potential as a natural alternative to synthetic antioxidants. Furthermore, the antioxidant activity likely results from a combination of bioactive constituents working synergistically, which may offer additional functional benefits beyond radical scavenging alone. This highlights the promise of *L. aromatica* as a valuable source of natural antioxidants for applications in food preservation, cosmetic formulations, and nutraceutical development, especially in contexts where the replacement of synthetic

additives with natural alternatives is desired due to consumer safety and regulatory considerations.

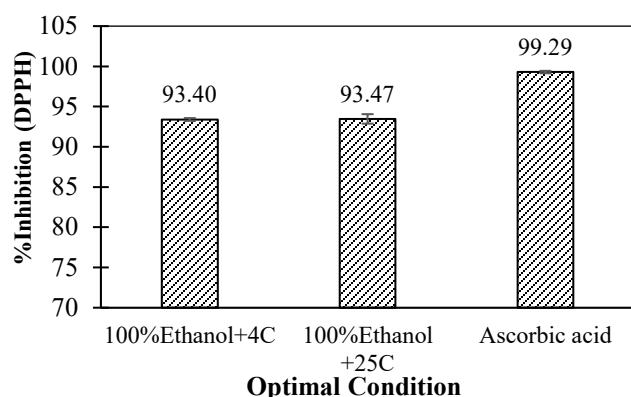


Fig. 3 Comparison of the antioxidant activity between the crude extract obtained under optimal extraction conditions and ascorbic acid standard, evaluated by DPPH radical scavenging assay. (Results are presented as mean $\pm$ SD, n = 3.)

In summary, the extraction solvent and maceration temperature strongly influence the antioxidant activity of *L. aromatica* crude extracts. Among the tested conditions, 100% ethanol and a moderate temperature of 25°C offered the most effective combination for maximizing DPPH radical scavenging. These findings provide a basis for optimizing this plant's extraction protocols and support its potential as a source of natural antioxidants for functional applications.

IV. CONCLUSION

This study provides compelling evidence that both solvent type and maceration temperature play pivotal roles in determining the antioxidant efficacy of *L. aromatica* crude extracts. Among the tested solvents, 100% ethanol demonstrated the highest extraction efficiency, yielding significantly greater DPPH radical scavenging activity than aqueous or acetone-based systems. When examining thermal effects, maceration at 25 °C and 4 °C resulted in similarly high antioxidant activities (93.47 $\pm$ 0.59% and 93.40 $\pm$ 0.17%, respectively), underscoring the preservation of thermolabile compounds at lower to moderate temperatures.

Importantly, extraction at ambient temperature (25 °C) offers practical advantages in terms of cost-effectiveness, energy efficiency, and industrial scalability, making it a more viable condition for real-world applications. Although the extract's antioxidant activity was marginally lower than that of the pure standard ascorbic acid (99.29 $\pm$ 0.15%), the minimal difference of less than 6% highlights the remarkable potential of *L. aromatica* as a natural antioxidant source. The synergistic effects among its phytochemical constituents may further contribute to its functional value beyond radical scavenging alone.

Overall, the findings of this study contribute to the growing body of knowledge on optimizing plant-based antioxidant extraction and lay the groundwork for future

research into process intensification, compound stability, and product formulation for use in the food, cosmetic, and nutraceutical industries.

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Response Surface Analysis of Dextrose and Peptone Influencing Bioactive Activities in *Phyllosticta capitalensis*

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Abstract. The present study analyzed the impact of dextrose (5–45 g/L) and peptone (5–15 g/L) concentrations on enhancement of antibacterial, antifungal, and antioxidant activities of *Phyllosticta capitalensis* extract using Box–Behnken design and response surface methodology (RSM). A quadratic model was developed and evaluated by ANOVA. For antibacterial activity, the model was significant ($p = 0.004$) with $R^2 = 0.876$, while lack-of-fit was not significant ($p = 0.232$), indicating adequate model fitness. For antifungal activity, the model was significant ($p = 0.007$) with high determination coefficient ($R^2 = 0.961$). However, lack-of-fit was significant ($p = 0.001$), suggesting that the quadratic model may not fully capture the system variability. For antioxidant activity, the model showed strong significance ($p < 0.001$) with $R^2 = 0.961$, but significant lack-of-fit ($p = 0.024$) was observed. Response surface analysis indicated that higher dextrose concentration combined with moderate peptone levels enhanced bioactivities, with antioxidant activity showing the strongest quadratic dependence. These findings demonstrate that carbon–nitrogen balance significantly influences bioactive metabolite production.

Index Terms— Bioactive activities, Dextrose, Peptone, *Phyllosticta capitalensis*, Response surface methodology

I. INTRODUCTION

Endophytic fungi are microorganisms that inhabit the internal tissues of plants for all or part of their life cycle without causing apparent disease symptoms or immediate adverse effects on the host. These fungi establish symbiotic, mutualistic, or commensal associations with plants, in which they gain access to nutrients and physical protection within plant tissues [1]. In return, endophytic fungi play important roles in enhancing plant survival and fitness through multiple biological mechanisms. They promote plant growth and development by producing phytohormones such as auxins, gibberellins, and cytokinins, as well as by facilitating the solubilization and uptake of essential minerals, including phosphorus and iron. In addition, endophytic fungi improve plant tolerance to abiotic stresses, such as drought, salinity, extreme temperatures, and heavy metal toxicity. They also protect plants from biotic stresses, including insect pests, herbivores, and microbial pathogens, through the production of antimicrobial compounds, competition for nutrients and ecological niches, and activation of the plant's induced systemic resistance [2, 3]. Beyond their ecological roles, endophytic fungi are increasingly recognized as prolific producers of structurally diverse bioactive secondary metabolites, including alkaloids, terpenoids, steroids, and polyketides. These metabolites exhibit a wide range of biological activities and hold considerable potential for

applications in medicine, such as anticancer, antimicrobial, antiviral, and antioxidant agents, as well as in sustainable agriculture as environmentally friendly alternatives to synthetic chemicals [1, 4].

Phyllosticta capitalensis is a remarkably versatile and widely distributed endophytic fungus that has attracted increasing attention due to its substantial potential in medicinal, agricultural, and ecological applications. Numerous studies have reported the production of diverse secondary metabolites from this species, highlighting its capacity as a prolific source of bioactive compounds. Among these metabolites, meroterpenoids such as guignardone A, J, and M have been isolated, with guignardone A demonstrating notable antibacterial activity against *Pseudomonas aeruginosa* [5]. In addition, guignardianone C, a meroterpenoid belonging to the guignardianone class, has exhibited potent antifungal activity against *Candida albicans* [6]. Several polyketide compounds produced by *P. capitalensis*, including xenofuranone B and regiolone, have been reported to possess significant antiviral activity against tobacco mosaic virus [5]. Furthermore, phenolic compounds such as 1-octacosanol have shown antioxidant and antibacterial properties, while 3,4-dihydroxybenzoic acid has been identified as a compound with broad-spectrum antimicrobial activity [5, 7]. Collectively, these findings underscore the remarkable chemical diversity and biological potential of secondary metabolites produced by

P. capitalensis.

Recently, we isolated an endophytic *P. capitalensis* strain MSSU017 from *Ocimum sanctum*. The ethyl acetate extract obtained from its culture in half-strength Sabouraud Dextrose broth exhibited broad-spectrum antibacterial activity [8]. The composition of the cultivation medium is a critical determinant of both the quantity and structural diversity of secondary metabolites produced by endophytic fungi. Alteration of cultivation parameters, particularly carbon and nitrogen sources, metal ions, and the addition of specific inducer molecules has been shown to activate silent biosynthetic gene clusters to produce novel bioactive compounds [9, 10]. In this study, medium composition was optimized to enhance the production of bioactive compounds by *P. capitalensis*, as evaluated by antibacterial, antifungal, and antioxidant activities, using response surface methodology.

II. METHODOLOGY

Microorganisms

Phyllosticta capitalensis MSSU017 was obtained from the culture collection of the Department of Microbiology, Faculty of Science, Silpakorn University. *Candida albicans* TISTR5239 and *Staphylococcus aureus* TISTR885 were acquired from the Thailand Institute of Scientific and Technological Research (TISTR). The fungal strains were maintained on Sabouraud dextrose agar (SDA; dextrose 40 g/L, peptone 10 g/L, agar 15 g/L, pH 5.6) at 30 °C, while *S. aureus* was maintained on nutrient agar (nutrient broth 13 g/L, agar 15 g/L, pH 7.2) at 37 °C. All cultures were subcultured monthly to ensure viability and purity.

Extraction of bioactive metabolites

P. capitalensis MSSU017 was initially cultivated on HS-SDA at 30 °C for 3 days. Five mycelial plugs (0.5 cm in diameter) were transferred into 500 mL of liquid medium with the designated composition in 1-L Erlenmeyer flasks. The cultures were incubated at 30 ± 2 °C for 21 days under shaking conditions at 80 rpm. Following incubation, the fungal biomass was separated from the culture broth by filtration through sterile gauze. The mycelia were dried in a hot-air oven at 60 °C until a constant weight was achieved. The culture filtrate was extracted with ethyl acetate at a 1:1 (v/v) ratio using a separatory funnel. The organic phase was collected and concentrated under reduced pressure using a rotary evaporator. The resulting crude extract was weighed, and extraction yield was calculated using Equation (1). The extract was subsequently dissolved in 10% (v/v) dimethyl sulfoxide (DMSO) to obtain a stock solution at 3.5 mg/mL, corresponding to the maximum solubility concentration.

$$\text{Extraction yield} = \frac{\text{Amount of crude extract (mg)}}{\text{volume of culture filtrate (mL)}} \quad (1)$$

Antimicrobial susceptibility assay

For antibacterial testing, *Staphylococcus aureus* TISTR885 was cultivated on nutrient agar at 37 °C for 24 h. Subsequently, one to three well-isolated colonies were transferred into 1 mL of sterile 0.85% (w/v) NaCl solution and adjusted to a turbidity equivalent to a 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL). For antifungal evaluation, *Candida albicans* TISTR5239 was grown on HS-SDA at 30 °C for 24 h. Yeast cells were harvested and suspended in sterile 0.85% NaCl, then standardized to a 0.5 McFarland turbidity (approximately 5×10^6 CFU/mL). The standardized suspensions were further diluted at a ratio of 1:2000 prior to use. In each well of a 96-well microtiter plate, 50 µL of culture medium, 50 µL of fungal extract (1 mg/mL), and 50 µL of the prepared microbial inoculum were combined. Plates were incubated at the respective optimal temperature for 18 h. Microbial growth was assessed by measuring optical density at 600 nm (OD₆₀₀). The percentage of growth inhibition was calculated according to Equation (2).

$$\% \text{ Inhibition} = \left(1 - \frac{\text{OD sample}}{\text{OD control}}\right) \times 100 \quad (2)$$

ABTS radical scavenging assay

ABTS radical cations were generated by mixing 7 mM ABTS solution with 2.45 mM potassium persulfate and incubating the mixture in the dark at 30 °C for 16 h. The resulting solution was diluted with 0.1 M sodium acetate buffer (pH 4.5) to obtain the ABTS working solution. The 0.5 mg/mL extracts (10 µL) were mixed with 200 µL of ABTS working solution in a 96-well plate and incubated in the dark for 7 min. Absorbance was measured at 734 nm. Trolox was used as a positive control. The percentage of ABTS radical scavenging activity was calculated using Equation (3).

$$\% \text{ ABTS Scavenging} = \frac{\text{Abs ABTS} - \text{Abs sample}}{\text{Abs ABTS}} \times 100 \quad (3)$$

Optimization of medium composition using response surface methodology

Response surface methodology (RSM) employing a Box–Behnken design (BBD) was used to optimize the culture medium composition for enhanced production of bioactive compounds by *P. capitalensis*. Two independent variables, dextrose (A) and peptone (B) concentrations, were selected as the main factors influencing metabolite production. A total of 13 experimental runs were generated at three coded levels (−1, 0, and +1) according to the BBD matrix (Tables 1 and 2). The evaluated responses included fungal dry biomass, extraction yield, antibacterial activity, antifungal activity, and antioxidant activity.

The experimental data were subjected to analysis of variance (ANOVA) and fitted to a second-order polynomial regression model using Microsoft Excel. The statistical significance of model coefficients was assessed at a confidence level of 95% ($p < 0.05$). Model adequacy was

evaluated based on the coefficient of determination (R^2) and lack-of-fit analysis. The relationship between the dependent responses (Y) and the independent variables (A: dextrose; B: peptone) was described by Equation (4).

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_{12} AB + \beta_{11} A^2 + \beta_{22} B^2 \quad (4)$$

TABLE I
RANGE AND LEVELS OF VARIABLES USED IN
BOX-BEHNKEN DESIGN

Variables	Range and Levels		
	-1	0	1
Dextrose (g/L)	5	25	45
Peptone (g/L)	5	10	15

III. RESULTS AND DISCUSSION

A Box–Behnken design (BBD) comprising 13 experimental runs was employed to evaluate the effects of dextrose (5–45 g/L) and peptone (5–15 g/L) on extraction yield and three bioactivities, i.e. antibacterial, antifungal, and antioxidant. The experimental responses are presented in Table 2.

The ethyl acetate extraction yield ranged from 205.6 to 2765.2 mg/L. However, ANOVA indicated that the quadratic model did not adequately describe the extraction response. The lack-of-fit was significant, and the predicted R^2 was not in reasonable agreement with the adjusted R^2 (data not shown). Additionally, several regression terms were statistically non-significant. These findings indicate that the extraction yield exhibited complex nonlinear behavior that could not be sufficiently captured by a second-order polynomial model within the investigated design space.

Therefore, the model was not used for predictive optimization of yield. Nevertheless, empirical observation showed that the combination of low dextrose (5 g/L) and high peptone (15 g/L) resulted in the highest extraction yield (2288.3 mg/L), suggesting that nitrogen-rich conditions may favor metabolite accumulation in this system. Importantly, no mechanistic inference is drawn here, as the statistical model was not adequate.

Antibacterial activity against *Staphylococcus aureus* was expressed as percentage inhibition and ranged from 37.5% to 69.7% (Table 2). ANOVA demonstrated that the quadratic regression model (Equation 5) was statistically significant ($F = 9.935$, $p = 0.004$). The lack-of-fit was not significant ($F = 2.190$, $p = 0.232$), indicating that the model adequately represents the experimental data relative to pure error. The coefficient of determination ($R^2 = 0.876$) suggests that 87.6% of the variability in antibacterial activity was explained by the model. The adjusted R^2 (0.788) and predicted R^2 (0.847) were in reasonable agreement (difference < 0.2), supporting acceptable predictive reliability. Among the model terms, both dextrose (A) and peptone (B) significantly influenced antibacterial activity ($p < 0.05$), whereas the interaction term (AB) was not significant (Table 3). This indicates that the effects of carbon and nitrogen sources were primarily additive rather than synergistic. The response surface (Fig. 1A) showed a distinct curvature, confirming the significance of quadratic terms. Antibacterial activity increased with increasing peptone concentration and was moderately influenced by dextrose concentration. The contour plot (Fig. 1B) displayed elliptical contours with no pronounced distortion, consistent with the non-significant interaction term. The model predicted maximum antibacterial activity at 45 g/L dextrose and approximately 15 g/L peptone within the tested range. Because the lack-of-fit was non-significant and the predicted R^2 was acceptable, this optimization can be considered statistically reliable within the design space.

TABLE II
BOX-BEHNKEN DESIGN AND RESULTS OF RESPONSE SURFACE METHODOLOGY

Run	Factors		Responses							
	A: Dextrose	B: Peptone	Extraction yield (mg/L)		Antibacterial activity (% Inhibition)		Antifungal activity (% Inhibition)		Antioxidant activity (% Inhibition)	
			Experimental Value	Predicted Value	Experimental Value	Predicted Value	Experimental Value	Predicted Value	Experimental Value	Predicted Value
1	-1	-1	205.6	221.9	37.5	39.5	41.9	44.2	77.1	78.6
2	-1	1	2765.2	2288.3	47.5	50.5	27.2	27.8	32.6	38.7
3	1	-1	1344.6	1264.4	54.5	54.9	71.8	62.6	67.7	64.6
4	1	1	1002.2	428.9	66.8	68.2	75.2	64.5	84.8	86.3
5	0	-1	748.4	812.3	55.5	53.1	82.6	89.5	98.3	99.8
6	0	1	377.6	1427.8	69.7	65.2	72.2	82.2	98.3	90.7
7	-1	0	1364.8	1825.4	40.7	35.7	33.9	30.9	35.3	27.7
8	1	0	763.4	1416.9	54.2	52.3	38.7	58.5	43.0	44.5
9	0	0	2087.6	1690.3	45.6	49.9	83.4	80.7	65.6	64.3
10	0	0	1975.2	1690.3	48.0	49.9	81.5	80.7	65.3	64.3
11	0	0	1791.0	1690.3	46.1	49.9	84.4	80.7	63.2	64.3
12	0	0	1847.6	1690.3	54.2	49.9	83.7	80.7	60.1	64.3
13	0	0	1864.4	1690.3	48.7	49.9	87.7	80.7	61.3	64.3

TABLE III
ANALYSIS OF VARIANCE (ANOVA) OF THE FITTED QUADRATIC MODEL FOR ANTIBACTERIAL, ANTIFUNGAL
AND ANTIOXIDANT ACTIVITY

Source	df	Antibacterial activity				Antifungal activity				Antioxidant activity			
		Sum of Squares	Mean Square	F-value	p-value	Sum of Squares	Mean Square	F-value	p-value	Sum of Squares	Mean Square	F-value	p-value
Model	5	890.176	178.035	9.935	0.004*	5127.010	1025.402	8.663	0.007*	5015.371	1003.074	34.749	0.000*
A (Dextrose)	1	412.420	412.420	23.100	0.002*	1137.001	1137.001	23.100	0.017*	423.571	423.571	23.100	0.006*
B (Peptone)	1	221.847	221.847	9.460	0.010*	79.162	79.162	9.460	0.440	125.166	125.166	9.460	0.076*
AB	1	1.269	1.269	0.700	0.798	82.519	82.519	0.700	0.431	948.812	948.812	0.700	0.001*
A ²	1	95.076	95.076	7.180	0.055	3587.075	3587.075	7.180	0.001*	2199.506	2199.506	7.180	0.000*
B ²	1	236.973	236.973	13.220	0.008*	71.486	71.486	13.220	0.463	2645.765	2645.765	13.220	0.000*
Residual	7	125.435	17.919			828.521	118.360			202.061	28.866		
Lack-of-fit	3	77.973	25.991	2.190	0.232	807.898	269.299	52.232	0.001*	178.939	59.646	10.318	0.024*
Pure error	4	47.462	11.865			20.624	5.156			23.123	5.781		
Total	12	1015.610				5955.531				5217.432			
R ²		0.876				0.860				0.961			
Adjusted R ²		0.788				0.761				0.933			
Predicted R ²		0.847				0.824				0.956			

