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Overview

Pertanika Journal of Tropical Agricultural Science is an official journal of Universiti Putra Malaysia. It is an open-access online scientific journal. It publishes the scientific outputs. It neither accepts nor commissions third party content.

Recognised internationally as the leading peer-reviewed interdisciplinary journal devoted to the publication of original papers, it serves as a forum for practical approaches to improving quality in issues pertaining to tropical agriculture and its related fields.

Pertanika Journal of Tropical Agricultural Science currently publishes 6 issues per year (*January, February*, *May*, *June, August*, and *November*). It is considered for publication of original articles as per its scope. The journal publishes in **English** and it is open for submission by authors from all over the world.

The journal is available world-wide.

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Pertanika Journal of Tropical Agricultural Science aims to provide a forum for high quality research related to tropical agricultural research. Areas relevant to the scope of the journal include agricultural biotechnology, biochemistry, biology, ecology, fisheries, forestry, food sciences, genetics, microbiology, pathology and management, physiology, plant and animal sciences, production of plants and animals of economic importance, and veterinary medicine.

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Pertanika was founded in 1978. Currently, as an interdisciplinary journal of agriculture, the revamped journal, *Pertanika* Journal of Tropical Agricultural Science now focuses on tropical agricultural research and its related fields.

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To publish journals of international repute.

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The abbreviation for *Pertanika* Journal of Tropical Agricultural Science is *Pertanika J. Trop. Agric. Sci*.

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Pertanika Journal of Tropical Agricultural Science: e-ISSN 2231-8542 (Online).

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Peer reviewers are experts chosen by journal editors to provide written assessment of the **strengths** and **weaknesses** of written research, with the aim of improving the reporting of research and identifying the most appropriate and highest quality material for the journal.

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Operating and review process

What happens to a manuscript once it is submitted to *Pertanika*? Typically, there are 7 steps to the editorial review process:

1. The journal's Chief Executive Editor and the Editor-in-Chief examine the paper to determine whether it is relevance to journal needs in terms of novelty, impact, design, procedure, language as well as presentation and allow it to proceed to the reviewing process. If not appropriate, the manuscript is rejected outright and the author is informed.

2. The Chief Executive Editor sends the article-identifying information having been removed, to 2 or 3 reviewers. They are specialists in the subject matter of the article. The Chief Executive Editor requests that they complete the review within 3 weeks.

   Comments to authors are about the appropriateness and adequacy of the theoretical or conceptual framework, literature review, method, results and discussion, and conclusions. Reviewers often include suggestions for strengthening of the manuscript. Comments to the editor are in the nature of the significance of the work and its potential contribution to the research field.

3. The Editor-in-Chief examines the review reports and decides whether to accept or reject the manuscript, invite the authors to revise and resubmit the manuscript, or seek additional review reports. In rare instances, the manuscript is accepted with almost no revision. Almost without exception, reviewers' comments (to the authors) are forwarded to the authors. If a revision is indicated, the editor provides guidelines to the authors for attending to the reviewers' suggestions and perhaps additional advice about revising the manuscript.

4. The authors decide whether and how to address the reviewers' comments and criticisms and the editor's concerns. The authors return a revised version of the paper to the Chief Executive Editor along with specific information describing how they have answered' the concerns of the reviewers and the editor, usually in a tabular form. The authors may also submit a rebuttal if there is a need especially when the authors disagree with certain comments provided by reviewers.

5. The Chief Executive Editor sends the revised manuscript out for re-review. Typically, at least 1 of the original reviewers will be asked to examine the article.

6. When the reviewers have completed their work, the Editor-in-Chief examines their comments and decides whether the manuscript is ready to be published, needs another round of revisions, or should be rejected. If the decision is to accept, the Chief Executive Editor is notified.

7. The Chief Executive Editor reserves the final right to accept or reject any material for publication, if the processing of a particular manuscript is deemed not to be in compliance with the S.O.P. of *Pertanika.* An acceptance notification is sent to all the authors.

   The editorial office ensures that the manuscript adheres to the correct style (in-text citations, the reference list, and tables are typical areas of concern, clarity, and grammar). The authors are asked to respond to any minor queries by the editorial office. Following these corrections, page proofs are mailed to the corresponding authors for their final approval. At this point, **only essential changes are accepted**. Finally, the manuscript appears in the pages of the journal and is posted on-line.

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Contents

Foreword

Welcome to the fourth issue of 2025 for the Pertanika Journal of Tropical Agricultural Science (PJTAS)!

PJTAS is an open-access journal for studies in Tropical Agricultural Science published by Universiti Putra Malaysia Press. It is independently owned and managed by the university for the benefit of the world-wide science community.

This issue contains 15 articles: two review articles; one short communication; and the rest are regular articles. The authors of these articles come from different countries namely Afghanistan, Indonesia, Ireland, Malaysia, Nigeria and Thailand.

A selected article entitled "Prevalence and Risk Factors of Bubaline Subclinical Mastitis in Selected Peninsular Malaysian States" assessed 12 buffalo farms in Selangor, Kedah, and Penang to determine the prevalence and risk factors of subclinical mastitis (SCM) in Malaysian dairy buffaloes. Using the California Mastitis Test, the SCM prevalence was found to be 29.7% at the quarter level and 40.1% at the animal level. Quarter-level prevalence varied significantly across states, with the highest in Penang. Significant risk factors associated with SCM included a history of mastitis (animal level), lack of pre- and post-teat dipping, and the absence of other dairy animals (farm level). The findings reflect a high prevalence of SCM in buffaloes and the factors that can be considered in developing effective SCM prevention and control measures. The detailed information of this article is available on the page 1071.

A study by Ying Ju Tan and team entitled "Acute and Subacute Toxicity Assessment of Crude Aqueous Extract of *Melastoma malabathricum* Leaves" evaluated the safety of *Melastoma malabathricum* (MM) crude aqueous extract through acute and subacute oral toxicity tests in female Sprague Dawley rats. A single acute dose of 3000 mg/kg showed no signs of toxicity or mortality over 14 days. In the subacute study, rats were administered daily doses of 100, 500, and 1000 mg/kg for 28 days, with no significant changes observed in behavior, physical appearance, body weight, blood parameters, organ weights, or liver histology compared to the control group. The results indicate that MM crude aqueous extract is non-toxic and safe for oral use at the tested doses. Full information on this study is presented on the page 1093.

A regular article entitled "Effects of Tamanu Kernel Cake from Plantation By-product on Ruminal Digestibility and Methane Emission" evaluated the use of tamanu kernel cake (TKC), a by-product of tamanu oil production, as a protein substitute in ruminant

concentrate diets. The concentrate was formulated to contain ~15% crude protein and ~65% total digestible nutrients, with TKC replacing conventional protein sources at 0% (T0), 50% (T1), and 100% (T2), and a fourth treatment (T3) adding 0.5% mineral salt to T2. Using in vitro rumen incubation (Tilley and Terry method), the results showed no significant differences in dry matter and organic matter digestibility or in ruminal total VFA and ammonia concentrations across treatments. However, T2 and T3 significantly reduced methane emissions compared to T0 ($p < 0.05$), with no additional effect from mineral salt in T3. The study concludes that TKC can replace up to 100% of conventional protein sources and reduce methane emissions without compromising digestibility or fermentation. Further details of this study are found on the page 1151.

We anticipate that you will find the evidence presented in this issue to be intriguing, thought-provoking and useful in reaching new milestones in your own research. Please recommend the journal to your colleagues and students to make this endeavour meaningful.

All the papers published in this edition underwent Pertanika's stringent peer-review process involving a minimum of two reviewers comprising internal as well as external referees. This was to ensure that the quality of the papers justified the high ranking of the journal, which is renowned as a heavily-cited journal not only by authors and researchers in Malaysia but by those in other countries around the world as well.

We would also like to express our gratitude to all the contributors, namely the authors, reviewers, Editor-in-Chief and Editorial Board Members of PJTAS, who have made this issue possible.

PJTAS is currently accepting manuscripts for upcoming issues based on original qualitative or quantitative research that opens new areas of inquiry and investigation.

Editor-in-Chief
Mohamed Thariq Hameed Sultan

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Prevalence and Risk Factors of Bubaline Subclinical Mastitis in Selected Peninsular Malaysian States

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ABSTRACT

Subclinical mastitis (SCM) is a common disease in dairy buffaloes worldwide, resulting in economic losses due to reduced milk production and quality. There is a dearth of information on the susceptibility to bubaline mastitis and the associated factors at farm and animal levels. This study determines bubaline mastitis's prevalence and risk factors in buffalo farms in Malaysia. A cross-sectional study was conducted at 12 buffalo farms across selected states in Peninsular Malaysia, including Selangor, Kedah and Penang. California Mastitis Test (CMT) was used to identify infected and non-infected buffaloes, whereas on-farm assessment and cross-sectional survey were conducted to collect farm and animal-based data. Data analysis was conducted using descriptive statistics, Chi-square test, and binary logistic regression. Overall, the prevalences of SCM at the quarter and animal levels were 29.7% (95% CI: 6.3%–49.0%) and 40.1% (95% CI: 0.0%–66.7%), respectively. While the animal-level prevalence of SCM was not significantly different across states, a significant difference was observed in the quarter-level prevalence (Selangor; 28.2% [95% CI 9.9–40.1], Kedah; 22.1% [95% CI: 6.3–33.3], Penang; 46.3% [95% CI: 43.2–49.0]). The prevalence of SCM bubaline was significantly associated with mastitis history ($p < 0.001$) at the animal level, pre- and post-teat dipping ($p = 0.041$) and the absence of other dairy animals ($p = 0.048$) at the

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farm level. The findings reflect a high prevalence of SCM in buffaloes and the factors that can be considered in developing effective SCM prevention and control measures.

Keywords: Bubaline mastitis, Malaysia, prevalence, risk factors

INTRODUCTION

Buffalo is the second most important dairy animal after cows. Asia has the largest population of buffaloes and is the leading producer of raw buffalo milk, accounting for 98.8% of the total production (Food and Agriculture Organization of the United Nations, 2022). According to the official data from the Department of Veterinary Services (DVS), Malaysia has 67,959 buffaloes. In Peninsular Malaysia, the buffalo population is estimated at around 50,221, comprising both swamp and Murrah buffaloes (Department of Veterinary Services Malaysia, 2022). Notably, the population of Murrah buffaloes in Peninsular Malaysia has consistently shown growth from 2013 to 2022. However, the specific population of Murrah buffaloes in the western region of Peninsular Malaysia, including Penang and Kedah, are among the lowest, while the population in Selangor is currently unknown. In Malaysia, the buffalo industry primarily focuses on meat rather than milk production. This industry predominantly consists of smallholder operations (Nor & Rosli, 2015). Unfortunately, smallholder farmers often confront various challenges, including limited herd sizes and inadequate husbandry practices. These challenges ultimately hinder productivity and market competitiveness, as Ariff et al. (2015) emphasised.

Mastitis, a condition characterised by mammary gland inflammation, is one of the most prevalent diseases affecting dairy animals worldwide. It causes severe economic losses due to reduced milk yield and quality (Costa et al., 2020). The disease is generally categorised into clinical (CM) and subclinical mastitis (SCM) (Singha et al., 2023). Subclinical mastitis is a disease that is concerning in dairy animals, especially buffaloes. It leads to significant economic losses because it is difficult to detect, with no visible changes in the milk or teat udders (Krishnamoorthy et al., 2021). Managing mastitis and reduced milk yield accounted for 55% and 16% of the loss, respectively (Malik & Verma, 2017). Ali et al. (2021) showed that the prevalence of SCM in buffaloes (66%) is higher compared to cows (53%). It is linked to the high nutrient content in buffalo milk, which promotes bacterial growth post-infection.

Somatic cell counts (SCC), California mastitis test (CMT) and bulk milk somatic cell count (BMSCC) are presently the common methods used in screening for SCM and udder health status at animal and farm levels (Costa et al., 2020; Hussain et al., 2018). Several studies have used CMT to investigate the prevalence of SCM, with the quarter-level prevalence ranging from 10% to 46% and widely variable between countries and regions (Islam et al., 2019; Preethirani et al., 2015; Singha et al., 2023). Given the

increasing consumption of buffalo milk and its substantial contribution to milk production in South Asian countries (Ali et al., 2021), it is crucial to understand the prevalence of SCM and associated risk factors to develop effective prevention and control strategies and adopt appropriate therapeutic approaches. Studies conducted in other countries such as Bangladesh, Pakistan, and India have shown that high-yield buffalo, intensively managed, limited availability of pasture, milking practices, farm management, age, parity number, lactation stage, quarters position, and morphology of the teat end increases the risk of SCM in buffalo population (Islam et al., 2019; Salvador et al., 2012; Singha et al., 2023). This information is crucial in developing effective control and prevention measures against mastitis at regional and national levels (Ali et al., 2021; Islam et al., 2019).

In Malaysia, the most common breed of buffalo is the Murrah buffalo, with the capacity to produce an average milk yield of 4.7–5.0 litres per day. This production level remains significantly below the potential of superior buffaloes at an average of 15–20 litres per day (Wahid & Rosnina 2016). Consequently, available data suggests that milk production from buffaloes is yet to meet the demand for milk and dairy products in Malaysia (Mohd Azmi et al., 2021). While diverse factors ranging from genetics to management practices may contribute to the low production level among buffaloes in Malaysia, there is data paucity on the prevalence of SCM and associated factors. Given the evident knowledge gap, it is vital to identify the farm, animal and quarter-related factors that further heighten the risk of SCM in water buffalo in Malaysia. In addition, knowledge of the causative agents and their antimicrobial susceptibility is essential for effective treatment protocols. Therefore, a cross-sectional study was conducted on water buffalo in west Peninsular Malaysia to determine both animal and quarter-level prevalence of SCM bubaline and their associated risk factors.

MATERIALS AND METHODS

Ethics Approval and Consent to Participate

The study was carried out with the approval of the Institutional Animal Care and Use Committee (IACUC) Universiti Putra Malaysia under the animal utilisation protocol (AUP) number UPM/IACUC/AUP-R009/2022. Meanwhile, the survey for collecting farm and animal-based data was approved by the Ethics Committee for Research involving Human Subjects (JKE) Universiti Putra Malaysia with the reference number JKEUPM-2022-054.

Study Design and Study Area

A total of 12 available dairy buffalo farms from the 56 buffalo farms registered and listed by the Department of Veterinary Services (DVS), Malaysia (Figure 1) across three selected states (Selangor, Kedah, and Penang) were recruited for the cross-sectional study from February 2022 to February 2023. These states were selected because they have buffalo

farms that are actively producing buffalo milk and are listed under the registered farms in Malaysia. Additional information on the studied farms is presented in Table 1.

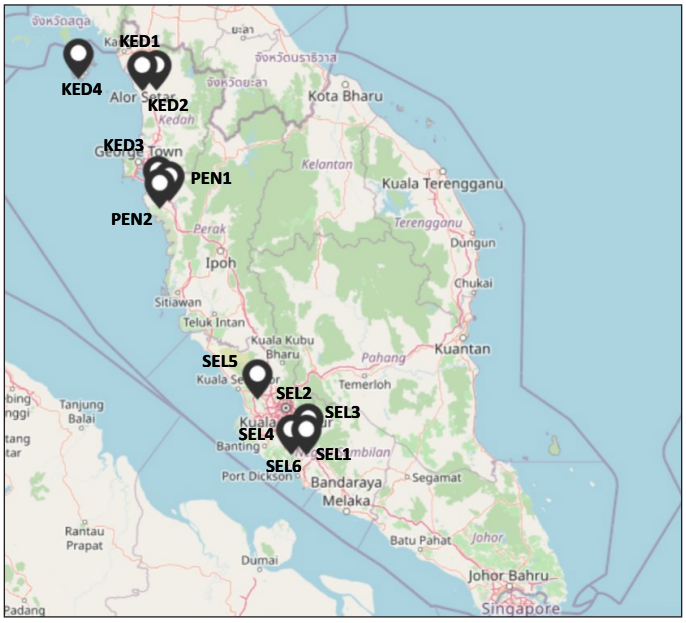

Figure 1. Map showing the distribution of selected farms in Peninsular Malaysia

Table 1
The information on 12 available dairy buffalo farms in Selangor, Kedah and Penang

Farm	District	Rainfall (%, mm)	Temperature (°C)	Animal population	Number of workers on the farm	Breeding system
SEL1	Hulu Langat	60.7, 7.2	27.7	124	5	Semi-intensive
SEL2	Hulu Langat	60.7, 1.5	28.7	5	1	Semi-intensive
SEL3	Hulu Langat	60.7, 1.5	28.9	40	4	Semi-intensive
SEL4	Sepang	60.7, 1.5	29.0	68	2	Semi-intensive
SEL5	Kuala Selangor	75.0, 4.2	29.2	63	5	Semi-intensive
SEL6	Hulu Langat	60.7, 1.5	27.7	650	15	Semi-intensive
KED1	Jitra	42.9, 7.2	28.1	18	2	Semi-intensive
KED2	Jitra	42.9, 7.2	28.2	9	1	Intensive
KED3	Bandar Baharu	42.9, 8.2	26.4	80	3	Semi-intensive
KED4	Langkawi	46.5, 0.0	27.5	49	4	Semi-intensive
PEN1	Seberang Perai	42.9, 0.0	26.6	31	2	Semi-intensive
PEN2	Seberang Perai	42.9, 0.0	26.6	20	5	Intensive

Note. The information about rainfall and temperature was collected from the official website of the Malaysian Meteorological Department (MET) by the Ministry of Natural Resources and Environmental Sustainability (MET, 2022), SEL = Selangor, KED = Kedah, PEN = Penang

Selection of Animals

The sample size for this study was determined using EpiTools Epidemiological Calculators (Ausvet). Based on a population size of 534 adult buffaloes in 12 farms across three selected states and an expected prevalence of 24.2% (Badua et al., 2020), with a 95% confidence interval (CI) and a precision level of 5% (Thrusfield, 2005), the minimum required sample size was computed as 185. The sample size was determined based on the population of 534 adult female buffaloes on the farms. The total population of Murrah buffaloes in the western region of Peninsular Malaysia, including Penang and Kedah, is among the lowest, while the population in Selangor is currently unknown. Since there were 217 dairy buffaloes available on the 12 farms from the three states, all of them were selected for the study. Specifically, 172 dairy buffaloes were selected from Selangor (six farms), 21 from Kedah (four farms), and 24 from Penang (two farms). During a single visit to each farm, all lactating buffaloes underwent the CMT test to diagnose SCM before milk sampling.

Animal and Farm Characteristics

Data on individual animals and farm management were obtained through interviews, farm records, on-farm assessment, and cow assessment. Based on the information gleaned from previous studies (Costa et al., 2020; Singha et al., 2023), nine animal-based and 18 farm-based variables were investigated as potential risk factors for SCM bubaline. As shown in Table 2, the research variables were categorised as described by Demil et al. (2022).

Table 2
Categories of animal-based and farm-based data and assessment methods used in the studied farms

	Animal-based data			
No.	**Variables**	**Categories**	**Methods**	**References**
1	Udder quarters position	Left front, right front, left hind, right hind	CA	Kashyap et al. (2019); Singh et al. (2023)
2	Lactation stage	Early lactation (≤90 days), mid-lactation (≥90-180 days), late lactation (≥180 days)	Interview, FR	Hameed et al. (2012); Srinivasan et al. (2013); Swami et al. (2017)
3	Age	≤6 years, ≥6–9 years, ≥9 years	Interview, FR	Hameed et al. (2012); Salvador et al. (2012); Swami et al. (2017)
4	Calving cycle	≤4 cycles, 4–7 cycles, ≥7 cycles	Interview, FR	Hameed et al. (2012); Vishwakarma et al. (2010)
5	Breed	Murrah, Nilli-ravi, Crossbreed Sawah	Interview, CA	Javed et al. (2022); Kashyap et al. (2019); Tiwari et al. (2022)
6	Calving interval	<1 year, >1-year	Interview	Chishty et al. (2007)

Table 2 *(continue)*

	Animal-based data			
No.	**Variables**	**Categories**	**Methods**	**References**
7	History of mastitis	Yes, no	Interview	Badua et al. (2020); Tiwari et al. (2022)
8	Milk yield	≤8L, 8–12L, ≥12L	Interview	Jaglan et al. (2022)
9	Milk letdown practice	Allowing the calf to suckle, oxytocin injection	CA	Hameed et al. (2012)
	Farm-based data			
No.	**Variables**	**Categories**	**Methods**	**References**
1	Farm size	<4 acres, 4–20 acres, >20 acres	Interview	Aliul et al. (2020)
2	Paddock size	<1000ft^2, 1000–10000ft^2, >10000ft^2	Interview	Badua et al. (2020)
3	Breeding system	Semi-intensive, intensive	Interview	Singh et al. (2023); Tiwari et al. (2022)
4	Milking method	Hand milking, machine milking	Interview, OA	Badua et al. (2020); Hameed et al. (2012); Ottalwar et al. (2018)
5	Frequency of milking	Once daily, twice daily	Interview, OA	Singha et al. (2024)
6	Milking place	Milking inside the paddock, milking outside the paddock or in a milking parlour	Interview, OA	García-Acevedo et al. (2023)
7	Practices of hand and teat hygiene before milking	Yes, no	Interview, OA	Badua et al. (2020); García-Acevedo et al. (2023); Malik and Verma (2017)
8	Towel drying of teats	Yes, no	Interview, OA	
9	Pre- and post-teat dipping	Yes, no	Interview, OA	Aliul et al. (2020)
10	Use of antibiotics for mastitis treatment and dry therapy	Yes, no	Interview	Badua et al. (2020); Bhandari et al. (2021)
11	Presence of other dairy animals	Yes, no	OA	Badua et al. (2020)
12	Type of milking utensils	Mix (using plastic and stainless steel) plastic, stainless steel	Interview, OA	Bomfim et al. (2023)
13	Frequency of cleaning milking utensils	Once daily, twice daily	Interview	
14	Frequency of paddock cleaning	Once daily, twice daily, three times daily	Interview	Bhandari et al. (2021)
15	Number of milkers	<3 milkers, >3 milkers	Interview	Singh et al. (2023)
16	Water source on the farm	Underground water, wastewater	Interview, OA	Zaki et al. (2010)

Note. CA = Cow assessment, FR = Farm records, OA = On-farms assessment

Detection of SCM-affected Animals

CMT was conducted to detect SCM at an early stage, following the procedures outlined by Schalm et al., 1971. Briefly, 2 mL of milk sample from clean teats were mixed with 2 mL of CMT reagent (Kruuse Bovivet CMT liquid, Kruuse, Denmark) on a CMT paddle. The paddle was gently rotated for 10 seconds, and any colour changes or formation of viscous gel were promptly observed. The visible reaction disappears after approximately 20 seconds, particularly for the weak reaction. The CMT results were recorded as follows: negative for no reaction (indicating uninfected or healthy), trace or 1+ score (indicating weak infection with SCM), and 2+ and 3+ scores (indicating strong infection with SCM). Buffaloes that tested negative and showed traces of the CMT were classified as healthy. Conversely, buffaloes that scored +1, +2, or +3 on the CMT in at least one quarter were classified as SCM.

Statistical Data Analysis

Descriptive analysis was performed to summarise the farm characteristics, prevalence of SCM, and farm and animal-based data. The association between the position of udder quarters and the prevalence of SCM bubaline was analysed using the Chi-square test. Logistic regression models were built to determine the factors associated with the prevalence of SCM bubaline. Parameter estimates were computed at a 95% confidence interval with crude odds ratio (COR) and adjusted odds ratio (AOR) at the univariable and multivariable levels, respectively. Model fit was assessed using the Hosmer-Lemeshow test. All the analyses were conducted using IBM SPSS Statistics version 27.0 (IBM Corp., USA).

RESULTS

Farm Characteristics and Screened Buffaloes

The study encompassed a range of lactating buffaloes, with a minimum of 2 animals per farm on the SEL5 farm and a maximum of 97 animals per farm on the SEL6 farm. The Murrah buffalo breeds constituted 83.9% of the sampled animals. Farms with a few animals were deliberately included to augment the sample size and gain a comprehensive understanding of the variations in animal and farm management data. The overall farm characteristics are presented in Table 3.

Prevalence of SCM Bubaline

The overall animal-level prevalence of SCM bubaline was 40.1% (87/217, 95% Confidence Interval (CI): 0.0%–66.7%). It included buffaloes with positive CMT results (+1, +2, and +3) in at least one quarter (Table 4). The mean prevalence of bubaline at the animal level on farms in Selangor, Kedah, and Penang was 41.3% (71/172, 95% CI: 0.0-53.6), 38.1% (8/21, 95% CI: 0.0–66.7), and 33.3% (8/24, 95% CI: 18.2–46.2), respectively.

Table 3
Characteristics of the 12 buffalo farms recruited in the study from three states in Peninsular Malaysia

Categories	Selangor (*n* = 172 buffaloes, 6 farms)	Kedah (*n* =21 buffaloes, 4 farms)	Penang (*n* =24 buffaloes, 2 farms)	Total (%)
Farm size (acres)				
<4	3	1	1	5 (41.7)
4–20	2	1	1	4 (33.3)
>20	1	2	0	3 (25.0)
Paddock size (ft^2)				
<1,000	2	2	2	6 (50.0)
1,000–10,000	3	2	0	5 (41.7)
>10,000	1	0	0	1 (8.3)
Breeding systems				
Semi-intensive	6	3	1	10 (83.3)
Intensive	0	1	1	2 (16.7)
Milking method				
Hand	4	3	2	9 (75.0)
Machine	2	1	0	3 (25.0)
Milking frequency				
One time	0	2	0	2 (16.7)
Two times	6	2	2	10 (83.3)
Milking place				
Milking inside the paddock	4	3	2	9 (75.0)
Milking outside the paddock	2	1	0	3 (25.0)
Hand hygiene before milking				
No	0	0	0	0 (0.0)
Yes	6	4	2	12 (100.0)
Teat hygiene before milking				
No	0	0	0	0 (0.0)
Yes	6	4	2	12 (100.0)
Pre and post-teat dipping				
No	5	3	1	9 (75.0)
Yes	1	1	1	3 (25.0)
Towel drying of teats				
Yes	0	0	0	0 (0.0)
No	6	4	2	12 (100.0)
Type of milking utensils				
Mix	6	4	2	12 (100.0)
Plastic	0	0	0	0 (0.0)
Stainless steel	0	0	0	0 (0.0)

Table 3 *(continue)*

Categories	Selangor (*n* = 172 buffaloes, 6 farms)	Kedah (*n* =21 buffaloes, 4 farms)	Penang (*n* =24 buffaloes, 2 farms)	Total (%)
Utensils cleaning frequency				
One time	0	2	0	2 (16.7)
Two times	6	2	2	10 (83.3)
Antibiotics used to treat mastitis				
No	3	3	2	8 (66.7)
Yes	3	1	0	4 (33.3)
Antibiotics used for dry therapy				
No	5	3	1	9 (75.0)
Yes	1	1	1	3 (25.0)
Cleaning farm frequency				
One time	1	3	0	4 (33.3)
Two times	5	1	0	6 (50.0)
Three times	0	0	2	2 (16.7)
Presence of other dairy animals				
No	3	3	0	6 (50.0)
Yes	3	1	2	6 (50.0)
Number of milkers on the farm				
<3	2	4	1	7 (58.3)
>3	4	0	1	5 (41.7)
Water source in the farm				
Underground water (pipe)	5	4	2	11 (91.7)
Wastewater (lake)	1	0	0	1 (8.3)

Table 4
The animal-level and quarter-level prevalence of SCM bubaline in selected states in Peninsular Malaysia

Prevalence		Selangor	Kedah	Penang	Total
Animal-level	Number examined	172	21	24	217
	Number of positive CMT	71	8	8	87
	Prevalence (%)	41.3	38.1	33.3	40.1
	95% confidence interval (%)	0.0–53.6	0.0–66.7	18.2–46.2	0.0–66.7
Quarter-level	Number examined	650	77	95	822
	Number of positive CMT	183	17	44	244
	Prevalence (%)	28.2	22.1	46.3	29.7
	95% confidence interval (%)	9.9–40.1	6.3–33.3	43.2–49.0	6.3–49.0

Meanwhile, the quarter-level prevalence of SCM bubaline was 29.7% (244/822, 95% CI: 6.3%–49.0%), including quarters with positive CMT results (trace, +1, +2, and +3). A total of 46 quarters that were blind were excluded from the examined quarters. The mean quarter-level prevalence of SCM bubaline on farms in Selangor, Kedah, and Penang was 28.2% (183/650, 95% CI: 9.9–40.1), 22.1% (17/77, 95% CI: 6.3–33.3), and 46.3% (44/95, 95% CI: 43.2–49.0), respectively. In addition, the CMT scores of SCM bubaline at the quarter level on farms in Selangor, Kedah, and Penang were presented in Table 5.

Table 5
The CMT scores of SCM bubaline at quarter level in selected states in Peninsular Malaysia

State	Number examined	CMT score (n) (proportion positive [%])				
		Negative	Trace	+1	+2	+3
Selangor	650	470 (72.3)	45 (6.9)	79 (12.2)	44 (6.8)	12 (1.8)
Kedah	77	60 (77.9)	6 (7.8)	11 (14.3)	0 (0)	0 (0)
Penang	95	51 (53.7)	27 (28.4)	10 (10.5)	3 (3.2)	4 (4.2)
Total	822	581 (70.7)	78 (9.5)	100 (12.2)	47 (5.7)	16 (1.9)

Risk Factors of SCM Bubaline

Udder Quarter Position Level

Table 6 shows results regarding the relationship between the investigated factors and the quarter-level prevalence of SCM. No significant association was detected between quarter position and the prevalence of SCM bubaline.

Table 6
Relationship analysis between the prevalence of SCM bubaline with udder quarters position

Quarters	Sample (*n*)	Positive SCM (*n*) (proportion positive (%)	Chi-square		
			X^2	*df*	*p*-value
Quarters position					
LF	206	63 (30.6)	0.795	3	0.851
RF	208	62 (29.8)			
LH	210	65 (31.0)			
RH	198	54 (27.3)			

Note. LF= left front, RF= right front, LH= left hind, RH= right hind

Animal Level

The association between the animal-based factors and the prevalence of SCM bubaline are presented in Table 7. In the univariable model, significant factors at the *p*-value of 0.10 include mastitis history ($p < 0.001$) and the daily milk yield ($p = 0.009$) at the animal

level. Buffaloes without a mastitis history were less likely (OR: 0.64, 95% CI: 0.002-0.019) to have SCM compared to those with a mastitis history. Buffaloes that produced more than 12L of milk were more likely (OR: 1.91, 95% CI: 1.01-3.64) relative to those producing less than 12L. However, only mastitis history was significantly associated with the prevalence of SCM ($p < 0.001$) at the multivariable level. Buffaloes without a mastitis history were less likely (OR: 0.005, 95% CI: 0.001-0.017) to have SCM than buffaloes with a mastitis history.

Table 7
Univariable and multivariable binary logistic regression analysis between animal-level factors and prevalence of SCM bubaline in selected states in Peninsular Malaysia

Categories	Sample (n) (sample frequency [%])	Positive SCM (n) (proportion positive [%])	Univariable level			Multivariable level		
			OR	95% Cl	*p*-value	OR	95% Cl	*p*-value
Lactation stage				*p*=0.659				
Early	105 (48.4)	42 (40.0)	Ref					
Mid	80 (36.9)	30 (37.5)	0.900	0.495–1.636	0.730			
Late	32 (14.7)	15 (46.9)	1.324	0.597–2.935	0.490			
Age				*p*=0.158				
<6 years	103 (47.5)	36 (35.0)	Ref					
6-9 years	95 (43.8)	40 (42.1)	1.354	0.762–2.404	0.302			
>9 years	19 (8.7)	11 (57.9)	2.559	0.945–6.933	0.065			
Mastitis history								
No	137 (63.1)	12 (8.8)	0.006	0.002–0.019	<0.001	0.005	0.001–0.017	<0.001
Yes	80 (36.9)	75 (93.8)	Ref			Ref		
The volume of milk produced				*p*=0.009			*p*=0.527	
<8L	61 (28.1)	21 (34.4)	Ref			Ref		
8-12L	48 (22.1)	12 (25.0)	0.635	0.274–1.471	0.289	1.812	0.467–7.025	
>12L	108 (49.8)	54 (50.0)	1.905	1.011–3.645	0.052	0.889	0.250–3.162	
Milk letdown practices								
Allow calf to suckle	192 (88.5)	74 (38.5)	Ref					
Oxytocin injection	25 (11.5)	13 (52.0)	1.727	0.748–3.988	0.200			

Farm Level

Table 8 depicts the relationship between the farm-level factors and the prevalence of SCM bubaline. At the univariable level, the prevalence of SCM was significantly associated ($p<0.1$) with paddock size (p=0.001), breeding system ($p=0.045$), milking method

Table 8
Univariable and multivariable binary logistic regression analysis between farm-level factors and the prevalence of SCM bubaline in selected states in Peninsular Malaysia

Categories	Sample (*n*)	Positive SCM (*n*) (proportion positive [%])	Univariate model			Multivariate model		
			OR	95% CI	*p*-value	OR	95% CI	*p*-value
Paddock size (ft^2)				*p*=0.001			*p*=0.420	
<1,000	65	18 (27.7)	Ref			Ref		
1,000–10,000	55	17 (30.9)	1.168	0.531–2.571	0.699	0.577	0.130–2.556	0.469
>1,0000	97	52 (53.6)	3.017	1.538–5.921	0.001	0.078	0.002–3.456	0.188
Breeding system								
Semi-intensive	202	85 (42.1)	Ref			Ref		
Intensive	15	2 (13.3)	0.212	0.047–0.963	0.045	0.000	-	0.999
Milking method								
Hand	111	32 (28.8)	Ref			Ref		
Machine	106	55 (51.9)	2.662	1.520–4.662	<0.001	0.000	-	0.999
Milking place								
Milking inside the paddock	111	32 (28.8)	Ref					
Milking outside the paddock	106	55 (51.9)	2.662	1.520–4.662	<0.001			
Hand hygiene before milking								
No	0	(0.0)	Ref					
Yes	217	87 (40.1)	0.669		0.004			
Teat hygiene before milking								
No	0	0 (0.0)	Ref					
Yes	217	87 (40.1)	0.669		0.004			
Pre and post-teat dipping								
No	102	30 (29.4)	0.424	0.242–0.743	0.003	0.033	0.001–0.870	0.041
Yes	115	57 (49.6)	Ref			Ref		

Table 8 *(continue)*

Categories	Sample (*n*)	Positive SCM (*n*) (proportion positive [%])	Univariate model			Multivariate model		
			OR	95% CI	*p*-value	OR	95% CI	*p*-value
Cleaning and drying teat using a towel								
No	217	87 (40.1)	0.669	-	0.004			
Yes	0	0 (0.0)	Ref					
Milking utensils type								
Mix	217	87 (40.1)	0.669		0.004			
Plastic	0	0 (0.0)	-		-			
Stainless steel	0	0 (0.0)	Ref					
Antibiotics used to treat mastitis								
No	78	22 (28.2)	Ref			Ref		
Yes	139	65 (46.8)	2.236	1.233–4.054	0.008	0.201	0.026–1.578	0.127
Antibiotics used for dry therapy								
No	102	30 (29.4)	0.424	0.242–0.743	0.003	0.000	-	0.999
Yes	115	57 (49.6)	Ref			Ref		
Presence of other dairy animals								
No	151	70 (46.4)	2.491	1.316–4.713	0.005	3.181	0.710–30.854	0.048
Yes	66	17 (25.8)	Ref			Ref		
Number of milkers								
<3 milkers	62	17 (27.4)	Ref			Ref		
>3 milkers	155	70 (45.2)	2.180	1.148–4.140	0.017	3.181	0.710–14.259	0.131

(p<0.001), milking place (p<0.001), pre-and post-teat dipping (p=0.003), antibiotics used to treat mastitis (p=0.008) and for dry therapy (p=0.003), and the presence of other dairy animals (p=0.005). The final factors associated with SCM prevalence at the multivariable level (p<0.05) were (pre- and post-teat dipping (p=0.041) and the presence of other dairy animals (p=0.048). Farms that kept only buffaloes were more likely to have SCM (OR: 3.18, 95%CI: 0.71–30.85) compared to farms with other dairy animals.

DISCUSSION

Mastitis in dairy animals can cause huge economic losses and pose serious public health concerns (Chakraborty et al., 2019). This study is the first attempt to investigate SCM bubaline's prevalence and risk factors in Peninsular Malaysia's selected states. The prevalence of SCM at the animal level and quarter level was 40.1% and 29.7%, respectively. No previous studies have been conducted on SCM bubaline in Peninsular Malaysia. Therefore, the only comparable research available is a recent study on bovine mastitis conducted in dairy farms across Selangor, Perak, Pahang, Negeri Sembilan, and Johor, which reported a higher prevalence of SCM in bovine populations at 67.6%, which is higher than the prevalence found in the current study on bubaline populations from various states (Ali et al., 2020). The use of different detection methods (bacterial culture vs CMT) and study locations might contribute to these discrepancies. Nevertheless, two previous studies conducted in Egypt on both buffaloes and cows reported a prevalence of SCM in buffaloes (44.3% and 44%), which was comparable to that in cows (49.9% and 52.1%) (Ahmed et al., 2018; Algammal et al., 2020). This finding suggests that both buffaloes and cows are equally susceptible to SCM. It is important to note that the prevalence of SCM in buffaloes can have significant economic implications, as it can lead to increased somatic cell counts, decreased milk production, and a significant decline in milk quality, leading to low demand. These implications are similar to those of bovine mastitis (Pizauro et al., 2014).

Numerous reports on the prevalence of SCM in buffaloes have been conducted in different countries. The prevalences reported in this study were higher compared to reports in Brazil (8.35%) (Pizauro et al., 2014), the Philippines (24.22%) (Badua et al., 2020), Bangladesh (27.9%) (Singha et al., 2023), and China (29.44%) (Zhang et al., 2023), but comparable to reports in India (41.51%) (Ottalwar et al., 2018), and Egypt (44.3%) (Ahmed et al., 2018). Additionally, our findings were lower compared to studies conducted in India (68.33%) (Kashyap et al., 2019), Nepal (70%) (Tiwari et al., 2022), and Pakistan (75.31%) (Javed et al., 2022). The variation in the prevalence of mastitis can be attributed to geographic location, climate, animal-level risk factors such as breed, lactation stage, age, parity, quarters, and teat shape, as well as farm-level risk factors such as breeding system, husbandry practices, and farm hygiene (Salvador et al., 2012; Tiwari et al., 2022).

The association between the prevalence of SCM bubaline and the position of the quarter was not significant in the present study. Some studies found that the left quarters had a higher prevalence of SCM than the right quarters (Hoque et al., 2022; Kaur et al., 2015; Singha et al., 2023). On the contrary, other studies conducted in Pakistan and Nepal reported that the left front quarter had a higher prevalence of SCM than the right hind quarter, left hind quarter, and right front quarter. The association between quarter position and SCM depends on the position of the farmers during milking, which may increase exposure to pathogens and pressure on the teats (Bhandari et al., 2021; Chishty et al., 2007). The position of the farmers during milking was related to the sequencing of teat milking. Milking the right quarter after the left quarter leaves the teat canal open, allowing pathogens to enter the mammary glands and lead to SCM (Singha et al., 2023). In addition, hindquarters are more susceptible to SCM due to their increased exposure to contamination from urine, faeces, and the tail (Kashyap et al., 2019; Kisku & Samad, 2013). As Kashyap et al. (2019) mentioned, the hindquarters are larger and more pendulous than the front quarters, increasing the risk of contamination from the milking area or barn floor. The lack of a significant association in our study is not fully understood. However, the similarity in the milking practices may annul the relative risk of greater exposure to SCM between left and right quarters and hind and front quarters.

In this study, the prevalence of SCM bubaline was higher in buffaloes with a history of mastitis compared to those without the history. This finding is consistent with previous studies conducted in Nepal (Bhandari et al., 2021; Tiwari et al., 2022) and the Philippines (Badua et al., 2020). Bhandari et al. (2021) suggested that buffaloes with a history of mastitis linked to a high prevalence of SCM due to the overuse or misuse of antibiotics in the treatment of previous mastitis cases, selection pressure, and poor farm hygiene (Ali et al., 2014). Incomplete or ineffective mastitis treatment in buffaloes may also lead to inflammation and damage to the mammary tissue, allowing residual bacteria to persist (Cheng & Han, 2020). It indicates that previous episodes of mastitis may contribute to persistent or recurrent infections, resulting in a higher prevalence of SCM in buffaloes with a history of mastitis.

This study also found significant differences in the prevalence of SCM in farms that practice teat dipping and those not practising the procedure. This finding may stem from several factors, such as the type of disinfectant used, the technique of application, and overall farm hygiene practices (Sharif & Ahmad, 2007; Singha et al., 2023). Improper teat dipping practices, including the use of ineffective disinfectants or inadequate application of the disinfectant, as well as the timing of teat dipping (preferably within 30 seconds after milking), could be the possible causes for the high prevalence of SCM in farms that do not practice teat dipping (Nickerson et al., 2019).

The prevalence of SCM bubaline was significantly higher in farms that kept only buffaloes compared to other dairy animals, such as cows and goats. This result is consistent with the report from the Philippines (Badua et al., 2020), which focused on the association between the presence of other animals on the farm and the prevalence of *methicillin-resistant S. aureus (MRSA)* in bubaline mastitis. On the other hand, the increased risk of SCM in farms that solely keep only one species may be due to factors like limited farm size, poor hygiene practices, and the role of milkers interacting with animals, which increase exposure to environmental and contagious pathogens (Abebe et al., 2023; Gantner et al., 2023; Pletinckx et al., 2011). Diversified livestock farms tend to have more comprehensive biosecurity measures. These measures effectively prevent the transmission of diseases between species (Msimang et al., 2022; Pozo et al., 2024; Renault et al., 2021). Biosecurity measures include separate fencing, control of animal movement, segregated equipment usage, proper manure management, isolation of sick animals, regular veterinary involvement, vaccination protocols, and rigorous testing of bulk tank milk. However, the relationship between the presence of other dairy animals and the prevalence of SCM bubaline requires further investigation.

Despite the strengths of this study in bridging the research gap towards identifying control and prevention measures for SCM bubaline, the research limitations are well-acknowledged. Samples were collected from farms located in three states in Peninsular Malaysia. Hence, the findings may not be generalisable to all buffalo farms in the country. Regarding the investigated factors, information on the biosecurity measures or specific teat dipping procedures implemented by the farms was not collected, including the timing and type of disinfection used. As a result, further investigation is required to elucidate the relationship between teat dipping, biosecurity measures, and the prevalence of SCM bubaline.

CONCLUSION

The current study found a high prevalence of SCM bubaline, associated with a history of mastitis at the individual animal level, pre- and post-teat dipping, and the absence of other dairy animals at the farm level. These findings provide valuable information for developing effective prevention and control measures against SCM, particularly in buffaloes in Peninsular Malaysia. Additionally, educating farmers on good farm management and proper milking practices could assist in reducing the occurrence of SCM in the farms.

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REFERENCES

Abebe, R., Markos, A., Abera, M., & Mekbib, B. (2023). Incidence rate, risk factors, and bacterial causes of clinical mastitis on dairy farms in Hawassa City, southern Ethiopia. *Scientific Reports*, *13*(1), 10945. https://doi.org/10.1038/s41598-023-37328-1

Ahmed, H. F., Straubinger, R. K., Hegazy, Y. M., & Ibrahim, S. (2018). Subclinical mastitis in dairy cattle and buffaloes among smallholders in Egypt: Prevalence and evidence of virulence of *Escherichia coli* causative agent. *Malaysian Society of Parasitology and Tropical Biomedicine*, *35*(2), 321-329.

Algammal, A. M., Enany, M. E., El-Tarabili, R. M., Ghobashy, M. O., & Helmy, Y. A. (2020). Prevalence, antimicrobial resistance profiles, virulence, and enterotoxins-determinant genes of MRSA isolated from subclinical bovine mastitis in Egypt. *Pathogens*, *9*(5), 362. https://doi.org/10.3390/pathogens9050362

Ali, B., Zunita, Z., Jesse, F. F., Ramanoon, S. Z., & Mohd-Azmi, M. L. (2020). *Prevalence and antimicrobial resistance profiles of bacterial isolates associated with intramammary infections in Malaysian dairy herds.* Research Square. https://doi.org/10.21203/rs.2.22292/v1

Ali, T., Kamran, R., Raziq, A., Wazir, I., Ullah, R., Shah, P., Ali, M. I., Han, B., & Liu, G. (2021). Prevalence of mastitis pathogens and antimicrobial susceptibility of isolates from cattle and buffaloes in Northwest Pakistan. *Frontiers in Veterinary Science*, *8*, 746755. https://doi.org/10.3389/fvets.2021.746755

Ali, T., Rahman, A., Qureshi, M. S., Hussain, M. T., Khan, M. S., Uddin, S., Iqbal, M., & Han, B. (2014). Effect of management practices and animal age on incidence of mastitis in Nili Ravi buffaloes. *Tropical Animal Health and Production*, *46*, 1279-1285. https://doi.org/10.1007/s11250-014-0641-2

Aliul, H., Kumar, P. A., Mahmood, R. M., Mizanur, R., & Selim, A. M. (2020). Investigation of prevalence and risk factors of subclinical mastitis of dairy buffaloes at Bhola district of Bangladesh. *Asian Journal of Medical and Biological Research*, *6*(4), 697–704. https://doi.org/10.3329/ajmbr.v6i4.51236

Ariff, O. M., Sharifah, N. Y., & Hafidz, A. W. (2015). Status of beef industry of Malaysia. *Malaysian Journal of Animal Science*, *18*(2), 1-21.

Badua, A. T., Boonyayatra, S., Awaiwanont, N., Gaban, P. B. V., & Mingala, C. N. (2020). Methicillin-resistant *Staphylococcus aureus* (MRSA) associated with mastitis among water buffaloes in the Philippines. *Heliyon*, *6*(12), e05799. https://doi.org/10.1016/j.heliyon.2020.e05799

Bhandari, S., Subedi, D., Tiwari, B. B., Shrestha, P., Shah, S., & Al-Mustapha, A. I. (2021). Prevalence and risk factors for multidrug-resistant *Escherichia coli* isolated from subclinical mastitis in the western Chitwan region of Nepal. *Journal of Dairy Science*, *104*(12), 12765-12772. https://doi.org/10.3168/jds.2020-19480

Bomfim, V. V. B. da S., Balieiro, A. C., Costa, A. C. M. de S. F. da, Romeiro, E. T., Franco, E. de S., Neves, M. L. M. W., Marinho, G. L. de O. C., & Farias M. P. O. (2023). Risk factors associated with bovine mastitis. *Revista Ibero-Americana De Humanidades, Ciências E Educação*, *9*(3), 1452–1459. https://doi.org/10.51891/rease.v9i3.8958

Chakraborty, S., Dhama, K., Tiwari, R., Yatoo, M. I., Khurana, S. K., Khandia, R., Munjal, A., Munuswamy, P., Kumar, M. A., Singh, R., Gupta, V. K., & Chaicumpa, W. (2019). Technological interventions and advances in the diagnosis of intramammary infections in animals with emphasis on bovine population: A review. *Veterinary Quarterly*, *39*(1), 76-94. https://doi.org/10.1080/01652176.2019.1642546

Cheng, W. N., & Han, S. G. (2020). Bovine mastitis: Risk factors, therapeutic strategies, and alternative treatments: A review. *Asian-Australasian Journal of Animal Sciences*, *33*(11), 1699-1713. https://doi.org/10.5713/ajas.20.0156

Chishty, M. A., Arshad, M., Avais, M., & Ijaz, M. (2007). Cross-sectional epidemiological studies on mastitis in cattle and buffaloes of tehsil Gojra Pakistan. *Buffalo Bulletin*, *26*(2), 50-55.

Costa, A., Neglia, G., Campanile, G., & De Marchi, M. (2020). Milk somatic cell count and its relationship with milk yield and quality traits in Italian water buffaloes. *Journal of Dairy Science*, *103*(6), 5485-5494. https://doi.org/10.3168/jds.2019-18009

Demil, E., Teshome, L., Kerie, Y., Habtamu, A., Kumilachew, W., Andualem, T., & Mekonnen, S. A. (2022). Prevalence of subclinical mastitis, associated risk factors, and antimicrobial susceptibility of the pathogens isolated from milk samples of dairy cows in Northwest Ethiopia. *Preventive Veterinary Medicine*, *205*, 105680. https://doi.org/10.1016/j.prevetmed.2022.105680

Department of Veterinary Services Malaysia. (2022). *Livestock statistics 2022/2023*. https://www.dvs.gov.my/index.php/pages/view/4564

Food and Agriculture Organization of the United Nations. (n.d.). *FAOSTAT: Crops and livestock products*. https://www.fao.org/faostat/en/#data/QCL

Gantner, V., Jožef, I., Gantner, R., Steiner, Z., & Zmaić, L. (2023). Estimation of prevalence, effect and cost of mastitis on Simmental dairy farms of different sizes. *Economics of Agriculture*, *70*(4), 1123-1139. https://:10.59267/ekoPolj23041123G

García-Acevedo, J. F., Tobón, J. D., Grisales, C. F., Gómez, C., Fernández-Silva, J. A., & Ramírez-Vásquez, N. F. (2023). Prevalence and risk factors of mastitis among dairy buffaloes from the departments of Antioquia and Córdoba, Colombia. *Journal of Buffalo Science*, *12*, 117-133. https://doi.org/10.6000/1927-520X.2023.12.14

Hameed, S., Arshad, M., Ashraf, M., Avais, M., & Shahid, M. A. (2012). Cross-sectional epidemiological studies on mastitis in cattle and buffaloes of Tehsil Burewala, Pakistan. *The Journal of Animal and Plant Sciences*, *22*(3 Supplement), 371-376.

Hoque, M. N., Talukder, A. K., Saha, O., Hasan, M. M., Sultana, M., Rahman, A. A., & Das, Z. C. (2022). Antibiogram and virulence profiling reveals multidrug resistant *Staphylococcus aureus* as the predominant aetiology of subclinical mastitis in riverine buffaloes. *Veterinary Medicine and Science*, *8*(6), 2631-2645. https://doi.org/10.1002/vms3.942

Hussain, A., Ahmad, M., Mushtaq, M. H., Chaudhry, M., Khan, M. S., Reichel, M., Hussain, T., Khan, A., Nisar, M., & Khan, I. A. (2018). Prevalence of overall and teatwise mastitis and effect of herd size in dairy buffaloes. *Pakistan Journal of Zoology*, *50*(3), 1107-1112. https://doi.org/10.17582/journal.pjz/2018.50.3.1107.1112

Islam, J., Rume, F. I., Liza, I. J., Chaudhary, P. K., & Anower, A. K. M. M. (2019). Assessment of subclinical mastitis in milch animals by different field diagnostic tests in Barishal district of Bangladesh. *Asian-Australasian Journal of Bioscience and Biotechnology*, *4*(1), 24-33.

Jaglan, K., Sukhija, N., George, L., Alex, R., & Verma, A. (2022). *Impact of non-genetic factors on clinical mastitis incidence in Murrah buffaloes.* Research Square. https://doi.org/10.21203/rs.3.rs-1967312/v1

Javed, S., McClure, J., Syed, M. A., Obasuyi, O., Ali, S., Tabassum, S., Ejaz, M., & Zhang, K. (2022). Epidemiology and molecular characterization of *Staphylococcus aureus* causing bovine mastitis in water buffaloes from the Hazara division of Khyber Pakhtunkhwa, Pakistan. *PLOS One*, *17*(5), e0268152. https://doi.org/10.1371/journal.pone.0268152

Kashyap, D. K., Giri, D. K., & Dewangan, G. (2019). Prevalence of subclinical mastitis (SCM) in she buffaloes at Surajpur district of Chhattishgarh, India. *Buffalo Bulletin*, *38*(2), 373-381.

Kaur, M., Verma, R., Bansal, B. K., Mukhopadhyay, C. S., & Arora, J. S. (2015). Status of sub-clinical mastitis and associated risk factors in Indian water buffalo in Doaba region of Punjab, India. *Indian Journal of Dairy Science*, *68*(5), 483-487.

Kisku, J. J., & Samad, M. A. (2013). Prevalence of sub-clinical mastitis in lactating buffaloesdetected by comparative evaluation of indirect tests and bacteriological methods with antibiotic sensitivity profiles in Bangladesh. *Buffalo Bulletin*, *32*(4), 293-306. https://doi.org/10.14456/ku-bufbu.2013.41

Krishnamoorthy, P., Goudar, A. L., Suresh, K. P., & Roy, P. (2021). Global and countrywide prevalence of subclinical and clinical mastitis in dairy cattle and buffaloes by systematic review and meta-analysis. *Research in Veterinary Science*, *136*, 561-586. https://doi.org/10.1016/j.rvsc.2021.04.021

Malaysian Meteorological Department. (2022). *Weather and climate*. https://data.gov.my/dashboard/weather-and-climate

Malik, M. H., & Verma, H. K. (2017). Prevalence, economic impact, and risk factors associated with mastitis in dairy animals of Punjab. *Indian Journal of Animal Sciences*, *87*(12), 1452-1456.

Mohd Azmi, A. F., Abu Hassim, H., Mohd Nor, N., Ahmad, H., Meng, G. Y., Abdullah, P., Abu Bakar, M. Z., & Zamri-Saad, M. (2021). Comparative growth and economic performances between indigenous swamp and Murrah crossbred buffaloes in Malaysia. *Animals*, *11*(4), 957. https://doi.org/10.3390/ani11040957

Msimang, V., Rostal, M., Cordel, C., Machalaba, C., Tempia, S., Bagge, W., Burt, F., Karesh, W., Pawęska, J., & Thompson, P. (2022). Factors affecting the use of biosecurity measures for the protection of ruminant livestock and farm workers against infectious diseases in central South Africa. *Transboundary and Emerging Diseases*, *69*, e1899 - e1912. https://doi.org/10.1111/tbed.14525

Nickerson, S. C., Kautz, F. M., Ely, L. O., & Ryman, V. E. (2019*). Germicidal efficacy testing of Forticept Udder Wash (pre-dip) and Forticept Udder Forte (post-dip) in reducing the new intramammary infection rate and SCC under natural exposure to mastitis pathogens, with additional in vitro testing of germicidal activity.* University of Georgia. https://animaldairy.uga.edu/content/dam/caes-subsite/animal-dairy-science/documents/departmental-reports/2019-departmental-reports/Germicidal_efficacy_testing_of_Forticept.pdf

Nor, N. A. A. M., & Rosali, M. H. (2015). The development and future direction of Malaysia's livestock industry. *Food and Fertilizer Technology Centre Agricultural Policy Articles*, *8*. https://ap.fftc.org.tw/article/960

Ottalwar, T., Roy, M., Roy, S., & Ottalwar, N. (2018). Prevalence of subclinical mastitis in buffaloes (*Bubalus bubalis*) in Chhattisgarh, India. *International Journal of Academic Scientific Research*, *8*(1), 9-16.

Pizauro, L. J. L., Silva, D. G., Santana, A. M., Clemente, V., Lara, G. H. B., Listoni, F. J. P., Vaz, A. C. N., Vidal-Martins, A. M. C., Ribeiro, M. G., & Fagliari, J. J. (2014). Prevalence and etiology of buffalo mastitis and milk somatic cell count in dry and rainy seasons in a buffalo herd from Analândia, São Paulo State, Brazil. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, *66*(6), 1703-1710. https://doi.org/10.1590/1678-4162-7414

Pletinckx, L. J., Verhegghe, M., Dewulf, J., Crombé, F., De Bleecker, Y., Rasschaert, G., Goddeeris, B. M., & De Man, I. (2011). Screening of poultry-pig farms for methicillin-resistant *Staphylococcus aureus*: Sampling methodology and within herd prevalence in broiler flocks and pigs. *Infection, Genetics and Evolution*, *11*(8), 2133-2137. https://doi.org/10.1016/j.meegid.2011.07.008

Pozo, P., Isla, J., Asiain, A., Navarro, D., & Gortázar, C. (2024). Contribution of herd management, biosecurity, and environmental factors to the risk of bovine tuberculosis in a historically low prevalence region. *Animal*, *18*(3), 101105. https://doi.org/10.1016/j.animal.2024.101105

Preethirani, P. L., Isloor, S., Sundareshan, S., Nuthanalakshmi, V., Deepthikiran, K., Sinha, A. Y., Rathnamma, D., Prabhu, K. N., Sharada, R., Mukkur, T. K., & Hegde, N. R. (2015). Isolation, biochemical and molecular identification, and *in vitro* antimicrobial resistance patterns of bacteria isolated from bubaline subclinical mastitis in South India. *PLOS One*, *10*(11), e0142717. https://doi.org/10.1371/journal.pone.0142717

Renault, V., Humblet, M. F., Pham, P. N., & Saegerman, C. (2021). Biosecurity at cattle farms: Strengths, weaknesses, opportunities and threats. *Pathogens*, *10*(10), 1315. https://doi.org/10.3390/pathogens10101315

Salvador, R. T., Beltran, J. M. C., Abes, N. S., Gutierrez, C. A., & Mingala, C. N. (2012). Prevalence and risk factors of subclinical mastitis as determined by the California Mastitis Test in water buffaloes (*Bubalus bubalis*) in Nueva Ecija, Philippines. *Journal of Dairy Science*, *95*(3), 1363-1366. https://doi.org/10.3168/jds.2011-4503

Schalm, O. W., Carroll, E. J., & Jain, N. C. (1971). *Bovine mastitis*. Lea & Febiger.

Sharif, A., & Ahmad, T. (2007). Prevalence of severity of mastitis in buffaloes in district Faisalabad, Pakistan. *Journal of Agriculture and Social Science*, *3*(4), 34-36.

Singha, S., Koop, G., Ceciliani, F., Derks, M., Hoque, M. A., Hossain, M. K., Howlader, M. M. R., Rahman, M. M., Khatun, M., Boqvist, S., & Persson, Y. (2023). The prevalence and risk factors of subclinical mastitis in water buffalo (*Bubalus bubalis*) in Bangladesh. *Research in Veterinary Science*, *158*, 17-25. https://doi.org/10.1016/j.rvsc.2023.03.004

Singha, S., Koop, G., Rahman, M. M., Ceciliani, F., Addis, M. F., Howlader, M. M. R., Hossain, M. K., Piccinini, R., Locatelli, C., Persson, Y., & Bronzo, V. (2024). Pathogen group-specific risk factors for intramammary infection in water buffalo. *PLOS One*, *19*(4), e0299929. https://doi.org/10.1371/journal.pone.0299929

Srinivasan, P., Jagadeswaran, D., Manoharan, R., Giri, T., Balasubramaniam, G. A., & Balachandran, P. (2013). Prevalence and etiology of subclinical mastitis among buffaloes (*Bubalus bubalus*) in Namakkal, India. *Pakistan Journal of Biological Sciences*, *16*(23), 1776-1780. https://doi.org/10.3923/pjbs.2013.1776.1780

Swami, S. V., Patil, R. A., & Gadekar, S. D. (2017). Studies on prevalence of subclinical mastitis in dairy animals. *Journal of Entomology and Zoology Studies*, *5*(4), 1297-1300.

Thrusfield, M. V. (2005). *Veterinary epidemiology* (3rd ed.). Blackwell Science Limited.

Tiwari, B. B., Subedi, D., Bhandari, S., Shrestha, P., Pathak, C. R., Chandran, D., Pandey, G., Mohankumar, P., & Dhama, K. (2022). Prevalence and risk factors of staphylococcal subclinical mastitis in dairy animals of Chitwan, Nepal. *Journal of Pure and Applied Microbiology*, *16*(2), 1392-1403. https://doi.org/10.22207/JPAM.16.2.67

Vishwakarma, P., Sushovan Roy, S. R., Manju Roy, M. R., & Shakya, S. (2010). Occurrence of sub clinical mastitis in buffaloes of Chhattisgarh. *Indian Journal of Veterinary Medicine*, *30*(2), 94-96.

Wahid, H., & Rosnina, Y. (2016). Management of dairy animals: Buffalo: Asia. In *Encylopedia of dairy sciences* (pp. 836-844). Academic Press. https://doi.org/10.1016/B978-0-08-100596-5.21231-6

Zaki, M. M., El-Zorba, H. Y., & Kaoud, H. A. (2010). Environmental organisms as risk factors in the occurrence of mastitis of dairy buffaloes with suggested methods of control: A field study. *Journal Global Veterinaria*, *5*, 97-105.

Zhang, D., Lu, X., Feng, X., Shang, X., Liu, Q., Zhang, N., & Yang, H. (2023). Molecular characteristics of *Staphylococcus aureus* strains isolated from subclinical mastitis of water buffaloes in Guangdong Province, China. *Frontiers in Veterinary Science*, *10*, 1-8. https://doi.org/10.3389/fvets.2023.1177302

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Acute and Subacute Toxicity Assessment of Crude Aqueous Extract of *Melastoma malabathricum* Leaves

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ABSTRACT

Melastoma malabathricum (MM), also known as "Senduduk," is a medicinal herb commonly used among the indigenous folks in Malaysia as traditional medicine for practical purposes. Many toxicity profile studies have been carried out on MM extract. However, very few studies have described the toxicity profile of MM aqueous extract. Therefore, this study aimed to investigate the acute and subacute oral toxicity of MM crude aqueous extract using Sprague Dawley rats. A total of 34 female Sprague Dawley rats were randomly divided into six groups for acute (n=5) and subacute (n=6) studies. The duration for the acute and subacute studies was 14 days and 28 days. A single dose of MM extract was fixed at 3000 mg/kg and did not show any signs of toxicity or mortality during 14 days of observation. In the 28 days repeated dose toxicity test, the rats were orally fed with three different doses, 100 mg/kg, 500 mg/kg, and 1000 mg/kg of body weight/day, revealed no major significant change ($p<0.05$) in their physical appearance, behaviour, haematology,

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serum biochemistry indicators, and relative organ weight when compared to the control group. The findings were supported by a microscopic histopathology examination of the liver after feeding with MM crude aqueous extract. This study suggests that oral application of 3000 mg/kg in acute toxicity assay and 100 mg/kg, 500 mg/kg, and 1000 mg/kg of MM crude aqueous extract in subacute toxicity assay do not induce adverse effects in rats.

Keywords: Acute toxicity, aqueous crude extract, *Melastoma malabathricum*, Senduduk, subacute toxicity

INTRODUCTION

Melastoma malabathricum (MM) is a popular herb among the indigenous folks in Malaysia for its antimicrobial (Choudhury et al., 2011), anti-inflammation (Mazura et al., 2007) and antioxidant properties (Kumar et al., 2013). It has been traditionally applied for medical practical purposes. However, assessing the plant's toxicity is crucial to ensure its safety before use in medicinal applications. Toxicity refers to poisonous compounds within the plant that can harm cells. Exposure to hazardous chemicals or constituents can result in negative health consequences for individuals. It is worth noting that plants utilised in alternative medicine might contain toxic substances. Thoroughly assessing these effects is essential to safeguarding public health.

In 2012, Alnajar et al. (2012) performed the first acute toxicity assessment on ethanol-extracted MM plant samples in 2000 and 5000 mg/kg concentrations. In the following years, experiments were conducted to explore the toxicity of MM ethanol and methanol extracts using concentrations of 500 and 1000 mg/kg (Kamsani et al., 2019; Zahi et al., 2017). On the other hand, Reduan et al. (2020) assessed the dermal acute toxicity of MM ethanolic extracts in tests. All these investigations collectively indicated that MM extracts did not induce immediate adverse reactions either systematically or on the skin.

Many studies have documented diverse outcomes, potentially resulting from multiple factors that incorporate variations in experimental protocols, methodologies, herbal extraction techniques, and pre-treatment procedures. Previous research has suggested that the ethanol and methanol extracts derived from MM leaves were non-toxic in concentrations up to 5000 mg/kg. Nevertheless, aqueous extracts should have a lower toxicity response when compared with other solvents. The question raised on MM, specifically in crude aqueous extracts obtained through different extraction techniques, might cause similar toxic responses. Therefore, this study was conducted to assess the acute and subacute toxicity of MM leaves' crude aqueous extract in Sprague Dawley rats for 14 and 28 days, respectively. The experiment will involve the observation of physical and behavioural changes. Analysis of blood and serum markers, along with a detailed examination of organ histopathology, will be conducted to thoroughly assess the level of MM toxicity effect. The outcomes of this study will offer valuable insights into the safety profile of MM crude aqueous extract.

MATERIALS AND METHODS

Preparation of Extract

Fresh MM leaves were collected from the headquarters of the Malaysian Agricultural Research and Development Institute (MARDI), Malaysia, located at longitude 101° 41' 26.2284", latitude: N 2° 59.8573'. The MM plant was deposited at Universiti Putra Malaysia (UPM) with voucher number SK3338/18. Freshly harvested leaves were washed and dried in a 50 °C dryer. The crude extract was prepared by adding 80 g of the plants' ground leaf material in 1600 ml boiling water and shaking at 200 rpm for 2 hours. The crude extract was then filtrated through No. 1001 (Whatman, Germany) filter paper. The solution was freeze-dried and kept in the freezer prior to use.

Experimental Animals

Healthy female Sprague Dawley rats with an approximate weight of 150-170 g were obtained from A Sapphire Enterprise (001303794-M), located at Seri Kembangan, Selangor, Malaysia. These rats were allowed to acclimatise for two weeks prior to this study. Standard commercial rat pellets (Gold Coin Mouse Pellet 702P, Malaysia) and water were provided *ad libitium* for the animals. Individual cages were provided for each rat within the same animal room. The housing environment was maintained under specific conditions: a temperature of 25 °C ± 2 °C and a light-dark cycle of 12 hours each. These controlled conditions were upheld at the Animal Metabolism, Toxicity, and Reproductive Centre (AMTREC) within the Malaysian Agricultural and Research Development Institute (MARDI) located in Serdang, Selangor. The study was approved by the Malaysian Agricultural Research and Development, MARDI Animal Ethnic Committee (AEC) under 20220422/R/MAEC00108. All procedures involving the care and treatment of the animals strictly adhered to the approval of the ethical guidelines.

Acute Oral Toxicity

Following the acclimatisation period, a total of ten female Sprague Dawley rats were randomly divided into two groups. One group was subjected to gavage with distilled water (vehicle) at a dosage of 5 ml/kg ($n = 5$). In contrast, the other group (as the treatment group) received a high dose of MM extract at a concentration of 3000 mg/kg ($n = 5$) administered using a force-feed needle. This oral force-feeding procedure was conducted once, and the MM extract was freshly prepared daily before each administration. The rats were monitored individually within 30 minutes, followed by periodic observations throughout the first 24 hours. Special attention was given to the first four hours of observation. The animals were subjected to daily monitoring over a total duration of 14 days. The study was conducted in accordance with the Organisation for Economic Co-operation and Development (OECD,

2001) guidelines for Acute Oral Toxicity—Fixed Dose Procedure with slight modifications as per system suitability.

Subacute Oral Toxicity

A total of 24 female Sprague Dawley rats were randomly allocated into four groups. The first group (Group 1) was the normal control, and the rats were administered distilled water (vehicle) at a dosage of 5 ml/kg (n = 6). The remaining rat groups were subjected to varying treatments, with each group receiving an equivalent volume of MM crude extract that had been dissolved in distilled water at concentrations of 100 mg/kg (Group 2), 500 mg/kg (Group 3), and 1000 mg/kg (Group 4) MM crude extract, respectively (n = 6). Throughout the study's 28 days, oral forced feed on rats was carried out daily. Throughout the 28-day experiment, the rats were subjected to daily observations. Rats were observed individually for the first 30 minutes after dosing and then periodically during the first 24 hours. Special attention was given during the first four hours. The study was conducted in accordance with the Organisation for Economic Co-operation and Development (OECD, 2008), and the Repeated Dose 28-Day Oral Toxicity procedure, with slight modifications as per system suitability.

Data Collection

Daily examinations were conducted to monitor any signs of toxicity in the rats, including changes in skin, fur, eyes, and mucous membranes, as well as somatomotor activity and behaviour. Any unusual or abnormal pattern was documented. Individual rat weights were recorded prior to the study's commencement and at intervals of seven days. The rats' food intake was calculated daily.

Haematological Analysis

At the end of the experiment, all rats were fasting for approximately 16 hours and were humanely euthanised using carbon dioxide inhalation. Once the rats were confirmed dead, a small blood sample was collected from the tail to assess blood glucose levels using an instant Glucometer (Accu-Chek Instant Blood Glucometer, India). Subsequently, the abdominal walls of the rats were carefully incised. Approximately 7–10 ml of whole blood was extracted from each rat through the vena cava. Bloods were preserved at ethylene diamine tetra acetic acid (EDTA)--containing vacutainer tubes (Becton, Dickinson and Company, UK) and were kept at 4°C. The blood samples were analysed using a fully automated Veterinary Haematology analyser (Exigo H400, Sweden). Parameters such as red blood cell count (RBC), haemoglobin (HGB), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), total white blood cell count (WBC), lymphocytes, monocytes, granulocytes, haematocrit

(HCT), platelets (PLT), mean platelet volume (MPV), and red cell distribution width (RDW) was obtained.

Serum Biochemistry Analysis

Freshly collected blood from the vena cava was kept in gel-activated plain clot tubes (non-EDTA-containing) vacutainers. These tubes were properly labelled and then centrifuged at 1500 g for 5 minutes to obtain the serum. The serum was placed in 1.5 ml tubes and stored at -20 °C. The serum tubes were sent to the Haematology & Clinical Biochemistry Laboratory within the Department of Veterinary Laboratory Diagnosis at the Faculty of Veterinary Medicine, UPM. The blood serum underwent the evaluation of parameters such as alkaline phosphatase (ALP), aspartate aminotransferase (AST), total protein (TP), albumin (Alb), globulin (Glo), total bilirubin (T. Bil), creatinine (Creat.), urea, and alanine aminotransferase (ALT).

Histopathology Study

The entire rat organs, including the kidney, liver, lungs, stomach, heart, and spleen, were harvested, and their organ weight was recorded. The kidney and liver were preserved in 10% buffered formalin and sent to the Veterinary Laboratory Service Unit (VLSU), Department of Veterinary Laboratory Diagnosis Faculty of Veterinary Medicine, UPM. Sections of these organs were subjected to staining using Haematoxylin and Eosin (H&E) and examined under a microscope at magnifications of 100×, 200×, and 400× to identify and analyse any potential pathological changes.

Statistical Analysis

All data were statistically analysed using Statistical Analysis Software (SAS) software SAS/STAT® 9.4 (Statistical Analysis Software, 2011). The values were presented as the mean ± standard error. Analysis of variance (ANOVA) was performed to assess variances between the data sets of different groups. Analysis of variance (ANOVA) was done to compare the differences in data between groups. A significance level of $p<0.05$ was considered significant in statistical terms.

RESULTS AND DISCUSSION

Natural products from plants have been used extensively across the globe to address diverse ailments. However, the components derived from the plants need to undergo initial assessments to evaluate their toxicity profile for safety assurance. According to previous investigations (Alnajar et al., 2012; Kamsani et al., 2019; Reduan et al., 2020; Zahi et al., 2017), MM extracts administered orally were considered safe in acute and sub-acute toxicity assessments.

Acute Toxicity

In the acute toxicity study, no mortality, morbidity, abnormal behaviour, or adverse clinical signs were observed at the tested dose of 3000 mg/kg throughout the 14-day study period. The postmortem examination showed no abnormal or gross pathology changes in any rats. Consequently, the estimated LD_{50} of the extract in rats was expected to be higher than 3000 mg/kg.

Subacute Toxicity

Behaviour and Clinical Signs of Intoxication

Throughout the 28-day duration of the experiment, all groups were found healthy, and no treatment-related mortality and morbidity were attributed to the treatment (Table 1).

Table 1
Mortality and clinical signs of acute toxicity of Melastoma malabathricum *(MM) crude extract administered orally to Sprague Dawley rats*

Dose of MM extract (mg/kg)	Mortality	Abnormal behaviour/physical	Toxic symptoms
0	0/6	None	None
100	0/6	None	None
500	0/6	None	None
1000	0/6	None	None

Body Weight, Weight Gain and Feed Intake

Over the investigation period in the study, the mean body weight and weight gain of rats in the treated groups were similar to those of the normal control group, as summarised in Table 2. Nonetheless, on Day 14, Group II and Group III displayed slightly elevated values (189.25 + 2.75 g and 187.20 + 1.77 g, respectively) in comparison to the normal control group (172.00 + 4.71 g) (Figure 1). However, on Days 21 and 28, the body weight of the treated rats showed no significant difference (p>0.05) compared to the normal control group. Hence, the minor fluctuation observed in the mid-study can be considered within the realm of normal variability and does not suggest any substantial issues with the growth rates of the rats.

These findings were inconsistent with the regular feed intake (Figure 2). Potentially harmful extracts may undergo metabolism, yielding end-products that could adversely affect gastric function and impede food conversion efficiency (Ping et al., 2013). Such outcomes could lead to reduced food intake, indicating a loss of appetite, ultimately resulting in decreased body weight due to disruptions in carbohydrate, protein, or fat metabolism (Klaassen, 2018). In the study, none of the measured parameters in the treated

Table 2
The weekly body weight changes of rats during 28 days of subacute toxicity test with Melastoma malabathricum *crude extract*

Day of experiment	Mean body weight, g			
	Group I (0 mg/kg)	**Group II (100 mg/kg)**	**Group III (500 mg/kg)**	**Group IV (1000 mg/kg)**
0	$158.25 \pm 3.61^{a}_{A}$	$165.25 \pm 7.59^{a}_{A}$	$167.00 \pm 3.46^{a}_{A}$	$162.75 \pm 2.88^{a}_{A}$
7	$166.00 \pm 4.06^{a}_{A}$	$180.50 \pm 5.23^{a}_{B}$	$175.50 \pm 3.50^{a}_{AB}$	$167.80 \pm 3.69^{a}_{AB}$
14	$172.00 \pm 4.71^{a}_{AB}$	$189.25 \pm 2.75^{b}_{BC}$	$187.20 \pm 1.77^{b}_{BC}$	$180.67 \pm 5.06^{ab}_{B}$
21	$184.79 \pm 5.65^{a}_{BC}$	$196.68 \pm 2.25^{a}_{CD}$	$192.77 \pm 3.91^{a}_{C}$	$194.27 \pm 4.27^{a}_{C}$
28	$191.27 \pm 5.59^{a}_{C}$	$206.78 \pm 4.15^{a}_{D}$	$195.26 \pm 7.31^{a}_{C}$	$198.66 \pm 4.13^{a}_{C}$

Note. ^{a,b} Means in the same row with different superscripts are significantly different at $p<0.05$,
$_{A,B,C,D}$ Means in the same column with different subscripts are significantly different at $p<0.05$

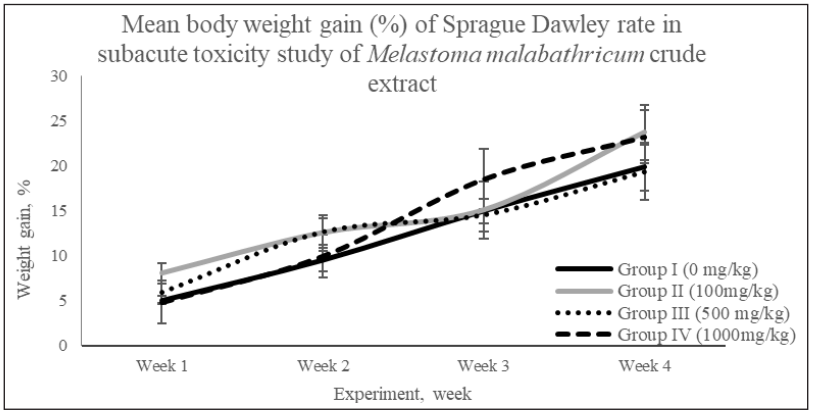

Figure 1. The weekly body weight gain changes of rats during subacute toxicity test with *Melastoma malabathricum* crude aqueous extract

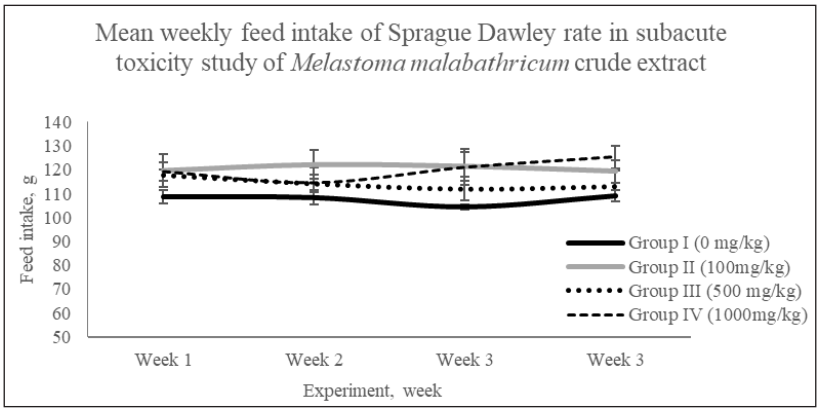

Figure 2. The weekly food intake changes of rats during subacute toxicity test with *Melastoma malabathricum* crude extract

groups exhibited noteworthy distinctions when compared to the normal control group. This indicates that MM extract did not influence carbohydrate, protein, or fat metabolism in the rats. Furthermore, the study revealed that the MM extract did not have a negative impact on weight gain, as seen in the control group when exposed to unrestricted food and water supply.