* Significant model terms were identified at $p < 0.05$

Importantly, the model is suitable for optimization within the studied range and should not be extrapolated beyond these factor levels.

$$Y = 49.885 + 8.2908A + 6.0807B + 0.5632AB - 5.8672A^2 + 9.2629B^2 \quad (5)$$

Antifungal activity against *Candida albicans* ranged from 27.2% to 87.7% (Table 2). The quadratic model (Equation 6) was statistically significant ($F = 8.633$, $p = 0.007$). The determination coefficient ($R^2 = 0.860$) indicated good explanatory power. The adjusted R^2 (0.761) and predicted R^2 (0.824) were reasonably close, suggesting acceptable predictive capability. However, the lack-of-fit was significant ($F = 52.232$, $p = 0.001$), indicating that systematic deviations between predicted and observed values remain. Therefore, although the model explains a large proportion of variability, it does not perfectly represent the experimental system (Table 3). The response surface (Fig. 1C) demonstrated pronounced curvature along the dextrose axis, indicating a significant quadratic effect of carbon concentration. In contrast, the contour plot (Fig. 1D) showed relatively parallel contours, supporting the weak interaction between dextrose and peptone. The model predicted that antifungal activity was maximized at approximately 25 g/L dextrose, with less sensitivity to peptone concentration. Given the significant lack-of-fit, the model should be interpreted as describing general trends rather than precise optima. Validation experiments would be required before practical implementation.

$$Y = 80.738 + 13.766A - 3.632B + 4.542AB - 36.038A^2 + 5.088B^2 \quad (6)$$

Antioxidant activity (ABTS scavenging) ranged from 32.6% to 98.4% (Table 2). The quadratic model (Equation 7)

was highly significant ($F = 34.749$, $p < 0.001$) with a high R^2 (0.961), indicating that 96.1% of response variability was explained by the model. The adjusted R^2 (0.933) and predicted R^2 (0.956) were closely aligned, demonstrating strong internal consistency and predictive potential. Both quadratic terms (A^2 and B^2) were highly significant ($p < 0.001$), indicating pronounced curvature in the response surface (Table 3). The 3D surface plot (Fig. 1E) showed steep response gradients, and the contour plot (Fig. 1F) exhibited closed elliptical patterns, confirming strong nonlinear effects. The model predicted maximum antioxidant activity at approximately 15 g/L dextrose across a broad range of peptone concentrations. Despite the high R^2 , the lack-of-fit was significant ($p = 0.024$), indicating residual structural inadequacy of the quadratic model. Therefore, while the model effectively describes response trends, precise quantitative prediction should be interpreted cautiously.

$$Y = 64.331 + 8.402A - 4.567B + 15.401AB - 28.220A^2 + 30.951B^2 \quad (7)$$

Culture medium composition is widely recognized as a critical determinant of fungal secondary metabolism. Variations in carbon and nitrogen sources can markedly influence both the quantity and spectrum of secondary metabolites produced. A classical illustration is penicillin biosynthesis, in which elevated dextrose concentrations repress the transcription of the penicillin biosynthetic gene cluster (*pcbAB*, *pcbC*, and *penDE*), leading to reduced antibiotic production [11, 12]. This phenomenon exemplifies carbon catabolite repression and underscores the regulatory sensitivity of secondary metabolic pathways to nutrient status. Beyond repression effects, numerous studies have demonstrated that specific carbon sources, particularly dextrose, can enhance the production of antimicrobial or

antioxidant metabolites depending on the fungal species and cultivation context. For example, optimization of fermentation parameters in *Aspergillus oryzae* identified 35.25 g/L dextrose as optimal for antifungal metabolite production [13]. Similarly, 49.0 g/L dextrose significantly

improved beauvericin yield in *Fusarium redolens* [14], while 12.72 g dextrose was reported as the most influential medium component affecting antioxidant activity in *Xylaria*

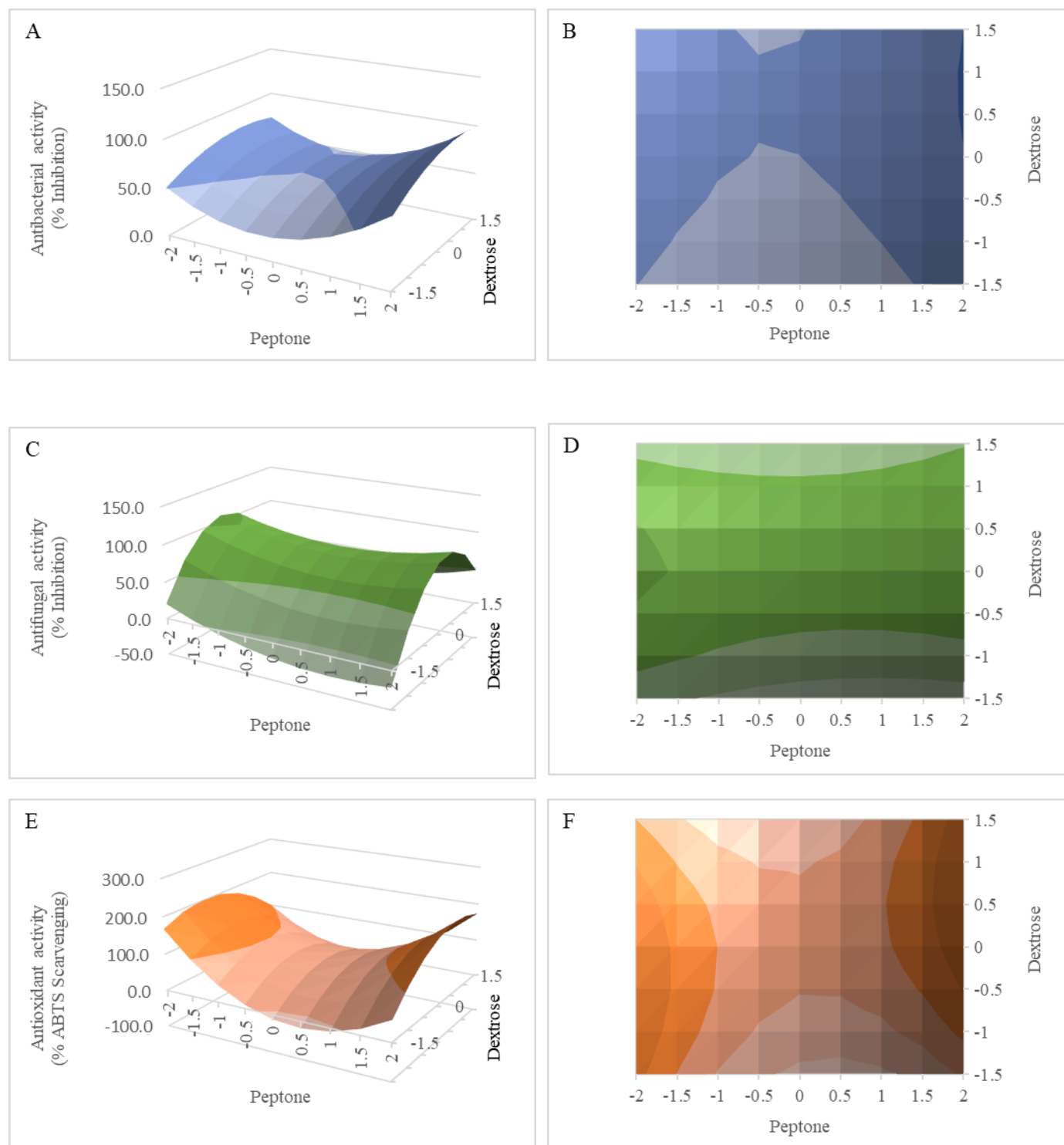


Fig. 1. RSM response surface plots (A, C, E) and contour plot (B, D, F) showing the effects dextrose and peptone on antibacterial activity (A, B), antifungal activity (C, D), and antioxidant activity (E, F).

feejeensis [15]. These findings collectively indicate that carbon concentration can modulate secondary metabolite output, although the optimal level is highly strain-dependent.

Nitrogen source quality and availability also play a pivotal role in regulating secondary metabolism. Complex organic nitrogen sources such as peptone frequently support enhanced metabolite production, whereas nitrogen limitation may act as a physiological trigger during late growth phases [16, 17]. For instance, peptone at 16.7 g/L, in combination with dextrose, maximized antifungal metabolite production in *Aspergillus oryzae* [13]. In *Fusarium redolens*, peptone was superior for both mycelial growth and beauvericin biosynthesis [14]. Moreover, substitution of yeast extract with peptone increased hirsutellone production in *Hirsutella nivea* [18], suggesting that nitrogen source composition can influence metabolic allocation toward specific secondary pathways.

In the present study, bioactivity responses varied according to dextrose and peptone concentrations. Maximum antibacterial activity was observed at higher nutrient levels (45 g/L dextrose and 20 g/L peptone), whereas antifungal activity was predicted to be highest at moderate dextrose concentration (25 g/L) across peptone levels. In contrast, antioxidant activity was predicted to peak at relatively lower dextrose concentration (15 g/L), largely independent of peptone concentration within the tested range. These distinct response patterns suggest that different classes of bioactive metabolites may be favored under different nutrient regimes. Importantly, while the statistical models revealed significant effects of medium components on bioactivity, the present study did not directly characterize the chemical profiles of the extracts. Therefore, although it is reasonable to hypothesize that nutrient availability influences central carbon flux, precursor supply, and regulatory signaling associated with secondary metabolite biosynthesis, such mechanistic interpretations remain inferential. The observed activity changes should thus be interpreted as phenotype-level responses to nutrient modulation rather than definitive evidence of specific pathway activation. Overall, the findings support the concept that carbon and nitrogen composition are key modulators of bioactivity expression in this fungal strain. However, further metabolomic or transcriptomic investigations would be required to elucidate the precise regulatory and biosynthetic mechanisms underlying these activity shifts.

IV. CONCLUSION

This study evaluated the influence of dextrose and peptone concentrations on the production of bioactive metabolites by *Phyllosticta capitalensis* MSSU017 using a Box–Behnken design coupled with response surface methodology. The results demonstrated that medium composition significantly affected antibacterial, antifungal, and antioxidant activities, although the magnitude and pattern of response varied among biological assays. For antibacterial activity, the quadratic model was statistically significant and exhibited a non-significant lack-of-fit, indicating acceptable model adequacy for describing the experimental data within the

studied range. In contrast, antifungal and antioxidant models, while statistically significant and characterized by relatively high R^2 values, showed significant lack-of-fit, suggesting that the second-order polynomial equation did not fully capture the complexity of these responses. Therefore, these models are appropriate for interpreting general response trends rather than for precise numerical prediction. The response surface analyses revealed distinct nutritional requirements for different bioactivities. Higher antibacterial activity was associated with elevated levels of both dextrose and peptone, whereas antifungal and antioxidant activities responded differently to carbon and nitrogen concentrations. These findings indicate that medium composition modulates the biological activity profile of fungal extracts, likely through alterations in secondary metabolite production. Overall, this work provides quantitative insight into the relationship between carbon and nitrogen sources and bioactivity in *P. capitalensis*. Although definitive global optimization was not achieved, the study establishes a rational experimental framework for medium optimization and highlights the need for further refinement, potentially incorporating additional factors or alternative modeling approaches to better capture system nonlinearity.

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Clove Extract (*Syzygium aromaticum*) Enhances Endoplasmic Reticulum Stress Tolerance in Aging *Saccharomyces cerevisiae* via UPR-Independent Proteostasis Modulation

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Abstract. The global population is rapidly transitioning toward an aging society. Aging is characterized by a progressive decline in biological processes driven by multiple factors. Loss of proteostasis refers to the disruption of the protein-balancing process, resulting in the accumulation of misfolded or unfolded proteins within cells. The endoplasmic reticulum (ER) plays a central role in protein quality control, and disruption of ER homeostasis induces ER stress. The unfolded protein response (UPR), an adaptive cellular pathway responding to misfolded protein accumulation and mitigating ER stress, becomes insufficient during aging. Our previous study demonstrated that clove extract (*Syzygium aromaticum*) extends the chronological lifespan of *Saccharomyces cerevisiae*, enhances multiple stress tolerances, and alleviates protein aggregation in aged yeast cells. In this present study, we investigated whether clove extract enhances ER stress tolerance through modulation of UPR activity during chronological aging, a potential underlying longevity-extending mechanism. Our results showed that while the UPR typically mitigates ER stress, its efficiency declines during aging. We report that clove extract enhances ER stress tolerance in chronologically aging *S. cerevisiae* despite significantly reducing UPR activity. Our results demonstrate a functional uncoupling between UPR signaling and stress survival, suggesting that clove extract promotes proteostasis via UPR-independent mechanisms that are likely achieved by reducing the primary accumulation of misfolded proteins. These findings highlight the anti-aging potential of clove extract for promoting healthy aging by maintaining cellular proteostasis.

Index Terms— Clove extract, Aging, Unfolded protein response (UPR), ER stress, Yeast

I. INTRODUCTION

The World Health Organization (WHO) anticipates that the proportion of the global population transitioning to an aging society will increase significantly by 2030 (1), a similar trend that is currently being observed in Thailand (2). Aging is a complex biological process driven by the accumulation of molecular and cellular damages (3), leading to the functional failure of tissues and organs. This deterioration contributes to the development of age-associated diseases such as neurodegenerative and cardiovascular diseases that can ultimately result in death (3-5). Multiple factors contribute to the initiation and progression of aging. Among these, loss of proteostasis resulting from a disruption of essential cellular processes involved in protein synthesis, folding, and degradation, leading to the accumulation of misfolded proteins, is one of the major aging hallmarks (6, 7).

The endoplasmic reticulum (ER) plays a critical role in protein quality control (8-10). Disruption of ER homeostasis induces ER stress, which activates the unfolded protein response (UPR), an adaptive signaling pathway that restores proteostasis. UPR activation leads to the induction of genes under the control of unfolded protein response element

(UPRE), including *KAR2* (BiP), *DER1*, *PDII*, and *INO1* genes (11-13) to improve proteostasis by ER-associated folding capacity, degradation, and induction of apoptosis (14-17). However, it has been shown that UPR activity markedly diminishes with aging (14, 18-20). This leads to impaired stress adaptation and increased accumulation of unfolded proteins in the ER lumen (20, 21).

Over the past century, numerous studies have explored strategies to promote healthy aging in the global population (22-25). Dietary natural extracts have emerged as promising approaches for anti-aging due to their biological properties, such as antioxidant, anti-inflammatory, and detoxification (23, 26). Clove (*Syzygium aromaticum*) is widely used in food flavoring and is recognized for its pharmacological activities (27). Its major bioactive component, eugenol, exhibited multiple biological activities, including antimicrobial, insecticidal, antioxidant, anti-inflammatory, and anti-tumorigenic (28, 29). Eugenol has been reported to preserve proteostasis by reducing protein carbonylation and oxidative damage (30), suggesting a potential role in mitigating age-associated proteotoxic stress.

Our previous findings demonstrated that clove extract significantly extends the chronological lifespan of the budding yeast model organism *Saccharomyces*

cerevisiae. In addition, clove extract enhances the survival of aged yeast cells under stress conditions, including oxidative and thermal

stress, and reduces protein aggregation during the aging of yeast (Suesattayapirom S. et al, unpublished data). Here in, we hypothesized that clove extract may enhance ER stress tolerance by modulating cellular proteostasis mechanisms. Among these, UPR represents a plausible pathway due to its central role in promoting proper protein folding capacity and maintaining ER protein quality control (14, 31).

In this study, we evaluated whether treatment with the clove extract enhances the tolerance to β -mercaptoethanol-induced ER stress in chronologically aged yeast. We next determine whether clove extract enhances ER stress tolerance through modulation of UPR activity by using a *UPRE*-driven *LacZ* reporter system, in which β -galactosidase activity encoded by the *LacZ* reporter serves as a quantitative indicator of UPR activation. These findings provided valuable insights into the anti-aging properties of clove extract and may contribute to the development of a natural intervention for promoting healthy aging.

II. METHODOLOGY

Yeast strains and plasmid

The wild-type strain *S. cerevisiae* utilized in this study is isogenic to haploid strains BY4742 (*Mat α* , *leu2 Δ 0*, *lys2 Δ 0*, *ura3 Δ 0*, *his3 Δ 1*). The *UPRE-lacZ* reporter plasmid pPW344, containing the *LacZ* gene under control of unfolded protein response element (*UPRE*) (15), was transformed into yeast cells using the lithium acetate procedure (32) and grown at 30 °C for 2 days (33). The transformants were selected using synthetic complete media lacking uracil (SC-Ura) supplemented with 2% glucose. This reporter plasmid quantifies UPR activity by measuring the expression of the *LacZ* gene encoding β -galactosidase. When the accumulation of unfolded proteins in the ER occurs, the *UPRE* is activated and transcribes the gene under the control of *UPRE* (15), resulting in the expression of β -galactosidase, which can be measured by an enzymatic assay.

Clove extract preparation

Clove bud powder was purchased from United Progress (Thailand) Ltd. (Bangkok, Thailand). Extraction of clove powder was performed using maceration in 95% ethanol. The resulting clove/ethanol mixtures were filtered and concentrated using a model R-1050 rotary evaporator (Henan Lanphan Industry Co., Ltd., Zhengzhou, China). The concentrated clove extract was mixed with maltodextrin and then lyophilized to obtain a dry powder. The lyophilized clove extract was reconstituted in dimethyl sulfoxide (DMSO) to prepare stock solutions and stored at -20 °C until use.

Culture conditions

The wild-type yeasts were cultured in SC medium supplemented with 2% glucose and treated with clove extract at 0.5, 1.0, and 1.5 μ g/ μ L or the vehicle control DMSO. Aged

yeast cells were cultured for 3 days to reach the aging phase (aging day 0) and were randomly sampled at days 0, 3, and 6 of chronological aging. To investigate UPR activity, yeast cells containing plasmid pPW344 were cultured in SC-Ura medium and treated with 1% DMSO as a vehicle control or clove extract at concentrations of 0.5, 1.0, and 1.5 μ g/ μ L. Cells were harvested on days 0, 3, and 6 of chronological aging to determine β -galactosidase activity.

ER stress sensitivity assay

At day 0 and 6 of chronological aging, the cell density under each condition was measured using a Genesys 20 visible spectrophotometer (Thermo Scientific, USA) at a wavelength of 600 nm. Dilution series of the cells were prepared and spotted on both the solid peptone-based medium supplemented with 2% glucose (YPD) containing 20 and 22 mM of β -mercaptoethanol (β -ME), an ER stress inducer (34), or on a solid YPD medium plate as a control. The plates were incubated at 30 °C for 4 days and imaged using a ChemiDoc XRS+ Imaging System (Bio-Rad, Hercules, USA) (34).