Haematology and Serum Biochemistry Analysis

The blood and serum obtained from the rats were subjected to haematological (Table 3) and serum biochemical (Table 4) analyses to validate the observations. The haematological parameter values can be measured to assess the adverse effects caused by compounds or extracts on the blood of tested animals. By evaluating haematological parameters, it is possible to determine the potential harm caused by the tested compounds or extracts on the blood (Kamsani et al., 2019).

Table 3
Haematological values of Sprague Dawley rats in Melastoma malabathricum's *crude extract-treated and control group*

Parameters	**Unit**	***Melastoma malabathricum*'s crude extract (mg/kg)**			
		Group I (0 mg/kg)	**Group II (100 mg/kg)**	**Group III (500 mg/kg)**	**Group IV (1000 mg/kg)**
Glucose	mmol/l	4.78 ± 0.14^{a}	5.45 ± 0.55^{a}	4.90 ± 0.16^{a}	4.70 ± 0.29^{a}
WBC	× 10^{9}/L	7.51 ± 0.67^{a}	9.93 ± 0.52^{a}	8.72 ± 0.65^{a}	7.98 ± 0.82^{a}
LYM	× 10^{9}/L	5.55 ± 0.53^{a}	7.33 ± 0.47^{a}	6.37 ± 0.52^{a}	5.96 ± 0.62^{a}
MONO	× 10^{9}/L	0.32 ± 0.06^{a}	0.33 ± 0.02^{a}	0.38 ± 0.10^{a}	0.40 ± 0.08^{a}
GRAN	× 10^{9}/L	1.65 ± 0.15^{a}	2.11 ± 0.25^{a}	1.96 ± 0.18^{a}	1.61 ± 0.18^{a}
LYM	%	73.95 ± 1.43^{a}	77.91 ± 2.52^{a}	72.85 ± 2.27^{a}	74.73 ± 1.88^{a}
MON	%	3.13 ± 0.52^{a}	2.60 ± 0.14^{a}	4.11 ± 0.77^{a}	4.16 ± 0.82^{a}
GRA	%	22.92 ± 1.45^{a}	19.48 ± 2.45^{a}	23.03 ± 1.94^{a}	22.76 ± 2.78^{a}
HGB	g/dl	17.98 ± 0.40^{a}	16.57 ± 0.55^{a}	16.85 ± 0.75^{a}	17.93 ± 0.29^{a}
HCT	%	48.46 ± 1.09^{a}	44.98 ± 1.58^{a}	46.05 ± 1.93^{a}	48.40 ± 0.64^{a}
RBC	× 10^{12}/L	9.46 ± 0.20^{a}	8.84 ± 0.28^{a}	8.94 ± 0.41^{a}	9.53 ± 0.11^{a}
MCV	Fl	51.18 ± 0.37^{a}	51.91 ± 0.42^{a}	51.67 ± 0.60^{a}	51.86 ± 0.93^{a}
MCH	pg	19.00 ± 0.09^{a}	19.13 ± 0.22^{a}	18.85 ± 0.21^{a}	18.70 ± 0.37^{a}
MCHC	g/dl	37.12 ± 0.14^{a}	36.85 ± 0.24^{a}	36.51 ± 0.19^{a}	36.63 ± 0.23^{a}
RDW	%	19.16 ± 0.27^{a}	19.05 ± 0.33^{a}	19.80 ± 0.47^{a}	19.35 ± 0.18^{a}
RDWa	Fl	36.90 ± 0.19^{a}	37.17 ± 0.61^{a}	38.10 ± 0.77^{a}	38.23 ± 0.91^{a}
PLT	× 10^{9}/L	951.50 ± 47.28^{a}	945.17 ± 91.74^{a}	947.67 ± 55.41^{a}	947.50 ± 39.71^{a}
MPV	fl	4.62 ± 0.09 ^{a}	4.96 ± 0.08 ^{b}	5.05 ± 0.08 ^{b}	4.85 ± 0.11 ^{ab}

Note. ^{a,b} Means in the same row with different superscripts are different significantly at $p<0.05$

Table 4
Serum biochemistry values of Sprague Dawley rats in Melastoma malabathricum's *crude extract-treated and control group*

Parameters	Unit	*Melastoma malabathricum*'s crude extract (mg/kg)			
		Group I (0mg/kg)	Group II (100 mg/kg)	Group III (500 mg/kg)	Group IV (1000 mg/kg)
ALP	U/L	164.05 ± 12.08[a]	205.74 ± 21.64[a]	232.09 ± 62.43[a]	202.08 ± 31.71[a]
AST	U/L	138.91 ± 5.92[a]	149.35 ± 7.51[a]	170.07 ± 21.39[a]	150.83 ± 8.11[a]
TP	g/L	68.06 ± 0.79[a]	65.73 ± 1.54[a]	66.96 ± 2.75[a]	70.13 ± 5.23[a]
ALB	g/L	41.33 ± 0.85[a]	40.52 ± 3.29[a]	37.58 ± 4.15[a]	43.85 ± 0.64[a]
Glo	g/L	26.75 ± 1.30[a]	25.22 ± 3.28[a]	29.38 ± 3.62[a]	26.90 ± 5.98[a]
A:G	Unit	1.57 ± 0.11[a]	1.81 ± 0.30[a]	1.50 ± 0.35[a]	1.80 ± 0.43[a]
TBil	umol/L	2.73 ± 0.34[a]	2.72 ± 0.38[a]	2.88 ± 0.77[a]	1.92 ± 0.20[a]
Creat	umol/L	59.00 ± 2.46[a]	59.00 ± 1.61[a]	57.17 ± 3.46[a]	51.25 ± 2.35[a]
Urea	mmol/L	7.80 ± 0.27[a]	8.23 ± 0.25[a]	7.95 ± 0.47[a]	7.90 ± 0.49[a]
ALT	U/L	51.00 ± 1.89[a]	51.83 ± 2.98[a]	51.93 ± 4.84[a]	45.00 ± 4.39[a]

Note. [a,b] Means in the same row with different superscripts are different significantly at $p<0.05$

The haematological parameters of the rats administered with various concentrations did not differ significantly ($p>0.05$) compared to the control group. However, a deviation was observed in the mean platelet volume (MPV) between the control group and groups II and III. These groups exhibited higher MPV values (4.96 ± 0.08 fl and 5.05 ± 0.08 fl, respectively) when compared to the normal control group (4.62 ± 0.09 fl) (Table 3). Nevertheless, Group IV (4.85 ± 0.11 fl) demonstrated no significant deviations when compared to any other groups ($p>0.05$). Despite the statistically significant disparities, the distinctions in MPV values between Group II, Group III, and the remaining groups were relatively small. These variances were minor and unlikely to lead to any irregularities in the rats under investigation. Petterino and Argetino-Storino (2006) reported a narrower range of normal MPV values for Sprague Dawley rats (3.60–4.40 fl) when compared to the measurements obtained in our study. Conversely, earlier investigations by He et al. (2017) and Lillie et al. (1996), focusing on the same rat species, presented considerably broader normal MPV ranges (6.60–8.30 fl and 5.38–5.90 fl, respectively). These variations could be attributed to differences in experimental settings, diets, or environmental factors. Furthermore, the highest concentration group (1000 mg/kg) exhibited no significant distinctions compared to the normal control group. Considering the slight variations observed in Group II and Group III, it is not conclusive that the tested herbs were toxic to the subjects. Overall, based on the sub-acute toxicity test, it can be reasonably concluded that MM can be deemed safe for oral administration in Sprague Dawley rats.

The liver and kidney are the vital organs that play a crucial role in determining an organism's overall well-being and survival (Zhang et al., 2016). Whether acute or

chronic, liver damage is often associated with elevated serum levels of AST (aspartate aminotransferase) and ALT (alanine aminotransferase). ALT, predominantly found in the liver, serves as a widely recognised biomarker for hepatotoxicity, and it is a specific indicator of liver dysfunction that potentially signals hepatocellular necrosis. In contrast, while AST can aid in identifying hepatocellular necrosis, it is regarded as a less specific biomarker for liver injury due to the possibility of its serum levels rising in response to dysfunction in other organs as well. Consequently, the assessment of ALT and AST levels, alongside total bilirubin (TBil), is a standard recommendation for evaluating hepatocellular injury in rodents during nonclinical investigations (Fishman, 1990; Kamsani et al., 2019; Sriuttha et al., 2018).

The urea, creatinine, and uric acid levels are among the parameters used to assess renal dysfunction. This also means if these indicators fall within the established normal range, it implies the absence of renal complications (Barnett and Cumming, 2018). The urea, creatinine, and uric acid levels within the groups treated with MM crude aqueous extract exhibited no difference compared to the control group. This suggests that the renal function remained unaffected in the treated groups during the toxicity analysis (Table 4). The results of the experiment provided sufficient evidence to conclude that oral administration of MM crude aqueous extract was safe and presented no toxicity effect even when consumed at high doses of 3,000 mg/kg and 100, 500 and 1000 mg/kg using acute and sub-acute toxicity tests, respectively.

Histopathology Study

The liver and kidney exhibited no adverse effects or clinical signs of toxicity during the histopathology analysis (Table 5). However, considering the importance of histopathology in completing the toxicology test, major organs, such as the liver and kidney, were selected. These organs are the focus of histopathology examinations due to their crucial functions in detoxifying and eliminating harmful substances from the body's metabolic processes. Consequently, these organs are susceptible to the impact of toxic compounds (Elufioye et al., 2009).

Relative organ weight percentage showed no significant difference in the kidney, liver, lung, heart, spleen, and stomach (Table 5). In subacute toxicity studies, microscopic assessments of kidneys and livers from both control and treated groups revealed normal histopathology features (Figure 3). The kidneys displayed typical histopathology characteristics, including glomeruli, tubules, interstitium, and blood vessels. Meanwhile, liver sections from the control and treatment rats exhibited intact hepatocellular structures, clearly visible central veins, and an absence of any anomalies. Importantly, the treatments for the rats did not yield any adverse impacts on the histopathology arrangement of hepatocytes.

Table 5
Effect of Melastoma malabathricum *crude extract on relative organ weight percentage of Sprague Dawley rats in subacute toxicity study*

Parameters	*Melastoma malabathricum*'s crude extract (mg/kg)			
	Group I (0 mg/kg)	Group II (100 mg/kg)	Group III (500 mg/kg)	Group IV (1000 mg/kg)
Kidney	0.71 ± 0.02^{a}	0.07 ± 0.03^{a}	0.73 ± 0.03^{a}	0.73 ± 0.03^{a}
Liver	2.94 ± 0.10^{a}	2.88 ± 0.08^{a}	3.16 ± 0.11^{a}	3.14 ± 0.07^{a}
Lung	0.60 ± 0.02^{a}	0.58 ± 0.03^{a}	1.08 ± 0.45^{a}	0.64 ± 0.03^{a}
Heart	0.36 ± 0.01^{a}	0.34 ± 0.01^{a}	0.36 ± 0.02^{a}	0.34 ± 0.02^{a}
Spleen	0.17 ± 0.01^{a}	0.34 ± 0.01^{a}	0.23 ± 0.06^{a}	0.21 ± 0.01^{a}
Stomach	0.61 ± 0.01^{a}	0.60 ± 0.04^{a}	0.63 ± 0.02^{a}	0.62 ± 0.01^{a}

Note. ^{a,b} Means in the same row with different superscripts are different significantly at $p<0.05$

Under microscopic observations, the histopathology slides under H&E staining did not yield significant deviant changes from toxicity effects attributed to the extract's oral administration (Figure 3). Treatment of MM crude aqueous extract did not yield any alterations in organ colour or appearance or signs of hypertrophy. No modifications in cell architecture or pathological abnormalities were detected under the light microscope. As Mirza and Pancha (2019) pointed out, relative organ weights in toxicity studies indicate particular organs in detecting significant toxicity-related changes in vital organs such as the kidneys, liver, heart, stomach, spleen, and lungs.

The MM crude aqueous extracts demonstrated good tolerance among the experimental animals under investigation. Throughout the study period, acute and sub-acute toxicity tests observed no mortality or significant changes in physical appearance, behaviour, food consumption, or body weight that deviated from the normal control group. Furthermore, assessments encompassing blood haematological analysis, serum biochemistry, and histopathological examination indicated the absence of any indications of toxic effects in rats subjected to acute and sub-acute toxicity tests.

Female Sprague Dawley rats were selected as the experimental subjects for this study because the intended population for the test substance was females. Utilising female rats exclusively can yield more pertinent data for risk assessment within the target population (Zahi et al., 2017). Furthermore, conducting toxicity studies can be resource-intensive and costly. Opting for a more streamlined approach that utilises fewer animals and causes reduced distress while maintaining the ability to rank the substance's toxicity comparably makes focusing solely on female rats.

Additionally, female rats generally exhibit greater sensitivity to toxic substances compared to males in toxicity studies (OECD, 2001; Zahi et al., 2017). Earlier preliminary screenings involving acute and sub-acute toxicity tests of similar extracts, utilising male

Figure 3. Histopathology assessment of the rat's organs (liver and kidney) for control and treated rats group with 3000 mg/kg (acute toxicity test) and 100, 500 and 1000 mg/kg (subacute toxicity test) of *Melastoma malabathricum* crude aqueous extract

and female rats, did not reveal significant discrepancies in toxicity based on sex (Alnajar et al., 2012; Kamsani et al., 2019; Manicam et al., 2013; Zahi et al., 2017). Based on a rational foundation and the existing evidence, the decision to exclusively use female rats for the study is a pragmatic choice to enhance cost-effectiveness, minimise redundant efforts, and allocate resources optimally while still producing valuable toxicity insights.

CONCLUSION

The oral administration of MM crude aqueous extract for 14 days in the acute study and 28 days in the subacute study did not cause any detrimental effects on behaviour, body weight, haematological and biochemical indicators, relative organ weight, or histopathology analysis. Consequently, no Observed Adverse Effect Level (NOAEL) was established at doses greater than 3000 mg/kg and 1000 mg/kg for the acute and subacute toxicity studies, respectively.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

Alnajar, Z. A. A., Abdulla, M A., Ali, H. M., Alshawsh, M. A., & Hadi, A. H. A. (2012). Acute toxicity evaluation, antibacterial, antioxidant and immunomodulatory effects of *Melastoma malabathricum*. *Molecules*, *17*(3), 3547-3559. https://doi.org/10.3390/molecules17033547

Barnett, L. M. A., & Cummings, B. S. (2018). Nephrotoxicity and renal pathophysiology: A contemporary perspective. *Toxicological Sciences*, *164*(2), 379–390. https://doi.org/10.1093/toxsci/kfy159

Choudhury, M. D., Nath, D., & Talukdar, A. D. (2011). Antimicrobial activity of *Melastoma malabathricum* L. *Biological and Environmental Sciences*, *7*(1), 76-78.

Elufioye, T. O., Alatise, F. A., & Fakoya, J. M. (2009). Toxicity studies of *Tithonia diversifolia* A. Gray (Asteraceae) in rats. *Journal of Ethnopharmacology*, *122*(2), 410-415. https://doi.org/10.1016/j.jep.2008.12.007

Fishman, W. H. (1990). Alkaline phosphatise isozymes: Recent progress. *Clinical Biochemistry*, *23*(2), 99-104. https://doi.org/10.1016/0009-9120(90)80019-F

He, Q., Su, G., Liu, K., Zhang, F., Jiang, Y., Gao, J., Liu, L., Jiang, Z, Jin, M., & Xie, H. (2017). Sex-specific reference intervals of hematologic and biochemical analytes in Sprague-Dawley rats using the

nonparametric rank percentile method. *PLoS ONE*, *12*(12), e0189837. https://doi.org/10.1371/journal.pone.0189837

Kamsani, N. E., Zakaria, Z. A., Nasir, N. L. M., Mohtarrudin, N. & Alitheen, N. B. M. (2019). Safety assessment of methanol extract of Melastoma malabathricum L. leaves following the subacute and subchronic oral consumptions in rats and its cytotoxic effect against the HT29 cancer cell line. *Hindawi Evidence-Based Complementary and Alternative Medicine*, *2019*(1), 5207958. https://doi.org/10.1155/2019/5207958

Klaassen, C. D. (2018). *Casarret and Doull's Toxicology: The basic science of poisons.* McGraw-Hill Education. https://accesspharmacy.mhmedical.com /book.aspx?bookid=2462

Kumar, V., Ahmed, D. M., Gupta, D. A., Anwar, F., & Mujeeb, M. (2013). Anti-diabetic, anti-oxidant and anti-hyperlipidemic activities of *Melastoma malabathricum* Linn. leaves in streptozotocin induced diabetic rats. *Complementary and Alternative Medicine*, *13*, 222. https://doi.org/10.1186/1472-6882-13-222

Lillie, L. E., Temple, N. J., & Florence Z. (1996). Reference values for young normal Sprague-Dawley rats: Weight gain, hematology and clinical chemistry. *Human & Experimental Toxicology, 15*(8), 612-616. https://doi.org/10.1177/096032719601500802

Manicam, C., Khoo, L. T., Abdullah, J. O., Tohit, E. R. M., Seman, Z., Sieo, C. C., & Hamid, M. (2013). Subacute toxic effects of *Melastoma malabathricum* Linn. aqueous leaf extract on liver and kidney histopathology of rats. *International Journal of Pharmacology*, *9*(6), 358-365. https://doi.org/10.3923/ijp.2013.358.365

Mazura, M. P., Susanti, D., & Rasadah, M. A. (2007). Anti-inflammatory action of components from *Melastoma malabathricum*. *Pharmaceutical Biology*, *45*(5), 372–375. https://doi.org/10.1080/13880200701214797

Mirza, A. C., & Pancha, S. S. (2019). Safety evaluation of syringic acid: Subacute oral toxicity studies in Winstar rats. *Heliyon*, *5*(8), e02129. https://doi.org/10.1016/j.heliyon.2019.e02129

Organisation for Economic Co-operation and Development. (2001). *Test No. 423: Acute oral toxicity – Acute toxic class method (OECD guidelines for the testing of chemicals, section 4).* OECD Publishing. https://doi.org/10.1787/9789264071001-en

Organisation for Economic Co-operation and Development. (2008). *Test No. 407: Repeated dose 28-day oral toxicity study in rodents (OECD guidelines for the testing of chemicals, section 4).* OECD Publishing. https://doi.org/10.1787/9789264070684-en

Petterino, C., & Argentino-Storino, A. (2006). Clinical chemistry and haematology historical data in control Sprague-Dawley rats from pre-clinical toxicity studies. *Experimental and Toxicologic Pathology*, *57*(3), 213-219. https://doi.org/10.1016/j.etp.2005.10.002

Ping, K. Y., Darah, I., & Chen Y. Sreeramanan S., & Sasidharan S. (2013). Acute and subchronic toxicity study of *Euphorbia hirta* L. methanol extract in rats. *BioMed Research International*, *2013*, 182064. https://doi.org/10.1155/2013/182064

Reduan, F. H., Shaari, R. M., Sayuti, N. S. A., Mustapha, N. M., Bakar, M. Z, A. B., Sithambaram, S., & Hamzah, H. (2020). Acute and subacute dermal toxicity of ethanolic extract of *Melastoma malabathricum* leaves in Sprague-Dawley rats. *Toxicology Research*, *36*, 203-210. https://doi.org/10.1007/s43188-019-00013-5

Sriuttha, P., Sirichanchuen, B., & Permsuwan, U. (2018). Hepatotoxicity of nonsteroidal anti-inflammatory drugs: A systematic review of randomized controlled trials. *International Journal of Hepatology*, *2018*(1), 5253623. https://doi.org/10.1155/2018/5253623

Zahi, A. K., Hamzah, H., Shaari, M. R., Widodo, R. T., Johnny, L., Noordin, M. M., & Sithambaram, S. (2017). Investigation and evaluation of acute and sub-acute dermal toxicity studies of ethanolic leaves extract of *Melastoma malabathricum* in Sprague Dawley rats. *International Journal of Current Research in Medical Science*, *3*(5), 84-99. http://doi.org/10.22192/ijcrms.2017.03.05.013

Zhang, Y., Guan, E., Zhao, X., Wang, B., Yin, L., Zhang, L., Huang, J., & Fu, X. (2016). A subchronic toxicity study of ethanol root extract of baked *Aconitum flavum* in rats. *Revista Brasileira de Farmacognosia*, *26*(4), 438-445. https://doi.org/10.1016/j.bjp.2016.03.004

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Short Communication

Extraction of Useful DNA from Different Parts of the Durian (*Durio zibethinus*) Fruit

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ABSTRACT

Successful deoxyribonucleic acid (DNA) extraction from the durian fruit allows effective molecular identification and genotyping of durian cultivars for quality assurance. While most genetic identification of durian samples used the leaf as the DNA source, studies exploring durian fruit as a source of DNA for molecular identification and genotyping are limited. In this study, four potential sources of genetic material: the peduncle, spine, carpel, and aril from the durian fruit were evaluated for DNA yield (in terms of concentration), quality (in terms of ratio of A_{260}/A_{280}), and suitability in PCR amplification (using genetic markers that target the chloroplast, nuclear, and microsatellite DNA regions). Results of this study show that the spine is a good potential source of genetic material alongside the peduncle.

Keywords: Cultivar genotyping, DNA extraction, fruit DNA, genetic resources

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INTRODUCTION

The fruit of the durian (*Durio zibethinus* L.; Malvaceae) is known for its unique smell, intense taste, and significant nutritional

value (Ali et al., 2020). Native to Southeast Asia, durian has a crucial role in the cultural and economic aspects of countries, including Malaysia, Thailand, and Indonesia (Thorogood et al., 2022). It is commonly referred to as the "King of Fruits" due to its extensive appeal and significant market value despite its divisive smell. In 2023, Thailand was the primary global durian supplier, with Vietnam and Malaysia following closely behind. On the other hand, China is the world's leading importer of durian, accounting for over 95% of all imports (Food and Agriculture Organization of the United Nations [FAO], 2023).

Consumer taste plays a pivotal role in determining the demand for durian, with preference for different cultivars changing through time as new cultivars emerge in the market; Malaysia, for example, currently has 129 durian cultivars registered with the National Crop List (http://pvpbkkt.doa.gov.my/NationalList/Search.php). Since different durian cultivars present different aromas, tastes, textures, and nutritional levels, the identification of the cultivar from which a durian fruit is derived has to be accurate and consistent (May et al., 2023) for quality assurance. Ensuring the authenticity and quality of exported durian fruit and its products is crucial for maintaining market confidence towards durian-producing countries.

So far, genotyping of DNA markers has shown potential in differentiating the various durian cultivars (Siew et al., 2018; Mursyidin et al., 2022). It has significant applications in the quality control and assurance of durian exports, given the international demand and high market price, by reducing cases of false cultivar claims or adulterated products, which can damage the reputation of the producing countries. Due to its size, durian fruits sold on the market do not have other tissues from the mother tree, such as leaves or branches attached. It means that if any DNA analyses were to be done on the fruit, the DNA would have to be extracted from the fruit itself. The DNA extraction and polymerase chain reaction (PCR) amplification steps are essential processes that ensure the success of a genetic analysis. However, obtaining high-quality DNA from plant tissues, especially from the fruit, poses distinct challenges due to high concentrations of polysaccharides, secondary metabolites, and other substances that complicate the extraction process and affect the quality and quantity of the DNA obtained (Japelaghi et al., 2011). Determining suitable parts of the high-value durian fruit as sources of quality genetic material would thus aid in efforts that enable molecular identification and genotyping of its different cultivars.

This study seeks to address the challenges in DNA extraction from the durian fruit by conducting a comparative analysis of the efficacy of DNA extraction from various parts of the durian fruit and using the extracted DNA for PCR amplification. By examining the quality and quantity of extracted DNA from the durian fruit peduncle, spine, carpel, and aril, as well as determining the suitability of each sample type for PCR amplification of chloroplast and nuclear DNA fragments and microsatellite DNA, this study provides vital information on the approach for extracting useful DNA from the durian fruit. We envision

that the findings in this study would enhance the efficiency of genetic investigations for the authentication of durian fruits at border controls or international trade platforms.

MATERIALS AND METHODS

Fruit Material and DNA Extraction

Three Musang King variety durian fruits were purchased from a local fruit seller. Prior to excising pieces ("sample") of the different parts of the fruit—the peduncle, spine, carpel, and aril (Figure 1)—the surfaces of each sample type were gently wiped and sterilised with a C-fold hand towel sprayed with 70% ethanol. After surface sterilisation, 100 mg of each sample type was obtained for DNA extraction.

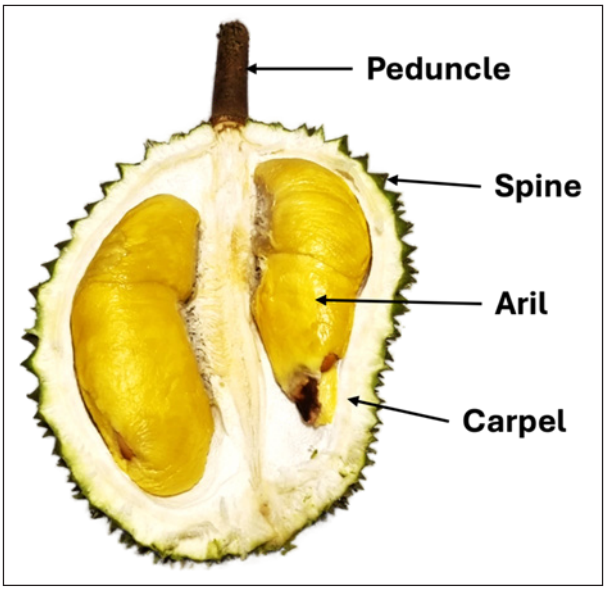

Figure 1. An opened durian fruit showing the different parts of the fruit sampled for DNA extraction: the peduncle, spine, carpel, and aril

Total genomic DNA was extracted using the FavorPrep Plant Genomic DNA Extraction Mini Kit (Favorgen Biotech, China) with minor modifications. Briefly, for the samples of the peduncle, spine, and carpel, the sample was cut into small cubes of 1mm×1mm×1mm or smaller using a sterile scalpel before being added into a 2 mL microcentrifuge tube containing 1 mL of lysis buffer (FAPG1 Buffer). In contrast, the sample of the aril was directly added to the lysis buffer. A sterile mini pestle was then used to grind the sample in the lysis buffer to ensure mixing and maximum contact of cells with the buffer. After adding 16 µL of RNase A solution (50 mg/mL), the mixture was vortexed vigorously and incubated at 65 ˚C for 2 h in a water bath. The mixture was let to cool down prior to

adding 280 µL FAPG2 Buffer and then incubated at -20 ˚C for 30 min. Subsequent steps to bind, wash, and elute the DNA were per the manufacturer's protocol. DNA extraction was conducted for each sample in duplicates as technical replicates. The quantity and quality of the extracted DNA were then determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

PCR Amplification and Verification of DNA Quality

Six primer pairs specific to the chloroplast and nuclear DNA regions were used to test for PCR amplification using the total genomic DNA extracted in this study. The marker loci were: (1) *mat*K (1R_KIM: 5'-ACCCAGTCCATCTGGAAATCTTGGTTC-3' and 3F_KIM: 5'-CGTACAGTACTTTTGTGTTTACGAG-3') by Kim (unpublished), (2) *trn*L-*trn*F intergenic spacer (trnL-e: 5'-GGTTCAAGTCCCTCTATCCC-3' and trnF-f: 5'-ATTTGAACTGGTGACACGAG-3') by Taberlet et al. (1991), (3) ITS (ITS-p5: 5'-CCTTATCAYTTAGAGGAAGGAG-3' and ITS-S3R: 5'-GACGCTTCTCCAGACTACAAT-3') by Chen et al. (2010) and Cheng et al. (2016), (4) ITS2 (ITS-p3: 5'-GCCRAGATATCCGTTGCCGAG-3' and ITS-S3R: 5'-GACGCTTCTCCAGACTACAAT-3') by Chen et al. (2010) and Cheng et al. (2016), and microsatellite markers (5) DZ05 (DZ05_F2: 5'-ACACATACACAACTCACCTC-3' and DZ05_R: 5'-ATGCCCGATGAAATTGTAAC-3') and DZ07 (DZ07_F: 5'-ACACACCATCTTCCCTTTG-3' and DZ07_R: 5'-TGCACATGTTGTTTGTATATATG-3') by Siew et al. (2018).

Based on literature, the chloroplast partial gene sequence *mat*K and intergenic spacer region *trn*L-*trn*F would produce amplifications of about 850 bp and 500 bp, respectively. In comparison, the nuclear ribosomal DNA internal transcribed spacer (ITS) and ITS2 would produce amplifications of about 900 bp and 500 bp, respectively. The nuclear microsatellite markers DZ05 and DZ07 were expected to produce amplifications of about 200 bp and 440 bp, respectively. PCR amplifications were carried out in a 25 µL total reaction volume containing Taq Plus Master Mix (Dye Plus; Vazyme, China), 0.4 µM of each primer, and 15 ng of genomic DNA as a template. A negative control for each marker was included, using sterilised distilled water in place of the DNA template. PCR amplifications were conducted on a SelectCycler II thermal cycler (Select-Bioproducts, USA), with initial denaturation at 95°C for 3 min, 35 cycles of denaturation at 95°C for 15 s, annealing at 50 °C for 15 s, and extension at 72°C for 60 s, and a final extension at 72°C for 5 min. PCR amplification was carried out twice for each marker and sample (equivalent to two technical replicates). The amplified products were separated on 1.0% agarose gels containing the FloroSafe DNA Stain (1st BASE, Malaysia) and visualised under ultraviolet (UV) light.

RESULTS

DNA Quality and Yield

Using the selected DNA extraction method, we found that all four types of samples from the durian fruit exhibited different levels of total genomic DNA purity and concentration (Table 1). The ratio of absorbance at 260 nm to absorbance at 280 nm (A_{260}/A_{280}) of between 1.5 and 2.0 is typically the "acceptable" purity of a DNA extract for downstream applications (Lucena-Aguilar et al., 2016). For the peduncle sample, at least one technical replicate from Durian 1 (D1) and Durian 3 (D3) returned good DNA purity, with DNA concentrations of 35.5 ng/µL and 12.5 ng/µL, respectively. The DNA purity extracted from the spine sample was only acceptable in D1 but not in Durian 2 (D2) or D3. The DNA concentrations for the spine samples from D1 were 25–31.3 ng/µL. Of the six carpel samples, only one displayed an acceptable purity of 1.64 from D1. When using the aril as the tissue sample, four tissue samples from the three durian samples returned with at least one technical replicate with an absorbance ratio between 1.5 and 2.0, with DNA concentrations ranging from 36.6 to 149.9 ng/µL.

Table 1
The purity and concentration of DNA extracts of four sample types from the durian fruit

		Durian 1		Durian 2		Durian 3	
		Technical replicate 1	**Technical replicate 2**	**Technical replicate 1**	**Technical replicate 2**	**Technical replicate 1**	**Technical replicate 2**
Peduncle	A_{260}/A_{280}	2.09	1.62	1.17	1.13	2.02	1.93
	Concentration (ng/µL)	7.40	35.50	34.10	148.70	12.40	12.50
Spine	A_{260}/A_{280}	1.89	1.79	2.47	1.47	2.26	2.72
	Concentration (ng/µL)	25.00	31.30	12.10	13.20	12.70	12.80
Carpel	A_{260}/A_{280}	1.64	1.20	1.37	2.22	2.08	1.29
	Concentration (ng/µL)	27.60	32.50	31.90	18.00	15.90	25.50
Aril	A_{260}/A_{280}	1.50	1.34	1.77	1.55	1.53	1.25
	Concentration (ng/µL)	36.60	71.60	149.90	75.50	68.40	104.20

PCR Amplification Using Different Genetic Markers

From the negative controls, all tested genetic markers showed no false signals from contamination (Figure 2). The success of the amplification varied among the samples and genetic markers. At the *mat*K locus, only the spine, carpel, and aril samples from D2 and D3 presented amplified DNA (including both technical replicates). For the peduncle

samples, both technical replicates from D2 showed positive amplifications for *mat*K, while D1 and D3 only showed one successful amplification in one of their technical replicates. All sample types (including both technical replicates) produced amplifications for the *trn*L-*trn*F and ITS2 loci. However, only one technical replicate for the peduncle sample of D1 and the carpel sample of D3 produced the amplicon of interest. DNA extracts from the peduncle of D1 and the carpels of all three durian samples did not produce amplicons for the ITS locus. Only one of the two technical replicates produced amplification for the peduncle sample of D2 and the spine sample of D3. At the microsatellite marker loci DZ05 and DZ07, PCR amplification was successful for both technical replicates of the peduncle sample of D3, the spine sample of D1 and D2, and the aril sample of D2. Only one replicate of the peduncle and aril samples from D2 and D3 produced amplification at the microsatellite loci. DNA extracts derived from the peduncle sample of D1, the spine sample of D3, the aril sample of D1, and all the carpel samples showed no amplification.

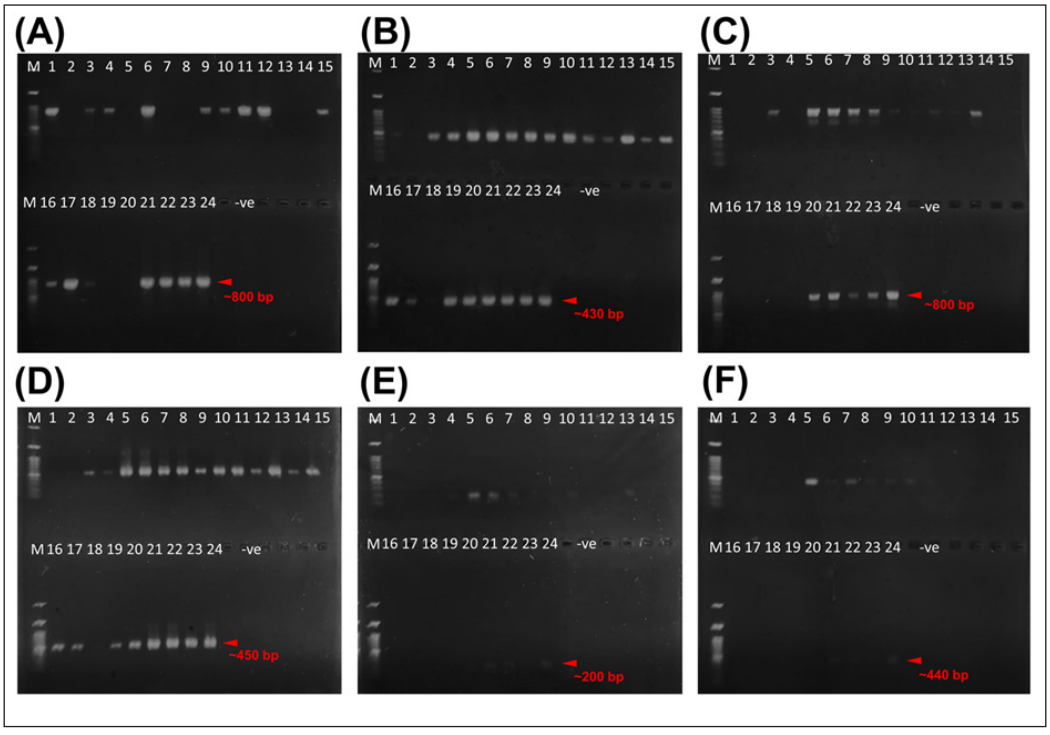

Figure 2. PCR amplification results for the genetic marker loci (A) *mat*K, (B) *trn*L-*trn*F intergenic spacer, (C) ITS, (D) ITS2, (E) DZ05, and (F) DZ07. Lane 1–2: D1 peduncle, 3–4: D1 spine, 5–6: D1 carpel, 7-8: D1 aril, 9–10: D2 peduncle, 11–12: D2 spine, 13–14: D2 carpel, 15–16: D2 aril, 17–18: D3 peduncle, 19–20: D3 spine, 21–22: D3 carpel, 23–24: D3 aril. Two technical replicates were produced for each sample. M:100 bp DNA ladder

DISCUSSION

To the best of current knowledge, this is the first study to report the evaluation of DNA yield and quality of the total genomic DNA extracted from the durian fruit. The results indicated that most sample types from the durian fruit did not achieve an acceptable absorbance ratio, whereas the DNA quality of the aril sample appeared promising. Generally, an A_{260}/A_{280} ratio of <1.5 could mean that there are proteins, phenols, or other contaminants that absorb strongly at or near 280 nm (Desjardins et al., 2010), while a ratio of >2.0 indicates the presence of RNA contamination that may inhibit amplification (Yuen et al., 2001).

As with many other plant species, genetic studies on durian usually involve using its leaf as the source of genetic material (Cheon et al., 2017; Nawae et al., 2023) due to its availability and ease of sample processing. However, as the fruits are marketed or exported, unlike some fruits, the durian leaves do not come with the fruit. Nonetheless, genetic studies using the durian fruit as a source of genetic material have been attempted in the past: Teh et al. (2017) used the peduncle as a source of genetic material for whole-genome sequencing of the durian, while Jantan et al. (2024) and Teh et al. (2017) also used the aril as the genetic material for transcriptomic studies on the durian fruit. The findings confirm that the peduncle and aril contain sufficient genetic material for genomic analysis. While the woody peduncle may seem to be lacking in chloroplasts, prior research has demonstrated that woody tissue can serve as a suitable source for obtaining extranuclear genetic material (Dumolin-Lapegue et al., 1999; Asif & Cannon, 2005).

Based on the findings in this study, the spine is proposed as a potential alternative source of genetic material for durian cultivar genotyping for its high success rate in PCR amplification of commonly used genetic markers. While the peduncle also produced good amplification results, not all durian fruit sold in the market come with a peduncle attached. On the other hand, the carpel and aril are impractical as sources of genetic material for quality assurance at checkpoints, as one has to open the fruit to obtain the samples. Thus, the spine (and, if available, the peduncle) would be an effortless and straightforward source of genetic material for total genomic DNA extraction.

Nonetheless, it is acknowledged that outcomes could vary when different DNA extraction protocols are used. As this study aimed to find a suitable source of sample from the durian fruit for quick and inexpensive DNA extraction, this work also did not consider an additional DNA purification step, which could improve the quality of the extracted DNA and, subsequently, the amplification success. Variations in the biochemical composition of fruit samples across different durian cultivars may also necessitate adjustments to the DNA extraction protocol to achieve optimal DNA yields.

CONCLUSION

This study evaluated the DNA yield and quality of total genomic DNA extracted from the durian fruit. While most sample types did not consistently achieve acceptable and/or consistent A_{260}/A_{280} ratios, DNA extracted from the durian aril showed promising DNA quality and concentration. Eventually, all sample types successfully amplified both the chloroplast *trn*L-*trn*F and nuclear ITS2 loci, demonstrating their suitability for downstream molecular applications.

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REFERENCES

Ali, M. M., Hashim, N., Abd Aziz, S., & Lasekan, O. (2020). Exploring the chemical composition, emerging applications, potential uses, and health benefits of durian: A review. *Food Control*, *113*, 107189. https://doi.org/10.1016/j.foodcont.2020.107189

Asif, M. J., & Cannon, C. H. (2005). DNA extraction from processed wood: a case study for the identification of an endangered timber species (*Gonystylus bancanus*). *Plant Molecular Biology Reporter*, *23*, 185-192. https://doi.org/10.1007/BF02772709

Chen, S., Yao, H., Han, J., Liu, C., Song, J., Shi, L., Zhu, Y., Ma, X., Gao, T., Pang, X., Luo, K., Li, Y., Li, X., Jia, X., Lin, Y., & Leon, C. (2010). Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. *PloS One*, *5*(1), e8613. https://doi.org/10.1371/journal.pone.0008613

Cheng, T., Xu, C., Lei, L., Li, C., Zhang, Y., & Zhou, S. (2016). Barcoding the kingdom Plantae: New PCR primers for ITS regions of plants with improved universality and specificity. *Molecular Ecology Resources*, *16*(1), 138-149. https://doi.org/10.1111/1755-0998.12438

Cheon, S.-H., Jo, S., Kim, H.-W., Kim, Y.-K., Sohn, J.-Y., & Kim, K.-J. (2017). The complete plastome sequence of Durian, *Durio zibethinus* L. (Malvaceae). *Mitochondrial DNA B Resources*, *2*(2), 763-764. https://doi.org/10.1080%2F23802359.2017.1398615

Desjardins, P., & Conklin, D. (2010). NanoDrop microvolume quantitation of nucleic acids. *Journal of Visualized Experiments*, *45*, e2565. https://doi.org/10.3791%2F2565

Dumolin-Lapègue, S., Pemonge, M. H., Gielly, L., Taberlet, P., & Petit, R. J. (1999). Amplification of oak DNA from ancient and modern wood. *Molecular Ecology*, *8*(12), 2137-2140. https://doi.org/10.1046/j.1365-294x.1999.00788.x

Food and Agriculture Organization of the United Nations. (2023). *Durian global trade overview 2023*. https://openknowledge.fao.org/server/api/core/bitstreams/84bbf2cb-d119-48c2-b4f1-f22bf802a7af/content

Jantan, S. Z., Poh, K. B., & Ginibun, F. C. (2024). Comparative transcriptomic study of matured fruit and post-fruit developmental stages in Malaysian durian varieties. *Plant Gene*, *39*, 100463. https://doi.org/10.1016/j.plgene.2024.100463

Japelaghi, R. H., Haddad, R., & Garoosi, G. A. (2011). Rapid and efficient isolation of high-quality nucleic acids from plant tissues rich in polyphenols and polysaccharides. *Molecular Biotechnology*, *49*, 129-137. https://doi.org/10.1007/s12033-011-9384-8

Lucena-Aguilar, G., Sánchez-López, A. M., Barberán-Aceituno, C., Carrillo-Avila, J. A., López-Guerrero, J. A., & Aguilar-Quesada, R. (2016). DNA source selection for downstream applications is based on DNA quality indicator analysis. *Biopreservation and Biobanking*, *14*(4), 264-270. https://doi.org/10.1089%2Fbio.2015.0064

May, O. S., Ismail, W. N. A. B. W., & Jun, C. (2023). The king of fruits dilemma in Malaysia: Discovering durian export challenges to China. *Journal of Engineering Research and Education*, *15*, 61-75. https://doi.org/10.58915/jere.v15.2023.652

Mursyidin, D. H., Makruf, M. I., & Noor, A. (2022). Molecular diversity of exotic durian (*Durio* spp.) germplasm: A case study of Kalimantan, Indonesia. *Journal of Genetic Engineering and Biotechnology*, *20*(1), 39. https://doi.org/10.1186/s43141-022-00321-8

Nawae, W., Naktang, C., Charoensri, S., U-thoomporn, S., Narong, N., Chusri, O., Tangphatsornruang, S., & Pootakham, W. (2023). Resequencing of durian genomes reveals large genetic variations among different cultivars. *Frontiers in Plant Science*, *14*, 1137077. https://doi.org/10.3389/fpls.2023.1137077

Siew, G. Y., Ng, W. L., Salleh, M. F., Tan, S. W., Ky, H., Alitheen, N. B. M., Tan, S. G., & Yeap, S. K. (2018). Assessment of the genetic variation of Malaysian durian varieties using inter-simple sequence repeat markers and chloroplast DNA Sequences. *Pertanika Journal of Tropical Agricultural Science*, *41*(1), 321-332.

Siew, G. Y., Ng, W. L., Tan, S. W., Alitheen, N. B., Tan, S. G., & Yeap, S. K. (2018). Genetic variation and DNA fingerprinting of durian types in Malaysia using simple sequence repeat (SSR) markers. *PeerJ*, *6*, e4266. https://doi.org/10.7717/peerj.4266

Taberlet, P., Gielly, L., Pautou, G., & Bouvet, J. (1991). Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology*, *17*, 1105-1109. https://doi.org/10.1007/bf00037152

Thorogood, C. J., Ghazalli, M. N., Siti-Munirah, M. Y., Nikong, D., Kusuma, Y. W. C., Sudarmono, S., & Witono, J. R. (2022). The king of fruits. *Plants, People, Planet*, *4*(6), 538-547. https://doi.org/10.1002/ppp3.10288

Yuen, P. S., Brooks, K. M., & Li, Y. (2001). RNA: a method to specifically inhibit PCR amplification of known members of a multigene family by degenerate primers. *Nucleic Acids Research*, *29*(6), e31-e31. https://doi.org/10.1093/nar/29.6.e31

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Identification of Novel Competitive Antagonists for Histamine H1 Receptor in Malaysian Kelulut Honey

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ABSTRACT

Current breakthroughs in molecular docking approaches have significantly contributed to discovering novel antagonists that competitively bind to histamine H1 receptor (H1R), thereby inhibiting histamine-mediated reactions. A reliable antagonist must possess a superior binding affinity with H1R compared to histamine while having the ability to be efficiently absorbed and traverse the intestinal barrier for systemic circulation. The consideration of human intestinal absorption (HIA) provides an extension for comprehending ligands' bioavailability before engaging in docking studies. In this study, the Brain Or IntestinaL EstimateD permeation method (BOILED-Egg) model was utilized to predict the HIA of the ligands, followed by molecular docking to target the H1R with compounds presented in Malaysian Kelulut honey that are impenetrable to the blood-brain barrier (BBB-) by using a virtual screening tool, Pyrx. The findings highlighted the importance of specific residues, including ASP 107, TYR 108, SER 111, and TYR 431, in H1R, as they interact with histamine within the binding spaces, demonstrating strong intermolecular forces. Among 69 BBB- compounds, Polydatin (PD), Sophoflavescenol, and Dendrocandin B exhibited favorable results in docking studies with H1R, indicating their potential to be used as competitive antagonists in treating allergic symptoms. A more promising candidate can be nominated to develop optimal antagonists by addressing absorption properties and binding affinity perspectives.

Keywords: Antihistamine, histamine H1 receptor, human intestinal absorption, kelulut, molecular docking

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INTRODUCTION

The prevalence of allergies and inflammation has recently increased worldwide due to inadequate exposure to allergens such as environmental microorganisms and food allergens (Renz & Skevaki, 2021). Human mast cells degrade upon exposure due to elevated intracellular calcium ion levels and release inflammatory mediators, including histamine, into the cytoplasm. Histamine is then bound to the Histamine H1 Receptor, a G protein-coupled receptor, which can trigger various signaling pathways, including the Phospholipase C (PLC) and protein kinase pathways. It then results in intracellular calcium ion influx that triggers vascular permeability and physiological allergic reactions such as airway constriction and vasodilation (Zhou et al., 2024).

To date, antihistamines have been effective in mitigating histamine-mediated reactions. However, antihistamines can exhibit adverse reactions while relieving allergic symptoms. First-generation antihistamines can cause sedative effects due to their ability to penetrate the blood-brain barrier (BBB) and act on the central nervous system (CNS) (Ince & Ruether, 2021). Second and third-generation antihistamines were then developed to reduce the sedative effects by targeting the peripheral nervous system (PNS), nevertheless, they still result in adverse effects such as headache and dizziness when overdosed (Ince & Ruether, 2021). To minimize adverse effects associated with medications, natural food products such as honey have been considered as alternatives in treatment because they are rich in phytochemical compounds that are potentially useful to combat the disease.

Honey contains a variety of phytonutrients with pharmacological benefits, including antibacterial, anti-inflammatory, antioxidant, and wound-healing properties (Aspar et al., 2020). However, due to limited honey production, most studies have focused on manuka honey instead of Malaysian honey, particularly Kelulut honey (Tuksitha et al., 2018). Kelulut honey is a Malaysian polyfloral honey that possesses phytochemical compounds such as flavonoids and phenolic acids. These polyphenols are significant contributors to pharmacologic and therapeutic properties, including anti-inflammatory properties. Additionally, it is found that Kelulut honey consists of 69 BBB- compounds in a previous study of prediction of BBB permeability, showing that compounds in Kelulut honey can be potentially utilized in managing allergies with lesser side effects on CNS (Edros et al., 2023a).

Allergic symptoms and inflammation mostly involve smooth muscle due to the presence of Histamine H1 Receptor (H1R). A desired ligand must be able to passively absorb into the human gastrointestinal while impermeable to BBB to be transported to the affected area, particularly H1R in smooth muscle cells (Kikiowo et al., 2023). Thus, Human Intestinal Absorption (HIA) assessment is crucial to classify the Kelulut honey compounds into good intestinal absorption (HIA+) and poor intestinal absorption (HIA-) before molecular docking, demonstrating the ability of a ligand to be absorbed by the intestinal barrier into

the bloodstream. It is to ensure the bioavailability of the ligand, where it can be absorbed and delivered to the affected area through the bloodstream while not passing across the BBB, which could damage the brain due to toxicity and adverse effects (Zekri et al., 2023). Once absorbed, the ligand can mitigate histamine-mediated reactions by competitively binding to the H1R binding site (Wang et al., 2021).

For a ligand to exhibit competitive antagonism, it should possess a structural analog while demonstrating higher binding affinity and better conformation compared to histamine when it interacts with active residues in the H1R active site (Conrad et al., 2023). Virtual screening was commonly utilized to investigate small molecules via structure-based screening, especially the molecular docking technique. Structure-based docking can provide valuable insights into the binding pattern of Kelulut Honey ligands at the H1R active site, identifying the potential antagonist that competitively interacts with the active residue that histamine did.

In this work, PyRx, a virtual screening tool, was utilized for molecular docking between H1R and BBB- compounds in Kelulut honey. BBB- ligands present in Kelulut honey were extracted from BBB permeability prediction in a previous study to identify their potential as an H1R antagonist via molecular docking (Edros et al., 2023a). The structure of the H1R was extracted from the protein data bank (PDB) and refined using PyMol to obtain the most robust structure, followed by HIA prediction using the BOILED-Egg model to obtain HIA+ ligands for docking (Daina & Zoete, 2016). Once protein and ligands were prepared, grid generation was performed to set the H1R binding site as docking space, and molecular docking could be started. The result of binding affinity was extracted, whereas the output of binding conformation was visualized in Biovia Discovery Studio Visualizer to analyze the interaction of H1R-ligand complexes. By correlating and comparing the binding affinity and interaction, potential antagonists among ligands in Kelulut honey were identified.

METHODS

Protein Preparation

The crystal structure of H1R (PDB ID: 3RZE) was retrieved from the Protein Data Bank (https://www.rcsb.org/) with a resolution of 3.10Å, determined by x-ray diffraction. The H1R-doxepin complex was preprocessed by using PyMol version 3.0.2 (The PyMOL Molecular Graphics System, Version 3.0 Schrödinger, LLC) to remove doxepin, water molecules, and the phosphate group, extracting the H1R structure without the presence of antagonists (Akram et al., 2024). Then, hydrogen atoms were added to demonstrate the hydrogen bond interaction with ligands, producing an H1R structure for molecular docking.

Ligands Preparation

BBB-compounds present in Kelulut honey from previous studies regarding BBB permeability prediction were used as ligands to identify their potential as an H1R antagonist via molecular docking (Edros et al., 2023b; Feng et al., 2024; Mohd et al., 2020). The structure of ligands was extracted from PubChem (https://pubchem.ncbi.nlm.nih.gov/) to ensure the availability of 3D structure. Before initializing the ligands for docking, gastrointestinal absorption of ligands was predicted by using the BOILED-Egg model based on topological polar surface area (TPSA) and water-octanol partition coefficient (Log P) (Daina & Zoete, 2016). HIA+ ligands that can be passively absorbed into the GI tract barrier were then prepared by inputting ligands structure in .pdb format into Open Babel in PyRx version 0.8 (https://pyrx.sourceforge.io/) for energy minimization with energy difference cutoff of 0.1 in 200 steps using Universal Force Field (uff) (Dallakyan & Olson, 2015).

Molecular Docking

After the optimization, a grid was generated specifically at the binding site of histamine with 30Å (X=15.98, Y=34.95, Z=22.47) to comparatively analyze the binding affinity and conformation between ligands and histamine. Molecular docking was performed using Autodock Vina integrated with PyRx with an exhaustiveness of eight, representing eight initial random runs for ligand conformation search within the space of the H1R binding site (Dallakyan & Olson, 2015).

Post Docking Analysis

Post-docking analysis involves binding affinities ranking among ligands and histamine, binding conformation with root mean square deviation (RMSD), and binding interaction between ligands and residues. This study generated and extracted the binding affinity and RMSD in a .csv file. H1R-ligands complexes were visualized in two-dimensional (2D) and three-dimensional (3D) views using PyMol and Biovia Discovery Studio Visualizer (version 21.1.0.20298, San Diego). It analyzes the binding conformation and interaction, including intermolecular and covalent forces between active residue and ligands (Ahmed et al., 2021). All results were organized and curated for correlation analysis to identify the potential H1R competitive antagonist among Kelulut honey ligands.

RESULTS AND DISCUSSION

H1R Active Site

In protein preparation, H1R embedded in the phospholipid bilayer consists of 12 receptor cavities and six active sites, as illustrated in Figure 1. Out of six active sites, it is found that histamine interacts with an active site containing residues including ASP107, TYR108,

SER111, THR112, TRP158, ASN198, TRP428, TYR431, PHE432, PHE435, and TYR458 in transmembrane (TM) 3, 5, and 6 of H1R (Feng et al., 2013; Xia et al., 2021). These residues are located in three extracellular loops that are extended from the hydrophobic TM and interact with the hydrophobic core of the phospholipid bilayer, making H1R an integral protein, as illustrated in Figure 1(A). Therefore, this binding site is accessible through extracellular domains, triggering the signaling transduction to the GCPR protein via intracellular domains (Kok et al., 2022).

Specifically, ASP107 and TYR431 form hydrogen bonds with protonated amine groups in histamine. At the same time, THR112 and TYR431 interact with 3-position nitrogen atom (N^{τ}) and 1-position nitrogen atom (N^{π}) in the imidazole ring, respectively. These hydrogen bonds stabilize the binding between histamine and the binding site (Riza et al., 2019). Not forgetting aromatic residues, including TRP428, PHE432, PHE435, and TRP158, which interact with histamine through hydrophobic interaction (Mehta et al., 2021). Contacts between hydrophobic surfaces of protein and ligands expel the water molecules from the binding spaces, resulting in the increment of entropy that contributes to the binding stability and specificity. Additionally, π-π stacking contributed by TYR108 and TRP158 in the upper aromatic region of the imidazole ring also anchored histamine in position, enhancing the stability and binding affinity (Xia et al., 2021). Therefore, the grid was generated based on this specific active site to identify ligands that potentially modulate H1R activities upon binding. Figures 1(B) illustrate the active site involved in molecular docking and the active residues interacting with histamine within the binding site.

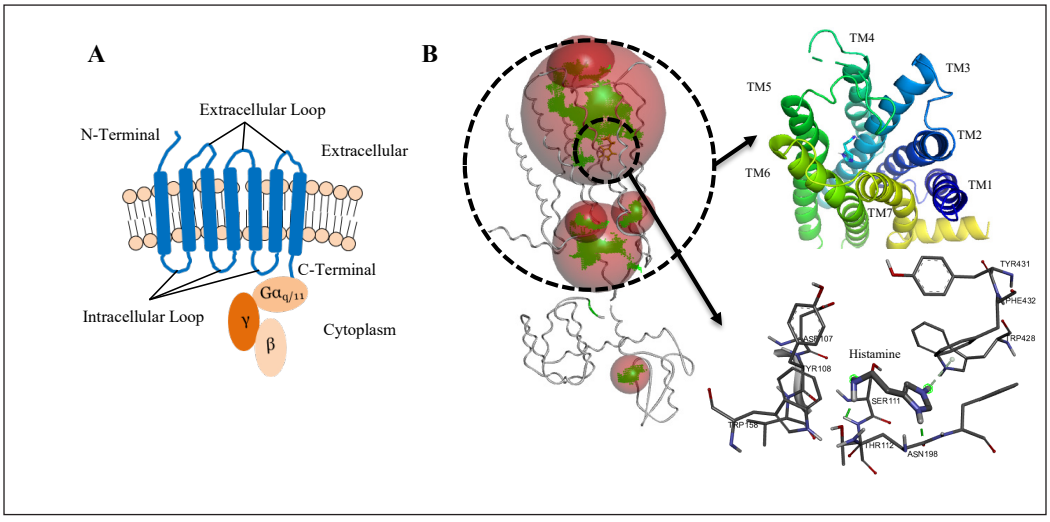

Figure 1. (A) Structure of H1R as an integral protein in the phospholipid bilayer and (B) Six binding sites are visualized by a red sphere along with the visualization of the H1R structure indicating seven TM involved in the binding site and active residue involved in the interaction between the H1R active site and histamine

HIA Assessment

In bioavailability assessment, HIA is one of the critical properties in Absorption, Distribution, Metabolism, and Excretion (ADME) to identify the ligands that can be passively absorbed by the human gastrointestinal (GI) tract (Laskar et al., 2023). HIA+ ligands are more likely to be absorbed by the GI tract after administration than HIA- ligands. A high absorption rate indicates that HIA+ ligands can effectively reach therapeutic concentrations in the systemic circulation (Zhang et al., 2020). Subsequently, the efficacy and consistency of HIA+ ligands in H1R inhibition can be enhanced. Among 69 BBB- ligands, 10 were predicted as HIA+ out of 50, as highlighted in Figure 2. The rationale for using BBB- ligands instead of ligands that are permeable to BBB (BBB+) is to minimize the potential adverse reactions in the CNS, like first-generation antihistamines. In contrast to first-generation antihistamines that may cause sedation, a second and third-generation antihistamine targets H1R in the PNS, thus causing fewer adverse reactions (Cevikbas & Lerner, 2020).

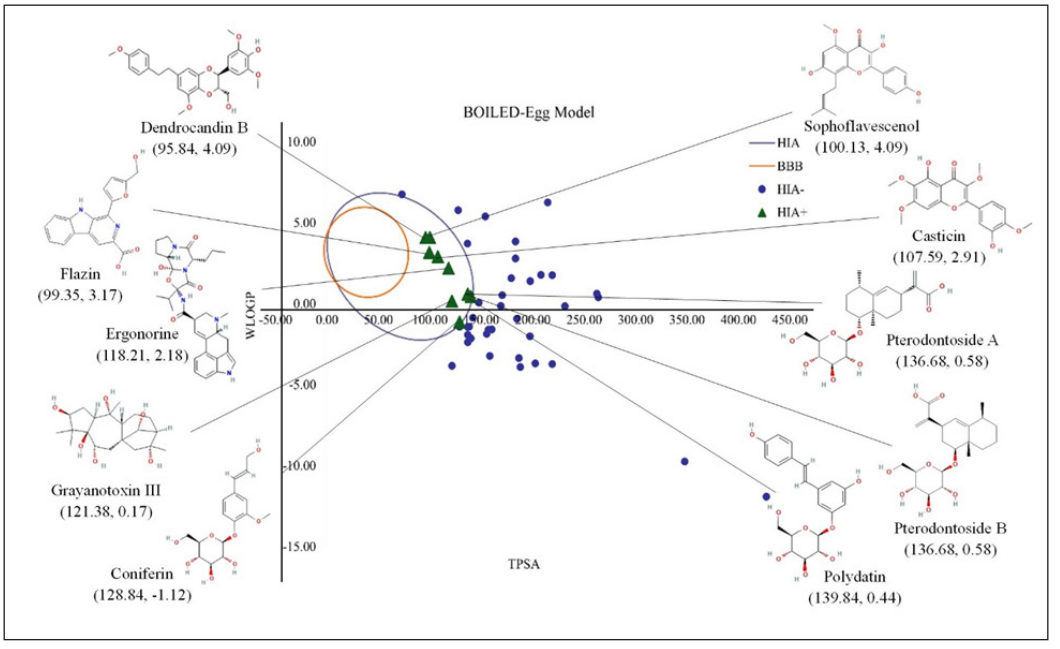

Figure 2. BOILED-Egg model was employed for HIA prediction and 2D structures of 10 HIA+ ligands at two sides of the model. A scatter plot shows the relationship between TPSA and Log P values for HIA+ and HIA- ligands, which are represented as triangle-shaped and bullet-shaped points, respectively. TPSA (x-axis) and WLOGP (y-axis) values for 10 HIA+ ligands are shown as coordinates beneath their respective structures

From the result, the TPSA of 10 ligands ranged from 95.84 to 139.84, whereas Log P ranged from -1.12 to 4.09. TPSA represents the surface of ligands that can interact with

the water molecules by forming hydrogen bonds, whereas Log P indicates the lipophilicity of a ligand. According to the findings, low TPSA and moderate Log P are preferred for a ligand to exhibit optimal intestinal membrane penetration (Ramirez et al., 2021). A lower TPSA value correlates with less interaction with the intestinal barrier, thus providing better membrane permeability and absorption across the phospholipid bilayer of the GI tract. From the perspective of lipophilicity, a moderate Log P value indicates that ligands that reach a balance between hydrophilicity and hydrophobicity are accessible to passive diffusion through the intestinal phospholipid bilayer and able to be soluble in the bloodstream after absorption (Chmiel et al., 2019).

Docking Analysis

In molecular docking, 10 HIA+ ligands were docked with H1R to obtain their binding affinities and binding conformation. The docking results with 0 in RMSD were tabulated in Table 1. During docking, it was observed that Polydatin (PD) has the highest binding affinity of -8.1 kcal/mol, followed by Sophoflavescenol with -7.9 kcal/mol. The binding affinity of Dendrocandin B and Flazin was recorded as -7.6 kcal/mol. The lowest binding affinity was -5.8 kcal/mol and -6.1 kcal/mol recorded by Pterodontoside A, Grayanotoxin III, and Pterodontoside B, respectively, as their binding site are not within the cavity. Other ligands also show good binding energy against H1R, suggesting their potential to be H1R antagonists. This work analyzed three ligands with the highest binding affinities based on their binding conformation, as visualized in Figure 3.

Table 1
Binding affinities of HIA ligands from the lowest to the highest binding energy

HIA+ ligands	Binding affinities (kcal/mol)
Polydatin	-8.1
Sophoflavescenol	-7.9
Dendrocandin B	-7.6
Flazin	-7.6
Coniferin	-7.4
Casticin	-7.1
Ergonorine	-6.2
Pterodontoside B	-6.1
Grayanotoxin III	-6.1
Pterodontoside A	-5.8

PD demonstrated the highest binding affinity among tested ligands, forming 11 interactions with H1R. These interactions included one unfavorable donor-donor interaction, one π-Anion, one π-Alkyl bond, three π-π T-shaped bonds, and five hydrogen bonds. Three hydrogen bonds were established between the hydroxyl group of phenol and ASN198, TYR458, and ILE454 with distances of 1.77Å, 2.76Å, and 2.80Å The cumulative effect of multiple hydrogen bonds bound to phenol contributes to structure stability because hydrogen bonds reinforce each other in the interaction (Shukla & Tripathi, 2020). The other two hydrogen bonds were found between terminal oxygen and LYS179 with distances of 1.78Å and 2.79Å. Lysine residue consists of a positively charged side chain, which can interact with negatively charged terminal oxygen, inducing a strong electrostatic force

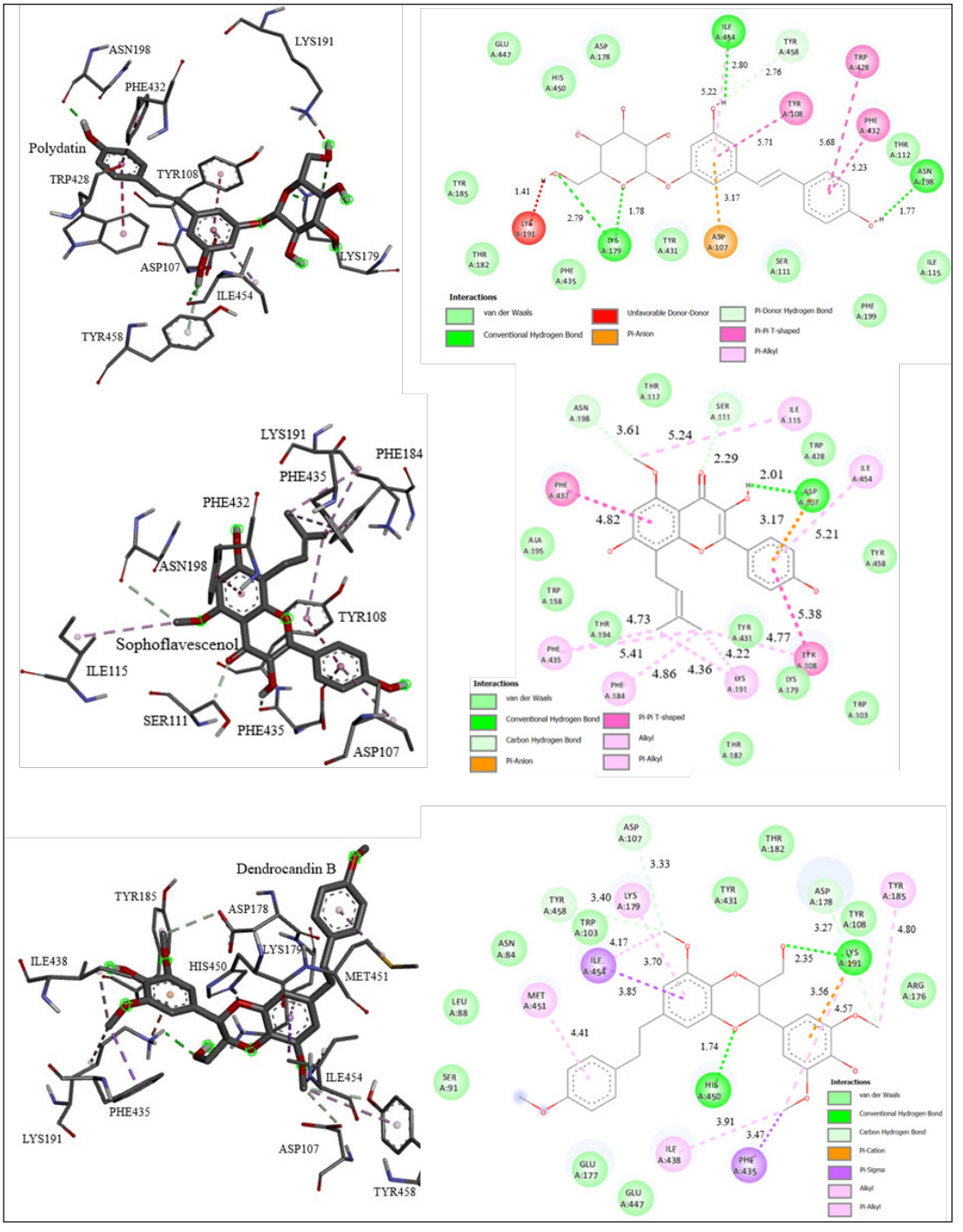

Figure 3. 3D and 2D view of H1R-ligand interaction for Polydatin (top), Sophoflavescenol (middle), and Dendrocandin B (bottom)

with a short bond distance (Dereka et al., 2021). π-π T-shaped interaction was observed between two phenol rings when approaching the H1R active site, interacting with TYR108, TRP428, and PHE432. Each distance was 5.23Å, 5.68Å, and 5.71Å, respectively. Other than π-π interaction, the phenol ring in the middle also interacts with the active site via the electrostatic bond, π-Anion with ASP107, and hydrophobic bond, π-Alkyl, yielding distances within 3.17Å and 5.22Å, respectively.

Previous studies have highlighted that PD, a resveratrol derivative with enhanced bioavailability, demonstrates its potential as a mast cell stabilizer. Unlike antihistamines, PD works as a mast cell stabilizer to inhibit the IgE-mediated allergic reaction. Depressing calcium influx in the mast cell can prevent degranulation, resulting in minimizing histamine release upon mast cell activation (Karami et al., 2022). This study suggest that PD can also function as an H1R antagonist as it exhibits the highest binding affinity amongst BBB-ligands in Kelulut Honey while interacting with prominent residues involved in the H1R histamine binding pattern. Additionally, PD has demonstrated anti-inflammatory properties that reduce inflammation. It makes PD an interesting candidate for a dual mechanism of action similar to a second-generation antihistamine, olopatadine, which acts as a mast cell stabilizer and selectively antagonizes H1R (Kaliner et al., 2010; Tamura et al., 2004). In short, PD has the potential for a dual mechanism of action, which allows it to stabilize most cells and block the binding of histamine in H1R.

The docking of Sophoflavescenol and H1R with the binding affinity of -7.9 kcal/mol showed 14 interactions, including 11 non-bond interactions and three hydrogen bonds. Among non-bond interactions, four π-alkyl were found in the terminal aliphatic chain and one within a benzene ring. Other than hydrophobic interaction, π-Anion also formed between ASP107 and the benzene ring. As the structure of Sophoflavescenol consists of two aromatic rings, π-π T-shaped is formed between two benzene rings with TYR108 and PHE432, respectively. The longest distance of these non-bond interactions was 5.41Å, whereas the shortest distance was 3.17Å. Nevertheless, the strongest interactions were contributed by hydrogen bonds between ligands with ASP107, SER111, and ASN198 at the distances 2.01Å, 2.29Å, and 3.61Å, respectively.

Sophoflavescenol is a flavonoid consisting of two aromatic rings connected by a heterocyclic ring, which is known for diverse pharmacological properties, including antioxidant and anti-inflammatory activities (Hamad, 2023). Previous studies indicate that flavonoids exhibit the potential to inhibit histamine release. For instance, quercetin and luteolin demonstrate the advantage of suppressing the release of pro-inflammatory mediators, including histamine, from human mast cells instead of inhibiting H1R because of low binding affinity compared to known antihistamines (Rakha et al., 2022). Thereby, it is reasonable to hypothesize that Sophoflavescenol can suppress the mast cell from releasing histamine due to structural similarity. From the perspective of binding interaction, unlike

other flavonoids, the presence of hydrophobic interactions towards terminal aliphatic chains in Sophoflavescenol contributes to the efficient packing of the structure within the binding site. Empty spaces between aliphatic chains and aromatic rings within the binding site were minimized to stabilize the structural conformation (Xiao & Woods, 2023). With that, the conformational flexibility of the structure can be minimized to become more specific and stable in the interaction, highlighting the possibility of Sophoflavescenol comparatively inhibiting H1R.

The interaction between Dendrocandin B and the H1R active site was recorded as -7.6 kcal/mol with 14 interactions. ASP107, ASP178, LYS191, HIS450, and TYR458 contributed five hydrogen bonds. Each distance from the longest to the shortest was 3.40Å, 3.33Å, 3.27Å, 2.35Å, and 1.74Å. Furthermore, nine non-bond interactions were observed during molecular docking. One electrostatic bond was recorded as a π-Cation interaction between LYS191 and the benzene ring. Meanwhile, four π-alkyl hydrophobic bonds were observed, and the distance ranged from 3.70Å to 4.80Å. Unlike PD and Sophoflavescenol, Dendrocandin B consists of two π-Sigma interactions with residue PHE435 and ILE454 at 3.47Å and 3.85Å, respectively, instead of π-π T-shaped, which have slightly stronger interactions in terms of binding distances. Dendrocandin B is a bibenzyl derivative commonly extracted from the *Dendrobium* species. Previous studies indicate that compounds extracted from *Dendrobium* species possess anti-inflammatory and anti-allergic properties (Wu et al., 2014). Nonetheless, there is a lack of studies that have directly examined the anti-allergic activity of Dendrocandin B on H1R. Since the interactions with H1R highlight the high binding affinity towards H1R among BBB-ligands, extensive studies are necessary to investigate the pharmacological activities, particularly anti-allergic properties, of Dendrocandin B.

Based on docking results, Sophoflavescenol and Dendrocandin B bind to prominent residues in extracellular domains extending from TM, demonstrating their potential to block histamine binding and inhibit histamine-mediated reactions. In summary, this study identifies Polydatin, Sophoflavescenol, and Dendrocandin B as potential natural H1R antagonists with strong binding affinity, with Polydatin and Sophoflavescenol also showing potential dual action as mast cell stabilizers to inhibit histamine release (Ye et al., 2017). Most observed bonding was non-covalent, involving interactions requiring lower energy. However, the combination of various non-covalent bonds, such as hydrogen bonds, electrostatic interactions, hydrophobic forces, and van der Waals forces, plays a significant role in determining the strength and stability of the interaction between ligands and the protein (Adhav & Saikrishnan, 2023; Chaubah et al., 2019). It enables the ligands to occupy binding sites within the active site, preventing histamine from binding and triggering signaling pathways that lead to allergic reactions. These findings suggest a natural alternative from Kelulut honey to synthetic antihistamines in allergy treatments.

CONCLUSION

This study employed a comprehensive approach involving molecular docking and the BOILED-Egg model for HIA prediction to identify potential H1R competitive antagonists from the ligands presented in Malaysian Kelulut Honey. Among 69 BBB- compounds, Polydatin, Sophoflavescenol, and Dendrocandin B exhibit the highest binding affinity with the optimal bioavailability profile. The docking results revealed the interactions between identified ligands and critical residues, including ASP107, TYR108, ASN198, PHE435, and TYR458, with the H1R active site. These ligands formed multiple non-covalent interactions such as hydrogen bonds, ionic bonds, π interactions, and van der Waals interactions, suggesting their capabilities to competitively bind to the identified active site.

However, further studies are required prior to the *in-vitro* and *in-vivo* assessment to ensure the efficacy and effectiveness of these ligands to discover the ligands with optimal antagonist profiles and minimum adverse reaction when having antagonism interactions with H1R. It is recommended that machine learning techniques be implemented to identify H1R antagonists and address the gap in this study. By employing an automated workflow of machine learning, the time taken for analyzing protein-ligand interactions can be reduced by training the machine learning model using a dataset. In addition, consideration of physicochemical properties, including Lipinski's rule of five in predicting the binding affinity, can significantly improve the accuracy of the docking analysis.

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REFERENCES

Adhav, V. A., & Saikrishnan, K. (2023). The realm of unconventional noncovalent interactions in proteins: Their significance in structure and function. *ACS Omega*, *8*(25), 22268–22284. https://doi.org/10.1021/acsomega.3c00205

Ahmed, S., Mahtarin, R., Ahmed, S. S., Akter, S., Islam, M. S., Mamun, A. Al, Islam, R., Hossain, M. N., Ali, M. A., Sultana, M. U. C., Parves, M. R., Ullah, M. O., & Halim, M. A. (2021). Investigating the binding affinity, interaction, and structure-activity-relationship of 76 prescription antiviral drugs targeting RdRp and Mpro of SARS-CoV-2. *Journal of Biomolecular Structure and Dynamics*, *39*(16), 6290–6305. https://doi.org/10.1080/07391102.2020.1796804

Akram, A., Su, C.-H., & Fu, C.-C. (2024). Targeting small druggable compounds against 3RZE histamine H1 receptor as potential of anti-allergic drug applying molecular modeling approach. *Future Journal of Pharmaceutical Sciences*, *10*, 76. https://doi.org/10.1186/s43094-024-00646-w

Aspar, M. M., Edros, R., & Hamzah, N. A. (2020). Antibacterial evaluation of malaysian kelulut, tualang and acacia honey against wound infecting bacteria. *IOP Conference Series: Materials Science and Engineering*, *991*(1), 012065. https://doi.org/10.1088/1757-899X/991/1/012065

Cevikbas, F., & Lerner, E. A. (2020). Physiology and pathophysiology of itch. *Physiological Reviews*, *100*(3), 945–982. https://doi.org/10.1152/physrev.00017.2019

Chaubah, M. A. L., Bontes, B. W., & Mulachelah, N. A. (2019). Molecular docking of polycyclic aromatic hydrocarbons as potentially carcinogenic molecules through binding with aryl hydrocarbon receptor. *Journal of Smart Bioprospecting and Technology*, *1*(1), 12–15. https://doi.org/10.21776/ub.jsmartech.2019.001.01.3

Chmiel, T., Mieszkowska, A., Kempińska-Kupczyk, D., Kot-Wasik, A., Namieśnik, J., & Mazerska, Z. (2019). The impact of lipophilicity on environmental processes, drug delivery and bioavailability of food components. *Microchemical Journal*, *146*, 393–406. https://doi.org/10.1016/j.microc.2019.01.030

Conrad, M., Horn, A. H. C., & Sticht, H. (2023). Computational analysis of histamine protonation effects on H1R binding. *Molecules*, *28*(9), 3774. https://doi.org/10.3390/molecules28093774

Daina, A., & Zoete, V. (2016). A BOILED-Egg to predict gastrointestinal absorption and brain penetration of small molecules. *ChemMedChem*, *11*(11), 1117–1121. https://doi.org/10.1002/cmdc.201600182

Dallakyan, S., & Olson, A. (2015). Small-molecule library screening by docking with PyRx. In J. Hempel, C. Williams & C. Hong (Eds.), *Chemical biology: Methods in molecular biology* (Vol. 1263, pp. 243–250). Humana Press. https://doi.org/10.1007/978-1-4939-2269-7_19

Dereka, B., Yu, Q., Lewis, N. H. C., Carpenter, W. B., Bowman, J. M., & Tokmakoff, A. (2021). Crossover from hydrogen to chemical bonding. *Science*, *371*(6525), 160–164. https://doi.org/10.1126/science.abe1951

Edros, R., Feng, T. W., & Dong, R. H. (2023a). Utilizing machine learning techniques to predict the blood-brain barrier permeability of compounds detected using LCQTOF-MS in Malaysian Kelulut honey. *SAR and QSAR in Environmental Research*, *34*(6), 475–500. https://doi.org/10.1080/1062936X.2023.2230868

Edros, R., Feng, T. W., & Dong, R. H. (2023b). Utilizing machine learning techniques to predict the blood-brain barrier permeability of compounds detected using LCQTOF-MS in Malaysian Kelulut honey. *SAR and QSAR in Environmental Research*, *34*(6), 475–500. https://doi.org/10.1080/1062936X.2023.2230868

Feng, T. W., Edros, R., Ghani, N. A., Mokhtar, S. U., & Dong, R. (2024). Prediction of blood-brain barrier permeability of compounds by machine learning algorithms. *Journal of Advanced Research in Applied Sciences and Engineering Technology*, *33*(2), 269–276. https://doi.org/10.37934/araset.33.2.269276

Feng, Z., Hou, T., & Li, Y. (2013). Docking and MD study of histamine H4R based on the crystal structure of H1R. *Journal of Molecular Graphics and Modelling*, *39*, 1–12. https://doi.org/10.1016/j.jmgm.2012.10.003

Hamad, R. S. (2023). Rutin, a flavonoid compound derived from garlic, as a potential immunomodulatory and anti-inflammatory agent against murine *Schistosomiasis mansoni*. *Nutrients*, *15*(5), 1206. https://doi.org/10.3390/nu15051206

Ince, M., & Ruether, P. (2021). Histamine and antihistamines. *Anaesthesia & Intensive Care Medicine*, *22*(11), 749–755. https://doi.org/https://doi.org/10.1016/j.mpaic.2021.07.025

Kaliner, M. A., Oppenheimer, J., & Farrar, J. R. (2010). Comprehensive review of olopatadine: The molecule and its clinical entities. *Allergy and Asthma Proceedings: The Official Journal of Regional and State Allergy Societies*, *31*(2), 112–119. https://doi.org/10.2500/aap.2010.31.3317

Karami, A., Fakhri, S., Kooshki, L., & Khan, H. (2022). Polydatin: Pharmacological mechanisms, therapeutic targets, biological activities, and health benefits. *Molecules*, *27*(19), 6474. https://doi.org/10.3390/molecules27196474

Kikiowo, B., Ahmad, I., Alade, A. A., Ijatuyi, T. T., Iwaloye, O., & Patel, H. M. (2023). Molecular dynamics simulation and pharmacokinetics studies of ombuin and quercetin against human pancreatic α-amylase. *Journal of Biomolecular Structure and Dynamics*, *41*(20), 10388–10395. https://doi.org/10.1080/07391102.2022.2155699

Kok, Z. Y., Stoddart, L. A., Mistry, S. J., Mocking, T. A. M., Vischer, H. F., Leurs, R., Hill, S. J., Mistry, S. N., & Kellam, B. (2022). Optimization of peptide linker-based fluorescent ligands for the histamine H1 receptor. *Journal of Medicinal Chemistry*, *65*(12), 8258–8288. https://doi.org/10.1021/acs.jmedchem.2c00125

Laskar, Y. B., Mazumder, P. B., & Talukdar, A. D. (2023). Hibiscus sabdariffa anthocyanins are potential modulators of estrogen receptor alpha activity with favourable toxicology: A computational analysis using molecular docking, ADME/Tox prediction, 2D/3D QSAR and molecular dynamics simulation. *Journal of Biomolecular Structure and Dynamics*, *41*(2), 611–633. https://doi.org/10.1080/07391102.2021.2009914

Mehta, P., Miszta, P., & Filipek, S. (2021). Molecular modeling of histamine receptors—recent advances in drug discovery. *Molecules*, *26*(6), 1778. https://doi.org/10.3390/molecules26061778

Mohd, M. A., Edros, R., & Hamzah, N. A. (2020). Antibacterial properties of Kelulut, Tualang and Acacia honey against fourteen clinically-isolated strains of bacteria-infecting wound. *AIP Conference Proceedings*, *2252*, 020001. https://doi.org/10.1063/5.0020300

Rakha, A., Umar, N., Rabail, R., Butt, M. S., Kieliszek, M., Hassoun, A., & Aadil, R. M. (2022). Anti-inflammatory and anti-allergic potential of dietary flavonoids: A review. *Biomedicine & Pharmacotherapy*, *156*, 113945. https://doi.org/10.1016/j.biopha.2022.113945

Ramirez, D. A., Federici, M. F., Altamirano, J. C., Camargo, A. B., & Luco, J. M. (2021). Permeability data of organosulfur garlic compounds estimated by immobilized artificial membrane chromatography: Correlation across several biological barriers. *Frontiers in Chemistry*, *9*, 690707. https://doi.org/10.3389/fchem.2021.690707

Renz, H., & Skevaki, C. (2021). Early life microbial exposures and allergy risks: Opportunities for prevention. *Nature Reviews Immunology*, *21*(3), 177–191. https://doi.org/10.1038/s41577-020-00420-y

Riza, Y. M., Parves, M. R., Tithi, F. A., & Alam, S. (2019). Quantum chemical calculation and binding modes of H1R ; a combined study of molecular docking and DFT for suggesting therapeutically potent H1R antagonist. *In Silico Pharmacology*, *7*(1), 1–9. https://doi.org/10.1007/s40203-019-0050-3

Shukla, R., & Tripathi, T. (2020). Molecular dynamics simulation of protein and protein–ligand complexes. In D. B. Singh (Ed.), *Computer-aided drug design* (pp. 133–161). Springer Singapore. https://doi.org/10.1007/978-981-15-6815-2_7

Tamura, T., Matsubara, M., Takada, C., Hasegawa, K., Suzuki, K., Ohmori, K., & Karasawa, A. (2004). Effects of olopatadine hydrochloride, an antihistamine drug, on skin inflammation induced by repeated topical application of oxazolone in mice. *British Journal of Dermatology*, *151*(6), 1133–1142. https://doi.org/10.1111/j.1365-2133.2004.06172.x

Tuksitha, L., Chen, Y.-L., Chen, Y.-L., Wong, K.-Y., & Peng, C.-C. (2018). Antioxidant and antibacterial capacity of stingless bee honey from Borneo (Sarawak). *Journal of Asia-Pacific Entomology*, *21*(2), 563–570. https://doi.org/10.1016/j.aspen.2018.03.007

Wang, Z., Bosma, R., Kuhne, S., van den Bor, J., Garabitian, W., Vischer, H. F., Wijtmans, M., Leurs, R., & de Esch, I. J. P. (2021). Exploring the effect of cyclization of histamine H1 receptor antagonists on ligand binding kinetics. *ACS Omega*, *6*(19), 12755–12768. https://doi.org/10.1021/acsomega.0c06358

Wu, C.-T., Huang, K.-S., Yang, C.-H., Chen, Y.-C., Liao, J.-W., Kuo, C.-L., Chen, C.-L., Lo, S.-F., Hsieh, C.-C., & Tsay, H.-S. (2014). Inhibitory effects of cultured Dendrobium tosaense on atopic dermatitis murine model. *International Journal of Pharmaceutics*, *463*(2), 193–200. https://doi.org/10.1016/j.ijpharm.2013.08.015

Xia, R., Wang, N., Xu, Z., Lu, Y., Song, J., Zhang, A., Guo, C., & He, Y. (2021). Cryo-EM structure of the human histamine H1 receptor/Gq complex. *Nature Communications*, *12*, 2086. https://doi.org/10.1038/s41467-021-22427-2

Xiao, Y., & Woods, R. J. (2023). Protein–Ligand CH−π Interactions: Structural Informatics, Energy Function Development, and Docking Implementation. *Journal of Chemical Theory and Computation*, *19*(16), 5503–5515. https://doi.org/10.1021/acs.jctc.3c00300

Ye, J., Piao, H., Jiang, J., Jin, G., Zheng, M., Yang, J., Jin, X., Sun, T., Choi, Y. H., Li, L., & Yan, G. (2017). Polydatin inhibits mast cell-mediated allergic inflammation by targeting PI3K/Akt, MAPK, NF-κB and Nrf2/HO-1 pathways. *Scientific Reports*, *7*, 11895. https://doi.org/10.1038/s41598-017-12252-3

Zekri, A., Harkati, D., Kenouche, S., Saleh, B. A., & Alnajjar, R. (2023). A computational study of potent series of selective estrogen receptor degraders for breast cancer therapy. *Journal of Biomolecular Structure and Dynamics*, *41*(20), 11078–11100. https://doi.org/10.1080/07391102.2022.2159877

Zhang, A., Meng, K., Liu, Y., Pan, Y., Qu, W., Chen, D., & Xie, S. (2020). Absorption, distribution, metabolism, and excretion of nanocarriers in vivo and their influences. *Advances in Colloid and Interface Science*, *284*, 102261. https://doi.org/10.1016/j.cis.2020.102261

Zhou, Z., An, Q., Zhang, W., Li, Y., Zhang, Q., & Yan, H. (2024). Histamine and receptors in neuroinflammation: Their roles on neurodegenerative diseases. *Behavioural Brain Research*, *465*, 114964. https://doi.org/ https://doi.org/10.1016/j.bbr.2024.114964

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Metabolic Dysfunctions and Dynamic of Antioxidant Enzymes Activity in Developing Recalcitrant Cacao (*Theobroma cacao*) Seeds at Different Storage Conditions

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ABSTRACT

A potential breakthrough in improving the short-term preservation of recalcitrant cacao (*Theobroma cacao*) seeds is the specific conditions with slow-drying procedures. Thus, this study aimed to elucidate the effect of storage conditions on the physio-chemical and reactive oxygen species (ROS) antioxidant enzymes with slow-drying processes in cacao seeds. Seeds from ripened cacao pods (clone PBC 123) were demucilaged, placed in a zip-lock polyethene bag, and stored at 14°C and 16°C (40% and 80% relative humidity [RH]), room temperature (RT; 25°C), and control (0 days of storage). Seeds at 14°C tend to retain the highest respiration rate during 12 days of storage. Their impaired respiratory activity is reflected through the highest accumulation of soluble sugar during the first 6 days after storage, lower protein content with the highest antioxidant enzyme activities, indicating increased ROS production. Antioxidant enzymes involved in the glutathione-ascorbate cycle, ascorbate peroxidase, and glutathione reductase activity were crucially responsive to the oxidative status within seed cells at 14°C. In contrast, seeds demonstrated decreasing moisture content during storage (RT and 16°C, 40% RH). They displayed higher ROS (hydrogen peroxide) signalling but within the oxidative concentration threshold, giving the advantage of holding seeds with lesser exposure to oxidative stress. As the condition of 16°C, 40% RH produced lesser germinated seeds (8% to 12%) than seeds at RT during storage, it is then suggested to be the alternative to minimise seed's physio-chemical changes, contributing to the maximum germination characteristics for 12 days of storage, and thus, increase the potential for further exploration.

Keywords: Carbohydrate reserves, hydrated storage, metabolic changes, osmotic cell homeostasis, oxidative stress

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INTRODUCTION

The interrelation of seed moisture content and storage temperature, listed as one of the major factors, strongly influences recalcitrant seed damage, and it is difficult to separate them (Shibata & Coelho, 2016). Moreover, tropical weather, predominance of high temperatures and relative humidity (RH) influence storage problems and consequently contribute to low-quality products (Lisboa et al., 2017). Several adaptive mechanisms that shield cells from harm during water loss are necessary for the optimal metabolic rate in seed storage and the capacity to withstand dehydration (Corbineau et al., 2024). Therefore, saturated RH hydrated storage will be required to sustain viability in the short to medium term. The loss in viability of cacao (*Theobroma cacao*) seed is abrupt; for instance, a temperature dropped from 17°C to 15°C may kill the seeds. The research on tropical recalcitrant-seeded species is still small, with most of their longevity only being measured in days or weeks.

With all detrimental reactions (chemical, reactive oxygen species [ROS], enzymatic, respiratory, or metabolic) likely to occur, keeping seeds at least 20% to 30% hydrated may cause them to degrade quickly, especially at higher temperatures. However, water is still present, allowing defence mechanisms to function in a fully hydrated molecular state (Juan et al., 2021). More so than water content per se, the consequences for seed quality at the wider range of moist and completely imbibed seeds are dependent on functional repairing mechanisms. Soon after imbibition starts, deoxyribonucleic acid (DNA) repair gets started, and depending on how well the functional repair mechanisms are functioning, damaged genes' transcription and/or function may be affected (Pagano et al., 2017). There may also be negative responses to lowering the temperature (chilling) which, usually physically considered, such as membrane changes, protein/enzyme dysfunction, and more physiological changes that lead to loss of structural integrity and overall quality if allowed to continue over a period (Liang et al., 2020).

In the plant life cycle, seed germination and early seedling development depend mainly on the storage of carbohydrates moving in the form of soluble sugars from seed tissue to various organs such as stem and radical, which are necessary for osmotic cell homeostasis growth and maintenance (Wolny et al., 2018). Stresses related to metabolism at a specific water content may result in a breakdown of metabolic coordination in cells, which might trigger uncontrollable attacks by ROS and reduce the protection that enzymes and non-enzymes provide against oxidative damage (Hasanuzzaman et al., 2020). The detoxifying enzymes and antioxidants that make up the mechanism of cell antioxidants underpin ROS's dual role in plants. These systems might eliminate possibly harmful ROS created under stressful conditions or strictly manage ROS concentrations to govern different signalling pathways (Bailly, 2004). Elevated oxidative stress has been associated with hydrated storage, and varying species' reactions to seed storage have been attributed to the severity of the stress (Chandra et al., 2019). The metabolic pathway to the formation

of ROS and enzyme machinery involved in detoxifying is restarted during seed imbibition and subsequent metabolism reactivation (hydrated storage). Following seed imbibition, the metabolic pathway for ROS generation and enzyme machinery involved in detoxifying is reactivated. In general, superoxide dismutase (SOD), catalases (CAT), ascorbate peroxidases (APX), glutathione peroxidase (GPX), glutathione reductases (GR), dehydroascorbate reductases (DHAR), monodehydroascorbate reductases (MDAR), thioredoxins (TRX), and peroxidases (POXs) are some of the various enzymes and signalling molecules involved in ROS detoxification. All enzymes and signalling molecules are produced and active at different levels during seed germination and maturation (Zandi & Schnug, 2022).

No comprehensive data is available on the dynamics of water content-dependent changes in cacao seed viability and vigour loss under slow-drying regimes, including changing storage temperatures and relative humidity over time. Furthermore, considering ROS's multiple roles in seeds, identifying the antioxidant machinery inside the cell, particularly in terms of detoxifying enzymes, may help clarify the consequences of excessive endogenous ROS generation. Therefore, this study aims to elucidate the changes in physio-chemical and accumulation of ROS antioxidant enzymes in cacao seeds during storage.

MATERIALS AND METHODS

Treatments and Experimental Design

The Malaysian Cocoa Board in Tawau, Sabah, Malaysia, gave the PBC 123 clone's ripened cacao (*Theobroma cacao*) pods. Pusat Penyelidikan dan Pembangunan Koko, Madai, Kunak, Sabah (4.7965000487779506, 117.9670069587197) is where the cacao plants were planted. As quickly as feasible, the collected pods were sent to the Universiti Malaysia Sabah laboratory in Sandakan, Sabah. Once there, seeds were taken out of their pods, sawdust was used to demucilage them, and a soft sponge was used to clean them. One hundred and sixty (160) cacao seeds were placed in each 23 × 15 cm zip-lock polyethylene (PE) bag and kept at the following temperatures: (1) air-conditioned room temperature, RT (25±2°C, 55±5% RH); (2) 16°C, 80% RH; (3) 16°C, 40% RH; (4) 14°C, 80% RH; (5) 14°C, 40% RH. Moreover, cacao seeds with 0 days of storage are the control. A microprocessor-controlled console-style germinator (Seedburo MPG-3000, USA) was used for seed hydration.

A split-plot arrangement was used in a completely randomised design (CRD), with 3 replications (160 seeds/ PE bag per replication) for the experiment assigned during storage (seed hydration process) in the germinator. Daily, all the PE bags were opened for one to two minutes, and the seeds were gently swirled to allow for the aerated state within the bag. For a duration of 12 days, the germination characteristics, physio-chemical and ROS enzyme activity changes of the seed cell were assessed every 48 hours, and all the parameters were also measured for the control seeds at 0 days of storage.

Seed Moisture Content (MC)

Ten seeds per replication were extracted and dried for sixteen hours at 103±2°C (Bonner, 1996). The seed MC was calculated using the following formula:

$$MC\ (\%) = (\text{Fresh weight} - \text{Dry weight}) / (\text{Fresh weight}) \times 100\% \quad [1]$$

Leachate Conductivity (LC)

LC was calculated using Bonner's (1996) procedure with modification. For every replication, five seeds were extracted, weighed, and immersed in 25 mL of ultrapure water. After 24 hours, the leachate was poured off, and its conductivity was measured with an LC meter (Eutech PC2700).

Respiration Rate (carbon dioxide; CO_2 evolved)

The respiratory rate was measured using a modified method of Raudiene et al. (2017). Twenty seeds were weighed and positioned in a sealed bottle (250 mL), with the CO_2 gas sensor and infrared detector attached to the probe attachment on top (CO_2-BTA Vernier, USA). Respiration rates were calculated as mL of CO_2 generated per gram of cacao seeds per hour.

Germination Percentage and Germination Rate Index (GRI)

Germination took place in trays at a laboratory temperature (25±2°C). Fifty seeds were placed between moist cloth and monitored for 14 days. Seeds were deemed germinated when a radicle protrusion of 2 mm was observed. GRI represents the speed of germination and is computed as follows:

$$GRI\ (\%\ \text{per day}) = G1/1 + G2/2 + \cdots + Gx/x \quad [2]$$

Where G1 is germination percentage × 100 on the first day after sowing.

Sucrose and Raffinose-family Oligosaccharides (RFOs)

Sucrose and raffinose-family oligosaccharides (RFOs) were measured using a diagnostic kit of Product K-RAFGL (Megazyme Int. Ireland Ltd., Bray, Ireland).

Antioxidant Capacity (DPPH Scavenging)

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) inhibition in cacao seeds was determined by Gangwar et al. (2014). In a centrifuge tube, 200 mg of sample was used. In addition to the samples, 200 µL of distilled water was used as a control. Following that, 1 mL of DPPH (8 mg/100 mL ethanol, 80%) solution was applied to the sample and blank. This configuration

was kept at room temperature for half an hour. The tubes were then centrifuged for 10 minutes at 4000 rpm. After that, 0.5 mL of the supernatant was added to new tubes holding 1 mL of ethanol (80% v/v), and the absorbance at 517 nm was measured using a spectrophotometer (DeNovix DS-11, USA) and compared to the ethanol.

Hydrogen Peroxide (H_2O_2)

The quantity of H_2O_2 was measured in three repetitions using the approach of Velikova et al. (2000). Seed tissues (200 mg) were homogenised in 2 mL of trichloroacetic acid 0.1% (w/v) (TCA) and centrifuged (12,000 × g) for 15 minutes at 25°C. To measure absorbance at 390 nm, one millilitre of supernatant was combined with 1 mL of sodium phosphate buffer (10 mM, pH 7) and 2 mL of potassium iodide (1M) in a spectrophotometer (DeNovix DS-11, USA). The H_2O_2 concentration was computed using an extinction value of 0.28 M^{-1} cm^{-1}.

Enzyme Extraction and Determination of Protein Content

Cacao seed tissues (250 mg) were pulverised using a pestle and mortar, then homogenised in 1.0 ml of phosphate buffer (100 mM, pH 7.8) containing polyvinylpolypyrrolidone (2%). The homogenate was centrifuged (16,000 × g) (4°C) for 18 minutes, and the supernatant served as an enzyme extract. The protein content was measured using the Bradford (1976) approach. A standard curve was created using bovine serum albumin (BSA), using the equation $y = 0.573x - 0.0131$ ($R^2 = 0.99$).

Assay of Antioxidant Ascorbate Peroxidase (APX) Enzyme Activity

The test combination included 0.1 mL of enzyme solution, potassium phosphate buffer (50 mM, pH 7.0), ascorbate (0.5 mM), hydrogen peroxide (1 mM), and ethylenediaminetetraacetic acid (EDTA) (0.1 mM) in a total volume of 1 mL. After introducing an enzyme to initiate the reaction, the optical density change was measured at 290 nm (Li & Sun, 1999). An attenuation coefficient of 2.8 mM^{-1} cm^{-1} was used to calculate the APX activity.

Assay of Antioxidant Superoxide Dismutase (SOD) Enzyme Activity

SOD activity was determined utilising a method developed by Dhindhsa et al. (1981). Three millilitres of the reaction mixture contained the following: 1.5 mL of potassium phosphate buffer (100 mM, pH 7.5), 0.1 mL of Na_2CO_3 (1.5 M), 0.2 millilitres of methionine (200 mM), 0.1 millilitres of EDTA (3 mM), 0.1 mL of *p*-nitroblue tetrazolium chloride (NBT) (2.25 mM) and 0.05 millilitres of enzyme samples. An enzyme-free tube served as the control. 0.1 mL of riboflavin (60 µM) was added to the tubes, and they were left under the light of two 15 W fluorescent lamps for 15 minutes to initiate the reaction. The reaction was

prevented by shutting off the light and covering the tubes with black cloth. The absorbance was measured at 560 nm. The unit of measurement for SOD activity was U mg^{-1} protein.

Assay of Antioxidant Glutathione Reductase (GR) Enzyme Activity

GR activity was determined according to Esterbauer & Grill (1978), which involved measuring the rate of nicotinamide adenine dinucleotide phosphate (NADPH) oxidation at 340 nm (extinction coefficient = 6.22 mM^{-1} cm^{-1}). The assay mixture included NADPH (0.5 mM), oxidised glutathione (GSSG) (10 mM), EDTA (10 mM) in phosphate buffer (0.1 M, pH 7.8), and 50–100 µL of enzyme extract.

Statistical Analysis

The data were analysed using analysis of variance (ANOVA) and Pearson's correlation, with the significant means separated by the least significant difference (LSD) test at $p< 0.05$.

RESULTS AND DISCUSSION

Recalcitrant seeds often preserve an active metabolism and organelle activity. After morphogenesis, developing seeds move into a phase known as "maturation," which appears to be more metabolically and genetically active than seed drying. Known also as "reserves accumulation" periods, they entail metabolic reorganisation and the creation of store compounds, such as starch and storage proteins (Angelovici et al., 2010).

Sugar Depletion and Metabolic Dysfunction

The prolonged chilling storage on recalcitrant seeds indicates the continuous internal physiological acclimation and alterations that might cause disturbances in the distribution and function of soluble sugars in seed cells. It could be a factor in the embryo axis having less available carbohydrates (sugar deprivation) and seeds germinating at a slower rate (Morkunas et al., 2012). Similarly, all storage treatments, other than room temperature (RT), started with the lower germination rate index (GRI) (Figure 1 B) at 2 days after storage (DAS), as compared to the control (control = 0 day [storage duration]). Seeds at 14°C were consistent with the lower GRI values until 12 DAS. Moreover, soluble sugars (sucrose content; Figure 1G) for seeds at 14°C also showed a similar pattern: higher during early storage and reduced mostly after 6 DAS. Seed reserve content was stated to be linked to germination percentages and/or rates (speed) (Zhao et al., 2018). In the present study, GRI is negatively correlated with soluble sugar (sucrose; r = -0.43**).

In contrast, the protein content showed a positive correlation with both germination characteristics measured: GRI (r = 0.55**) and final germination percentage (FGP) (r = 0.63**) (Table 1). It might have to do with the depletion of reserves in the seeds meant

to sustain the developing embryo. At the same time, they are being stored (Maldonado et al., 2015), which further causes significant physio-chemical changes to occur to maintain respiration and other metabolic processes. Sugar-starved cells first adjust to the absence of carbohydrates by progressively substituting glucose with protein metabolism (Morkunas et al., 2012). During the present study, the quantification of protein (cytosolic) (Figure 1 F) concentration displayed a dramatic decrease as early as at 2 DAS for all storage treatments.

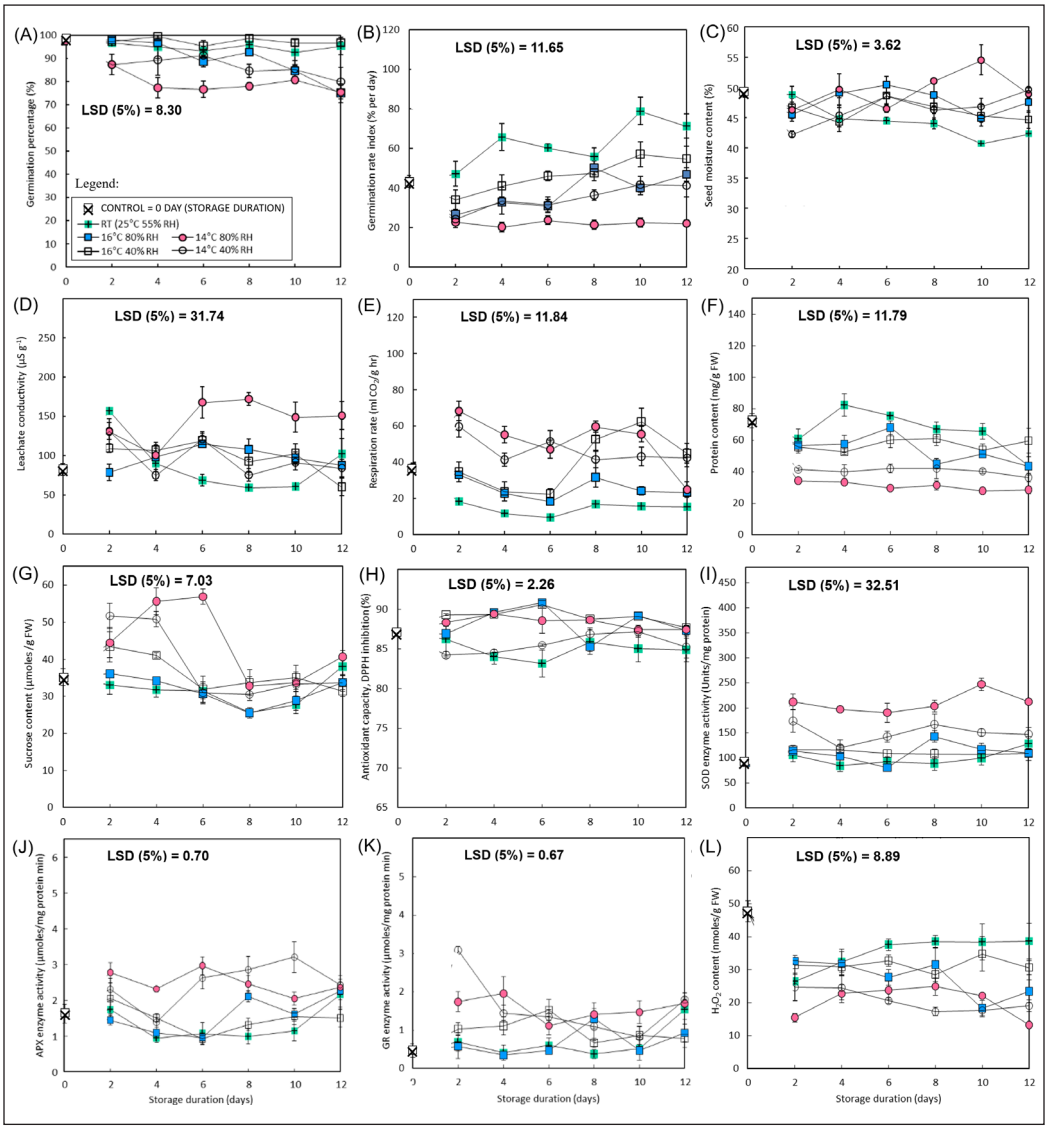

Figure 1. Effect of storage conditions of cacao seeds for 12 days of storage on the (A) final germination percentage (FGP); (B) germination rate index (GRI); (C) moisture content (MC); (D) leachate conductivity (LC); (E) respiration rate; (F) protein content; (G) sucrose content; (H) antioxidant capacity (DPPH scavenging); (I) SOD enzyme activity; (J) APX enzyme activity; (K) GR enzyme activity; (L) H_2O_2 content

Table 1
Pearson's correlation table

Correlations	LC	Respiration Rate	Sucrose Content	Protein content
FGP	-0.38**	-0.31*	-0.25*	0.63**
GRI	-0.50**	-0.54**	-0.43**	0.55**
LC	-			
Respiration rate	0.31*	-		
Sucrose content	0.31*	0.39**	-	
Protein content	-0.52**	-0.50**	-0.42**	-

Note. **, significant at $p \leq 0.001$; *, significant at $p \leq 0.05$; ns, not significant. FGP: final germination percentage, GRI: germination rate index, LC: leachate conductivity

Since those seeds are in the germination mode, de novo protein synthesis is needed. Thus, the insufficient free amino acids in cells might induce signals to start the degradation of a protein storage in the initial hours of imbibition (Rosental et al., 2014). In parallel, the decrease in cytosolic proteins is correlated with the increase of soluble sugars (sucrose content; r = -0.42**) (Table 1), which further supports their substitutional function of carbohydrates.

The other indicator of sugar starvation would be the decline in respiration rate, as shown in previous studies on yellow lupine by Borek et al. (2011). The absence of respiratory substrates is the cause of the lowered respiration rate (Morkunas et al., 2012). Most of the storage conditions showed a decreasing pattern of respiration rate (Figure 1E) at 2 until 6 DAS. It is in parallel with the positive correlation of respiration rate with sucrose content (r = 0.39**) (Table 1). However, many genes are activated in carbohydrate-depleted tissues, resulting in increased activity of relevant enzymes (Borek et al., 2013). It further explains the negative correlation shown by protein content with both respiration rate (r = -0.50**) and sucrose content (r = -0.42**) (Table 1). Seeds at chilling conditions (14°C), which maintained the higher respiration rate (Figure 1E), also displayed the lowest protein content (Figure 1F) and maintained the low values until 12 DAS. According to Da Silva et al. (2018), the increased respiratory activity throughout storage in *Jatropha curcas* seed further led to protein degradation due to the decreased pattern of soluble sugars quantified. Moreover, the increasing pattern and maintaining the highest LC (Figure 1D) (membrane damages) with the consistently lower FGP and GRI (Figure 1 A–B) showed for seeds at 14°C. It supported the significant negative correlations of LC with the germination characteristics: FGP (r = -0.38**) and GRI (r = -0.50**) (Table 1). This parallel adverse situation happened to signify the maintained low protein content (Figure 1 F) from 2 until 12 DAS at 14°C, which might be due to a sudden drop in many of the enzyme's activity, protein deterioration, and modifications to the phospholipids in the membrane that result in the loss of membrane integrity (Maldonado et al., 2015). It explains the negative correlation showed by protein content with the leachate conductivity (LC) (r = -0.52**) (Table 1).

Plants need energy and resources to deal with both biotic and abiotic stress situations; when their metabolic processes slow down due to sugar starvation, they become more susceptible to external factors (Morkunas et al., 2012). The possible reduction of adenosine triphosphate (ATP) due to impaired respiration during storage might disrupt the cells and induce the imbalance of metabolic events, further developing into more progressive deterioration within seed cells. Abiotic stress results in a rise in the radical production, or ROS, which suggests that the stressor is more influential and can cause oxidative damage (Rosental et al., 2014). In anhydrobiosis, oxygen serves as the 'fuel' to produce ROS (Bailly, 2019), but there might be lesser conditions of anhydrobiosis within seed cells during the present study.

As soon as the re-hydrated seed begins to absorb oxygen, ROS are produced and the redox state changes. However, dissolved oxygen concentration lowers when temperature increases, thus bringing about oxygen shortage at high temperatures (Brennan et al., 2016). It explains the consistency of the lower respiration rate (Figure 1E) displayed by seeds at RT. Similarly, for seeds at 16°C, both treatments displayed a lower respiration rate than seeds at 14°C. Plants always produce ROS, which can be detrimental to cells due to various metabolic pathways within individual cellular compartments or as an unavoidable consequence of electron leakage onto oxygen from the electron transport functions of mitochondria, chloroplasts, and plasma membranes (Sharma et al., 2012).

ROS attacks enzymes, damages molecules, and reduces sugar concentrations in cells, which plays a vital part in lowering antioxidant activity (Juan et al., 2021). Similar events might have happened during the present study when seeds at all storage treatments displayed decreasing soluble sugar (sucrose) (Figure 1G) at 8 DAS and kept the low values until 12 DAS. It is widely established that reduced tissue sucrose or glucose levels accelerate lipid, protein, and starch degradation (Borek et al., 2013).

Oxidative Stresses: Dynamic of Antioxidant Enzymes Activity in Developing Cacao Seeds at Different Storage Conditions

The seed moisture content (MC) and metabolic activity change significantly from the beginning of development until the final step of germination. Oxidative stress is an imbalance in forming reactive oxygen species (ROS) and an organism's ability to tolerate antioxidants (Apak et al., 2016). In this study, the increment of DPPH radical scavenging (Figure 1H) within seed cells responded more to the stress conditions during storage. Seeds in most of the storage treatments showed a higher percentage of DPPH scavenging than the control, and they further stagnated at those values during the 12 days of storage. Seeds at 14°C, 40% RH showed the gradually increasing pattern of DPPH radical scavenging activity along 12 days of storage, as well as seeds at RT, which showed a quite stagnant and lower pattern of those scavenging activities. These comparatively low initial DPPH radical

scavenging levels in seeds exposed to high temperatures point to an intentional strategy to enable ROS required for cell membrane loosening and other development processes during the early stages of germination.

The primary mechanisms of delaying ageing are cell membrane repair and enzymatic detoxification. During the present study, in such hypoxic surrounding seed cells, the overly accumulated glycolysis by-products may induce stress conditions and increased ROS production, which is reflected through the increment of antioxidant enzymes. Seeds at both 14°C, 40% RH, and 80% RH displayed quite a similar pattern: to record the higher antioxidant enzyme activity (Figure 1 I-K) than the other storage treatments, along 12 days of storage.

Results displayed the significant positive correlations of all antioxidant enzyme activity quantified with leachate conductivity (LC); APX ($r = 0.35$**), SOD ($r = 0.57$**), and GR ($r = 0.38$**) (Table 2). These indicate the significant cause of increased ROS, probably due to the fast-uncoupling oxidative phosphorylation (impaired respiration). Respiration is the primary source of electron leakage to oxygen, which produces free radicals (Francini et al., 2006). It supported the significant positive correlations of all antioxidant enzyme activity with respiration rate; APX ($r = 0.52$**), SOD ($r = 0.58$**), and GR ($r = 0.44$**) (Table 2). The seed's life span is determined by its capacity to evoke antioxidative enzyme activity to detoxify excess ROS levels (Sahu et al., 2017). Moreover, the consistently significant negative correlations of all antioxidant enzymes (APX, SOD and GR) with the germination characteristics (final germination percentage [FGP] and germination rate index [GRI] (Table 2) indicated that there might be insufficient functional antioxidant enzymes reactions on recovering oxidative stresses within seed cells. Those patterns were significantly displayed for seeds stored at 14°C. Moreover, according to Da Silva et al. (2018), one of the causes and indicators of deterioration is decreased enzyme activity during seed ageing. The stagnant or reducing only slow pattern of most antioxidant enzyme activity quantified along 12 days of storage reflected deterioration development within seed cells.

Typically, hydroxyl radical (OH^-), superoxide (O_2^-), and hydrogen peroxide (H_2O_2) are the ROS found in imbibed seeds (Pehlivan, 2017). The plasma membrane NADPH

Table 2
Pearson's correlation table

Correlations	FGP	GRI	LC	Respiration rate	H_2O_2 content
APX enzyme activity	-0.50**	-0.43**	0.35**	0.52**	-0.50**
SOD enzyme activity	-0.61**	-0.56**	0.57**	0.58**	-0.64**
GR enzyme activity	-0.38**	-0.39**	0.38**	0.44**	-0.44**

Note. **, significant at $p \leq 0.001$; *, significant at $p \leq 0.05$; ns, not significant. FGP: final germination percentage, GRI: germination rate index, LC: leachate conductivity, APX: ascorbate peroxidase, SOD: superoxide dismutase, GR: glutathione reductase, H_2O_2: hydrogen peroxide

oxidases transmit electrons from cytoplasmic NADPH to oxygen, producing O_2^-, which is then dismutated into H_2O_2 (Ishibashi et al., 2010). Hypoxia creates the O_2^- radical, an uncoupled electron molecule that can stabilise its energy through reactions with other molecules (Pehlivan, 2017). Approximately 1-2% of the oxygen absorbed by mitochondria generates O_2^- anion at the electron transport chain (complexes I and II), producing oxygen and H_2O_2 (Bailly, 2019). Those conditions might occur within seed cells in all storage treatments in this study, as they reflected the hypoxic conditions due to the higher seed MC recorded (>40%). SOD enzyme is known to be highly responsive to the changes and increase of O_2^- to normal living cells in catalysing the conversion of O_2^- to the harmless component's oxygen and H_2O_2 (Daniel & Mani, 2016). Previous studies found that higher SOD activity is necessary both at the start and end of the seed maturation process (Sharma et al., 2012).

However, as those recalcitrant seeds did not go through the maturation drying process, thus the SOD activity (Figure 1 I) within seeds displayed consistently higher activities than the control, led by seeds at 14°C, 80% RH, and followed by 14°C, 40% RH. It suggests that its role in ROS defence is particularly crucial when the seeds are immature and exhibit high levels of metabolic and respiratory activity, as well as when they are almost mature and offer protection against the production of ROS during desiccation (Sharma et al., 2012). In the present study, seeds at 14°C, 80% RH showed the highest and stagnant SOD activity along 6 DAS before increasing onwards. On the contrary, seeds at 14°C, 40% RH followed to display the lower but with a stagnant pattern of SOD activity, even though with the gradual increase of MC along 12 days of storage. The mitochondrial respiratory chain produces ROS naturally and continuously. When the re-hydrated seed begins to absorb oxygen, it produces ROS and shifts its redox state (Bouranis et al., 2007).

Higher SOD activity increases the concentration of H_2O_2 in cells, which increases the activity of antioxidant enzymes to eliminate this ROS (De Souza et al., 2018). APX involved in scavenging H_2O_2 are found in plant cells at the source of H_2O_2 production. H_2O_2 is mentioned as a possible inducer of the expression of many genes, including enzymes involved in ROS synthesis or degradation (Pehlivan, 2017). In parallel with the 15% increment of SOD activity (Figure 1I) was the higher increasing pattern of APX activity (Figure 1J) (47% increment), starting at 6 DAS, especially for seeds at 14°C, 40% RH. The synergy of functional SOD and APX activity in seed cells is further evidenced through the highly negative correlations with H_2O_2 content (SOD; r = -0.64**, APX; r = -0.50**) (Table 2). By oxidising lipids, proteins, and nucleic acids, H_2O_2 may readily pass through membranes and cause cell damage (Suresh et al., 2019), which explains more than doubled accumulation of propagated H_2O_2 (Figure 1L) within seed cells in this study. Because of its high affinity for H_2O_2, APX helps cells detoxify by controlling the intracellular H_2O_2 in each cell compartment (Sahu et al., 2017).

The later higher APX activity might also be due to their slower activity, especially during the first 6 DAS. The sugar starvation events might further have induced the stress conditions to activate more antioxidant enzymes to react. Many times, it is said that the antioxidant system becomes active towards the end of the germination or growth phase. Antioxidant enzymes are only activated when ROS levels are above a certain threshold to preserve ROS homeostasis inside the oxidative window for germination (Bailly, 2019). The rehydration process or increasing MC (Figure 1C) might cause the activation and manifestation of those ROS and further added to the newly produced ROS, which contributed to the respiratory burst; that explains the sharp increment of APX activity of seeds at 14°C, 40% RH during 6 DAS, and the levels maintained higher than the other treatments until 12 DAS. Moreover, the low pattern of LC (Figure 1D) further explains that the lower attack of ROS might be due to their lesser damage on membrane cells and lesser leaked molecules, which reduced the potential of converting H_2O_2 to more harmful molecules. Furthermore, each transition increasing or decreasing pattern of MC might contribute to the unregulated generation of ROS and is likely to occur during intermediate hydration levels where metabolic down-regulation becomes uncoordinated. Seeds at 14°C, 40% RH further recorded the consistency to accumulate among the lowest level of H_2O_2 (Figure 1 L), indicating the function of those antioxidant enzymes to reduce ROS.

The above reasons might further explain the decreasing GR activity (Figure 1 K), in parallel with the increased pattern of APX activity (Figure 1 J), for seeds at 14°C, 40% RH. GR activity, which is actively needed to complete the glutathione-ascorbate cycle, might be the pivotal indicator of the significant effects of lower protein content or depletion on sets of enzyme activity that take part in the glutathione-ascorbate cycle, or specifically in reducing oxidised glutathione to glutathione (GSH). As both processes might be needed to counter-back the increasing production of H_2O_2, the gradual reducing GR activity indicated the limited bases or sources (reduced glutathione or NADPH) for those reactions to have occurred. Moreover, the needs of NADPH usually arise from the pentose phosphate pathway; the metabolic pathway parallels glycolysis. Sugar starvation for seeds at 14°C, 40% RH, however, might cause a significant decrease in the activities of all the pentose phosphate pathway enzymes except for glucose 6-phosphate dehydrogenase. As for seeds at 14°C, 40% RH, both conditions might have occurred together to cause the gradual decreasing GR activity in this study.

During the present study, the imbibitional chilling injury seems to dominate for seeds at 14°C, 80% RH, and more than 40% RH, especially at 8 DAS onwards. Compared to seeds at 14°C, 40% RH, seeds at 80% RH showed many significant consequences on ROS attacks during storage. It displayed a gradual decreasing pattern of germination (Figure 1 A-B) and eventually worsened at 8 DAS onwards. Higher MC retention from

the earlier days of storage, with the combination of the chilling temperature, may cause stressful storage conditions or accelerated ageing, leading to ultracellular changes leading to membrane damages, solution leakage, transcription damage, and damage that cause defective or incomplete protein (enzyme) synthesis essential to germination of seed (Suresh et al., 2019). On the other hand, with the increasing pattern of SOD activity (Figure 1I), seeds at 14°C, 80% RH, however, parallelly show the uneven pattern of APX activity (Figure 1J), or increasing only slowly pattern, which indicates that these effects are the result of RNA synthesis damage, which finally leads to reduced protein synthesis and enzyme deactivation. The absence of a repair mechanism might further result in high cellular H_2O_2.

Seeds at 16°C, 80% RH showed quite a similar pattern of damages as at 14°C, 80% RH, except the ones stored in the later conditions displayed faster deterioration due to chilling injury development within seed cells. Both conditions also showed an increasing pattern and maintained to be among the highest MC (Figure 1C), especially during the first 6 DAS, which caused any damaging symptom manifested indirectly through their declining trend of germination performance (Figure 1 A–B) along 12 days of storage. As for seeds at 16°C, 80% RH, dehydration might occur only slowly (at 8 DAS onwards); thus, metabolism is thought to become imbalanced. Surprisingly, high water concentrations can cause significant intracellular damage and seed/embryo mortality (Berjak & Pammenter, 2008). In the present study, all the antioxidant enzyme activity (SOD, APX and GR) (Figure 1 I-K) measured displayed the increasing pattern at 8 DAS. Seeds at 16°C, 80% RH practically displayed the slower development of oxidative stress, with the significant ROS attack observed after the visible reducing MC at 8 DAS and the significant reducing protein content at 10 DAS.

In the present study, seeds at RT and 16°C, 40% RH, are seen to be at their best ranges, preserving the ROS concentration below the detrimental threshold of deterioration. As suggested by Bailly (2019), ROS homeostasis is directly related to a seed's capacity to germinate. When a seed's ROS concentration is within ranges that allow ROS signalling but do not injure the seed, germination is most likely to occur. Germination, on the other hand, is blocked when the level of ROS is either too low or too high. Together with the reducing MC (Figure 1C) for seeds at RT (at 4 DAS onwards) and 16°C, 40% RH (at 8 DAS onwards), however, seeds at both storage conditions displayed the lower and almost maintained a stagnant pattern of all the antioxidant enzymes activity (APX, SOD and GR) (Figure 1I–K) measured in this study. Their differences were only displayed in the higher germinability for seeds at RT than the ones at 16°C, 40% RH. However, it came together with the constraints of about 8% to 30% (RT) and 8% to 12% (16°C, 40% RH) of seeds germinated during storage. During germination, ROS signalling is thought to be a part of a complex signalling network that involves a variety of actions, such as interaction

with cytoplasmic signalling pathways, oxidative modification of gene expression inside the nucleus, and further weakening of the cell wall (Bailly, 2019). Production sources primarily determine ROS homeostasis, and if ROS production does not exceed their concentration threshold, it will not produce oxidative damage, which may further influence their germinability.

During this study, the ranges differences between 19% to 47% of H_2O_2 (Figure 1L) for seeds at RT and 16°C, 40% RH with the control seeds was recorded, with those seeds consistently exhibiting their good germination performances, along 12 days of storage. Moreover, Juan et al. (2021) added that the time needed for the repair of membrane damage and damage to other regions of the cell, as well as the initiation of antioxidant system activity to avoid the build-up of oxidative stress conditions, are likely the main causes of the delay in the germination of aged seeds. On the other hand, seeds at both storage treatments showed the least changes in their antioxidant enzyme activity, except for the elevated GR activity (Figure 1K) since 2 DAS was recorded for seeds at 16°C, 40% RH. The accumulation of GSH build-up might increase GR activity in cells, resulting in stress tolerance. Besides, at 16°C, 40% RH also displayed a considerable increase in respiration rate (Figure 1E) at 8 DAS, which coincidently occurred with the reducing MC pattern (Figure 1C). Imbibed seeds are extremely sensitive to little changes in environmental variables. Thus, environmental information must be correctly conveyed to seeds and translated into endogenous germination signals (Bailly, 2019). Considering the sugar starvation mode to have occurred at a similar timeline, the increased respiration rate but with maintained antioxidant enzyme activity and the evidence of good performance in germination indicated the efficiency of respiratory activity of seed cells at 16°C, 40% RH in response to their surrounding changes.

CONCLUSION

The present study discovered the alternatives to the easily handled short-term storage of locally produced recalcitrant cacao seeds that can be beneficial for improving the seed producer's awareness and understanding of maintaining good seed quality. Seeds at 14°C, 80% RH, and 16°C, 80% RH were found to be potentially threatened by the oxidative damages, which caused their faster deterioration development during 12 days of storage. While seeds at 14°C, 40% RH were found to respond more to starvations and depletions of their reserved food, which reduced their ability to produce sufficient energy to counter-back the strikes of oxidative stress, especially at 8 DAS onwards. In contrast, the consistency of cacao seeds quality with the minimal physio-chemical changes exhibited along 12 days of storage at 16°C, 40% RH, also demonstrated the highest germinability, lesser seeds germinated during storage (8% to 12%) with lesser fungi infestation (as compared to seeds at RT), and thus, contribute to the higher potential for further exploration. However, as this

research was limited to a specific cacao clone, specific target of fruit maturity, specific target of physio-chemical character and within a specific storage time frame, more alternatives and investigations need to be explored to amend their storability potential, and thereby, will be more practical to be used in the future.

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REFERENCES

Angelovici, R., Galili, G., Fernie, A. R., & Fait, A. (2010). Seed desiccation: A bridge between maturation and germination. *Trends in Plant Science*, *15*(4), 211–218. https://doi.org/10.1016/j.tplants.2010.01.003

Apak, R., Özyürek, M., Güçlü, K., & Çapanoɨlu, E. (2016). Antioxidant activity/capacity measurement. Classification, physicochemical principles, mechanisms, and electron transfer (ET)-based assays. *Journal of Agricultural and Food Chemistry, 64*(5), 997–1027. https://doi.org/10.1021/acs.jafc.5b04739

Bailly, C. (2019). The signalling role of ROS in the regulation of seed germination and dormancy. *Biochemical Journal*, *476*(20), 3019–3032. https://doi.org/10.1042/BCJ 20190159

Berjak, P., & Pammenter, N. W. (2008). From Avicennia to Zizania: Seed recalcitrance in perspective. *Annals of Botany*, *101*(2), 213–228. https://doi.org/10.1093/aob/mcm168

Bonner, F. T. (1996). Responses to drying of recalcitrant seeds of *Quercus nigra* L. *Annals of Botany*, *78*(2), 181–187. https://doi.org/10.1006/anbo.1996.0111

Borek, S., & Nuc, K. (2011). Sucrose controls storage lipid breakdown on gene expression level in germinating yellow lupine (*Lupinus luteus* L.) seeds. *Journal of Plant Physiology*, *168*, 1795–1803. https://doi.org/10.1016/j.jplph.2011.05.016

Borek, S., Kubala, S., & Kubala, S. (2013). Diverse regulation by sucrose of enzymes involved in storage lipid breakdown in germinating lupin seeds. *Acta Physiologiae Plantarum*, *35*, 2147-2156. https://doi.org/10.1007/s11738-013-1251-8

Bouranis, D. L., Chorianopoulou, S. N., Siyiannis, V. F., Protonotarios, V. E., & Hawkesford, M. J. (2007). Lysigenous aerenchyma development in roots – triggers and cross-talks for a cell elimination program. *International Journal of Plant Developmental Biology, 1*(1), 127–140.

Bradford, M. M. (1976). A rapid sensitive method for the quantification of microgram quantities of protein utilising the principle of protein-dye binding. *Analytical Biochemistry*, *72*, 248-254. https://doi.org/10.1006/abio.1976.9999

Brennan, C. E., Blanchard, H., & Fennel, K. (2016). Putting temperature and oxygen thresholds of marine animals in context of environmental change: A regional perspective for the scotian shelf and gulf of St. Lawrence. *PLoS ONE*, *11*(12), e0167411. https://doi.org/10.1371/journal.pone.0167411

Chandra, J., Sershen, Varghese, B., & Keshavkant, S. (2019). The potential of ROS inhibitors and hydrated storage in improving the storability of recalcitrant *Madhuca latifolia* seeds. *Seed Science and Technology*, *47*(1), 33–45. https://doi.org/10.15258/sst. 2019. 47.1.04

Corbineau, F. (2024). The effects of storage conditions on seed deterioration and ageing: How to improve seed longevity. *Seeds*, *3*(1), 56-75. https://doi.org/10.3390/seeds3010005

Da Silva, L. J., Dias, D. S., Sekita, M. C., & Finger, F. L. (2018). Lipid peroxidation and antioxidant enzymes of *Jatropha curcas* seeds stored at different maturity stages. *Acta Scientiarum–Agronomy*, *40*(1), 1–10. https://doi.org/10.4025/actasciagron.v40i1.34978

Daniel, G., & Mani, S. (2016). Determination of enzymatic and non-enzymatic antioxidants in fresh beans of *Theobroma cacao* (L.) and *Coffee arabica* (L.). *International Journal of Advanced Research and Development*, *1*(11), 13–17.

De Souza, G. A. Dias, D. C. F. S., Pimenta, T. M., Cardoso, A. A., Pires, R. M. O., Alvarenga, A. P., & Pícoli, E. A. T. (2018). Morpho-anatomical, physiological and biochemical changes in rubber tree seeds. *Annals of the Brazilian Academy of Sciences*, *90*(2), 1625–1641. https://doi.org/10.1590/0001-3765201820170340

Dhindhsa, R. A., Plumb-Dhindsa, P., & Thorne, T. A. (1981). Leaf senescence: Correlated with increased permeability and lipid peroxidation and decreased levels of superoxide dismutase and catalase. *Journal of Experimental Botany*, *32*, 93–101. https://doi.org/10.1093/jxb/32.1.93

Esterbauer, H., & Grill, D. (1978). Seasonal variation of glutathione and glutathione reductase in needles of *Picea abies*. *Plant Physiology*, *61*, 119–121. https://doi.org/10.1104/pp.61.1.119

Gangwar, M., Gautam, M. K., Sharma, A. K., Tripathi, Y. B., Goel, R. K., & Nath, G. (2014). Antioxidant capacity and radical scavenging effect of polyphenol rich *Mallotus philippenensis* fruit extract on human erythrocytes: An in vitro study. *The Scientific World Journal*, *2014*(1), 279451.https://doi.org/10.1155/2014/279451

Hasanuzzaman, M., Bhuyan, M. H. M. B., Parvin, K., Bhuiyan, T. F., Anee, T I., Nahar, K., Hossen, M. S., Zulfiqar, F., Alam, M. M., & Fujita, M. (2020). Regulation of ROS metabolism in plants under environmental stress: A review of recent experimental evidence. *International Journal of Molecular Science*, *21*, 8695. https://doi.org/10.3390/ijms21228695

Ishibashi, Y., Tawaratsumida, T., Zheng, S. H., Yuasa, T., & Iwaya-Inoue, M. (2010). NADPH oxidases act as key enzyme on germination and seedling growth in barley (*Hordeum vulgare* L.). *Plant Production Science*, *13*(1), 45–52. https://doi.org/10.1626/pps.13.45

Juan, C. A., Lastra, J. M., Plou, F. J., & Pérez-Lebeña, E. (2021). The chemistry of reactive oxygen species (ROS) revisited: Outlining their role in biological macromolecules (DNA, lipids and proteins) and induced pathologies. *International Journal of Molecular Sciences*, *22*(9), 4642. https://doi.org/10.3390/ijms22094642

Li, C., & Sun, W. Q. (1999). Desiccation sensitivity and activities of free radical-scavenging enzymes in recalcitrant *Theobroma cacao* seeds. *Seed Science Research*, *9*(3), 209–217. https://doi.org/10.1017/S0960258599000215

Liang, S., Kuang, J., Ji, S., Chen, Q., Deng, W., Min, T., Shan, W., Chen, J., & Lu, W. (2020). The membrane lipid metabolism in horticultural products suffering chilling injury. *Food Quality and Safety*, *4*(1), 9–14. https://doi.org/10.1093/fqsafe/fyaa001

Lisboa, C. F., Araújo, R. S. L. De, Teixeira, I. R., Mota, J. H., Da Silva, A. G., De Araújo, M. E. V., Silva, D. D. A., França, E. E., Silva, I. L., & De Camargo, F. R. T. (2017). Influence of water content on the quality of pigeonpea seeds. *American Journal of Plant Sciences*, *8*(10), 2397–2406. https://doi.org/10.4236/ajps.2017.810162

Maldonado, M. F. E., Lasprilla, D. M., Magnitskiy, S., & Melgarejo, L. M. (2015). Germination, protein contents and soluble carbohydrates during storage of sugar apple seeds (*Annona squamosa* L.). *Journal of Applied Botany and Food Quality*, *88*, 308–313. https://doi.org/10.5073/JABFQ.2015.088.044

Morkunas, I., Borek, S., Formela, M., & Ratajczak, L. (2012). Plant responses to sugar starvation. In C. F. Chang (Eds.), *Carbohydrates - comprehensive studies on glycobiology and glycotechnology* (p. 572). InTechOpen. https://doi.org/10.5772/51569

Pagano, A., Araújo, S., Macovei, A., Leonetti, P., & Balestrazzi, A. (2017). The seed repair response during germination: disclosing correlations between DNA repair, antioxidant response, and chromatin remodelling in *Medicago truncatula*. *Frontier Plant Science*, *8*, 1972. https://doi.org/10.3389/fpls.2017.01972

Pehlivan, F. E. (2017). Free radicals and antioxidant system in seed biology. In J. C. Jimenez-Lopez (Eds.), *Advances in seed biology* (p. 350). InTechOpen. https://doi.org/10.5772/intechopen.70837

Raudiene, E., Rusinskas, D., Balciunas, G., Juodeikiene, G., & Gailius, D. (2017). Carbon dioxide respiration rates in wheat at various temperatures and moisture contents. *Mapan–Journal of Metrology Society of India*, *32*(1), 51–58. https://doi.org/10.1007/s12647-016-0202-4

Rosental, L., Nonogaki, H., & Fait, A. (2014). Activation and regulation of primary metabolism during seed germination. *Seed Science Research*, *24*(1), 1–15. https://doi.org/10.1017/S0960258513000391

Sahu, B., Sahu, A. K., Thomas, V., & Naithani, S. C. (2017). Reactive oxygen species, lipid peroxidation, protein oxidation and antioxidative enzymes in dehydrating karanj (*Pongamia pinnata*) seeds during storage. *South African Journal of Botany*, *112*, 383–390. https://doi.org/10.1016/j.sajb.2017.06.030

Sharma, P., Jha, A. B., Dubey, R. S., & Pessarakli, M. (2012). Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *Journal of Botany*, *2012*(1), 217037. https://doi.org/10.1155/2012/217037

Shibata, M., & Coelho, C. M. M. (2016). Early harvest increases post-harvest physiological quality of *Araucaria angustifolia* (Araucariaceae) seeds. *Revista de Biología Tropical, 64*(2), 885–896. https://doi.org/10.15517/rbt.v64i2.19254

Suresh, A., Shah, N., Kotecha, M., & Robin, P. (2019). Evaluation of biochemical and physiological changes in seeds of *Jatropha curcas* under natural aging, accelerated aging and saturated salt accelerated aging. *Scientia Horticulturae*, *255*, 21–29. https://doi.org/10.1016/j.scienta.2019.05.014

Velikova, V., Yordanov, I., & Edreva, A. (2000). Oxidative stress and some antioxidant systems in acid rain treated bean plants: Protective role of exogenous polyamines. *Plant Science, 151*, 59–66. https://doi.org/10.1016/S0168-9452(99)00197-1

Wolny, E., Betekhtin, A., Rojek, M., Braszewska-Zalewska, A., Lusinska, J., & Hasterok, R. (2018). Germination and the early stages of seedling development in *Brachypodium distachyon*. *International Journal of Molecular Sciences*, *19*(10), 2916. https://doi.org/10.3390/ijms19102916

Zandi, P., & Schnug, E. (2022). Reactive oxygen species, antioxidant responses and implications from a microbial modulation perspective. *Biology (Basel)*, *11*(2), 155. https://doi.org/10.3390/biology11020155

Zhao, L. J., Liu, W., Xiong, S. H., Tang, J., Lou, Z. H., Xie, M. X., Xia, B. H., Lin, L. M., & Liao, D. F. (2018). Determination of Total Flavonoids Contents and Antioxidant Activity of Ginkgo biloba Leaf by Near-Infrared Reflectance Method. *International Journal of Analytical Chemistry*, 1-7. https://doi.org/10.1155/2018/8195784

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Effects of Tamanu Kernel Cake from Plantation By-product on Ruminal Digestibility and Methane Emission

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ABSTRACT

The study investigates the effects of tamanu kernel cake (TKC) as protein substitution in the dietary concentrate on ruminal digestibility and methane emission. TKC is a by-product of the plantation industry of tamanu oil. The dietary concentrate consisted of wheat pollard, rice bran, corn grain, palm kernel cake, and soybean meal. The concentrate was formulated to contain crude protein and total digestible nutrients of approximately 15% and 65%, respectively. In the present study, TKC was used to substitute protein sources at different levels, such as 0% (T0), 50% (T1), and 100% (T2). Another dietary treatment was also prepared by adding 0.5 mineral salt to T2 (T3). All dietary treatments were incubated in rumen buffer according to the method of Tilley and Terry for 48 h at 39°C. In the results, the digestibility of dry matter and organic matter from dietary T1, T2, and T3 were not different compared to T0. In ruminal fermentation, dietary treatment did not affect total VFA and ammonia. Dietary T2 and T3 resulted in lower methane emissions than dietary T0 (p<0.05). Additional mineral salt in dietary T3 did not affect methane emission compared to dietary T2. The present study concluded that the substitute of protein source with TKC at 30% reduced methane production effectively without negatively affecting ruminal digestibility and fermentation.

Keywords: Methane, protein source, rumen, substitution, tamanu kernel cake

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INTRODUCTION

Recently, the development of the ruminant industry has been considered to create a green farming model by reducing methane (CH_4) production. It occurred because high emissions of methane can contribute to the accumulation of greenhouse gases, which drive global warming and climate change

(Appuhamy et al., 2016; Johnson & Johnson, 1995). Methane results naturally during ruminal enteric fermentation by methanogen bacteria (Johnson & Johnson, 1995). Generally, 30% of total anthropogenic methane emission was contributed by the ruminant sector (Shibata & Terada, 2010). In addition, the ruminant industry in developing countries also faces the limited preference and availability of a protein source, which is relatively expensive, especially for local farmers. Thus, alternative protein sources must be studied with the consideration of local based. Interestingly, the by-product of industry plantation could be an option to supply the requirement of protein source for ruminant feed.

Tamanu (*Calophyllum inophyllum*) kernel cake (TKC) is a by-product from the plantation industry of tamanu crude oil (TCO) (Paradhipta et al., 2023a). In Indonesia, the development of TCO is increasing year by year due to the high demand for alternative biofuel following government regulation, which also increases the production of TKC. Tamanu trees are cultivated in the plantation industry in Indonesia, can be maintained easily in tropical areas, and are spread from Sumatra and Papua islands. In fact, the tamanu kernel can produce similar amounts of crude oil to palm kernel cake (Leksono et al., 2014). Therefore, the development of the TCO industry has high prospects. Kernel cake is the by-product that has high results during oil pressing. Tamanu kernel results in a cake and crude oil at a 1:1 ratio (Leksono et al., 2014). Without any utilization, tamanu cake is the only waste that can contaminate the environment. TKC is potentially used as an alternative feed for ruminants. A previous study reported that TKC contains crude protein (CP), ether extract (EE), and neutral detergent fiber (NDF) approximately at 20.1%, 15.3%, and 53.3%, respectively (Paradhipta et al., 2023a). In addition, TKC also contains total phenol 3.73%–6.47%, total flavonoid 1.13%–1.70%, saponins 0.57%–0.90%, and tannins 0.47%–0.93% (Paradhipta et al., 2023a; Umroni et al., 2024). The chemical compositions of TKC are similar to palm kernel cake (PKC), which contains a CP of around 20%–22% (Paradhipta et al., 2023a). In addition, the price of PKC and TKC is similar in the market. With the massive production of TCO in the future, the price of TKC will be cheaper. Three TCO industries have been established in Central Java Province to supply the market demand.

As a single feed, TKC has a similar ruminal digestibility to PKC and copra cake but results in a lower methane emission (Paradhipta et al., 2023a). Tamanu kernel cake contains the plant's secondary metabolites, which have a role as rumen modifiers that can inhibit the growth of *methanogens archaea* in the rumen (Bodas et al., 2012; Lee et al., 2021; Paverini et al., 2012). It could effectively decrease methane emissions in the rumen. As a by-product of oil extraction, PKC also contains secondary metabolites, but it is lower than TKC. The total phenolic content as main plant secondary metabolites in PKC is lower than 0.55% (Tsouko et al., 2019). Protein sources are commonly applied

as mixture feed as concentrate. However, the use of TKC as a mixture feed was limited in the study. Moreover, the effectiveness of TKC to reduce methane emissions was unknown. This information is important to increase the value of plantation by-products and support the development of integrated farming models among plantation and livestock industries. The present study applied TKC to substitute a protein source, such as PKC. In addition, the use of TKC as a rich source of plant secondary metabolites was combined with the supplementation of mineral salt to have beneficial effects on ruminal fermentation. Previous studies reported that supplementing mineral salt containing Se, Fe, Mn, Zn, Cu, and Co could help decrease methane emissions, especially for lactating animals (Li et al., 2017). The mineral salt could help to reduce the acetate-to-propionate ratio and increase the use of free H^+ ions for propionate production (Li et al., 2017). In addition, trace minerals such as Se and Zn potentially have an antioxidant effect, which might affect rumen methanogens diversity (Cortinhas et al., 2010; Hendawy et al., 2022; Parashuramulu et al., 2015). Therefore, the present study investigated the effect of TKC as a mixture feed on the ruminal digestibility, fermentation, and methane emission by *in vitro* technique.

MATERIALS AND METHODS

Preparation of Diet

The concentrate consisted of wheat pollard, rice bran, corn grain, soybean meal, PKC, and TKC. The TKC was collected from Purworejo Districts and was sub-sampled for laboratory analyses. TKC was used to replace the protein source in the diet. There were four dietary treatments as follows in Table 1. PKC replaced TKC at 0% (T0), 50% (T1) and 100% (T2). In dietary T3, the commercial mineral mix (Booster, PT. Agromix, Indonesia)

Table 1
Ingredients and formulation of dietary treatments (%)

Ingredients	T0	T1	T2	T3
Wheat pollard	30	30	30	30
Rice bran	20	20	20	20
Corn grain	10	10	10	10
Soybean meal	10	10	10	10
Palm kernel cake	30	15	0	0
Tamanu kernel cake	0	15	30	30
Total	100	100	100	100
Mineral salt*	-	-	-	0.5

Note. T0 = control diet; T1= the use of TKC to substitute 50% of palm kernel cake; T2 = the use of TKC to substitute 100% of palm kernel cake; T3 = dietary T2 with an additional 0.5% of trace mineral; *Fe 12.5 g/kg, Mg 1.8 g/kg, Zn 439.0 mg/kg, Se 131 µg/kg

was added as a supplement. The supplementation of commercial mineral mix was chosen and conducted based on field observation. The commercial mineral mix contained trace minerals such as Fe, Mg, Zn, Se and complex vitamins (A, D, E). All dietary treatments were formulated to contain CP approximately at 15% and total digestible nutrient (TDN) at 65%. The ingredients and formulation of dietary treatments are shown in Table 1.

Chemical Compositions of Diet

Both TKC and PKC, and all dietary treatments were sub-sampled at approximately 500 g for proximate analyses following the procedure of the Association of Official Analytical Chemists (AOAC, 2016). In the preparation, samples from TKC and dietary treatments were dried using the dry oven (Memmert UN55, Germany) at 55°C for 48 h and then ground using a Wiley mill to pass a 1 mm screen as a standard for proximate analysis. The grinding process also helped to homogenize samples. In the determination of dry matter (DM), the sample was dried at 105°C for 24 h (method 934.01). A Muffle furnace (Advantec KM-420, Japan) was used to measure the organic matter (OM), conducted at 550°C for 5 h. The CP was determined by a Nitrogen Analyzer (B-324, 412, 435 and 719 S Titrino, BUCHI, Flawil, Switzerland) following the procedure of Kjeldahl (method 984.13). The EE was analyzed according to the Soxhlet procedure (method 920.39). The fiber fractions, such as neutral detergent fiber (NDF) and acid detergent fiber (ADF), were determined by using an Ankom 200 fiber analyzer (Ankom Technology, Macedon, NY, USA) (method 2002.04 and method 973.18, respectively). The TDN was estimated based on Dahlke's model (2020) calculations.

Plant secondary metabolites from TKC and all dietary treatments were determined using Spectrophotometry UV-vis (UV-1800 Shimadzu, Japan). In this investigation, the methods of Chaovanalikit and Wrolstad (2004) and Gonzalez and Herrador (2007) were utilized for total phenol and total flavonoid, respectively. The methods of Makkar et al. (1993) and Pramono (2005) were followed for the measurements of tannin and saponins, respectively.

***In Vitro* Digestibility**

The rumen fluid was collected from two fistulated Bali cattle (*Bos indicus*) before morning feeding. In general, cattle had an average weight of 310 kg. The diet of fistulated cattle contained CP at 12.5% and metabolizable energy (ME) at 10 kcal/kg, which consisted of *Pennisetum purpureum* grass and commercial concentrate at a 7:3 ratio. Collected rumen fluid was placed in an anaerobic container at 39°C before being transferred to the laboratory. The collected rumen fluid was filtered in the laboratory using three cheesecloth layers. The rumen buffer consisted of a rumen fluid and buffer solution at a ratio of 1:4, following the procedure of McDougall (1948).

All samples were incubated in rumen buffer according to Tilley and Terry's (1963) procedure. In the incubation, a 0.5 g sample was placed in a 100 mL glass serum bottle and added with 40 mL of rumen buffer. Each treatment used quadruplicate using four incubation bottles along with four blanks. Incubation was conducted at 39°C for 48 h in the aerobic incubator. Incubation was conducted over three different periods in different weeks. After 48 h incubation, a syringe collected 10 mL of gas from each bottle. The gas was transferred into the vacuum tube for storage before methane analysis. Gas Chromatography (Shimidzu GC-2010, Japan) was used to determine methane concentration following Paradhipta et al.'s setup (2023a). In addition, helium was used as a carrier gas. The concentration of methane was expressed as ppm.

All bottles were opened and filtered using a gooch crucible to separate a sample from the rumen buffer. The rumen buffer, which consisted of pH, ammonia-N, and total volatile fatty acid (VFA), was used to evaluate ruminal fermentation. The filtered sample was used to evaluate the digestibility of a diet consisting of *in vitro* dry matter digestibility (IVDMD) and *in vitro* organic matter digestibility (IVOMD). The difference in concentrations of DM and OM, before and after ruminal incubation, were used to calculate the IVDMD and IVOMD. Collected rumen buffer was used to measure rumen pH using a digital pH meter (Ohaus AB23PH-F, China). The concentration of ammonia-N was analyzed using the colorimetric method by Chaney and Marbach (1962). The analysis of VFA using gas chromatography (GC 8A, Shimadzu Crop., Japan) is according to the description of Hidayah et al. (2023).

Ethical Approval

The animal care and *in vitro* procedures were carried out in accordance with the ethical standards of the Ethics Committee of the Integrated Laboratory for Research and Testing, Universitas Gadjah Mada (No. 00007/III/UN1/LPPT/EC/2024).

Data Analysis

Data was analyzed based on a completely randomized design using PROC GLM of Statistical Analysis Software (SAS), version 9. The model applied in the present study was $Y_{ij} = \mu + T_i + e_{ij}$, where Y_{ij} is the response variable, μ is the overall mean, T_i is the effect of dietary treatment, and e_{ij} is the error mean. Tukey test was applied for mean separation, and the significant differences were declared at $p \leq 0.05$.

RESULTS

Chemical Compositions

In the present study, the concentrations of DM, OM, CP, EE, NDF, ADF, and TDN from TKC were 89.5, 91.4, 22.6, 16.8, 52.6, 37.4, and 61.6%, respectively (Table 2). Generally,

the chemical compositions of TKC were similar to PKC, especially in the contents of CP, NDF, and ADF. The PKC contained DM, OM, CP, EE, NDF, ADF, and TDN approximately at 91.5, 91.8, 21.7, 10.4, 54.1, 36.4, and 62.4%, respectively. In addition, the concentration of total phenol, total flavonoid, total saponin, condensed tannin, and hydrolysable tannin from TKC were 6.23, 1.56, 0.96, 0.42, and 0.45%, respectively (Table 3).

Different substitution levels of TKC do not affect the chemical compositions of dietary treatments (Table 4). The means of DM, OM, CP, EE, NDF, ADF, and TDN from all treatments were 86.5, 88.4, 15.9, 2.53, 45.1, 27.3, and 68.7%.

The present study reported that dietary T2 and T3 had higher total phenol (p=0.001; 1.32 and 1.33 vs. 0.11 and 0.88) and total flavonoid (p=0.001; 0.35 and 0.38 vs. 0.09 and ND) compared to T0 and T1 (Table 5). In addition, saponin and tannin were not detected or in less concentration for measurement.

Table 2
Chemical compositions of tamanu kernel cake and palm kernel cake in the present study (%, DM)

Items	Feedstuffs[1]	
	TKC	PKC
Chemical compositions		
Dry matter	89.5	91.5
Organic matter	91.4	91.8
Crude protein	22.6	21.7
Ether extract	16.8	10.4
Neutral detergent fiber	52.6	54.1
Acid detergent fiber	37.4	36.4
Total digestible nutrient[2]	61.6	62.4

Note. [1]TKC = Tamanu kernel cake; PKC = Palm kernel cake; DM = Dry matter; NA = Not available

Table 3
Plant secondary metabolites of tamanu kernel cake in the present study (%, DM)

Items	Feedstuffs[1]
Total phenol	6.23
Total flavonoid	1.56
Total saponin	0.96
Total tannin	0.87
Condensed tannin	0.42
Hydrolysable tannin	0.45

Table 4
Chemical compositions of dietary treatments (%, DM)

Items	Dietary treatment[1]				SEM	p-value
	T0	T1	T2	T3		
Dry matter	86.4	86.3	86.4	86.9	0.640	0.667
Organic matter	88.5	88.7	88.1	88.4	1.174	0.952
Crude protein	15.8	16.2	16.0	15.5	1.097	0.871
Ether extract	3.04	2.38	2.30	2.41	0.719	0.587
Neutral detergent fiber	44.4	45.6	45.4	44.9	2.021	0.879
Acid detergent fiber	27.3	27.8	27.2	27.1	1.792	0.945
Total digestible nutrient[2]	68.7	68.4	68.8	68.9	1.254	0.944

Note. [1]T0 = Control diet; T1 = The use of TKC to substitute 50% of palm kernel cake; T2 = The use of TKC to substitute 100% of palm kernel cake; T3 = Dietary T2 with an additional 0.5% of trace mineral; [2]Total digestible nutrient is based on calculation; DM = Dry matter

Table 5
Secondary metabolites of dietary treatments (%, DM)

Item	Dietary treatment				SEM	*p*-value
	T0	T1	T2	T3		
Total phenol	0.11[b]	0.88[b]	1.32[a]	1.33[a]	0.044	0.001
Total flavonoid	ND[b]	0.09[b]	0.35[a]	0.38[a]	0.093	0.001
Saponin	ND	ND	ND	ND	NA	NA
Tannin	ND	ND	ND	ND	NA	NA

Note. [1]T0 = Control diet; T1= The use of TKC to substitute 50% of palm kernel cake; T2 = The use of TKC to substitute 100% of palm kernel cake; T3 = Dietary T2 with an additional 0.5% of trace mineral; DM = Dry matter; ND = Not detected (less concentration); Means with different superscript letters in a row are significantly different (p<0.05)

Digestibility

The present study reported that the application of TKC at different levels of substitution did not affect IVDMD and IVOMD during 48 h of incubation (Figure 1). T1, T2, and T3 resulted in similar results to T0. The average IVDMD was 42.9%, while the average IVNDFD was 65.7% of all treatments.