β -galactosidase (*LacZ*) Assay for UPR activity

The aging yeast cells were resuspended in the Z-buffer, consisting of 107 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, and 1.75 mM DTT, prepared in distilled water. The samples were incubated with the o-nitrophenyl- β -D-galactopyranoside (ONPG) substrate (33). β -galactosidase activity was assayed by quantitatively measuring a yellow chromophore o-nitrophenol produced by the hydrolysis of ONPG by β -galactosidase using a Genesys 20 visible spectrophotometer (Thermo Scientific, USA) at a wavelength of 420 nm. The protein content was measured by the Bradford protein assay according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). The results from three independent experiments were reported in Miller units, which is a unit of β -galactosidase enzyme activity to quantify the expression of genes fused to the *lacZ* reporter gene (34).

Statistical Analysis

Experimental data were shown as the mean values and standard deviations (error bars) of three independent experiments. Normality was assessed using the Shapiro-Wilk test. Differences between groups were analyzed using One-way ANOVA followed by Tukey's post-hoc test for multiple comparisons. Statistical significance was defined as $p < 0.05$.

III. RESULTS AND DISCUSSION

Clove extract treatment enhances ER Stress tolerance in chronologically aged yeast

Under homeostatic conditions, the endoplasmic reticulum (ER) functions as a central organelle responsible for several

essential cellular processes, including nascent protein folding, post-translational modification, lipid biosynthesis,

and calcium homeostasis (35, 36). Maintenance of ER integrity is therefore critical for cellular health and longevity (8). However, the aging process is associated with a progressive decline in these processes (3, 5), leading to the disruption of ER homeostasis and the accumulation of misfolded protein, a condition referred to as ER stress. Prolonged ER stress can ultimately promote apoptotic cell death (37). To determine whether clove extract enhances ER stress tolerance during aging, the yeast treated with different concentrations of clove extract was analyzed at days 0 and 6 of aging. Cells were spotted onto solid YPD containing 20 and 22 mM of the ER stress inducer β -mercaptoethanol (β -ME). As shown in Fig. 1, clove extract-treated cells and vehicle controls displayed comparable growth under non-stress conditions at day 0 of aging. This suggests that clove extract treatment does not adversely affect the basal cell viability or growth of aged yeast cells. In contrast, under ER stress conditions, clove extract-treated cells exhibited enhanced survival compared with vehicle-treated controls at day 6 of aging. These findings suggest that clove extract enhances the ability of aged yeast cells to tolerate ER stress induced by chemical inducers of protein misfolding. This enhanced survival suggests that clove extract mitigates proteotoxic stress associated with aging, which may be a result of the reduction in the accumulation of misfolded protein, thereby alleviating ER stress at its source.

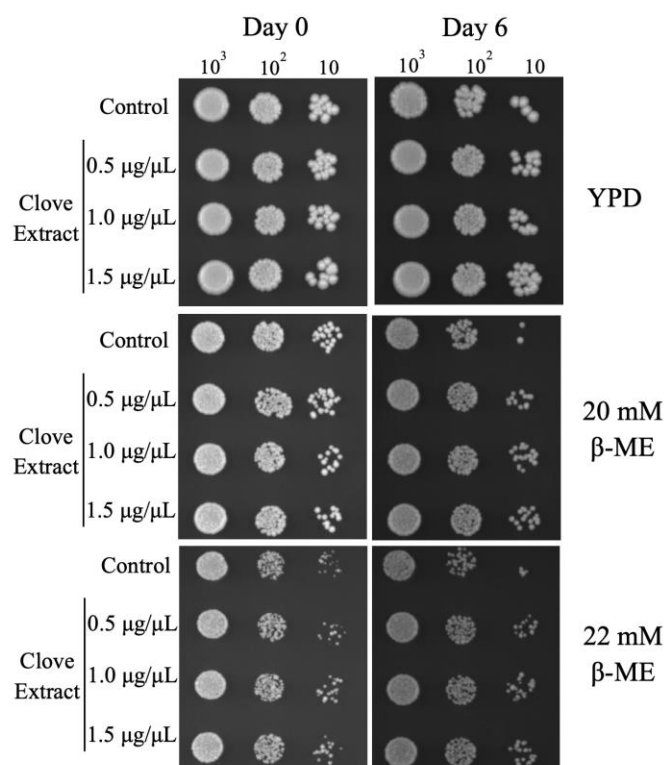


Fig.1 The growth of yeast cells with clove extract treatment under ER stress during days 0 and 6 of aging. Chronologically aged *Saccharomyces cerevisiae* was monitored by spotting 10^3 , 10^3 , and 10 cells onto a solid YPD plate (control) and YPD containing different concentrations of ER stress inducer β -mercaptoethanol (β -ME: 20 and 22 mM). The plates were photographed after incubation at 30 °C for 4 days. The image shown is a representative of three independent experiments.

Clove extract treatment attenuates the activation of unfolded protein response in aged cells

During aging, proteostasis progressively declines, leading to the alteration of protein structure and function and the accumulation of unfolded proteins inside the cells (18, 19). Among the cellular proteostasis systems, the unfolded protein response (UPR) within the ER plays an important role in sensing and responding to the accumulation of misfolded or unfolded proteins in the ER lumen (14, 20). A previous study has shown that early-stage chronologically aged yeast cells upregulate UPR activity compared to younger cells, suggesting that UPR activation is required for the recovery of aging loss of proteostasis (38). Consistent with this, our observation indicated that vehicle control aged yeast cells display increased sensitivity to ER stress (Fig.1), which may reflect impaired protein homeostasis. In order to better understand the effect of clove extract on UPR activity during chronologically aged yeast cells, we examined UPR activity using the reporter plasmid containing the *lacZ* gene under the control of UPR elements (*UPRE*) (Fig. 2A).

As shown in Fig 2B, the vehicle-treated aged yeast showed a progressive decline in UPR activity across multiple aging time points, with a significant reduction on day 6 of aging compared with days 0 and 3. This finding is consistent with previous reports demonstrating that aging diminishes the cellular capacity to sustain an effective UPR response to manage misfolded or unfolded proteins (38, 39). Interestingly, UPR activity of aged yeast cells treated with 1.0 and 1.5 $\mu\text{g}/\mu\text{L}$ clove extract was significantly reduced during day 0. In addition, on day 3 of aging, treatment with all clove extract led to a significant reduction in UPR activation compared with the vehicle control. This effect may be related to our previous findings that clove extract-treated yeast exhibited reduced protein aggregations beginning at day 0 of aging (Suesattayapirom S. et al, unpublished data) and thus, these results suggest that clove extract may reduce the accumulation of misfolded or unfolded proteins, thereby lowering the requirement for UPR activation rather than directly activating the UPR pathway to eliminate proteotoxic stress inside the cells. Notably, despite reduced UPR activity, clove extract-treated cells exhibited an enhanced capacity of cells to grow under ER stress conditions (Fig.1). This enhanced survival observed suggests a functional uncoupling between UPR activation and ER stress tolerance, suggesting that clove extract reduces the burden of misfolded proteins, thereby alleviating ER stress at its source and minimizing the requirement for a sustained UPR pathway. Our previous findings demonstrated that clove extract reduces ROS levels and protein carbonylation in aged yeast. The reduction in UPR activity observed here suggests that the extract may act as a proteostasis stabilizer, preventing the initial accumulation of misfolded proteins. By

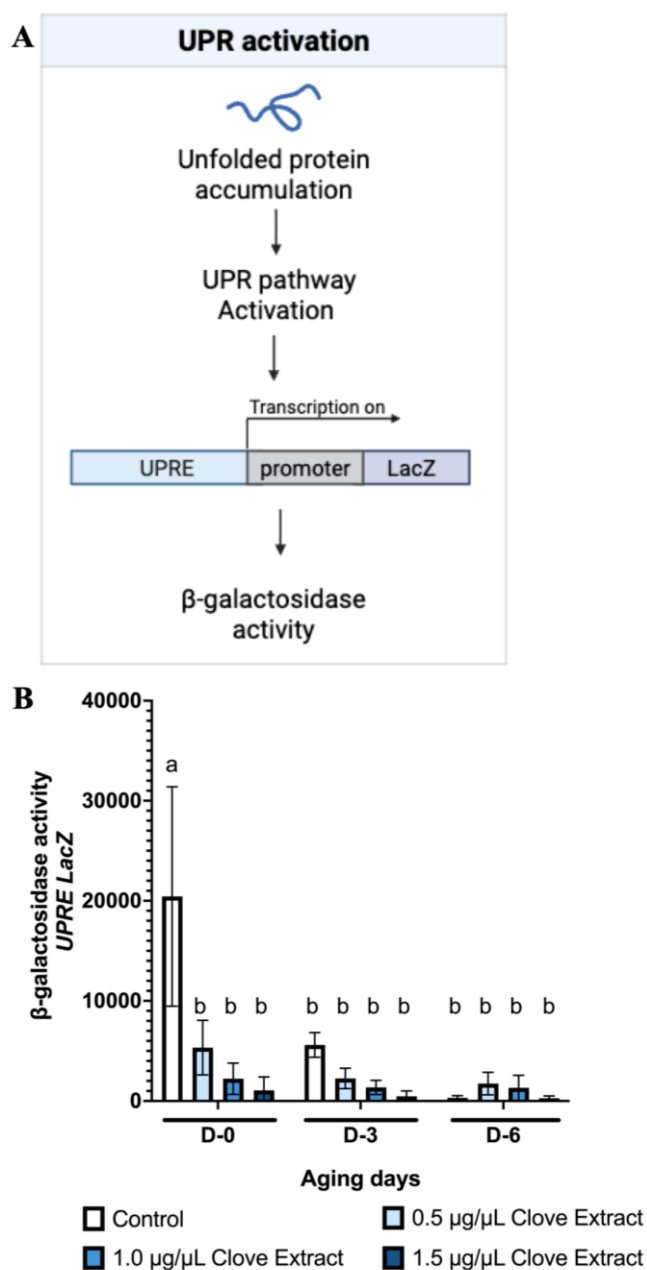


Fig.2 **A**) Schematic representation of the *UPRE-LacZ* reporter system used for monitoring unfolded protein response activity in *Saccharomyces cerevisiae* (created in <https://BioRender.com>). **B**) UPR activity decreased in aged yeast cells treated with clove extract compared with the vehicle control. The data were shown as the mean values and standard deviations (error bars) of three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test. Values with different letters indicate statistically significant differences ($p < 0.05$), whereas values that share the same letter are not significantly different ($p > 0.05$).

neutralizing oxidative stress at the source, the extract reduces the cellular burden on the ER, making high-level UPR activation unnecessary. While the UPR is initially adaptive, prolonged or chronic activation in aging can trigger apoptotic cell death (21, 40). By attenuating the UPR signal, clove extract may prevent aged cells from reaching the pro-

apoptotic threshold of ER stress, thereby extending the chronological lifespan.

Limitations and future perspectives

While our study demonstrates that UPR activity naturally declines as aging progresses, treatment with clove extract was found to further attenuate this signaling while simultaneously enhancing tolerance to ER stress. This suggests a functional uncoupling where the survival benefits of the extract do not rely on the induction of the UPR pathway. However, this study is limited by the use of a single β -galactosidase reporter system, which may not fully reflect the complexities of UPR regulation. Future research should incorporate transcriptional analysis of target genes and proteins to provide a more comprehensive assessment. Furthermore, while clove extract likely promotes proteostasis via UPR-independent mechanisms, possibly by reducing the initial burden of misfolded proteins through its known antioxidant properties, the specific involvement of alternative pathways such as autophagy or proteasomal degradation remains unclear. Subsequent investigations exploring these proteostasis-regulating processes are warranted to fully elucidate the underlying mechanisms by which clove extract promotes healthy aging and mitigates proteotoxic stress

IV. CONCLUSION

Our study demonstrates that UPR activity declines as aging progresses, a finding consistent with the age-associated impairment of proteostasis. We found that clove extract treatment significantly reduces UPR activity while simultaneously enhancing tolerance to ER stress, suggesting that the survival benefits provided by the extract do not rely on the induction of the UPR pathway. When integrated with our previous findings, which showed that clove extract extends chronological lifespan by reducing ROS levels and protein oxidative damage and aggregation, this study suggests that the extract promotes longevity and ER stress tolerance through alternative protective mechanisms that operate independently of UPR signaling. Although the specific nature of these mechanisms remains to be fully elucidated, these findings highlight the anti-aging potential of clove extract and its promise as a natural intervention for promoting healthy aging by maintaining cellular proteostasis.

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Ethanollic Extraction and Comparative Evaluation of Total Flavonoid Content and Antibacterial Activity of Leaf and Branch of *Cratoxylum formosum* Against *Streptococcus mutans*

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Abstract. Dental caries is a prevalent oral disease primarily associated with *Streptococcus mutans*. Natural plant-derived compounds have gained increasing attention as alternative antimicrobial agents. This study aimed to compare the total flavonoid content (TFC) and antibacterial activity of ethanol extracts from the leaf and branch of *Cratoxylum formosum*. TFC was determined using the aluminum chloride colorimetric method, and antibacterial activity was evaluated using the disc diffusion assay. The leaf extract exhibited significantly higher TFC (11.41 ± 1.23 mg/g) compared to the branch extract (5.44 ± 2.15 mg/g) ($p < 0.05$). However, the branch extract showed slightly greater antibacterial activity against *S. mutans*, with inhibition zones of 7.1 ± 0.3 mm at 24 h and 6.5 ± 0.3 mm at 48 h, compared to 6.7 ± 0.2 mm and 6.2 ± 0.1 mm for the leaf extract, respectively. Despite the higher flavonoid content in the leaf extract, no direct correlation between total flavonoid content and antibacterial activity was observed. This suggests that antibacterial activity may depend on specific bioactive compounds or synergistic interactions rather than total flavonoid content alone. However, both extracts exhibited lower activity than chlorhexidine. These findings highlight the complexity of plant-derived antibacterial agents and support further investigation into their active constituents.

Index Terms— *Streptococcus mutans*, *Cratoxylum formosum*, Total Flavonoid Content, Disc Diffusion Test

I. INTRODUCTION

Dental caries remains one of the most prevalent oral diseases worldwide and is primarily associated with *Streptococcus mutans*, a key bacterium responsible for biofilm formation and acid production leading to enamel demineralization. Although chemical antimicrobial agents such as chlorhexidine are widely used, their long-term use may cause adverse effects and contribute to antimicrobial resistance. Natural products derived from medicinal plants have gained increasing attention as alternative antibacterial agents. Among these, flavonoids are widely recognized for their antimicrobial properties, including membrane disruption, inhibition of nucleic acid synthesis, and

interference with bacterial metabolism. *Cratoxylum formosum*, a medicinal plant commonly found in Southeast Asia, has been reported to contain flavonoids and other phenolic compounds with biological activities. However, comparative information regarding the distribution of bioactive compounds between different plant parts and their relationship with antibacterial activity remains limited. Importantly, the correlation between total flavonoid content and antibacterial activity against *S. mutans* has not been clearly established. Therefore, this study aims to compare the total flavonoid content and antibacterial activity of leaf and branch extracts of *C. formosum*, and to evaluate whether flavonoid content is directly associated with antibacterial efficacy.

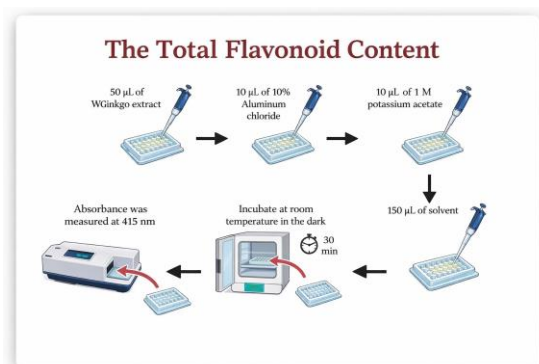
II. METHODOLOGY

Plant extraction

Dried leaf and branch samples of *Cratoxylum formosum* were ground into powder and extracted using 80% ethanol by maceration at a ratio of 1:10 (w/v) for 72 h at room temperature with occasional shaking. The extracts were filtered and concentrated under reduced pressure using a rotary evaporator.

Total Flavonoid Content

Total flavonoid content (TFC) was determined using the aluminum chloride colorimetric method and expressed as mg quercetin equivalent per gram of extract (mg QE/g).

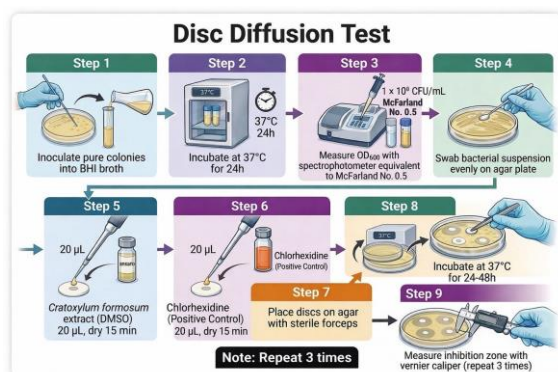


Bacteria and culture condition

The bacterial strain *S. mutans* ATCC 27175 used in this study was kindly provided by the Faculty of Medicine, Srinakharinwirot University, Thailand. The bacteria were cultured on brain heart infusion (BHI) agar and incubated at 37 °C under aerobic conditions. A few colonies from well-isolated plates were inoculated into BHI broth and incubated overnight (24 h) at 37 °C. The culture turbidity was adjusted to an optical density at 600 nm (OD₆₀₀) of 0.10–0.12, which corresponded to approximately 10⁸ colony-forming units CFU/mL.

Disc diffusion assay

Antibacterial activity was evaluated using the disc diffusion method. Sterile paper discs (6 mm diameter) were impregnated with 20 μ L of extract (100 mg/mL) and placed on inoculated agar plates. Chlorhexidine (0.12%) was used as a positive control and DMSO as a negative control. Plates were incubated at 37°C for 24–48 h, and inhibition zones were measured in millimeters.

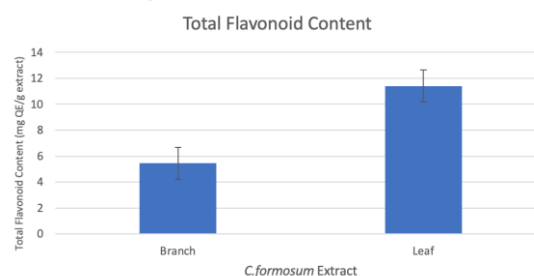


All experiments were performed in triplicate (n = 3).