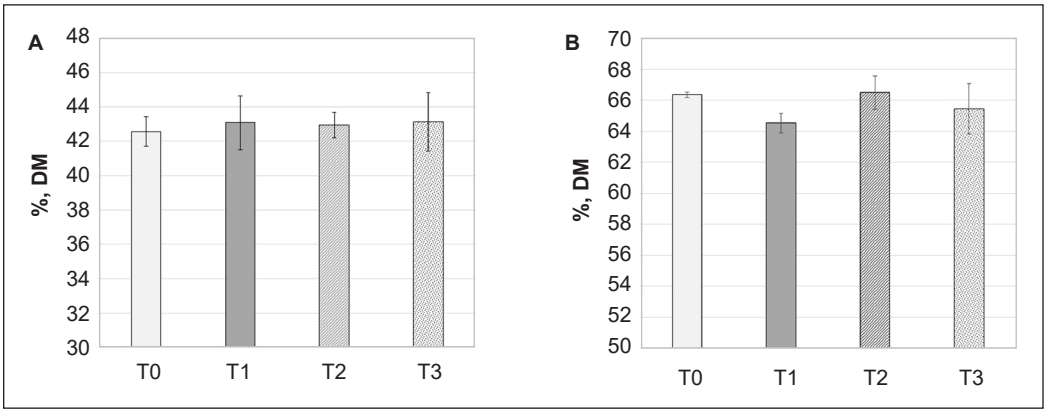

Figure 1. The *in vitro* dry matter digestibility (A) and *in vitro* organic matter digestibility (B) of dietary treatments
Note. T0 = Control diet, T1 = The use of TKC to substitute 50% of palm kernel cake, T2 = The use of TKC to substitute 100% of palm kernel cake, T3 = Dietary T2 with an additional 0.5% of trace mineral

Ruminal Fermentation

The present study reported that the application of TKC in different levels of substitution did not affect rumen pH, ammonia-N, total VFA, VFA profiles, and acetate-to-propionate ratio (Table 6). The T1, T2, and T3 had the same result compared to T0. Generally, the means of pH, ammonia-N, and total VFA from all treatments were 7.03, 7.53 mg/dL, and 1235.6

Table 6
Effects of dietary treatments on ruminal fermentation

Items	Dietary treatment[1]				SEM	*p*-value
	T0	T1	T2	T3		
pH	7.03	7.04	7.04	7.03	0.034	0.985
Ammonia-N, mg/dL	7.60	7.69	7.29	7.53	0.251	0.290
Total VFA, mg/L	1214.5	1260.1	1236.7	1231.3	136.31	0.981
Acetate, % molar	77.7	78.1	74.9	78.0	2.003	0.158
Propionate, % molar	13.5	13.6	14.3	13.0	0.728	0.176
Butyrate, % molar	8.83	8.30	10.76	9.01	1.500	0.205
Acetate: Propionate	5.75	5.79	5.24	6.01	0.441	0.159

Note. [1]T0 = Control diet, T1 = The use of TKC to substitute 50% of palm kernel cake, T2 = The use of TKC to substitute 100% of palm kernel cake, T3 = Dietary T2 with an additional 0.5% of trace mineral

mg/L. In addition, the means of acetate, propionate, and butyrate from all treatments were 77.2%, 13.6%, and 9.23%, while the mean of acetate to propionate ratio was 5.70.

Methane Emission

Dietary treatments affected ruminal methane emissions (Figure 2). The application of T2 and T3 resulted in lower methane emissions than T0 (p=0.002; 49684.6 and 50340.3 ppm vs. 55658.7 ppm). The application of T1 was not different from other dietary treatments.

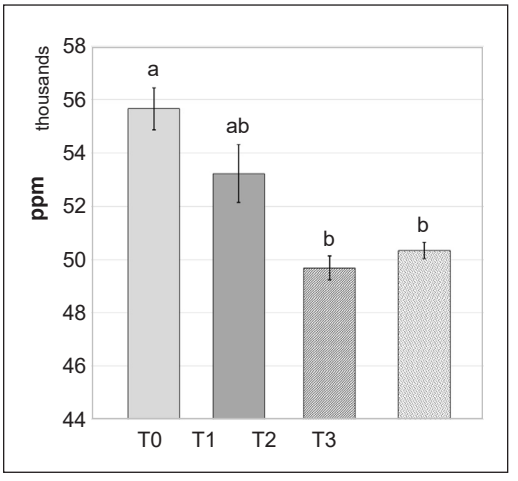

Figure 2. Ruminal methane emission by dietary treatments
Note. T0 = Control diet, T1 = The use of TKC to substitute 50% of palm kernel cake, T2 = The use of TKC to substitute 100% of palm kernel cake, T3 = Dietary T2 with an additional 0.5% of trace mineral; [a,b]Mean with different superscripts differ significantly (p<0.05)

DISCUSSION

All dietary treatments were formulated to have similar chemical compositions, such as OM, CP, EE, NDF, ADF, and TDN. However, the concentration of plant secondary metabolites was different in dietary treatments. According to our previous study, TKC is a source of total phenol and total flavonoid (Paradhipta et al., 2023a; Umroni et al., 2024), and it could be higher than PKC (Tsouko et al., 2019). TCO was produced using the mechanical pressing method (Leksono et al., 2014), where secondary metabolites might still remain in their by-product, such as TKC. Therefore, a higher proportion of TKC in dietary treatment was reported to increase the concentration of plant secondary metabolites (Table 5). Dietary treatments T2

and T3 had a similar concentration of secondary metabolites due to the same proportion of TKC in the diet. In contrast, dietary T1 had a low concentration of secondary metabolites due to a proportion of TKC in the diet.

In general, the different levels of TKC as substitution were unaffected by the rumen's digestibility and fermentation. The combination of TKC and trace minerals did not affect it. It could occur due to similar chemical compositions among dietary treatments (Table 4). However, dietary T2 and T3 could effectively decrease methane emissions. The high concentration of total phenol and total flavonoid could be a reason for the decrease in methane production by these dietaries in the present study. Previous studies reported that the use of phenol and flavonoid in proper concentration could inhibit the growth of methanogens archaea without affecting the activity of fibrinolytic microbes in the rumen (Bodas et al., 2012; Lee et al., 2021; Paverini et al., 2012). According to a previous study, plant secondary metabolites, particularly phenolic compounds, could control archaea methanogens populations. Phenolic compounds, like flavonoids and phenolic acid, interact with bacterial membrane cells due to their lipophilicity. Flavonoids inhibit cytoplasmic membrane function, cell wall synthesis, and cell wall synthesis, while phenolic acid acidifies the cytoplasm, leading to cell death (Nørskov et al., 2023). In a low dose, the effect of plant secondary metabolites has no impact on rumen fermentation, including methane emission. However, in high doses, it could present a toxic response for ruminal microbes and hoses (Bodas et al., 2012; Paverini et al., 2012). In the *in vitro* study, the decrease in digestibility is a sign of toxicity by planting secondary metabolites. This condition could inhibit the activity of fibrinolytic bacteria from degrading fiber in the diet (Bodas et al., 2012; Lee et al., 2021; Vasta et al., 2019). Results of the present study indicated that the use of TKC on dietary T2 and T3 did not affect a toxic condition in the ruminal ecosystem. This confirmed that TKC is a viable protein replacement source, such as PKC while reducing methane emissions without negatively affecting the rumen ecosystem. However, the use of TKC at 50% for substitution is still in low doses of plant secondary metabolites, which could not yet have a big impact on methane emission.

A combination of TKC and mineral salt had no effects on methane emissions. This can be seen from the results of methane production by dietary T2 and T3. Mineral salt was expected to reduce the ratio of acetate to propionate, which could decrease methanogenesis (Li et al., 2017). However, in the present study, dietary treatments did not affect the acetate-to-propionate ratio. The previous study also reported that using mineral salt could help increase feed efficiency and animal performance, but it does not always promise the reduction of methane emissions (Grešáková et al., 2021; Son et al., 2023). The dose of trace minerals in the present study was in range according to many previous studies (Grešáková et al., 2021; Hendawy et al., 2022; Son et al., 2023; Li et al., 2017). The difference in physiological status, animal breed, nutrients in the diet, feeding strategies, and utilization of mineral salt might be the factors that affect the variety of results in methane production

(Grešáková et al., 2021; Li et al., 2017; Son et al., 2023). Especially the ingredient and nutrient content of the diet in the present study were different compared to previous studies (Grešáková et al., 2021; Hendawy et al., 2022; Son et al., 2023; Li et al., 2017), which could be a main reason for none effect of dietary T3 on methane reduction. In general, the reduction of methane emission in the present study was mainly affected by using TKC as a protein substitution in the concentrate diet.

CONCLUSION

The present study concluded that TKC could be used to substitute a protein source in the concentrated diet without presenting any negative effects on ruminal digestibility and fermentation through *an in vitro* technique. Substituting protein sources in the concentrate with TKC at 30% could reduce ruminal methane emission. However, combining TKC and mineral salt did not effectively reduce methane emissions.

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REFERENCES

Appuhamy, J. A. D. R. N., France, J., & Kebreab, E. (2016). Models for predicting enteric methane emissions from dairy cows in North America, Europe, and Australia and New Zealand. *Global Change Biology*, *22*(9), 3039-3056. https://doi.org/10.1111/gcb.13339

Association of Official Analytical Chemists. (2016). *Official methods of analysis* (20th ed.). AOAC.

Bodas, R., Prieto, N., Garcia-Gonźalez, R., Andrés, S., Giráldez, F. J. & López, S. (2012). Manipulation of rumen fermentation and methane production with plant secondary metabolites. *Animal Feed Science and Technology*, *176*, 78-93. https://doi.org/10.1016/j.anifeedsci.2012.07.010

Chaney, A. L., & Marbach, E. P. (1962). Modified reagents for determination of urea and ammonia. *Clinical Chemistry*, *8*(2), 130-132. https://doi.org/10.1093/clinchem/8.2.130

Chaovanalikit, A., & Wrolstad, R. E. (2004). Total anthocyanins and total phenolics of fresh and processed cherries and their antioxidant properties. *Food Chemistry and Toxicology*, *69*(1), 67–72. https://doi.org/10.1111/j.1365-2621.2004.tb17858.x

Cortinhas, C. S., Botaro, B. G., Sucupira, M. C. A., Renno, F. P., & Santos, M. V. (2010). Antioxidant enzymes and somatic cell count in dairy cows fed with organic source of zinc, copper and selenium. *Livestock Science*, *127*(1), 84–87. https://doi.org/10.1016/j.livsci.2009.09.001

Dahlke, G. R., Jakub, D., & Goeser, J. (2020). A comparison of TDN and net energy calculations for estimating empty body weight change for beef cows using ADF, NRC-01 Lignin and TTNDFd Methodology. *Iowa State University Animal Industry Report*, *17*(1), 14445. https://doi.org/10.31274/air.11805

Gonzalez, A. G., & Herrador, M. A. (2007). A practical guide to analytical method validation, including measurement uncertainty and accuracy profiles. *Trends in Analytical Chemistry*, *26*(3), 227-238. https://doi.org/10.1016/j.trac.2007.01.009

Grešáková, Ľ., Holodová, M., Szumacher-Strabel, M., Huang, H., Ślósarz, O., Wojtczak, J., Sowińska, N., & Cieślak, A. (2021). Mineral status and enteric methane production in dairy cows during different stages of lactation. *BMC Veterinary Research*, *17*, 287. https://doi.org/10.1186/s12917-021-02984-w

Hendawy, A. O., Sugimura, S., Sato, K., Mansour, M. M., Abd El-Aziz, A. H., Samir, H., Islam, M. A., Bostami, A. B. M. R., Mandour, A. S., Elfadadny, A., Ragab, R. F., Abdelmageed, H. A., & Ali, A. M. (2022). Effects of selenium supplementation on rumen microbiota, rumen fermentation, and apparent nutrient digestibility of ruminant animals: A review. *Fermentation, 8*(1), 4. https://doi.org/10.3390/fermentation8010004

Hidayah, N., Kustantinah, K., Noviandi, C. T., Astuti, A., Hanim, C. & Suwignyo, B. (2023). Evaluation of rumen *in vitro* gas production and fermentation characteristics of four tropical seaweed species. *Veterinary Integrative Sciences*, *21*(1), 229–238. https://doi.org/10.12982/VIS.2023.018

Johnson, K. A., & Johnson, D. E. (1995). Methane emissions from cattle. *Journal of Animal Science*, *73*(8), 2483-2492. https://doi.org/10.2527/1995.7382483x

Lee, S. J., Kim, H. S., Eom, J. S., Choi, Y. Y., Jo, S. U., Chu, G. M., Lee, Y., Seo, J., Kim, K. H. & Lee, S. S. (2021). Effects of olive (*Olea europaea* L.) leaves with antioxidant and antimicrobial activities on *in vitro* ruminal fermentation and methane emission. *Animals*, *11*(7), 2008. https://doi.org/10.3390/ani11072008

Leksono, B., Windyarini, E., & Hasnah, T. M. (2014). *Budidaya Tanaman Nyamplung (Calophyllum inophyllum* L*.) untuk Bioenergi dan Prospek Pemanfaatan Lainnya* [Cultivation of Nyamplung Plants (*Calophyllum inophyllum* L.) for Bioenergy and Other Utilization Prospects]. IPB press.

Li, X., Liu, C., Chen, Y., Shi, R., Cheng, Z., Dong, H. (2017). Effects of mineral salt supplement on enteric methane emissions, ruminal fermentation, and methanogen community of lactating cows. *Animal Science Journal*, *88*(8), 1049-1057. https://doi.org/10.1111/asj.12738

Makkar, H. P. S., Bluemmel, M., Borowy, N. K. & Becker, K. (1993). Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods. *Journal of the Science of Food and Agriculture*, *61*(2), 161–165. https://doi.org/10.1002/jsfa.2740610205

McDougall, E. I. (1948). Studies on ruminant saliva. I. The composition and output of sheep's saliva. *Biochemical Journal*, *43*(1), 99-109.

Nørskov, N. P., Battelli, M., Curtasu, M. V., Olijhoek, D. W., Chassé, E., & Niselsen M. O. (2023). Methane reduction by quercetin, tannic, salicylic acids: Influence of molecular structures on methane formation and fermentation *in vitro*. *Scientific Reports*, *13*, 16023. https://doi.org.org/10.1038/s41598-023-4341-w

Paradhipta, D. H. V., Hanim, C., Agus, A., Leksono, B., Umroni, A., Maharani, S., Wardani, A.R.D., & Anam, M.S. (2023a). Study of nyamplung (*Calophyllum inophyllum*) kernel cake as an alternative protein source for ruminant feed and its effect on methane emission through *in vitro*. *Livestock Research for Rural Development*, *35*(11), 105.

Paradhipta, D. H. V. P., Seo, M. J., Jeong, S. M., Joo, Y. H., Lee, S. S., Seong, P. N., Lee, H. J., & Kim, S. C. (2023b). Antifungal and carboxylesterase-producing bacteria applied into corn silage still affected the fermented total mixed ration. *Animal Bioscience*, *36*(5), 720-730. https://doi.org/10.5713/ab.22.0232

Parashuramulu, S., Nagalakshmi, D., Rao, D. S., Kumar, M. K., & Swain, P. S. (2015). Effect of zinc supplementation on antioxidant status and immune response in buffalo calves. *Animal Nutrition and Feed Technology*, *15*(2), 179–88. https://doi.org/10.5958/0974-181X.2015.00020.7

Pavarini, D. P., Pavarini, S. P., Niehues, M. & Lopes, N. P. (2012) Exogenous influences on plant secondary metabolites levels. *Animal Feed Science and Technology*, *176*, 5-16. https://doi.org/10.1016/j.anifeedsci.2012.07.002

Pramono, S. (2005). Antiinflammatory effect of several *Umbelliferae* species. *Hayati*, *12*(1), 7-10. https://doi.org/10.1016/S1978-3019(16)30316-3

Shibata, M., & Terada, F. (2010). Factors affecting methane production and mitigation in ruminants. *Animal Science Journal*, *81*(1), 2–10. https://doi.org/10.1111/j.1740-0929.2009.00687.x

Son, A. R., Islam, M., Kim, S. H., Lee, S. S., & Lee, S. S. (2023). Influence of dietary organic trace minerals on enteric methane emissions and rumen microbiota of heat stressed dairy streers. *Journal of Animal Science and Technology*, *65*(1), 132-148. https://doi.org/10.5187/jast.2022.e100

Tilley, J. M. A., & Terry, R. A. (1963). A Two-stage technique for the *in vitro* digestion of forage crops. *Grass and Forage Science*, *18*(2), 104-111. https://doi.org/10.1111/j.1365-2494.1963.tb00335.x

Tsouko, E., Alexandri, M., Fernandes, K. V., Freire, D. M. G., Mallouchos, A. & Koutinas, A. A. (2019). Extraction of phenolic compounds from palm oil processing residues ad their application as antioxidants. *Food Technology and Biotechnology*, *57*(1), 29-38. https://doi.org/10.17113%2Fftb.57.01.19.5784

Umroni, A., Rianawati, H., Rahayu, A. A. D., Krisnawati, Leksono, B., & Paradhipta, D. H. V. (2024). Chemical compositions and plant secondary metabolites of nyamplung (*Calophyllum inophyllum L*) Oilseed press-cake from different locations. In *IOP Conference Series: Earth and Environmental Science* (p. 012001). IOP Publishing. https://doi.org/10.1088/1755-1315/1360/1/012001

Vasta, V., Daghio, M., Cappucci, A., Buccioni, A., Serra, A., Viti, C. & Mele, M. (2019). Invited review: Plant polyphenols and rumen microbiota responsible for fatty acid biohydrogenation, fiber digestion, and methane emission: Experimental evidence and methological approaches. *Journal of Dairy Science*, *102*(5), 3781-3804. https://doi.org/10.3168/jds.2018-14985

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Antidiabetic and Pharmacokinetic Properties of *Shorea macrophylla* Fruits' Extracts in Borneo

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ABSTRACT

The global rise in diabetes prevalence has intensified the search for effective and safer natural antidiabetic agents. *Shorea macrophylla* fruits, known for their lipogenesis effects, present a promising avenue. This study explores the antidiabetic properties of *S. macrophylla* fruits' crude extracts through *in vitro* assays for α-amylase and advanced glycation end-products (AGEs) inhibition, alongside molecular docking for inhibitor prediction and *in silico* pharmacokinetic evaluation. While all extracts exhibited mild inhibitory effects on α-amylase, they are significantly less effective than acarbose. Methanolic (MeOH) extract demonstrated the strongest inhibitory effects on AGEs, surpassing other extracts at 100 µg/ml. However, it exhibits no significant differences compared to Aminoguanidine (AG), suggesting its potential to become an alternative antiglycation source. Molecular docking revealed that five compounds, methyl stearate, methyl palmitate, methyl arachidate, methyl oleate, and methyl linoleate, had higher binding energies than acarbose for Human pancreatic alpha-amylase (HPA) (PDB ID: 5E0F). However, their binding energies with the receptor for advanced glycation end-products (RAGE) (PDB ID: 3O3U) were lower than AG (-3.515 kcal/mol), ranging from -5.760 to -6.510 kcal/mol with amino acid residue ARG-66 consistently involved in hydrogen bonding interactions. Analysis of pharmacokinetic properties confirmed that these compounds adhere to Lipinski's Rule of Five, indicating their drug-like properties despite generally poor solubility and potential skin irritation. In summary, *S. macrophylla* fruits' crude extracts, particularly the MeOH extract, show promise as antiglycation agents, necessitating further *in vivo* studies to validate these findings for drug development.

Keywords: α-amylase, antidiabetic, molecular docking, pharmacokinetic, *Shorea macrophylla*

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INTRODUCTION

Diabetes has a substantial impact on socioeconomic development and community health globally. Based on the International Diabetes Federation (IDF), the number of individuals (aged 20–79) worldwide with diabetes was 536.6 million in 2021. Additionally, it is projected that by 2030 and 2045, the number will rise to 643 million and 783 million, respectively (Magliano & Boyko, 2021). Malaysia has the greatest prevalence of diabetes in the Western Pacific, costing the country about 600 million USD annually (Ganasegeran, 2021). For instance, there was a total of 4.5 million adults in Malaysia who had diabetes in 2021, with a prevalence of 20%, posing a serious risk to public health.

Though synthetic drugs like insulin, insulin sensitisers, insulin secretagogues, and reactive oxygen species (ROS) inhibitors have been used to treat diabetes, concerns have been raised about their adverse effects (Alam et al., 2022). These include hallucinations, memory loss, drowsiness (Arora et al., 2021), renal failure, and weight gain (Blahova et al., 2021). Despite the potential of traditional medicinal plants, the antidiabetic and pharmacokinetic properties of *Shorea macrophylla* remain largely unexplored. Thus, exploring *S. macrophylla* fruits' crude extracts could offer the possibility of discovering a safer and more effective antidiabetic agent.

Addressing postprandial hyperglycemia (PPHG) is crucial for diabetes treatment. By lowering PPHG, compounds that hinder the natural carbohydrases α-glucosidase and α-amylase can be effective treatments for diabetes (Lankatillake et al., 2021). According to Luo et al. (2019), enzyme inhibitors are common targets in drug discovery for metabolic diseases, including diabetes. Similar research on medicinal plants for diabetes treatment had been performed in a variety of *in vitro* approaches, such as α-amylase, α-glucosidase, β-glucosidase (Bouyahya et al., 2021), and advanced glycation end products (AGEs) (Nur Akmal et al., 2021). Consequently, screening plant extracts for enzyme inhibition is a typical method for finding antidiabetic agents (Rajan et al., 2020). *Shorea macrophylla*, a tropical plant from Kalimantan and Borneo Malaysia, is known for its high fat content. Chew (2023) noted that methanolic and diethyl ether extracts of *S. macrophylla* fruits promote lipogenesis, that linked to obesity and Type 2 diabetes (T2DM). Thus, it is hypothesised that *S. macrophylla* fruit extracts could assist in T2DM management. This study aims to examine the antidiabetic properties of *S. macrophylla* fruits' crude extracts and evaluate the pharmacokinetic properties through *in vitro* and *in silico* approaches respectively.

MATERIALS AND METHODS

In Vitro Inhibition of α-amylase

A total of four different *S. macrophylla* fruit crude extracts (hexane, dichloromethane, ethyl acetate, and methanol) were obtained from the Animal Biotechnology Lab at Universiti Malaysia Sarawak. This assay was carried out according to Wickrmaratne et al. (2016)

with slight modifications. Different concentrations of test extracts ranging from 62.5 to 1000 µg/ml, α-amylase enzyme (2 U /mL) (Sigma-Aldrich, US), and 1 % soluble starch (Sigma-Aldrich, US) were dissolved in 0.02 M sodium phosphate buffer containing 6 mM NaCl (pH 6.9). Exactly 200 µL of α-amylase was added to each test tube containing 200 µL of sample or acarbose at various concentrations. The mixture was incubated at 37°C for 15 minutes before adding 200 µL of starch. After vortexing and further incubation at 37°C for another 15 minutes, 200 µL of 3,5-Dinitrosalicylic acid (DNSA) colour reagent was added. The reaction mixture was boiled (85-90°C) for 5 minutes, cooled to room temperature, and diluted. Acarbose served as a positive control, and a negative control with 100% enzyme activity was prepared without the sample. A blank using only DNSA was also prepared. Absorbance readings were read at 540 nm using a microplate reader (Infinite M200 PRO / TECAN, Switzerland). Triplicates were performed. Raw data were adjusted by subtracting the absorbance of the blank. Results were expressed as the percentage inhibition of α-amylase, calculated by: ([Absorbance reading of negative control-Absorbance reading of sample]/[Absorbance reading of negative control]) ×100.

In Vitro Inhibition of AGEs

This assay was carried out by referring to the experiment by Sekhon-Loodu and Rupasinghe (2019) with minor modifications. The incubation mixtures were dissolved in 0.2 M sodium phosphate buffer (pH 7.4) containing sodium azide (0.02% w/v) to make up a final volume of 600 µL, consisting of 200 µL of each BSA (5 mg/ml), D-glucose (36 mg/ml), and sample extracts (ranging from 5–100 µg/ml) or aminoguanidine (AG), a known inhibitor for AGEs. Negative control was performed when the samples were omitted. The mixtures were then subjected to incubation at 37◦C for a week. After a week, the fluorescence readings were obtained utilising a microplate reader (Infinite M200 PRO/, TECAN, Switzerland) at 360 nm and 420 nm of the excitation and emission wavelengths (Starowicz & Zielinski, 2019). Triplicates were done. The final result was expressed in terms of the percentage of inhibition of AGEs (%), calculated by using the equation: Percentage Inhibition of AGEs (%) = 1- ([Fluorescence of the test sample]/ [Fluorescence of control]) ×100%.

Molecular Docking Analysis

The natural compounds shared across all four extracts, previously identified by Chew (2023) via Gas chromatography-mass spectrometry (GC-MS) analysis, were selected as ligands to be docked with diabetes-related proteins. The chosen proteins were Human pancreatic alpha-amylase in complex with mini-montbretin A (HPA) (PDB ID: 5E0F) and the Crystal Structure of Human Receptor for Advanced Glycation End-products (RAGE) (PDB ID: 3O3U). Molecular docking was performed using UCSF Chimera v1.17.3 and AutoDock Vina. Protein structures were obtained from the Protein Data Bank (PDB), and the canonical

SMILES of the selected ligands were retrieved from the PubChem database before being checked for their most stable molecular geometry by performing energy minimisation using Gaussian 16. For HPA (PDB ID: 5E0F), the grid box was centred at coordinates -7.20, 5.69, -23.42 (Ahmed et al., 2023) with dimensions of 30, 27, 24.75 (Belaiba et al., 2020). For RAGE (PDB ID: 3O3U), the grid box was centred at coordinates 19.898, 17.096, and 69.161 (Tambe et al., 2022) with dimensions of 42, 42, and 42.

In Silico Pharmacokinetics Analysis

The pharmacokinetic properties of the compounds consistently found in various crude extracts of *S. macrophylla* fruit were analysed using two freely accessible web-based ADMET predictor platforms: SwissADME and ADMETlab2.0. The results were summarised and interpreted based on their physicochemical features, pharmacokinetic properties, toxicity, and drug-likeness.

Statistical Analysis

All the *in vitro* antidiabetic assays were conducted in triplicates, and the resulting data were presented as mean ± standard deviation (n=3). Statistical analysis was performed using Microsoft Excel for Microsoft 365 MSO (Version 2403 Build 16.0.17425.20176) 64-bit. One-way analysis of variance (ANOVA) and Tukey tests were used to evaluate significant differences (p<0.05) between the extracts and their respective control.

RESULTS AND DISCUSSION

In Vitro Inhibition of α-amylase Assay

Shorea macrophylla, locally known as engkabang, is well known for its high-fat content. A study by Chew (2023) revealed that the methanolic and diethyl ether crude extracts of *S. macrophylla* fruits stimulate lipogenesis, a process closely linked to Type 2 Diabetes Mellitus (T2DM) (Imamura et al., 2020). Excess lipogenesis leads to increased adiposity and ectopic fat deposition, further contributing to insulin resistance and dysregulated glucose homeostasis, the hallmarks of T2DM (Ahmed et al., 2021). Despite this, specific compounds within these extracts may offer therapeutic potential for managing T2DM by regulating glucose uptake. Therefore, *in vitro* antidiabetic assays targeting the enzyme α-amylase and advanced glycation end-products (AGEs) were conducted to elucidate their potential underlying antidiabetic mechanisms. In this study, the inhibitory effects of four distinct crude extracts of *S. macrophylla* fruit, namely hexane (Hex), ethyl acetate (EA), dichloromethane (DCM), and methanolic (MeOH), were examined on α-amylase activity. A range of concentrations from 62.5 µg/ml to 1000 µg/ml was applied to each extract. Acarbose, a known inhibitor, was used for comparison at the same concentrations.

Figure 1 demonstrates that all samples (Hex, EA, DCM, MeOH) exhibited mild inhibition of α-amylase compared to acarbose. The color of the 3,5-dinitrosalicylic acid (DNSA) shift from yellow to orange indicated the presence of reducing sugars, with intensity corresponding to their concentration (Jain et al., 2020). The orange colouration in *S. macrophylla* extracts suggests the partial formation of reducing sugars, indicating intermediate α-amylase inhibition. Figure 2 shows that the inhibitory effects of both acarbose and *S. macrophylla* fruit's crude extracts increase with rising concentrations, indicating a dose-dependent response. Acarbose achieved 60.86% inhibition at 62.5 µg/ml, 77.45% at 125 µg/ml, and plateaued at 83.9% at 250 µg/ml, double the inhibition of the other extracts at the same concentration. At the highest tested concentration (1000 µg/ml), the inhibition effects were 42.40% (EA), 43.68% (DCM), 48.24% (Hex), and 48.42% (MeOH). Among the extracts, MeOH exhibited the strongest inhibitory effects on α-amylase. However, statistical analysis revealed that their efficacy is significantly lower (p<0.01) than that of acarbose (refer to Table 1).

Though the inhibitory effects of *S. macrophylla* fruits' crude extracts were significantly less effective (p<0.01) than acarbose (as shown in Table 1), the similarly mild inhibition effects across all the extracts can be discussed based on their compounds analysed through Gas chromatography-mass spectrometry (GC-MS). According to the GC-MS list revealed by Chew (2023), five main compounds appeared consistently across the four extracts. The compounds were hexadecanoate <methyl-> (methyl palmitate), 9,12-octadecadienoic acid (Z,Z)-, methyl ester (methyl linoleate) 9-octadecenoic acid, methyl ester, (E) (methyl oleate), methyl stearate, and eicosanoate <methyl-> (methyl arachidate).

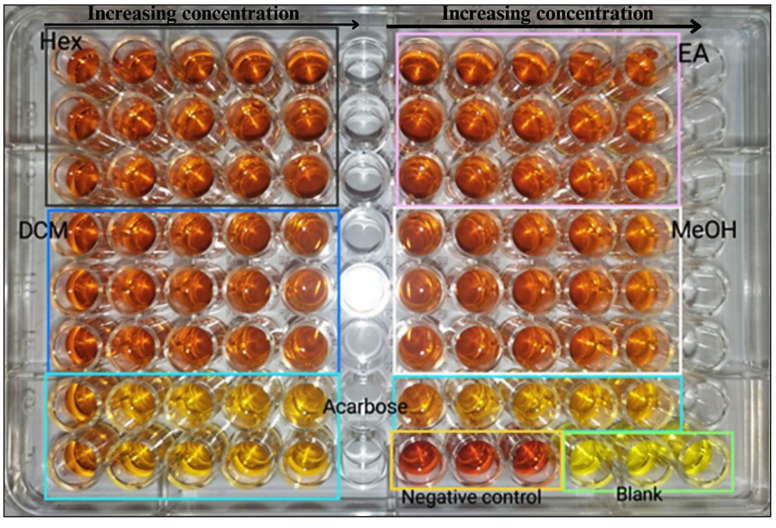

Figure 1. Result of the *in vitro* inhibition of α-amylase assay. The microplate is filled with samples and acarbose in increasing concentration (62.5-1000 µg/ml) from left to right except for the negative control and blank

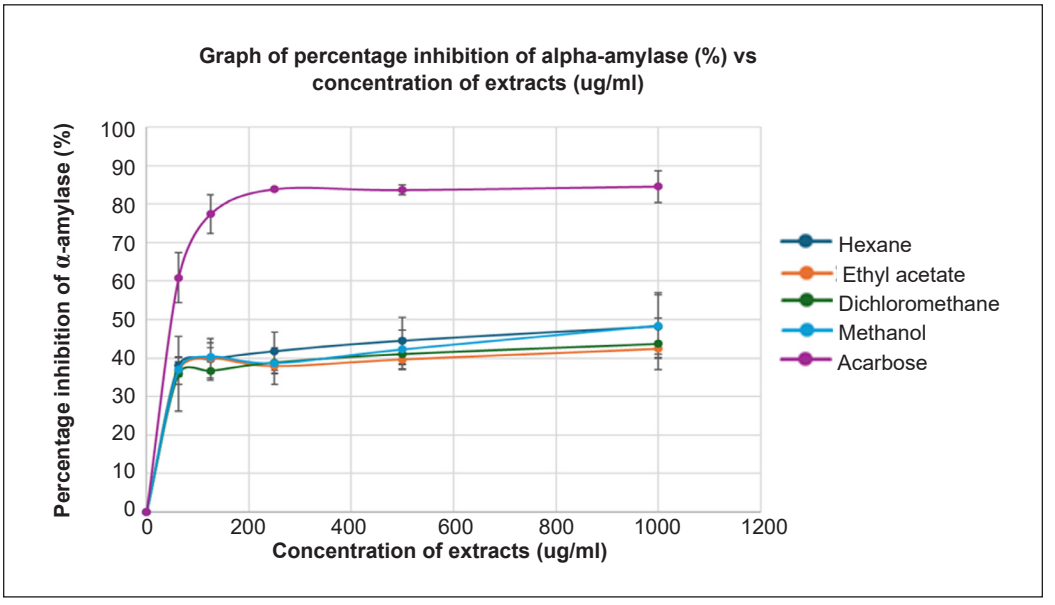

Figure 2. The inhibitory effects of α-amylase by different extracts at various concentrations. All the findings were displayed as mean ± SD (n = 3)

Table 1
Percentage of inhibition of alpha-amylase (%) by different Shorea macrophylla *extracts*

Extracts	Percentage of inhibition of alpha-amylase (%)				
	62.5 (ug/ml)	**125 (ug/ml)**	**250 (ug/ml)**	**500 (ug/ml)**	**1000 (ug/ml)**
Hex	38.12±2.02^{b}	39.68±2.93^{b}	41.76±4.90^{b}	44.49±6.11^{b}	48.24±8.15^{b}
EA	36.70±3.62^{b}	39.92±5.19^{b}	37.90±4.76^{b}	39.64±1.35^{b}	42.40±1.44^{b}
DCM	35.90±9.65^{b}	36.66±2.40^{b}	38.83±2.85^{b}	41.03±4.00^{b}	43.68±6.74^{b}
MeOH	37.07±1.88^{b}	40.34±3.70^{b}	38.65±2.82^{b}	42.21±4.96^{b}	48.42±8.58^{b}
Acarbose	60.86±6.52^{a}	77.45±5.05^{a}	83.9±0.53^{a}	83.69±1.35^{a}	84.59±4.13^{a}

Note. Statistical analysis of differences between each type of extract and acarbose (control). Data with different letters indicates the pair has a significant difference (p<0.01)

Hexadecanoic acid, or palmitic acid, along with its esters, has been recognised by Sivagurunathan and Xavier (2014) for their anti-androgenic properties. Moreover, palmitic acid and 9,12-Octadecadienoic acid (Z, Z) have demonstrated hypocholesterolemic effects, thereby reducing HDL cholesterol levels. Given the paramount importance of maintaining optimal cholesterol levels to mitigate cardiovascular risks (Kim et al., 2022), particularly in T2DM patients prone to dyslipidemia (Ahmmed et al., 2021), the regulation of cholesterol levels is important in the management of T2DM and associated cardiovascular complications. However, no study directly reveals that methyl palmitate and methyl linoleate show inhibition effects on α-amylase.

On the other side, research conducted by Ryan et al. (2000) suggests that consumption of oleic acid and stearic acid can reduce blood glucose levels. Findings by Paulraj et al. (2014) also concluded that the ethanolic extract of *Passiflora foetida* L. (bitter gourd) exhibited inhibitory effects on both α-amylase and α-glucosidase activity. Their GC-MS analysis reveals the presence of palmitic acid, methyl linoleate, oleic acid, stearic acid, and linolenic acid. Given this evidence, four of the five main compounds in *S. macrophylla* fruit, that are methyl palmitate, methyl linoleate, methyl oleate, and methyl stearate may contribute to the antidiabetic properties observed. Those compounds share the chemical structures and functional groups with their parent acids, which have been proven to have antidiabetic properties.

However, adding a methyl group (-CH3), which distinguishes them from their parent acid, may alter some chemical properties. Nevertheless, it is noted that before GC-MS analysis, Chew (2023) conducted derivatisation steps for their samples rich in fatty acids. It is to increase the volatility of the fatty acids for Gas Chromatography- mass spectrometry (GCMS) data collection. Consequently, the fatty acid methyl esters (FAMEs) detected (methyl stearate, methyl palmitate, methyl arachidate, methyl oleate, and methyl linoleate) could predominantly represent their respective parent acids, such as stearic acid, palmitic acid, arachidic acid, oleic acid, and linoleic acid. Therefore, further research is required to validate the antidiabetic potential of these fatty acids, particularly their impact on α-amylase.

In Vitro Inhibition of AGEs

Advanced glycation end-products (AGEs) constitute a diverse group of compounds formed from various mechanisms and precursors, both endogenously and exogenously. Typically, they are formed through non-enzymatic condensation (Twarda-Clapa et al., 2022). Accumulating in tissues and organs, AGEs disrupt normal cellular processes and trigger related inflammatory pathways, thereby promoting ageing-related conditions and chronic diseases. These include diabetes complications and cardiovascular diseases (Khan et al., 2020). Thus, inhibitors of AGEs can help manage diabetes complications.

Based on the results in Figure 3, the Hexane (Hex) extract showed the least inhibitory effect, with a slight increase in inhibition from 15.84% at 5 µg/ml to 18.49% at 100 µg/ml. Conversely, methanolic (MeOH) extract demonstrated the highest efficacy in inhibiting AGEs, steadily increasing from 5 µg/ml to 100 µg/ml, reaching 41.11% inhibition at the highest concentration, over twice that of other extracts. Notably, MeOH's 41.11% inhibition at 100 µg/ml surpassed the reference drug's 34.53% at the same concentration. Statistical analysis (Table 2) revealed that Hex, Ethyl acetate (EA), and Dichloromethane (DCM) were significantly less effective ($p<0.05$) compared to Aminoguanidine (AG), making them less desirable. In contrast, MeOH's inhibition effects on AGEs were not significantly different from AG, indicating its potency *in vitro*.

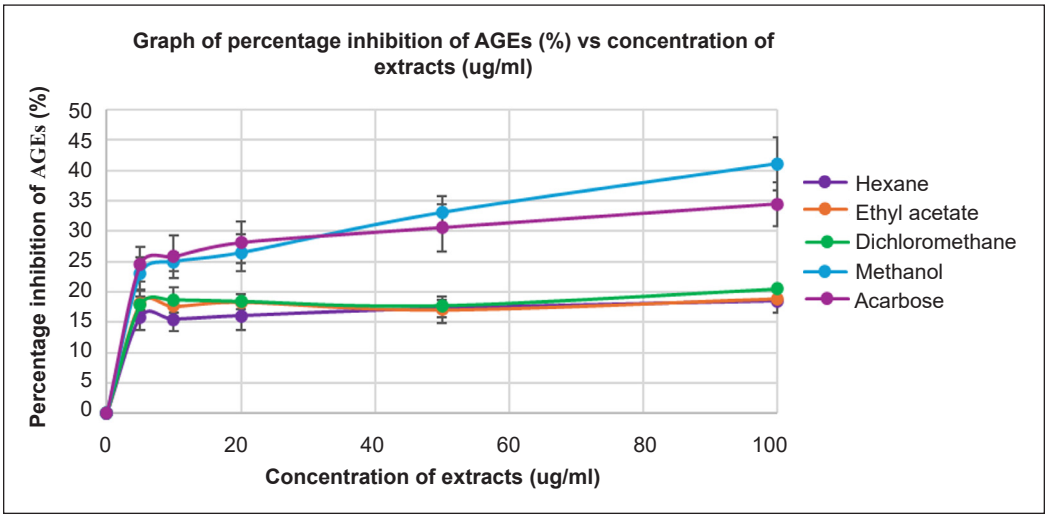

Figure 3. Comparison of AGEs inhibition by different extracts. The data presented are in the form of mean ± SD (n = 3)

Table 2
Percentage inhibition of AGEs (%) by different Shorea macrophylla *extracts*

Extracts	Percentage Inhibition of AGEs (%)				
	5 (ug/ml)	10 (ug/ml)	20 (ug/ml)	50 (ug/ml)	100 (ug/ml)
Hex	15.84±2.04^{b**}	15.49±1.90^{b**}	16.05±2.35^{b**}	17.29±1.45^{b**}	18.49±1.97^{b**}
EA	18.08±1.22^{b*}	17.62±1.11^{b**}	18.32±1.22^{b**}	17.05±2.13^{b**}	18.89±0.34^{b**}
DCM	17.88±2.40^{b*}	18.68±2.15^{b*}	18.47±0.25^{b**}	17.77±0.27^{b**}	20.49±0.47^{b**}
MeOH	23.01±2.77^{a}	25.00±1.54^{a}	26.47±3.07^{a}	33.07±2.65^{a}	41.11±4.38^{a}
AG	24.58±2.85^{a}	25.83±3.57^{a}	28.14±3.44^{a}	30.62±3.91^{a}	34.53±3.61^{a}

Note. Statistical analysis of differences between the extracts and AG (control). Different letters denote the presence of significant differences with *= p<0.05, **= p<0.01

The comparable inhibitory effects of MeOH extract with AG suggest that MeOH can be an alternative to AGE inhibitors. Furthermore, the inhibitory effects imply that the effectiveness of the MeOH extract may be attributed to the compounds with an antiglycation effect. Thus, MeOH extract's primary components (more than 1% GC-MS abundance) were discussed. It included the five compounds mentioned previously (methyl stearate, methyl palmitate, methyl arachidate, methyl oleate, and methyl linoleate) (Chew, 2023).

A study by Sowmiya et al. (2021) identified the potential of methyl linoleate in anti-cancer and anti-inflammatory responses, positing it as a probable contributor to AGEs inhibition in the current investigation. Furthermore, several investigations have underscored these compounds' anti-cancer, antioxidant, and anti-inflammatory attributes. For example, both antioxidant and anti-inflammatory properties have been ascribed to

palmitic acid (Odu et al., 2023; Paulraj et al., 2014; Sivagurunathan & Xavier, 2014). Similarly, methyl oleate has exhibited antioxidant and anti-cancer activities (Yu et al., 2005). However, no study has been exploring the antiglycation properties of methyl stearate and methyl arachidate.

There is a correlation between these properties (anti-cancer, antioxidant, and anti-inflammatory) and the inhibition of AGEs. Certain anti-cancer agents may possess anti-glycation properties due to the shared signalling pathways, such as the nuclear factor (NF)-κB pathway, implicated in both cancer and diabetes (Dariya & Nagaraju, 2020). Additionally, chronic inflammation has been closely associated with AGE formation (Salazar et al., 2021), underscoring the potential of compounds endowed with anti-inflammatory properties to attenuate inflammatory responses, thus potentially reducing AGE formation. Moreover, compounds exhibiting antioxidants help neutralise reactive oxygen species (ROS) (Parcheta et al., 2021), reducing oxidative stress and inhibiting AGEs formation. Given that the previously mentioned compounds either exhibit indirect antiglycation properties or are the parent acid with those identified in this study (such as methyl palmitate), their potential contribution to AGE inhibition is suggested. It is also noteworthy that other compounds in relatively low abundances within the undiscovered crude extracts might also contribute to AGE inhibition. Hence, further investigation is required to examine the antiglycation properties of these compounds.

Molecular Docking with Human Pancreatic Alpha-amylase (PDB ID: 5E0F)

Enzyme α-amylase plays a key role in breaking down starch into sugars. Inhibiting its activity can help control blood sugar levels in diabetic patients (Chigurupati et al., 2022). Generally, more negative binding energies indicate stronger binding affinities between the ligands and the protein. The docking results, summarised in Table 3, indicate that methyl oleate exhibited the lowest binding energy (-5.431 kcal/mol) and highest affinity to Human Pancreatic alpha-amylase (HPA), followed by methyl linoleate (-5.126 kcal/mol), methyl arachidate (-5.103 kcal/mol), methyl stearate (-5.099 kcal/mol), and methyl palmitate (-4.821 kcal/mol). Figure 4 presents the optimal positions for molecular interactions between these ligands and HPA. Notably, acarbose showed significantly lower binding energy (-8.116 kcal/mol) and stronger affinity compared to all the ligands.

Studies on HPA's X-ray crystal structure and enzyme kinetics have revealed three critical amino acid residues in its active site: ASP-197, GLU-233, and ASP-300. These residues are essential for starch hydrolysis, with ASP-197 serving as a catalytic nucleophile, GLU-233 as an acid-base catalyst, and ASP-300 optimising substrate orientation (Kikiowo et al., 2020). In the current docking study with HPA (PDB id: 5E0F), methyl palmitate, methyl arachidate, and methyl oleate formed H-bonds with THR-163 at bond distances of 2.632 Å, 2.463 Å, and 2.399 Å, respectively (refer to Figure 5).

Table 3
Molecular docking result of the FAMEs with HPA (PDB ID: 5E0F)

Compounds/Proteins	Pubchem CID	Binding energy (kcal mol^{-1})	Number of hydrogen bonds	Best hydrogen bonding position
Methyl Stearate Canonical SMILES CCCCCCCCCCCCCC CCCC(=O)OC	8201	-5.099	2	HIS 201 (2.240) TYR 151 (2.387)
Methyl Palmitate Canonical SMILES: CCCCCCCCCCCCC CCC(=O)OC	8181	-4.821	1	THR 163 (2.632)
Methyl Arachidate Canonical SMILES: CCCCCCCCCCCCC CCCCCCC(=O)OC	14259	-5.103	1	THR 163 (2.463)
Methyl Oleate Canonical SMILES: CCCCCCCCC=CCC CCCCCC(=O)OC	5364509	-5.431	1	THR 163 (2.399)
Methyl Linoleate Canonical SMILES: CCCCCC=CCC=CC CCCCCCC(=O)OC	5284421	-5.126	1	HIS 299 (2.168)
Acarbose Canonical SMILES: CC1C(C(C(C(O1)O C2C(OC(C(C2O)O) OC3C(OC(C(C3O)O) O)CO)CO)O)O)NC4 C=C(C(C(C4O)O)O)CO	41774	-8.116	4	ALA 106 (2.412) HIS 305 (2.380) GLU 233 (2.238) LYS 200 (2.711)

Methyl linoleate, on the other hand, interacted with HIS-299 at a bond distance of 2.168 Å. The same amino acid residues THR-163 and HIS-299 at the active site of 5E0F were also identified in the study by Ghannay et al. (2020). Only methyl stearate formed two H-bonds in this study, interacting with HIS-201 (2.240 Å) and TYR-151 (2.387 Å). These amino acid residues (THR-163, HIS-201, TYR-151) were the same as those revealed in Mohamed and Ibrahim's (2022) study. However, all FAMEs had higher predicted binding energies than acarbose, indicating their relatively weaker binding affinities towards HPA, according to Table 3. The docking scores of acarbose in this study closely resembled those reported by Chigurupati et al. (2021) (-8.10 kcal/mol), with four same interacting residues (LYS-200, GLU-233, ASP-300, and HIS-305) observed, except for ASP-300.

In short, the docking scores for the compounds with HPA in this study ranged from -4.821 kcal/mol to -5.431 kcal/mol. A comparison with a similar investigation conducted by Ahmed et al. (2023) reveals that these scores are relatively lower. It indicated that

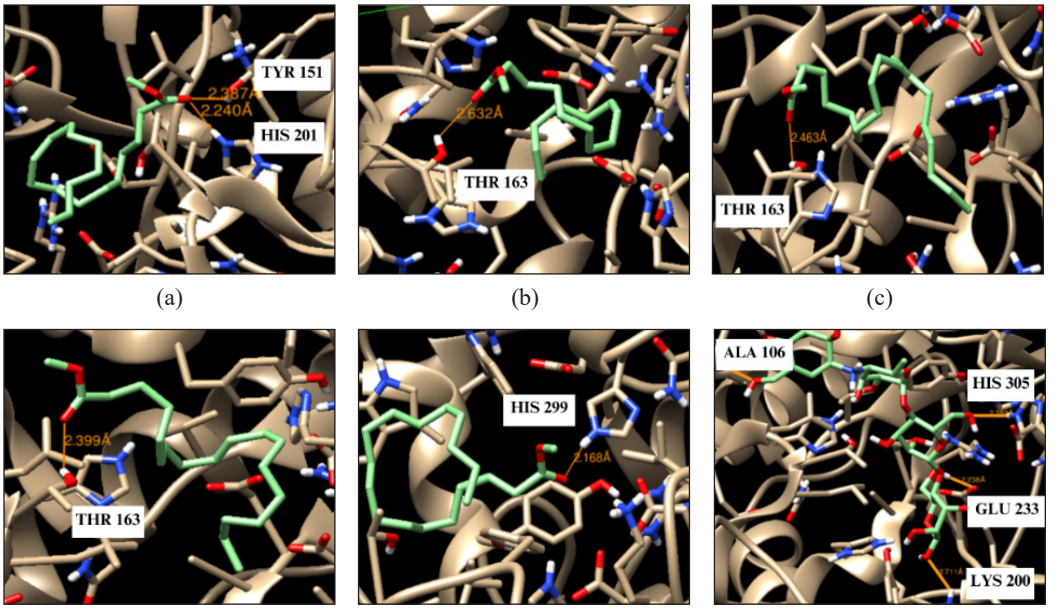

Figure 4. Compilation of top binding poses for the FAMEs and acarbose docked to HPA (PDB ID: 5E0F): (a) Methyl stearate; (b) Methyl palmitate; (c) Methyl arachidate; (d) Methyl oleate; (e) Methyl linoleate; and (f) Acarbose

the five selected compounds are predicted to have a higher binding affinity, potentially offering greater efficacy in managing blood glucose levels. However, in contrast to studies conducted by Belaiba et al. (2023) and Aroua et al. (2023), the binding scores obtained herein are higher, implying weaker interactions with HPA compared to those compounds. Hence, additional investigation and analysis are needed to enhance the potential of these compounds for improved efficacy.

Molecular Docking with RAGE (PDB ID: 3O3U)

The receptor for advanced glycation end-products (RAGE) is a transmembrane receptor resembling immunoglobulins and has multiple isoforms. It binds various endogenous extracellular ligands and intracellular effectors, initiating signalling cascades that produce reactive oxygen species (ROS), inflammatory responses, cell proliferation, or apoptosis and upregulates RAGE (Bongarzone et al., 2017). Extensive research has established correlations between RAGE activity and various pathological conditions, including diabetes (Ramasamy et al., 2011). Given its involvement in numerous pathological states, RAGE has become an appealing target for therapeutic strategy.

The docking scores for the ligands with RAGE were summarised in Table 4. Methyl linoleate among the ligands posed the lowest binding energy (-6.510 kcal/mol) and highest

Table 4
Molecular docking result of the FAMEs with RAGE (PDB ID: 3O3U)

Compounds/Proteins	Puchem CID	Binding Energy (kcal mol−1)	Number of Hydrogen Bonds	Best Hydrogen Bonding position
Methyl Stearate Canonical SMILES CCCCCCCCCCCCCCCCC CC(=O)OC	8201	-5.760	1	ARG 66 (2.264)
Methyl Palmitate Canonical SMILES: CCCCCCCCCCCCCCCC (=O)OC	8181	-6.210	3	ARG 66 (2.351) ARG 66 (2.209) ARG 66 (2.547)
Methyl Arachidate Canonical SMILES: CCCCCCCCCCCCCCCCC CCC(=O)OC	14259	-6.194	1	ARG 66 (1.989)
Methyl Oleate Canonical SMILES: CCCCCCCCC=CCCCCC CCC(=O)OC	5364509	-6.191	2	ARG 66 (2.176) ARG 66 (2.241)
Methyl Linoleate Canonical SMILES: CCCCCC=CCC=CCCCCC CCC(=O)OC	5284421	-6.510	2	ARG 66 (2.348) ARG 66 (2.295)
Aminoguanidine Canonical SMILES: C(=NN)(N)N	2146	-3.515	3	LEU 151 (2.151) ALA 206 (2.195) ILE 348 (2.134)

affinity to RAGE, followed by methyl palmitate (-6.210 kcal/mol), methyl arachidate (-6.194 kcal/mol), methyl oleate (-6.191 kcal/mol) and methyl stearate (-5.760 kcal/mol). The best position for molecular interactions between these ligands with RAGE is presented in Figure 5. The binding energy of Aminoguanidine (AG) for RAGE was higher (-3.638 kcal/mol) compared to all the compounds. It indicates that the tested ligands have a stronger affinity with RAGE than AG, emphasising their possibility and efficacy as a therapeutic agent in managing diabetes complications.

In docking simulations with RAGE, all five compounds engaged in hydrogen bonding (H-bond) interactions with ARG-66 of the 3O3U protein, displaying varying numbers and bond distances (Table 4). Notably, these interactions consistently involved the nitrogen atom of arginine and the oxygen atom of the ligand. Concordant with the findings of Abbas et al. (2014), ARG-66 emerged as a pivotal amino acid residue in the binding interactions of 3O3U, alongside LYS-15, ARG-66, MET-330, GLU-44, GLU-45, GLU-54, GLU-111, GLU-153, ASP-14, and ASP-65. All the FAMEs tested formed H-bonds with ARG-66, indicating that this residue is a crucial interaction site on the 3O3U protein.

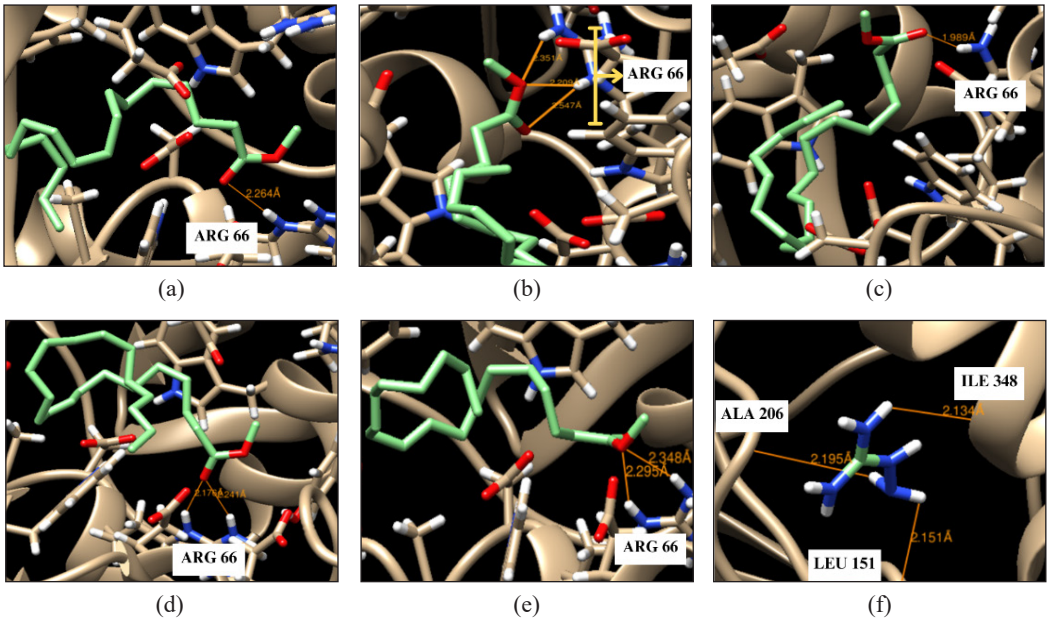

Figure 5. Best binding poses of selected FAMEs when interacting with RAGE (PDB ID: 3O3U): (a) Methyl stearate; (b) Methyl palmitate; (c) Methyl arachidate; (d) Methyl oleate; (e) Methyl linoleate; and (f) Aminoguanidine

Besides, the involvement of ARG-66 across different ligands suggests a conserved binding mechanism. Due to specific location and properties, this residue might be part of a highly conserved binding pocket or active site that interacts favourably with these ligands. According to Sokalingam et al. (2012), arginine is known for forming strong H-bonds and electrostatic interactions due to its positively charged guanidinium group. This could explain why it frequently forms H-bonds with various ligands. In short, the binding energies of the compounds in this study, ranging from -5.760 kcal/mol to -6.510 kcal/mol, show notable affinity towards RAGE. However, the binding energy is generally higher compared to those reported in the Tambe et al. (2022) study.

Overall, the docking results with 5E0F and 3O3U demonstrated that the five FAMEs exhibited considerable affinity towards the active sites of HPA and RAGE. Both methyl oleate (-5.431 kcal/mol) and methyl linoleate (-6.510 kcal/mol) showed the lowest binding affinities for HPA and RAGE respectively. This suggests their strongest inhibitory potential among other compounds, making them a promising candidate for further study.

Evaluation of the *In Silico* Pharmacokinetic Properties

In extending to the observed biological activities demonstrated by *S. macrophylla* fruit's crude extracts in previous *in vitro* and molecular docking studies, the five FAMEs were subjected to comprehensive *in silico* analysis. Utilising SwissADME and ADMETlab2.0,

this evaluation encompassed medicinal chemistry, physicochemical properties, and pharmacokinetic (ADME) profiles. According to Sadeghi et al. (2021), an imperative criterion in selecting a chemical for therapeutic candidacy is its absence of toxicity. Thus, the toxicity of these FAMEs was also predicted using ADMETlab2.0. The aim was to determine their suitability as potential antidiabetic drug candidates.

The analysis of physicochemical properties showed in Table 5 predicts that all tested FAMEs exhibited consistent molecular structures, characterised by two H-bond acceptors and no H-bond donors. Their shared topological polar surface area (TPSA) value of 26.30 Å indicates uniform polarity, influencing interactions with biological targets. However, solubility varied among the FAMEs. Good water solubility is crucial for optimal absorption and oral bioavailability (Agrawal et al., 2024). Using the estimated solubility (ESOL) model, all compounds were classified as moderately soluble, except methyl arachidate, which was poorly soluble. Further refinement with Log S (SILICOS-IT) confirmed methyl linoleate as moderately soluble, while others were downgraded to poor solubility. This highlights the importance of multiple predictive models. Besides, lipophilicity, which is crucial for drug absorption across cell membranes, was calculated using five models (XLOGP3, WLOGP, MLOGP, SILICOS-IT, and iLOGP), and summarised into consensus prediction of the logarithm of the partition coefficient (P) of a compound between n-octanol and water (consensus Log Po/w) (Daina et al., 2017). Methyl arachidate showed the highest lipophilicity (6.96), indicating a strong affinity for

Table 5
The physicochemical properties of the FAMEs

Compounds	Methyl Stearate	Methyl Palmitate	Methyl Arachidate	Methyl Oleate	Methyl Linoleate
Formula	$C_{19}H_{38}O_2$	$C_{17}H_{34}O_2$	$C_{21}H_{42}O_2$	$C_{19}H_{36}O_2$	$C_{19}H_{34}O_2$
MW	298.5	270.45	326.56	296.49	294.47
nHA	2	2	2	2	2
nHD	0	0	0	0	0
TPSA	26.30	26.30	26.30	26.30	26.30
Log S (ESOL)	-5.83	-5.18	-6.47	-5.32	-4.97
ESOL class	Moderately soluble	Moderately soluble	Poorly soluble	Moderately soluble	Moderately soluble
Log S (SILICOS-IT)	-6.81	-6.01	-7.61	-6.09	-5.37
SILICOS-IT class	Poorly soluble	Poorly soluble	Poorly soluble	Poorly soluble	Moderately soluble
Consensus Log $P_{o/w}$	6.24	5.54	6.96	5.95	5.69

Note. MW: Molecular Weight, nHA: Number of H-bond acceptors, nHD: number of H-bond donors, TPSA: Topological polar surface area, Log S: solubility, ESOL: estimated solubility, Consensus Log $P_{o/w}$: consensus prediction of the logarithm of the partition coefficient (P) of a compound between n-octanol and water, key measures of lipophilicity

lipid environments, while methyl palmitate had the lowest lipophilicity (5.54), suggesting a preference for water solubility.

When developing antidiabetic drugs, the focus is on targeting organs involved in glucose metabolism, such as the liver, muscles, adipose tissue, and pancreas. Therefore, pharmacological properties should also prioritise factors such as GI absorption, distribution to target organs, metabolic stability, and excretion. Based on Table 6, all compounds, except methyl arachidate, were predicted to have high GI absorption, indicating their potential for intestinal absorption following oral administration (Al-Ghamdi et al., 2021). Besides, P-glycoprotein (P-gp) is critical in drug absorption and elimination (Pirzada et al., 2024). The selected FAMEs in this study are not considered P-gp substrates, and this is significant because P-gp inducers and inhibitors can alter absorption and excretion processes, potentially affecting drug efficacy or toxicity (Yamazaki et al., 2019). All tested FAMEs except methyl palmitate were non-blood-brain-barrier (BBB) permeants, aligning with the common traits of antidiabetic drugs, which typically do not need to cross the BBB. However, the BBB permeation of methyl palmitate suggests potential for central nervous system (CNS) targeting, offering opportunities for neurological therapeutic interventions. Notably, all compounds are cytochrome P450 1A2 enzyme inhibitors (CYP1A2), while only methyl linoleate is CYP2C9 inhibitor. None of the tested FAMEs inhibit CYP2C19, CYP2D6, or CYP3A4.

Regarding skin permeability, the current study found that the compounds have low skin permeability overall (Alade et al., 2023), indicating their unsuitability for transdermal

Table 6
The pharmacokinetic properties (ADME) of selected compounds predicted using SwissADME

Compounds	Methyl Stearate	Methyl Palmitate	Methyl Arachidate	Methyl Oleate	Methyl Linoleate
GI absorption	High	High	Low	High	High
Pgp-substrate	No	No	No	No	No
BBB penetration	No	Yes	No	No	No
CYP 1A2 inhibitor	Yes	Yes	Yes	Yes	Yes
CYP 2C19 inhibitor	No	No	No	No	No
CYP 2C9 inhibitor	No	No	No	No	Yes
CYP 2D6 inhibitor	No	No	No	No	No
CYP 3A4 inhibitor	No	No	No	No	No
Log Kp	-2.190 cm/s	-2.710cm/s	-1.690 cm/s	-2.820 cm/s	-3.250 cm/s
CL (mL/min/kg)	4.767	4.995	4.655	5.771	7.742
Half-life ($T_{1/2}$)	0.201	0.281	0.143	0.261	0.343

Note. GI absorption: gastrointestinal absorption, P-gp substrate: P-glycoprotein substrate, BBB penetration: blood-brain barrier penetration, CYP inhibitors: cytochrome P450 enzymes inhibitors, Log Kp: skin permeation, CL: Clearance rates

drug products. Clearance rates (CL) and half-life ($T_{1/2}$) highlighted pharmacokinetic differences, with methyl oleate (5.771 mL/min/kg) and methyl linoleate (7.742 mL/min/kg) showing moderate CL, while the others showed low CL. ADMETlab2.0 predictions indicated that methyl linoleate had the highest probability (0.343) of having a long $T_{1/2}$. A high CL indicates rapid elimination, while a long $T_{1/2}$ suggests prolonged drug exposure, potentially reducing dosing frequency (Adepu & Ramakrishna, 2021). Since the probabilities of having a long half-life for all tested FAMEs ranged from 0.143 to 0.343, which were relatively low, this suggests that frequent dosing may be necessary to maintain therapeutic levels.

The *in silico* pharmacokinetic assessment of the compounds in Table 7 revealed several key characteristics. No Pan Assay Interference Compounds (PAINS) or Brenk violations were identified across the tested compounds, indicating a favourable medicinal chemistry profile, as Brenk signals denote hazardous, reactive, and metabolically unstable fragments (Ononamadu & Ibrahim, 2021). The absence of PAINS alerts suggests a lack of structural features commonly associated with false positives in computational assays (Ahmad et al., 2023). However, all FAMEs displayed two lead-likeness criteria violations, indicating potential issues with pharmacokinetic behaviour and drug-likeness. All FAMEs exhibited a very low probability of human hepatotoxicity (H-HT) and Ames test for mutagenicity (AMES), suggesting minimal toxicity risk and reduced likelihood of liver damage, enhancing their suitability for drug development (Flores-Holguín et al., 2021). Nonetheless, high skin sensitisation scores across all compounds highlight the importance of early skin irritation assessment. Carcinogenicity probabilities ranged from 0.042 to 0.467, with methyl linoleate having the highest risk. Despite these variations, all tested FAMEs adhered to the Lipinski Rule of Five, suggesting drug-like properties (Rao & Hariprasad, 2021).

Table 7
The medicinal chemistry, toxicity, and drug-likeness of the selected compounds

Compounds	Methyl stearate	Methyl palmitate	Methyl arachidate	Methyl oleate	Methyl linoleate
PAINS	0	0	0	0	0
Brenk	0	0	0	0	0
Lead-likeness # violations	2	2	2	2	2
H-HT	Low (0.025)	Low (0.026)	Low (0.023)	Low (0.018)	Low (0.011)
AMES Toxicity	Low (0.006)	Low (0.006)	Low (0.006)	Low (0.005)	Low (0.016)
Skin sensitisation	High (0.96)	High (0.955)	High (0.964)	High (0.97)	High (0.975)
Carcinogenicity	Low (0.05)	Low (0.063)	Low (0.042)	Low (0.113)	Moderate (0.467)
Lipinski #violations	Accepted	Accepted	Accepted	Accepted	Accepted

Note. PAINS = Pan Assay Interference Compounds, Brenk = Brenk's Rule, AMES toxicity = Ames test for mutagenicity, H-HT = Human hepatotoxicity

Linking the *in silico* molecular docking results with pharmacokinetic predictions, methyl oleate and methyl linoleate are the two insightful antidiabetic drug candidates. Both of them comply with Lipinski's Rule of Five, indicating good potential for oral administration due to favourable gastrointestinal absorption. However, these two compounds are less suitable for transdermal use, as a high risk of skin sensitisation is predicted. With moderate renal clearance, they can avoid overly fast or slow elimination issues. Nevertheless, formulation adjustments are recommended to improve the solubility of methyl oleate, while methyl linoleate needs modification to address its predicted carcinogenicity risk.

CONCLUSION

The study revealed that all crude extracts of *S. macrophylla* fruits (Hex, EA, DCM, and MeOH) exhibited varied inhibitory effects on both α-amylase and AGE formation *in vitro*. While their effects on α-amylase were mild and significantly less effective ($p<0.01$) than acarbose, the MeOH extract showed the strongest effect on AGE formation, doubling the efficacy of other extracts at 100 µg/ml. However, no significant differences were found between the MeOH extract and the control AG, suggesting its potential as a natural alternative with similar therapeutic benefits. Molecular docking and *in silico* pharmacokinetic analyses indicated considerable affinity of the five compounds towards the active sites of HPA and RAGE. Although these compounds had lower affinity for HPA compared to acarbose, their affinities for RAGE were higher than AG, indicating a stronger interaction. Furthermore, ADMET analysis suggested drug-like properties for all tested FAMEs, with concerns regarding poor solubility and skin irritation, emphasising the need for compound modification before drug development. In short, MeOH crude extract of *S. macrophylla* fruits has the potential to act as an antiglycation agent. Further research is needed to validate whether individual compounds or interactions between multiple compounds are responsible for the observed antiglycation effects.

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REFERENCES

Abbas, A., Kalsoom, S., Hadda, T. B., & Naseer, M. M. (2014). Evaluation of 4-alkoxychalcones as a new class of antiglycating agents: A combined experimental and docking study. *Research on Chemical Intermediates*, *41*(9), 6443–6462. https://doi.org/10.1007/s11164-014-1752-0

Adepu, S., & Ramakrishna, S. (2021). Controlled drug delivery systems: Current status and future directions. *Molecules/Molecules Online/Molecules Annual*, *26*(19), 5905. https://doi.org/10.3390/molecules26195905

Agrawal, S. A., Dhamne, S. R. & Shinde, P. S. (2024). *In-silico* pharmacokinetic properties prediction of coumarins present in *Aegle marmelos* L. *International Journal of Pharmaceutical Sciences and Research*, *15*(2), 800-07. https://doi.org/10.13040/IJPSR.0975-8232.15(3).800-07

Ahmad, I., Kuznetsov, A. E., Pirzada, A. S., Alsharif, K. F., Daglia, M., & Khan, H. (2023). Computational pharmacology and computational chemistry of 4-hydroxyisoleucine: Physicochemical, pharmacokinetic, and DFT-based approaches. *Frontiers in Chemistry*, *11*, 1-15. https://doi.org/10.3389/fchem.2023.1145974

Ahmed, B., Sultana, R., & Greene, M. W. (2021). Adipose tissue and insulin resistance in obese. *Biomedicine & Pharmacotherapy*, *137*, 111315. https://doi.org/10.1016/j.biopha.2021.111315

Ahmed, J., Khan, M. A., Khalid, M. E., Ahmad, I., Pervaiz, I., Khurshid, U., Khaliq, S., Khan, K. U. R., Arshad, M. A., Albadrani, G. M., Altyar, A. E., Sayed, A. A., Germoush, M. O., & Abdel-Daim, M. M. (2023). A comprehensive approach to derivatization: elemental composition, biochemical, and *in silico* studies of metformin derivatives containing copper and zinc complexes. *Molecules/Molecules Online/Molecules Annual*, *28*(3), 1406. https://doi.org/10.3390/molecules28031406

Ahmmed, M. S., Shuvo, S. D., Paul, D., Karim, M. R., Kamruzzaman, M., Mahmud, N., Ferdaus, J., & Elahi, M. T. (2021). Prevalence of dyslipidemia and associated risk factors among newly diagnosed Type-2 Diabetes Mellitus (T2DM) patients in Kushtia, Bangladesh. *PLOS Global Public Health*, *1*(12), e0000003. https://doi.org/10.1371/journal.pgph.0000003

Alade, A. A., Ahmed, S. A., Mujwar, S., Kikiowo, B., Akinnusi, P. A., Olubode, S. O., Olufemi, O. M., & Ohilebo, A. A. (2023). Identification of levomenthol derivatives as potential dipeptidyl peptidase-4 inhibitors: A comparative study with gliptins. *Journal of Biomolecular Structure and Dynamics*, *8*(42), 4029-4047. https://doi.org/10.1080/07391102.2023.2217927

Alam, S., Sarker, M. M. R., Sultana, T. N., Chowdhury, M. N. R., Rashid, M. A., Chaity, N. I., Zhao, C., Xiao, J., Hafez, E. E., Khan, S. A., & Mohamed, I. N. (2022). Antidiabetic phytochemicals from Medicinal plants: Prospective candidates for new drug discovery and development. *Frontiers in Endocrinology*, *13*, 800714. https://doi.org/10.3389/fendo.2022.800714

Al-Ghamdi, A. Y., Fadlelmula, A. A., Abdalla, M. O., & Zabin, S. A. (2021). Phytochemical screening, chemical composition, antimicrobial activity and *in silico* investigation of the essential oil of *Coleus forskohlii* L. collected from the Southwestern Region of Saudi Arabia. *Journal of Essential Oil-bearing Plants*, *24*(1), 120–133. https://doi.org/10.1080/0972060x.2021.1901613

Arora, K., Tomar, P. C., & Mohan, V. (2021). Diabetic neuropathy: an insight on the transition from synthetic drugs to herbal therapies. *Journal of Diabetes & Metabolic Disorders*, *20*, 1773-1784. https://doi.org/10.1007/s40200-021-00830-2

Aroua, L. M., Alosaimi, A. H., Alminderej, F. M., Messaoudi, S., Mohammed, H. A., Almahmoud, S. A., Chigurupati, S., Albadri, A. E., & Mekni, N. H. (2023). Synthesis, molecular docking, and bioactivity study of novel hybrid benzimidazole urea derivatives: A promising α-amylase and α-glucosidase inhibitor candidate with antioxidant activity. *Pharmaceutics*, *15*(2), 457. https://doi.org/10.3390/pharmaceutics15020457

Belaiba, M., Aldulaijan, S., Messaoudi, S., Abedrabba, M., Dhouib, A., & Bouajila, J. (2023). Evaluation of biological activities of twenty flavones and *in silico* docking study. *Molecules*, *28*(6), 2419. https://doi.org/10.3390/molecules28062419

Blahova, J., Martiniakova, M., Babikova, M., Kovacova, V., Mondockova, V., & Omelka, R. (2021). Pharmaceutical drugs and natural therapeutic products for the treatment of Type 2 Diabetes Mellitus. *Pharmaceuticals (Basel, Switzerland)*, *14*(8), 806. https://doi.org/10.3390/ph14080806

Bongarzone, S., Savickas, V., Luzi, F., & Gee, A. D. (2017). Targeting the receptor for Advanced Glycation Endproducts (RAGE): A medicinal chemistry perspective. *Journal of Medicinal Chemistry*, *60*(17), 7213–7232. https://doi.org/10.1021/acs.jmedchem.7b00058

Bouyahya, A., Omari, N., Elmenyiy, N., Guaouguaou, F., Balahbib, A., Belmehdi, O., Salhi, N., Imtara, H., Mrabti, H. N., Shazly, M., & Bakri, Y. (2021). Moroccan antidiabetic medicinal plants: Ethnobotanical studies, phytochemical bioactive compounds, preclinical investigations, toxicological validations, and clinical evidences; Challenges, guidance, and perspectives for future management of diabetes worldwide. *Trends in Food Science & Technology*, *115*, 147-254, https://doi.org/10.1016/j.tifs.2021.03.032.

Chew, I. Y. Y. (2023). *In-silico* and *in-vitro* analysis of lipogenesis effect of *Shorea macrophylla* fruits' crude extracts. [Unpublished Master Dissertation]. Universiti Malaysia Sarawak.

Chigurupati, S., Alharbi, F. S., Almahmoud, S., Aldubayan, M., Almoshari, Y., Vijayabalan, S., Bhatia, S., Chinnam, S., & Venugopal, V. (2021). Molecular docking of phenolic compounds and screening of antioxidant and antidiabetic potential of *Olea europaea* L. ethanolic leaves extract. *Arabian Journal of Chemistry*, *14*(11), 103422. https://doi.org/10.1016/j.arabjc.2021.103422

Daina, A., Michielin, O., & Zoete, V. (2017). SwissADME: A free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. *Scientific Reports*, *7*(1). https://doi.org/10.1038/srep42717

Dariya, B., & Nagaraju, G. P. (2020). Advanced glycation end products in diabetes, cancer and phytochemical therapy. *Drug Discovery Today*, *25*(9), 1614–1623. https://doi.org/10.1016/j.drudis.2020.07.003

Flores-Holguín, N., Frau, J., & Glossman-Mitnik, D. (2021). *In silico* pharmacokinetics, ADMET study and conceptual DFT analysis of two plant cyclopeptides isolated from Rosaceae as a computational peptidology approach. *Frontiers in Chemistry*, *9*, 708364. https://doi.org/10.3389/fchem.2021.708364

Ganasegeran, K., Hor, C. P., Jamil, M. F. A., Suppiah, P. D., Noor, J. M., Hamid, N. A., Chuan, D. R., Manaf, M. R. A., Ch'ng, A. S. H., & Looi, I. (2021). Mapping the scientific landscape of diabetes research in Malaysia (2000-2018): A systematic scientometrics study. *International Journal of Environmental Research and Public Health*, *18*(1), 318. https://doi.org/10.3390/ijerph18010318

Ghannay, S., Snoussi, M., Messaoudi, S., Kadri, A., & Aouadi, K. (2020). Novel enantiopure isoxazolidine and C-alkyl imine oxide derivatives as potential hypoglycemic agents: Design, synthesis, dual inhibitors of α-amylase and α-glucosidase, ADMET and molecular docking study. *Bioorganic Chemistry*, *104*, 104270. https://doi.org/10.1016/j.bioorg.2020.104270

Imamura, F., Fretts, A. M., Marklund, M., Korat, A. V. A., Yang, W., Lankinen, M., Qureshi, W., Helmer, C., Chen, T., Virtanen, J. K., Wong, K., Bassett, J. K., Murphy, R., Tintle, N., Yu, C. I., Brouwer, I. A., Chien, K., Chen, Y., Wood, A. C., Forouhi, N. G. (2020). Fatty acids in the *de novo* lipogenesis pathway and incidence of type 2 diabetes: A pooled analysis of prospective cohort studies. *PLoS Medicine*, *17*(6), e1003102. https://doi.org/10.1371/journal.pmed.1003102

Jain, A., Jain, R., & Jain, S. (2020). Quantitative analysis of reducing sugars by 3,5-dinitrosalicylic acid (DNSA Method). In *Basic techniques in biochemistry, Microbiology and molecular biology* (pp. 181-183). Springer. https://doi.org/10.1007/978-1-4939-9861-6_43

Khan, R., Ooi, X. Y., Parvus, M., Valdez, L., & Tsin, A. (2019). Advanced Glycation End Products: formation, role in diabetic complications, and potential in clinical applications. In *IntechOpen eBooks*. https://doi.org/10.5772/intechopen.89408

Kikiowo, B., Ogunleye, J. A., Iwaloye, O., & Ijatuyi, T. T. (2020). Therapeutic potential of *Chromolaena odorata* phyto-constituents against human pancreatic α-amylase. *Journal of Biomolecular Structure and Dynamics*, *40*(4), 1801–1812. https://doi.org/10.1080/07391102.2020.1833758

Kim, K., Hong, S., Han, K. D., & Park, C. (2022). Assessing the validity of the criteria for the extreme risk category of atherosclerotic cardiovascular disease: A nationwide population-based study. *Journal of Lipid and Atherosclerosis*, *11*(1), 73. https://doi.org/10.12997/jla.2022.11.1.73

Lankatillake, C., Luo, S., Flavel, M., Lenon, G. B., Gill, H., Huynh, T., & Dias, D. A. (2021). Screening natural product extracts for potential enzyme inhibitors: Protocols, and the standardisation of the usage of blanks in α-amylase, α-glucosidase and lipase assays. *Plant Methods*, *17*, 3. https://doi.org/10.1186/s13007-020-00702-5

Luo, S., Gill, H., Dias, D. A., Li, M., Hung, A., Nguyen, L. T., & Lenon, G. B. (2019). The inhibitory effects of an eight-herb formula (RCM-107) on pancreatic lipase: enzymatic, HPTLC profiling and *in silico* approaches. *Heliyon*, *5*(9), e02453. https://doi.org/10.1016/j.heliyon.2019.e02453

Magliano, D. J., & Boyko, E. J. (2021). *IDF diabetes atlas*. https://www.ncbi.nlm.nih.gov/books/NBK581940/

Mohamed, G. A., & Ibrahim, S. R. M. (2022). Garcixanthone E and Garcimangophenone C: New metabolites from *Garcinia mangostana* and their cytotoxic and alpha amylase inhibitory potential. *Life*, *12*(11), 1875. https://doi.org/10.3390/life12111875

Odu, N. M., Akor, J., Eze, L. A., Nweze, J. A., Nweze, J. E., Orjiocha, S. I., Ejembi, D. O., Onyeyilim, E. L., & Nwanguma, B. C. (2023). Anti-inflammatory and antioxidant activities of selected fractions of *Parinari kerstingii* leaf extract. *Tropical Journal of Natural Product Research*, *7*(10), 4954–4960. http://www.doi.org/10.26538/tjnpr/v7i10.35

Ononamadu, C., & Ibrahim, A. (2021). Molecular docking and prediction of ADME/drug-likeness properties of potentially active antidiabetic compounds isolated from aqueous-methanol extracts of *Gymnema sylvestre* and *Combretum micranthum*. *BioTechnologia*, *102*(1), 85–99. https://doi.org/10.5114/bta.2021.103765

Parcheta, M., Świsłocka, R., Orzechowska, S., Akimowicz, M., Choińska, R., & Lewandowski, W. (2021). Recent developments in effective antioxidants: The structure and antioxidant properties. *Materials*, *14*(8), 1984. https://doi.org/10.3390/ma14081984

Paulraj, J. A., Subharamanian, H., Suriyamoorthy, P., & Kanakasabapathi, D. (2014). Phytochemical screening, GC-MS analysis and enzyme inhibitory activity of *Passiflora foetida* L. *Indo American Journal of Pharmaceutical Research*, *4*(8), 3526-3534. https://www.researchgate.net/publication/279961040_PHYTOCHEMICAL_SCREENING_GC-

Pirzada, A. S., Khan, H., Alam, W., Darwish, H. W., Elhenawy, A. A., Kuznetsov, A., & Daglia, M. (2024). Physicochemical properties, pharmacokinetic studies, DFT approach, and antioxidant activity of nitro and chloro indolinone derivatives. *Frontiers in Chemistry*, *12*, 1360719. https://doi.org/10.3389/fchem.2024.1360719

Rajan, L., Palaniswamy, D., & Mohankumar, S. K. (2020). Targeting obesity with plant-derived pancreatic lipase inhibitors: A comprehensive review. *Pharmacological Research*, *155*, 104681. https://doi.org/10.1016/j.phrs.2020.104681

Ramasamy, R., Yan, S. F., & Schmidt, A. M. (2011). Receptor for AGE (RAGE): Signaling mechanisms in the pathogenesis of diabetes and its complications. *Annals of the New York Academy of Sciences*, *1243*(1), 88–102. https://doi.org/10.1111/j.1749-6632.2011.06320.x

Rao, M. M. V., & Hariprasad, T. P. N. (2021). In silico analysis of a potential antidiabetic phytochemical erythrin against therapeutic targets of diabetes. *In Silico Pharmacology*, *9*(5), 2-12. https://doi.org/10.1007/s40203-020-00065-8

Ryan, M., McInerney, D., Owens, D., Collins, P., Johnson, A., & Tomkin, G. H. (2000). Diabetes and the Mediterranean diet: A beneficial effect of oleic acid on insulin sensitivity, adipocyte glucose transport and endothelium-dependent vasoreactivity. *QJM: An International Journal of Medicine*, *93*(2), 85-91. https://doi.org/10.1093/qjmed/93.2.85

Sadeghi, M., Moradi, M., Madanchi, H., & Johari, B. (2021). *In silico* study of garlic (*Allium sativum* L.)-derived compounds molecular interactions with α-glucosidase. *In Silico Pharmacology*, *9*, 11. https://doi.org/10.1007/s40203-020-00072-9

Salazar, J., Navarro, C., Ortega, Á., Nava, M., Morillo, D., Torres, W., Hernández, M., Cabrera, M., Angarita, L., Ortíz, R., Chacín, M., D'Marco, L., & Bermúdez, V. (2021). Advanced glycation end products: New clinical and molecular perspectives. *International Journal of Environmental Research and Public Health/International Journal of Environmental Research and Public Health*, *18*(14), 7236. https://doi.org/10.3390/ijerph18147236

Sekhon-Loodu, S., & Rupasinghe, H. P. V. (2019). Evaluation of antioxidant, antidiabetic and antiobesity potential of selected traditional medicinal plants. *Frontiers in Nutrition*, *6*, 53. https://doi.org/10.3389/fnut.2019.00053

Sivagurunathan, A., & Xavier, B. I. (2014). GC-MS evaluation of bioactive compounds of *Marsilea quadrifolia* Linn. aquatic Fern. *International Journal for Pharmaceutical Research Scholars*, *3*(1), 2277-7873.

Sokalingam, S., Raghunathan, G., Soundrarajan, N., & Lee, S. (2012). A study on the effect of surface lysine to arginine mutagenesis on protein stability and structure using green fluorescent protein. *PloS One*, *7*(7), e40410. https://doi.org/10.1371/journal.pone.0040410

Sowmiya, V., Sakthi Priyadarsini, S., & Kumar, P. R. (2021). GC-MS Profiling of different fractions of *Physalis minima* Linn. leaves. *Natural Volatiles and Essential Oils*, *8*(5), 12852-12859. https://www.nveo.org/index.php/journal/article/view/4063/3343

Starowicz, M., & Zieliński, H. (2019). Inhibition of advanced glycation end-product formation by high antioxidant-leveled spices commonly used in european cuisine. *Antioxidants*, *8*(4), 100. https://doi.org/10.3390/antiox8040100

Tambe, V., Kherade, D., Wagh, A., & Kothawade, P. (2022). A comparative molecular docking study of crocetin with multiple receptors for the treatment of Alzheimer's disease. *Biomedical and Biotechnology Research Journal*, *6*(2), 230. https://doi.org/10.4103/bbrj.bbrj_6_22

Twarda-Clapa, A., Olczak, A., Białkowska, A., & Koziołkiewicz, M. (2022). Advanced Glycation End-Products (AGEs): Formation, chemistry, classification, receptors, and diseases related to AGEs. *Cells*, *11*(8), 1312. https://doi.org/10.3390/cells11081312

Yamazaki, S., Costales, C., Lazzaro, S., Eatemadpour, S., Kimoto, E., & Varma, M. V. (2019). Physiologically-based pharmacokinetic modeling approach to predict Rifampin-mediated intestinal P glycoprotein induction. *CPT: Pharmacometrics and Systems Pharmacology*, *8*(9), 634–642. https://doi.org/10.1002/psp4.12458

Yu, F., Lian, X., Guo, H., McGuire, P., Li, R., Wang, R., & Yu, F. (2005). Isolation and characterization of methyl esters and derivatives from *Euphorbia kansui* (Euphorbiaceae) and their inhibitory effects on the human SGC-7901 cells. *PubMed*, *8*(3), 528–535.

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The Antifungal Potential of Seven Plant Species Against Selected Plant Pathogen

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ABSTRACT

The antifungal activities of methanol extract from seven plant species, namely *Plectranthus amboinicus* (Mexican mint), *Morinda citrifolia* (noni), *Clitoria ternatea* (butterfly pea), *Passiflora suberosa* (corkystem passionflower), *Azadirachta indica* (neem), *Moringa oleifera* (ben oil tree) and *Vernonia amygdalina* (bitter leaf) were assessed against six fungal plant pathogens using poison agar technique at concentrations of 5%, 10%, 15% and 20%. The *in vitro* study revealed that the effectiveness of these plant extracts varied in suppressing the growth of *Ganoderma boninense*, *Rhizoctonia solani*, *Rigidoporus microporous* (basidiomycetes), *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (Foc TR4), Pyricularia oryzae (ascomycetes), and *Phytophthora palmivora* (oomycetes). Notably, *A. indica* demonstrated complete inhibition of all pathogens across all concentration levels, except against Foc TR4 at 5%, where it achieved 48.05% inhibition. This highlights the broad-spectrum antifungal potential of *A. indica*, as it proved effective against all the selected fungal plant pathogens. Complete inhibition of Foc TR4 was achieved solely with extracts from *P. amboinicus*, *M. citrifolia*, *C. ternatea*, *A. indica* and *V. amygdalina* at concentrations of 10% and above, which indicate higher concentrations required for effective inhibition of Foc TR4. Nevertheless, most pathogens were effectively suppressed at 5% concentration, and *A. indica*, *V. amygdalina* and *C. ternatea* demonstrated inhibition against all tested pathogens starting at 10%. These findings suggest these plant species could be developed into bio-fungicides for controlling major plant diseases in Malaysia, with *A. indica* being the

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most promising candidate. Further field trials are needed to validate their commercial viability compared to synthetic fungicides.

Keywords: Antifungal potential, botanical plant extracts, plant diseases, plant protection, poison agar

INTRODUCTION

Malaysia's agriculture sector is crucial for sustaining its population and driving economic growth through produce exports, which contribute approximately 8% to the country's gross domestic product (GDP). The key crops planted in Malaysia include oil palm, rubber, cocoa, rice, and tropical fruits (International Trade Administration, 2024). Despite its fertile land and favourable climate, the sector contends with challenges such as pest and disease outbreaks, climate change impacts, extreme weather events, labour shortages, and rapid population growth. Among these, insect infestation and disease pose a considerable menace to global food security, trade and livelihoods. Annually, as much as 40% of global crop production is lost due to pests and diseases (Food and Agriculture Organization of the United Nations [FAO], 2024), with fungal diseases alone accounting for 70-80% of crop losses (Peng et al., 2021). For instance, rice blast and sheath blight caused by *Pyricularia oryzae* and *Rhizoctonia solani* account for approximately 30% of losses in global rice production (Nalley et al., 2016; Zhu et al., 2019). The economic impact of *Ganoderma* disease on oil palm is estimated at around RM 1.5 billion (Malaysian Palm Oil Board [MPOB], 2022). Meanwhile, the disease incidence of *Rigidoporus microporus* in Malaysia ranges from 5% to 40%, based on random sampling conducted in rubber plantations across five states (Andrew, 2020). Recent estimates suggest that tropical race 4 of the fungal pathogen *Fusarium oxysporum* f. sp. *cubense* (Foc TR4) could potentially spread across 1.7 million hectares of banana plantations in 29 countries by 2040 if no significant interventions are instituted, with annual production losses projected at around 10 billion dollars (Scheerer et al., 2018). Furthermore, *Phytophthora palmivora* affects all parts of the cocoa tree, causing 20%–30% pod losses through black pod rot and killing up to 10% of trees annually through stem cankers (Guest, 2007; Perrine-Walker, 2020).

Fungicides are widely used by growers as a primary approach in disease management, with various active ingredients (a.i.) such as propiconazole, tricyclazole, azoxystrobin and tebuconazole that are commonly employed (Mohiddin et al., 2021). While chemical fungicides provide rapid action and accessibility at lower costs, their extensive use raises environmental and health concerns (Wong, Hamid et al., 2020). Developing fungal resistance to these chemicals urges ongoing efforts to explore alternative solutions. The persistence of fungicide residues in food and the environment further emphasises the need for safer alternatives (Baibakova et al., 2019). Decades of conventional agricultural practices have clearly had a significant environmental impact. Balancing the present-day imperative to enhance food production and supply without compromising the health of the

planet and the ability of future generations to meet their needs is essential (Çakmakçı et al., 2023). In response, sustainable agriculture has emerged as a solution to address these challenges (Coulibaly et al., 2021). Therefore, implementing and adopting best practices for managing fungal plant pathogens in crops while minimising environmental impact is critical for sustainable agriculture and ensuring food security.

Environment-friendly solutions are available, such as biological control, natural compounds, crop rotation, resistant varieties, cultural practices, Integrated Pest Management (IPM), soil amendments, and precision agriculture (Çakmakçı et al., 2023). Humans can minimise their reliance on synthetic chemicals through sustainable disease management practices, thereby reducing pollution and environmental harm while preserving biodiversity by safeguarding beneficial organisms and habitats typically affected by chemical pesticides. Additionally, they contribute to soil health and water quality preservation (Sofo et al., 2022). Despite potentially higher initial costs, adopting sustainable practices tends to reduce the dependence on costly chemical inputs and enhances crop resilience, ultimately boosting profitability. By prioritising the health and safety of agricultural workers and communities, sustainable farming also fosters resilience to environmental stresses and climate change. The improved soil health and biological control methods reduce the likelihood of disease outbreaks and crop failures and are vital for ensuring food security amidst evolving environmental conditions.

In recent decades, there has been a rapid surge in interest regarding the exploration of natural compounds derived from medicinal or botanical plants as potential substitutes for chemical fungicides (Hernández-Ceja et al., 2021; Kursa et al., 2022; Lee et al., 2022). For instance, Ali et al. (2024) highlighted the significant antifungal efficacy of methanol and ethanol extracts derived from *Azadirachta indica* seeds and leaves against various fungal pathogens such as *Colletotrichum coccodes*, *Cladosporium fulvum*, *R. solani* and *F. oxysporum*. Additionally, research by Ilondu (2013) demonstrated the antifungal properties of ethanolic leaf extracts from *V. ambigua*, *V. amygdalina* and *V. cinerea* against groundnut leafspot diseases caused by *Cercosporella apersica* and *Curvularia lunatus*. These extracts contain bioactive compounds, including flavonoids, terpenoids, glycosides, phenols, alkaloids, saponins and tannins, that have been shown to possess antimicrobial properties (Ali et al., 2024; Ilondu, 2013).

Despite that, there remains a gap in understanding the antifungal efficacy of plant extracts from families such as Lamiaceae, Rubiaceae, Fabaceae, Passifloraceae, Meliaceae, Moringaceae and Asteraceae against the important fungal plant pathogens in Malaysia. These pathogens, which affect major crops, include *Ganoderma* basal stem rot in oil palm (Isha et al., 2020), white root rot in rubber (Go et al., 2023), rice blast and sheath blight in rice (Wong, Surendran et al., 2020), cocoa pod rot and durian fruit rot caused by *Phytophthora* species (Latifah et al., 2018), and *Fusarium* wilt in banana (Clement et al., 2019). Addressing this gap, the present study assesses the crude extracts of seven plant

species for their antifungal activity under laboratory conditions against important plant pathogens, namely *G. boninense*, *R. solani*, *R. microporous*, Foc TR4, *P. oryzae*, and *P. palmivora*, which commonly affect the crops in Malaysia. This study explores safer and sustainable bio-fungicides as alternatives to synthetic chemicals for crop protection.

MATERIALS AND METHODS

Collection of Plant Materials

Leaf samples of *P. amboinicus*, *M. citrifolia*, *C. ternatea*, *P. suberosa*, *A. indica*, *M. oleifera* and *V. amygdalina* were collected from various sites within Universiti Putra Malaysia (UPM), located in Seri Kembangan, Selangor, Malaysia (Table 1). The collected leaves were brought to Makmal Serbaguna B, situated in the Department of Plant Protection, Faculty of Agriculture, UPM, for further processing.

Table 1
Location of plant material collection

Plant specimen (Common name)	Location of plant material
Plectranthus amboinicus (Mexican mint)	Ladang 10, Universiti Putra Malaysia
Morinda citrifolia (noni)	Institute of Bioscience, Universiti Putra Malaysia
Clitoria ternatea (butterfly pea)	Faculty of Agriculture, Universiti Putra Malaysia
Passiflora suberosa (corkystem passionflower)	Faculty of Agriculture, Universiti Putra Malaysia
Azadirachta indica (neem)	Taman Herba, Universiti Putra Malaysia
Moringa oleifera (ben oil tree)	Taman Herba, Universiti Putra Malaysia
Vernonia amygdalina (bitter leaf)	Taman Herba, Universiti Putra Malaysia

Fungal Cultures

The selected fungal plant pathogens, namely *G. boninense, R. solani, R. microporous, Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (Foc TR4), *P. oryzae* and *P. palmivora* used in this study were obtained from the Fungal Culture Collection Unit, Department of Plant Protection, Faculty of Agriculture, UPM. Each fungal strain was sub-cultured and maintained using different selected sterilised media: *R. solani, R. microporous*, Foc TR4 and *P. oryzae* on Potato Dextrose Agar (PDA) (Oxoid, UK), *G. boninense* on Malt Extract Agar (MEA) (Oxoid, UK) and *P. palmivora* on Corn Meal Agar (CMA) (Oxoid, UK). Subsequently, all culture plates were maintained in a culture chamber at a temperature of 26 ± 2 °C under laboratory conditions.

Preparation of Plant Crude Extracts

The leaf specimens of *P. amboinicus*, *M. citrifolia*, *C. ternatea*, *P. suberosa*, *A. indica*, *M. oleifera* and *V. amygdalina* were washed to eliminate dirt and soil particles. Subsequently, all

leaves were air-dried at room temperature (26 ± 2 °C) for three days before being grounded separately into a fine powder (<100 µm) using a grinder (Cross Beater Mill SK 100, Retsch, Germany). Following this, 50 g of the grounded leaves were separately macerated in 300 ml of methanol (Fisher Chemical™, USA) in a conical flask and stirred at 120 rpm for 24 hr using an orbital shaker (Forma 420 Orbital Shaker, Thermo, USA). Each mixture was filtered using Whatman No. 1 filter paper (Cytiva, UK) and concentrated using a rotary evaporator (R215W, Buchi, Switzerland) at 145 rpm (42 °C). The resulting dried extracts were collected in a beaker with 5 ml of methanol (Fisher Chemical™, USA) and stored in an airtight container at 4 °C, following the method described by Wong, Hamid, Shah et al. (2020).

Screening of Antifungal Activities

The poison agar technique, as described by Balouiri et al. (2016), was used to assess the antifungal efficacy of *P. amboinicus*, *M. citrifolia*, *C. ternatea*, *P. suberosa*, *A. indica*, *M. oleifera* and *V. amygdalina* against six selected fungal plant pathogens, namely *G. boninense*, *R. solani*, *R. microporous*, *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (Foc TR4), *P. oryzae* and *P. palmivora*. Stock solutions from the crude plant extracts were prepared and diluted in methanol (Fisher Chemical™, USA) at a ratio of 1:10, following the method described by Abu et al. (2017). Nineteen millilitres of sterilised PDA (Oxoid, UK) and 1 ml of plant extract were mixed together and plated onto a sterilised Petri dish (Brandon, USA) to achieve a 5% concentration. This procedure was repeated to attain concentrations of 10%, 15%, and 20%, with the amount of plant extract added into the sterilised PDA (Oxoid, UK), as detailed in Table 2. Each concentration had five replications, and 0% served as the negative control. The same step was repeated for other plant extracts.

Table 2
The quantity of methanol extract utilised to obtain various concentration levels for the poison agar of the tested plants

Percentage of plant extract (%)	The methanol extract (ml)	PDA agar (ml)	Final concentration (mg/ml)
5	1	19	100
10	2	18	200
15	3	17	300
20	4	16	400

The poison agar medium was prepared individually at 100, 200, 300, and 400 mg/ml concentrations for each crude extract. A fungal disc with a diameter of 5 mm diameter, obtained from the actively grown pure culture of the selected pathogens, was placed at the centre of Petri dishes (Brandon, USA) containing various concentrations of the crude

extracts, with five replications for each concentration (Yusoff et al., 2020). This process was repeated for the other crude plant extracts. Subsequently, all plates were incubated at 26 ± 2 °C. The antifungal activity of the tested plants against the pathogens was assessed using the percentage inhibition of diameter growth (PIDG). This involved measuring the diameter of mycelial growth daily until the Petri dishes (Brandon, USA) with 0% concentration of the tested plants were completely covered with pathogen mycelia. The mycelial growth of the pathogens on different treatment concentrations was observed and recorded daily, following the PIDG formula described by Wong et al. (2020c).

$$\text{PIDG (\%)} = \frac{D1-D2}{D1} \times 100$$

Where, D1 is the average mycelial growth in control plates, and D2 is the average mycelial growth in treatment plates.

Experimental Design and Statistical Analysis

The effect of plant extracts and their antifungal activities were conducted *in vitro* using a completely randomised design (CRD) with five treatments (0%, 5%, 10%, 15%, 20%), each replicated five times. Statistical analysis was conducted using SAS® Software (SAS Institute, North Carolina State University, USA, Version 9.4, 2012). Mean comparisons were determined using the Least Significant Difference (LSD) at a 5% probability level.

RESULTS

Effect of *P. amboinicus* Crude Extract on Mycelial Growth of Selected Fungal Plant Pathogens

The Percentage Inhibition of Diameter Growth (PIDG) of selected fungal plant pathogens treated with *P. amboinicus* (Mexican mint) extract at concentrations of 5%, 10%, 15% and 20% is presented in Figure 1. Mexican mint extract showed high efficacy against *G. boninense* and *R. microporous* (basidiomycetes), as well as *P. oryzae* (ascomycetes), achieving PIDG values of 100% across all concentrations tested (Figure 1). All pathogens were completely inhibited by the 10% concentration of *P. amboinicus* extract, except for *P. palmivora* (84.25%) within the oomycetes group. Furthermore, concentrations of 15% and 20% consistently displayed 100% suppression against all pathogens, with no significant difference observed (Figure 1).

Effect of *M. citrifolia* Crude Extract on Mycelial Growth of Selected Fungal Plant Pathogens

The antifungal activity of *M. citrifolia* (noni) methanol extract against selected fungal plant pathogens is presented in Figure 2. The results illustrated that *G. boninense*, classified

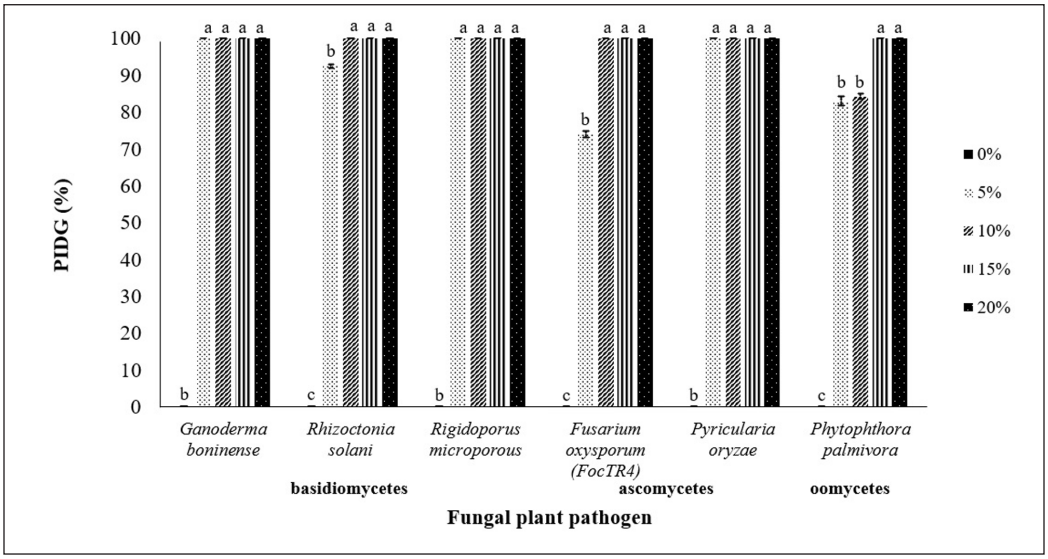

Figure 1. Percentage inhibition of diameter growth (PIDG) of *Plectranthus amboinicus* against selected fungal plant pathogens

Note. Measurements were taken at different intervals: Three days after inoculation (DAI) for *Rhizoctonia solani*; 8 DAI for *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (Foc TR4) and *Phytophthora palmivora*; 9 DAI for *Ganoderma boninense*; and 11 DAI for *Pyricularia oryzae* and *Rigidoporus microporous*. Values are the means of five replicates. Means with the same letter are not significantly different at $p = 0.05$

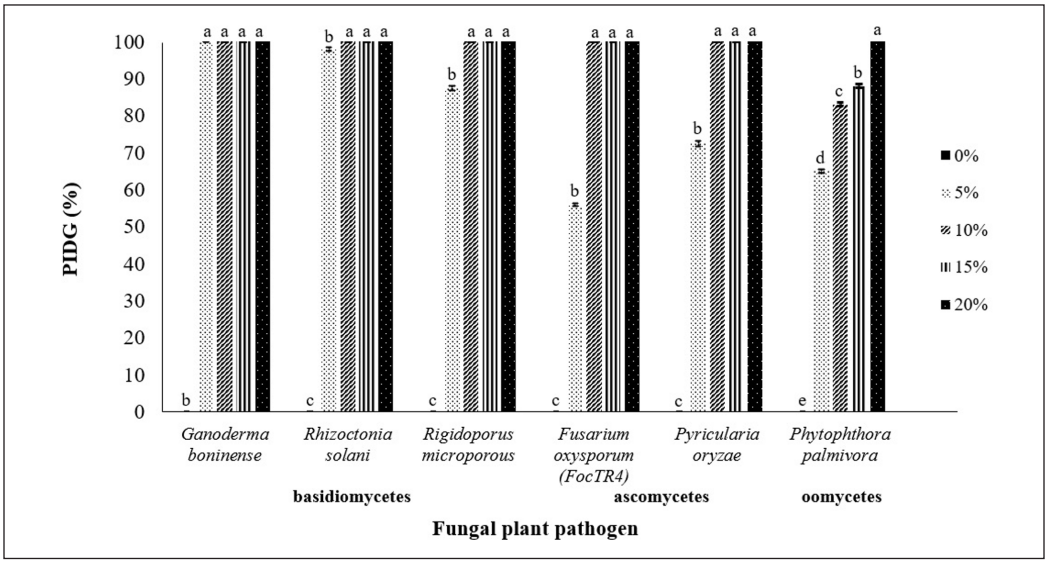

Figure 2. Percentage inhibition of diameter growth (PIDG) of *Morinda citrifolia* against selected fungal plant pathogens

Note. Measurements were taken at different intervals: Three days after inoculation (DAI) for *Rhizoctonia solani*; 8 DAI for *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (Foc TR4) and *Phytophthora palmivora*; 9 DAI for *Ganoderma boninense*; and 11 DAI for *Pyricularia oryzae* and *Rigidoporus microporous*. Values are the means of five replicates. Means with the same letter are not significantly different at $p = 0.05$

under basidiomycetes, was the only pathogen effectively inhibited by noni extracts across all concentrations (ranging from 5% to 20%). Another two basidiomycetes (*R. solani* and *R. microporous*) and two ascomycetes (Foc TR4 and *P. oryzae*) were entirely inhibited by noni extract starting from 10% and above concentrations. Meanwhile, the most effective inhibition (100%) against *P. palmivora* (oomycetes) was achieved only with a 20% concentration, whereas for the concentrations of 10% and 15%, the PIDG values were recorded at 83% and 88%, respectively. At 20%, noni plant extract demonstrated the ability to suppress the mycelial growth of all tested pathogens (Figure 2).

Effect of *C. ternatea* Crude Extract on Mycelial Growth of Selected Fungal Plant Pathogens

Figure 3 depicts the antifungal efficacy of *C. ternatea* (butterfly pea) methanol extract against six selected fungal plant pathogens. Notably, butterfly pea extract significantly inhibited *G. boninense* (basidiomycetes) *and P. palmivora* (oomycetes) growth, achieving a PIDG of 100% across all concentrations. Additionally, *R. solani* and *R. microporous* (basidiomycetes), as well as Foc TR4 and *P. oryzae* (ascomycetes), revealed complete suppression by butterfly pea extract at concentrations of 10% and higher (Figure 3). This indicated that butterfly pea extracts effectively controlled all pathogens at 10%.

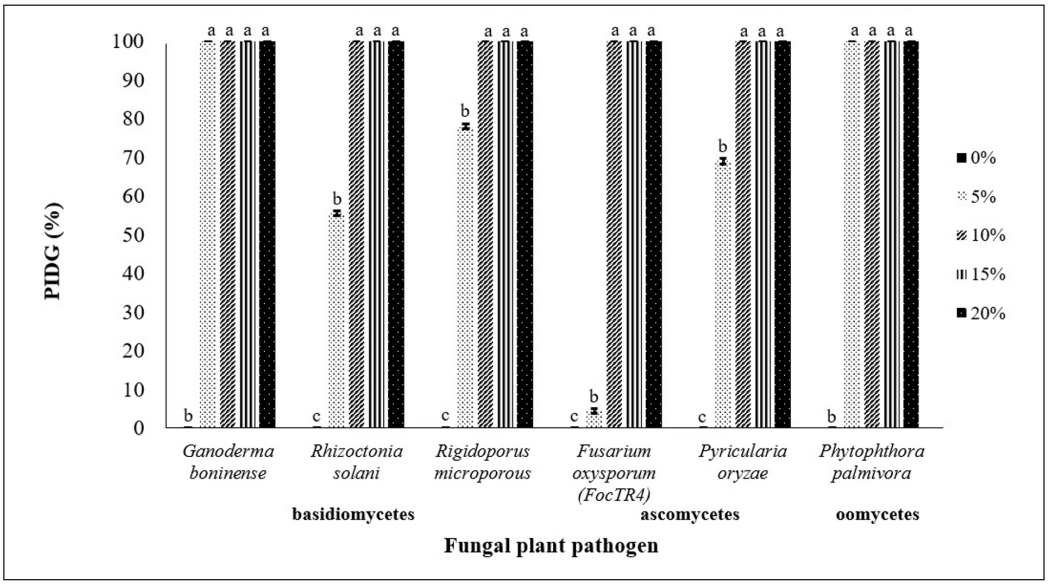

Figure 3. Percentage inhibition of diameter growth (PIDG) of *Clitoria ternatea* against selected fungal plant pathogens

Note. Measurements were taken at different intervals: Three days after inoculation (DAI) for *Rhizoctonia solani*; 8 DAI for *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (Foc TR4) and *Phytophthora palmivora*; 9 DAI for *Ganoderma boninense*; and 11 DAI for *Pyricularia oryzae* and *Rigidoporus microporous*. Values are the means of five replicates. Means with the same letter are not significantly different at $p = 0.05$

Effect of *P. suberosa* Crude Extract on Mycelial Growth of Selected Fungal Plant Pathogens

The antifungal efficacy of *P. suberosa* (corkystem passionflower) methanol extract against the selected pathogen was revealed in Figure 4. Among the tested pathogens, *P. oryzae* (ascomycetes) and *P. palmivora* (oomycetes) were completely inhibited by corkystem passionflower extracts across all concentrations, as shown in Figure 4. Furthermore, complete inhibition of *R. solani* (basidiomycetes) occurred at concentrations above 10%, while *G. boninense* and *R. microporus*, also from the same class, were entirely inhibited at concentrations above 15%. Nevertheless, 5% and 10% *P. suberosa* extract against *G. boninense* still achieved a PIDG value of more than 80% (Figure 4). Conversely, corkystem passionflower extract exhibited the lowest effect against Foc TR4 (ascomycetes) across all concentrations, with significant differences observed between concentration levels (Figure 4).

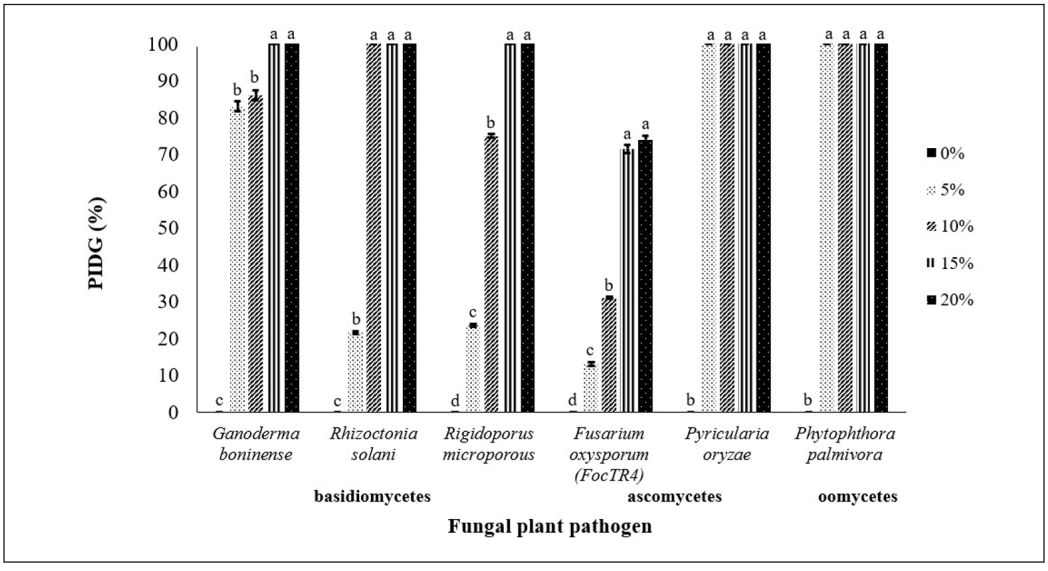

Figure 4. Percentage inhibition of diameter growth (PIDG) of *Passiflora suberosa* against selected fungal plant pathogens

Note. Measurements were taken at different intervals: Three days after inoculation (DAI) for *Rhizoctonia solani*; 8 DAI for *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (Foc TR4) and *Phytophthora palmivora*; 9 DAI for *Ganoderma boninense*; and 11 DAI for *Pyricularia oryzae* and *Rigidoporus microporous*. Values are the means of five replicates. Means with the same letter are not significantly different at $p = 0.05$

Effect of *A. indica* Crude Extract on Mycelial Growth of Selected Fungal Plant Pathogens

Figure 5 shows the efficacy of *A. indica* (neem) extract against six selected pathogens. Neem extract exhibited high efficacy against all the tested pathogens, namely *G. boninense*, *R. solani* and *R. microporus* (basidiomycetes), as well as *P. oryzae* (ascomycetes) and *P.*

palmivora (oomycetes), except Foc TR4 (ascomycetes) at 5%. The plant extract achieved 100% inhibition against these pathogens at all concentrations, with no significant difference observed between concentration levels (Figure 5). Concerning Foc TR4 (ascomycetes), complete suppression by neem extract was attained at concentrations of 10% and above. *A. indica* demonstrated the ability to suppress mycelial growth of all the tested pathogens at 10% onwards (Figure 5).

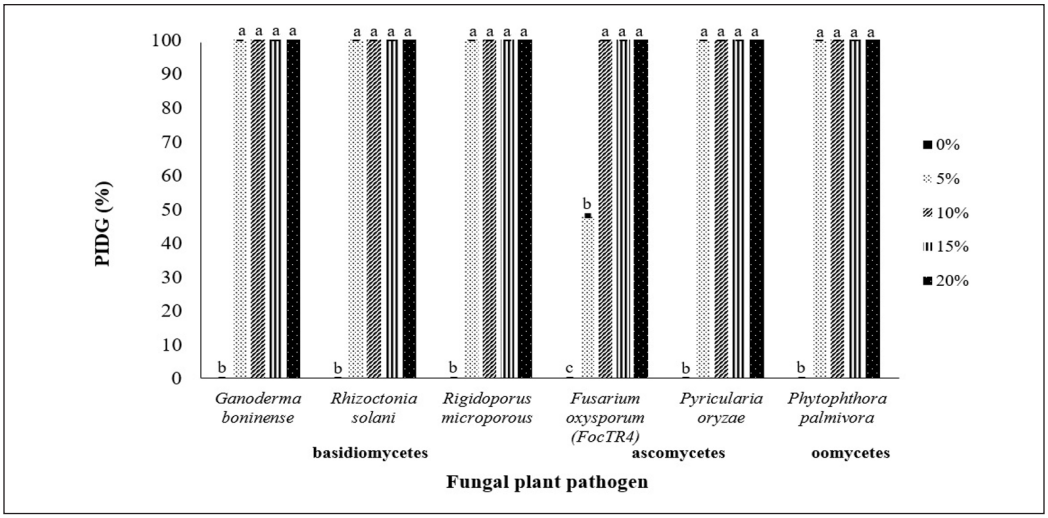

Figure 5. Percentage inhibition of diameter growth (PIDG) of *Azadirachta indica* against selected fungal plant pathogens

Note. Measurements were taken at different intervals: Three days after inoculation (DAI) for *Rhizoctonia solani*; 8 DAI for *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (Foc TR4) and *Phytophthora palmivora*; 9 DAI for *Ganoderma boninense*; and 11 DAI for *Pyricularia oryzae* and *Rigidoporus microporous*. Values are the means of five replicates. Means with the same letter are not significantly different at $p = 0.05$

Effect of *M. oleifera* Crude Extract on Mycelial Growth of Selected Fungal Plant Pathogens

The results in Figure 6 demonstrated the variable antifungal activity of *M. oleifera* (ben oil tree) methanol extract against selected fungal plant pathogens at different concentrations. The methanol extract of the ben oil tree possessed high potential in suppressing the growth of *G. boninense* and *R. solani* (both basidiomycetes), achieving 100% inhibition across all concentrations (Figure 6). The results further indicate that the plant extract exhibited 100% inhibition at 10% and above concentrations against *R. microporus* (basidiomycetes) and *P. palmivora* (oomycetes). In contrast, the methanol extract of the ben oil tree did not entirely inhibit the growth of fungal species classified under ascomycetes, namely Foc TR4 and *P. oryzae*, across the concentrations. However, 10% and above concentrations of the plant extract could score PIDG more than 80% against both pathogens, with their highest PIDG values recorded at 93.25% and 85%, respectively, at 20% concentration (Figure 6).

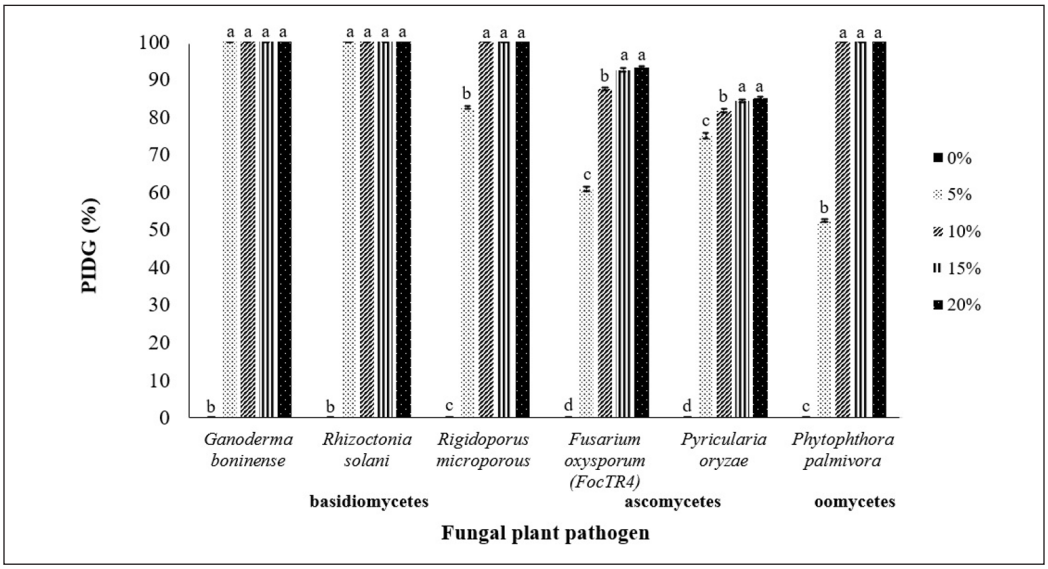

Figure 6. Percentage inhibition of diameter growth (PIDG) of *Moringa oleifera* against selected fungal plant pathogens

Note. Measurements were taken at different intervals: Three days after inoculation (DAI) for *Rhizoctonia solani*; 8 DAI for *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (Foc TR4) and *Phytophthora palmivora*; 9 DAI for *Ganoderma boninense*; and 11 DAI for *Pyricularia oryzae* and *Rigidoporus microporous*. Values are the means of five replicates. Means with the same letter are not significantly different at $p = 0.05$

Effect of *V. amygdalina* Crude Extract on Mycelial Growth of Selected Fungal Plant Pathogens

Figure 7 reveals the antifungal activity of *V. amygdalina* (bitter leaf) against selected pathogens from three distinct groups: basidiomycetes, ascomycetes and oomycetes. Bitter leaf extract exhibited significant potential in inhibiting the growth of *G. boninense* and *R. solani* (both basidiomycetes), as well as *P. oryzae* (ascomycetes), achieving complete inhibition (100%) across all concentrations (5%, 10%, 15% and 20%). The growth of *R. microporous* (basidiomycetes), Foc TR4 (ascomycetes) and *P. palmivora* (oomycetes) was entirely inhibited by the bitter leaf extract at concentrations of 10% and above. Notably, bitter leaf extract exhibited the capacity to suppress the mycelial growth of all tested pathogens at concentrations of 10% and higher (Figure 7).

Comparison of the Antifungal Activity of Plant Extracts Against Selected Fungal Plant Pathogens at Five Concentration Levels

Table 3 summarises the antifungal activities of several plant methanol extracts against selected fungal plant pathogens. The growth of *G. boninense* was completely inhibited by nearly all plant extracts (*A. indica*, *V. amygdalina*, *C. ternatea*, *P. amboinicus*, *M. citrifolia* and *M. oleifera*) at concentrations as low as 5%, except for *P. suberosa*, which required

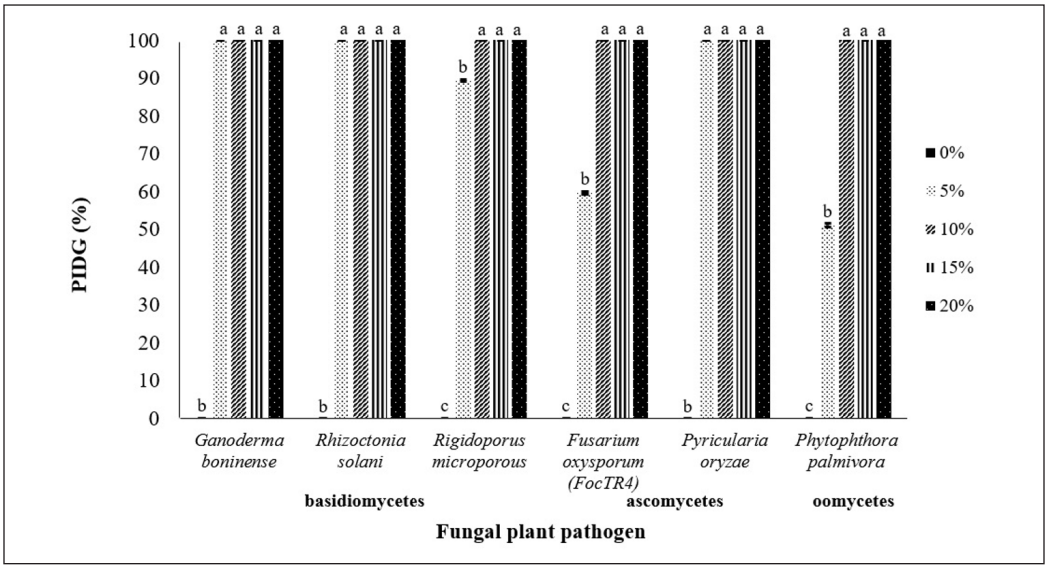

Figure 7. Percentage inhibition of diameter growth (PIDG) of *Vernonia amygdalina* against selected fungal plant pathogens

Note. Measurements were taken at different intervals: Three days after inoculation (DAI) for *Rhizoctonia solani*; 8 DAI for *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (Foc TR4) and *Phytophthora palmivora*; 9 DAI for *Ganoderma boninense*; and 11 DAI for *Pyricularia oryzae* and *Rigidoporus microporous*. Values are the means of five replicates. Means with the same letter are not significantly different at $p = 0.05$

a concentration of 15% or higher to achieve 100% inhibition. For *R. solani*, complete inhibition was observed with three plant extracts (*A. indica*, *V. amygdalina* and *M. oleifera*) at 5% concentration, while four other extracts (*C. ternatea*, *P. amboinicus*, *M. citrifolia* and *P. suberosa*) achieved 100% inhibition at 10% or higher. *R. microporous* was fully inhibited by 5% extracts of *A. indica* and *P. amboinicus*, while four other plant extracts (*V. amygdalina*, *C. ternatea*, *M. citrifolia* and *M. oleifera*) required a 10% concentration, and *P. suberosa* needed 20%. Complete inhibition of *P. oryzae* was achieved by the 5% extracts of *A. indica*, *V. amygdalina*, *P. amboinicus* and *P. suberosa*, followed by *C. ternatea* and *M. citrifolia*, achieving 100% inhibition at 10%, while *M. oleifera* at 20% resulted in only 85% inhibition (Table 3). The growth of *P. palmivora* was fully inhibited by *A. indica*, *C. ternatea* and *P. suberosa* at 5% concentration, followed by *V. amygdalina* and *M. oleifera* at 10%, *P. amboinicus* at 15%, and *M. citrifolia* at 20%. Regarding Foc TR4, nearly all plant extracts achieved 100% inhibition at a concentration of 10% or higher, except for *M. oleifera* and *P. suberosa*, which did not reach complete inhibition even at 20%, with PIDG values recorded at 74% and 93.25%, respectively (Table 3).

Comparing between plant species, the current findings indicate that higher concentrations (15% and 20%) generally resulted in better inhibition across all plant extracts. *A. indica* (neem) exhibited remarkable antifungal activity against all pathogens

Table 3
Maximum inhibition of seven methanol leaf extracts on the mycelial growth of selected fungal plant pathogens at different concentrations

	Fungal plant pathogen					
	Ganoderma boninense	***Rhizoctonia solani***	***Rigidoporus microporous***	***Pyricularia oryzae***	***Phytophthora palmivora***	***Fusarium oxysporum* (Foc TR4)**
Plant species	***PIDG %** (plant extract concentration)					
Azadirachta indica	100.00 ± 0.00[a] (5% and above)	100.00 ± 0.00[a] (5% and above)	100.00 ± 0.00[a] (5% and above)	100.00 ± 0.00[a] (5% and above)	100.00 ± 0.00[a] (5% and above)	100.00 ± 0.00[a] (10% and above)
Vernonia amygdalina	100.00 ± 0.00[a] (5% and above)	100.00 ± 0.00[a] (5% and above)	100.00 ± 0.00[a] (10% and above)	100.00 ± 0.00[a] (5% and above)	100.00 ± 0.00[a] (10% and above)	100.00 ± 0.00[a] (10% and above)
Clitoria ternatea	100.00 ± 0.00[a] (5% and above)	100.00 ± 0.00[a] (10% and above)	100.00 ± 0.00[a] (10% and above)	100.00 ± 0.00[a] (10% and above)	100.00 ± 0.00[a] (5% and above)	100.00 ± 0.00[a] (10% and above)
Plectranthus amboinicus	100.00 ± 0.00[a] (5% and above)	100.00 ± 0.00[a] (10% and above)	100.00 ± 0.00[a] (5% and above)	100.00 ± 0.00[a] (5% and above)	100.00 ± 0.00[a] (15% and above)	100.00 ± 0.00[a] (10% and above)
Morinda citrifolia	100.00 ± 0.00[a] (5% and above)	100.00 ± 0.00[a] (10% and above)	100.00 ± 0.00[a] (10% and above)	100.00 ± 0.00[a] (10% and above)	100.00 ± 0.00[a] (20%)	100.00 ± 0.00[a] (10% and above)
Moringa oleifera	100.00 ± 0.00[a] (5% and above)	100.00 ± 0.00[a] (5% and above)	100.00 ± 0.00[a] (10% and above)	85.00 ± 0.49[c] (20%)	100.00 ± 0.00[a] (10% and above)	93.25 ± 0.24[b] (20%)
Passiflora suberosa	100.00 ± 0.00[a] (15% and above)	100.00 ± 0.00[a] (10% and above)	100.00 ± 0.00[a] (15% and above)	100.00 ± 0.00[a] (5% and above)	100.00 ± 0.00[a] (5% and above)	74.00 ± 1.00[d] (20%)

*Values are the means of five replicates. Means with the same letter are not significantly different at $p = 0.05$

except Foc TR4 at 5%, achieving complete inhibition at 10% and above concentrations. This suggests that neem is a potent natural broad-spectrum antifungal agent, demonstrating maximum effectiveness even at relatively low concentrations (5%–10%). A similar trend was observed with *V. amygdalina* (bitter leaf), which displayed strong antifungal activity, particularly at concentrations of 10% or higher, achieving 100% inhibition against all fungal plant pathogens. However, at 5%, the extracts were less effective against *R. microporous*, *P. palmivora* and Foc TR4 (Table 3). *C. ternatea* (butterfly pea) also revealed promising results, with moderate antifungal activity at 5% but achieving 100% inhibition for all fungal plant pathogens at concentrations above 10%. *P. amboinicus* (Mexican mint) showed comparable efficacy, reaching complete inhibition at concentrations above 15%, with strong antifungal activity even at lower concentrations (Table 3). *M. citrifolia* (noni) exhibited good effectiveness at all concentrations, achieving full inhibition at 20%. Meanwhile,

M. oleifera (ben oil tree) revealed consistent antifungal performance, though it did not inhibit completely at 20% against *P. oryzae*. Lastly, *P. suberosa* (corkystem passionflower) demonstrated the mildest antifungal activity among the plants tested but still showed significant inhibition at higher concentrations.

DISCUSSION

In recent years, there has been a notable surge in the use of plant extracts as environmentally friendly treatments for plant diseases. Methanol has been recognised as the best solvent for extracting bioactive substances like phenolics, flavonoids, alkaloids, and terpenoids from plants (Truong et al., 2019). Despite limited research on the efficacy of the plant extracts used in this study against *G. boninense* and *R. microporus*, methanol typically yields higher concentrations of bioactive compounds compared to alternatives like ethanol or distilled water (Andleeb et al., 2020). The strong inhibitory effects observed in the plant extracts against *G. boninense* and *R. microporus* in this study are likely attributed to the efficacy of methanol extraction.

This study found that *A. indica* (neem), *M. oleifera* (ben oil tree) and *V. amygdalina* (bitter leaf) extracts achieved complete suppression of *R. solani* at all concentrations (5%, 10%, 15% and 20%). Ali et al. (2024) highlighted the susceptibility of *R. solani* to the methanol extract of A. indica, with an inhibition zone of 23 ± 00 mm. A. indica is also utilised in foliar spray at 3% neem oil concentration to manage sheath blight caused by *R. solani* (Kumar, 2020). Goss (2018) demonstrated significant suppression of *R. solani* and *F. solani* in lettuce through aqueous Moringa leaf, seed and bark extracts in greenhouse and field studies. Additionally, *V. amygdalina* methanol extract showed mild activity against *R. solani* (Ohigashi et al., 1991). Conversely, extracts from *P. amboinicus* (Mexican mint), *M. citrifolia* (noni), *C. ternatea* (butterfly pea) and *P. suberosa* (corkystem passionflower) inhibited *R. solani* by 100% at higher concentrations (10% onwards). Islam et al. (2023) reported that finotin, a protein from butterfly pea seeds, effectively inhibits various fungal pathogens, including *R. solani*. Meela et al. (2019) highlighted *P. suberosa*'s promising antifungal activity against multiple pathogens, including *R. solani*. The findings of this study align with those of Ali et al. (2024), Goss (2018), Islam et al. (2023), Kumar (2020), Meela et al. (2019) and Ohigashi et al. (1991), demonstrating the efficacy of these plant extracts against *R. solani* at different concentrations.

It was observed that *P. amboinicus* (Mexican mint), *M. citrifolia* (noni), *C. ternatea* (butterfly pea), *A. indica* (neem) and *V. amygdalina* (bitter leaf) extracts achieved 100% inhibition of Foc TR4 at concentrations ranging from 10% to 20%. However, *P. suberosa* (corkstem passionflower) and *M. oleifera* (ben oil tree) extracts did not achieve complete inhibition across all concentrations tested. Comparing these results to those of Malik et

al. (2021), M. oleifera has been reported to give the best results (59.33%), followed by *A. indica* (55.00%) in disease reduction at 40% concentration against *Fusarium oxysporum* f. sp. *capsica*. Meela et al. (2019) reported the promising antifungal activity of P. suberosa against various pathogens, including *F. oxysporum*. The findings reported by Meela et al. (2019) and Malik et al. (2021) contradict the results observed in this study for Foc TR4. These dissimilarities could arise from species diversity and varying virulence levels within *F. oxysporum*, a species complex with numerous formae speciales (f. sp.) and races specialised to infect specific host plants (Rana et al., 2017). Each forma specialis causes distinct diseases in different host plants, highlighting the pathogen's variability among these specialised forms. Overall, the efficacy of the plant extracts against Foc TR4 varied significantly across concentrations, suggesting the potential for increased efficacy at higher concentrations to inhibit Foc TR4 mycelial growth.

Based on the findings of this study, 100% suppression of *P. oryzae* was consistently achieved across all concentrations in the plant extracts of *P. amboinicus* (Mexican mint), *P. suberosa* (corkstem passionflower), *A. indica* (neem) and *V. amygdalina* (bitter leaf). This aligns with the findings by Agbowuro et al. (2020), who similarly reported the antifungal properties of neem extracts against rice blast disease caused by *P. oryzae*. However, *M. citrifolia* (noni) and *C. ternatea* (butterfly pea) extracts achieved complete inhibition at 10% and above concentrations, indicating high efficacy but slightly varied potency compared to the aforementioned extracts. Meanwhile, *M. oleifera* (ben oil tree) extract did not achieve complete inhibition across all concentrations tested but demonstrated inhibition above 75%. *M. oleifera* extracts have been reported by Ilanko et al. (2019) as weak inhibitors of bacterial growth when used alone but show significantly enhanced antibacterial efficacy when combined with conventional antibiotics.

Plant extracts of *C. ternatea* (butterfly pea), *P. suberosa* (corkstem passionflower) and *A. indica* (neem) achieved 100% suppression on *P. palmivora* across all concentrations tested. *M. oleifera* (ben oil tree) and *V. amygdalina* (bitter leaf) extracts showed full inhibition at 10%–20%, revealing their efficacy albeit at slightly higher concentrations. Yousaf et al. (2018) corroborated these findings, demonstrating that methanol leaf extracts of *A. indica* and *M. oleifera* effectively controlled the mycelial growth of *P. palmivora* at concentrations of 5%, 10% and 15%. Additionally, Chainanta et al. (2023) highlighted the potential of *V. amygdalina* ethyl acetate to suppress the growth of *P. palmivora*. In contrast, achieving complete inhibition of *P. palmivora* with *P. amboinicus* (Mexican mint) (15-20%) and *M. citrifolia* (noni) (20%) requires higher concentrations of methanol extracts. Yusoff et al. (2020) found that the concentration of the crude extract had the most significant impact on reducing mycelial growth and influencing the percentage of fungal inhibition in the modified PDA medium. This effect is likely related to the concentration of antifungal compounds in the crude extract.

CONCLUSION

The screening of seven plant extracts for fungitoxic potential showed promising results, with *A. indica* (neem), *V. amygdalina* (bitter leaf) and *C. ternatea* (butterfly pea) achieving complete inhibition against all tested fungal plant pathogens at concentrations of 10% and above (15% and 20%). Some pathogens required higher concentrations for effective suppression, particularly *Foc TR4*. Notably, *A. indica* demonstrated broad-spectrum efficacy, fully inhibiting five out of six pathogens at concentrations as low as 100 mg/ml, highlighting its potential to be developed as a versatile bio-fungicide. These findings suggest that these plant extracts could be eco-friendly alternatives to synthetic fungicides. Given the availability of these plant species in Malaysia, further research to isolate bioactive compounds and conduct field trials are recommended to assess their practical application as sustainable bio-fungicides to reduce reliance on agrochemicals.

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REFERENCES

Abu, F., Mat Taib, C. N., Mohd Moklas, M. A., & Mohd Akhir, S. (2017). Antioxidant properties of crude extract, partition extract, and fermented medium of *Dendrobium sabin* flower. *Evidence-Based Complementary and Alternative Medicine*, *2017*, 2907219. https://doi.org/10.1155/2017/2907219

Agbowuro, G. O., Aluko, M., Salami, A. E., & Awoyemi, S. O. (2020). Evaluation of different aqueous plant extracts against rice blast disease fungus (*Magnaporthe oryzae*). *Ife Journal of Science*, *22*(3), 198–202. https://doi.org/10.4314/ijs.v22i3.17

Ali, J., Hussain, A., Siddique, M., & Rahman, Z. U. (2024). Evaluation of some bioactive compounds of *Azadirachta indica* extracts and its application as safe fungicide against different plant pathogenic fungi. *International Journal of Engineering, Science and Technology*, *16*(1), 44–54. http://doi.org/10.4314/ijest.v16i1.5

Andleeb, S., Alsalme, A., Al-Zaqri, N., Warad, I., Alkahtani, J., & Bukhari, S. M. (2020). In-vitro antibacterial and antifungal properties of the organic solvent extract of *Argemone mexicana* L. *Journal of King Saud University-Science*, *32*(3), 2053–2058. https://doi.org/10.1016/j.jksus.2020.01.044

Andrew, B. (2020). *Characterization and genetic diversity of* Rigidoporus microporus *associated with white root rot disease in rubber tree* (Hevea brasiliensis müll.arg.). [Unpublished Master thesis]. Universiti Putra Malaysia.

Baibakova, E. V., Nefedjeva, E. E., Suska-Malawska, M., Wilk, M., Sevriukova G.A., & Zheltobriukhov, V. F. (2019). Modern fungicides: Mechanisms of action, fungal resistance and phytotoxic effects. *Annual Research & Review in Biology*, *32*(3), 1–16. https://doi.org/10.9734/ARRB/2019/V32I330083

Balouiri, M., Sadiki, M., & Ibnsouda, S. K. (2016). Methods for in vitro evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis*, *6*(2), 71–79. https://doi.org/10.1016/j.jpha.2015.11.005

Çakmakçı, R., Salık, M. A., & Çakmakçı, S. (2023). Assessment and principles of environmentally sustainable food and agriculture systems. *Agriculture*, *13*(5), 1073. https://doi.org/10.3390/agriculture13051073

Chainanta, J., Yakajay, K., Chinda, C., Intaparn, P., To-anun, C., Tipduangta, P., Sirithunyalug, B., Haituk, S., Nguanhom, J., Pusadee, T., Karunarathna, A., & Cheewangkoon, R. (2023). *Vernonia amygdalina* extract loaded microspheres for controlling *Phytophthora palmivora*. *Sustainability, 15*(14), 10842. https://doi.org/10.3390/su151410842

Clement, W. K. F., Vadamalai, G., Saidi, N. B., & Zulperi D. (2019). Research progress, challenges, future perspectives on the management of *fusarium* wilt of banana in Malaysia: A review: Disease management of *fusarium* wilt of banana in Malaysia. *Malaysian Journal of Science*, *38*(2), 47–66. https://doi.org/10.22452/mjs.vol38no2.4

Coulibaly, T. P, Du, J-G., & Diakité, D. (2021). Sustainable agricultural practices adoption. *Agriculture (Poľnohospodárstvo)*, *67*(4), 166−176. https://doi.org/10.2478/agri-2021-0015

Food and Agriculture Organization of the United Nations (2024). *Plant production and protection*. https://www.fao.org/plant-production-protection/about/en

Go, W. Z., Chin, K. L., H'ng, P. S., Wong, M. Y., Lee, C. L., & Khoo, P. S. (2023). Exploring the biocontrol efficacy of *Trichoderma* spp. against *Rigidoporus microporus*, the causal agent of white root rot disease in rubber trees (*Hevea brasiliensis*). *Plants*, *12(5)*, 1066. https://doi.org/10.3390/plants12051066

Goss, M. (2018). *The efficacy of Moringa oleifera plant extracts against selected fungal and bacterial plant pathogens infecting selected vegetable crops in Zimbabwe* [Doctoral dissertation, University of Kwazulu-Natal]. DSpace. https://researchspace.ukzn.ac.za/handle/10413/16712

Guest, D. (2007). Black pod: Diverse pathogens with a global impact on cocoa yield. *Phytopathology*, *97*, 1650–1653. https://doi.org/10.1094/PHYTO-97-12-1650

Hernández-Ceja, A., Loeza-Lara, P. D., Espinosa-García, F. J., García-Rodríguez, Y. M., Medina-Medrano, J. R., Gutiérrez-Hernández, G. F., & Ceja-Torres, L. F. (2021). *In vitro* antifungal activity of plant extracts on pathogenic fungi of blueberry (*Vaccinium* sp.). *Plants*, *10*, 852. https://doi.org/10.3390/plants10050852

Ilanko, P., McDonnell, P. A., van Vuuren, S., & Cock, I. E. (2019). Interactive antibacterial profile of *Moringa oleifera* Lam. extracts and conventional antibiotics against bacterial triggers of some autoimmune inflammatory diseases. *South African Journal of Botany*, *124*, 420–435. https://doi.org/10.1016/j.sajb.2019.04.008

Ilondu, E. M. (2013). Phytochemical composition and efficacy of ethanolic leaf extracts of some *Vernonia* species against two phytopathogenic fungi. *Journal of Biopesticides*, *6*(2), 165–172. https://doi.org/10.57182/jbiopestic.6.2.165-172

International Trade Administration (2024). *Malaysia - country commercial guide: Agriculture sector*. https://www.trade.gov/country-commercial-guides/malaysia-agricultural-sector

Isha, A., Yusof, N. A., Shaari, K., Osman, R., Abdullah, S. N. A., & Wong, M. Y. (2020). Metabolites identification of oil palm roots infected with *Ganoderma boninense* using GC–MS-based metabolomics. *Arabian Journal of Chemistry*, *13*(7), 6191–6200. https://doi.org/10.1016/j.arabjc.2020.05.026

Islam, M. A., Mondal, S. K., Islam, S., Shorna, M. N. A., Biswas, S., Uddin, M. S., Zaman, S., & Saleh, M. A. (2023). Antioxidant, cytotoxicity, antimicrobial activity, and in silico analysis of the methanolic leaf and flower extracts of *Clitoria ternatea*. *Biochemistry Research International*, *2023*, 8847876. https://doi.org/10.1155/2023/8847876

Kumar, C. V. (2020). Use of neem extracts (*Azadirachta indica*) in control of plant diseases: A review. *International Journal of Current Microbiology and Applied Sciences*, *11*, 3481–3487.

Kursa, W., Jamiołkowska, A., Wyrostek, J., & Kowalski, R. (2022). Antifungal effect of plant extracts on the growth of the cereal pathogen *Fusarium* spp.—An in vitro study. *Agronomy*, *12*, 3204. https://doi.org/10.3390/agronomy12123204

Latifah, M., Kamaruzaman, S., Zainal Abidin, M. A., & Nusaibah, S. A. (2018). Identification of *Phytophthora* spp. from perennial crops in Malaysia, its pathogenicity and cross-pathogenicity. *Sains Malaysiana*, *47*(5), 909–921. http://doi.org/10.17576/jsm-2018-4705-06

Lee, S. H., Oh, Y. T., Lee, D-Y., Cho, E., Hwang, B. S., & Jeon, J. (2022). Large-scale screening of the plant extracts for antifungal activity against the plant pathogenic fungi. *The Plant Pathology Journal*, *38*(6), 685–691. https://doi.org/10.5423/PPJ.NT.07.2022.0098

Malaysian Palm Oil Board. (2022). *Basal stem rot (BSR) disease of oil palm*. http://sawitsecure.mpob.gov.my/ganoderma-basal-stem-rot/

Malik, N., Habib, A., Malik, M. K., Zubair, M., Ali, S., Khan, Q. A. T., Qamar, H., Hanif, K., Iqbal, S., & Ul Haq, M. A. (2021). Efficacy of different plant extracts on *Fusarium* wilt caused by *Fusarium oxysporum* in chillies under field condition. *International Journal of Advanced Research in Biological Sciences*, *8*(12), 118–122. http://doi.org/10.22192/ijarbs.2021.08.12.012

Meela, M. M., Mdee, L. K., Masoko, P., & Eloff, J. N. (2019). Acetone leaf extracts of seven invasive weeds have promising activity against eight important plant fungal pathogens. *South African Journal of Botany*, *121*, 442–446. https://doi.org/10.1016/j.sajb.2018.12.007

Mohiddin, F. A., Bhat, N. A., Wani, S. H., Bhat, A. H., Ahanger, M. A., Shikari, A. B., Sofi, N. R., Parveen, S., Khan, G. H., Bashir, Z., Vachova, P., Hassan, S., & Sabagh, A. E. (2021). Combination of Strobilurin and Triazole chemicals for the management of blast disease in Mushk Budji -Aromatic rice. *Journal of Fungi*, *7*(12), 1060. https://doi.org/10.3390/jof7121060

Nalley, L., Tsiboe, F., Durand-Morat, A., Shew, A., & Thoma, G. (2016). Economic and Environmental Impact of Rice Blast Pathogen (*Magnaporthe oryzae*) Alleviation in the United States. *PLoS One*, *11*(12), e0167295. https://doi.org/10.1371/journal.pone.0167295

Ohigashi, H., Takagaki, T., Koshimizu, K., Watanabe, K., Kaji, M., Hoshino, J., Nishida, T., Huffman, M. A., Takasaki, H., Jato, J., & Muanza, D. N. (1991). Biological activities of plant extracts from tropical Africa. *African Studies Monographs*, *12*, 201–210.

Peng, Y., Li, S. J., Yan, J., Tang, Y., Cheng, J. P., Gao, A. J., Yao, X., Ruan, J. J., & Xu, B. L. (2021). Research progress on phytopathogenic fungi and their role as biocontrol agents. *Frontiers in Microbiology*, *12*, 670135. https://doi.org/10.3389/fmicb.2021.670135

Perrine-Walker, F. (2020). *Phytophthora palmivora*-Cocoa Interaction. *Journal of Fungi*, *6*, 167. https://doi.org/10.3390/jof6030167

Rana, A., Sahgal, M., & Johri, B. N. (2017). *Fusarium oxysporum*: Genomics, diversity and plant–host interaction. In T. Satyanarayana, S. Deshmukh & B. Johri (Eds.), *Developments in fungal biology and applied mycology*. Springer. https://doi.org/10.1007/978-981-10-4768-8_10

Scheerer, L., Pemsl, D., Dita, M., Vicente, L. Perez & Staver, C. (2018). A quantified approach to projecting losses caused by *Fusarium* wilt Tropical race 4. *Acta Horticulturae*, *1196*, 211-218. https://doi.org/10.17660/ActaHortic.2018.1196.26

Sofo, A., Zanella, A., & Ponge, J. F. (2022). Soil quality and fertility in sustainable agriculture, with a contribution to the biological classification of agricultural soils. *Soil Use and Management*, *38*(2), 10851112. https://doi.org/10.1111/sum.12702

Truong, D. H., Nguyen, D. H., Ta, N. T. A., Bui, A. V., Do, T. H., & Nguyen, H. C. (2019). Evaluation of the use of different solvents for phytochemical constituents, antioxidants, and in vitro anti-inflammatory activities of *Severinia buxifolia*. *Journal of Food Quality*, *2019*, 8178294. https://doi.org/10.1155/2019/8178294

Wong, M. Y., Hamid, S., Iskandar Shah, N. A., & Ab Razak, N. H. (2020). Botanical extracts as biofungicides against fungal pathogens of rice. *Pertanika Journal of Tropical Agricultural Science*, *43*(4), 457–466. https://doi.org/10.47836/pjtas.43.4.03

Wong, M. Y., Surendran, A., Saad, N. M., & Burhanudin, F. (2020). Chitosan as a biopesticide against rice (*Oryza sativa*) fungal pathogens, *Pyricularia oryzae* and *Rhizoctonia solani*. *Pertanika Journal of Tropical Agricultural Science*, *43*(3), 275–287.

Wong, M. Y., Hamid, S., Shah, N. A. I., Rahim, N. M., Kasim, Z. Z., & Razak, N. H. A. (2020

). Antifungal potential of six herbal plants against selected plant fungal pathogens. *Pertanika Journal of Tropical Agricultural Science*, *43*(2), 107–117.

Yousaf, Q., Subhani, M. N., Iqbal, M. Z., & Chattha, M. B. (2018). Effectiveness of fungicides, methanolic botanical extracts and antagonists against invasive *Phytophthora palmivora* inciting leaf spots of chinese fan palm. *Bionature*, *38*(6), 376–385.

Yusoff, S. F., Haron, F. F., Tengku Muda Mohamed, M., Asib, N., Sakimin, S. Z., Abu Kassim, F., & Ismail, S. I. (2020). Antifungal activity and phytochemical screening of *Vernonia amygdalina* extract against *Botrytis cinerea* causing gray mold disease on tomato fruits. *Biology*, *9*(9), 286. https://doi.org/10.3390/biology9090286

Zhu, G., Liang, E. X., Lan, X., Li, Q., Qian, J. J., Tao, H. X., Zhang, M. J., Xiao, N., Zuo, S. M., Chen, J. M., & Gao, Y. (2019). ZmPGIP3 gene encodes a polygalacturonase-inhibiting protein that enhances resistance to sheath blight in rice. *Phytopathology*, *109*, 1732–1740. https://doi.org/10.1094/PHYTO-01-19-0008-R

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Review Article

Freshwater Snails in Malaysia: Diversity, Roles in Trematode Transmission, and Agricultural Impacts

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ABSTRACT

Freshwater snails play a critical role as intermediate hosts in the transmission of trematode diseases, affecting human and animal health and posing challenges to agriculture. This review summarises studies on freshwater snails and trematode diseases in Malaysia from 1973 to 2024, based on comprehensive literature searches across multiple databases, including Web of Science, PubMed, Scopus, and Google Scholar. A total of 32 snail species were identified, with *Radix rubiginosa* hosting five types of cercariae and *Robertsiella silvicola* acting as a host for furcocercous cercariae. Economically significant species, such as *Pomacea maculata* and *Pomacea canaliculata*, were highlighted for their agricultural impact. While treatments for trematode infections are available, prevention through controlling snail populations remains essential due to the complex transmission involving reservoir hosts. This study underscores the zoonotic potential of trematode parasites in Malaysia and identifies a significant research gap regarding intermediate host snails.

Keywords: Freshwater snails, trematode, Malaysia, overview

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INTRODUCTION

Snails are prevalent in freshwater environments across tropical and subtropical regions, notably Southeast Asia (Böhm et al., 2021). Scholars suggest that more than 350 snail species may have medical or

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veterinary significance (Madsen & Hung, 2014). Around 45 species from 26 genera and seven families in Southeast Asia are implicated in transmitting trematodes (Lu et al., 2018; Madsen & Hung, 2014).

Mud and freshwater snails act as obligatory intermediate hosts for approximately 71 trematode parasites (Lu et al., 2018; Phiri et al., 2007). These parasites include members of the families Clinostomidae, Echinostomatidae, Fasciolidae, Schistosomatidae, Paramphistomidae, and Pronocephalidae (Tookhy et al., 2023; Saijuntha et al., 2021). In humans and animals, snails, for example, the *Bulinus, Biomphalaria, Lymñaea* and *Oncomelania* (Tookhy et al., 2023; Martin & Cabera, 2018; Dida et al., 2014; Dung et al., 2013) serve as the common intermediate parasitic hosts. Furthermore, according to Yahaya et al. (2017) and Greene (2018), some particular species of snails, for example, apple snails, constitute a major challenge as they can destroy rice plantations.

As reported by Lu et al. (2018), Prasopdee et al. (2015), and Tookhy et al. (2023), infections by some trematodes are an example of parasitic diseases transmitted by snails, and they represent an important health challenge to people and livestock, which can lead to socioeconomic losses. According to Martin and Cabera (2018), the exchange of nutrients between aquatic and terrestrial systems is being expedited by the roles played by some freshwater snails in the aquatic environment.

Among the foregoing is a critical fascioliasis disease by *Fasciola hepatica* and *Fasciola gigantica*, from which man and livestock suffer (World Health Organization [WHO], 2020). Almost two million and four hundred thousand people in about 700 nations of the world, by estimation, are victims of fascioliasis (WHO, 2018). In the same vein, other zoonoses and neglected tropical diseases, such as schistosomiasis, kill about 200 million people yearly, affect around 230 million individuals, and more than 700 million people are at higher risk of being infected globally (Colley et al., 2014; WHO, 2016). The diagnosis of schistosomiasis has been made more difficult by the massive human population drift to the western part of the globe.

Although there are reports on human and livestock trematode infections in Malaysia, there is, however, grossly inadequate research that specifically addresses the role and impact of snails in the transmission of this parasite. Attwood et al. (2005), Greer et al. (1988), and Tookhy et al. (2023) have documented the significance of human medical and veterinary trematodes. In-depth findings into the roles played by snails in the transmission of this parasite in the environment are lacking. This is a knowledge gap that has hampered the proper understanding and implementation of appropriate snail control measures to manage and prevent disease transmission.

Therefore, this work aims to provide a comprehensive overview of Malaysian freshwater snails and their role as intermediate hosts in the transmission of trematode parasites. By a critical review of the ecology and epidemiology of freshwater snails, their importance in human and livestock health will be discussed, as well as specific control measures.

OVERVIEW OF TREMATODE INFECTIONS IN MALAYSIA

Different species of trematodes, such as *Fasciola* spp., have been documented in various locations and have been encountered in many states in Malaysia. Among the cattle in Terengganu, and according to studies by Khadijah et al. (2017), Rita et al. (2017), and Saad et al. (2019), *Fasciola* infection has been established and reported; the infection has also been established in buffalo along with cattle in Sabah (Kamaruddin et al., 2021). In Pahang, as documented by Rosilawati et al. (2017), *Fasciola* infections were predominant in buffalo, while the study of Diyana et al. (2020) has reported the infection in cattle and buffalo in Selangor and Perak. In Kuala Lumpur, *Fasciola* infections have been reported in humans by Naresh et al. (2006). Infection by the rumen fluke has been reported in cattle and buffalo from Terengganu (Kahdijah et al., 2021; Rita et al., 2017; Saad et al., 2019); Sarawak (Harizt et al., 2021) and Perak (Tookhy et al., 2024). Furthermore, in Perak, a 2% mortality rate was reported among local cattle (Debbra et al., 2018), and in Johor, among buffalo (Jamnah et al., 2013). Infection due to *Paragonimus westermani* was documented in Selangor when *Brotia costula* was established as an intermediate host (Kim, 1978); this infection has also been found in tigers in Selangor (Lee, 1965).

Schistosoma spp. infections have been documented across different regions and hosts, with *Schistosoma malayensis* infections in humans reported in Selangor and Pahang, the latter also showing infections with *Robertsiella* spp. (Attwood et al., 2005; Greer et al., 1988; Latif et al., 2013). *Schistosoma japonicum* infections have been observed in humans in Peninsular Malaysia (Greer & Anuar, 1984), while a variety of *Schistosoma* spp. and other trematodes have been reported in wildlife, with no associated mortality. Notably, the intermediate host is unknown in Malaysia for most of the above trematodes.