III. RESULTS AND DISCUSSION

Extract	Total Flavonoid Content
	QE (mg/g)
Branch *	5.44 $\pm$ 2.15
Leaf *	11.41 $\pm$ 1.23

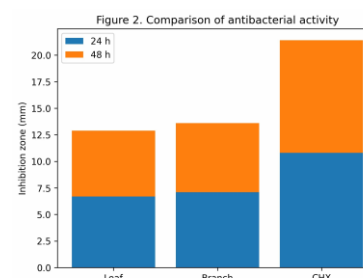
Table 1 Total Flavonoid Content (TFC) of ethanol extracts from branch and leaf of *Cratoxylum formosum*
*Values are mean of five samples $\pm$ SD



The results of total flavonoid content (TFC) analysis of the ethanol (EtOH) extract from *C. formosum* showed that the leaf contained a higher flavonoid content than the branch. The leaf extract showed 11.41 $\pm$ 1.23 mg/g, while the branch extract showed 5.44 $\pm$ 2.15 mg/g. These results indicate that flavonoids are more abundant in the leaves than in the branches. Flavonoids are well-known plant secondary metabolites that possess significant antibacterial properties. According to Shamsudin et al., (2022) flavonoids can inhibit bacterial growth through several mechanisms, including disruption of the bacterial cell membrane, inhibition of nucleic acid synthesis, and interference with energy metabolism. In addition, flavonoids have been reported to inhibit the adhesion and biofilm formation of *S. mutans*, which is a critical step in the development of dental plaque and dental caries. The inhibition of biofilm formation may occur through the suppression of glucosyltransferase enzymes that are responsible for synthesizing extracellular polysaccharides, which are essential for bacterial attachment to tooth surfaces. Therefore, the presence of flavonoids in *C. formosum* extracts may contribute to the antibacterial activity observed against *S. mutans* in this study.

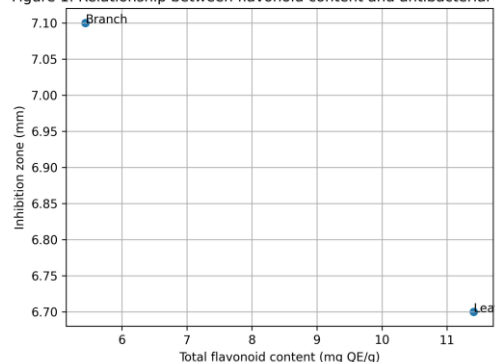
Extract	Inhibition Zone (mm)	
	24 h.	48 h.
Branch *	7.13 $\pm$ 0.31	6.53 $\pm$ 0.25
Leaf *	6.67 $\pm$ 0.15	6.20 $\pm$ 0.10
Chlorhexidine*	10.77 $\pm$ 0.15	10.77 $\pm$ 0.12

Table 2 antibacterial activity of ethanol extracts from branch and leaf of *Cratoxylum formosum* against *Streptococcus mutans*
*Values are mean of three samples



The antibacterial activity of ethanol extracts from the branch and leaf of *C. formosum* against *S. mutans* was evaluated using the disc diffusion test. The branch extract showed inhibition zones of 7.13 ± 0.31 mm at 24 hours and 6.53 ± 0.25 mm at 48 hours, while the leaf extract showed 6.67 ± 0.15 mm at 24 hours and 6.20 ± 0.10 mm at 48 hours. The positive control, chlorhexidine, exhibited significantly larger inhibition zones of 10.77 ± 0.15 mm at 24 hours and 10.77 ± 0.12 mm at 48 hours. These results indicate that both branch and leaf extracts possess antibacterial activity against *S. mutans*, although their inhibitory effects are lower than that of chlorhexidine.

Figure 1. Relationship between flavonoid content and antibacterial activity



Although the leaf extract contained a significantly higher total flavonoid content, the branch extract demonstrated slightly stronger antibacterial activity against *S. mutans*. This indicates that total flavonoid content alone may not be a reliable predictor of antibacterial efficacy. This discrepancy may be explained by several factors. First, antibacterial activity may depend on specific bioactive compounds rather than the total flavonoid content. Previous studies have reported that *Cratoxylum* species contain xanthenes and other phenolic compounds with potent antimicrobial activity, which may be more abundant in woody tissues such as branches. Second, the structural diversity of flavonoids plays a critical role in their biological activity. Therefore, the qualitative composition of flavonoids may be more important than their total quantity. Third, synergistic interactions among phytochemicals may contribute to antibacterial activity. Such interactions may enhance the biological effect even when the total concentration of flavonoids is relatively low. Additionally, the relatively small inhibition zones observed (~6–7 mm) suggest weak antibacterial activity compared to chlorhexidine, which is consistent with the limitations of crude plant extracts.

IV. CONCLUSION

This study demonstrated that both leaf and branch extracts of *C. formosum* possess antibacterial activity against *S. mutans*. Although the leaf extract contained higher total flavonoid content, the branch extract exhibited slightly stronger antibacterial activity. These findings indicate that

total flavonoid content does not directly correlate with antibacterial efficacy. The observed activity may be attributed to specific bioactive compounds or synergistic interactions. However, the antibacterial activity of both extracts was relatively weak compared to chlorhexidine. Therefore, further studies including MIC/MBC determination, biofilm inhibition assays, and phytochemical profiling are required to identify active compounds and enhance their potential application.

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The author would like to express sincere gratitude to the Center for Excellence in Plant and Herbal Innovation Research (CEPHIR) and the Strategic Wisdom and Research Institute of Srinakharinwirot University for their generous contributions and collaboration, which have been crucial in enabling the completion of this study. The author would also like to express heartfelt appreciation to our family for their continuous encouragement, support, and inspiration throughout this project. In addition, sincere thanks are extended to Phuket wittayalai school and teachers for their guidance and support in facilitating this research.

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Product Development of Healthy Snack Bar from Milky Mushroom (*Calocybe indica*): Effect of Microwave-Assisted Extraction on Antioxidant Properties

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Abstract. Milky mushroom (*Calocybe indica*) is a nutritious edible mushroom with reported medicinal properties. This study aimed to investigate the effect of microwave-assisted extraction (MAE) using ethanol at various concentrations (0, 20, 40, 60, 80, and 100%) on the extraction yield, total phenolic content (TPC), and antioxidant activities of milky mushroom extracts, as well as to develop a healthy snack bar product. The fresh milky mushroom contained 87.56% moisture and 0.94% ash. Among the extraction conditions, 80% ethanol yielded the highest extraction yield (38.32%). The water extract exhibited the highest TPC (3.91 mg GAE/g extract). The 40% ethanol extract showed the greatest DPPH radical scavenging activity (114.55 $\mu\text{mol TE/g}$ extract), while the 80% ethanol extract demonstrated the highest ABTS radical scavenging activity (523.53 $\mu\text{mol TE/g}$ extract). The 100% ethanol extract exhibited the highest ferric reducing antioxidant power (1595.42 $\mu\text{mol TE/g}$ extract), and the 60% ethanol extract had the greatest metal chelating activity (1109.72 $\mu\text{mol EDTA/g}$ extract). For snack bar development, Formula 3 (40% dried milky mushroom and 20% oatmeal) exhibited the lowest water activity (0.31) and moisture content (4.69%), along with the highest lightness ($L^* = 67.06$) and total color difference ($\Delta E = 30.32$). These results suggest that milky mushroom is a rich source of antioxidants suitable for incorporation into functional food products.

Index Terms—Antioxidant activity, *Calocybe indica*, microwave-assisted extraction, milky mushroom, snack bar development

I. INTRODUCTION

(Milky mushroom (*Calocybe indica*) is a tropical edible mushroom belonging to the family Lyophyllaceae. Originally identified in West Bengal, India, it has since been commercially cultivated across tropical and subtropical regions [1]. This mushroom species is characterized by its large fruiting body, firm and chewy texture, mild chicken-like flavor, and appealing milky white appearance. These desirable characteristics have contributed to its growing popularity among consumers and food processors [2]. Milky mushroom is nutritionally rich, containing approximately 3.22% protein, 1.11% dietary fiber, 6.80% carbohydrates, 2.30% ash, and notable amounts of vitamins A, C, and E on a fresh weight basis [1]. Furthermore, it has been reported to possess anti-hyperglycemic and anti-hyperlipidemic properties [3].

Despite its nutritional merits, fresh milky mushroom has a relatively short postharvest shelf life due to rapid physical, chemical, and microbiological deterioration, including browning, off-flavor development, and spoilage [4]. These quality changes result in significant economic losses for mushroom growers. Therefore, processing fresh milky mushroom into value-added food products represents a promising approach to extend shelf life, enhance economic value, and provide additional income for farmers.

Snack bars have gained considerable popularity as convenient, ready-to-eat food items with appealing taste and attractive appearance [5]. The incorporation of mushroom powder into snack bar formulations has recently emerged as a strategy to develop functional foods with enhanced nutritional and bioactive properties [6], [7]. Mushroom-based ingredients contribute antioxidant compounds, dietary fiber, and protein, thereby improving the health-promoting attributes of conventional snack products [8].

Microwave-assisted extraction (MAE) is an efficient technique for recovering bioactive compounds from plant and fungal materials. MAE offers advantages over conventional extraction methods, including shorter extraction time, reduced solvent consumption, and higher extraction efficiency [9]. The choice of solvent and its concentration are critical factors influencing the yield and composition of extracted bioactive compounds [10].

Therefore, the objectives of this study were to: (1) determine the proximate chemical composition of fresh milky mushroom; (2) evaluate the effect of ethanol concentration on the extraction yield, total phenolic content, and antioxidant activities of milky mushroom extracts obtained by MAE; and (3) develop a healthy snack bar product incorporating dried milky mushroom.

II. METHODOLOGY

A. Chemical Composition Analysis of Fresh Milky Mushroom

Fresh milky mushrooms were obtained from the mushroom cultivation plots at the Faculty of Agricultural Production, Maejo University, Chiang Mai, Thailand. The samples were washed, air-dried, and trimmed into pieces of approximately 0.5×0.5 cm. Moisture and ash contents were determined according to the standard methods of AOAC [11].

B. Microwave-Assisted Extraction of Milky Mushroom

MAE was performed following the modified method of Chen and Xiao-Feng [9]. Dried milky mushroom powder (7 g) was mixed with 210 mL of ethanol at concentrations of 0, 20, 40, 60, 80, and 100% (v/v). Extraction was carried out using a microwave oven (EME2024MW, Electrolux, Sweden) at 350 W for 10 min. The extract was filtered through Whatman No. 1 filter paper and the ethanol was removed using a rotary evaporator (Büchi, Switzerland) at 50 °C. The concentrated extract was subsequently freeze-dried (Dura-Dry II MP, Kinetecs, Thailand) and stored at -18 °C in sealed containers until further analysis. Extraction yield was calculated as a percentage of extract weight relative to the initial fresh mushroom weight.

C. Total Phenolic Content (TPC)

TPC was determined using the Folin-Ciocalteu method as described by Buamard and Benjakul [12]. Mushroom extract (10 mg/mL, 200 μ L) was mixed with 1.5 mL of 10% Folin-Ciocalteu reagent, allowed to stand for 5 min, and then 1.5 mL of 6% (w/v) sodium carbonate was added. The mixture was incubated in the dark at room temperature for 1 h. Absorbance was measured at 765 nm using a spectrophotometer (VIS-723G, Rayleigh, China). Results were expressed as mg gallic acid equivalents (GAE) per gram of extract.

D. Antioxidant Activity Assays

1) *DPPH Radical Scavenging Activity*: The DPPH assay was conducted following the method of Wu et al. [13].

Mushroom extract (1.5 mL) was mixed with 1.5 mL of 0.15 mM DPPH solution and incubated in the dark at room temperature for 30 min. Absorbance was measured at 517 nm. Results were expressed as μ mol Trolox equivalents (TE) per gram of extract.

2) *ABTS Radical Scavenging Activity*: The ABTS assay was performed according to Binsan et al. [14]. ABTS radical cation was generated by mixing 7.4 mM ABTS solution with 2.6 mM potassium persulfate (1:1, v/v) and incubating in the dark for 12 h at room temperature. The stock solution was diluted with distilled water (1:25, v/v) to obtain an absorbance of 1.1 ± 0.02 at 734 nm. Mushroom extract (150 μ L) was mixed with 2850 μ L of diluted ABTS solution. Results were expressed as μ mol TE per gram of extract.

3) *Ferric Reducing Antioxidant Power (FRAP)*: The FRAP assay was conducted as described by Benzie and Strain [15]. The FRAP reagent was prepared by combining 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mM HCl, and 10 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$. The mixture was incubated at 37 °C for 30 min. Mushroom extract (150 μ L) was mixed with 2850 μ L of FRAP reagent and incubated in the dark for 30 min. Absorbance was measured at 593 nm. Results were expressed as μ mol TE per gram of extract.

4) *Metal Chelating Activity*: Metal chelating activity was determined according to Thiansilakul et al. [16]. Mushroom extract (2.35 mL) was mixed with 0.05 mL of 2 mM FeCl_2 and 0.1 mL of 5 mM ferrozine, then incubated for 20 min at room temperature. Absorbance was measured at 562 nm. Results were expressed as μ mol EDTA equivalents per gram of extract.

E. Snack Bar Development

Three snack bar formulations were prepared with different ratios of dried milky mushroom to oatmeal, adapted from a previously described method [17]. The formulations are shown in Table I. Dried milky mushroom was prepared by cutting fresh mushroom into small pieces and drying in a hot air oven (Binder Model ED53, Germany) at 70 °C for 1 h, followed by light pan-roasting to reduce residual moisture. The dried mushroom was then combined with other ingredients (syrup, honey, salt, raisins, and cranberries) and pressed into bar-shaped molds. The finished snack bars were evaluated for water activity (a_w) using a water activity meter (AquaLab Series 3, Meter Group, USA), color parameters (L^* , a^* , b^* , and ΔE) using a colorimeter (ColorFlex EZ, HunterLab, Virginia, USA), and moisture content following AOAC methods [11].

III. RESULTS AND DISCUSSION

A. Chemical Composition of Fresh Milky Mushroom

The chemical composition of fresh milky mushroom is presented in Table II. The moisture content was $87.56 \pm 0.38\%$, and the ash content was $0.94 \pm 0.21\%$. The high moisture content is characteristic of fresh edible mushrooms

TABLE I

SNACK BAR FORMULATIONS WITH VARYING PROPORTIONS OF MILKY MUSHROOM AND OATMEAL

Formula	Dried milky mushroom (%)	Oatmeal (%)	Other ingredients (%)
1	20	40	40
2	30	30	40
3	40	20	40

Other ingredients include syrup, honey, salt, raisins, and cranberries in equal proportions across all formulations.

mushrooms and is attributable to the humid growing conditions typical of enclosed mushroom cultivation facilities [1]. The relatively low ash content corresponds to the high-water proportion in fresh mushrooms, which dilutes the concentration of mineral residues. These findings are consistent with previously reported values for tropical milky mushrooms, where compositional variations may arise from differences in substrate, cultivation conditions, mushroom age, and the specific portion of the fruiting body analyzed [1], [3].

TABLE II

CHEMICAL COMPOSITION OF FRESH MILKY MUSHROOM

Parameters	Content (%)
Moisture	87.56 ± 0.38
Ash	0.94 ± 0.21

Values are expressed as mean ± SD (n = 3).

B. Extraction Yield

The extraction yields of milky mushroom extracts obtained using different ethanol concentrations are shown in Table III. The yields ranged from 1.69% (water extraction) to 38.32% (80% ethanol). The 80% ethanol extract produced the highest yield, followed by 100% ethanol (26.86%), 40% ethanol (10.67%), 60% ethanol (7.96%), 20% ethanol (4.77%), and

water (1.69%). The influence of ethanol concentration on extraction yield can be attributed to the polarity differences of the solvent system. Water preferentially extracts polar compounds, whereas ethanol at higher concentrations facilitates the co-extraction of both polar and non-polar bioactive constituents [10], [18]. The optimal yield at 80% ethanol suggests that this concentration provides a favorable balance of solvent polarity for extracting the broadest range of compounds from milky mushroom.

TABLE III

EXTRACTION YIELD OF MILKY MUSHROOM EXTRACTS AT VARIOUS ETHANOL CONCENTRATIONS

Ethanol concentrations (%)	Extraction yield (%)
0 (water)	1.69
20	4.77
40	10.67
60	7.96
80	38.32
100	26.86

C. Total Phenolic Content

The TPC values of milky mushroom extracts obtained with ethanol at concentrations of 0, 20, 40, 60, 80, and 100% were 3.91, 1.69, 0.90, 1.09, 0.35, and 0.52 mg GAE/g extract, respectively (Fig. II). The water extract (0% ethanol) exhibited the highest TPC. Phenolic compounds are predominantly polar molecules that are readily soluble in aqueous solvents [19]. This finding is consistent with previous reports indicating that water is an effective solvent for extracting phenolic compounds from mushroom matrices [3], [20]. The decreasing TPC trend with increasing ethanol concentration suggests that the phenolic constituents of milky mushroom are primarily hydrophilic in nature.

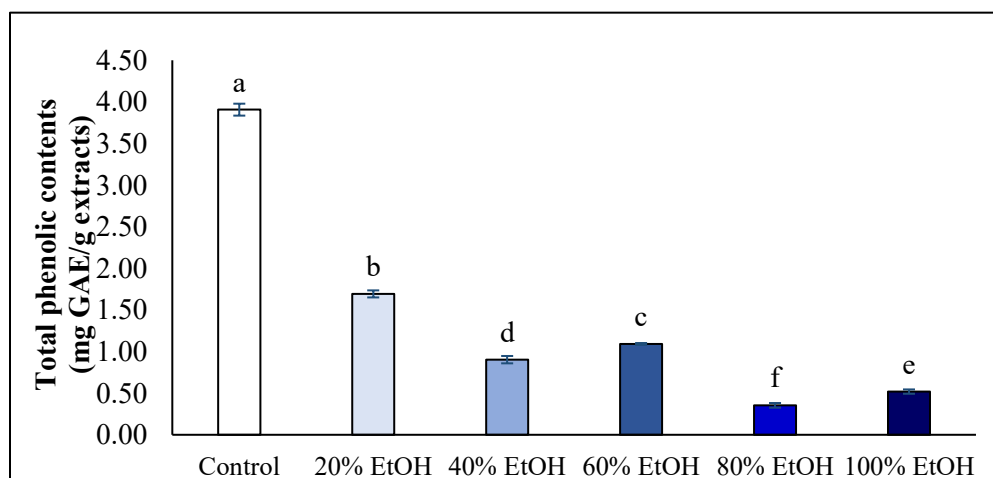


Fig. I. Total phenolic content of milky mushroom extracts obtained by microwave-assisted extraction using ethanol at various concentrations

D. Antioxidant Activities

The antioxidant activities of milky mushroom extracts at various ethanol concentrations are summarized in Fig. II- V. Different assay methods yielded distinctly different optimal ethanol concentrations, reflecting the diversity of antioxidant mechanisms and the varying polarities of bioactive compounds present in milky mushroom.