The overview of the pattern of distribution and identification methods, comprising conventional morphological assessment and molecular analyses such as polymerase chain reaction (PCR), for different species of snails found in various aquatic ecosystems across Malaysia is shown in Table 1. Details such as species nomenclature, geographical location, preferred habitats (wetlands, rivers or streams), and identifiable types of cercaria, where possible. Notably, this study from Peninsular Malaysia highlighted a relatively small

Table 1
Snail species reported in different water resources based on different methods and their larval stage

Snail	Location	Water resources	Cercarial	Identification	References
Radix rubiginosa	Perak	Wetlands	*Echinostome, gymnocephalus, xiphidiocercariae, furcocercous*	Morphological ID and PCR (COI)	Tookhy et al. (2023)
Robertsiella silvicola	Perak	Stream	*Furcocercous cercaria*	Morphological ID	Attwood et al. (2005)

Table 1 *(continue)*

Snail	Location	Water resources	Cercarial	Identification	References
Brotia costula	Kuala Lumpur	Stream		Morphological ID	Kim (1978)
Melanoides tuberculata	Perak	Stream			Attwood et al. (2005)
Brotia costula *Thiara scabra* *Pila ampullaceal* *Bithynia* *Pygmaea* *Stenothyra cambodiensis* *Gyraulus chinensis* *R. gismanni* *R. kaporensis*					
Pomacea maculata *Pomacea canaliculata*	Peninsular	Rivers, streams, lakes, ponds	-	PCR (COI)	Hah et al. (2022)
Pomacea maculata *Pomacea canaliculata*	Selangor	Wetlands	-	PCR (COI)	Phoong et al. (2018)
P. canaliculate *P. maculata*	Peninsular	Pond, lakes, rice fields	-	SM and PCR (COI)	Rao et al. (2018)
Pomacea maculata	Selangor,	Rice fields	-	Morphological ID	Arfan et al. (2014)
Pomacea canaliculata	Perak, Penang, Kedah and Kelantan				
Physella acuta *Indoplanorbis exustus* *Melanoides tuberculata* *Sinotaia guangdungensis* *Pila ampullacea* *Pila scutate* *Clea bangueyensis* *Neritina pulligera* *Septaria porcellana* *Vittina variegate* *Coromandeliana* *Sulcospira pageli* *Paludomus everetti* *Paludomus luteus* *Mieniplotia scabra* *Tarebia granifera*	Sabah	Different water bodies	-	Morphological ID	Ng et al. (2017)

number of freshwater snail species, with only 15 species identified through a combination of molecular and morphological approaches. Through morphological evaluation, 17 species were identified in Sabah, a region in Malaysia rich in a variety of freshwater snails. It was observed that cercarial types were reported in only two species, *R. rubiginosa* and *R. silvicola*, suggesting that they play a role in Malaysian freshwater ecosystems. All the cercaria reported are zoonotic, suggesting they are of public importance.

THE IMPORTANT SNAIL FAMILIES PRESENT IN MALAYSIAN FRESHWATER ENVIRONMENTS

Family: Lymnaidae
Genus: Lymnea
Species: *Radix rubiginosa*

Earlier, the presence of *R. rubiginosa* and cercariae in the wetlands of Perak was established using both morphological and molecular (PCR, ITS2) methodologies by Tookhy et al. (2023). The identification of cercariae, such as *Echinostome, Gymnacephalous, Xiphidiocercariae,* and *Furocercous,* corroborates the ecological diversity within this environment (Tookhy et al., 2023). Additionally, Tookhy et al. (2023) have reported that *R. rubiginosa* can accommodate the larval stage of *Schistosoma* spp., which causes human cercarial dermatitis in Malaysia. The freshwater snail of the *Lymnaeidae* family has gained enormous interest because of its involvement in the life cycle of different trematodes, which have great biomedical and veterinary importance (Bargues et al., 2016; Saijuntha et al., 2021).

Family: Pomatiopsidae
Genus: Robertsiella
Species: *Robertsiella silvicola*

As reported by Attwood et al. (2005), the small size, lack of a defined spiral macrosculpture on the shell, and distinctive style characteristics are suggestive of *R. silvicola,* differentiating it from other species in the *Robertsiella* genus. This species of snail can be found in different aquatic environments ranging from diminutive, spring-fed, first-order streams to meagre rivulets originating from forest hillsides. The occurrence of this snail species is restricted to limestone regions surrounding the mountainous bases of the Perak and Kedah states in West Malaysia. It is important that *R. silvicola* display affinity to serve as an intermediate host for the parasitic blood fluke *S. malayensis* that infects humans and livestock (Attwood et al., 2005).

Family: Ampullariidae
Genus: Pomacea
Species: *Pomacea maculata* and *Pomacea canaliculate*

It has been reported that *Pomacea maculate* and *P. canaliculate* have been isolated in diverse bodies of water such as streams, rivers, lakes, wetlands and ponds within Peninsular

Malaysia (Hah et al., 2022; Rao et al., 2018) as well as in Selangor (Arfan et al., 2014; Phoong et al., 2018). In this study, PCR (COI) confirmed the presence of these two species of snails, suggesting that they can adapt and be found in various ecosystems.

The financial loss and damage caused by apple snails in rice farming are enormous; hence, their management has taken centre stage (Yahaya et al., 2017). The feeding patterns of these two snail species, which consume aquatic plants, thereby altering the food chain, may significantly impact the community structure, water quality, and functionality of aquatic ecosystems in wetlands (Horgan et al., 2014).

Family: Thiaridae
Genus: Melanoides
Species: *Melanoides tuberculate*

The *M. tuberculate,* commonly known as red-rimmed Melania or the Malaysia Trumpet Snail (Müller, 1774), is a benthic freshwater gastropod of the *Thiaridae* family. According to a report by Krailas et al. (2014), *M. tuberculate* is indigenous to Asia and Africa. Naturally, this species is widely distributed, including Africa, the East Indies, southern Asia, northern Australia, Madagascar, the Mediterranean, and the Pacific Islands (Maciaszek et al., 2019; Facon et al., 2003).

The presence of *M. tuberculata* (Müller, 1774) has been documented by Piechocki et al. (2003) in Europe (Hungary, Germany, Malta, the Netherlands, Spain and Austria), where it has been identified as an intermediate host for many trematode species, some of these trematodes are of paramount importance in human and livestock health (Francis, 2012; Lopes et al., 2021; Post et al., 2022).In Malaysia, and as documented by Ng et al. (2017), the prevalence of *M. tuberculata* cut across various regions, such as offshore islands like Tenom, Kota Kinabalu, Tuaran, Pulau Gaya, Tawau, Pulau Tiga, Kota Belud, Pulau Bodgaya near Semporna, and Pulau Bohey Dulang. It inhabits rivers, paddy fields, and man-made concrete drains.

Family: Planorbidae
Genus: Indoplanorbis
Species: *Indoplanorbis exustus*

Indoplanorbis exustus is widely found throughout Southeast Asia, especially in rice plantations, and it plays the role of an intermediate host for some trematodes (Dumidae et al., 2024; Bawm et al., 2022; Nguyen et al., 2022). Following harvest, farmers often introduce ducks into their rice fields to consume these snails, thereby facilitating the maintenance and completion of the parasitic trematode life cycle through a zoonotic route. Thailand has reported a notable prevalence of *Echinostoma* infections in free-ranging ducks, as documented by Saijuntha et al. (2013). *Indoplanorbis exustus* also act as intermediate host

of furcocercous cercaria (*Schistosome cercariae*) (Dumidae et al., 2024). In Peninsular Malaysia, this species has been demonstrated to harbour *S. spindale*, which is responsible for causing cercarial dermatitis in humans who become infected (Chiew et al., 2009).

ROLE OF SNAILS IN AGRICULTURE

Freshwater snails exhibit a dual role in agriculture, beneficial and detrimental, depending on the context. Economically, they are valued in rural communities as a source of food and traditional medicine (Gupta & Khanal, 2024). Nonetheless, a species such as *Pomacea canaliculate,* commonly called the golden apple snail, is a stubborn pest and a threat to agricultural success, predominantly in rice cultivation, by extensively damaging the shoots of young rice plantations, which results in a gross reduction in crop yields (Joshi et al., 2020). The combination of these roles highlights the complication of their impacts on the environment and the economy.

Yusa et al. (2006) have highlighted that *Pomacea canaliculata* is a main pest in Southeast Asia; however, it is less encountered, with less associated damage in other regions with lower annual precipitation, like parts of South Asia. Variations in climate, crop management, or the presence of a predator could have been the reason for this. Therefore, this suggests that the environment and geographical locations might have meaningfully impacted the level of infestation and resultant damage occasioned by freshwater snails.

Considering the level of loss in high-yielding crops, as reported by Kumar (2020) and Yusa et al. (2006), this urgently calls for effective and operational control measures. However, the differences in loss levels across regions and studies could be attributed to variations in farming systems, snail population densities, and mitigation strategies. For instance, integrated pest management methods that incorporate natural predators and manual removal are promising in reducing infestations (Thakuri et al., 2019). The report states that snails contaminate agricultural by-products with zoonotic parasites after they have damaged the by-product (Kumar, 2020). In the area of food security and livestock health, this perspective could have broader implications, especially in resource-limited settings. It is believed that if this study is compared with other studies on the dynamics of parasite transmission in other snail species, there could be better clarity. Unexpectedly, some studies have reported increased snail populations during dry spells within the rainy season (Faiz, 2010). It could indicate adaptive behaviour or resilience in certain species, necessitating further research on the triggers and ecological factors that enable such growth.

One potential strategy for mitigating the negative impacts of freshwater snails while leveraging their benefits involves utilising them as food and animal feed sources. This approach reduces their population and provides economic incentives for farmers. There could be better insights if studies on the nutritional and economic feasibility of snail-derived feed were compared against those of traditional livestock feed.

THE ROLE OF SNAIL IN MEDICINE AND VETERINARY PRACTICE

From time immemorial, snails have been known for their potent therapeutic properties, principally in traditional medicine. For instance, the mucus of some snail species, such as *Helix aspersa* and *Eremina desertorum,* is rich in bioactive compounds that have anti-inflammatory and antimicrobial effects by regulating transforming growth factor beta 1 (TGF-β1) and vascular endothelial growth factor (VEGF) gene expression that promotes wound healing (El-Zawawy & Mona, 2021). Also, Noothuan et al. (2021) have reported the antibacterial, antioxidant, and anti-tyrosinase properties demonstrated by mucus from *Hemiplecta distincta* and *Lissachatina fulica*, while available documents on *Helix aspersa* show it is effective in the treatment of gastric ulcers and reduction of oxidative stress, inflammation, and skin conditions (Gugliandolo et al., 2021).

Furthermore, lipids in *Bellamya benghalensis*, comprising oleic acid and cyclopropane fatty acids, possess anti-inflammatory potential for managing immune-related diseases, such as rheumatism, through the modulation of macrophage activity (Bhattacharya et al., 2014). Antimicrobial properties against infection, such as whooping cough, have been demonstrated in snail meat (Cobbinah et al., 2008). Furthermore, cancer-combating potential has been raised in the mucus of *Achatina fulica* (EFSA Panel on Nutrition, Novel Foods and Food Allergens et al., 2024). However, concerns about the consistency and safety of *Helix aspersa* maxima mucus as a novel food, according to the European Food Safety Authority (Adeyeye et al., 2020). Despite these challenges, snails are promising for medicinal purposes and as a novel food or feed source.

In animal production, snails can serve as an unconventional protein source, as they can be raised on low-grade biowastes and bioresources (Pathak et al., 2024). Therefore, they are a valuable and sustainable option for animal feed, as they reduce reliance on conventional feed resources (International Feed Industry Federation, 2021). Conversely, these snails also serve as a reservoir for zoonotic parasites and other pathogenic microbes, constituting health threats such as Fascioliasis, Schistosomiasis, Angiostrongyliasis and microbial diseases like Gastroenteritis and Rat-bite fever (Tookhy et al., 2024; Pathak et al., 2024). Specifically, ruminants and humans are susceptible to liver fluke disease (Lee et al., 2017). While snail farming presents opportunities for sustainable food and feed production, these health risks will necessitate careful and effective management to ensure the safe commercialisation of snails.

GENETIC CHARACTERISATION OF SNAIL

The extinction of some species has been a continuous event in the long history of nature, driven by various natural processes and human activities (Ceballos et al., 2017; Barnosky et al., 2011). Currently, the five leading drivers of species extinction are well-documented: habitat loss, invasive species, exploitation, disease, and climate change (Sodhi et al., 2009).

The most diverse group of animals, next to arthropods, is the molluscs, which are principally vulnerable, with an estimated number of known species falling between 70,000 and 120,000. The International Union for Conservation of Nature (IUCN) Red List has documented about 83,125 mollusc species, out of which 8,934 have been evaluated, with about 2,340 species (11%) currently on the verge of extinction (Chapman, 2009; IUCN, 2021).

Freshwater snails are a critical component of marine bionetworks. However, their environment is highly fragile, and coupled with the rising pressures due to man's activities, they can easily become extinct (Johnson et al., 2013; Strayer & Dudgeon, 2010). For example, lymnaeid snails display considerable eco-phenotypic plasticity with highly varying shell morphology, which is intensely affected by the environment (Hurtrez-Bouss et al., 2005). This variability can confound the taxonomy using only shell characteristics, as morphological variations may lead to misclassification (Vinarski et al., 2020).

The standard tools for accurately identifying species, particularly when morphological characteristics are insufficient, are molecular phylogenetics and molecular taxonomy (Japa et al., 2021). The commonly employed genetic markers like the internal transcribed spacer 2 (ITS2), mitochondrial cytochrome c oxidase subunit I (COI) and 16s rRNA genes were known to be reliable in the study of lymnaeid snails (Tookhy et al., 2023; Japa et al., 2021). These are valuable molecular markers for species identification, investigating genetic divergence, and understanding the structural relationships within snail populations. This methodology offers a more accurate and reliable understanding of the taxonomy and genetics of freshwater snails (Mirfendereski et al., 2021; Vinarski et al., 2020).

SNAIL CONTROL

Methods for the control of snails include physical, chemical, and biological methods (Lu et al., 2018). Physical control measures target the reduction of snail populations by effective management of the environment (Garba Djirmay et al., 2024). For example, the removal of natural bodies of water, like the marshes and ponds, and controlling human settlements in high-risk zones are effective approaches. In other areas, the spread of *Schistosoma haematobium* and *Schistosoma japonicuma* has been significantly brought low employing appropriate drainage systems and environmental engineering, such as upsetting epilithic snail habitats by the use of boat-mounted rototillers or tractors with rakes, which is also effective in eliminating a substantial portion of snail populations (Li et al., 2016). Other methods, such as removing bird roosting sites, introducing mechanised farming, and rotating between aquatic and xerophytic crops, help further reduce snail populations (Leighton et al., 2000).

In chemical control, molluscicides are a widely used method since they rapidly decimate snail populations, thereby reducing disease transmission (Xia et al., 2014). However, its long-lasting effect is a function of sustained application (Maes et al., 2021).

Chemical control measures typically involve the use of synthetic or natural molluscicides, and this is one of the most effective methods for managing snail populations (Xia et al., 2014). Since the 1950s through the 1970s, compounds such as sodium pentachlorophenate (NaPCP), copper sulfate, Ntritylmorpholine, and niclosamide (Bayluscide) were widely used, especially in the control of snails responsible for the transmission of schistosomiasis in regions of Asia, Africa, and South America (King et al., 2015).

The biological control of molluscs is a more sustainable approach, where natural predators, parasites, or competitors to subdue the number of snails are introduced (Younes et al., 2017). Predatory fish, ducks, or some kind of insects can be employed to control the snail population; nonetheless, success is a function of the availability of appropriate predators and the ability to maintain ecological balance (Sokolow et al., 2015). Predatory prawns, like *Macrobrachium vollenhoveni*, have been used to reduce infected snails and schistosomiasis transmission in Senegal (Sokolow et al., 2014). The water bug Sphaerodema urinator and black carp (*Mylopharyngodon piceus*) have been reported to effectively control snails that host parasitic diseases (Younes et al., 2017). Even though these techniques display potential, careful management is required to prevent negative impacts on human health. Biological control benefits humans and the environment (Hung et al., 2013).

CONCLUSION

This literature review on freshwater snails and trematode diseases in Malaysia has highlighted the complex roles these snails play in human and animal health, as well as in agriculture. The findings of this study underscore the multiplicity of snail species in the region and their association with diverse trematode infections, including zoonotic parasites. Moreover, the economic importance of certain snail species in agriculture stresses the need for effective control procedures to prevent the spread of risks. Conversely, despite advancements in medical treatments for trematode diseases, preventive schemes that target intermediate hosts are of great importance, given the complexity of transmission dynamics involving the final hosts. The paucity of research centred on snails as intermediate hosts suggests an essential area for further exploration to improve the understanding of disease transmission, hence facilitating concerted control efforts. Employing integrated control methods, physical, chemical, and biological, can help avoid the negative impacts of snails while promoting sustainable practices. A well-adjusted approach is fundamental to maximising the benefits of freshwater snails while minimising their challenges.

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REFERENCES

Adeyeye, S. A. O., Bolaji, O. T., Abegunde, T. A., & Adesina, T. O. (2020). Processing and utilization of snail meat in alleviating protein malnutrition in Africa: A review. *Nutrition & Food Science*, *50*(6), 1085-1097. https://doi.org/10.1108/NFS-10-2019-0303

Arfan, A., Muhamad, R., Omar, D., Azwady, A., & Manjeri, G. (2014). Distribution of two *Pomacea* spp. in Rice Fields of Peninsular Malaysia. *Annual Research and Review in Biology*, *4*(24), 4123-4136. https://doi.org/10.9734/arrb/2014/11398

Attwood, S. W., Lokman, H. S., & Ong, K. Y. (2005). *Robertsiella silvicola*, a new species of triculine snail (Caenogastropoda: Pomatiopsidae) from peninsular Malaysia, intermediate host of *Schistosoma malayensis* (Trematoda: Digenea). *Journal of Molluscan Studies*, *71*(4), 379-391. https://doi.org/10.1093/mollus/eyi040

Bargues, M. D., Malandrini, J. B., Artigas, P., Soria, C. C., Velásquez, J. N., Carnevale, S., Mateo, L., Khoubbane, M., & Mas-Coma, S. (2016). Human fascioliasis endemic areas in Argentina: Multigene characterisation of the lymnaeid vectors and climatic-environmental assessment of the transmission pattern. *Parasites and Vectors*, *9*(1), 1-20. https://doi.org/10.1186/s13071-016-1589-z

Bawm, S., Khaing, N. H. E., Win, S. Y., Thein, S. S., Khaing, Y., Thaw, Y. N., Soe, N. C., Chel, H. M., Hmoon, M. M., Hayashi, N., Htun, L. L., Katakura, K., Nonaka, N., & Nakao, R. (2022). Morphological and molecular identification of trematode cercariae related with humans and animal health in freshwater snails from a lake and a dam in Myanmar. *Parasitology Research*, *121*(2), 653-665. https://doi.org/10.1007/s00436-022-07428-4

Barnosky, A. D., Matzke, N., Tomiya, S., Wogan, G. O., Swartz, B., Quental, T. B., & Ferrer, E. A. (2011). Has the Earth's sixth mass extinction already arrived? *Nature*, *471*(7336), 51-57. https://doi.org/10.1038/nature09678

Bhattacharya, S., Chakraborty, M., Bose, M., Mukherjee, D., Roychoudhury, A., Dhar, P., & Mishra, R. (2014). Indian freshwater edible snail *Bellamya bengalensis* lipid extract prevents T cell-mediated hypersensitivity and inhibits LPS-induced macrophage activation. *Journal of Ethnopharmacology*, *157*, 320-329. https://doi.org/10.1016/j.jep.2014.09.012

Böhm, M., Dewhurst-Richman, N. I., Seddon, M., Ledger, S. E. H., Albrecht, C., Allen, D., Bogan, A. E., Cordeiro, J., Cummings, K. S., Cuttelod, A., Darrigran, G., Darwall, W., Fehér, Z., Gibson, C., Graf, D. L., Köhler, F., Lopes-Lima, M., Pastorino, G., Perez, K. E., Smith, K … Collen, B. (2021). The conservation status of the world's freshwater molluscs. *Hydrobiologia*, *848*(12-13), 3231-3254. https://doi.org/10.1007/s10750-020-04385-w

Ceballos, G., Ehrlich, P. R., & Dirzo, R. (2017). Biological annihilation via the ongoing sixth mass extinction signaled by vertebrate population losses and declines. *Proceedings of the National Academy of Sciences*, *114*(30), E6089-E6096. https://doi.org/10.1073/pnas.1704949114

Chapman, A. D. (2009). *Number of living species in Australia and the world* (2nd ed.). Department of the Environment, Water, Heritage and the Arts, Australian Government: Canberra, Australia. https://www.dcceew.gov.au/science-research/abrs/publications/other/numbers-living-species

Cobbinah, J. R., Vink, A., & Onwuka, B. (2008). *Snail farming: Production, processing and marketing*. Agromisa. https://publications.cta.int/en/publications/publication/1497/post-harvest-processing/index.html

Chiew, E. W., Lim, L. H. S., & Ambu, S. (2009). Cercarial dermatitis in Kelantan, Malaysia an occupation related health problem. *International e-Journal of Science, Medicine and Education, 1*(2), 69-73.

Colley, D. G., Bustinduy, A. L., Secor, W. E., & King, C. H. (2014). Human schistosomiasis. *The Lancet*, *383*(9936), 2253-2264. https://doi.org/10.1016/S0140-6736(13)61949-2

Debbra, M., Premalatha, B., Nurulaini, R., Azima, L.. Erwanas, A., & Sohayati, A. R. (2018). Rumen paramphistomosis in Bos indicus from a sample received by Veterinary Research Institute of Ipoh, Perak. *Malaysian Journal of Veterinary Research*, *9*(2), 154-159. https://doi.org/doi/pdf/10.5555/20193004793

Dida, G. O., Gelder, F. B., Anyona, D. N., Matano, A. S., Abuom, P. O., Adoka, S. O., Ouma, C., Kanangire, C. K., Owuor, P. O., & Ofulla, A. V. O. (2014). Distribution and abundance of schistosomiasis and fascioliasis host snails along the Mara River in Kenya and Tanzania. *Infection Ecology and Epidemiology*, *4*(1), 24281. https://doi.org/10.3402/iee.v4.24281

Diyana, J. N. A., Mahiza, M. I. N., Latiffah, H., Fazila, S. H. N., Lokman, I. H., Hazfalinda, H. N., Chandrawathani, P., Ibitoye, E. B., & Juriah, K. (2020). Occurrence, morphometric, and molecular investigation of cattle and buffalo liver adult fluke in Peninsular Malaysia main abattoirs. *Journal of Parasitology Research*, 9-14. https://doi.org/10.1155/2020/5436846

Dumidae, A., Subkrasae, C., Ardpairin, J., Pansri, S., Polseela, R., Thanwisai, A., & Vitta, A. (2024). Population genetic structure of Indoplanorbis exustus (Gastropoda: Planorbidae) in Thailand and its infection with trematode cercariae. *PloS One*, *19*(1), e0297761. https://doi.org/10.1371/journal.pone.0297761

Dung, B. T., Doanh, P. N., The, D. T., Loan, H. T., Losson, B., & Caron, Y. (2013). Morphological and molecular characterization of lymnaeid snails and their potential role in transmission of *Fasciola* spp. in Vietnam. *Korean Journal of Parasitology*, *51*(6), 657-662. https://doi.org/10.3347/kjp.2013.51.6.657

EFSA Panel on Nutrition, Novel Foods and Food Allergens, Turck, D., Bohn, T., Castenmiller, J., De Henauw, S., Hirsch-Ernst, K. I., & Knutsen, H. K. (2024). Safety of HelixComplex snail mucus (HSM) as a novel food pursuant to Regulation (EU) 2015/2283. *EFSA Journal*, *22*(1), e8492. https://doi.org/10.2903/j.efsa.2024.8492

El-Zawawy, N. A., & Mona, M. M. (2021). Antimicrobial efficacy of Egyptian *Eremina desertorum* and *Helix aspersa* snail mucus with a novel approach to their anti-inflammatory and wound healing potencies. *Scientific Reports*, *11*(1), 24317. https://doi.org/10.1038/s41598-021-03751-8

Facon, B., Pointier, J. P., Glaubrecht, M., Poux, C., Jarne, P., & David, P. (2003). A molecular phylogeography approach to biological invasions of the New World by parthenogenetic Thiarid snails. *Molluscan Ecology*, *12*, 3027-3039. https://doi.org/10.1046/j.1365-294X.2003.01972.x

Faiz, L. Z. (2020). Diversity and damage assessment of snail in cultivated crops of Neelabut Bagh, Azad Jammu and Kashmir (Pakistan). *Journal of Bioresource Management*, *7*(4), 11. https://doi.org/10.35691/JBM.0202.0149

Francis, R. A. (Ed.). (2012). *A handbook of global freshwater invasive species*. Routledge. https://doi.org/10.4324/9780203127230

Garba Djirmay, A., Yadav, R. S., Guo, J., Rollinson, D., & Madsen, H. (2024). Chemical control of snail vectors as an integrated part of a strategy for the elimination of Schistosomiasis review of the state of

knowledge and future needs. *Tropical medicine and infectious disease*, *9*(9), 222. https://doi.org/10.3390/tropicalmed9090222

Greene, S. D. (2008). Extending integrated pest management to the golden apple snail: Examining a community centre approach in northeast Thailand. *International Journal of Pest Management*, *54*(2), 95-102. https://doi.org/10.1080/09670870701621282

Greer, G. J., Ow-Yang, C. K., & Yong, H. S. (1988). *Schistosoma malayensis* n.sp.: A *Schistosoma japonicum*-complex schistosome from Peninsular Malaysia. *Journal of Parasitology*, *74*(3), 471-480. https://doi.org/10.2307/3282058

Greer, G. J., & Anuar, H. (1984). Serological evidence of schistosomiasis among Orang Asli from three areas of Peninsular Malaysia. *Southeast Asian Journal of Tropical Medicine and Public Health*, *15*, 303-12.

Gugliandolo, E., Cordaro, M., Fusco, R., Peritore, A. F., Siracusa, R., Genovese, T., & Crupi, R. (2021). Protective effect of snail secretion filtrate against ethanol-induced gastric ulcer in mice. *Scientific Reports*, *11*(1), 3638. https://doi.org/10.1038/s41598-021-82921-4

Gupta, A., & Khanal, P. (2024). The potential of snails as a source of food and feed. *Journal of Agriculture and Food Research, 18*, 101330. https://doi.org/10.1016/j.jafr.2024.101330

Hurtrez-Boussès, S., Pendino, A., Bernabé, C., Rondelaud, D., Durand, C., Meunier, C., Hurtrez-Boussès, J. E., & Renaud, F. (2005). Comparison between shell morphology and genetic diversity in two sympatric lymnaeid snails, vectors of fasciolosis. *Canadian Journal of Zoology*, *83*(11), 1643–1648. https://doi.org/10.1139/z05-150

Hung, N. M., Duc, N. V., Stauffer, J. R., & Madsen, H. (2013). Use of black carp (*Mylopharyngodon piceus*) in biological control of intermediate host snails of fish-borne zoonotic trematodes in nursery ponds in the Red River Delta, Vietnam. *Parasites and Vectors*, *6*(1), 142. https://doi.org/10.1186/1756-3305-6-142

Harizt, A. M., Malahubban, M., Syed-Hussain, S. S., Ramanoon, S. Z., Sadiq, M. B., Sarbini, S. R., Hassan, M. N., Mustafa, S., Aziz, N. A. A., Isa, N. M. M., Nating, W. R., Asut, R., & Kamaludeen, J. (2021). Gastrointestinal parasitic infections of buffaloes (*Bubalus bubalis*) in Sarawak Borneo: Prevalence, risk factors, and farming practices. *Tropical Biomedicine*, *38*(3), 318-326. https://doi.org/10.47665/TB.38.3.072

Hah, H. E., Liew, T. S., Rama Rao, S., Yow, Y. Y., & Ratnayeke, S. (2022). Distribution and environmental associations of invasive freshwater Pomacea snails in Peninsular Malaysia. *Biological Invasions*, *24*(1), 189-204. https://doi.org/10.1007/s10530-021-02634-0

Horgan, F. G., Stuart, A. M., & Kudavidanage, E. P. (2014). Impact of invasive apple snails on the functioning and services of natural andmanaged wetlands. *Acta Oecologica*, *54*, 90-100. https://doi.org/10.1016/j.actao.2012.10.002

International Union for Conservation of Nature and Natural Resources. (2021). *The IUCN Red List of Threatened Species* (Version 2021-2). https://www.iucnredlist.org

International Feed Industry Federation. (2021). *Global feed statistics*. https://ifif.org/global-feed/statistics

Japa, O., Suwancharoen, C., Bunsong, T., & Phuangsri, C. (2021). Parasitological and molecular characterization of the avian schistosomatid cercariae infecting lymnaeidae snails in Phayao, Northern Thailand. *Veterinary World*, *14*(11), 2655–2661. https://doi.org/10.14202/vetworld.2021.2655-2661

Johnson, P. D., Bogan, A. E., Brown, K. M., Burkhead, N. M., Cordeiro, J. R., Garner, J. T., & Strong, E. E. (2013). Conservation status of freshwater gastropods of Canada and the United States. *Fisheries*, *38*(6), 247-282. https://doi.org/10.1080/03632415.2013.785396

Jamnah, O., Marliah, A., Norazura, A. H., Maizatul Azlina, A. M., Premaalatha, B., Erwanas, A. I., Ainani, A., Mohamad Bohari, J., Mohd Rostan, A. A., Norhafiza, A. H., Naheed, M., Chandrawathani, P., & Ramlan, M. (2013). Parasites in local buffaloes – incidental findings from mortalities in nili-ravi buffaloes. *Malaysian Journal of Veterinary Research*, *4*(2), 47-49.

Joshi, R. C., Cowie, R. H., & Sebastian, L. S. (Eds.). (2017). Biology and management of invasive apple snails. *Philippine Rice Research Institute* (PhilRice). 406 pp. https://ri.conicet.gov.ar/bitstream/handle/11336/108894/CONICET_Digital_Nro.6add5657-f918-46ab-b63c-3146fd3c10e8_r.pdf.pdf?sequence=5

Kamaruddin, N. C., Razali, M. A. I., Busayo, I. E., Hamzah, N. H., Idris, L. H., & Md Isa, N. M. (2021). The first report of ruminant fascioliasis in Sabah, East Malaysia. *Journal of Parasitology Research*, *1*(2021), 6691483. https://doi.org/10.1155/2021/6691483

Kim, J. S. (1978). An ecological study of Paragonimus in Malaysia. *Kisaengch'unghak chapchi. The Korean Journal of Parasitology*, *16*(1), 47-53. https://doi.org/10.3347/kjp.1978.16.1.47

Krailas, D., Namchote, S., Koonchornboon, T., Dechruksa, W., & Boonmekam, D. (2014). Trematodes obtained from the thiarid freshwater snail *Melanoides tuberculata* (Müller, 1774) as vector of human infections in Thailand. *Zoosystematics and Evolution*, *90*, 57. https://doi.org/10.3897/zse.90.7306

Khadijah, S., Ariff, Z., Nurlaili, M. R., Sakiinah, A., Izzudin, A. H., Mursyidah, A. K., Rita, N., & Nur Aida, H. (2017). Fasciola and Paramphistomum infection in large ruminants. *International Journal of Agronomy and Agricultural Research International*, *10*, 19-26.

King, C. H., Sutherland, L. J., & Bertsch, D. (2015). Systematic review and meta-analysis of the impact of chemical-based molluscicide for control of *Schistosoma mansoni* and *S. haematobium* transmission. *PLoS Neglected Tropical Diseases*, *9*(12), e0004290. https://doi.org/10.1371/journal.pntd.0004290

Kumar, P. (2020). A review—on molluscs as an agricultural pest and their control. *International Journal of Food Science and Agriculture*, *4*(4), 383-389. https://doi.org/10.26855/ijfsa.2020.12.013

Lee, J. H., Quan, J. H., Choi, I. W., Park, G. M., Cha, G. H., Kim, H. J., & Lee, Y. H. (2017). *Fasciola hepatica*: Infection status of freshwater snails collected from Gangwon-do (Province), Korea. *The Korean Journal of Parasitology*, *55*(1), 95-100. https://doi.org/10.3347/kjp.2017.55.1.95

Leighton, B. J., Zervos, S., & Webster, J. M. (2000). Ecological factors in schistosome transmission, and an environmentally benign method for controlling snails in a recreational lake with a record of schistosome dermatitis. *Parasitology International*, *49*(1), 9–17. https://doi.org/10.1016/S1383-5769(00)00035-7

Li, Z. J., Ge, J., Dai, J. R., Wen, L. Y., Lin, D. D., & Madsen, H., et al. (2016). Biology and control of snail intermediate host of *Schistosoma japonicum* in the People's Republic of China. *Advances in Parasitology*, *92*, 197–236. https://doi.org/10.1016/bs.apar.

Latif, B., Heo, C. C., Razuin, R., Shamalaa, D. V., & Tappe, D. (2013). Autochthonous human schistosomiasis, Malaysia. *Emerging Infectious Diseases*, *19*(8), 1340-1341.

Lee, H. (1965). *Paragonimus westermani* infection in wild mammals and crustacean host in Malaysia. *The American Journal of Tropical Medicine and Hygiene*, *14*(4), 581.

Lopes, A. S., Pulido-Murillo, E. A., López-Hernández, D., Melo, A. L. de, & Pinto, H. A. (2021). First report of *Melanoides tuberculata* (Mollusca: Thiaridae) harboring a xiphidiocercaria in Brazil: A new parasite introduced in the Americas? *Parasitology International*, *82*(October 2020). https://doi.org/10.1016/j.parint.2021.102284

Lu, X. T., Gu, Q. Y., Limpanont, Y., Song, L. G., Wu, Z. D., Okanurak, K., & Lv, Z. Y. (2018). Snail-borne parasitic diseases: An update on global epidemiological distribution, transmission interruption and control methods. *Infectious Diseases of Poverty*, *7*(1), 1-16. https://doi.org/10.1186/s40249-018-0414-7

Maes, T., Hammoud, C., Volckaert, F. A., & Huyse, T. (2021). A call for standardised snail ecological studies to support schistosomiasis risk assessment and snail control efforts. *Hydrobiologia*, *848*(8), 1773-1793. https://doi.org/10.1007/s10750-021-04547-4

Madsen, H., & Hung, N. M. (2014). An overview of freshwater snails in Asia with main focus on Vietnam. *Acta Tropica*, *140*, 105-117. https://doi.org/10.1016/j.actatropica.2014.08.005

Martin, G. L., & Cabrera, E. C. (2018). Morphological characterization of emerging cercariae among lymnaeid snails from Barangay Cawongan, Padre Garcia, Batangas, Philippines. *Journal of Parasitology Research*, *2018*(1), 1-13. https://doi.org/10.1155/2018/5241217

Maciaszek, R., Marcinek, D., & Sosnowski, W. (2019). Tropical snail, the red-rimmed melania *Melanoides tuberculata* Müller, 1774 (*Thiaridae*) from artificial lake in Warsaw, Central Poland. *World Science News*, *122*, 285-290.

Mirfendereski, R., Hashemi, S., Shirali, S., Shemshadi, B., & Lawton, S. P. (2021). DNA barcoding of Iranian radicine freshwater snails begins to untangle the taxonomy and phylogeography of intermediate hosts of schistosomiasis and fasciolosis from the Middle East and across Central Asia. *Infection, Genetics and Evolution, 89*, 104728. https://doi.org/10.1016/j.meegid.2021.104728

Naresh, G., Gomez, P. A., Salmah, B., & Suryati, M. Y. (2006). Fasciolosis (liver fluke) of the breast in a male patient: A case report. *Breast*, *15*(1), 103-105. https://doi.org/10.1016/j.breast.2005.02.009

Ng, T. H., Dulipat, J., Foon, J. K., Lopes-Lima, M., Zieritz, A., & Liew, T. S. (2017). A preliminary checklist of the freshwater snails of Sabah (Malaysian Borneo) deposited in the BORNEENSIS collection, Universiti Malaysia Sabah. *ZooKeys*, *673*, 105-123. https://doi.org/10.3897/zookeys.673.12544

Nguyen, H. M., Greiman, S. E., Van Hoang, H., Ngoc, C. N., Van Nguyen, H., Pham, T. C., & Madsen, H. (2022). Morphological and molecular identification of trematode cercariae related with humans and animal health in freshwater snails from a lake and a dam in Myanmar. *The Journal of Parasitology*, *108*(6), 601-612. https://doi.org/10.1645/21-125

Noothuan, N., Apitanyasai, K., Panha, S., & Sutcharit, C. (2021). Snail mucus from the mantle and foot of two land snails, *Lissachatina fulica* and *Hemiplecta distincta*, exhibits different protein profile and biological activity. *BMC Research Notes, 14*, 138. https://doi.org/10.1186/s13104-021-05557-0

Pathak, C. R., Luitel, H., Utaaker, K. S., & Khanal, P. (2024). One-health approach on the future application of snails: A focus on snail-transmitted parasitic diseases. *Parasitology Research, 123*(1), 28. https://doi.org/10.1007/s00436-023-07975-y

Phiri, A. M., Phiri, I. K., Chota, A., & Monrad, J. (2007). Trematode infections in freshwater snails and cattle from the Kafue wetlands of Zambia during a period of highest cattle-water contact. *Journal of Helminthology*, *81*(1), 85-92. https://doi.org/10.1017/S0022149X07387786

Phoong, M. J. C., Hah, H. E., Rao, S. R., Yow, Y. Y., & Ratnayeke, S. (2018). Invasive apple snails in wetlands of Selangor, Malaysia: Species, Distribution, and ecological associations. *Journal of Tropical Biology and Conservation (JTBC)*, *15*, 43-60. https://doi.org/10.51200/jtbc.v15i0.1474

Piechocki, A., Wawrzyniak-Wydrowska, B., & Zdanowski, B. (2003). *Melanoides tuberculatus* (O. F. Müller, 1774) (Orthogastropoda: Thiaridae), a gastropod species new for the fauna of Poland. *Folia Malacologica*, *11*, 39-41. https://doi.org/10.12657/folmal.011.004

Post, J. M., Reasch, R. J., & Bailey, E. S. (2022). Detection of trematodes from the host exotic aquatic snail *Melanoides tuberculata* in an urban stormwater system. *Zoonotic Diseases*, *2*(4), 258-266. https://doi.org/10.3390/zoonoticdis2040021

Prasopdee, S., Tesana, S., Cantacessi, C., Laha, T., Mulvenna, J., Grams, R., Loukas, A., & Sotillo, J. (2015). Data set from the proteomic analysis of Bithynia siamensis goniomphalos snails upon infection with the carcinogenic liver fluke *Opisthorchis viverrini. Data in Brief*, *2*, 16-20. https://doi.org/10.1016/j.dib.2014.09.005

Rao, S. R., Liew, T. S., Yow, Y. Y., & Ratnayeke, S. (2018). Cryptic diversity: Two morphologically similar species of invasive apple snail in Peninsular Malaysia. *PLoS ONE*, *13*(5), 1-16. https://doi.org/10.1371/journal.pone.0196582

Rita, N., Mursyidah, A. K., & Khadijah, S. (2017). The prevalence of helminthiasis in cattle, Terengganu, Peninsular Malaysia. *Tropical Biomedicine*, *34*(2), 324-331.

Rosilawati, K., Ramli S., & Saipul, B. A. R. (2017). Fascioliasis in an adult draught buffalo in Malaysia: A case report. *Malaysian Journal of Veterinary Research*, *8*(1), 169-172.

Saad, K., Mohammad Faiz, M., Nadarasan, R., Mursyidah, A. K., & Hassan, Q. N. (2019). High prevalence fluke infection at four cattle farms located in Kuala Terengganu, Malaysia. *Tropical Life Sciences Research*, *30*(3), 59-67. https://doi.org/10.21315/tlsr2019.30.3.4

Saijuntha, W., Andrews, R. H., Sithithaworn, P., & Petney, T. (2021). *Biodiversity of Southeast Asian parasites and vectors causing human disease*. In T. N. Petney, W. Saijuntha, H. Mehlorn (Eds.)., Parasitology research monographs. Springer. https://doi.org/10.1007/978-3-030-71161-0_1

Sodhi, N. S., Brook, B. W., & Bradshaw, C. J. (2009). Causes and consequences of species extinctions. *The Princeton Guide to Ecology, 1*(1), 514-520.

Sokolow, S. H., Huttinger, E., Jouanard, N., Hsieh, M. H., Lafferty, K. D., Kuris, A. M., Riveau, G., Senghor, S., Thiam, C., N'Diaye, A., Faye, D. S., & De Leo, G. A. (2015). Reduced transmission of human schistosomiasis after restoration of a native river prawn that preys on the snail intermediate host. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(31), 9650–9655. https://doi.org/10.1073/pnas.1502651112

Sokolow, S. H., Lafferty, K. D., & Kuris, A. M. (2014). Regulation of laboratory populations of snails (*Biomphalaria* and *Bulinus* spp.) by river prawns, *Macrobrachium* spp. (Decapoda, Palaemonidae):

Implications for control of schistosomiasis. *Acta Tropica, 132*, 64–74. https://doi.org/10.1016/j.actatropica.2013.12.021

Strayer, D.L., & Dudgeon, D. (2010). Freshwater biodiversity conservation: recent progress and future challenges. *Journal of The North American Benthological Society*, *29*(1), 344-359. https://doi.org/10.1899/08-171.1

Thakuri, B., Acharya, B. K., & Sharma, G. (2019). Population density and damage of invasive giant African snail *Achatina fulica* in an organic farm in East Sikkim, India. *Indian Journal of Ecology, 46*(3), 631-635.

Tookhy, N. A., Isa, N. M. M., Mansor, R., Rahaman, Y. A., Ahmad, N. I., Bui, D. T., Idris, L. H., Hamzah, N. H., & Zulkifli, N. (2023). Morphological and molecular identification of lymnaeid snail and trematodes cercariae in different water bodies in Perak, Malaysia. *Parasitology Research*, *122*(7), 1475-1488. https://doi.org/10.1007/s00436-023-07845-z

Tookhy, N. A., Mahiza, M. I. N., Mansor, R., Yasmin, A. R., Ahmad, N. I., Hamzah, N. H., & Idris, L. H. (2022). Rumen Fluke in Cattle and Buffaloes in Asia: A Review. *Pertanika Journal of Tropical Agricultural Science*, *45*(3), 781-803. https://doi.org/10.47836/pjtas.45.3.15

Tookhy, N. A., Isa, N-M. M., Rahaman, Y. A., Ahmad, N. I., Sharma, R. S. K., Idris, L. H., Mansor, R., Bui, D. T., Hamzah, N. H., & Shakhes, S. A. (2024). Epidemiology of rumen fluke infection in selected buffalo farms in Perak, Malaysia: Prevalence, molecular species identification, and associated risk factors. *Parasitology Research*, *123*, 199. https://doi.org/10.1007/s00436-024-08219-9

Vinarski, M., Bolotov, I. N., Aksenova, O. V., Babushkin, E. S., Bespalaya, Y. V., et al. (2020). Freshwater Mollusca of the Circumpolar Arctic: A review on their taxonomy, diversity, and biogeography. *Hydrobiologia*, 1–28. https://doi.org/10.1007/s10750-020-04345-5

World Health Organization. (2016). *Schistosomiasis: WHO reports substantial treatment progress for scool age children.* https://www.who.int/news/item/08-12-2017-schistosomiasis-who-reports-substantial-treatment-progress-for-school-age-children

World Health Organization. (2018). *Human fascioliasis: Review provides fresh perspectives on infection and control.* https://www.who.int/news/item/26-07-2018-human-fascioliasis-review-provides-fresh-perspectives-on-infection-and-control

World Health Organization. (2020). *Neglected tropical diseases.* https://www.who.int/news-room/questions-and-answers/item/q-a-on-fascioliasis

Xia, J., Yuan, Y., Xu, X., Wei, F., Li, G., Liu, M., ... & Nie, S. (2014). Evaluating the effect of a novel molluscicide in the endemic schistosomiasis japonica area of China. *International Journal of Environmental Research and Public Health*, *11*(10), 10406-10418.

Yahaya, H., Amzah, B., Sivapragasam, A., Nordin, M., Muhamad Hisham, M. N., & Misrudin, H. (2017). Invasive apple snails in Malaysia. *Biology and Management of Invasive Apple Snails*, *1*, 169195.

Yi, X. L., Liu, J., Cao, M. L., Xiong, J., Deng, Y. P., Wang, H. M., Ma, P. P., Liu, G. H., & Yang, H. (2024). Population genetics and genetic variation of *Pomacea canaliculata* (Gastropoda: Ampullariidae) in China revealed by sequence analyses of three mitochondrial genes. *Ecology and evolution*, *14*(1), e10836. https://doi.org/10.1002/ece3.10836

Yusa, Y., Sugiura, N., & Wada, T. (2006). Predatory potential of freshwater animals on an invasive agricultural pest, the apple snail *Pomacea canaliculata* (Gastropoda: Ampullariidae), in southern Japan. *Biological Invasions, 8*, 137–147. https://doi.org/10.1007/s10530-004-1790-4

Younes, A., El-Sherief, H., Gawish, F., & Mahmoud, M. (2017). Biological control of snail hosts transmitting schistosomiasis by the water bug, *Sphaerodema urinator*. *Parasitology Research*, *116*(4), 1257–1264. https://doi.org/10.1007/s00436-017-5402-5

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Optimal Time to Harvest Brine Shrimp (*Artemia salina*) Nauplii as Live Food for Aquatic Animals

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ABSTRACT

Brine shrimp (*Artemia salina*) are commonly utilized as aquatic live food, but only a few studies have investigated the optimal harvesting time. In the present study, brine shrimp eggs were hatched in artificial seawater and harvested at different periods (22, 24, 26, 28, 30, 32, 34, and 36 h). Harvested nauplii from quadruplicate tanks were analyzed for total carotenoid content, digestive enzyme activities, proximate chemical composition and fatty acids. Economically significant fish were used to test *in vitro* digestibility using their digestive enzymes. Total carotenoid contents significantly decreased with post-hatch time as the equation $y = –0.0108x + 0.6265$ ($p < 0.05$, –65.3% of final value). The trypsin-specific activity was elevated in brine shrimp harvested at 26 to 36 h and was lowest at 24 h ($p < 0.05$). However, there was no difference in the specific activities of chymotrypsin, lipase, and amylase over the eight harvesting periods. Ash contents were significantly lower at 34 to 36 h after hatching, compared to earlier times. Crude lipid contents fluctuated without showing a trend. In the fatty acid profile, levels of highly unsaturated, monounsaturated, polyunsaturated fatty acids, omega-3 and omega-6, were highest at 22 h after hatching. *In vitro* digestibility indicated suitable times for feeding out brine shrimp to guppy (22 h), striped catfish (22 to 24 h), Nile tilapia (22 to 26 h), and swordtail (22 to 30 h). Our investigation of the optimal time for harvesting brine shrimp as live food will help improve the quality of aquaculture produce.

Keywords: *Artemia*, digestive enzyme, fatty acid, *in vitro* digestibility, sea monkeys

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INTRODUCTION

Brine shrimp (*Artemia salina*) are zooplankton that can survive in a salinity range from 9 to 340 g/l (Gajardo & Beardmore, 2012; Sellami et al., 2020). They develop and grow rapidly within 48 h after hatching (Sleet & Brendel, 1983). Brine shrimp nauplii are commonly used as live food in the aquaculture industry, since they are smaller than the mouth size of aquatic animal larvae. In addition, they contain enzymes and nutrients that are necessary for the growth of young aquatic animals (Sellami et al., 2020; Sorgeloos et al., 2001). They are also widely available and easily cultivated at low cost, have a high reproductive rate, and tolerate difficult environmental conditions (McLaughlin et al., 1991; Turan & Mammadov, 2021; Yun et al., 2020).

Brine shrimps are red or orange. Only the keto-carotenoids canthaxanthin and echinenone (in a 19:1 ratio) contribute to their coloring (Krinsky, 1965), which improves the coloration and immune status of fish reared on a diet of *A. salina* (Biswas et al., 2024). Additionally, the digestive enzymes in *A. salina* also affect animals that consume it. They are quickly absorbed by the body, enhancing the digestion, growth, and color of the feeder. In general, brine shrimp comprise five main digestive enzymes: protease, trypsin, and chymotrypsin contribute to the digestion of protein; amylase contributes to the digestion of carbohydrates; and lipase contributes to the digestion of fat (Solorzano et al., 2009).

Many fish fry require highly unsaturated fatty acids (HUFAs) for normal growth and survival (Rainuzzo et al., 1997). As fish fry cannot synthesize this fatty acid sufficiently, they require additional fatty acids. The composition of fatty acids in brine shrimp has been studied in depth. Since they can be deficient in certain essential fatty acids necessary for fish larvae, an enrichment protocol is always applied to improve their lipid profile (Choi et al., 2021; Morshedi et al., 2022; Pham et al., 2023). Therefore, the fatty acid composition of a supply of brine shrimp must be determined, as well as the development of their fatty acid profiles up to harvesting.

The standard incubation period for brine shrimp cysts is 24 to 48 h (Sorgeloos et al., 1986), but the appropriate harvesting time of the nauplii has not been thoroughly investigated. Proximate chemical composition assessment indicated that feeding out *Artemia* nauplii should take place less than 48 h post-hatching, ideally as soon as they hatch (Sanders, 2008). The current knowledge should be expanded to provide the most effective nutrition for aquatic animals. Thus, this study aims to determine the optimal period for harvesting brine shrimp to raise aquatic animals without an enrichment protocol. Total carotenoid content, digestive enzyme activities, fatty acid profile, and proximate chemical composition were used as assessment criteria. To select the appropriate harvesting time, enzymes extracted from the guppy (*Poecilia reticulata*), swordtail (*Xiphophorus hellerii*), Nile tilapia (*Oreochromis niloticus*) and striped catfish (*Pangasianodon hypophthalmus*), which are economically important species, were used in *in vitro* digestibility tests. The results of these *in vitro* assays

bear significant relationships with *in vivo* digestibility, can be analyzed quickly, and are reliable (Hahor et al., 2022). The current investigation results can direct a practical protocol for harvesting brine shrimp nauplii in live-feed fish farming. Subsequently, aquatic animals will grow healthily if this live food is fed.

MATERIALS AND METHODS

Animal Ethics

This research was authorized by the Prince of Songkla University Institutional Animal Care and Use Committee (Project Code 2024-SCI15-067). Animal rearing, sampling, and euthanasia were based on the "Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes" of the National Research Council of Thailand (Application No. U1-06514-2560).

Brine Shrimp Rearing and Collection

Containers were designed for brine shrimp rearing by dividing a 6 L plastic bottle (15 cm long, 12.5 cm in width, and 35 cm in height) into two parts. The upper part (roughly conical) was covered with a black plastic bag 11 cm long and served as the container for hatching brine shrimp cysts. The lower part of the bottle served as the base for the upper part, which was inverted and placed inside the lower part. Approximately 4 g of cysts (Snow Mountain Eagle, Tianjin, China) were hatched in a quadruplicate set of plastic containers, holding 2 L of 30 g/l artificial seawater within a pH range of 8 to 9. A two-way oxygen pump supplied aeration. Incubation proceeded under a 12-h dark/12-h light photoperiod. Under our incubation conditions, the nauplii broke out of the cysts after 15 to 20 h, passing into the Instar I phase. Therefore, the harvesting period began at 22 h and ended at 36 h, when the brine shrimp reached the Instar II phase. At that point, suspension feeding would normally start. Before sampling the nauplii at 22, 24, 26, 28, 30, 32, 34 and 36 h, empty and unhatched cysts and envelopes of hatched cysts were removed using a strainer so that only nauplii ($n = 4$) were collected. Before determining total carotenoid contents, digestive enzyme activities, chemical compositions, and *in vitro* protein digestibility, specimens were rinsed in distilled water to remove seawater and any contaminants.

Total Carotenoid Determination

The extraction technique outlined in Thongprajukaew et al. (2014) was used to extract total carotenoids. For three days, mixing at least once daily, a known weight (~3 mg) of ground, dried material was extracted using 1 ml of acetone at 4°C in the dark. For 10 min, the mixture was centrifuged at 5,000 × *g*. The extinction coefficient, E (1%, 1 cm) = 1,900, was used to determine the total carotenoid content after the absorbance of the supernatant was measured at 474 nm (Foss et al., 1984).

Digestive Enzyme Activities

Enzyme Extraction and Protein Quantification

The sampled brine shrimp were extracted using a tissue homogenizer (THP-220; Omni International, Kennesaw, GA, USA) at a ratio of 1:3 (w/v) in cold 0.2 M Na_2HPO_4-NaH_2PO_4 buffer (pH 7.5). For 30 min, the homogenate was centrifuged at 15,000 × *g* at 4°C. Prior to analysis, supernatants were gathered and stored at −20°C. Using bovine serum albumin as the protein standard, the standard method of Lowry et al. (1951) was used to determine the protein concentration in the crude enzyme extracts.

Digestive Enzyme Assays

The activities of trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), lipase (EC 3.1.1.3), and α-amylase (EC 3.2.1.1) were assessed using crude enzyme extracts. Trypsin and chymotrypsin activities were evaluated using *N*-benzoyl-*L*-Arg-*p*-nitroanilide and *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide as substrates, respectively, in accordance with the method of Rungruangsak-Torrissen et al. (2006). The outcomes were compared with the typical *p*-nitroanilide linear range. Using *p*-nitrophenyl palmitate as a substrate and comparing the results to the linear range of standard *p*-nitrophenol, lipase activity was evaluated using the Winkler and Stuckmann (1979) method. Using soluble starch as a substrate, Areekijseree et al.'s (2004) method assessed the activity of α-amylase and contrasted it with the linear range of regular maltose. By dividing each sample's amylase activity by its trypsin activity, the amylase/trypsin ratios were determined.

Chemical Composition Analysis

The methods described in the Association of Official Analytical Chemists (2005) recommendations were used in the proximate composition analysis to determine the amounts of moisture, crude protein, crude fat, and ash. Brine shrimp nauplii were dried for 24 h at 105°C in a hot air oven (WOF155; Wisd Laboratory Instruments, Wertheim, Germany) to determine the moisture content. The crude protein level was ascertained using a Kjeldahl analyzer (KjeltecTM 8100; Foss, Höganäs, Sweden). Petroleum ether was used as the solvent in a Soxhlet extraction equipment (Soxtec™ 8000; Foss, Suzhou, China) to measure the crude lipid content. Samples were burned for 2 h at 600°C in a muffle furnace (E30-HT; Thai Furnaces Engineering, Lampang, Thailand) to quantify the ash content gravimetrically.

Fatty Acid Measurement

Brine shrimp nauplii samples weighing 50 mg were extracted. The methanolysis of fatty acids in the extract was conducted through direct transesterification. In a reaction vial, 1

ml of the precisely weighed sample was mixed with 0.5 M KOH in methanol solution (RCI Labscan, Bangkok, Thailand). The mixture was vortexed for 1 min, followed by sonication for 20 min and hydrolyzed in a thermoreactor at 100°C for 20 min. Then, 0.4 ml of HCl (Merck, Darmstadt, Germany)/methanol was added at a 4:1 v/v ratio to the reaction vial, vortexed for 1 min, and then placed in the thermoreactor for another 20 min at 100°C. Once the time had elapsed, the mixture was cooled to room temperature, and the fatty acids were extracted with 2 ml of deionized water and 3 ml of petroleum ether (RCI Labscan, Bangkok, Thailand), vortexed for 1 min, and left to separate. After passing through a 0.22 µm membrane filter, the upper phase—petroleum ether and fatty acid—was gathered in a vial. Fatty acids were extracted using petroleum ether once more, and the resulting extracts were mixed. Nitrogen gas was evaporated at all extraction stages until they were completely dry.

The fatty acid residue was dissolved using 0.6 ml of heptane solvent (RCI Labscan, Bangkok, Thailand) in an injection vial, and 1.0 µl of the solution was injected for analysis by gas chromatograph-flame ionization detector (GC-FID, 7890A; Agilent Technologies, CA, USA). The carrier was helium gas, which was employed at a steady flow rate of 1 ml/min. The FID gases were the nitrogen makeup at 25 ml/min, H_2 at 30 ml/min, and air zero at 300 ml/min. All samples were injected using a split mode (25:1 ratio) with an injector. Temperature settings for the FID detector were 290°C and 300°C, respectively. The oven temperature was first kept at 140°C for 5 min, then raised to 210°C at a rate of 10°C/min, held there for 5 min, and finally raised to 250°C at a rate of 5°C/min, held there for 8 min. The fatty acid concentration was calculated using the following equation:

$$C_t = \frac{A_t \times C_{std} \times V_{solvent}}{A_{std} \times Wt} \times 1{,}000$$

where, C_t = Fatty acids interest content (mg/g); A_t = Peak areas of the fatty acid interests; C_{std} = Reference solution concentration (Methyl heptadecanoate, C17: 0) (mg/ml); A_{std} = Peak areas of reference solution (Methyl heptadecanoate, C17: 0); $V_{solvent}$ = Volume of heptane solvent that dissolves the sample (ml); Wt = Sample weight (mg).

In Vitro Digestibility

Extraction of Fish Digestive Enzymes

Nile tilapia (5.73 ± 0.05 g), striped catfish (3.52 ± 0.02 g), swordtails (0.16 ± 0.01 g), and guppy (0.18 ± 0.01 g) were bought from a private farm in Songkhla Province, Thailand. The intestines of the fish were removed and placed on ice. They were then combined with 0.2 M Na_2HPO_4-NaH_2PO_4 buffer at pH 8 (1:3 w/v), and the digestive enzymes were extracted as detailed for brine shrimp.

In Vitro Protein Digestibility

For use as substrates, nauplii samples were pulverized, freeze-dried for 48 h, and then sieved. According to Thongprajukaew et al. (2011), the enzymatic reactions were carried out. The reaction mixtures included 125 µl of dialyzed crude enzyme extract, 50 µl of 0.5% chloramphenicol, 10 ml of 50 mM Na_2HPO_4-NaH_2PO_4 buffer at pH 8, and 5 mg of dried brine shrimp nauplii. The mixtures were agitated at 200 rpm for 24 h while incubated at 25 °C. The increase in released reactive amino groups of cleaved peptides at 420 nm was used as a spectrophotometric measure of *in vitro* protein digestibility. For comparison, *DL*-alanine linear ranges were employed.

Statistical Analysis

A desired power test of 0.8 (Cohen, 1988) was used to establish the minimum sample size ($n = 4$) using R 3.6.0 Software. Our completely randomized design experiment consisted of four distinct tanks. The collected results were displayed as means and standard error of the mean (SEM). Version 22 of the Statistical Package for Social Sciences (SPSS Inc., Chicago, USA) was used to calculate all statistical values. Duncan's multiple range test was used as a *post hoc* test in a one-way analysis of variance to assess differences in means between treatment groups. If the *p*-value was less than 0.05, the hypothesis was disproved.

RESULTS AND DISCUSSION

Total carotenoid contents of brine shrimp nauplii are illustrated in Figure 1. Their concentrations significantly decreased with post-hatch time ($y = –0.0108x + 0.6265$, $r = –0.853$, $p = 0.007$). Our results corroborate the observation made by Gilchrist and Green (1960) that carotenoid content substantially declines during development. Generally, the only keto-carotenoids found in brine shrimp are canthaxanthin and echinenone, which are present in a 19:1 ratio (Krinsky, 1965). Quantitative changes in *cis*- and, to a lesser extent, all-*trans*-canthaxanthin are strongly connected to the elementary carotenoid in specific developmental stages (Nelis et al., 1988). No feed was available to the nauplii during our trial. Hence, there were no effects of dietary nutrients. In aquaculture, brine shrimp are essentially fed microalgae with high carotenoid contents, such as *Haematococcus pluvialis*, *Isochrysis galbana*, *Myrmecia incisa, Dunaliella salina*, and *Spirulina* sp. Apart from their positive impacts on fish larvae, carotenoids have also been shown to enhance the growth and health of brine shrimp, improving growth, survival rate, antioxidant capacity, and immunity (Gui et al., 2022).

The progressive change in carotenoid content and caloric value is associated with the breakdown and excretion of the yolk sac of newly hatched *Artemia* nauplii (Sanders, 2008; Treece, 2000). Only trypsin activity among all protein-digesting enzymes examined was impacted by post-hatch time (Table 1, $p < 0.05$). Specific activity of chymotrypsin was

not affected ($p > 0.05$). Since trypsin is a key enzyme in the development and digestion of proteins, substantial changes in its activity between 24 and 32 h may be related to some physiological responses. Also, trypsin activates itself and certain zymogens involved in the intestinal digestion of proteins (Moraes & Almeidam, 2020). However, based on dry matter, crude protein contents did not change with time post-hatching (Table 2, $p > 0.05$). In this study, empty, unhatched cysts and hatched cyst envelopes were removed before samples were collected, since the non-protein nitrogen contents from chitinous materials could result in false-positive results when crude protein is analyzed using the Kjeldahl method.

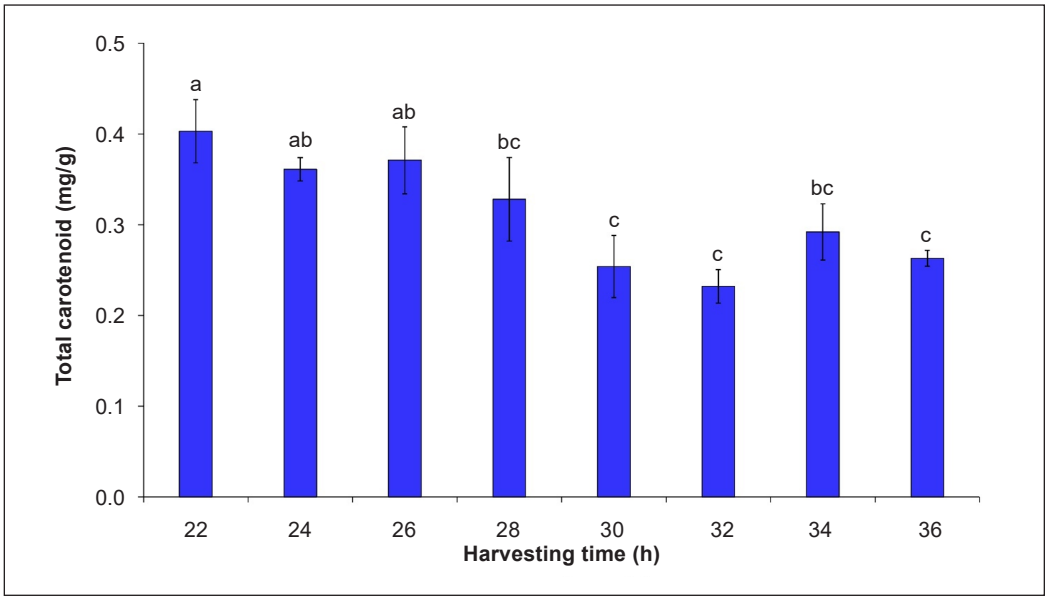

Figure 1. Total carotenoid contents ($n = 4$, based on dry weight) of brine shrimp collected at various harvesting times. Significant differences between treatments are shown by different superscripts ($p < 0.05$)

Table 1
Digestive enzyme activities of brine shrimp nauplii collected at various harvesting times (n = 4)

Digestive enzyme	Harvesting time (h)								Pooled SEM	*p*-value
	22	24	26	28	30	32	34	36		
Amylase (U/mg protein)	1.63	1.26	1.93	1.56	1.84	1.86	1.63	1.97	0.08	0.452
Trypsin (mU/mg protein)	6.76^{bc}	4.95^{c}	7.86^{ab}	8.59^{ab}	8.60^{ab}	9.45^{a}	6.44^{bc}	7.70^{ab}	0.51	0.021
Chymotrypsin (mU/mg protein)	5.77	3.74	6.75	7.41	6.74	7.66	6.50	5.65	0.44	0.052
Lipase (mU/mg protein)	1.67	1.61	0.87	1.18	1.40	1.01	0.82	1.00	0.12	0.156
Amylase/trypsin ratio	214	236	231	219	208	203	246	260	7.06	0.373

Note. $^{a–c}$ Means in the same row with different superscripts indicate significant differences ($p < 0.05$)

Table 2
The proximate chemical compositions of brine shrimp nauplii collected at various harvesting times (n = 4)

Component	Harvesting time (h)								Pooled SEM	*p*-value
	22	24	26	28	30	32	34	36		
Moisture (% FW)	88.1	90.9	89.2	89.9	87.6	91.1	91.7	92.5	0.61	0.072
Crude protein (% DW)	53.2	51.6	53.6	53.0	54.1	54.5	53.1	53.4	0.27	0.138
Crude lipid (% DW)	12.5^{bc}	12.5^{bc}	13.0^{ab}	12.2^{bcd}	11.1^{d}	11.3^{cd}	14.2^{a}	13.4^{ab}	0.36	0.005
Ash (% DW)	9.49^{a}	9.88^{a}	10.1^{a}	10.7^{a}	10.8^{a}	9.51^{a}	6.57^{b}	6.74^{b}	0.58	0.005

Note. FW = fresh weight, DW = dry weight, $^{a–d}$ Means in the same row with different superscripts indicate significant differences ($p < 0.05$)

No variations occurred in the activity of the carbohydrate-digesting amylase and the marker of carbohydrate utilization per protein unit, the amylase/trypsin ratio (Table 1, $p > 0.05$). Similarly, the lipase activity, a lipid-digesting enzyme, was unaffected by post-hatch time (Table 1, $p > 0.05$). Crude lipid contents fluctuated with no clear change trend (Table 2, $p < 0.05$), indicating some changes in lipid breakdown during the 36-h investigation. The fatty acid analysis showed that post-hatch time did not affect levels of the fatty acids C15:0, C16:0, C17:0, C20:0 and C23:0, total saturated fatty acids (SFAs), and arachidonic acid/eicosapentaenoic acid (ARA/EPA) (Table 3, $p > 0.05$). Nevertheless, the fatty acid levels

C20:1*n*9 significantly increased ($p < 0.05$) with time post-hatching. Meanwhile, the concentrations of all other fatty acids were significantly higher in brine shrimp nauplii at 22 h ($p < 0.05$), including C14:0, C16:1*n*7, C17:1*n*7, C18:1*n*9t, C18:1*n*9c, C18:2*n*6t, C18:2*n*6c, C18:3*n*3, C20:4*n*6, C20:5*n*3, total unsaturated fatty acids (UFAs), monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs), total *n*-3, total *n*-6, *n*-3/*n*-6, total *n*-9, and HUFAs.

Crustaceans lose energy during molting and use stored energy for growth, reducing certain fatty acid levels. Molting is a crucial biological process closely related to crustaceans' growth and organ development. It requires significant energy to absorb water and swell the outer shell (Huang et al., 2015; Jung et al., 2013). Not only does this involve the metabolism of nutrients (particularly fats and fatty acids), but the molting process demands energy production to maintain energy balance and survival post-molt (Fang et al., 2021; Huang et al., 2015; Wang et al., 2014). While certain fatty acids, such as C18:0 and C20:1*n*9, increase depending on the rearing conditions (Peykaran Mana et al., 2014), brine shrimp nauplii molt twice within 24 h after hatching (Benijts et al., 1976). When they molt, the depletion of energy and nutrients for growth could reduce their nutritional value compared to younger nauplii. The proportion of fatty acids is lower in Instar–II+ *Artemia* than in Instar–I (McKay & Jeffs, 2023). The balance of nutrients, such as UFAs and PUFAs, is important to establish when determining the optimal harvesting time to

Table 3
Fatty acid profiles of brine shrimp nauplii collected at various harvesting times (n = 4)

Fatty acid	Harvesting time (h)								Pooled SEM	*p*-value
	22	24	26	28	30	32	34	36		
C14:0	2.75^{a}	2.76^{a}	2.68^{a}	2.73^{a}	2.44^{b}	2.43^{b}	2.35^{b}	2.42^{b}	0.27	0.009
C15:0	0.39	0.38	0.39	0.39	0.36	0.36	0.36	0.38	0.11	0.288
C16:0	16.5	16.5	16.3	16.7	15.2	15.3	15.1	15.7	0.65	0.098
C16:1*n*7	17.1^{a}	16.8^{a}	15.8^{ab}	14.9^{bc}	13.1^{d}	13.2^{d}	12.8^{d}	13.6^{cd}	0.73	0.001
C17:0	0.66	0.66	0.67	0.66	0.63	0.64	0.67	0.68	0.16	0.809
C17:1*n*7	0.30^{a}	0.28^{a}	0.21^{b}	0.15^{c}	0.12^{d}	0.12^{d}	0.11^{d}	0.11^{d}	0.08	0.005
C18:0	4.68^{b}	4.76^{b}	4.91^{b}	5.13^{ab}	4.75^{b}	4.91^{b}	5.08^{b}	5.53^{a}	0.37	0.023
C18:1*n*9t	18.8^{a}	18.6^{a}	17.5^{ab}	16.6^{bc}	14.8^{d}	15.0^{cd}	14.9^{cd}	16.0^{bcd}	0.74	0.002
C18:1*n*9c	11.5^{a}	11.4^{ab}	10.9^{ab}	10.3^{bc}	9.29^{c}	9.51^{c}	9.66^{c}	10.4^{abc}	0.62	0.009
C18:2*n*6t	3.94^{a}	3.65^{a}	2.65^{b}	1.66^{c}	1.24^{d}	1.09^{d}	0.95^{d}	1.11^{d}	0.28	0.005
C18:2*n*6c	0.86^{a}	0.79^{b}	0.53^{c}	0.30^{d}	0.22^{e}	0.19^{ef}	0.15^{f}	0.16^{ef}	0.13	0.005
C18:3*n*3	12.8^{a}	11.8^{b}	7.73^{c}	4.01^{d}	2.96^{e}	2.38^{ef}	1.96^{f}	2.15^{ef}	0.53	0.005
C20:0	0.14	0.14	0.14	0.14	0.13	0.13	0.14	0.15	0.07	0.171
C20:1*n*9	0.89^{d}	1.02^{d}	1.47^{c}	1.98^{b}	1.99^{b}	2.11^{ab}	2.19^{a}	2.22^{a}	0.20	0.005
C20:4*n*6	1.29^{a}	1.18^{a}	0.77^{b}	0.42^{c}	0.15^{d}	0.29^{cd}	0.24^{cd}	0.27^{cd}	0.23	0.005
C20:5*n*3	13.4^{a}	12.3^{b}	7.88^{c}	3.92^{d}	3.05^{de}	2.55^{e}	2.05^{e}	2.30^{e}	0.59	0.005
C22:1*n*9	0.22^{bc}	0.21^{c}	0.21^{c}	0.27^{ab}	0.23^{bc}	0.22^{bc}	0.25^{bc}	0.30^{a}	0.11	0.017
C23:0	0.99	1.06	1.09	1.04	0.99	1.00	1.01	1.01	0.21	0.504
SFAs	26.1	26.3	26.2	26.8	24.4	24.8	24.7	25.9	0.85	0.229
UFAs	81.1^{a}	78.0^{a}	65.6^{b}	54.4^{c}	47.2^{d}	46.6^{d}	45.3^{d}	48.7^{d}	1.31	0.005
MUFAs	48.9^{a}	48.4^{ab}	46.1^{abc}	44.1^{bcd}	39.5^{d}	40.1^{cd}	39.9^{d}	42.7^{cd}	1.22	0.004
PUFAs	32.3^{a}	29.7^{b}	19.6^{c}	10.3^{d}	7.63^{d}	6.50^{d}	5.36^{d}	5.99^{d}	0.84	0.005
Total *n*-3	26.2^{a}	23.8^{b}	15.6^{c}	7.93^{d}	6.01^{de}	4.93^{e}	4.01^{e}	4.45^{e}	0.76	0.005
Total *n*-6	6.10^{a}	5.62^{b}	3.95^{c}	2.37^{d}	1.62^{e}	1.57^{e}	1.34^{e}	1.54^{e}	0.31	0.005
n-3/*n*-6	31.5^{a}	31.4^{a}	30.0^{b}	29.1^{bc}	26.1^{c}	26.8^{c}	27.0^{c}	29.0^{bc}	0.94	0.019
n-9	4.29^{a}	4.24^{a}	3.95^{ab}	3.32^{abc}	3.72^{ab}	3.15^{bc}	2.98^{bc}	2.90^{c}	0.40	0.002
HUFAs	14.7^{a}	13.4^{b}	8.65^{c}	4.33^{d}	3.20^{de}	2.84^{e}	2.30^{e}	2.57^{e}	0.59	0.005
ARA/EPA	0.10	0.10	0.10	0.11	0.05	0.11	0.12	0.12	0.11	0.428

*Note***.** SFA = saturated fatty acids, UFA = unsaturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, HUFA = highly unsaturated fatty acids, ARA = arachidonic acid, EPA = eicosapentaenoic acid, $^{a–f}$ Means in the same row with different superscripts indicate significant differences ($p < 0.05$)

ensure the nutrient content of nauplii is complete and suitable for fish larvae and juvenile aquatic animals.

Ash contents in *Artemia* range from 4.20 to 21.4%, and their contents increase with molting (Léger et al., 1986). In the current investigation, ash contents were substantially reduced in brine shrimp nauplii harvested at 34 to 36 h after hatching compared to earlier times (Table 2, $p < 0.05$). This probably indicates a loss of minerals after starvation.

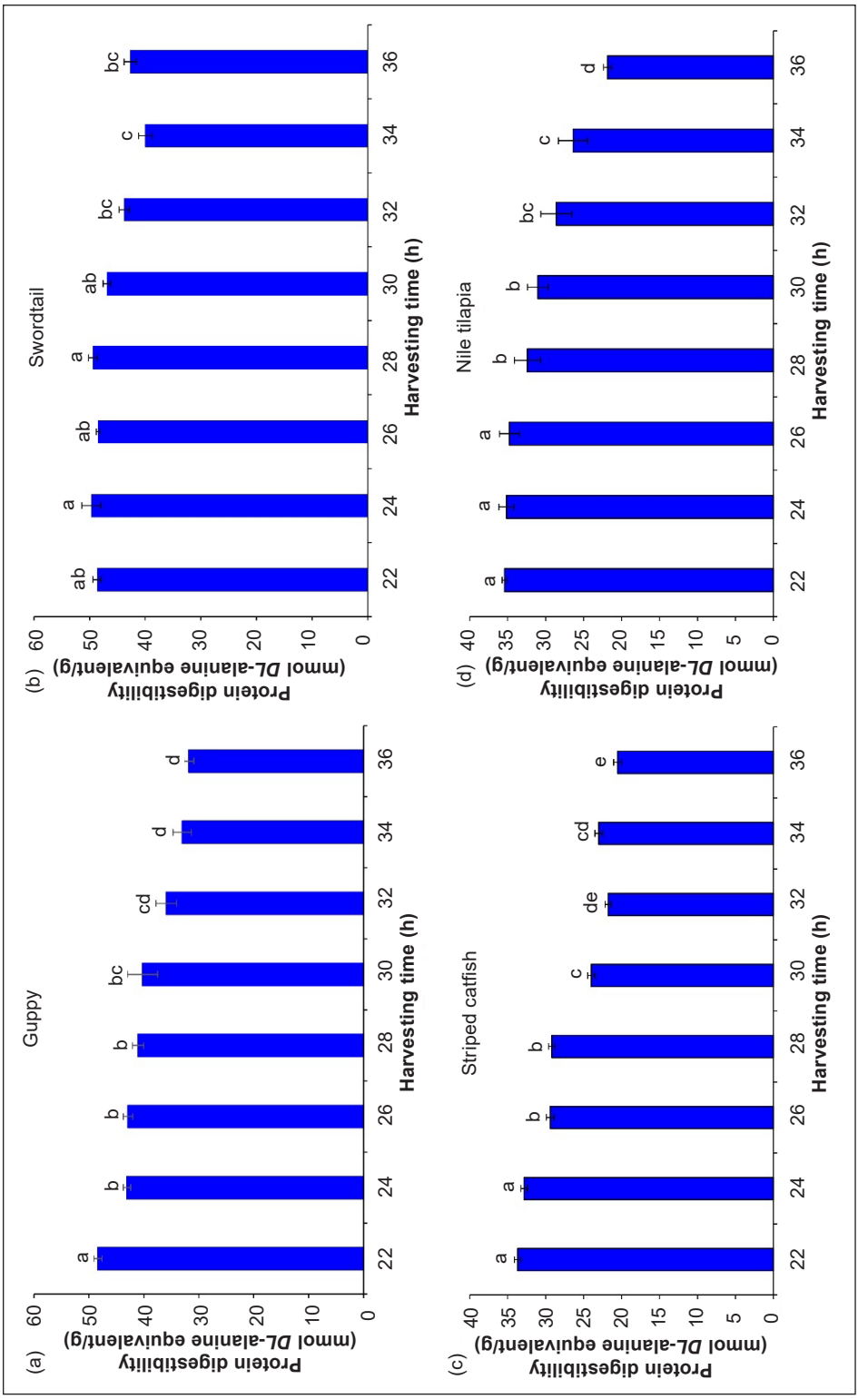

Figure 2. In vitro digestibility of protein from brine shrimp collected at various harvesting times ($n = 4$). The crude digestive enzymes utilized in digestibility tests were taken from the guppy (a), swordtail (b), striped catfish (c), and Nile tilapia (d). Significant differences between treatments are shown by different superscripts ($p < 0.05$)

Protein digestibility in the ornamental fish (guppy and swordtails) and food fish (striped catfish and Nile tilapia) decreased significantly with post-hatch time (Figure 2, $p < 0.05$). The guppy better utilized protein from brine shrimp nauplii harvested soon after hatching (22 h), whereas striped catfish (22 to 24 h) and Nile tilapia (22 to 26 h) better utilized protein from nauplii harvested 2 to 4 h later. A wider range of harvesting times (22 to 30 h) was suitable for swordtails. As brine shrimp age, there may be changes in their biochemical composition, such as an increase in chitin content. This limits larval fish's ability to digest them and reduces absorption. The strong exoskeleton of crustaceans can hinder access to the nutrient-rich tissues contained within (Cara et al., 2003; Luizi et al., 1999; Schipp et al., 1999), and digestive enzymes of small or juvenile fish may not be concentrated enough to digest the complex chitin structure. The simple intestine of small or juvenile fish may also limit their digestive capacity. Insufficient digestive enzyme quantity, activity and variety may further limit the digestion of chitin. The findings of this experiment provide information that could improve the nutritional value of live aquafeed, and the evaluation of the nutrient content of developing brine shrimp could promote more efficient nutrient utilization in fish larvae.