The DPPH assay measures the ability of antioxidant compounds to donate hydrogen atoms or electrons to neutralize the stable DPPH radical [13]. The 40% ethanol extract exhibited the highest DPPH radical scavenging activity (114.55 $\mu\text{mol TE/g extract}$), whereas the 60% and 80% ethanol extracts showed markedly lower activities (21.15 and 20.22 $\mu\text{mol TE/g extract}$, respectively). This pattern suggests that the hydrogen-donating antioxidants in milky mushroom, such as flavonoids and certain phenolic acids, are moderately polar compounds that partition most effectively into a 40% aqueous-ethanol mixture. At higher ethanol concentrations, these polar hydrogen donors are less soluble, leading to reduced extraction efficiency [10].

In contrast, the ABTS assay evaluates antioxidant capacity through both electron transfer and hydrogen atom donation mechanisms, and is applicable to both hydrophilic and lipophilic antioxidants [21], [22]. The 80% ethanol extract demonstrated the highest ABTS scavenging capacity (523.53 $\mu\text{mol TE/g extract}$), a value approximately 4.6-fold greater than the second-highest concentration (100% ethanol, 100.58 $\mu\text{mol TE/g extract}$). This exceptionally high activity at 80% ethanol indicates the presence of unique semi-polar antioxidant compounds, potentially including terpenoids, sterols, or non-polar phenolic derivatives, that are selectively extracted under this condition [23]. The ABTS radical cation is soluble in both aqueous and organic media, allowing it to react with a broader range of antioxidant species than the DPPH radical, which explains why the optimal concentration differs between the two assays.

The FRAP assay quantifies the electron-donating capacity of antioxidants by measuring the reduction of a ferric tripyridyltriazine complex to its ferrous form [15]. The 100% ethanol extract exhibited the highest FRAP value (1595.42

$\mu\text{mol TE/g extract}$), approximately 2.3-fold greater than the second-highest concentration (20% ethanol, 683.53 $\mu\text{mol TE/g extract}$). This result demonstrates the presence of potent reducing agents with low polarity in milky mushroom, such as tocopherols, carotenoids, or ergosterol derivatives, that are preferentially dissolved in absolute ethanol [24]. Notably, the FRAP values followed a non-linear pattern across the ethanol gradient, with 60% ethanol yielding the lowest activity (82.74 $\mu\text{mol TE/g extract}$), suggesting a complex interplay between solvent polarity and the solubility of different classes of reducing compounds.

The metal chelating activity assay measures the ability of antioxidant compounds to sequester pro-oxidant transition metal ions, particularly Fe^{2+} , thereby preventing metal-catalyzed oxidative reactions [25]. The 60% ethanol extract showed the highest metal chelating activity (1109.72 $\mu\text{mol EDTA/g extract}$), approximately 2.6-fold greater than the second-highest concentration (100% ethanol, 500.47 $\mu\text{mol EDTA/g extract}$). This finding suggests that the chelating compounds in milky mushroom, such as certain peptides, organic acids, or phosphorylated compounds, have intermediate polarity and are most efficiently extracted at a moderate ethanol concentration [25].

The divergent optimal conditions across assays highlight that no single ethanol concentration simultaneously maximizes all antioxidant mechanisms. Nevertheless, from a comprehensive perspective, the 80% ethanol extract demonstrated the best overall antioxidant profile. This condition not only provided the highest extraction yield (38.32%) and the highest ABTS scavenging capacity (523.53 $\mu\text{mol TE/g extract}$), but also produced moderate FRAP activity (392.76 $\mu\text{mol TE/g extract}$) and metal chelating activity (298.73 $\mu\text{mol EDTA/g extract}$). When considering the combined efficiency of extraction and antioxidant capacity, 80% ethanol represents the most practical and industrially applicable solvent condition for recovering antioxidant-rich extracts from milky mushroom via MAE. This concentration achieves an optimal balance between solvent polarity and co-extraction of both hydrophilic and lipophilic bioactive constituents [10], [23].

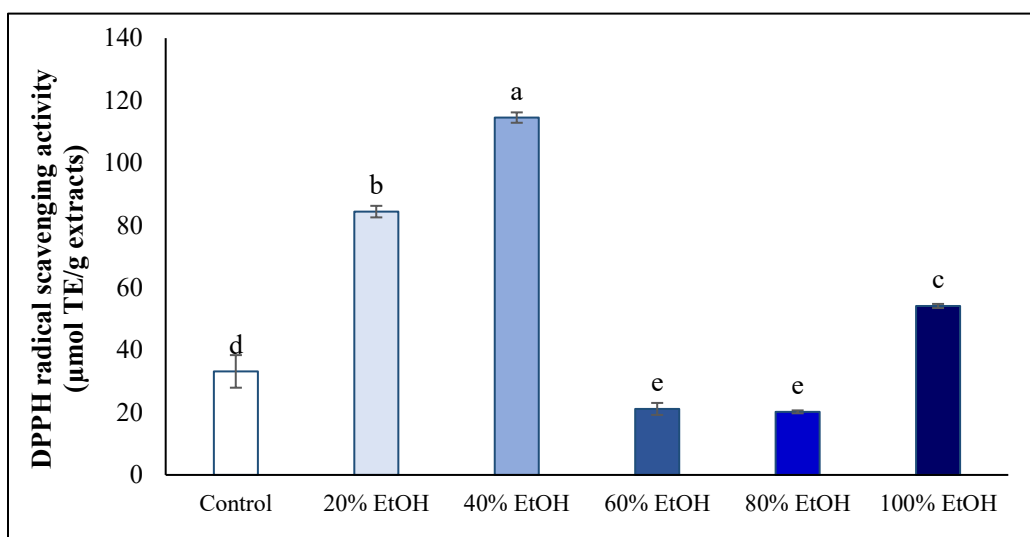


Fig. II. DPPH radical scavenging activity of milky mushroom extracts obtained by microwave-assisted extraction using ethanol at various concentrations.

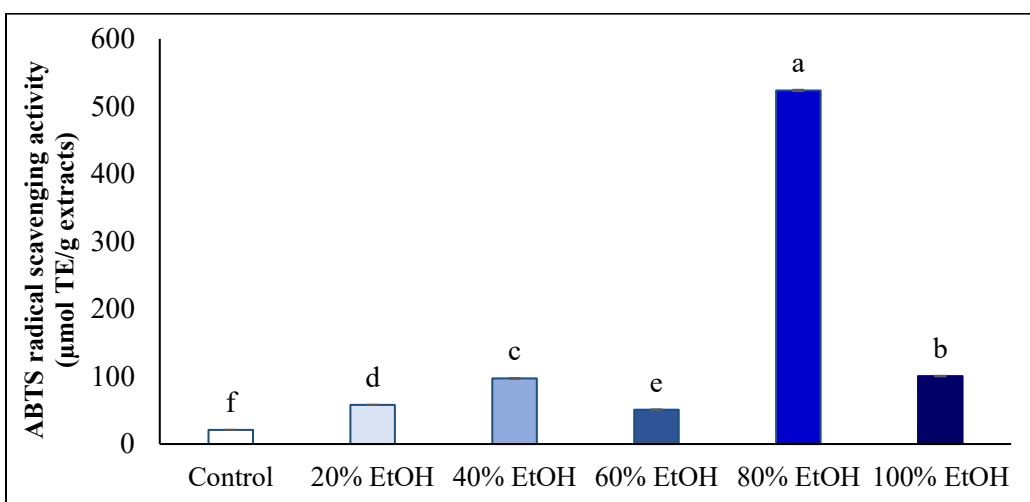


Fig. III. ABTS radical scavenging activity of milky mushroom extracts obtained by microwave-assisted extraction using ethanol at various concentrations.

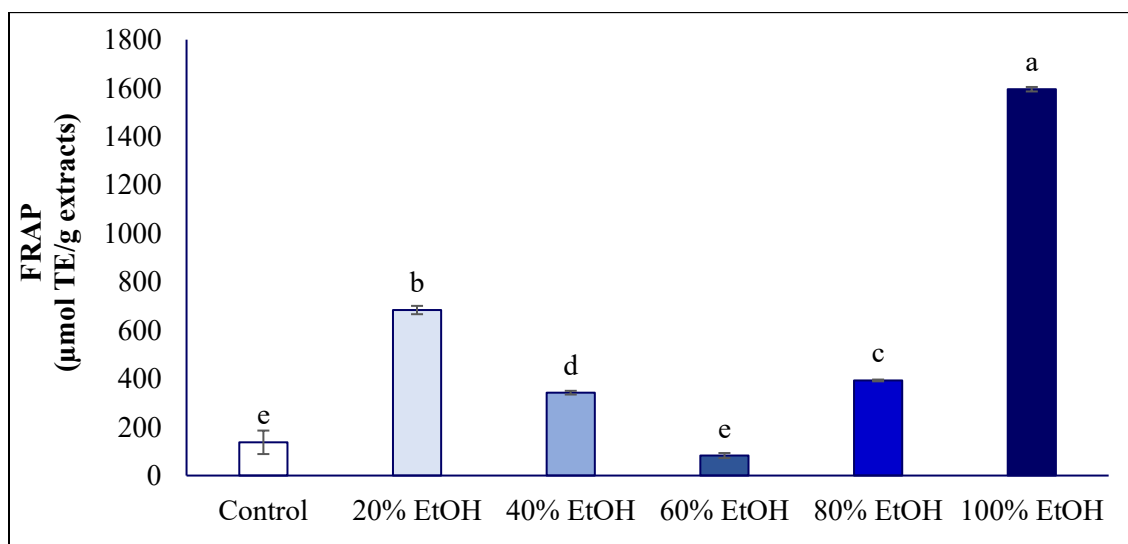


Fig. IV. Ferric reducing antioxidant power (FRAP) of milky mushroom extracts obtained by microwave-assisted extraction using ethanol at various concentrations.

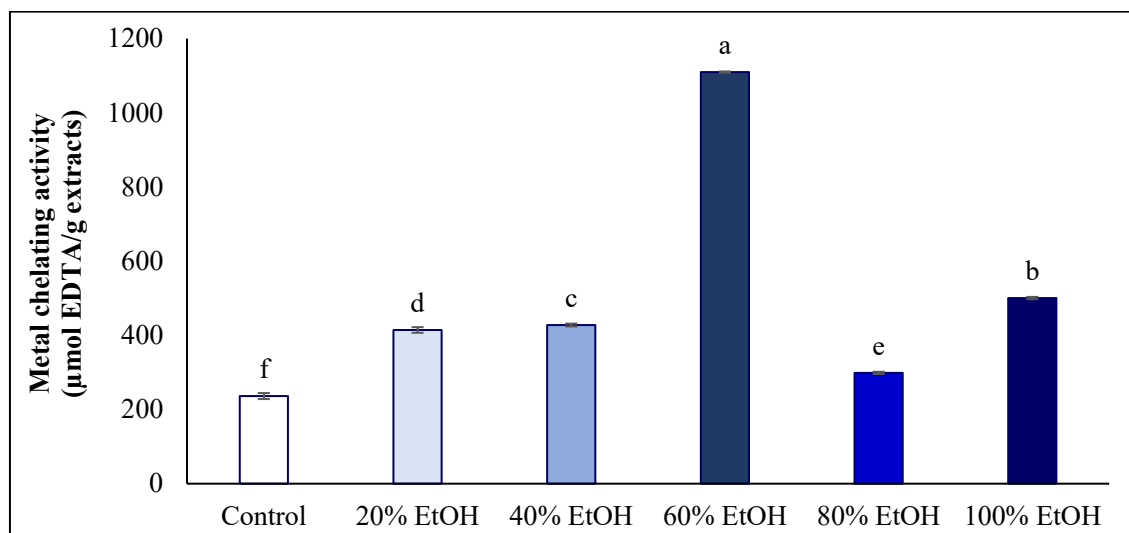


Fig. V. Metal chelating activity of milky mushroom extracts obtained by microwave-assisted extraction using ethanol at various concentrations.

E. Snack Bar Quality Characteristics

The physical and chemical quality parameters of milky mushroom snack bars are presented in Table IV. The water activity (a_w) values of Formulas 1, 2, and 3 were 0.39, 0.37, and 0.31, respectively. All formulations exhibited a_w values below 0.6, classifying them as dry food products that are microbiologically stable [26]. Formula 3, which contained the highest proportion of dried mushroom (40%) and the lowest proportion of oatmeal (20%), exhibited the lowest a_w . This can be attributed to the pre-drying treatment applied to the mushroom component. In contrast, oatmeal may have absorbed moisture from liquid ingredients such as honey and syrup.

Formula 3 exhibited the highest lightness ($L^* = 67.06$) and total color difference ($\Delta E = 30.32$), while Formula 1 showed

the highest redness ($a^* = 6.47$) and yellowness ($b^* = 26.29$). The higher L^* value of Formula 3 is attributable to the greater proportion of milky mushroom, which possesses a naturally pale-yellow color. The elevated a^* and b^* values of Formula 1 can be explained by its higher oatmeal content, which absorbed honey and underwent Maillard browning during thermal processing [27].

The moisture contents of Formulas 1, 2, and 3 were 6.21, 5.41, and 4.69%, respectively. Formula 3 had the lowest moisture content, consistent with its lowest a_w value. Low moisture content is desirable in snack bar products as it inhibits microbial growth and extends product shelf life [26], [28].

TABLE IV
PHYSICAL AND CHEMICAL QUALITY CHARACTERISTICS OF MILKY MUSHROOM SNACK BARS

Formula	L^*	a^*	b^*	ΔE	a_w	Moisture (%)
1 (20:40)	62.76 ± 1.98	6.47 ± 0.57	26.29 ± 1.07	25.95 ± 2.01	0.39 ± 0.00	6.21 ± 0.62
2 (30:30)	64.84 ± 1.14	6.11 ± 0.30	24.51 ± 0.57	28.02 ± 1.10	0.37 ± 0.06	5.41 ± 0.23
3 (40:20)	67.06 ± 1.89	5.69 ± 0.36	23.68 ± 0.57	30.32 ± 1.97	0.31 ± 0.01	4.69 ± 0.27

L^* = lightness; a^* = redness; b^* = yellowness; ΔE = total color difference; a_w = water activity. Formula ratios shown as mushroom:oatmeal (%). Values are expressed as mean $\pm$ SD

IV. CONCLUSION

This study demonstrated that milky mushroom (*Calocybe indica*) is a nutritious edible fungus with considerable antioxidant potential. The antioxidant activities of milky mushroom extracts obtained by MAE varied significantly depending on the ethanol concentration employed, with each assay yielding a different optimal concentration due to the distinct mechanisms and polarity preferences involved. The water extract yielded the highest phenolic content (3.91 mg GAE/g), the 40% ethanol extract showed the greatest DPPH radical scavenging activity (114.55 μ mol TE/g), the 80% ethanol extract exhibited the highest ABTS scavenging capacity (523.53 μ mol TE/g) and extraction yield (38.32%),

the 100% ethanol extract demonstrated the highest ferric reducing power (1595.42 μ mol TE/g), and the 60% ethanol extract possessed the strongest metal chelating ability (1109.72 μ mol EDTA/g). Considering the combined extraction efficiency and broad-spectrum antioxidant capacity, 80% ethanol is recommended as the most suitable solvent condition for MAE of milky mushroom bioactive compounds. Among the three snack bar formulations, Formula 3 (40% dried mushroom and 20% oatmeal) demonstrated the most favorable quality attributes, including the lowest water activity and moisture content and the highest lightness. Therefore, milky mushroom represents a

promising functional ingredient for the development of health-promoting snack products.

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Zero-Waste Green Extraction of *Mitragyna speciosa* Korth. Using Coconut Milk: Characterization of Bioactive-Rich Oil and Nutrient-Dense By-product

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Abstract. This study proposes a sustainable, zero-waste approach for the recovery of bioactive compounds from *Mitragyna speciosa* Korth. (Kratom) leaves using coconut milk as a natural bio-solvent via a cold extraction technique. The process was designed to achieve total biomass utilization by partitioning the extracts into two distinct functional streams: coconut oil with Kratom extract (COK) and cream coconut-kratom extract residue (CCR). Experimental results demonstrated that a weight ratio of 1:0.4 (coconut meat to Kratom leaves) provided the optimal equilibrium for bioactive transfer. At this ratio, the COK exhibited a significant total phenolic content of 18.76 ± 0.61 mg GAE/g and a DPPH radical scavenging activity of $34.73 \pm 0.25\%$. Remarkably, the CCR, characterized as a "nutrient-dense super-byproduct," outperformed the oil fraction in antioxidant capacity with an inhibition of $39.15 \pm 0.31\%$. Proximate analysis revealed that the CCR is a rich source of macronutrients, containing 19.70% crude protein and 24.79% total fat, with a total energy value of 303.43 kcal/100g. These findings confirm that coconut milk-based cold extraction successfully partitions potent antioxidants into the residue matrix while enriching the oil phase with lipophilic bioactives. This dual-product strategy redefines traditional extraction waste as a novel, high-protein, antioxidant-rich material for upcycling into functional animal feed or nutritional supplements. The study provides a robust framework for green, zero-waste botanical processing that maximizes both the commercial and nutritional value of *M. speciosa*.