CONCLUSION

The harvesting time had a relatively small effect on overall digestive enzyme activities and chemical composition. However, total carotenoid contents, fatty acid profiles, and *in vitro* protein digestibility showed a clear decreasing trend with harvesting time. The results of this study conflict with the standard practice of feeding out brine shrimp nauplii to aquatic animals at 48 h after hatching. Although the digestibility assays indicated an optimum harvesting range, these data may not be consistent with the evolution of the key nutrients of brine shrimp. Therefore, the nauplii should be fed out 22 h after hatching from their cysts, or as early as possible, depending on the hatching conditions. However, further experiments on feeding brine shrimp to aquatic animals are still needed, as this study was conducted only *in vitro*.

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REFERENCES

Areekijseree, M., Engkagul, A., Kovitvadhi, U., Thongpan, A., Mingmuang, M., Pakkong, P., & Rungruangsak-Torrissen, K. (2004). Temperature and pH characteristics of amylase and proteinase of adult freshwater pearl mussel, *Hyriopsis* (*Hyriopsis*) *bialatus* Simpson 1900. *Aquaculture*, *234*, 575–587. https://doi.org/10.1016/j.aquaculture.2003.12.008

Association of Official Analytical Chemists. (2005). *Official methods of analysis* (18th ed.). AOAC International.

Benijts, F., Van Voorden, E., & Sorgeloos, P. (1976). Changes in the biochemical composition of the early larval stages of the brine shrimp, *Artemia salina* L. *Proceedings of the 10th European Symposium on Marine Biology*, *1*(1), 1–9.

Biswas, P., Singh, S. K., Debbarma, R., Dey, A., Waikhom, G., Deb, S., & Patel, A. B. (2024). Effects of carotenoid supplementation on colour, growth and physiological function of the endemic dwarf chameleon fish (*Badis badis*). *Journal of Animal Physiology and Animal Nutrition*, *108*(1), 126–138. https://doi.org/10.1111/jpn.13873

Cara, J. B., Moyano, F. J., Cárdenas, S., Fernández-Díaz, C., & Yúfera, M. (2003). Assessment of digestive enzyme activities during larval development of white bream. *Fish Biology*, *63*(1), 48–58. https://doi.org/10.1046/j.1095-8649.2003.00120.x

Choi, J., Han, G. S., Lee, K. W., Byun, S. G., Lim, H. J., Lee, C. H., Lee, D. Y., & Kim, H. S. (2021). Effects of feeding differentially enriched *Artemia* nauplii on the survival, growth, fatty acid composition, and air exposure stress response of Pacific cod (*Gadus macrocephalus*) larvae. *Aquaculture Reports*, *21*, 100829. https://doi.org/10.1016/j.aqrep.2021.100829

Cohen, J. (1988). *Statistical power analysis for the behavioral sciences* (2nd ed.). Lawrence Erlbaum. https://doi.org/10.4324/9780203771587

Fang, F., Yuan, Y., Jin, M., Shi, B., Zhu, T., Luo, J., Lu, J., Wang, X., Jiao, L., & Zhou, Q. (2021). Hepatopancreas transcriptome analysis reveals the molecular responses to different dietary *n*-3 PUFA lipid sources in the swimming crab *Portunus trituberculatus*. *Aquaculture*, *543*, 737016. https://doi.org/10.1016/j.aquaculture.2021.737016

Foss, P., Storebakken, T., Schiedt, K., Liaaen-Jensen, S., Austreng, E., & Streiff, K. (1984). Carotenoid in diets for salmonids I: Pigmentation of rainbow trout with the individual optical isomers of astaxanthin in comparison with canthaxanthin. *Aquaculture*, *41*(3), 213–226. https://doi.org/10.1016/0044-8486(84)90284-9

Gajardo, G. M., & Beardmore, J. A. (2012). The brine shrimp *Artemia*: Adapted to critical life conditions. *Frontiers in Physiology*, *3*, 185. https://doi.org/10.3389/fphys.2012.00185

Gilchrist, B. M., & Green, J. (1960). The pigments of *Artemia. Proceedings of the Royal Society of London*, *152*(946), 118–136. https://doi.org/10.1098/rspb.1960.0028

Gui, L., Xu, L., Liu, Z. Y., Zhou, Z. G., & Sun, Z. (2022). Carotenoid-rich microalgae promote growth and health conditions of *Artemia* nauplii. *Aquaculture*, *546*, 737289. https://doi.org/10.1016/j.aquaculture.2021.737289

Hahor, W., Thongprajukaew, W., Nuntapong, N., Saekhow, S., Rungruangsak-Torrissen, K., Dumrongrittamatt, T., & Phonchai, A. (2022). Partial pretreatment of ingredient mixture effectively improved feed chemical composition, physicochemical properties and *in vitro* digestibility. *Animal Feed Science and Technology*, *285*, 115216. https://doi.org/10.1016/j.anifeedsci.2022.115216

Huang, S., Wang, J., Yue, W., Chen, J., Gaughan, S., Lu, W., Lu, G., & Wang, C. (2015). Transcriptomic variation of hepatopancreas reveals the energy metabolism and biological processes associated with molting in Chinese mitten crab, *Eriocheir sinensis*. *Scientific Reports*, *5*, 14015. https://doi.org/10.1038/srep14015

Jung, H., Lyons, R. E., Hurwood, D. A., & Mather, P. B. (2013). Genes and growth performance in crustacean species: A review of relevant genomic studies in crustaceans and other taxa. *Reviews in Aquaculture*, *5*(2), 77–110. https://doi.org/10.1111/raq.12005

Krinsky, N. I. (1965). The carotenoids of the brine shrimp, *Artemia salina*. *Comparative Biochemistry and Physiology*, *16*(2), 181–187. https://doi.org/10.1016/0010-406X(65)90057-5

Léger, P., Bengtson, D. A., Simpson, K. L., & Sorgeloos, P. (1986). The use and nutritional value of *Artemia* as a food source. *Oceanography and Marine Biology: An Annual Review*, *24*, 521–623.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, *193*(1), 265–275. https://doi.org/10.1016/S0021-9258(19)52451-6

Luizi, F. S., Gara, B., Shields, R. J., & Bromage, N. R. (1999). Further description of the development of the digestive organs in Atlantic halibut (*Hippoglossus hippoglossus*) larvae, with notes on differential absorption of copepod and *Artemia* prey. *Aquaculture*, *176*, 101–116. https://doi.org/10.1016/S0044-8486(99)00054-X

McKay, W., & Jeffs, A. (2023). Optimization of artemia feed regimes for larval giant kōkopu (*Galaxias argenteus*). *Fishes*, *8*, 183. https://doi.org/10.3390/fishes8040183

McLaughlin, J. L., Chang, C. J., & Smith, D. L. (1991). Bench-top bioassays for the discovery of bioactive natural products: An update. In A. U. Rhaman (Ed.), *Studies in natural products chemistry* (pp. 383–409). Elsevier.

Moraes, G., & de Almeidam, L. C. (2020). Nutrition and functional aspects of digestion in fish. In B. Baldisserotto, E. C. Urbinati & J. E. P. Cyrino (Eds.), *Biology and physiology of freshwater neotropical fish* (pp. 251–271). Academic Press. https://doi.org/10.1016/C2017-0-03766-7

Morshedi, V., Mozanzadeh, M. T., Hamedi, S., Naserifard, I., Ebrahimi, H., Agh, N., Nafisi, M., Azodi, M., & Rashidian, G. (2022). Enrichment of live feed with very low level of docosahexaenoic acid (DHA) is enough for yellowtail sea bream (*Acanthopagrus latus*) larvae. *Aquaculture Reports*, *26*, 101310. https://doi.org/10.1016/j.aqrep.2022.101310

Nelis, H. J., Lavens, P., Van Steenberge, M. M., Sorgeloos, P., Criel, G. R., & De Leenheer, A. P. (1988). Qualitative and quantitative changes in the carotenoids during development of the brine shrimp *Artemia. Journal of Lipid Research*, *29*(4), 491–499. https://doi.org/10.1016/S0022-2275(20)38522-9

Peykaran Mana, N., Vahabzadeh, H., Seidgar, M., Hafezieh, M., & Pourali, H. R. (2014). Proximate composition and fatty acids profiles of *Artemia* cysts, and nauplii from different geographical regions of Iran. *Iranian Journal of Fisheries Sciences*, *13*(3), 761–775. http://hdl.handle.net/1834/11783

Pham, H. D., Siddik, M. A. B., Rahman, Md. A., Huynh, L. T., Nahar, A., & Vatsos, I. N. (2023). Effects of *n*-3 HUFA-enriched *Artemia* on growth, biochemical response, skeletal morphology and stress resistance of Asian sea bass (*Lates calcarifer*) larvae reared at high temperature. *Aquaculture*, *574*, 739732. https://doi.org/10.1016/j.aquaculture.2023.739732

Rainuzzo, J. R., Reitan, K. I., & Olsen, Y. (1997). The significance of lipids at early stages of marine fish: A review. *Aquaculture*, *155*, 103–115. https://doi.org/10.1016/S0044-8486(97)00121-X

Rungruangsak-Torrissen, K., Moss, R., Andresen, L. H., Berg, A., & Waagb, R. (2006). Different expressions of trypsin and chymotrypsin in relation to growth in Atlantic salmon (*Salmo salar* L.). *Fish Physiology and Biochemistry*, *32*(1), 7–23. https://doi.org/10.1007/s10695-005-0630-5

Sanders, J. M. (2008). *Time post-hatch caloric value of Artemia salina* [Senior Honors Projects]. University of Rhode Island. https://digitalcommons.uri.edu/srhonorsprog/83

Schipp, G. R., Bosmans, J. M. P., & Marshall, A. J. (1999). A method for hatchery culture of tropical calanoid copepods, *Acartia* spp. *Aquaculture*, *174*, 81–88. https://doi.org/10.1016/S0044-8486(98)00508-0

Sellami, I., Charmantier, G., Naceur, H. B., Kacem, A., & Lorin-Nebel, C. (2020). Osmoregulatory performance and immunolocalization of Na^+/K^+-ATPase in the branchiopod *Artemia salina* from the Sebkha of Sidi El Hani (Tunisia). *Tissue and Cell*, *63*, 101340. https://doi.org/10.1016/j.tice.2020.101340

Sleet, R. B., & Brendel, K. (1983). Improved methods for harvesting and counting synchronous populations of *Artemia* nauplii for use in developmental toxicology. *Ecotoxicology and Environmental Safety*, *7*(5), 435–446. https://doi.org/10.1016/0147-6513(83)90082-9

Solorzano, Y., Viana, M. T., López, L. M., Correa, J. G., True, C. C., & Rosas, C. (2009). Response of newly hatched *Octopus bimaculoides* fed enriched *Artemia salina*: Growth performance, ontogeny of the digestive enzyme and tissue amino acid content. *Aquaculture*, *289*, 84–90. https://doi.org/10.1016/j.aquaculture.2008.12.036

Sorgeloos, P., Dhert, P., & Candreva, P. (2001). Use of the brine shrimp, *Artemia* spp., in marine fish larviculture. *Aquaculture*, *200*, 147–159. https://doi.org/10.1016/S0044-8486(01)00698-6

Sorgeloos, P., Lavens, P., Léger, P., Tackaert, W., & Versichele, D. (1986). *Manual for the culture and use of brine shrimp in aquaculture.* State University of Ghent. https://api.semanticscholar.org/CorpusID:87385939

Thongprajukaew, K., Kovitvadhi, S., Kovitvadhi, U., & Rungruangsak-Torrissen, K. (2014). Pigment deposition and *in vitro* screening of natural pigment sources for enhancing pigmentation in adult male Siamese fighting fish (*Betta splendens* Regan, 1910). *Aquaculture Research*, *45*(4), 709–719. https://doi.org/10.1111/are.12009

Thongprajukaew, K., Kovitvadhi, U., Kovitvadhi, S., Somsueb, P., & Rungruangsak-Torrissen, K. (2011). Effects of different modified diets on growth, digestive enzyme activities and muscle compositions in juvenile Siamese fighting fish (*Betta splendens* Regan, 1910). *Aquaculture*, *322–323*, 1–9. https://doi.org/10.1016/j.aquaculture.2011.10.006

Treece, G. D. (2000). *Artemia production for marine larval fish culture*. Southern Regional Aquaculture Center. https://hatcheryfm.com/documents/20/1325052010_2.pdf

Turan, M., & Mammadov, R. (2021). Evaluation of the toxicity activity of bioactive compounds of some geophytes against brine shrimp (*Artemia salina* L.). *Acta Aquatica Turcica*, *17*(2), 195–201. https://doi.org/10.22392/actaquatr.791297

Wang, W., Wu, X., Liu, Z., Zheng, H., & Cheng, Y. (2014). Insights into hepatopancreatic functions for nutrition metabolism and ovarian development in the crab *Portunus trituberculatus*: Gene discovery in the comparative transcriptome of different hepatopancreas stages. *PloS One*, *9*(1), e84921. https://doi.org/10.1371/journal.pone.0084921

Winkler, U. K., & Stuckmann, M. (1979). Glycogen, hyaluronate and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens*. *Journal of Bacteriology*, *138*(3), 663–670. https://doi.org/10.1128/jb.138.3.663-670.1979

Yun, S., Yoon, S. Y., Hong, E. J., Giri, S. S., Kim, S. G., Kim, S. W., Han, S. J., Kwon, J., Oh, W. T., Lee, S. B., & Park, S. C. (2020). Effect of plasma-activated water, used as a disinfectant, on the hatch rate of dormant cysts of the *Artemia salina*. *Aquaculture*, *523*, 735232. https://doi.org/10.1016/j.aquaculture.2020.735232

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Understanding *Rafflesia zollingeriana*: A Comprehensive Study of Bud Development, Growth, Mortality, and Life Cycle

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ABSTRACT

Rafflesia zollingeriana is one of the endemic species in the eastern Java Region of Indonesia that has gained much attention from researchers. *R. zollingeriana* has not been studied for its flower bud development, mortality, and population dynamics. The present study investigated the flower growth, mortality, flowering success, sex ratio, and life cycle of *R. zollingeriana* in the Papring Forest, Kalipuro Banyuwangi, East Java, Indonesia. Five populations of *R. zollingeriana* with 412 individuals were observed visually every two weeks for 12 months. Each bud was observed to determine the growth dynamics, growth rate, life cycle, distribution size, bud sustainability, mortality, and sex ratio of *R. zollingeriana*. The bud phase development was recorded to determine its population structure and life cycle. The results showed *that R. zollingeriana* required between two and three years to complete its life cycle. The smaller buds grew more slowly than the larger buds. The bud mortality rate was quite high at 73.54% and occurred during the cupule and bract transition phase. The blooming success rate of its buds was only 17.71%, with a higher proportion of female flowers than male flowers. *R. zollingeriana* in Papring Forest was flowering throughout the year, with the most frequent flowering incidents occurring during the high rainfall months of January, February, March, and June. There is a relationship between the number of flowering incidents and environmental

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factors such as rainfall, temperature, and length of irradiation. This study underscores the species' vulnerability, low blooming success, and high mortality, which inform conservation strategies for *R. zollingeriana* in Papring Forest, Banyuwangi.

Keywords: Growth dynamics, phenology, *Rafflesia zollingeriana*, sex ratio

INTRODUCTION

Rafflesia is a genus of parasitic plants with no leaves, stems, or true roots and only has flowers. *Rafflesia* flowers are known as the largest single flower in the plant kingdom. This unique appearance of *Rafflesia* attracts significant attention among biologists. These plants are only found in Southeast Asia, from Southern Thailand, Peninsular Malaysia, Sumatra, Java, Kalimantan, and several islands of the Philippines (Maezulpah et al., 2019). Unfortunately, not all *Rafflesia* species have their conservation status recorded by the International Union for Conservation of Nature (IUCN) (Janra, 2019). The IUCN considers only *Rafflesia magnifica* as Critically Endangered (CR) (Malabrigo et al., 2023). Researchers in Southeast Asia have developed a conservation status classification based on IUCN criteria from 2001 to 2024. Nine species of *Rafflesia* were classified in the CR category (Nais, 2001), with only one species listed in the Vulnerable category (Susatya, 2011). Some endemic *Rafflesia* species in Gunung Lauser National Park, Sumatra, Indonesia, including *R. lawangensis*, are classified as CR, and *R. micropylora* is classified as Endangered (Mahyuni et al., 2024). Renjana et al. (2022) categorize all Indonesian *Rafflesia* as CR, except for *Rafflesia arnoldii*, which is in the vulnerable category. Kusuma et al. (2022) showed that all Java-endemic *Rafflesia,* consisting of *Rafflesia rochussenii*, *Rafflesia patma*, and *Rafflesia zollingeriana*, experienced higher extinction risks compared to the others due to global warming and climate change, and the high pressure of human activities. According to the classification of Nais (2001), three types of *Rafflesia* endemic to the island of Java are included in the vulnerable category. Furthermore, according to the 2011 criteria, it is included in the critically endangered category (Susatya, 2011).

More research related to *Rafflesia*'s life history is necessary to provide complete information regarding its mortality according to the flower development phase (Wicaksono et al., 2021a). *Rafflesia bengkuluensis*, *Rafflesia conrueloae,* and *Rafflesia patma* have a mortality rate of 67%–100%, 77.3%, and 75%, with a flowering success of 7.9%, 19.7%, and 6.7%–12.5%, respectively (Mohd-Elias et al., 2021). Another study has reported the mortality of *R. zollingeriana* in Meru Betiri National Park, which is one of five known geographical sites of this species. The mortality of *R. zollingeriana* was 43.5% in 2003, 49.1% in 2006, and 28.95%–57.14% in 2014 (Hikmat, 2006). More information on mortality and population dynamics of *R. zollingeriana* at other sites, such as Papring forest, Banyuwangi, is crucial to provide a better understanding and

comprehensive ecology of the species *R. zollingeriana*, endemic to the East Java region, was first discovered in 1918 in Puger Jember by Kooders. It has since been confirmed in several areas, including Meru Betiri National Park, Pasirian and Tempursari Lumajang, and most recently in the Papring Forest area in northern Banyuwangi. Several studies have assessed its population profile. In 2012, the distribution and population structure of *R. zollingeriana* in Meru Betiri National Park were assessed (Kusuma et al., 2023). In 2019, the population structure development of *R. zollingeriana* in Meru Betiri National Park was studied (Maezulpah et al., 2019), including the morphological comparisons of *R. zollingeriana* from Meru Betiri National Park and Lumajang, as well as morphological variations in the Papring Forest area (Lestari & Susatya, 2022). Life history is important for understanding the interaction between the *Rafflesia* and its environment to ensure maximum fitness. The fitness results from the interaction between growth and mortality at various growth and development phases (Susatya, 2020). Not many life history studies have been conducted for *Rafflesia* species. Hidayati et al. (2000) initiated a life history study for *R. patma*. A more detailed study was then carried out by Susatya (2020) for *R. arnoldii* and Wee et al. (2024) for *R. cantleyi*.

The study of *R. zollingeriana* life history is important because it has been considered an endemic *Rafflesia* in eastern Java Island, including Watang Nature Conservation Area, Meru Betiri National Park, Jember, Pasirian and Tempursari, Lumajang, and Papring, Northern Banyuwangi (Lestari & Susatya, 2022). All these locations are isolated and fragmented habitats. The life history study results will help find a proper alternative conservation scheme for *R. zollingeriana*. Therefore, the present study aimed to investigate the flower growth, mortality, flowering success, sex ratio, and life history of *R. zollingeriana* in the Papring Forest, Kalipuro Banyuwangi, East Java, Indonesia.

MATERIALS AND METHODS

Study Site

This research was conducted in the forest area of North Banyuwangi Forest Management Unit (KPH), located at Papring Village, Kalipuro sub-district, Banyuwangi. The research site was a hilly terrain covered by mixed teak and pine plantation forests with canopy coverage ranging from 62.57% to 98.35% (Table 1). The percentage of canopy was measured using the hemispherical photography method, which uses the front of a cell phone camera directed perpendicular to the sky. The trick of taking photos is between trees with several takes. Furthermore, it was analyzed using ImageJ software. This area was covered with bamboo sprouts, which sometimes became a dominant vegetation in the open area of the site. Five populations of *Rafflesia zollingeriana* were selected with latitude and longitude of S 08°06’34.52” E 114°21’31.42” (location 1), S 08°06’33.71” E 114°21’29.43” (location 2), S 08°06’33.55” E 114°21’29.90” (location 3), S 08°06’33.98” E

114°21’34.13” (location 4) and S 08°09’34.83” E 114°21’30.52” (location 5). Each location was observed every two weeks from January to December 2023 (Figure 1). The habitat has an elevation ranging from 283–436 m above sea level (ASL) and various slopes ranging from mild slope (18°) to moderate heavy slope (30°). The nearest and farthest distances of the habitat to the river were 10 m and 200 m, respectively (Table 1).

Table 1
Physical attributes of the habitat of R. zollingeriana *at Papring Forest, Banyuwangi Regency*

Site Location	Site Characteristic			
	Elevation (above sea level/asl)	Slope	Distance from River (m)	Percentage of Canopy Cover (%)
Location 1	357	18°	50	71.36
Location 2	376	28°	10	62.47
Location 3	379	21°	25	95.38
Location 4	436	30°	200	70.81
Location 5	283	23°	50	84.57

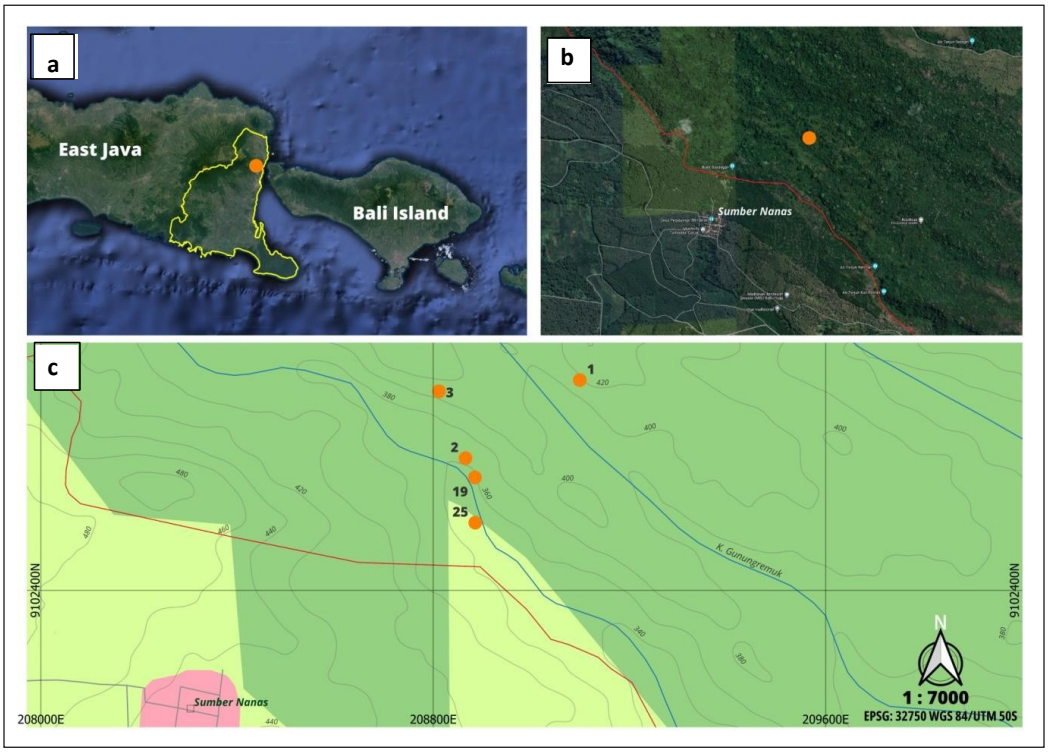

Figure 1. Map of the research location: (a) Papring Forest area, which is part of Kalipuro Subdistrict located in Banyuwangi regency, East Java, Indonesia; (b) Focus of Papring Forest Area, which is in the Forest area adjacent to the Sumber Nanas area; (c) Observation location of *R. zollingeriana* life history is shown with orange circles. Five locations are marked 1, 2, 3, 19, and 25

Morphological Identification of *R. zollingeriana*

The species of *Rafflesia* in Papring Forest was determined as *R. zollingeriana* based on the identification of morphological characteristics according to Susatya et al. (2023). The identification results show that the diameter of the fully bloomed flower is only 24–34 cm. Its perigone consists of five lobes with whitish orange or creamy white warts, numerous, small in size, close to the edge and become larger towards the center of the lobes, denser towards the diaphragm, and large, rectangular or circular warts. The diaphragm is convex towards the aperture with many creamy white, irregularly shaped warts, surrounded by orange color. Wart with discontinuous concentric apertures meeting has a thin ring of solid dark orange and white with no windows. It has ramenta from the rim of the lower surface of the diaphragm to the base of the perigone tube. The upper ramenta are tuberculate and have several variations from the top to the inside of the diaphragm. In the center, the type is swallowed lobes ramenta; in the lower diaphragm, the type is branched ramenta; in the lower part of the perigone tube, the type is simple tubercle ramenta. Processus truncated cone-shaped with various blunt spikes at its top, light orange at its base, and darker at its top. The processus forms four rings from the outside to the center of 20, 17, 8, and 4 processus, respectively. It has a well-developed interior annulus and a poorly developed exterior annulus. These characteristics correspond to the morphological characteristics of *R. zollingeriana*.

Growth Dynamics of *R. zollingeriana*

The study collected 412 flower buds from five populations of *R. zollingeriana*. Buds of *R. zollingeriana* were located on the soil surface and identified by visual observation. Observation was conducted every two weeks from January to December 2023 at all five populations. Each bud in each population was labeled, mapped, and photographed for every observation. The diameters were measured using vernier calipers for diameters less than 10 cm and a tape measure for diameters greater than 10 cm. The growth dynamics of *R. zollingeriana* were determined by observing the flower development stage, the blooming process, and the development stage of fruit formation. The development of *R. zollingeriana* flowers was determined by observing bud changes from the swollen host to the anthesis stage.

Flowering Pattern and Phenological Stage of *R. zollingeriana*

The flowering pattern of *R. zollingeriana* was determined by counting the flowers that successfully bloomed in all plots and associated with environmental factors. The fruit formation stage was observed from when the flower bloomed until it decayed and lost its perigone, and only the disk containing the fruit ovule remained. Phenological stage observations were made to see the type of perigone opening during the blossoming process. These observations were made on fully covered perigones that were ready to bloom, and

then observed every two days to see the process of opening perigones one by one until they opened completely.

The phenological stages of *R. zollingeriana* flowering were analyzed using multiple linear regression to assess the significant effects of environmental factors such as rainfall, air temperature, and duration of irradiation. Multiple linear regression was carried out with R software. The regression model used the mathematical form Y = a + b1X1 + b2X2 + b3X3, where Y represents the predicted value, a is a constant, b1 to b3 are regression coefficients, and X1 to X3 are the environmental variables. The notation X1 is rainfall, X2 is air temperature, and X3 is the duration of irradiation.

The Growth rate of *R. zollingeriana*

The growth rate of *R. zollingeriana* was observed based on the results of measuring the diameter of the bud every two weeks, recording the size of the smallest and largest diameters in each phase. The flower's diameter growth rate was determined by subtracting two consecutive measurements. Growth rates were computed by comparing the diameter at each developmental stage over the observation period. The formula for determining growth rates is as follows:

$$\text{Growth Rate} = \frac{\text{Average}}{\text{length of each observation period}} = \frac{\frac{\sum_{i=1}^{n} x_i}{n}}{14} \quad [1]$$

Life History Reconstruction

The largest and smallest diameter sizes for each phase were also used to determine the life cycle of *R. zollingeriana*. The life history reconstruction of *R. zollingeriana* buds utilized an exponential growth model, similar to models used for *R. patma* and *R. arnoldii* (Hidayati et al., 2000; Susatya, 2020). The exponential equation $Yt = c\ e^{kX}$ was applied, where Yt represents bud diameter at time t, c is a constant, e is the base of the natural logarithm (~2.719), k denotes the growth rate constant, and X signifies the time required for buds to reach a specific diameter during development. Constants c and k were derived by transforming the exponential equation into a linear form and conducting regression analysis (Susatya, 2020). The regression model was built using bud diameters observed biweekly, encompassing the entire developmental span from initial stages to just before blooming. Based on empirical observation data, this approach aimed to provide a comprehensive life history reconstruction of *R. zollingeriana* at each developmental stage.

Distribution Size, Mortality, Bud Sustainability, and Sex Ratio Determination

The distribution size of *R. zollingeriana* was observed by counting the number of buds at each study site every two weeks. Furthermore, the population size difference was

accumulated every three months to determine the population structure. The population structure was seen from the changes in the number of buds found alive. The population structure illustrates the sustainability of buds, as indicated by changes in buds due to bud death and the emergence of new buds. The population's structure was constructed according to its flower development phases and consists of (a) swollen host; (b) cupule; (c) cupule bract transition (CBT); (d) bract; (e) bract perigone transition with witish (BPT Witish); (f) bract perigone transition with pinkish (BPT pinkish); (g) perigone full covered; and (h) anthesis stage. All photographed buds were accordingly assigned to their flower growth phase.

The mortality of each bud was recorded during observation and calculated using the formula:

$$\text{Mortality Rate} = \frac{\text{Total number of Dead Bud}}{\text{Total number of Bud}} \times 100\% \quad [2]$$

Observations of mortality were also included on the cause of death. The observations also recorded the bite marks, damage, and other signs of rot to assess the causes of the bud mortality. Each bud found was also categorized based on size: < 2 cm, 2–5 cm, 5–10 cm, 10–15 cm, 15–20 cm, and > 20 cm. The < 2 cm size class indicates the swollen host and cupule phase size, and the 5–10 cm size class corresponds to CBT and bract. Size class 10–15 cm corresponds to bract to perigone. Class 15–20 cm corresponds to BPT until the perigone and a small part of the anthesis. The >20 cm class corresponds to buds that have fully bloomed and entered the ovule formation stage. Bud sustainability was also observed by calculating the percentage of buds that survived, those that died before blooming, those that successfully bloomed, and those that died after passing through the decay process. The sex ratios between male and female flowers were determined based on the presence of anthers at the disc of flowering *Rafflesia* by palpating the underside of the disk. The male and female flowers found are counted, and the ratio is calculated.

Environmental Factors Measurement

Environmental factors were measured based on weather factors, including air temperature, air humidity, length of irradiation, and rainfall. Air temperature and humidity were measured using a thermohydrometer. Measurements were taken every two weeks. Rainfall and length of irradiation data were obtained from Meteorology, Climatology, and Geophysical Agency for one year of observation. The weather data were then averaged every month.

RESULTS AND DISCUSSION

Growth Dynamics of *R. zollingeriana* Bud

Bud growth is divided into two stages: flower and fruit development. The flower development stage begins with the appearance of buds from *Tetrastigma* and continues

until the blooming phase. The fruit development stage begins with the aging of flowers until the formation of fruit and seeds. This stage includes the rotten stage and the formation of mature fruit containing seeds.

Flower Development Stage

The flower development stage of *R. zollingeriana* begins with the appearance of swelling on the *Tetrastigma* root called a swollen host (Figure 2a). The swollen host was measured from 1.06 to 1.98 cm high. The second stage is the cupule stage, where the true *Rafflesi*a body is still completely covered by *Tetrastigma* bark (Figure 2b). The size of the cupule ranges from 1.53 to 4.97 cm in diameter. The next stage is characterized by an increase in the diameter of the *Rafflesia* body, which causes the *Tetrasigma* bark to crack and gives rise to a visible whitish bract of *Rafflesia*. This condition indicates the bud enters the transition phase from cupule to bract or cupule-bract transition (CBT) (Figure 2c). The diameter of the CBT of *R. zollingerian*um in Papring Forest varies from 3.56 cm to 7.91cm.

The bract phase is marked by a whitish bract covering the whole surface of the flower bud (Figure 2d). The flower bud is 3.56 to 12.65 cm in diameter at this phase. The whitish Bract Perigone Transition (BPT) phase is indicated by emerging whitish perigone lobes, which slowly replace bracts (Figure 2e) and are 4.51 to 17 cm in diameter. The next phase is the Pinkish BPT phase, characterized by changing the color of perigone lobes from white to pinkish (Figure 2f). It ranges from 7.26 to 8.76 cm in diameter. Bract is still seen in these two last phases. When the perigone lobes completely cover the upper surface of the bud,

Figure 2. Stage of flower development of *R. zollingeriana*: (a) swollen host; (b) cupule; (c) Cupule Bract Transition (CBT); (d) bract; (e) bract perigone transition with whitish (BPT whitish); (f) Bract Perigone Transition with pinkish (BPT Pinkish); (g) perigone full covered; and (h) anthesis stage

then it becomes a perigone-fully covered phase with a diameter ranging from 15.09 to 20.9 cm. This phase also indicates that the flower bud will be flowering soon. The flowering or anthesis phase occurs at buds with a 24.43 to 36.42 cm diameter.

Blossoming Process and Phenology. The blossoming process of *Rafflesia* shows a certain pattern, which can be observed at the perigone phase bud. Two types of *Rafflesia* bud blooming process patterns exist, including spiral and flap types (Tolod et al., 2020). The flowering pattern of *R. zollingeriana* also follows these two processes. The first flowering was the spiral type, indicated by the simultaneous opening of the perigone lobes. The second type of flowering pattern of *R. zollingeriana* was the flap type. The blooming process in the type one-by-one perigone lobe will open from the outermost to the innermost lobes (Figure 3).

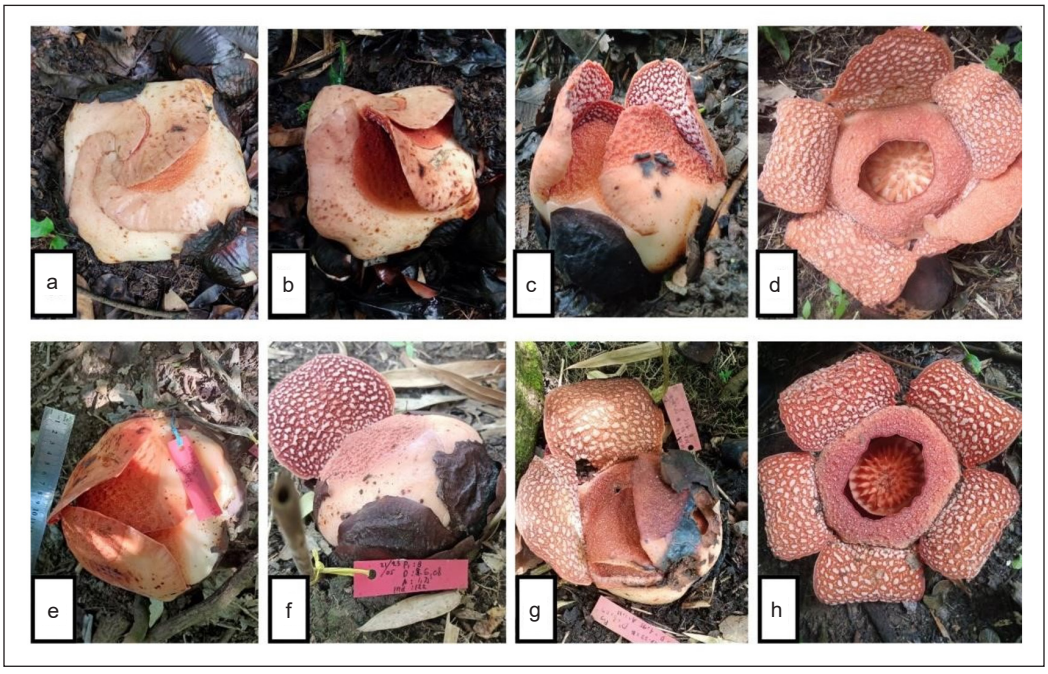

Figure 3. The flowering pattern in *R. zollingeriana*: (a–d) spiral type flowering pattern; (e–h) flap

Flowering Pattern. The observations for 12 months revealed variations in the number of flowers that successfully bloomed each month. During the 12 months of observation, 73 *R. zollingeriana* in the Papring Forest have bloomed. The study obtained the multiple regression model of $Y=-96.11916 + 0.45501X_1 + 3.10024X_2 + 1.40725X_3$. The results of multiple linear regression showed that rainfall, air temperature, and length of irradiation significantly influenced the success of *R. zollingeriana* flower blooms. There is a relationship between

the rainfall, air temperature, and length of irradiation variable and the number of blooming *R. zollingeriana* flowers. Every 1-unit increase in air temperature leads to a rise in the number of *R. zollingeriana* blooming flowers by 3.10. Also, every 1-unit change in the duration of irradiation causes the number of blooming flowers to increase by 1.41. The variables of rainfall, air temperature, and length of irradiation also have significant effects (p<0.05) on the number of blooming flowers. Figure 4 shows a linear pattern, indicating that the number of blooming flowers was influenced by rainfall, temperature, and length of irradiation.

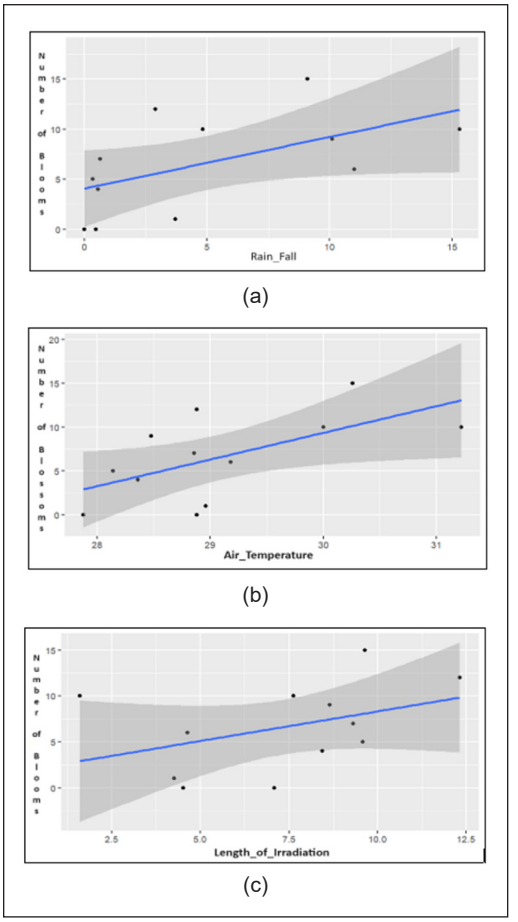

Figure 4. Graph of the relationship between the number of blooming flowers and environmental factors: (a) the relationship between the number of blooming flowers and rainfall (R^2 0.45501); (b) the relationship between blooming flowers and air temperature (R^2 3.10024); and (c) the relationship between blooming flowers and the length of exposure ($R^2$1.40725)

Flowering is generally influenced by temperature, water, and light availability (Tolod et al., 2020). The results revealed optimum rainfall conditions, air temperature, and length of irradiation support the flowering of *R. zollingeriana*. *R. zollingeriana* buds that successfully bloomed in Papring Forest occurred throughout the year. However, flowering occurs more frequently in months with rainfall and temperatures above average monthly values. This study found that the optimum air temperature to support the blooming of *R. zollingeriana* buds in Papring Forest ranged from 28°C to 31°C. The average temperature at the study site was 29.1°C. Flowering frequently occurred in months with higher temperatures than the average, including January, March, April, and July (Figure 5a). In those months, 6, 20, 15, 10, and 9 flowering incidences were recorded. High temperatures facilitate enzyme activity to support the bud development into flowers. A similar pattern is also found in *R. consueloae*, whose flowering occurs in the hottest period of the year, including January to June (Tolod et al., 2020).

Susatya (2020) stated that the optimum temperature for the development and blooming of *Rafflesia* is about 25–30°C. The blooming of the *Rafflesia* flower is a complex process

influenced by light and the availability of nutrients. The average monthly rainfall was 4.91 $mm^{3,}$ and flowering often occurred in months with rainfall above the monthly average (Figure 5c). The most frequent flowering incidence was recorded in February. Rainfall becomes an important factor that triggers the flowering process of tropical species (Satake et al., 2021). The high flowering pattern of *R. cantleyi* was recorded at higher rainfall (Wee et al., 2024). The duration of irradiation is also an important factor in the flowering of *Rafflesia.* Related to the duration of irradiation, flowering of *R. zollingeriana*

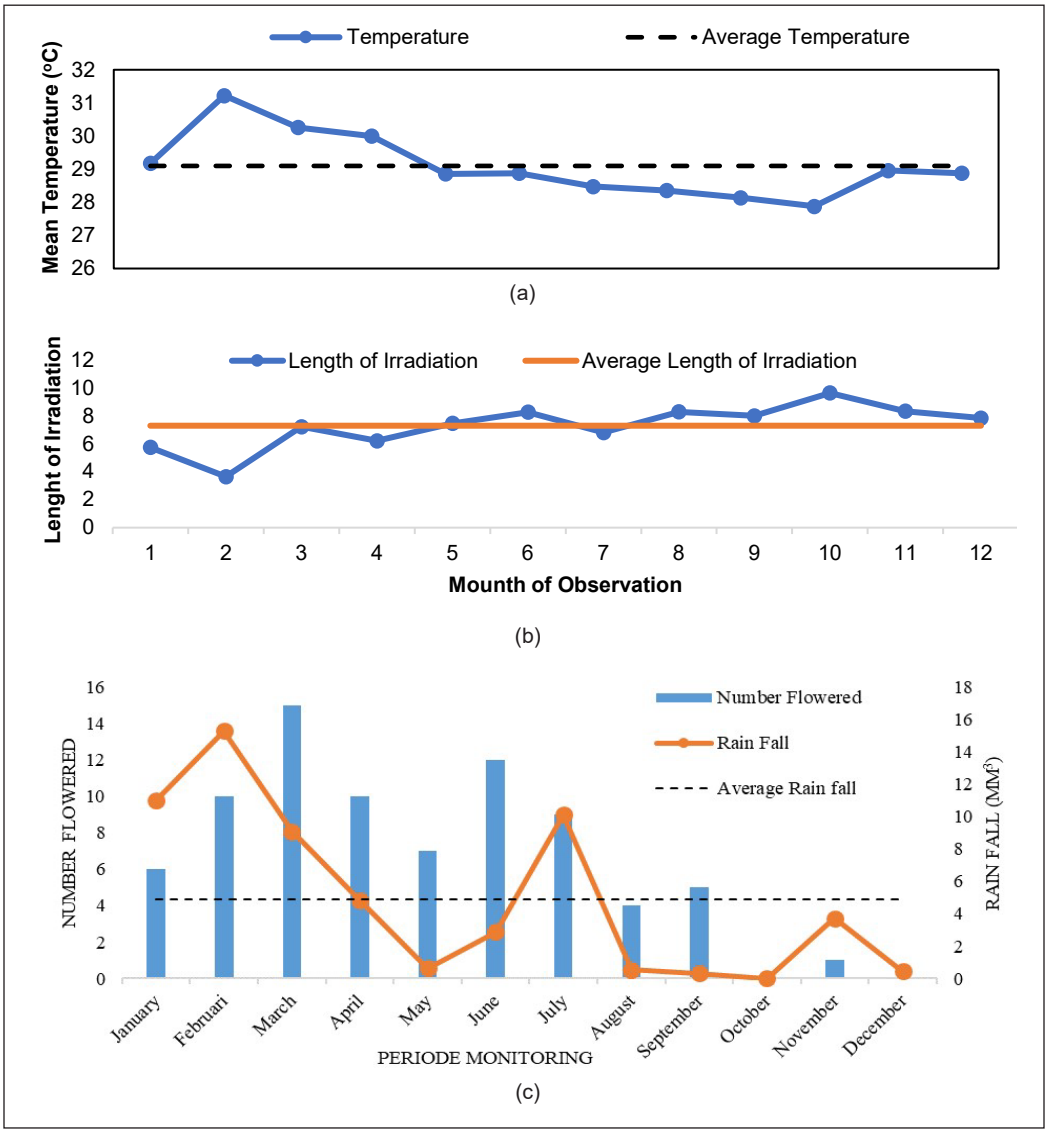

Figure 5. Flowering phenology of *R. zollingeriana* is shown as the total number of flowers blooming each month, in relation to: (a) average temperature; (b) length of irradiation; and (c) monthly rainfall

in Papring forest often occurs when the irradiation is below the average daily irradiation length that occurs every month, which was 7.3 hours (Figure 5b). This result shows that *R. zollingeriana* has low adaptation to light conditions, like other types of *Rafflesia*. The duration of irradiation indirectly affects temperature, humidity, and host health by providing nutrients for the flowering process (Nurchayati et al., 2024).

Fruit Development Stage

The fruit development stage in *R. zollingeriana* starts immediately after the flower enters the senescence stage. This stage was characterized by a change in color from red to blackish. The perigone lobes slowly turn blackish, decay, and eventually enter the rotten phase. The female flower has a disc and column that does not undergo a decay process and turns into fruit (Figure 6D). However, the male flower has both its disc and column undergo. All female flowers are assumed to produce ripe fruits if no decay occurs (Mohd-Elias et al., 2021).

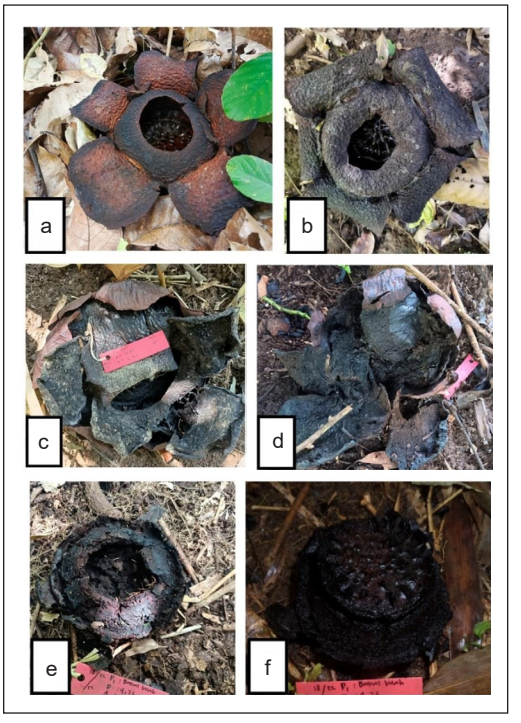

Figure 6. Stage of fruit development: (a) flower begins to turn blackish red after 2 to 3 days of blooming; (b) flower begins to enter the stage of decay or rotten stage after 5 to 7 days of blooming; (c) perigon dries up and begins to shed after 7 to 14 days of blooming; (d) perigon is fully decay after 14 to 20 days of blooming; (e) remain parts of the diaphragm and disc after 20 to 40 days of blooming; (f) leaves the discus and the fruit after 45 to 70 days of blooming

Growth Rate of *R. zollingeriana*

The growth of *R. zollingeriana* in Papring Forest was analyzed based on data on its diameter and size. The average bud growth rate reflects the variation of growth at different flower bud phases. Figure 7 shows that the growth pattern follows the exponential growth models and fits the growth model of *R. arnoldii* (Susatya, 2020), *R. patma* (Mursidawati et al., 2019; Mursidawati & Wicaksono, 2020), and both *R. cantley* and *R. azlanii* (Kamal et al., 2022). The bud growth model is expressed as Y (diameter)= $3.897e^{(0.0067x)}$, where Y represents the bud diameter and X represents the time (R^2=0.984). The model shows that the smaller buds have a slower growth rate, while the larger ones have faster rates. The cupule phase had the slowest growth rate (0.0099 cm/day). Meanwhile, the fastest was the perigone phase (1.1971 cm/day). The growth rate value showed the lowest value relative to the other stages. The developmental stage with

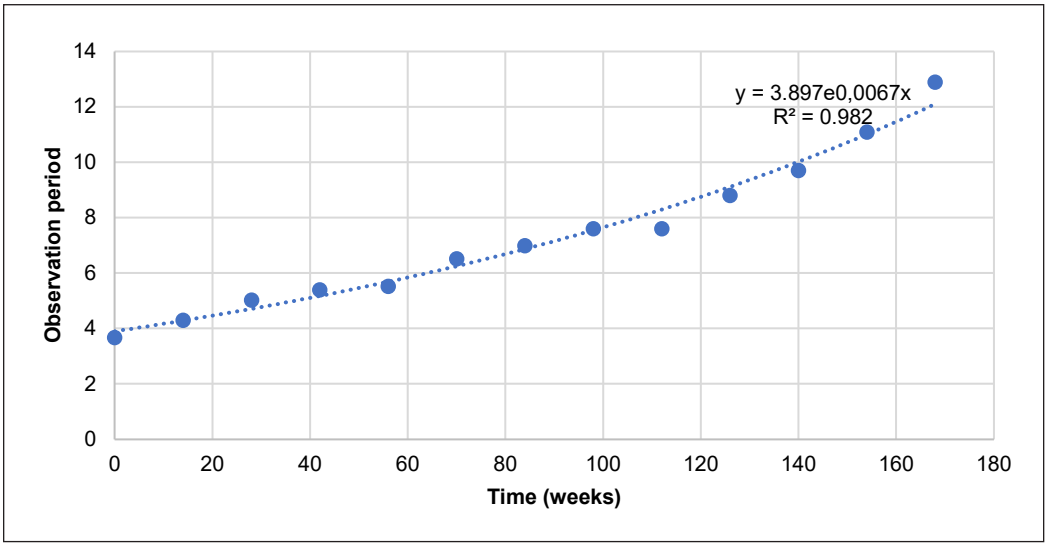

Figure 7. Growth Rate of *R. zollingeriana* based on time and observation period

Table 2
Growth rate and coefficient of variation of bud diameter by growth stage

Flower Development Stage of *R. zollingeriana* (Diameter Range in cm)	Growth Rate (cm day^{-1})	Standard Deviation (cm day^{-1})	Variation Coefficient (%)
Swollen Host (1.06–1.98)	-		
Cupule (1.53–4.97)	0.0099	0.0100	101.4239
Cupule Bract Transition (3.56–7.91)	0.0291	0.0319	91.3867
Bract (3.56–12.65)	0.0292	0.0278	105.3223
BPT with Witish (4.51–17)	0.0668	0.0557	119.9845
BPT with Pinkish (7.26–18.76)	0.0866	0.0706	122.5660
Perigon (15.09–20.19)	0.1971	0.1079	9.0113

the highest growth rate is the perigone stage, which was 1.1971 cm/day (Table 2 and Figure 7). The perigone phase is 19 times faster than the cupule phase.

Life Cycle of *R. zollingeriana*

The life history of *Rafflesia* is very important to investigate because it is both an invisible and a visible stage. The invisible stage is hard to study and starts from seeds infecting the cambium tissues of the host plant, *Tetrastigma*. The seed then grows its endophytes and radially spreads into the host's vascular tissues. It later develops into the expansion of the tissue network toward the xylem and phloem, which leads to the formation of new *Rafflesia* shoot protrusions or flower buds (De Rybel et al., 2016; Wicaksono et al., 2020; Wicaksono et al., 2021b). This intricate development process contributes to the formation

of new shoot protrusions, which play a vital role in the reproductive cycle of *Rafflesia*. This part is the beginning of the generative stage, which is part of the visible stage of *Rafflesia* (Kamal et al., 2021; Mursidawati et al., 2019; Wicaksono et al., 2020).

The vegetative development during the invisible stage is hard to observe due to the whole invisible stage taking approximately 2 to 3 years (Lestari & Susatya, 2022; Nurchayati et al., 2024). The visible stage was observable and divided into eight developmental phases: swollen host, cupule, CBT, bract, BPT with whitish, BPT with pinkish, perigone, and anthesis. The fruit development stage encompasses the stages of rotting and ripening fruit. The swollen host phase is the first sign of the emergence of the *Rafflesia* and takes 36 days to reach the CBT stage. From the CBT stage, a bud needs 21 to 70 days to enter the Bract stage (Figure 8).

The time required for buds in the bract phase to turn to the Bract Perigone Transition with whitish (BPT Whitish) phase is 21 to 43 days. The total time needed from the first stage to the BPT whitish stage is 219 days. The next stage is BPT pinkish. The analysis results show that the time required to reach the BPT Pinkish stage is 234 days. The time required for development from BPT whitish to BPT pinkish is approximately 15 to 71 days. The change from the BPT pinkish phase to the perigone stage takes 11 to 30 days (Figure 8). The time required for the perigone to develop to the anthesis stage is 28 to 118 days.

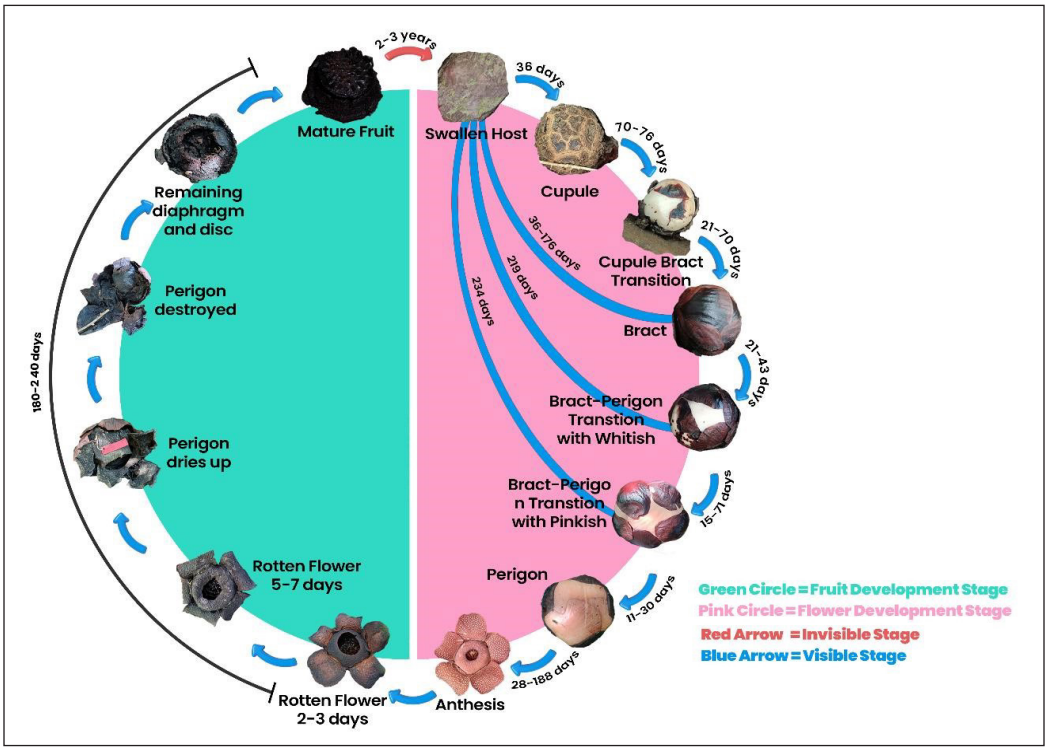

Figure 8. Life cycle reconstruction of *R. zollingeriana*

The anthesis phase or flower stage of *R. zollingeriana* bloomed perfectly for only 5 to 7 days and then blackened and entered the rotten stage. During the rotten stage, it takes 180 to 240 days to shed all the perigones and leave the ripe fruit (Figure 8).

Distribution Size, Bud Sustainability, Mortality, and Sex Ratio

The observation was made on five populations, with 9 to 171 buds in each. This condition changed as the observation time increased over 12 months (Table 3). During the 12 months, differences in mortality and bud emergence were observed. The buds that managed to stay alive and successfully bloom to form mature fruit without decaying until the end of the observation varied from different populations. The change in the population structure of the number of buds at each stage of development is shown in Figure 9.

Table 3
Condition of R. zollingeriana *buds during all observation periods in Papring Forest, Kalipuri Subdistrict, Banyuwangi, East Java, Indonesia*

Monitoring Period	Location	Total Buds Founded	Total dead Buds (%)	Blossom			Still in Buds Condition (%)
				Anthesis (%)	Rotten (%)	Mature Fruit (%)	
January–March 2023	1	86	24.42	1.16	5.81	4.65	63.95
	2	5	0.00	0.00	11.11	0.00	100.00
	3	11	72.73	0.00	0.00	0.00	27.27
	4	59	18.64	3.39	15.25	0.00	62.71
	5	67	16.42	1.49	14.93	1.49	65.67
Mean		45.50	26.38	1.21	9.42	1.23	63.92
April–June 2023	1	103	53.40	0.00	8.47	0.00	37.86
	2	9	100.00	0.00	0.00	0.00	0.00
	3	9	88.89	0.00	0.00	0.00	11.11
	4	58	77.59	0.00	3.45	0.00	18.97
	5	74	67.57	0.00	6.76	0.00	25.68
Mean		50.60	77.49	0.00	3.74	0.00	18.92
July–September 2023	1	83	54.22	2.41	10.84	0.00	32.53
	2	0	0.00	0.00	0.00	0.00	0.00
	3	12	16.67	0.00	0.00	0.00	83.33
	4	24	62.50	0.00	0.00	0.00	37.50
	5	34	67.65	2.94	11.76	0.00	17.65
Mean		30.60	40.21	1.07	4.52	0.00	34.20
October–December 2023	1	49	59.18	0.00	0.00	0.00	40.82
	2	0	0.00	0.00	0.00	0.00	0.00
	3	25	52.00	0.00	4.00	0.00	44.00
	4	11	45.45	0.00	0.00	0.00	54.55
	5	21	66.67	0.00	0.00	0.00	33.33
Mean		21.20	44.66	0.00	0.80	0.00	34.54

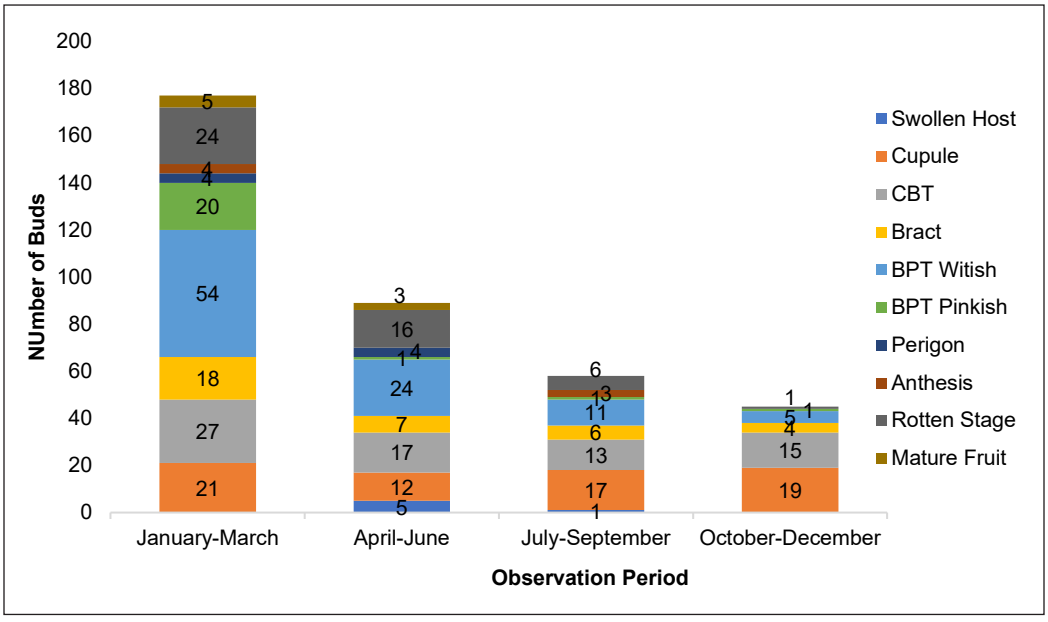

Figure 9. Population structure of *R. zollingeriana* buds in each phase after three months (January–March), six months (April–June), nine months (July–September), and 12 months (October–December)

The population size of *R. zollingeriana* in Papring Forest, Banyuwangi, has undergone varied dynamic changes across five populations. Initially, at the start of the observation, there were 148 individuals, predominantly in the bract phase, with 44 individuals, 38 in the CBT phase, and 23 each in the cupule and BPT whitish phases. Other phases, such as BPT pinkish (8 individuals), perigone (2), anthesis (2), rotten stage (6), and mature fruit (2), were present in smaller numbers. This initial phase series in Papring Forest was more comprehensive than the one found in Meru Betiri National Park, which only consisted of cupule, CBT, and bract phases (Lestari et al., 2014).

Over the following three months, the population structure of *R. zollingeriana* changed with the incoming 29 new buds, buds in growth development from a certain phase to the next growth stages, and bud mortality. Some buds in the cupule, CBT, bract, and BPT whitish phases died, while others progressed to the next phases. By the end of the three months in January to March, the number of buds in the BPT whitish phase increased to 54 individuals, whereas there were decreases in the cupule (21), CBT (27), and bract phases (18). There was an increase in the percentage of buds in the BPT pinkish (20), perigone (4), anthesis (4), rotten stage (24), and mature fruit phases (5). The observed mortality after three months was 51 buds, approximately 22.57% of the total observed (Figure 9).

Subsequent observations after six months, from April to June, revealed further changes in population size. New buds emerged, starting at the swollen host stage with five individuals. There were 89 live buds, including those in bloom or post-bloom stages.

Increases were noted in the cupule (12), CBT (17), perigon (4), and rotten stage conditions (16). The number of BPT whitish buds decreased as some transitioned to the rotten stage, resulting in 24 remaining individuals. BPT pinkish was represented by only one individual, and mature fruit by three. The observed mortality after six months rose to 167 buds, approximately 53.6% of the total buds.

From July to September, nine-month observations indicated a reduction in buds, leaving 58 individuals alive in stages such as anthesis, the rotten stage, and mature fruit. Increases were observed in the cupule stage (17), while decreases occurred in the swollen host stage (1), CBT (13), bract (6), and anthesis (3). The number of BPT whitish buds remained stable, with only one individual. Notably, perigon and mature fruit phases were absent at this stage due to either progression to subsequent phases or mortality, which claimed 85 buds, approximately 55.56% of the total.

Significant changes were evident by the end of twelve months, from October to December, with only 45 live buds remaining, mostly in the rotten stage. The swollen host stage disappeared as buds progressed, resulting in increased cupules (19) and CBT (15). Decreases were noted in the bract (4), BPT whitish (5), and rotten stage phases (1). One individual represented BPT pinkish, while the perigon, anthesis, and mature fruit phases were absent. The observed mortality after twelve months amounted to 51 buds, approximately 52.96% of the total observed during the entire period.

The total population size within one year of observation substantially varies among the five populations. The average population size, flowering success rate, and date rate were 82.13% and 78%, respectively (Table 4). The second population was recorded as the smallest population size, with nine buds, and also experienced the highest mortality within one year. All its buds had died. The first population has 171 flower buds, with a success rate of 16.4%, or 28 flowering buds. Flowering success was considered to vary from 0 to 28% and is not influenced by the population size. Similar to the success rate, the death rate does not seem to be influenced by population size. However, the death rate is considered high and ranges from 72.1% to 100%.

Table 4
Percentage of successful blooms versus percentage of deaths that occurred during the 12 months of observation

Location	Total Buds found	Number of Blooms	% Success Blooming	Number of dead	% Mortality
Plot 1	171	28	16.4%	124	72.5%
Plot 2	9	0	0	9	100%
Plot 3	43	1	2.3%	31	72.1%
Plot 19	82	14	17.1%	62	75.6
Plot 25	107	30	28%	77	72%
Average	82	15	13	61	78

Figure 10 shows that mortality was observed at various stages. The highest mortality was found in the CBT stage (27%), then followed by the rotten stage (21%), bract (16%), and cupule stages (15%). The rotten stage is dead when the bud has passed the blooming period and dies when no mature fruit has formed. So, its death was the end of the phase. The greatest mortality before blooming occurs in the CBT, bract, and cupule phases (1.53–4.97 cm, 3.56–4.97 cm, and 3.56–12.65 cm, respectively). All this high mortality was observed in small bud sizes. Susatya et al. (2017) revealed that buds smaller than 9 cm have high mortality. The buds with more than 15 cm exhibited zero mortality, leading to the blooming or flowering stage. The small bud sizes have a greater susceptibility to death. However, larger buds survived the conditions that caused death (Figure 11).

The high mortality rate of *R. zollingeriana* and the unbalanced ratio of male and female flowers pose significant challenges to the existence of *R. zollingeriana* populations in the future (Hidayati et al., 2000). Studies have shown that the mortality rates of *R. patma* and *R. bengkuluensis* buds are notably high at 44% and 49%, respectively (Mohd-Elias et al., 2021; Pelser et al., 2016). Additionally, *Rafflesia* bud mortality always occurs in various endemic areas. This high mortality is often considered a form of natural population control, linked to the host plant's ability to meet the nutritional needs of *Rafflesia* (Teppabut et al., 2018). The flowering success of *R. zollingeriana* has been observed to be relatively low, with a cumulative success rate of 17,71%. Female flowers tend to dominate the blooming flowers, with a ratio of 55% female flowers to 45% male flowers. This unbalanced ratio of male and female flowers is a natural phenomenon that has also been observed in *R. cantleyi*, where female flowers are more dominant than male flowers. However, the exact mechanism behind this imbalance in the ratio of male and female flowers remains unclear

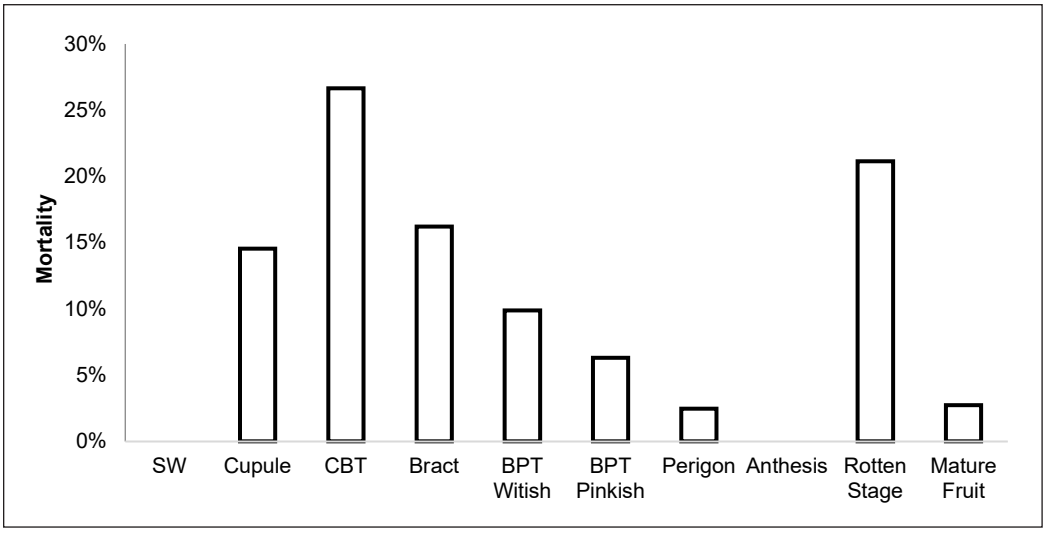

Figure 10. Mortality percentage at each time point during the 12 months of observation

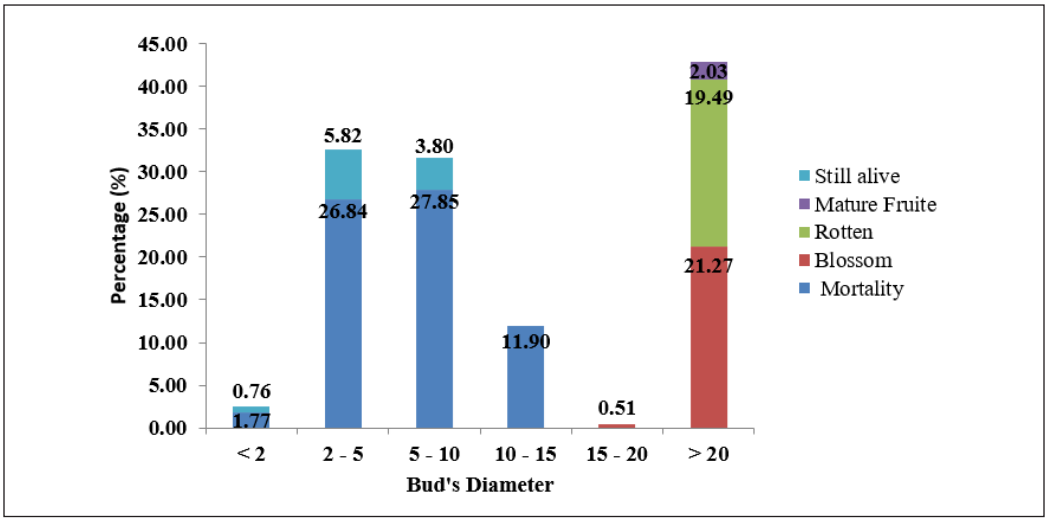

Figure 11. Condition of *R. zollingeriana* based on the distribution of bud size categories during the 12-month observation period in Papring Forest, Kalipuro subdistrict, Banyuwangi

due to limited research on the life history of *Rafflesia* (Hidayati et al., 2000). Future research is crucial to conduct a more comprehensive study of bud growth in relation to environmental factors to gain a clearer understanding of the impact of environmental conditions on bud development. DNA-based research is also needed to elucidate the mechanism behind the unbalanced sex ratio in *R. zollingeriana*. This information is vital for the *in situ* conservation of *R. zollingeriana* species in the Papring Forest, Banyuwangi Regency (Susatya, 2020).

Death of the buds was observed at different phases with different causes (Figure 12). The cause of death occurs due to environmental factors, such as drought or excessive water, and biological factors. In general, drought conditions cause the buds to dry out and disintegrate. Death was also found in the swollen host phase, as well as root damage to the host. Damage to the roots in the form of openings on the surface of the swollen host causes the cessation of its growth. This also happens to buds at the cupule and CBT phase (Figure 12b). The death of buds during the phase of CBT is probably a result of submerging buds in rainwater during the rainy season (Figure 12e). Submerging in excessive water will increase the infection of pathogenic organisms, which leads to the acceleration of the decay process (Figure 12c). Interestingly, the death caused by animal predation is also recorded at the site. Animal bites generally occur at large bud sizes or in BPT and Perigone stages (Figure 12g). Suspected animals were monkeys, wild boar, and rats. Dry conditions caused the CBT to dry and disintegrate (Figure 12d).

The condition of dead bracts in the Papring Forest often occurs because they are submerged in water and rot (Figure 12e). Excess water in *Rafflesia* buds can inhibit oxygen access to plant tissues, causing tissue death. Some BPT whitish phases were dead with holes

in the center due to being eaten by animals (Figure 12f). Injury to the bud surface can also cause death when the buds have entered the BPT with a pinkish phase (Figure 12g). The condition is similar to *R. cantleyi,* which died due to herbivory activity. Perigone phase *R. zollingeriana* buds that died due to dry conditions showed clear damage (Figure 12h).

The bract that protects the bud starts to dry out and lose its moisture. The thick and fleshy part of the perigone turns wrinkled and blackened. The inner part, the candidate flower part, decomposes and dries so that the bud can no longer open. In *Rafflesia*, normal death is at the end of the phase, namely after the flower blooms and enters the rotten stage (Figure 12i).

After blooming, *Rafflesia* flowers undergo a natural decay process essential for the ecosystem. This decay stage is marked by changes in the flower's appearance and smell. The previously large, fleshy, and reddish perigone transitions to a dark brown-to-black

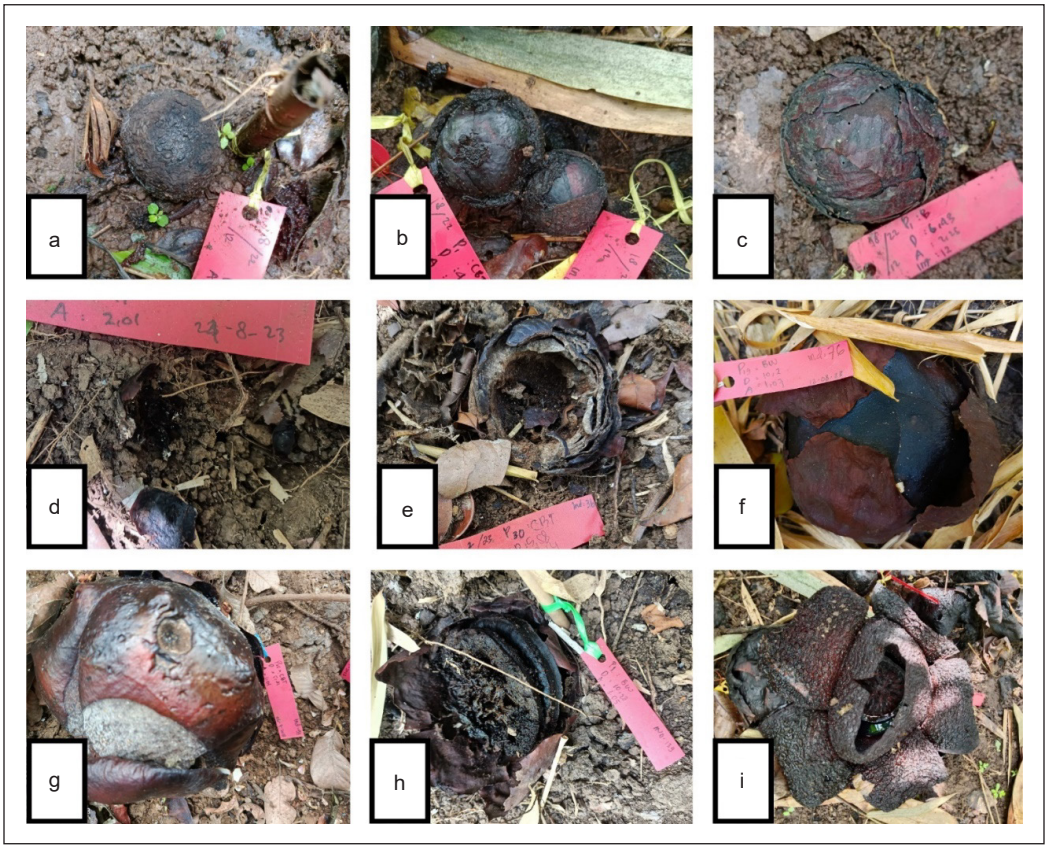

Figure 12. Various cases of death in *R. zollingeriana*: (a) Cupule that dies blackened because the *Tetrastigma* roots of its host are broken and damaged so that the supply of food intake is stopped; (b) CBT that rot because they are submerged in water during rain; (c) CBT that is destroyed due to drought conditions; (d) Bract that dies due to rotting submerged in water; (e) BPT Whitish that dies because monkeys eat it; (f) BPT Pinkish that died due to drought; (g) Perigon that died because of bite marks on its surface; (h) Perigon that dies due to drought; (i) Dead condition in flowers that have bloomed because they have entered the rotten stage phase

color, losing its thick texture. The flower's surface becomes slimy and watery, leading to the disintegration of the flower structure. This decay process is crucial as it contributes to the restoration of soil nutrients and supports the surrounding ecosystem (Mohd-Elias et al., 2021). The decay of *Rafflesia* flowers is part of their life cycle, where they play a role in nutrient recycling and ecosystem functioning. The decay process aids in returning organic matter and nutrients to the soil, enriching it and supporting the growth of other plants in the ecosystem. Additionally, the decay of *Rafflesia* flowers contributes to the overall biodiversity and functioning of the ecosystem by providing resources for various organisms (Pelser et al., 2016). Furthermore, the decay of *Rafflesia* flowers is a natural phenomenon that has been observed in other plant species as well. Studies on other flowers, such as Oenothera, have shown changes in petal color and chemical components during senescence, highlighting the natural progression of flower decay in different plant species (Teppabut et al., 2018). This senescence process is common in flowers and essential for nutrient recycling and ecosystem sustainability.

CONCLUSION

These findings underscore the species' vulnerability, low blooming success, and high mortality, which inform conservation strategies for *R. zollingeriana* in Papring Forest, Banyuwangi.