Index Terms— Antioxidant activity, Coconut milk, Green extraction, *Mitragyna speciosa* Korth

I. INTRODUCTION

Kratom (*Mitragyna speciosa* Korth.), a medicinal plant indigenous to Southeast Asia, has transitioned from traditional local use to gaining global recognition for its remarkable pharmacological potential. This international interest is primarily driven by its diverse profile of phytochemicals, most notably bioactive indole alkaloids and phenolic compounds. Mitragynine, the predominant alkaloid, plays a pivotal role in these biological activities, including potent antioxidant effects achieved through the scavenging of free radicals and the mitigation of cellular oxidative stress [1]. Furthermore, *M. speciosa* exhibits significant anti-inflammatory properties by downregulating the expression of key pro-inflammatory mediators at the genetic level, such as cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE2), and various cytokines [2], [3]. Given these multifaceted biological activities, Kratom leaves

represent a high-value raw material for the development of innovative functional foods and modern health products. Despite its therapeutic promise, the commercial and clinical application of mitragynine is severely constrained by its inherent physicochemical properties. Mitragynine is a highly lipophilic compound characterized by exceptionally poor aqueous solubility, particularly under neutral pH conditions [4]. This solubility constraint serves as a critical rate-limiting barrier during the extraction process and contributes significantly to its low oral bioavailability; insoluble compounds struggle to effectively permeate the intestinal epithelium to reach systemic circulation [5]. Additionally, mitragynine is susceptible to acid-induced degradation in the gastric environment, leading to chemical instability prior to absorption [6]. Therefore, developing specialized delivery systems or alternative solvents capable of enhancing encapsulation and solubilization is essential to ensure the consistent efficacy of Kratom-based formulations. Traditional extraction methods for recovering Kratom

alkaloids typically employ synthetic organic solvents such as methanol, ethanol, or chloroform. While efficient, these solvents present significant challenges regarding regulatory compliance and consumer safety. A primary concern is the potential for residual solvent toxicity; incomplete removal during purification can leave harmful traces that pose health risks and compromise pharmaceutical purity standards [7]. Furthermore, industrial-scale use of synthetic chemicals entails a substantial environmental footprint and energy-intensive recovery processes, which conflict with the principles of green chemistry [8]. Consequently, research has increasingly shifted toward identifying natural "green" solvents to mitigate these adverse effects and develop food-grade extracts that ensure both human safety and environmental sustainability [9]. In response to these challenges, this study proposes the utilization of coconut milk as a natural bio-solvent to replace conventional organic chemicals. This innovative approach leverages the unique extraction mechanisms provided by medium-chain fatty acids (MCFAs) and natural proteins inherent in coconut milk. These constituents' function as biological emulsifiers, reducing interfacial tension and enhancing the solubility of lipophilic mitragynine through natural encapsulation [10], [11]. The selection of coconut milk not only aligns with sustainable eco-friendly practices but also facilitates the preparation of extracts with potentially enhanced bioavailability. By integrating these bioactive components into a stable, food-grade matrix, this method ensures the final product is optimized for physiological uptake without the presence of hazardous residues (Chemat et al., 2012). A distinguishing feature of this research is its "Zero-Waste" framework. Unlike traditional extraction which often results in large quantities of solvent and biomass waste, our proposed method partitions the entire biomass into two high-value streams: a bioactive-rich Kratom-infused coconut oil (COK) and a nutrient-dense coconut-kratom residue (CCR). By characterizing both the oil and the byproduct, this study aims to demonstrate a circular economy model where every component of the extraction process is upcycled into functional ingredients—minimizing environmental impact while maximizing economic value.

II. MATERIALS AND METHODS

2.1 Materials

Mature coconuts (*Cocos nucifera* L., 10–12 months old) were purchased from local markets in Thung Song District, Nakhon Si Thammarat, Thailand. Fresh Kratom leaves were obtained from a private farm in the same province. The leaves were selected based on uniformity in maturity, washed with deionized water, and air-dried at room temperature before extraction. All chemicals and reagents used in this study, including 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Folin-Ciocalteu reagent, were of analytical grade, purchased from J.T. Baker (Phillipsburg, NJ, USA) [12].

2.2 Production of COK and CCR

The extraction was based on green chemistry and zero-waste principles, using coconut milk as a natural bio-solvent for *M.*

speciosa leaves through a cold-extraction and fractionation approach.

2.2.1 Preparation of Coconut Milk and Kratom Slurry

Freshly grated coconut meat was homogenized with distilled water (1:1 w/w) and mechanically pressed to obtain coconut milk. Simultaneously, fresh *M. speciosa* leaves were washed and blended for 10–20 min to form a leaf slurry.

2.2.2 Ultrasound-Assisted Extraction (UAE)

To enhance bioactive recovery, the leaf slurry underwent ultrasonication for 20 min. The mixture was filtered through muslin cloth. The remaining pulp was re-blended and re-filtered to ensure exhaustive extraction of bound phenolics [13].

2.2.3 Fractionation and Solid-Liquid Separation

The Kratom extract was integrated with coconut milk and incubated at 4–5 °C for 12 h to allow natural phase separation and preserve heat-sensitive compounds [14]. The mixture was then centrifuged at 3,500 rpm for 10 min, yielding two fractions: COK: A lipid-rich fraction containing lipophilic bioactive compounds, CCR: A nutrient-dense cream residue containing protein-bound phenolics.

2.2.4 Storage and Quality Preservation The COK was filtered and stored in light-protected amber bottles at 4 °C to prevent photo-oxidation. The CCR byproduct was collected and stabilized for further applications, such as sustainable aquatic feed [15].

2.3 Determination of Total Phenolic Content (TPC)

The total phenolic content of COK and CCR was quantified using the Folin-Ciocalteu spectrophotometric method, based on the electron transfer from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes [16].

2.3.1 Sample Extraction

Ten grams (10 g) of COK were dissolved in 20 mL of hexane and transferred to a separatory funnel. Phenolic compounds were extracted using 10 mL of a methanol-water mixture (80:20 v/v) by vigorous shaking for 3 min. The lower methanolic phase was collected, and the process was repeated twice. All methanolic fractions were pooled for analysis.

2.3.2 Colorimetric Assay and Measurement

A 1 mL aliquot of the methanolic extract was mixed with 1 mL of Folin-Ciocalteu reagent (1:10 dilution) and 4 mL of 7.5% (w/v) sodium carbonate (Na_2CO_3) solution in a 10 mL volumetric flask. The volume was adjusted to 10 mL with deionized water. After 30 min of incubation in the dark at room temperature, the absorbance was measured at 725 nm using a UV-Vis spectrophotometer. TPC was calculated using a gallic acid standard curve (0.1–0.5 mg/mL) and expressed as mg gallic acid equivalents per gram of sample (mg GAE/g).

2.4 Determination of Antioxidant Activity (DPPH Assay)

The antioxidant capacity was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, modified from Zunin et al. (2010) for lipid-based samples [17]. One gram (1 g) of COK was dissolved in ethyl acetate and adjusted to 10 mL. A 2 mL aliquot of this solution was mixed with a 10^{-4} M DPPH solution in ethyl acetate to reach a final volume of 10 mL. The mixture was vortexed for 10 s and incubated in the dark at room temperature for 30 min.

The absorbance was measured at 515 nm against an ethyl acetate blank. A control (DPPH without oil) and a sample blank (oil without DPPH to account for background absorbance) were also measured. All samples were analyzed in triplicate. The percentage of DPPH radical inhibition was

III. RESULTS AND DISCUSSION

3.1 Extraction yield and process efficiency

The extraction yields observed in this study (13.00% – 14.75%; Table 1) demonstrate the high efficiency of coconut milk as a natural, bio-based solvent for *M. speciosa* extraction. The peak yield of $14.75 \pm 0.35\%$ at the 1:0.4 (w/w) ratio suggests an optimal mass transfer of Kratom's phytochemicals into the coconut oil matrix. While traditional organic solvents like methanol can achieve higher crude yields—often exceeding 20% [1]—they necessitate intensive purification to eliminate toxic residues. In contrast, this "Green Extraction" framework provides a ready-to-use infused oil, aligning with [18], who noted that natural emulsions effectively trap lipophilic compounds directly, reducing energy consumption and solvent waste. The consistent efficiency is largely attributed to Ultrasound-Assisted Extraction (UAE). Acoustic cavitation facilitates the disruption of plant cell walls, enhancing the release of intracellular mitragynine and alkaloids into the solvent [19], [20]. Mechanistically, the coconut milk emulsion system overcomes the solubility barriers typically faced in aqueous extractions [6]. The enhanced interfacial surface area in the oil-in-water (O/W) system allows alkaloids to partition rapidly from the cell homogenate into micro-sized lipid droplets. This micellar-enhanced transfer is consistent with McClements (2012) [10], who demonstrated that colloidal systems drastically reduce diffusion distances for hydrophobic bioactives compared to bulk lipid phases. The observed plateau in yield at the 1:0.4 ratio indicates a saturation point where the solvent reaches its maximum loading capacity. Furthermore, the "Zero-Waste" approach sets this study apart; unlike traditional methods that discard plant residue, our process partitions the biomass into high-value COK and CCR. This supports the Circular Economy model proposed by Ojha et al. (2020) [15], ensuring total biomass utilization and environmental sustainability.

TABLE I
YIELD OF KRATOM LEAF EXTRACT IN COCONUT
OIL AT VARIOUS RATIOS

Sample	Extraction Yield (% Yield)
COK1 (at 1:0.1 w/w of coconut meat to Kratom leaves)	13.25 ± 0.35^a
COK2 (at 1:0.2 w/w of coconut meat to Kratom leaves)	13.00 ± 0.71^a
COK3 (at 1:0.3 w/w of coconut meat to Kratom leaves)	14.75 ± 0.35^b
COK4 (at 1:0.4 w/w of coconut meat to Kratom leaves)	14.75 ± 0.35^b

Values are expressed as Mean $\pm$ SD (n=3). Different superscripts in the same column indicate significant differences ($p < 0.05$).

3.2 Antioxidant Activity and Scavenging Capacity of COK

The antioxidant activity of Kratom-infused coconut oil (COK) exhibited a significant dose-dependent increase corresponding to the *M. speciosa* to coconut meat ratios ($p < 0.05$). The DPPH scavenging activity rose from $24.55 \pm 0.26\%$ at a 1:0.1 ratio to a peak of $34.73 \pm 0.25\%$ at a 1:0.4 ratio (Table 2). Interestingly, while the total extraction yield reached a plateau at the 1:0.3 ratio ($14.75 \pm 0.35\%$), the antioxidant potency continued to climb significantly. This divergence suggests that the lipid phase maintained a high selective capacity for potent bioactive molecules, such as mitragynine and phenolic compounds, even as total mass transfer approached saturation. The superior efficiency of this emulsion-mediated approach can be attributed to the natural micelles formed by coconut milk proteins and medium-chain triglycerides (MCTs). These micelles provide a protective microenvironment that stabilizes and shields alkaloids and phenolics from oxidative degradation during the cold extraction process [21], [22]. Compared to conventional water-based extractions, which often result in poor recovery of lipophilic mitragynine due to its low aqueous solubility [6], the current green extraction framework provides a more effective vehicle for bioactive recovery. These results confirm that cold extraction successfully preserves the functional integrity of Kratom's phytochemicals, producing a potent, solvent-free antioxidant oil suitable for functional food and high-value aquafeed applications.

TABLE II
YIELD OF KRATOM LEAF EXTRACT IN COCONUT
OIL AT VARIOUS RATIOS

Sample	% Inhibition
Virgin Coconut Oil	10.28 ± 0.21^a
COK1	24.55 ± 0.26^b
COK2	26.91 ± 0.00^c
COK3	29.28 ± 0.77^d
COK4	34.73 ± 0.25^e

Values are expressed as Mean $\pm$ SD (n=3). Different superscripts in the same column indicate significant differences ($p < 0.05$).

The progressive enhancement of DPPH radical scavenging activity in COK samples highlights the successful recovery of bioactive compounds from *Mitragyna speciosa* into the lipid phase. The marked increase from the base VCO (10.28%) to the 1:0.4 COK (34.73%) suggests that the infusion process successfully transferred potent secondary metabolites—primarily mitragynine and phenolic compounds—into the oil. According to Goh et al. (2021) [14], the antioxidant properties of Kratom are attributed to its diverse alkaloid and flavonoid profile, which act as hydrogen donors to stabilize DPPH radicals.

The efficiency of this Green Extraction using coconut milk as a natural solvent is consistent with the findings of Ravisankar and Agila (2019) [23], who stated that natural emulsions can effectively stabilize plant-derived antioxidants. The use of ultrasonication further intensified this effect by ensuring the exhaustive release of these compounds from the leaf matrix [19]. Similar dose-dependent trends have been reported in other herbal-infused oils. For instance, Zunin et al. (2010) [17] observed that the antioxidant capacity of virgin oils significantly improves with the addition of botanical extracts rich in polyphenols. The 34.73% inhibition achieved in this study is noteworthy, as it was obtained using a cold-extraction technique that preserves the bioactivity of heat-sensitive compounds, unlike traditional hot-infusion methods that may lead to thermal degradation [24]. These results confirm that a 1:0.4 ratio is the most effective formulation for producing a bioactive-rich oil. This high scavenging activity not only provides potential health benefits for consumers but also helps extend the oxidative stability of the coconut oil itself.

3.3 Comparative Analysis of Phenolic Content and Antioxidant Activity

The comparative results of Total Phenolic Content (TPC) and DPPH radical scavenging activity among Virgin Coconut Oil (VCO), coconut oil with kratom extract (COK) and cream coconut-kratom extract residue (CCR) are summarized in Table 3.

3.3.1 Impact of *M. speciosa* Infusion on Lipid Properties (VCO vs. COK)

The incorporation of *M. speciosa* at a 1:0.4 ratio significantly enhanced the functional properties of the oil ($p < 0.05$). The TPC increased from 8.41 ± 0.24 mg GAE/g in VCO to 18.76 ± 0.61 mg GAE/g in COK, while the antioxidant activity nearly tripled, rising from 10.28 % to 34.73 %. This improvement confirms the successful mass transfer of lipophilic alkaloids (such as mitragynine) and phenolic compounds into the lipid matrix. These findings are consistent with Zunin et al. (2010) [17], who reported that infusing virgin oils with polyphenol-rich botanical extracts significantly improves oxidative stability. Furthermore, the efficiency of this green extraction, using coconut milk as a natural solvent, aligns with Ravisankar and Agila (2019) [23], who stated that natural emulsions can effectively stabilize plant-derived antioxidants. Finally, the cold-extraction technique employed in this study preserves the bioactivity of heat-sensitive compounds, avoiding the thermal degradation typically associated with traditional hot-infusion methods [24].

3.3.2 Efficiency of the Zero-Waste Recovery (Cream Residue vs. CCR)

Following the Zero-Waste model, phytochemicals that did not partition into the oil phase were successfully captured within the solid cream fraction. The CCR demonstrated a marked improvement over its un-infused counterpart (Cream Residue), with TPC rising from 8.49 ± 0.38 mg GAE/g to 15.95 ± 1.01 mg GAE/g, and DPPH inhibition increasing from 13.07 % to 39.15 %. The substantial retention of phenolics in the CCR justifies its upcycling into functional ingredients or bioactive supplements for aquafeed, as

supported by circular economy principles [18, 25]. As noted by Ojha et al. (2020) [15], biomass residues from green extraction often retain significant bioactive potency, thereby transforming what was previously considered waste into a high-value co-product

3.3.3 Partitioning Dynamics and Antioxidant Superiority of CCR

A critical observation in this study was the **inverse relationship** between TPC and antioxidant activity when comparing COK and CCR. Although COK exhibited a higher TPC than CCR (18.76 and 15.95 mg GAE/g), the CCR fraction yielded the highest overall antioxidant efficacy (39.15 ± 0.31 %), significantly outperforming the oil phase (34.73 ± 0.25 %; $p < 0.05$). This phenomenon can be attributed to three primary factors:

Phase Affinity: According to Chear et al. (2021) [13], certain polar or semi-polar bioactive compounds in Kratom possess a higher affinity for the aqueous or solid proteinaceous phase than the bulk lipid phase. Consequently, the protein-rich matrix of CCR likely traps these potent radical scavengers more effectively during the fractionation process.

Micellar Protection: The natural oil-in-water (O/W) emulsion system of coconut milk facilitates the formation of micelles. These structures, stabilized by natural proteins and phospholipids, provide a robust protective microenvironment that shields bioactives from oxidative degradation more effectively than a bulk lipid phase [26].

Bound Phenolics: CCR contains bound phenolics covalently attached to the dietary fiber of the coconut meat. As suggested by Ojha et al. (2020) [15], while bound phenolics may exhibit lower values in standard TPC assays due to limited solubility in the extraction solvent, they can exert powerful antioxidant responses when measured directly in a radical scavenging system.

TABLE III
TOTAL PHENOLIC CONTENT AND %INHIBITION OF
KRATOM EXTRACT IN COCONUT OIL AND RESIDUE

Sample	TPC (mg GAE/1 g)	% inhibition (DPPH)
Virgin Coconut oil (VCO)	8.41 ± 0.24^a	10.28 ± 0.21^a
COK (at 1:0.4 w/w)	18.76 ± 0.61^d	34.73 ± 0.25^c
Cream Coconut residue (Control)	8.49 ± 0.38^a	13.07 ± 0.63^b
CCR (at 1:0.4 w/w)	15.95 ± 1.01^c	39.15 ± 0.31^d

Values are expressed as Mean $\pm$ SD ($n=3$). Different superscripts in the same column indicate significant differences ($p < 0.05$).

3.4 Proximate Composition and Upcycling Potential of CCR

The proximate analysis (Table 2) reveals that the Coconut-Kratom Residue (CCR) is a nutrient-dense matrix, characterized by a substantial crude protein content (19.70%) and total fat (24.79%). This high protein level is particularly significant, as it suggests that coconut milk proteins—specifically globulins and albumins—were effectively

concentrated within the residue during the fractionation process. According to Onsaard (2012) [21], these proteins possess a well-balanced amino acid profile, further enhancing the potential of CCR as a high-value protein supplement. The total fat content of 24.79% indicates that a significant portion of medium-chain triglycerides (MCTs) remains embedded within the residue matrix. These lipids contribute to a high total energy value (303.43 kcal/100g) and serve as a stabilized carrier for the lipophilic alkaloids and antioxidants recovered from the *M. speciosa* leaves. The remarkably low carbohydrate (0.38%) and ash (0.88%) contents further distinguish CCR as a high-purity, energy-dense material with minimal inorganic impurities. The classification of CCR as a 'super-byproduct' is justified by its unique dual-functionality: superior antioxidant activity (39% radical scavenging) coupled with a robust nutritional profile. Unlike traditional extraction residues, which are often nutrient-depleted, the cold-extraction process preserves the high-energy lipid fraction that further stabilizes Kratom-derived antioxidants. This synergy transforms CCR into a novel functional ingredient rather than mere waste. From a circular economy perspective, this residue provides a sustainable solution for the aquaculture industry, where the combination of potent bioactives and 20% protein can significantly enhance growth performance and oxidative stress resistance in aquatic species [15], [25]. As a byproduct of the fresh coconut milk extraction, the CCR maintains a high moisture level of 54.25%, which is typical for fresh cream-based residues. From a practical standpoint, the high moisture content suggests that proper stabilization, such as drying or cold storage, is required to prevent microbial spoilage for long-term applications. Overall, the composition confirms the economic viability of this zero-waste extraction model [18].

TABLE IV
PROXIMATE COMPOSITION OF CCR

Protein	19.70	In-house method TE-CH-012 based on AOAC (2016) 981.10 [27]
Total Fat	24.79	AOAC (2016) 920.39 [28]
Carbohydrate	0.38	Journal of AOAC INTERNATIONAL; 1993. p 106 [29]
Moisture	54.25	AOAC (2019) 930.15 [30]
Ash	0.88	AOAC (2016) 942.05 [31]
Total Energy	303.43 kcal/100g	AOAC (2019) 930.15 [30]

IV. CONCLUSION

This study established a sustainable, zero-waste green extraction model for *Mitragyna speciosa* using coconut milk

as a biocompatible bio-solvent. By leveraging an emulsion-based system, the process effectively recovered Kratom's bioactive constituents without synthetic chemicals. An optimal ratio of 1:0.4 (coconut meat to leaves) maximized both phytochemical recovery and nutrient density. The technique successfully partitioned bioactives into two high-value streams: COK, featuring a significant phenolic content (18.76 ± 0.61 mg GAE/g), and CCR. Remarkably, the CCR outperformed the oil fraction in antioxidant capacity (39.15 ± 0.31 %) and demonstrated a robust nutritional profile (19.70 % protein, 24.79 % fat). This integrated approach achieves 100% biomass utilization, upcycling CCR into a potent, high-protein supplement for aquaculture to enhance growth and oxidative stress resistance. Overall, this methodology provides a robust framework for a circular economy in the herbal and agricultural industries.

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Determination of Biosurfactant Activity from Endophytic Bacteria Isolated from Passion Fruit

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Abstract. Endophytic bacteria are microorganisms that live within plant tissues in beneficial relationships. They are considered a valuable source of bioactive compounds, particularly substances that protect plants from pathogens, making them promising tools for biocontrol. This experiment aimed to isolate and identify endophytic bacteria capable of producing biosurfactants. Primary screening of biosurfactant activity revealed that isolate PFy2 was the strongest biosurfactant producer. This isolate showed positive results in both the hemolytic assay and the drop collapse test. The cell-free supernatant from isolate PFy2 exhibited oil displacement activity, producing a clear zone of 44.06 ± 0.15 mm in the oil spreading assay. Furthermore, the crude biosurfactant obtained by acid precipitation from isolate PFy2 at a concentration of 1% (w/v) efficiently emulsified and maintained emulsion stability against hexane and palm oil after 24 h, with emulsion index (E24) values of 62.68 and 52.67, respectively. For antifungal activity against the growth of *Aspergillus flavus* IMI 242684, the crude biosurfactant from isolate PFy2 at concentrations of 50 and 25 mg/mL demonstrated inhibitory zones of 11.57 ± 0.07 and 7.15 ± 0.13 mm, respectively. Finally, analysis of the 16S rRNA gene sequence showed that isolate PFy2 was highly similar to *Bacillus siamensis* KCTC 13613^T and *B. velezensis* CR-502^T, with 99.86% sequence identity.

Index Terms—Biosurfactant, Endophytic bacteria, Passion fruit

I. INTRODUCTION

Endophytic bacteria are a group of bacteria that are capable of colonizing and living within the plant tissue. These bacteria have influenced plants and also their pathogens through symbiotic relationships with the host plants, ranging from mutualism to commensalism, as a result, enhance plant health and suppress pathogens [1]. The characteristics of endophytes are adapted for survival and symbiosis, including the production of secondary metabolites. Diverse natural products are synthesized, ranging from alkaloids and polyketides to lipopeptides with a variety of bioactivities for agricultural and pharmaceutical potential. [2]

Biosurfactants are natural surface-active compounds produced from several organisms such as plants, animals and microorganisms. Biosurfactants with a variety of chemical structures are amphiphilic molecules that combine surface activity with bioactivity. Potential applications of biosurfactants include environmental bioremediation, oil recovery, and pharmaceutical applications. [3, 4] Microbial biosurfactants have been reported for several distinguishing

properties such as their stability at high temperatures, pH, and salinity, the exceptional emulsifying capabilities, and the antimicrobial and antifungal activities. They interfere with pathogen growth using their surface activity by interacting with the lipid components of target microbes, proposing alternatives to conventional antibiotics. Among the various biosurfactant producing microorganisms, the foremost bacterial genera include *Rhodococcus*, *Pseudomonas*, *Bacillus*, and *Acinetobacter* etc have been reported. Some of these potential strains were endophytic bacteria including *Pseudomonas aeruginosa*, and *Bacillus* spp. [5]

In this study, endophytic bacteria of passion fruit was isolated and identified. The biosurfactant properties, including hemolytic activity, drop collapse, oil displacement, and emulsification index, were determined for potential use as biocontrol agents. In addition, antifungal activity was evaluated against *Aspergillus flavus*, a major cause of several plant diseases.

II. METHODOLOGY

Isolation of endophytic bacteria

Endophytic bacteria were isolated from the inedible outer rind of yellow passion fruit (*Passiflora edulis* f. *flavicarpa* Deg.) according to the method described by Shweta et al.

(2013), with some modifications [6]. The rind was cut into 0.5 cm² segments, washed under running tap water, and surface-sterilized by sequential immersion in 70% (v/v) ethanol for 5 min, followed by washes twice with sterilized distilled water. The samples were then disinfected with 4% (v/v) sodium hypochlorite solution for 5 min and subsequently washed three times with sterilized water. The samples were transferred into 10 mL of Brain Heart Infusion broth (BHI) and incubated at 30 °C for 48 h. After incubation, the culture broth was diluted with Phosphate Buffer Saline (PBS, pH 7.4) to perform 10-fold serial dilutions (10⁻¹ to 10⁻⁵). A 100 µL aliquot of each dilution was plated on Trypticase Soy Agar (TSA) and incubated at 30 °C for 48 h. All bacterial colonies were isolated and purified on TSA for further experiments.

Primary screening of biosurfactant activity

Hemolytic assay

The isolated colony was streaked on TSA with 5% v/v sheep blood and incubated at 30 °C for 24 h. The ability to rupture red blood cells was detected as the presence of a clear zone surrounding the colony [7].

Drop collapse test

A single colony of the isolate exhibiting hemolytic activity was grown in 5 mL of Trypticase Soy Broth (TSB) for 48 h at 30°C with shaking at 150 rpm. The cell-free supernatant was collected for the collapsing test using the method described by Nayariseri and coworkers, with some modifications. The cover of the 96-well microtiter plate was thoroughly coated with palm oil and left to equilibrate at room temperature for 24 h. Two microliters of cell-free supernatant were dropped onto the surface of the oil-coated area and left for 1 min. The supernatant that produced a flat drop was considered positive for biosurfactant production [8].

Oil displacement test

This experiment was performed using the methods of Hassanshahian (2014), with some modifications. Briefly, 10 mL of distilled water was added to a Petri dish (φ 90 mm), followed by the addition of 50 µL of palm oil to the water surface. Ten microliters of cell-free culture broth were then added to the oil surface. The diameter of the oil-free zone was measured (φ mm) [7].

Production and extraction of biosurfactant

The isolate exhibiting the highest biosurfactant activity was selected for biosurfactant production. A 2.5 mL aliquot of overnight culture suspension was inoculated into 250 mL of TSB and incubated at 30 °C with shaking at 150 rpm. After 48 h, bacterial cells were removed by centrifugation at 8,000 rpm for 15 min at 4 °C, and the cell-free supernatant was filtered through a 0.45 µm membrane filter. The obtained

supernatant was acidified to pH 2.0 using 6 M HCl and stored at 4 °C for 12 h. The solution was centrifuged at 10,000 rpm for 30 min at 4 °C. The precipitate was resuspended in distilled water (pH 8), and the suspension was concentrated by

freeze-drying. The frozen sample was freeze-dried for 18 h and stored at -20 °C for evaluation of emulsification index [9].

Determination of emulsification index

This experiment was designed to measure the ability of crude biosurfactant to stabilize an oil-in-water emulsion, using the methods of Varadavenkatesan and Murty (2013) with some modifications. To determine the emulsification index, 2 mL of hexane and 2 mL of palm oil were added into separate test tubes, followed by the addition of an equal volume of crude biosurfactant at a concentration of 1% (w/v). The tubes were vigorously mixed using a vortex mixer for 2 min. The emulsion index (E₂₄) was calculated after 24 h using the following equation [10].

$$E_{24} = (\text{Height of emulsion})/(\text{Total height of liquid}) \times 100$$

Bacterial identification based on 16s rRNA sequence

An isolate that showed the strongest activity was subjected to 16S rRNA sequence analysis for identification of its genus and species. The genomic DNA was extracted using the Hipure™ Bacterial Genomic DNA Purification Kit (HiMedia Laboratory Pvt. Ltd.). The 16S rRNA gene was amplified using the universal primers 27F and 1492R. Amplification was carried out in 50 µL reaction mixtures containing 20 mM Tris-HCl, 10 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM Mg₂SO₄, 0.1% Triton X-100, 20 pmol of each primer, 0.25 mM of each of the four dNTPs, 0.5 U of Taq DNA polymerase (NEB, USA), and 10 ng of DNA template. The thermocycling program started with an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 60 s, and 72 °C for 90 s. Finally, the extension step was performed at 72 °C for 3 min. The PCR products were separated using 1.0% agarose gel electrophoresis, and the amplicon (~1.5 kb) was purified. The nucleotide sequences were analyzed and compared with the 16S rRNA sequences of bacterial type strains in the EzBioCloud database (www.ezbiocloud.net/eztaxon).

Antifungal activity

The antifungal activity of the crude biosurfactant was determined using the agar well diffusion assay. The sample was dissolved in sterile distilled water to obtain final concentrations of 12.5, 25, and 50 mg/mL, respectively. Twenty milliliters of molten Potato Dextrose Agar (PDA) were poured into a 90 mm sterile petri dish and allowed to solidify as the agar base. Subsequently, 10 mL of molten PDA containing *A. flavus* IMI 242684 spores at a concentration of 10⁶ spores/mL was overlaid onto the agar base. After the medium solidified, a well with a diameter of 7.5 mm was made, and 100 µL of the test solution at each concentration was loaded into the well. The plates were incubated at room temperature for 5 days, after which the antifungal activity was evaluated. The zone of inhibition was measured using a vernier caliper.

III. RESULTS AND DISCUSSION

The seven isolates of endophytic bacteria were subjected to primary screening for biosurfactant production. Only two isolates, PFy2 and PFy4, exhibited hemolytic activity, showing clear zones surrounding their colonies as presented in Table 1. Hemolytic activity is commonly used as a primary screening method for biosurfactant production because many bacterial biosurfactants such as rhamnolipid, surfactin, and trehalose lipid can disrupt erythrocyte membranes. They act on the phospholipid bilayer, leading to loss of membrane integrity and pore formation [11-12].

However, in the drop collapse and oil displacement tests, six of the seven isolates did not show positive results, with the exception of isolate PFy2. The cell-free supernatant from isolate PFy2 caused the drop to collapse into a flat shape as a result of reduced surface tension between oil (a hydrophobic surface) and liquid [13]. Moreover, the cell-free supernatant of isolate PFy2 also exhibited strong oil displacement activity, producing a clear zone of 44.06 ± 0.15 mm, as shown in Table 1 and Figure 1.

TABLE 1
PRIMARY SCREENING OF BIOSURFACTANT
ACTIVITY FROM ENDOPHYTIC BACTERIA

Isolate	Hemolytic assay	Drop collapse test	Oil displacement test (ϕ mm)
PFy1	-	-	0
PFy2	+	+	44.06 ± 0.15
PFy3	-	-	0
PFy4	+	-	16.66 ± 0.25
PFy5	-	-	0
PFy6	-	-	0
PFy7	-	-	0

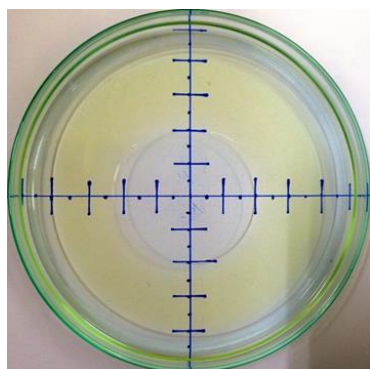


Fig. 1. Oil displacement activity from cell free supernatant of isolate PFy2.

The crude biosurfactant from isolate PFy2 at a concentration of 1% (w/v) efficiently emulsified and maintained emulsion stability against hexane and palm oil after 24 h (Fig. 2). The emulsion index (E24) values were 62.68 and 52.67, respectively. Biosurfactants are amphiphilic molecules that reduce the interfacial tension

between oil and water, making it easier for the two phases to mix and form stable droplets. Emulsification is achieved

through mechanical mixing, which breaks down and disperses the droplets. The surfactant molecules position themselves at the oil–water interface, with their hydrophilic portion oriented toward the water phase and their hydrophobic portion oriented toward the oil phase.



Fig. 2. The emulsification index of crude biosurfactant from isolate PFy2 with hexane (A) and palm oil (B).

This arrangement creates an elastic interfacial layer that enhances emulsion stability by preventing droplet coalescence [14].

According to the molecular identification based on nucleotide sequences of the 16S rRNA gene demonstrated that the sequence from isolate PFy2 was highly similar to those of *Bacillus siamensis* KCTC 13613^T and *B. velezensis* CR-502^T, showing 99.86% identity. However, further studies, including genotypic, phenotypic, and chemotypic characterizations, are required to distinguish isolate PFy2 from its closest relatives within the genus *Bacillus*. Several types of biosurfactants produced by *Bacillus* species have been reported, such as lichenysin, surfactin, iturin, and subtilisin [15].

For antifungal activity, the crude biosurfactant from isolate PFy2 at concentrations of 50 mg/mL and 25 mg/mL could inhibit the growth of *A. flavus* IMI 24684 with the diameter of inhibition zone at 11.57 ± 0.07 mm and 7.15 ± 0.13 mm, respectively as the results in Table 2.

TABLE 2
ANTIFUNGAL ACTIVITY OF CRUDE
BIOSURFACTANT

Isolate	Crude biosurfactant concentration (mg/ mL)	Zone of inhibition (mm)
PFy2	50	11.57 ± 0.07
	25	7.15 ± 0.13
	12.5	-

The mechanism of action of biosurfactants is generally associated with the disruption of the fungal plasma membrane. They bind to the membrane surface, and the hydrophobic portion inserts into the phospholipid bilayer.

This interaction induces structural alterations, leading to loss of membrane integrity and pore formation, which in turn causes leakage of cellular components [16].

IV. CONCLUSION

According to the results of this study, the endophytic bacterium PFy2, isolated from the inedible outer rind of yellow passion fruit, produces extracellular substances that exhibit both biosurfactant activity and antifungal activity against the growth of *Aspergillus flavus* IMI 242684. However, chemical separation and purification processes are required to extract and identify the chemical structure of the target biosurfactant. Furthermore, the anti-adhesion properties of the biosurfactant represent an interesting approach for its preventative application as a biocontrol agent.

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Papain–Bromelain Injection Modulates Texture and Quality of Salmon Steak for Elderly-Friendly Products

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Abstract. The aging population has driven demand for texture-modified foods that are easy to chew and swallow. This study investigated the effect of papain–bromelain injection on the physicochemical properties, protein degradation, and sensory quality of salmon (*Salmo salar*) steak for elderly food applications. Salmon steaks were injected with enzyme solutions containing papain (0–5%) combined with bromelain (0.25%), sodium tripolyphosphate (0.5%), and NaCl (2%), followed by incubation at 25 °C for 30 min. The results showed that increasing papain concentration significantly reduced hardness from 861.97 g.f (control) to 182.26 g.f at 5% ($p \leq 0.05$), due to extensive proteolysis of myofibrillar proteins, particularly myosin heavy chain, as confirmed by SDS-PAGE analysis. However, excessive enzymatic hydrolysis resulted in increased cooking loss (up to 13.92%) and the formation of bitter peptides, negatively affecting sensory quality. The treatment with 3% papain provided the optimal balance between sufficient tenderization and acceptable sensory attributes, particularly in terms of tenderness and ease of swallowing. These findings demonstrate that controlled proteolytic enzyme injection is an effective strategy for developing elderly-friendly fish products with improved texture while maintaining overall product quality.

Index Terms—Papain–bromelain injection, Enzymatic tenderization, Salmon steak, Texture modification, Protein degradation, Elderly-friendly food

I. INTRODUCTION

The rapid growth of the global aging population has created significant challenges in food design and nutrition. Elderly individuals commonly experience physiological deterioration, including reduced masticatory efficiency, tooth loss, decreased saliva secretion, and impaired swallowing (dysphagia), which collectively limit their ability to consume conventional foods [1]. As a result, the development of texture-modified foods that are easy to chew and swallow has become an important research focus [2]. Protein-rich foods, particularly meat and fish, are essential in elderly diets due to their high nutritional value. However, these foods often possess textures that are too firm for elderly consumers. Although fish is generally softer than red meat, its muscle structure—composed of organized myofibrillar proteins such as myosin and actin—can still present challenges for individuals with reduced oral processing ability [3].

Among various strategies to improve food texture, enzymatic tenderization has been widely explored due to its

efficiency and controllability. Proteolytic enzymes such as papain and bromelain are widely used in the food industry to improve meat tenderness by hydrolyzing muscle proteins [4]. Both enzymes belong to the cysteine protease group and exhibit broad substrate specificity toward myofibrillar proteins, including myosin and actin, which are critical for muscle structure [5]. The combined use of these enzymes may enhance protein hydrolysis through complementary catalytic mechanisms, resulting in more efficient tenderization [6]. Despite their effectiveness, enzymatic treatments must be carefully controlled. Excessive proteolysis can lead to undesirable quality changes, including excessive softening, increased cooking loss, and the generation of bitter peptides. The latter is particularly associated with the formation of low-molecular-weight hydrophobic peptides during protein hydrolysis [7]. Furthermore, extensive degradation of myofibrillar proteins can reduce water-holding capacity (WHC), resulting in higher cooking loss and reduced product yield [8]. In addition to enzyme selection and concentration, the method of enzyme application also plays a critical role in determining tenderization effectiveness. Conventional

surface marination may result in limited enzyme penetration, leading to non-uniform texture modification. In contrast, injection techniques allow direct delivery of enzymes into the muscle matrix, promoting more uniform proteolysis and consistent texture throughout the product [2]. However, studies focusing on enzyme injection in fish muscle, particularly for elderly food applications, remain limited. Despite the recognized effectiveness of proteolytic enzymes, limited information is available on their application in fish muscle via injection techniques, particularly regarding the balance between tenderization efficiency and sensory quality. Moreover, the relationship between protein degradation, texture modification, and sensory acceptability in enzyme-treated fish products remains insufficiently understood. Therefore, the objective of this study was to investigate the effects of papain and bromelain proteolytic enzyme injections on the texture, physicochemical properties, protein degradation, and sensory characteristics of salmon (*Salmo salar*) steak.

II. METHODOLOGY

Raw material and sample preparation

Atlantic salmon (*Salmo salar*) with an average body weight of 3–4 kg was obtained from a commercial supplier under chilled conditions. The fish were filleted, skinned, and cut into steak portions measuring approximately 1.5–2.0 cm thick and weighing 110–130 g each. All samples were individually vacuum-packed to minimize oxidation and microbial contamination and stored at –20 °C until further use. Prior to the experiment, samples were thawed at 4 °C for 12 h to ensure uniform temperature distribution and to minimize drip loss. The preparation procedure was designed to maintain the integrity of muscle structure before enzymatic treatment, as pre-processing conditions can significantly influence subsequent proteolysis and texture modification.

Preparation of enzyme solution

Proteolytic enzyme solutions were prepared using papain (Beijing Aoboxing Biotechnology Co., Ltd., China) at concentrations of 0, 0.10, 0.25, 0.50, 1.00, 3.00, and 5.00% (w/v). Bromelain (0.25%, w/v) was added to all treatments as a co-protease. The enzyme solution also contained sodium tripolyphosphate (STPP, 0.5%) and sodium chloride (NaCl, 2%). STPP was included to enhance water-holding capacity via protein solubilization and ionic-strength modification, while NaCl facilitated protein extraction and improved enzyme penetration into muscle tissue [9]. Papain and bromelain are cysteine proteases known to hydrolyze myofibrillar proteins, including myosin and actin, which are key structural components of muscle tissue [4]. The selected concentration range was based on preliminary studies to cover both sub-optimal and excessive enzymatic conditions.

Enzyme injection and incubation

The prepared enzyme solution was injected into salmon

steaks using a manual syringe. Each sample was injected at 20 evenly distributed sites, with 0.25 mL of solution per injection, resulting in uniform enzyme distribution within the muscle matrix. The injection technique was selected to ensure rapid, homogeneous diffusion of enzymes throughout the muscle, as diffusion-limited enzyme activity can lead to uneven tenderization. After injection, samples were incubated at 25 °C for 30 min. This incubation condition was selected based on previous findings indicating that moderate temperature enhances enzymatic activity without causing significant thermal denaturation of muscle proteins [4].

Cooking procedure

After incubation, samples were pan-seared at approximately 180 °C for 1 min on each side until well done. Cooking was performed to simulate typical culinary preparation and to evaluate the impact of enzymatic treatment on thermal stability, water retention, and final texture. Thermal processing is known to influence protein denaturation and water loss, which are critical factors in determining product quality [3].

Determination of cooking loss

Cooking loss was determined using a modified method described by Sriket, et al. [10]. The measurements were based on the weight of salmon samples after cooking compared with the weight after storage. All measurements were conducted in triplicate. Cooking loss (%) was calculated using the following equation:

$$\text{Cooking loss (\%)} = \frac{(\text{Weight before cooking (g)} - \text{Weight after cooking (g)})}{(\text{Weight before cooking (g)})} \times 100 \quad (1)$$

Texture analysis

Texture analysis of salmon samples was performed after incubation, followed by cooking to a well-done state using a grilling method. The measurements were conducted using a Texture Analyzer equipped with a cylindrical plunger probe. Texture was measured after cooking to reflect the product's actual consumption conditions, as thermal processing significantly influences protein denaturation and texture [11]. Texture was evaluated at two positions per sample: one on the dorsal muscle and one on the ventral muscle. The results were expressed as hardness in gram-force (g.f). Samples were selected for lower hardness than the control, while maintaining an external appearance comparable to the control.

Color measurement

Color analysis of salmon samples was performed before and after incubation. A colorimeter (Minolta, model CR-400, Japan) was used for all measurements. Color values were determined at six positions on each salmon steak, consisting of three positions on the dorsal muscle and three positions on the ventral muscle. The color parameters were expressed in

terms of L* (lightness), a* (redness), and b* (yellowness). The measured color values were compared with those of the control sample. Color stability is an important quality attribute influencing consumer acceptance of fish products.

SDS-PAGE analysis

Protein degradation patterns were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to standard procedures. Muscle proteins were extracted, denatured, and separated based on molecular weight. The intensity of protein bands, particularly myosin heavy chain, was used to evaluate the extent of proteolysis. SDS-PAGE is a widely used technique for analyzing protein degradation and has been extensively applied in studies of enzymatic tenderization [12].

Sensory evaluation

Sensory evaluation was conducted using 30 elderly panelists aged 60–70 years. Samples were evaluated for tenderness, swallowability, taste, and overall acceptability using a 5-point hedonic scale (1 = Dislike Extremely and 5 = Like Extremely). All samples were coded and presented in randomized order to minimize bias. The use of elderly panelists is critical for evaluating the suitability of texture-modified foods, as their perceptions differ from those of younger consumers [1]. Selected samples for sensory evaluation were based on preliminary texture analysis and visual assessment. Treatments showing excessive softening or minimal improvement were excluded, and representative samples (control, 0.1%, 1%, and 3%) were chosen to cover a practical range while minimizing panelist fatigue.

Statistical analysis

All experiments were conducted in triplicate, and results were expressed as mean $\pm$ standard deviation. Statistical analysis was performed using analysis of variance (ANOVA), followed by Duncan's multiple-range test to determine significant differences between treatments at the $p \leq 0.05$ confidence level.

III. RESULTS AND DISCUSSION

Effect of proteolytic enzymes on texture modification

The effect of proteolytic enzyme treatment on texture is summarized in Table I. The hardness of salmon steaks decreased significantly ($p \leq 0.05$) with increasing papain concentration. The control sample exhibited high hardness values of 861.97 g.f (dorsal muscle) and 959.15 g.f (ventral muscle), whereas samples treated with 5% papain showed a substantial reduction to 182.26 g.f and 369.16 g.f, respectively. Similar proteolytic effects of papain and bromelain on myofibrillar proteins have also been reported in fish muscle systems [11]. This pronounced decrease in hardness indicates extensive degradation of skeletal muscle's structural proteins. Papain and bromelain, both classified as cysteine proteases, act synergistically to hydrolyze myofibrillar proteins such as myosin heavy chain (MHC) and

actin, which are essential for maintaining the structural integrity of the sarcomere [4]. The cleavage of peptide bonds within these proteins disrupts the actomyosin network, reducing resistance to compression and improving tenderness. Similar mechanisms have been reported in enzyme-treated muscle systems, where proteolytic degradation leads to weakening of muscle fiber structure [3]. Interestingly, the hardness values at 3% papain were slightly higher than those at 1% papain, particularly in the dorsal muscle. This non-linear trend suggests that enzyme activity is influenced not only by concentration but also by enzyme diffusion and interaction within the muscle matrix. Uneven proteolysis or localized over-hydrolysis may lead to structural heterogeneity, as previously reported in enzyme-treated meat systems [6]. Overall, the results demonstrate that enzyme concentration plays a critical role in controlling texture modification. While increasing papain concentration enhances proteolysis and tenderness, excessive enzymatic activity may lead to structural instability and undesirable quality changes.

TABLE I
EFFECT OF MIXED ENZYME SOLUTION (PAPAIN
AND 0.25% BROMELAIN) ON COOKING LOSS (%)
AND HARDNESS OF SALMON SAMPLES
INCUBATED AT 25 °C FOR 30 MIN

Papain (%)	Cooking loss (%)	Hardness (g.f)*	
		Dorsal muscle	Ventral muscle
control [†]	5.43 $\pm$ 1.43 ^a	861.97 $\pm$ 60.23 ^{e†}	959.15 $\pm$ 65.84 ^c
0	7.28 $\pm$ 4.63 ^{ab}	598.69 $\pm$ 53.89 ^d	634.64 $\pm$ 233.94 ^b
0.10	9.38 $\pm$ 3.27 ^{ab}	356.02 $\pm$ 35.38 ^c	364.51 $\pm$ 19.58 ^a
0.25	9.94 $\pm$ 5.63 ^{ab}	333.11 $\pm$ 18.58 ^{bc}	382.89 $\pm$ 56.29 ^a
0.50	7.15 $\pm$ 1.00 ^{ab}	355.74 $\pm$ 11.04 ^c	402.18 $\pm$ 124.95 ^a
1.00	11.11 $\pm$ 4.24 ^{ab}	285.80 $\pm$ 27.90 ^b	345.84 $\pm$ 129.52 ^a
3.00	10.73 $\pm$ 2.11 ^{ab}	315.87 $\pm$ 23.60 ^{bc}	319.57 $\pm$ 32.66 ^a
5.00	13.92 $\pm$ 5.03 ^b	182.26 $\pm$ 9.79 ^a	369.16 $\pm$ 4.24 ^a

* Values are expressed as mean $\pm$ standard deviation (n = 3). [†] Control sample without incubation treatment. [‡] Different superscript letters within the same column indicate significant differences ($p \leq 0.05$). Cooking loss (%) and hardness (g.f) were determined from salmon samples cooked to a well-done state.

Cooking loss and water-holding capacity

Cooking loss values are also shown in Table I. A gradual increase in cooking loss was observed with increasing papain concentration, reaching a maximum value of 13.92% at 5% papain. The increase in cooking loss with higher enzyme concentrations is likely due to excessive proteolysis, which disrupts the muscle network responsible for water retention. The degradation of structural proteins reduces the muscle matrix's ability to bind water, leading to greater moisture loss during thermal processing [8]. This trend reflects a reduction in water-holding capacity (WHC) due to the degradation of myofibrillar proteins. Myosin plays a crucial role in water retention by forming a three-dimensional protein matrix that can entrap water molecules. Proteolytic degradation of these proteins disrupts this network, leading to increased water release during thermal processing [3]. Similar findings have been reported in enzyme-treated meat systems, where

excessive proteolysis resulted in structural collapse and increased cooking loss [8, 10]. At moderate enzyme concentrations ($\leq 3\%$), cooking loss remained relatively stable, suggesting that partial proteolysis can improve tenderness without significantly compromising WHC. This balance is essential in product development, as excessive cooking loss negatively affects yield and juiciness. These results are consistent with previous studies reporting increased cooking loss in enzyme-treated meat systems, attributed to the structural weakening of muscle fibers [8, 13].

Color stability

The effect of enzymatic treatment on color parameters (L^* , a^* , and b^*) is presented in Table II. No significant differences ($p > 0.05$) were observed among treatments, indicating that enzyme injections had minimal impact on the visual appearance of salmon steak. This suggests that enzymatic hydrolysis primarily affects internal protein structures rather than pigment components, such as astaxanthin, which is responsible for salmon's characteristic color. Color stability is advantageous for consumer acceptance, as the visual appearance remains comparable to that of untreated samples.

TABLE II
EFFECT OF MIXED PROTEOLYTIC ENZYME
SOLUTION (PAPAIN AND 0.25% BROMELAIN) ON
THE COLOR PARAMETERS (L^* , a^* , b^*) OF SALMON
AFTER INCUBATION AT 25 °C FOR 30 MIN.

Papain (%)	Color [§]		
	L^*_{ns}	a^*_{ns}	b^*_{ns}
Control [†]	49.39 ± 0.56	16.40 ± 0.04	24.18 ± 0.83
0	49.19 ± 0.36	16.49 ± 0.16	24.60 ± 0.95
0.10	49.05 ± 0.55	16.50 ± 0.04	24.27 ± 0.62
0.25	49.43 ± 0.67	16.53 ± 0.17	24.61 ± 1.13
0.50	49.86 ± 0.37	16.56 ± 0.38	24.52 ± 1.69
1.00	49.45 ± 0.77	16.75 ± 0.17	25.15 ± 0.46
3.00	48.99 ± 0.64	16.47 ± 0.46	24.62 ± 1.53
5.00	48.68 ± 0.58	16.31 ± 0.95	24.49 ± 0.98

[§] Values are expressed as mean ± standard deviation ($n = 3$). [†] Control sample without incubation treatment. ^{ns} indicates no significant difference ($p > 0.05$). Color values were determined from salmon samples after incubation and storage at -20 °C for 2 weeks.

Protein degradation and tenderization mechanism

The SDS-PAGE profiles of salmon muscle after enzymatic treatment are shown in Fig. 1. A progressive decrease in the intensity of myosin heavy chain (MHC) bands (~200 kDa) was observed with increasing papain concentration, confirming the degradation of myofibrillar proteins. Both papain and bromelain are cysteine proteases that catalyze the hydrolysis of peptide bonds through nucleophilic attack at the carbonyl carbon, leading to fragmentation of high-molecular-weight proteins into smaller peptides [4, 5]. Treatment with bromelain alone (0.25%) resulted in noticeable degradation of MHC, indicating its activity toward myofibrillar proteins. When papain was combined with bromelain, the extent of protein degradation increased in a concentration-dependent manner, suggesting a synergistic effect between the two enzymes.

Similar observations have been reported in muscle protein systems, where combined protease treatments accelerated protein breakdown and increased the formation of low-molecular-weight peptides [2, 11]. In contrast, actin (~45 kDa) exhibited relatively minor changes, suggesting that MHC is more susceptible to enzymatic hydrolysis than actin. This observation is consistent with previous studies reporting that MHC is more readily degraded during proteolytic treatment (Ramirez et al., 2008). Additional protein bands corresponding to paramyosin (~110 kDa), tropomyosin (~40 kDa), troponin T (~38 kDa), and myosin light chain (~20 kDa) were observed, consistent with typical muscle protein profiles [9]. Bands observed around 50 and 75 kDa were attributed to sarcoplasmic proteins commonly found in marine species. As enzyme concentration increased, the formation of lower-molecular-weight fragments (e.g., 170 kDa, 50 kDa, and 21–30 kDa) became more pronounced, indicating progressive proteolysis of myofibrillar proteins. The accumulation of these peptides reflects extensive disruption of the muscle protein network. Importantly, low-molecular-weight peptides, particularly those containing hydrophobic amino acid residues, are known to contribute to bitterness perception [7]. This structural degradation is directly associated with the hardness reduction observed in Table I. The degradation of MHC disrupts the sarcomere structure, resulting in reduced mechanical strength and increased tenderness. However, excessive proteolysis also results in the formation of low-molecular-weight peptides, particularly hydrophobic peptides, which are associated with bitterness [8].

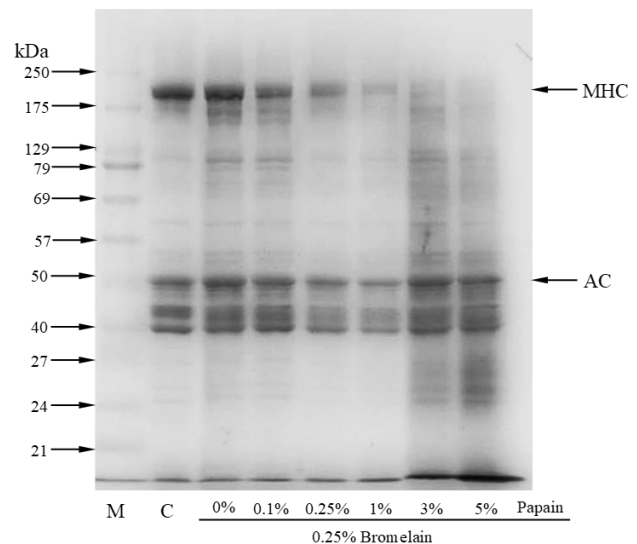


Fig. 1. SDS-PAGE profiles of salmon muscle (C) after incubation with a mixed enzyme solution containing 0.25% bromelain and varying concentrations of papain at 25 °C for 30 min. M, MHC, and AC denote molecular weight marker, myosin heavy chain, and actin, respectively.

Sensory evaluation and optimal condition

The sensory evaluation results are presented in Table III. Samples treated with 3% papain received the highest scores for texture (tenderness) and ease of swallowing, with mean values of 3.76 and 3.88, respectively ($p \leq 0.05$). These results indicate that enzymatic treatment at this concentration

effectively improved the textural properties of salmon steak, making it more suitable for elderly consumption. The improvement in sensory texture attributes is consistent with the reduction in hardness reported in Table I, indicating a strong correlation between instrumental measurements and sensory perception, as commonly observed in enzyme-treated muscle systems [4, 11]. No significant differences ($p > 0.05$) were observed among treatments in appearance, odor, flavor, and overall acceptability. This suggests that enzymatic treatment did not adversely affect the product's overall sensory characteristics. The overall acceptability scores ranged from "neither like nor dislike" to "slightly like," indicating acceptable consumer perception. At higher enzyme concentrations, excessive proteolysis promoted the formation of low-molecular-weight peptides, particularly hydrophobic peptides, which are commonly associated with bitter taste perception. However, this effect was inferred based on protein degradation patterns rather than directly confirmed by sensory evaluation [7, 8]. Therefore, controlling enzyme concentration is critical to achieving a balance between tenderization efficiency and sensory acceptability.

TABLE III
SENSORY ATTRIBUTES OF SALMON STEAK
TREATED WITH MIXED PROTEOLYTIC ENZYME
SOLUTION (PAPAIN AND 0.25% BROMELAIN)
AFTER INCUBATION AT 25 °C FOR 30 MIN,
EVALUATED BY 30 ELDERLY PANELISTS (60–70
YEARS OLD)[§]

Papain (%)	Control [†]	0.1	1	3
Appearance ^{ns}	3.42 ± 0.97	3.36 ± 0.90	3.27 ± 1.03	3.27 ± 0.91
Odor ^{ns}	3.57 ± 0.94	3.55 ± 0.90	3.45 ± 0.94	3.36 ± 0.89
Texture (Tenderness)	2.94 ± 1.03 ^{as}	3.15 ± 1.03 ^a	3.33 ± 1.14 ^{ab}	3.76 ± 0.87 ^b
Swallowability	3.0 ± 1.03 ^{as}	3.27 ± 1.07 ^a	3.45 ± 1.15 ^{ab}	3.88 ± 0.78 ^b
Taste ^{ns}	3.18 ± 0.80	3.15 ± 0.87	3.27 ± 1.10	3.21 ± 0.93
Overall acceptability	3.40 ± 1.09	3.42 ± 1.12	3.51 ± 1.12	3.88 ± 0.74

[§] Values are expressed as mean ± standard deviation of sensory evaluation scores. [†] Control sample without incubation treatment. [‡] Different superscript letters within the same row indicate significant differences ($p \leq 0.05$). ns indicates no significant difference ($p > 0.05$).

IV. CONCLUSION

Proteolytic enzyme injection with papain and bromelain effectively improved the tenderness of salmon steak by degrading myofibrillar proteins, particularly myosin heavy chain, thereby weakening muscle tissue structure. Increasing papain concentration significantly reduced hardness but also increased cooking loss and promoted the formation of bitter peptides at excessive levels. Among the tested conditions, 3% papain combined with 0.25% bromelain provided the optimal balance between effective tenderization and acceptable sensory quality, particularly in terms of tenderness and ease of swallowing. These findings highlight the importance of controlling enzymatic hydrolysis to achieve desirable texture while minimizing adverse quality changes. The results demonstrate the potential of controlled

enzyme injection as a practical approach for developing elderly-friendly seafood products with improved texture and swallowing properties.

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