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REFERENCES

De Rybel, B., Mähönen, A. P., Helariutta, Y., & Weijers, D. (2016). Plant vascular development: From early specification to differentiation. *Nature Reviews. Molecular Cell Biology*, *17*(1), 30–40. https://doi.org/10.1038/nrm.2015.6

Hidayati, S. N., Meijer, M., Baskin, J. M., & Walck, J. L. (2000). A contribution to the life history of the rare Indonesian holoparasite *Rafflesia patma* (Rafflesiaceae). *Biotropica*, *32*(3), 408-414. http://doi.org/10.1111/j.1744-7429.2000.tb00487.x

Hikmat, A. (2006). Kecenderungan populasi *Rafflesia zollingeriana* Kds. di Taman Nasional Meru Betiri, Jawa Timur [Population Trend of *Rafflesia zollingeriana* Kds. in Meru Betiri National Park, East Java]. *Media Konservasi*, *11*(3), 105-108. https://doi.org/10.29243/medkon.11.3

Janra, M. N. (2019). Birding backyard: Birdwatching in Andalas University. *IOP Conference Series. Earth and Environmental Science*, *327*(1), 1-12. https://doi.org/10.1088/1755-1315/327/1/012025

Kamal, S. H. S., Suratman, M. N., Khamis, S., Hassan, A. N. N., & Mohammad, M. S. (2021). Host-parasitic relationships between *Tetrastigma rafflesiae* and *Rafflesia azlanii* and *Rafflesia cantleyi* in Belum-Temenggor Forest Complex, Perak, Malaysia. *Pertanika Journal of Tropical Agricultural Science*, *44*(4), 755-771. https://doi.org/10.47836/pjtas.44.4.04

Kamal, S. H. S., Suratman, M. N., Khamis, S., Hassan, A. N. N., & Mohammad, M. S. (2022). Growth rate, mortality rate and life cycle of *Rafflesia azlanii* and *R. cantleyi* in Belum-Temenggor Forest Complex, Perak, Malaysia. *Sains Malaysiana*, *51*(4), 943–957. https://doi.org/10.17576/jsm-2022-5104-01

Kusuma, Y. W. C., Matsuo, A., Suyama, Y., Wanke, S., & Isagi, Y. (2022). Conservation genetics of three *Rafflesia* species in Java Island, Indonesia using SNP markers obtained from MIG-seq. *Conservation Genetics*, *23*(6), 1039–1052. https://doi.org/10.1007/s10592-022-01470-6

Kusuma, Y. W. C., Matsuo, A., Wanke, S., Suyama, Y., & Isagi, Y. (2023). How many species of *Rafflesia* exist in Java, Indonesia and what are the implications for conservation? *Plants People Planet*, *2023*, 1-9. https://doi.org/10.1002/ppp3.10447

Lestari, D., & Susatya, A. (2022). Morphological variation of *Rafflesia zollingeriana* Koord. and its recent distribution in East Java, Indonesia. *Biosaintifika*, *14*(1), 117–124. https://doi.org/10.15294/biosaintifika.v14i1.33089

Lestari, D., Hilmat, A., & Zuhud, E. A. (2014). New distribution and population structure *Rafflesia zollingeriana* Koord. in Meru Betiri National Park, East Java. *Buletin Kebun Raya*, *17*(2), 69-78.

Maezulpah, N., Briliawan, B. D., Fairuz, R., Iman, D. T., Pratama, M., Ahsania, D. A., Nurhayati, N., Saidah, I., Nurhaeni, W., Hidayati, S. N., Khoirunnisa, E., Tyas, A., & Hikmat, A. (2019). Population structure and conservation strategy of *Rafflesia zollingeriana* Koord. in Bandealit Resort, Meru Betiri National Park. *IOP Conference Series. Earth and Environmental Science*, *394*(1), 1-8. https://doi.org/10.1088/1755-1315/394/1/012008

Mahyuni, R., Dalimunthe, S. H., Ritonga, Y. E., & Kusuma, Y. W. C. (2024). *Rafflesia* in Mount Leuser National Park, Indonesia: An updated distribution, habitat characteristics, and conservation status. *Journal of Asia-Pacific Biodiversity*, *17*(1), 179–183. https://doi.org/10.1016/j.japb.2023.11.011

Malabrigo, P., Tobias, A. B., Witono, J., Mursidawati, S., Susatya, A., Siti-Munirah, M. Y., Wicaksono, A., Raihandhany, R., Edwards, S., & Thorogood, C. J. (2023). Most of the world's largest flowers (genus *Rafflesia*) are now on the brink of extinction. *Plants People Planet*, *2023*, 1-16. https://doi.org/10.1002/ppp3.10431

Mohd-Elias, N., Rosli, K., Alias, H., Juhari, M., Abu-Bakar, M., Md-Isa, N., Mat-Isa, M., Haji-Adam, J., Goh, H., & Wan, K. (2021). Transcriptome analysis of *Rafflesia cantleyi* flower stages reveals insights into the regulation of senescence. *Scientific Reports*, *11*(1), 1-11. https://doi.org/10.1038/s41598-021-03028-x

Mursidawati, S., & Wicaksono, A. (2020). Tissue differentiation of the early and the late flower buds of *Rafflesia patma* blume. *Journal of Plant Development*, *27*, 19–32. https://doi.org/10.33628/jpd.2020.27.1.19

Mursidawati, S., Wicaksono, A., & Da Silva, J. a. T. (2019). Development of the endophytic parasite, *Rafflesia patma* Blume, among host plant (*Tetrastigma leucostaphylum* (Dennst.) Alston) vascular cambium tissue. *South African Journal of Botany*, *123*, 382–386. https://doi.org/10.1016/j.sajb.2019.03.028

Nais, J. (2001). *Rafflesia of the world.* Natural History Publications.

Nurchayati, N., Batoro, J., Hakim, L., & Azrianingsih, R. (2024). Ethnobotanical study of *Rafflesia zollingeriana* and its host (*Tetrastigma* spp.) at Papring forest, Kalipuro, Banyuwangi, East Java, Indonesia. In *Bio Web of Conferences/BIO Web of Conferences* (Vol. 117, p. 01019). EDP Sciences. https://doi.org/10.1051/bioconf/202411701019

Pelser, P. B., Nickrent, D. L., & Barcelona, J. F. (2016). Untangling a vine and its parasite: Host specificity of Philippine *Rafflesia* (Rafflesiaceae). *Taxon*, *65*(4), 739–758. https://doi.org/10.12705/654.4

Renjana, E., Astuti, I. P., Munawaroh, E., Mursidawati, S., Witono, J. R., Yuzammi, N., Fijridiyanto, I. A., Raharjo, P. D., Solihah, S. M., Robiansyah, I., Cropper, W. P., & Yudaputra, A. (2022). Assessing potential habitat suitability of parasitic plant: A case study of *Rafflesia arnoldii* and its host plants. *Global Ecology and Conservation*, *34*, 1-12. https://doi.org/10.1016/j.gecco.2022.e02063

Satake, A., Nagahama, A., & Sasaki, E. (2021). A cross-scale approach to unravel the molecular basis of plant phenology in temperate and tropical climates. *New Phytologist*, *233*(6), 2340–2353. https://doi.org/10.1111/nph.17897

Susatya, A. (2011). *Rafflesia* pesona bunga terbesar di dunia [*Rafflesia* the charm of the largest flower in the world]. Direktorat Kawasan Konservasi dan Bina Hutan Lindung.

Susatya, A. (2020). The growth of flower bud, life history, and population structure of *Rafflesia arnoldii* (Rafflesiaceae) in Bengkulu, Sumatra, Indonesia. *Biodiversitas*, *21*(2), 792-798. https://doi.org/10.13057/biodiv/d210247

Susatya, A., Lestari, D., Mahyuni, R., Kusuma, Y. W. C., Dalimunthe, S. H., Nurchayati, N., Ardiyansyah, F., As'ari, H. (2023). Morphological variation and geographical distribution of a newly recorded *Rafflesia zollingeriana* (Rafflesiaceae). *Journal of Tropical Forest Science*, *35*(4), 465-475.

Susatya, A., Prandeka, F., Saprinurdin, S., & Rahman, N. (2017). Population attributes of the very rare *Rafflesia bengkuluensis* at Kaur Regency, Southern Bengkulu. *Botanic Gardens Bulletin*, *20*(1), 1-9. https://doi.org/10.13057/biodiv/d210247

Teppabut, Y., Oyama, K., Kondo, T., & Yoshida, K. (2018). Change of petals′ color and chemical components in oenothera flowers during senescence. *Molecules/Molecules Online/Molecules Annual*, *23*(7), 1-8. https://doi.org/10.3390/molecules23071698

Tolod, J. R., Galindon, J. M. M., Atienza, R. R., Duya, M. V., Fernando, E. S., & Ong, P. S. (2020). Flower and Fruit Development and Life History of *Rafflesia consueloae* (Rafflesiaceae). *Philippine Journal of Science*, *150*(S1), 321-334. https://doi.org/10.56899/150.S1.23

Wee, S. L., Tan, S. B., Tan, S. H., & Lee, B. K. B. (2024). Bud development, flower phenology and life history of holoparasitic *Rafflesia cantleyi*. *Journal of Plant Research*, *137*, 423–443. https://doi.org/10.1007/s10265-024-01522-7

Wicaksono, A., Cristy, G. P., Raihandhany, R., Mursidawati, S., Da Silva, J. a. T., & Susatya, A. (2021a). Rhizanthes, the forgotten relative of *Rafflesia* in the Rafflesiaceae. *Botanical Review*, *88*(1), 130–143. https://doi.org/10.1007/s12229-021-09261-3

Wicaksono, A., Mursidawati, S., & Molina, J. (2020). A plant within a plant: Insights on the development of the *Rafflesia* endophyte within its Host. *Botanical Review*, *87*(2), 233–242. https://doi.org/10.1007/s12229-020-09236-w

Wicaksono, A., Trần, H. Đ., & Kamal, S. H. S. (2021b). Preliminary histoanatomical study of *Sapria himalayana* Griff. f. *albovinosa* flower buds and its intersection with its host plant *Tetrastigma laoticum* gagnep. *Journal of Plant Development*, *28*, 23–31. https://doi.org/10.47743/jpd.2021.28.1.888

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Review Article

A Review of Pre-treatments, Drying Methods, and Processing of High-protein Insect Products

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ABSTRACT

Pre-treatment and drying techniques are vital in producing high-quality insect products. Pre-treatments ensure that moisture and undesirable elements, such as gut contents and microorganisms, are removed while preserving the nutritional values of insects. Drying techniques are also crucial to reducing moisture levels, halting bacterial and fungal growth, and extending product shelf life. Several studies have reported on pre-treatment and oven, freeze, sun, smoke, fluidised bed, vacuum, and microwave drying. High-quality insect products, including powders and flakes, have been successfully produced through pre-treatment and drying technique combinations. Generally, insect pre-treatment and drying approaches depend upon the intended usage and desired attributes of the products. This study reviewed pre-treatment and drying methods for insect products to enhance the effectiveness of the insect-based food sector, and their potential applications as sustainable options for both animal feed and human food, while briefly discussing insects as a solution to global protein shortages, their nutritional benefits and associated health risks.

Keywords: Drying, feed, insects, pre-treatment, processing

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INTRODUCTION

Approximately 80% of known species worldwide are insects, with an estimated 5.5 million species having been identified (Stork, 2018). Their abundance renders

insects an excellent alternative protein source. Insects are also a sustainable protein option for the growing global population as they offer significant environmental benefits, such as rearing on organic waste, requiring minimal land and water, and producing low levels of greenhouse gases (Rumpold & Schlüter, 2015).

Insects are rich in essential nutrients, including protein, iron, and calcium, providing additional nutrients to support human growth and development (Nowakowski et al., 2021). The rapid reproduction rates and minimal space requirements of insects offer a viable solution to future food security issues and further enhance their reliability as a protein source (De Matos et al., 2024; Mohamad et al., 2021). Consequently, insects are being processed into various food items such as protein bars, powders, and snacks, to cater for the demands of health-conscious consumers (Melgar-Lalanne et al., 2019).

In rural areas where traditional agriculture is not feasible, processing insects creates job opportunities, thereby reducing poverty and improving livelihoods (Rumpold & Schlüter, 2013; Van Huis et al., 2013). The initiative also supports local economies by fostering small-scale enterprises and promoting innovative food solutions, developing communities, and increasing food security and resilience against economic fluctuations (Food and Agriculture Organisation of the United Nations [FAO], 2013).

Insects are pre-treated before undergoing processing. Commonly, insect pre-treatments include blanching and freezing (Ciurzyńska et al., 2021; Rumpold & Schlüter, 2013; Van Huis et al., 2013). Nevertheless, the processing stages, which might involve drying, could damage and/or modify the vitamins, minerals, antioxidants, pigments, and other bioactive chemicals that provide various health advantages offered by insects (Ssepuuya et al., 2020). Undesired colour changes on the final products have also been reported (Ssepuuya et al., 2020). Various pre-treatments have been applied to reduce nutrient losses and enhance the nutritional and sensory values of dried insects (Calín-Sánchez et al., 2020; Yegrem & Ababele, 2022).

Currently, processing insect techniques rely on mechanical (grinding, pressing, and milling), thermal treatments (blanching, boiling, drying, cooling, freezing, and freeze drying), and fractionation processes (extraction, purification, separation, and centrifugation) (Parniakov et al., 2021). Drying is defined as vaporising and eliminating moisture from a substance and its surfaces, typically with the assistance of a carrier gas travelling through or over the material (Keey, 1992). The technique is the oldest, most popular, and most practical method of insect processing (Parniakov et al., 2021).

Drying insect products offers several advantages. The treatment improves preservation by reducing microbial growth and spoilage-enzyme activity in insects (Morgan et al., 2006). Nonetheless, the types of drying methods employed affect the quality of the final product (Bogusz et al., 2023). Drying is the most preferred approach, even though the procedure affects the colour, density, dimension, hardness, and nutritional values of the

insects (Purschke et al., 2018). Common drying methods applied during insect processing include sun, oven, freeze, and microwave drying (Van Huis & Tomberlin, 2017). Solar, fluidised bed, and vacuum drying and sand roasting have also been documented (Parniakov et al., 2021).

Insect pre-treatment and drying process optimisation are necessary to enhance and preserve the nutritional values of the resultant products. Moreover, information on insect pre-treatment and drying procedures is critical to producing insects as feed and food. This review aims to summarise available insect pre-treatment, drying techniques and the associated drying parameters based on the region or countries that primarily implement modern and traditional insect pre-treatment and drying methods. All nations could benefit from the application of various drying procedures, particularly in regions where insect processing equipment is not easily accessible. Furthermore, the present review might offer broader research paths, including pre-treatment and drying methods, for industrial-scale insect processing applications.

METHODOLOGY

In this review, a comprehensive literature search was conducted through online databases, including PubMed, ScienceDirect, and Google Scholar. The search criteria employed were insect pre-treatment, blanching, freezing, pulsed electric field applications, insect drying and sun-drying methods, and insect-based products. Manual searches of key publications were also performed by reviewing the reference lists of relevant papers.

The articles reviewed were selected based on their relevance to the objectives of the study. Peer-reviewed journal-published empirical research focusing on drying methods, insect pre-treatment, and products derived from insects was considered for inclusion. Quantitative and qualitative articles were also eligible. Nonetheless, only scientific reports, conference proceedings, books, abstracts, and theses written in English and Malay were included. Publications not providing information on insects, published in languages other than English or Malay, possessed insufficient data descriptions (such as incomplete pre-treatment and drying method descriptions), and did not align with the review objectives, were excluded.

The present review extracted data from the selected articles, including study characteristics (such as author, year, and study design), sample details (including types of samples), intervention specifics (such as type of pre-treatment and, drying method), outcome measures, and principal findings related to the quality and production of insect-based products. Subsequently, the information was categorised into major topics and subtopics to allow a comprehensive assessment of the influences of pre-treatment and drying interventions on the quality of the final product. A total of 108 articles published between 2000 and June 2024 were selected based on the inclusion criteria previously

mentioned. Bibliographies from the selected articles also led to the identification of other relevant studies.

RESULTS AND DISCUSSIONS

Insects as a Solution to Global Protein Shortages

The global demand for protein is projected to surge by 20% by 2050 as the population surpasses nine billion, particularly in emerging nations where economic growth drives increased consumption of animal protein (Smith et al., 2024). Insects offer a promising, sustainable alternative protein source that could alleviate the environmental and ethical impacts associated with conventional livestock production, addressing global protein shortages through reduced greenhouse gas emissions, minimal land and water use, preservation of biodiversity, and improved energy efficiency, all while considering animal welfare implications (FAO, 2014).

Greenhouse gas emissions per kilogram of insect protein were lower than those for beef and pork but higher than those for chicken and fish (van Huis et al., 2013). Similar findings were reported by van Loon et al. (2018), showing that greenhouse gas emissions per kilogram of mealworm protein were lower than those for beef and pork but higher than those for chicken and fish. Animal welfare is another element that is becoming more significant. In comparison to conventional livestock production, insect farming could provide a more compassionate method (Smetana et al., 2023). In contrast to larger animals, insects may be raised in smaller settings that better suit their natural preferences, resulting in less stress and easier access to food (van Huis et al., 2013). This change not only addresses ethical concerns related to intensive animal farming but also meets consumer demand for more humane methods of food production.

Insect protein is gaining recognition as a sustainable alternative to traditional protein sources. Insects are exceptionally high in protein, fat, and micronutrients (Rumpold & Schlüter, 2013). Edible insects have a greater protein concentration than plant protein sources, including wheat, soybeans, and lentils, ranging from 35% to 60% dry weight or 10% to 25% fresh weight on average (Melo et al., 2011; Schlüter et al., 2017). At the upper range, insects provide more protein than even meat and chicken eggs (Mlcek et al., 2014). Orthopteran edible insects, such as locusts, grasshoppers, and crickets, are very high in protein (Rumpold & Schlüter, 2013). However, because insects have an extremely rigid exoskeleton, their protein digestibility varies greatly (van Huis, 2016). High chitin content exoskeletons are particularly challenging to digest (Schlüter et al., 2017). Muzzarelli et al. (2012) indicate that the digestibility of chitin in humans remains uncertain. However, the exoskeleton can be effectively removed during processing (Rumpold & Schlüter, 2013). As summarised in Figure 1, various insect processing methods help address these problems.

Figure 1 outlines the methods of processing insects, which are divided into several steps. The first phase involves harvesting the insects. Subsequent stages involve subjecting the insects to pre-treatment processes, drying, and preparing them for commercialisation. The details of each phase are discussed in the following sections.

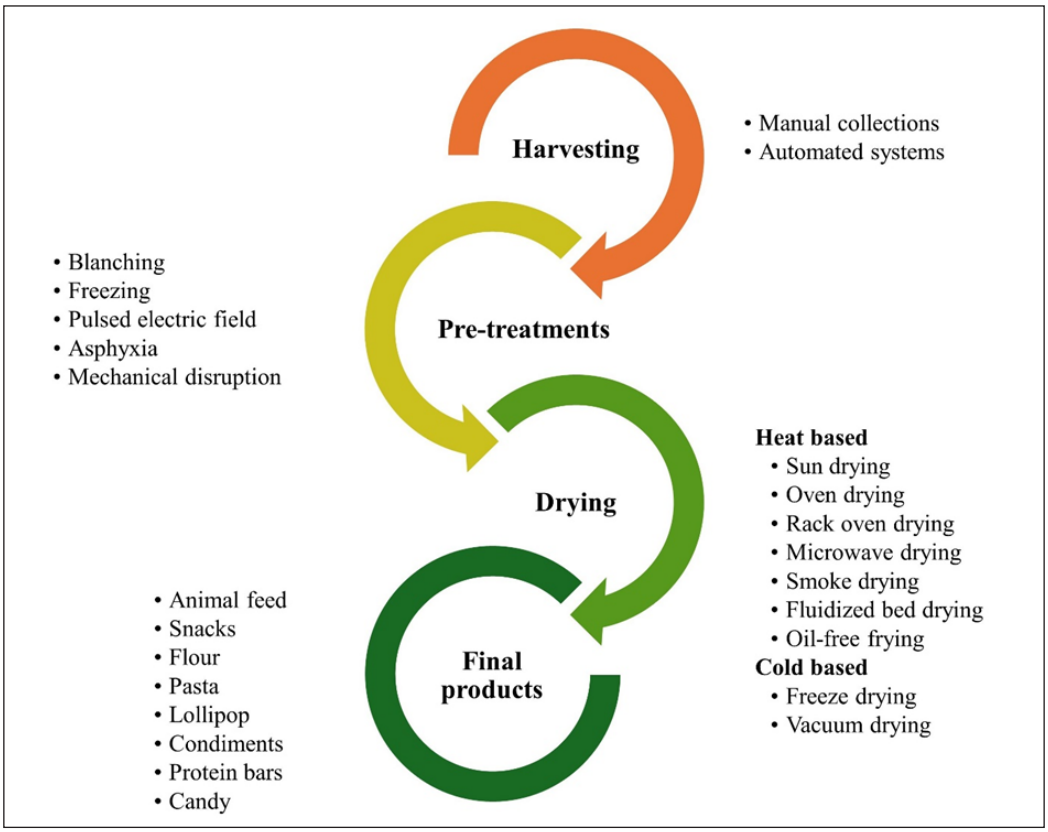

Figure 1. Summary of insect processing steps

Harvesting Process

Based on Figure 1, the initial phase of insect processing is harvesting the insects. The step is fundamental to the production chain. Harvesting is typically performed manually at small-scale production levels (Cerritos & Cano-Santana, 2008). Nevertheless, automation and industrial-scale production have become indispensable due to increasing demands for insect-based products (Berggren et al., 2018).

Automated insect harvesting systems include advanced technologies (Droukas et al., 2022). The innovations enhance productivity by allowing continuous and efficient insect collection (Sindermann et al., 2021). The approaches also facilitate production scalability without significantly increasing labour costs (Sindermann et al., 2021). Furthermore, insect

farming automation boosts production volumes and ensures consistent product quality by minimising handling stress and reducing contamination risks during harvesting (Ojha et al., 2021). Advances in the industry also allowed the integration of automation into insect harvesting processes, hence driving innovation, efficiency, and sustainability in the agriculture and biotechnology sectors (Rumpold & Schlüter, 2013).

Pre-treatment Phase

Harvested insects are pre-treated (Alles et al., 2020). Pre-treatment, or the insect-killing method, reduces microbial contamination and facilitates long-term storage of the final products (Adamek et al., 2018). Commonly employed insect pre-treatment procedures include blanching, freezing, and pulsed electric fields (Table 1). The pre-treatment methods extend the shelf life of insect-based products and maintain their nutritional contents and quality attributes, supporting the growth of the insect food industry (Larouche et al., 2019).

Table 1
Summarisation of pre-treatment methods for insect

Insect species	Pre-treatment	Condition and Results	References
Hermetia illucens larvae	Blanching	Condition: 100 °C, 40 seconds. • Reduced protein content, potentially lowering overall nutritional content. • Reduces protein browning caused by phenol oxidase, improves protein extractability, and increases susceptibility to enzymatic digestion.	Zhen et al. (2020) Leni et al. (2019)
Tenebrio molitor larvae	Blanching	Condition: 60 °C, 5 minutes. • Reduces microbial loads and stops the browning effect. Condition: 100 °C, 40 seconds. • Small decrease in protein and ash content. • Increase in water content. • Significant log reductions in Enterobacteriaceae, lactic acid bacteria, yeasts, moulds, and psychrotrophs. • Aerobic endospores are not affected.	Mancini et al. (2019) Lenaerts et al. (2018) Vandeweyer et al. (2017)
Hermetia illucens larvae	Blanching	Condition: 100 °C, 40 seconds. • Minimises lipid oxidation. • Reduces microbial contamination. • Initiates dehydration.	Larouche et al. (2019)
Polyrhachis vicina Roger	Freezing + Sun drying	Condition: -20 °C, 24 hours. • The sun-dried black ant had 28 organic components. • Combining freezing and sun drying speeds up lipid oxidation and hydrolytic rancidity.	Li et al. (2009)

Table 1 *(continue)*

Insect species	Pre-treatment	Condition and Results	References
Hermetia illucens larvae	Pulsed electric field	Condition: Monopolar pulses with an interval of 0.5 s (2 Hz), pulse duration 40 ms, specific energy intake of 10, 15, and 20 kJ/kg. • Reduces drying time and oil droplet size. • Enhances oil extraction from insects within specific power ranges.	Alles et al. (2020)
		Condition: Electric field strength: E= 1 to 3 kV/cm, specific energy: 1 to 20 kJ/kg, time: 3 h. • Optimal drying temperature is between 81-84°C and 11.2-13.1 kJ/kg of specific PEF energy input.	Shorstkii et al. (2020)
Tenebrio molitor larvae	Pulsed electric field	Condition: Peak voltage up to 30 kV and provided monopolar, rectangular pulses with a duration of 40 µs and frequency of 2 Hz. • Improved infrared drying kinetics. • Reduced browning in T. molitor infrared dried larvae biomass. • Improved microbial quality compared to untreated samples.	Bogusz et al. (2022)
Acheta domesticus	Pulsed electric field	Condition: Treatments were carried out at 1.5 kV/cm. The nominal pulse width and the frequency were kept constant at 15 µs and 20 Hz, respectively, with energy input between 4.9 and 49.1 kJ/kg. • Increased protein and fat yield. • Improved techno-functional properties. • Positively impacted chitin structure, increasing oil binding and emulsifying capacity.	Psarianos et al. (2022)
Drosophila melanogaster larvae	Asphyxia	Condition: The larvae were exposed to 100% CO_2 in a sealed petri dish. • Lethal for the larvae in 30 minutes.	Badre et al. (2005)
Adult grasshopper species	Asphyxia	Condition: The samples were placed in nitrogen gas-bubbled spring water (completely deoxygenated water). • Adult grasshopper species may live between 7.5 to 22 hours • The nymph of the same species can only survive between 3 to 13 hours.	Brust et al. (2007)
Musca domestica	Mechanical disruption	Condition: Grinding. • Most environmentally friendly technique.	Erens et al. (2012)

Blanching and freezing are the most utilised insect pre-treatment procedures (Mutungi et al., 2019). Blanching involves treating food with heat, such as steam or boiling water (Karim et al., 2023). The time and temperature parameters employed during the procedure depend upon the type of raw material and the final processing method (Lee, 1958). Typically, blanching is conducted at 60℃ to 100℃ for a brief period (between 40 s and 5 min), depending on the insects being processed (Hernández-Álvarez et al., 2021). Nevertheless, insects should be cooled rapidly after blanching to avoid the growth of surviving yeast and bacteria (Karim et al., 2023).

Blanching inactivates enzymes and microorganisms in insects, preventing undesirable deterioration in flavour, odour, nutrient content, pH, and colour (Mancini et al., 2019). *Tenebrio molitor* larvae exhibited a significantly diminished microbial load and halted browning, evidencing enzymatic inhibition following blanching at 60 ℃ for 5 min (Mancini et al., 2019). Similarly, Saucier et al. (2020) reported that blanching *Hermetia illucens* larvae at 100 ℃ for 40 s reduced their microbial load from 3.21 to 4.83 log in the dry product. In another study, Vandeweyer et al. (2017) investigated the effects of rapid blanching (10 to 40 s) followed by chilled storage or commercial microwave-drying on the microbial load in mealworms. The report revealed that blanching (with or without microwave-drying) destroyed vegetative cells but not bacterial spores. The blanched mealworms could be kept in refrigerators for six days without significant microbial development.

Blanching has several effects on insects, including microbial load reduction, thus lowering microbiological risks in the final products (Kouřimská & Adámková, 2016). Blanching also diminishes insect lipid oxidation primarily through enzyme deactivation and oxygen availability reduction (Belluco et al., 2013). Furthermore, blanching aids in preserving the natural colour of insects by inactivating polyphenol oxidase and peroxidase, which are responsible for the browning process, preventing discolouration (Yi et al., 2013). Blanching also softens exoskeletons, increasing the palatability of the insects (Rumpold & Schlüter, 2013).

During freezing, insects are exposed to extremely low temperatures, typically below −20°C (Hernández-Álvarez et al., 2021). The procedure ultimately kills or inactivates the insects due to tissue damage or basic function alterations (Lee, 1991). Nonetheless, the success of the treatment depends on the duration and freezing depth, as different insects possess differing degrees of tolerance to freezing temperatures. Low freezing temperatures lead to considerable ice crystal formations, resulting in significant water loss and inactivation of beneficial microorganisms, yielding low-quality end products (Larouche et al., 2019). Larouche et al. (2019) also reported that freezing *H. illucens* larvae at −20°C only reduced *Pseudomonas* spp. counts, requiring additional decontamination steps.

Low temperatures during freezing inhibit the growth of microorganisms already present in the environment and slow biochemical reactions that occur even after the insects are killed (Ščetar & Galić, 2017). Nevertheless, freezing does not lessen microbial load or

deactivate several hydrolytic enzymes responsible for quality degradation, which might lead to unfavourable flavour, colour, and textural changes in the insects (Montevecchi et al., 2020). In *Alphitobius diaperinus*, freezing negatively affected protein solubility, forming fat-protein aggregates that intensified browning before and after processing and raised phenol-oxidase activities during storage (Wessels et al., 2020).

Pulsed electric field (PEF) pre-treatment is a novel approach that temporarily permeabilises insect cell membranes with high-voltage pulses (Shorstkii et al., 2020). The process results in small pores in the cell membranes, leading to reversible or irreversible electroporation (Alles et al., 2020). The pre-treatment was successfully applied to *H. illucens* and *T. molitor* larvae and *Acheta domesticus* (Alles et al., 2020; Bogusz et al., 2022; Shorstkii et al., 2020).

According to Bogusz et al. (2022), *H. illucens* and *T. molitor* larvae biomass infrared drying kinetics improved following a PEF treatment at 5 kJ/kg, which modified some water binding capabilities. The treatment also increased browning in dried *H. illucens* biomass but decreased dried *T. molitor* biomass during infrared drying (Bogusz et al., 2022). Dried insects subjected to PEF also exhibited superior microbiological quality compared to untreated samples (Bogusz et al., 2022).

Psarianos et al. (2022) demonstrated that the protein and fat yields in *A. domesticus* treated with PEF increased by 18.62% and 41.75%, respectively. The article also noted that PEF enhanced the oil-binding and emulsifying abilities of the organism by approximately 40% and 70%, respectively, thereby improving techno-functional qualities. Furthermore, the chitin structure of the *A. domesticus* (10 g/100 g d.w.) was favourably influenced by PEF treatment.

Mahnič-Kalamiza et al. (2014) reported that PEF facilitated the release of intracellular components by creating pores in cell membranes. The phenomenon improved lipid, protein, and other crucial component extractions from insects. The pre-treatment procedure also reduced insect microbial loads, enhancing the safety and shelf life of insect-based products (Barba et al., 2015). PEF could also increase insect drying efficiency by enhancing their permeability and shortening the drying time (Alles et al., 2020). Moreover, cell membrane disruption during the pre-treatment reduces lipid oxidation (Barba et al., 2015).

Asphyxiation involves exposing insects to an environment with low oxygen levels or full oxygen deprivation, which leads to suffocation and eventually death. Carbon dioxide (CO_2) is commonly employed for anaesthetising invertebrates, with exposure times ranging between 3 min and 60 min, depending on the insect species (Erens et al., 2012). A CO_2 concentration exceeding 40% causes neuron depolarisation without altering conductance, immobilising insects (Clark & Eaton, 1983). Nevertheless, prolonged exposure could be fatal due to the lowering of haemolymph pH and insect dehydration initiation by promoting spiracle opening (Wong-Corral et al., 2013).

Saturating the air with nitrogen (N_2) is another method of asphyxiation, which necessitates under 3% oxygen content (Hashem et al., 2014). Vacuum packing and drowning are also applicable since large-scale air saturation might be challenging. Insect resistance to hypoxia varies according to species and developmental stages (Fernandez-Cassi et al., 2019). For instance, a study found that *Ephestia cautella* (Lepidoptera) larvae mortality rate exposed to 98% N_2 considerably varied, typically occurring within 96 h to 144 h, which was one to two days longer than the time recorded by larvae in aerobic environments of 60% CO_2 (Hashem et al., 2014).

The grinding pre-treatment entails mechanically reducing insects to tiny particles or powder with grinding equipment. Halloran et al. (2018) indicated that grinding breaks down insect structural integrity, reducing them into smaller particles or powder, allowing further processing and integration into food products. The procedure ensures particle size homogeneity, necessary for producing insect-based goods with consistent texture, flavour, and appearance (Rumpold & Schlüter, 2013). Nonetheless, grinding fresh insects has resulted in browning in numerous insects due to enzymatic and non-enzymatic reactions with polyphenols, which could reduce product quality (Janssen et al., 2017; Janssen et al., 2019; Yi et al., 2013). Accordingly, grinding should be conducted after stabilising the product.

Drying Methods

The techniques for drying insects are categorised into heat- and cold-based methods (Melgar-Lalanne et al., 2019). Heat-based approaches include sun, oven, microwave, smoke, and fluidised bed drying. On the other hand, cold-based approaches comprise freeze and vacuum drying. The information on the techniques is summarised in Table 2.

Generally, heat-based drying methods are preferred over their cold-based counterparts (Hernández-Álvarez et al., 2021). According to Omari et al. (2018), heat-based techniques are rapid and effective. Moreover, hot air convection and infrared radiation allow rapid drying (Mujumdar, 2006; Sharma et al., 2005). Heat drying is also more economical than energy-intensive methods, such as freeze drying (Novak & Lewicki, 2004). Moreover, heat-based drying is simple to manage, offering better drying process regulation than other approaches, including temperature adjustments required by different materials (Stramarkou et al., 2021).

Every strategy has different drawbacks and advantages. For instance, heat-based drying techniques lower product quality by causing shrinkage, warping, or cracking (Andharia et al., 2023). The process involves heating a material continuously, leading to excessive energy consumption and energy expenditure. Moreover, heat-based drying might pose safety hazards, such as fire threats, if performed inappropriately.

Table 2
Summarisation of drying methods for insect

Insect species	Common name	Drying method and condition	Main findings	References
Rhynchophorus phoenicis	Palm weevil	Solar: 5 days Oven: 50 °C (48 hours) Smoke: 6 hours	The best preservation technique for lipids was discovered to be smoking.	Tiencheu et al. (2012)
Sternocera orissa	Giant jewel beetle	Oven: 66 °C /24 hours Freeze: -55 °C /24 hours Frying pan: 130-cm diameter, 50-mL tap water. Fried without cooking oil.	Oven drying and cooking methods improved the proximate chemical composition.	Shadung et al. (2012)
Imbrasia epimethea	African moth	Oven: 80 °C /8 hours Solar: 3 days	Both drying methods showed a slight reduction in monounsaturated fatty acids.	Lautenschläger et al. (2017)
Ruspolia differens	Longhorn grasshopper	Freeze dry: Phase (1) −50 °C/0.40 bars/48 hours Phase (2) −55 °C/0.021 bars/48 hours Oven: 60 °C /24 hours	Both drying methods produced the same nutritional quality.	Fombong et al. (2017)
Tenebrio molitor	Mealworm larvae	Microwave assisted drying: 8, 10, 13, 16, 20 min/ 2 kw	Drying for 16 or 20 minutes produced average water activity of 0.16 and 0.23, which is required to completely stop microbial development.	Vandeweyer et al. (2017)
Polyrhachis vicina Roger	Black ant	Sun: 20–35 °C	Sun drying speeds up lipid oxidation and hydrolytic rancidity.	Li et al. (2009)
Musca domestica	Housefly larvae	Oven and sun dry	Oven drying yields higher protein larvae while sun drying yields higher fat content.	Aniebo and Owen (2010)
Hermetia illucens	Black soldier fly larvae	Oven: 60 °C Microwave drying: 500 W/15 min	Conventional and microwave-dried larvae have an essential amino acid to total amino acid ratio greater than 40%.	Huang et al. (2019)

Table 2 *(continue)*

Insect species	Common name	Drying method and condition	Main findings	References
Tenebrio molitor	Mealworm larvae	Hot air: 60 °C/7 hours and 24 hours, 80 °C/7 hours and 24 hours. Freeze-dry: 0.2 mbar/48 hours. Fluidised bed: bed temperature 60 °C, air outlet temperature 55 °C, differential pressure bed 15 bar, differential pressure filter -1.3 bar, air flow 500 $m^3 h^{-1}$/2 hours.	Due to browning reactions and tissue collapsing, drying at high temperatures resulted in considerable darkening and shrinkage.	Purschke et al. (2018)
Tenebrio molitor	Mealworm	Rack oven: 120 °C /1 hour/ ventilation stage 2. Freeze dry: -50 °C /24 hours Vacuum: vacuum oven 60 °C/ 24 hours	The mealworm powder with the best solubility was obtained via vacuum drying.	Kröncke et al. (2019)

Sun drying is a classic method of drying, which has been applied to vegetables, meats, seafood, fruits, and insects for a long time (Malakar et al., 2022). Nevertheless, direct sunlight drying necessitates a considerable open space, is significantly dependent on daylight, exposes items to insects, birds, and rodents, and is prone to contamination from foreign contaminants, including litter and dust (Agbede et al., 2023). Nevertheless, Yisa et al. (2022) suggested sun drying as a cost-efficient and effective alternative to oven and freeze drying for preserving edible insects, such as grasshoppers (*Ruspolia differens*), crickets (*Gryllus bimaculatus*), and caterpillars (*Bunea alcinoe*).

Prolonged sun drying might lead to heat-sensitive vitamin degradation, such as vitamins C and B (Halloran et al., 2018). According to Nguyen and Nguyen (2015), sun drying might render insect lipids vulnerable to oxidation, which could result in lipid-soluble vitamin breakdowns and unpleasant smell and taste developments. Water-soluble substances leaching, such as several B vitamins and minerals, might also occur from extended exposure to heat and sunshine during drying, specifically if the insects are not properly shielded (Halloran et al., 2018).

Oven drying is the most employed method due to its cost-effectiveness and adaptability to industrial operations (Table 2). Oven drying is a controlled dehydration process that utilises heat to remove moisture from objects or materials. Although various systems, including vacuums and rotating ovens, are applicable, their impacts on the products might differ (Kröncke et al., 2018). Employing high temperatures during oven drying also could

have detrimental effects on several functional qualities of the insects when turned into ingredients, particularly proteins (Azagoh et al., 2016; Kröncke et al., 2019).

Low temperatures are preferable in protein solubility preservation and Maillard reaction, shrinkage, and tissue collapse reduction (Kröncke et al., 2018; Melis et al., 2018; Purschke et al., 2018). Generally, temperatures between 50°C and 120°C are applied from 1 h to a few days (Kröncke et al., 2018; Melis et al., 2018; Purschke et al., 2018). Azzollini et al. (2016) found that the optimum drying temperature that minimised negative impacts and shortened the drying time was 60°C. In another study, Tiencheu et al. (2012) reported that oven-dried *Rhynchophorus phoenicis* exhibited raised peroxide values, particularly when boiled before being dehydrated. Conversely, oven-dried *Sternocera orissa* significantly recorded increased essential and non-essential amino acids (Shadung et al., 2012).

Microwave drying relies on the interactions between the electromagnetic radiation in the microwave frequency range and the moisture content of the material being dried. Primarily, microwave drying is more rapid than oven and freeze drying regarding drying insects (Kröncke et al., 2019). Bawa et al. (2020) noted that microwave drying was considered the most suitable for *A. domesticus* (crickets), considering their notable mineral element levels. The process improved colour parameters and had less microbiological load than the oven-dried samples. Nonetheless, the approach might denature the proteins and affect the functional qualities of the resulting components, similar to oven drying (Shorstkii et al., 2020).

Microwave drying requires 10 to 15 min, subject to microwave parameters, to completely dry insects (Lenaerts et al., 2018). Although the technique yields inflated, complete, and dried larvae, it permits browning in *T. molitor* larvae (Lenaerts et al., 2018). Furthermore, microwave-dried mealworm protein solubility documented a 40% reduction (Kröncke et al., 2018). Compared to oven-drying at 60 °C, microwave drying led to protein polymerisation in BSF larvae, which lowered the digestible amino acid score and digestibility, producing a powder with larger particle sizes (Huang et al., 2019).

Smoke drying is another food preservation technique which utilises heat and smoke. According to Ledesma et al. (2017), the method frequently preserves meat, fish, and other foods across civilisations globally. The raw products are exposed to smoke from wood pyrolysis, and the procedure is commonly coupled with salting. The entire process integrates heating, drying, salting, and smoking in a smoking chamber (Hernández-Álvarez et al., 2021). Nevertheless, the procedure could adversely affect the flavour of the final products (Hernández-Álvarez et al., 2021).

Smoke-dried insects are less documented than other drying processes (Table 2). Tiencheu et al. (2012) investigated the method on *Rhynchophorus phoenicis*, where a 6-h smoke drying procedure resulted in high-quality products. Nonetheless, smoke drying is less preferred due to health hazards from the smoke, which might contain carcinogenic compounds (Essumang et al., 2013). Studies have also reported that smoke-dried products

could have polycyclic aromatic hydrocarbons linked to increased cancer risks (Alexander et al., 2010).

A fluidised or fluid bed dryer is a type of machinery commonly utilised in the chemical, food, and pharmaceutical industries to dry heat-sensitive materials that tend to clump together rapidly (Bhakar, 2023). The technique was designed to reduce the moisture content of wet flakes, granules, and powders. During fluidised bed drying, heated air is introduced under high pressure into a perforated bed containing wet solid particles. Subsequently, the damp solids are lifted from the bottom and suspended in a fluidised state, floating within an airstream. Heat is transferred through the direct contact between the wet material and the hot gases. The drying gases transport the vapourised liquid. Kröncke et al. (2019) extensively studied the approach utilising *T. molitor* larvae. The article reported that the fluidised bed-dried mealworms exhibited lower water solubility (19.25% ± 0.21%) than their freeze-dried counterparts (40.65% ± 0.21%).

Fluidised bed drying offers comparatively lower drying times and requires larger-scale continuous manufacturing than vacuum drying and freeze drying (Bayrock et al., 1997). Nevertheless, fluidised bed drying might lead to protein denaturation at high temperatures, potentially affecting their functional qualities when utilised as ingredients (Kröncke et al., 2019). Although fluidised bed drying at 130°C for 110 min could slightly increase lipid oxidation, the procedure might also cause browning (Kröncke et al., 2018).

Although the heat-based drying method is commonly preferred, certain insect species require cold-based drying approaches (Table 2). Cold-based drying procedures employ dry and cold air to remove moisture from a material or item. After reaching the necessary moisture content, a material dries when subjected to circulating low-humidity air (Kilic, 2009). The preservation technique is frequently employed in industrial settings as it offers a less harmful alternative to heat drying (Kilic, 2009). The strategy is commonly applied in food processing, electronics production, and the storage of sensitive chemicals. Examples of cold-based drying methods include freeze-drying and vacuum drying.

Cold-based drying processes yield fewer risks of product damage or quality degradation (Kilic, 2009). Cold air drying also contributes to reduced product oxidation, whereas high temperatures might result in oxidation and spoiling, which could affect the colour and flavour of the products (Van Loey et al., 2005). Furthermore, drying at a low temperature preserves the nutritious contents of the products (Shonte et al., 2020).

Although cold air drying provides numerous food preservation advantages, the technique has some drawbacks. For instance, cold air requires a longer period to dry compared to hot air. Furthermore, cold air drying requires large spaces for air circulation. Several products benefit from cold air drying, while materials containing considerable water content might necessitate heat or a specific drying atmosphere. Moreover, inappropriately managed cold-dried materials might increase humidity and increase mould formation risks (Erkmen & Bozoglu, 2016).

Table 2 shows that freeze drying is the most utilised cold-based drying method. The technique is sometimes referred to as lyophilisation, which eliminates moisture from a product without affecting its composition or characteristics. For instance, Yi et al. (2013) documented that the essential amino acid levels of freeze-dried *A. domesticus*, *A. diaperinus*, *T. molitor*, *Zophobas morio*, and *Blaptica dubia* were equivalent to those in soybean proteins. In freeze drying, a material is frozen before it is placed in a vacuum atmosphere to sublimate the frozen water (Kröncke et al., 2019). Biological samples and perishable products, including fruits, vegetables, meats, and insects, are frequently preserved through freeze-drying (Kröncke et al., 2019).

Freeze drying is one of the best drying processes for retaining insect colour since it does not induce the Maillard reaction, hence yielding the whitest powder (Lenaerts et al., 2018). Furthermore, freeze-dried larvae appear inflated rather than shrunken, which might increase consumers' acceptance (Larouche et al., 2019). Nevertheless, the technique is expensive, with a minimum of 24 h to 53 h required to completely dry insects (Kröncke et al., 2018; Lenaerts et al., 2018). The approach also reduced protein solubility by 10% (Kröncke et al., 2018). Although freeze-drying causes lipid oxidation, leading to significant quality loss, blanching decreases the effects by half when applied before freeze-drying (Kröncke et al., 2018; Lenaerts et al., 2018).

Establishing a low-pressure atmosphere to remove moisture from a product is the procedure involved in vacuum drying (Kröncke et al., 2019). A material is placed in a vacuum chamber, which removes the pressure, and evaporation eliminates moisture. Vacuum drying requires less time and energy, considering the lower temperature and swifter pace of water evaporating in a vacuum than in normal air circumstances (Earle, 1969). Seho et al. (2021) demonstrated that blanching and vacuum drying *T. molitor* larvae reduced over 5 log *Escherichia coli* load and optimally preserved the brightness of the larvae.

Vacuum drying preserves heat-sensitive chemicals at lower temperatures than fluidised bed and oven drying. Nevertheless, the technique is time-consuming and possesses limited applicability on a broad scale due to the time required to dry insects. Vacuum drying employs low temperatures and pressures to reduce lipid oxidation and browning, retaining insect quality (Rumpold & Schlüter, 2013). Furthermore, vacuum drying has been proven to preserve the protein content and overall nutritional value of edible insects better than conventional drying techniques (Ssepuuya et al., 2017).

Insect Processing and the Resultant Products

According to Melgar-Lalanne et al. (2019), insects are gaining attention as prospective dietary components for sports dietary supplements, such as protein concentrates or isolates, flours, energy bars, protein shakes, and hydrolysates, due to their notable protein contents and well-balanced amino acid profile. Mealworms were the first species to be authorised

as food following the risk assessments on insects by the European Food Safety Authority (EFSA) in 2018 (Turck et al., 2021). Consequently, edible insects have been sold as flours, heat-dried larvae, pupae, and dried and powdered adult insects (Table 3). Edible insects are also available in various forms, including bulk goods in powder and flour forms, candy, chocolate-covered snacks, and liquor infusions (Melgar-Lalanne et al., 2019).

Table 3
Insect processing and products according to the countries

Insect species	Drying method	Product	Countries	Sources
Tenebrio molitor	Freeze dried	Feed	United States	https://www.etsy.com/market/freeze_dried_mealworm?ref=lp_queries_internal_bottom-8
Acheta domesticus	Roasting	Snacks	Malaysia	https://www.ento.my/collections/catalog
Hermetia illucens larvae	Microwave dried	Feed	Malaysia	https://biovae.com.my/shop/biovaegrubs-dried-bsfl/
Haplopelma albostriatum	Oven drying	Snacks	Thailand	https://www.thailandunique.com/canned-edible-tarantula-spider
Platylomia Radah	Microwave dried	Snacks	Thailand	https://www.thailandunique.com/edible-insects-bugs/edible-cicadas-for-sale
Bombyx mori pupae	Sun dried	Feed	China	https://www.alibaba.com/product-detail/Sun-Dried-Silkworm-Pupae_234965973.html?spm=a2700.7724857.0.0.12363cfbqzy23X
Tenebrio molitor larvae, *Acheta domesticus*, *Locusta migratoria*	Microwave dried	Lollipops	United Kingdom	https://www.crunchycritters.com/shop/buy-edible-insects/lollipops/lollipops-4-x-30g/
Alphitobius diaperinus	Freeze dried	Lollipops	United Kingdom	https://www.crunchycritters.com/shop/buy-edible-insects/lollipops/lollipops-4-x-30g/
Bombyx mori pupae	Oven dried	Candy	Thailand	https://www.thailandunique.com/chocolate-covered-insects/chocolate-covered-silkworms
Zophobas morio	Oven dried	Candy	Thailand	https://www.thailandunique.com/chocchoco-covered-insects/white-chocolate-superworms
Acheta domesticus	Freeze dried	Protein bars	Singapore	https://altimatenutrition.com/products/chocolate-banana-cricket-protein-bar

In the European Union, only four insect species have been approved as food: (1) dried *T. molitor* larvae, (2) *Locusta migratoria* (frozen, dried, and in powder form), (3) *A. domesticus* (frozen, dried, powder form, and partially defatted powder), and (4) *A. diaperinus* larvae (frozen, dried, and paste and powder forms) (Parniakov et al., 2021). Meanwhile, approximately 200 insect species are consumed in Thailand (Mongkolvai et al., 2009). The insects are prepared in creative ways, such as curries, dipping (combined with chilli paste), and salted, in addition to the standard roasting, frying, and steaming (Halloran et al., 2015; Raheem et al., 2018). Nonetheless, consumption is localised. For instance, although crickets are a northeastern Thailand speciality, they are rare in Bangkok restaurants as they are perceived as a rural poverty sign (Halloran et al., 2016).

Only the Borneo regions of Malaysia practice entomophagy. Durst et al. (2010) documented that *Rhynchophorus ferrugineus* larvae are valued for their significant protein content and consumed stir-fried or roasted in Borneo. Various species of grasshoppers, such as pointed-nose, short-horned, leaf-like, and *Valanga* sp., are also consumed in that region. Typically, the grasshoppers are boiled, lightly seasoned, and simmered until dry, and occasionally stir-fried, deep-fried, and roasted (Durst et al., 2010).

Numerous meals, including pastries and items that resemble meat, have been fortified with *T. molitor* larvae (Melgar-Lalanne et al., 2019). The insect has also been added (2%, w/w) to corn tortillas. In a study, Aguilar-Miranda et al. (2001) reported that maize tortillas with 7.14% *T. molitor* larval flour recorded a 2% greater protein content. The emulsification capabilities of freeze-dried *T. molitor* larvae-enriched emulsified sausages were also enhanced (Kim et al., 2016). Meat batter incorporated with edible silkworm pupae (*Bombyx mori*) and transglutaminase exhibited enhanced protein and ash content and reduced cooking loss, improving its physicochemical attributes (Park et al., 2017).

Kinyuru et al. (2015) reported that Winfood Classic, a complementary food containing oven-dried termites and dagaa fish had higher levels of protein, energy, fat, and zinc than the control samples, which had no termites and dagaa fish. The findings confirmed that the product can be consumed as a complementary food. In a separate study, Koffi et al. (2013) indicated that biscuits manufactured from up to 25% deffated *Macrotermes subhyalinus* flour mixed with sorghum flour recorded improved protein and mineral contents. Cinereous cockroach (*Nauphoeta cinerea*) flour was also adequately sanitary with a good nutritional profile (De Oliveira et al., 2017). Furthermore, the technical and sensory qualities of wheat bread were unaltered by the inclusion of insect flours (De Oliveira et al., 2017).

Approximately 70% to 95% of animal species are insects, making them the most diverse category (Chapman, 2009). Consequently, insects are the most suitable feedstock for human consumption. India, Thailand, China, and Mexico are the top four nations that consume insects (Jongema, 2017). Nevertheless, the extent, species, and methods of consuming insects as food or feed differ due to geographical and cultural variances.

Insects are particularly valued for their nutritional content. For instance, *T. molitor* and *H. illucens* larvae, are commonly applied as feed as they offer significant lipid and protein levels. *Tenebrio molitor* larvae contain approximately 24.3%-27.6% protein and 12%-12.5% lipids comparable to meat (Ghaly & Alkoaik, 2009). Meanwhile, the nutritional quality of *H. illucens* larvae varies with the substrate employed during rearing. For example, larvae supplied with palm kernel meal recorded crude protein and lipid levels of 42.1%-45.8% and 27.5%, respectively (Rachmawati et al., 2010). Consequently, adjusting the substrate allows the customisation of crude protein and lipid content to meet specific customer requirements.

Acheta domesticus are freeze-dried to produce protein bars, flour and pasta. The freeze-drying method preserves the nutritional content of the insect despite being expensive (Ratti, 2001). *Acheta domesticus* contains one of the highest protein values among edible insects at 64.4%–70.8% dry weight, considerably higher than conventional protein sources (Mariod et al., 2017). In India, *Oecophylla smaragdina* and *Samia ricini* larvae are sun-dried in particular regions, which could reach up to 40°C. The nation also receives ample sunlight throughout the year (Oldenborgh et al., 2017). Sun drying is also cost-effective and does not require any equipment.

Insects sold as snacks, lollipops, and candies are common in regions with years of practice in entomophagy, including Thailand, Central African countries, Cambodia, and China. In China, edible insect consumption is significantly common, as over 324 species from 11 groups of insects have been classified as edible (Feng et al., 2017). Gahukar (2018) reported that more than 82 insect species from nine orders are consumed regularly in Indian cuisines.

Using insects as animal feed and human food requires distinct quality and safety regulations. Key considerations for animal feed include microbial hazards, chemical hazards, and allergens, with regions such as the European Union, North America, East Asia, Australia, and Nigeria implementing regulations on insect feed to support its potential as a novel feed resource in the future (Lee et al., 2022). Conversely, human food must adhere to more stringent laws prioritising nutritional value, sensory qualities, and cultural acceptance. For human use, factors including microbiological safety, allergenicity, and heavy metal residues become even more crucial (Henderson, 2022; Lee et al., 2022). This differentiation needs specialised processing, pre-treatment, and storage techniques for insect products intended for certain applications.

Health Risks and Safety Considerations of Insect Protein

Insect protein is increasingly recognised as a sustainable and nutritional alternative to traditional protein sources. However, its intake poses a number of health hazards and safety concerns that must be addressed to assure consumer safety. The potential food safety

problems related to edible insects may be categorised into three categories: chemical, biological, and allergic (Murefu et al., 2019).

Among these, chemical hazards are a major concern, particularly due to pesticide residues, mycotoxins, and heavy metals (Murefu et al., 2019). Wild-harvested edible insects may contain pesticide residues due to their uncontrolled feeding on pesticide-sprayed vegetation, leading to the accumulation of residues in their body and eventually increasing the risk of food poisoning for consumers. For example, Murefu et al (2019) found that insect samples contained high levels of chlorinated and organophosphorus pesticides, with concentrations reaching 49.2 µg/kg and 740.6 µg/kg, respectively. Pesticide residues have been reported in edible insects such as *T. molitor* larvae, *Musca domestica* larvae and *H. illucens* larvae (Charlton et al., 2015; Gao et al., 2014; Van der Fels-Klerx et al., 2016). However, with the current development of edible insect farming, which controls their nutrition, it is feasible to create pesticide-free edible insects (Murefu et al., 2019).

Mycotoxins are a category of harmful secondary metabolites generated in the food chain by fungi that infect crops before and after harvest (Evans et al., 2022). The mycotoxins that have been studied include aflatoxins, fumonisins, zearalenone (ZEN), vomitoxin or deoxynivalenol (DON), and ochratoxins (OTAs) (Evans et al., 2022). Mycotoxins may be found in the feed substrate on which edible insects are cultivated (Muresu et al., 2019). Presence of mycotoxins with varying concentration has been reported in *Imbrasia belina* caterpillar, *Bunaea alcinoe* caterpillar, stink bugs and *T. molitor* larvae (Braide et al., 2011; Musundire et al., 2016; Simpanya et al., 2009; Wynants et al., 2017).

Heavy metals also pose significant concerns as chemical hazards in edible insects. Heavy metals such as lead, mercury, arsenic, and cadmium can cause systemic toxicities at low exposure levels, leading to adverse health effects in humans and animals (D'Souza & Peretiatko, 2002; Jan et al., 2015). Heavy metal accumulation in edible insects is influenced by various factors such as insect species, growth phase, and feed substrate (Van Huis, 2021). Cadmium, lead, mercury, and arsenic are essential heavy metals that accumulate in edible insects based on the metal element, insect species, and development stage (Diener et al., 2015; Van der Fels-Klerx et al., 2018). Cadmium and arsenic have been found to accumulate in black soldier fly larvae and yellow mealworm larvae (Van der Fels-Klerx et al., 2018). In Thailand, mercury, lead, cadmium and arsenic were detected at low concentrations in four edible insects, which are the mulberry silkworm, scarab beetle, house cricket and Bombay locust (Köhler et al., 2019).

Beyond chemical risks, biological contaminants also pose serious issues when it comes to the consumption of edible insects. Both spoilage and pathogenic microorganisms can be inherent in the level of contamination depending on the insect type, processing methods, handling procedures, and hygiene practices (Rumpold & Schlüter, 2013). Pathogenic bacterial genera such as *Escherichia*, *Staphylococcus*, and *Bacillus* can infect both humans

and invertebrates, including insects, posing health hazards to consumers of edible insects, even in the absence of contamination from other sources (Grabowski & Klein, 2017). Pathogenic microorganisms have been identified from a variety of edible insect species and are frequently linked to foodborne illness outbreaks (Murefu et al., 2019).

Edible insects processed and sold in Thailand harbour many potentially human pathogenic bacterial genera, including *Vibrio*, *Streptococcus*, *Staphylococcus*, *Clostridium* and *Bacillus* (Osimani et al., 2017). Moreover, consumption of edible insects may also lead to the transmission of parasitic foodborne diseases, with wild-gathered insects having a higher risk of transmission than farmed insects due to their unconfined eating habits (Murefu et al., 2019). Boye et al. (2012) reported that consumption of ants transmits *Dicrocoelium dendriticum*, a zoonotic parasite to humans.

In addition to chemical and biological risks, allergenicity also poses a significant concern in insect consumption. Due to their high protein content, edible insects, particularly those containing arginine kinase, may pose allergy risks (Murefu et al., 2019). Since insects and crustaceans are linked, it is possible that they might result in food allergies (Francis et al., 2019). In addition to arginine kinase, α-amylase and tropomyosin are other often found allergens associated with edible insects (Srinroch et al., 2015). A broad population may be at risk for allergic responses, as evidenced by research done in Belgium that indicated 19% of persons were sensitised to grilled samples of *A. domesticus* and *T. molitor* insects (Francis et al., 2019).

Although there are limited studies on adverse effects, eating insects has been connected to 7.6% of allergic responses in Laos and 18% of fatal food reactions in China (Barennes et al., 2015; Ji et al., 2009). Insects such as mealworm, silkworm, sago worms, caterpillars, grasshopper, locust, bee, cicada, *Bruchus lentis* and *Clanis bilineata* have been reported to cause food allergy (de Gier & Verhoeckx, 2018). Allergic responses to edible insects have been reported in various regions, including silkworm pupa in China, mopane caterpillars (*Imbrasia belina*) in Africa, and locusts and grasshoppers in India (Chakravorty et al., 2011; Ji et al., 2008; Kung et al., 2011). Additionally, carmine, a food pigment made from female cochineal insects (*Dactylopius coccus*), is the only insect-derived food additive linked to allergy reactions to date (de Gier & Verhoeckx, 2018). In order to protect consumers, such responses emphasise the necessity of additional allergenicity research and explicit labelling of items derived from insects.

Future Prospects

The widespread practice of consuming insects as feedstocks and snacks in the Asia-Pacific region reflects the cultural practices of the population in the region. Moreover, the action aligns with several Sustainable Development Goals (SDGs). In the Asia-Pacific region, particularly in rural areas, entomophagy is an accepted diet. The practice meets the SDG 2

objective, Zero Hunger, as insects provide a nutrient-rich source of proteins and essential nutrients to combat hunger and malnutrition (FAO, 2013; Yen, 2015).

Insect commercialisation creates job opportunities, fulfilling the eighth SDG, decent work and economic growth (United Nations Development Programme, n.d). The industry promotes livelihoods, economic growth, and poverty reduction through employment opportunities, particularly in rural regions, and fosters entrepreneurship and innovation in food production. The evolving methods and cultural practices in insect consumption highlight the necessity to explore sustainable food systems, hence meeting the SDG 12 aim of responsible consumption and production.

Various factors influence consumer acceptance of dried insects as food products. Studies indicated that customer approval from different areas and demographic groups varies significantly (Van Huis et al., 2013). Several Western nations indicated a major cultural barrier to consuming insects, frequently owing to the "yuck" factor or unfamiliarity with insects as food (Verbeke, 2015). Nonetheless, in regions where entomophagy is common, such as Africa, Asia, and Latin America, dried insects are widely tolerated and even regarded as a delicacy (Van Huis et al., 2013). Positive sensory experiences, such as good taste and texture, might improve acceptance (Caparros Megido et al., 2014). Knowledge of insect nutritional advantages, including their considerable protein content, vital amino acids, vitamins, and minerals, might also have positive effects on customer perceptions (Van Huis et al., 2013).

The Islamic perspective on entomophagy is crucial in understanding its compatibility with Islamic dietary laws (Halal). Proper pre-treatments, drying processes, and handling methods are essential in ensuring food safety. Furthermore, environmental and sustainability considerations within Islamic principles require consideration. Further research is also necessary to clarify the Islamic stance on entomophagy and promote its adoption within the Muslim community.

Understanding the Kosher perspective on entomophagy is equally crucial for those observing Jewish dietary laws (kashrut). The Jewish dietary laws known as kashrut impose certain limitations on the types of animals, including insects, that can be eaten. According to certain traits in Jewish scripture, eight kinds of insects are kosher (Jagadeesan et al., 2024). It has been shown that locust eating was quite common throughout the time of the Mishnah and Talmud. However, the custom was only maintained among Yemeni Jews and in some regions of northern Africa due to the lack of clarity on insects' wings and swarming animals, which caused a significant reduction (Hewamanage, 2016). All crawling animals on the ground, including insects with wings and swarming critters, are not kosher (Mclaughlin, 2017).

CONCLUSION

Applicable pre-treatment and drying methods depend upon insect type and desired final product. Generally, blanching is preferred due to its immediate effects compared to other pre-treatment processes. Although microwave and freeze-drying are the preferred methods, freeze-drying stands out as the optimal technique for preserving insect quality and appearance across species. Nevertheless, despite extensive exploration of different pre-treatments and drying approaches, no universally established guidelines are available.

Differences in edible insect species and their developmental phases necessitate species- and stage-specific pre-treatment and processing techniques to guarantee optimal quality and safety. Considerations must include nutritional composition, structural qualities, and possible pollutants. For instance, high-fat larvae may need lipid stabilisation, but adults with rigid exoskeletons may require deshelling or grinding. Safety precautions like blanching or gut-emptying can address microbial loads and pollutants. For applications involving human food and animal feed, efficient processing is ensured by adapting techniques to these variances.

Future studies might consider investigating novel pre-treatment and drying processes for insects that have yet to be extensively researched. Insect protein offers a promising solution to global protein shortages, providing nutritional, environmental, and ethical benefits. To ensure safety, addressing chemical residues, biological contaminants, and potential allergens through controlled farming, processing, and clear labelling is essential. With these safeguards in place, insect protein, along with other new options, can meet the rising protein demand while reducing the environmental impact of food production.

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REFERENCES

Agbede, O. O., Odewale, I. S., Aworanti, O. A., Alagbe, S. O., Ogunkunle, O., & Laseinde, O. T. (2023). Solar and open sun drying of untreated and pretreated banana stalk chips biomass: A sustainable processing of biomass using renewable solar energy. *Discover Food*, *3*(1), 17. https://doi.org/10.1007/s44187-023-00058-4

Aguilar-Miranda, E. D., López, M. G., Escamilla-Santana, C., & De La Rosa, A. P. B. (2001). Characteristics of maize flour tortilla supplemented with ground *Tenebrio molitor* larvae. *Journal of Agricultural and Food Chemistry*, *50*(1), 192-195. https://doi.org/10.1021/jf010691y

Alexander, D. D., Miller, A. J., Cushing, C. A., & Lowe, K. A. (2010). Processed meat and colorectal cancer: A quantitative review of prospective epidemiologic studies. *European Journal of Cancer Prevention*, *19*(5), 328-341. https://doi.org/10.1097/CEJ.0b013e32833b48fa

Alles, M. C., Smetana, S., Parniakov, O., Shorstkii, I., Toepfl, S., Aganovic, K., & Heinz, V. (2020). Bio-refinery of insects with pulsed electric field pre-treatment. *Innovative Food Science & Emerging Technologies*, *64*, 102403. https://doi.org/10.1016/j.ifset.2020.102403

Andharia, J. K., Solanki, J. B., & Maiti, S. (2023). Performance evaluation of a mixed-mode solar thermal dryer with black pebble-based sensible heat storage for drying marine products. *Journal of Energy Storage*, *57*, 106186. https://doi.org/10.1016/j.est.2022.106186

Aniebo, A. O., & Owen, O. J. (2010). Effects of age and method of drying on the proximate composition of housefly larvae (*Musca domestica* Linnaeus) meal (HFLM). *Pakistan Journal of Nutrition*, *9*(5), 485-487. https://doi.org/10.3923/pjn.2010.485.487

Azagoh, C., Ducept, F., Garcia, R., Rakotozafy, L., Cuvelier, M.E., Keller, S., Lewandowski, R., & Mezdour, S. (2016). Extraction and physicochemical characterization of *Tenebrio molitor* proteins. *Food Research International*, *88*, 24-31. https://doi.org/10.1016/J.FOODRES.2016.06.010

Azzollini, D., Derossi, A., & Severini, C. (2016). Understanding the drying kinetic and hygroscopic behaviour of larvae of yellow mealworm (*Tenebrio molitor*) and the effects on their quality. *Journal of Insects as Food Feed*, *2*(4), 233-243. https://doi.org/10.3920/jiff2016.0001

Badre, N. H., Martin, M. E., & Cooper, R. L. (2005). The physiological and behavioral effects of carbon dioxide on *Drosophila melanogaster* larvae. *Comparative biochemistry and physiology. Part A, Molecular & integrative physiology*, *140*(3), 363-376. https://doi.org/10.1016/j.cbpb.2005.01.019

Barba, F. J., Parniakov, O., Pereira, S. A., Wiktor, A., Grimi, N., Boussetta, N., Saraiva, J. A., Raso, J., Martin-Belloso, O., Witrowa-Rajchert, D., Lebovka, N., & Vorobiev, E. (2015). Current applications and new opportunities for the use of pulsed electric fields in food science and industry. *Food Research International*, *77*, 773-798. https://doi.org/10.1016/j.foodres.2015.09.015

Barennes, H., Phimmasane, M., & Rajaonarivo, C. (2015). Insect consumption to address undernutrition, a national survey on the prevalence of insect consumption among adults and vendors in Laos. *PloS one*, *10*(8), e0136458. https://doi.org/10.1371/journal.pone.0136458

Bava, L., Jucker, C., Gislon, G., Lupi, D., Savoldelli, S., Zucali, M., & Colombini, S. (2019). Rearing of *Hermetia illucens* on different organic by-products: Influence on growth, waste reduction, and environmental impact. *Animals*, *9*(6), 289. https://doi.org/10.3390/ani9060289

Bawa, M., Songsermpong, S., Kaewtapee, C., & Chanput, W. (2020). Effects of microwave and hot air oven drying on the nutritional, microbiological load, and color parameters of the house crickets (*Acheta domesticus*). *Journal of Food Processing and Preservation*, *44*(5), e14407. https://doi.org/10.1111/JFPP.14407

Bayrock, D., & Ingledew, W. M. (1997). Fluidized bed drying of baker's yeast: Moisture levels, drying rates, and viability changes during drying. *Food Research International*, *30*(6), 407-415.

Belluco, S., Losasso, C., Maggioletti, M., Alonzi, C. C., Paoletti, M. G., & Ricci, A. (2013). Edible insects in a food safety and nutritional perspective: A critical review. *Comprehensive Reviews in Food Science and Food Safety*, *12*(3), 296-313. https://doi.org/10.1111/1541-4337.12014

Berggren, Å., Jansson, A., & Low, M. (2018). Using current systems to inform rearing facility design in the insect-as-food industry. *Journal of Insects as Food and Feed*, *4*(3), 167-170.

Bhakar, N. (2023, May 14). *Fluidized bed dryer (FBD): Principle, working, troubleshooting, and components*. Pharmaguddu. https://pharmaguddu.com/principle-and-working-fluidized-bed-dryer-fbd

Bogusz, R., Smetana, S., Wiktor, A., Parniakov, O., Pobiega, K., Rybak, K., & Nowacka, M. (2022). The selected quality aspects of infrared-dried black soldier fly (*Hermetia illucens*) and yellow mealworm (*Tenebrio molitor*) larvae pre-treated by pulsed electric field. *Innovative Food Science and Emerging Technologies*, *80*, 103085. https://doi.org/10.1016/j.ifset.2022.103085

Boye, J. I., Danquah, A. O., Lam Thang, C., & Zhao, X. (2012). Food allergens. *Food biochemistry and food processing*, *3*(3), 798-819.

Braide, W., Oranusi, S., Udegbunam, L. I., Oguoma, O., Akobondu, C., & Nwaoguikpe, R. N. (2011). Microbiological quality of an edible caterpillar of an emperor moth, *Bunaea alcinoe*. *Journal of Ecology and the Natural Environment*, *3*(5), 176-180. https://doi.org/10.5897/jene.9000001

Brust, M. L., Hoback, W. W., & Wright, R. J. (2007). Immersion tolerance in rangeland grasshoppers (Orthoptera: Acrididae). *Journal of Orthoptera Research*, *16*(2), 135-138. https://doi.org/10.1665/1082-6467(2007)16

Calín-Sánchez, Á., Lipan, L., Cano-Lamadrid, M., Kharaghani, A., Masztalerz, K., Carbonell-Barrachina, Á. A., & Figiel, A. (2020). Comparison of traditional and novel drying techniques and its effect on quality of fruits, vegetables and aromatic herbs. *Foods*, *9*(9), 1261. https://doi.org/10.3390/foods9091261

Caparros Megido, R., Gierts, C., Blecker, C., Brostaux, Y., Haubruge, E., Alabi, T., & Francis, F. (2014). Consumer acceptance of insect-based alternative meat products in Western countries. *Food Quality and Preference*, *52*, 237-243. https://doi.org/10.1016/j.foodqual.2016.05.004

Cerritos, R., & Cano-Santana, Z. (2008). Harvesting grasshoppers *Sphenarium purpurascens* in Mexico for human consumption: A comparison with insecticidal control for managing pest outbreaks. *Crop Protection*, *27*(3-5), 473-480. https://doi.org/10.1016/j.cropro.2007.08.001

Chapman, A. D. (2009). *Numbers of living species in Australia and the world* (2nd Ed.). Australian Biodiversity Information Services.

Charlton, A. J., Dickinson, M., Wakefield, M.E., Fitches, E., Kenis, M., Han, R., Zhu, F., Kone, N., Grant, M., Devic, E., & Bruggeman, G. (2015). Exploring the chemical safety of fly larvae as a source of protein for animal feed. *Journal of Insects as Food and Feed*, *1*(1), 7-16. https://doi.org/10.3920/jiff2014.0020

Ciurzyńska, A., Falacińska, J., Kowalska, H., Kowalska, J., Galus, S., Marzec, A., & Domian, E. (2021). The effect of pre-treatment (blanching, ultrasound and freezing) on quality of freeze-dried red beets. *Foods*, *10*(1), 132. https://doi.org/10.3390/foods10010132

Clark, M. A., & Eaton, D. C. (1983). Effect of CO2 on neurons of the house cricket, *Acheta domestica*. *Journal of Neurobiology*, *14*(3), 237-250. https://doi.org/10.1002/neu.480140307

D'Souza, C., & Peretiatko, R. (2002). The nexus between industrialization and environment: A case study of Indian enterprises. *Environmental Management and Health*, *13*(1), 80-97. https://doi.org/10.1108/09566160210417859

de Gier, S., & Verhoeckx, K. (2018). Insect (food) allergy and allergens. *Molecular immunology*, *100*, 82-106. https://doi.org/10.1016/j.molimm.2018.03.015

De Matos, F. M., Rasera, G. B., & De Castro, R. J. S. (2024). Insects as a sustainable source of emerging proteins and their processing to obtain bioactive compounds: An updated review. *Sustainable Food Technology*, *2*(1), 19-31. https://doi.org/10.1039/d3fb00097d

De Oliveira, L. M., da Silva Lucas, A. J., Cadaval, C. L., & Mellado, M. S. (2017). Bread enriched with flour from cinereous cockroach (*Nauphoeta cinerea*). *Innovative Food Science and Emerging Technologies*, *44*, 30-35. https://doi.org/10.1016/J.IFSET.2017.08.015

Droukas, L., Doulgeri, Z., Tsakiridis, N. L., Triantafyllou, D., Kleitsiotis, I., Mariolis, I., Giakoumis, D., Tzovaras, D., Kateris, D. & Bochtis, D. (2023). A survey of robotic harvesting systems and enabling technologies. *Journal of Intelligent & Robotic Systems*, *107*(2), 21. https://doi.org/10.1007/s10846-022-01793-z

Durst, P. B., Johnson, D. V., Leslie, R. N., & Shono, K. (2010). Forest insects as food: Humans bite back. In P. B Durst, D. V. Johnson, R. N. Leslie & K. Shono (Eds.), *Proceedings of a workshop on Asia-Pacific resources and their potential for development.* Food and Agriculture Organization of the United Nations. https://www.fao.org/4/i1380e/i1380e00.htm

Earle, R. (1969). *Unit operations in food processing*. https://ci.nii.ac.jp/ncid/BA4982911X

Erens, J., Es Van S, H. F., Kapsomenou, E., & Luijben, A. (2012). *A bug's life. Large-Scale Insect Rearing in Relation to Animal Welfare.* Wageningen University and Research.

Erkmen, O., & Bozoglu, T. F. (2016). *Food microbiology, 2 volume set: Principles into practice* (Vol. 2). John Wiley & Sons.

Essumang, D. K., Dodoo, D. K., & Adjei, J. K. (2012). Polycyclic aromatic hydrocarbon (PAH) contamination in smoke-cured fish products. *Journal of Food Composition and Analysis*, *27*(2), 128-138. https://doi.org/10.1016/j.jfca.2012.04.007

Evans, N. M., & Shao, S. (2022). Mycotoxin metabolism by edible insects. *Toxins*, *14*(3), 217. https://doi.org/10.3390/toxins14030217

Feng, Y., Chen, X. M., Zhao, M., He, Z., Sun, L., Wang, C. Y., & Ding, W. F. (2017). Edible insects in China: Utilization and prospects. *Insect Science*, *25*(2), 184-198. https://doi.org/10.1111/1744-7917.12449

Fernandez-Cassi, X., Supeanu, A., Vaga, M., Jansson, A., Boqvist, S., & Vagsholm, I. (2019). The house cricket (*Acheta domesticus*) as a novel food: A risk profile. *Journal of Insects as Food and Feed*, *5*(2), 137-157. https://doi.org/10.3920/jiff2018.0021

Fombong, F. T., Van Der Borght, M., & Vanden Broeck, J. (2017). Influence of freeze-drying and oven-drying post blanching on the nutrient composition of the edible insect *Ruspolia differens*. *Insects*, *8*(3), 102. https://doi.org/10.3390/insects8030102

Food and Agriculture Organization of the United Nations. (2013). *The state of food insecurity in the world 2013: The multiple dimensions of food security*. https://www.fao.org/4/i3434e/i3434e00.htm

Food and Agriculture Organization of the United Nations. (2014). *SAFA sustainability assessment of food and agriculture systems: Guideline's version 3.0.* https://openknowledge.fao.org/handle/20.500.14283/i3957e

Francis, F., Doyen, V., Debaugnies, F., Mazzucchelli, G., Caparros, R., Alabi, T., Blecker, C., Haubruge, E., & Corazza, F. (2019). Limited cross reactivity among arginine kinase allergens from mealworm and cricket edible insects. *Food chemistry*, *276*, 714-718. https://doi.org/10.1016/j.foodchem.2018.10.082

Gahukar, R. (2018). Entomophagy in traditional healthcare practiced by indigenous communities: Potential, implications and constraints. *International Journal of Basic and Applied Sciences*, *7*(4), 11434. https://doi.org/10.14419/ijbas.v7i4.11434

Gao, Y., Wang, H., Qin, F., Xu, P., Lv, X., Li, J., & Guo, B. (2014). Enantiomerization and enantioselective bioaccumulation of metalaxyl in *Tenebrio molitor* larvae. *Chirality*, *26*(2), 88-94. https://doi.org/10.1002/chir.22269

Ghaly, A. E., & Alkoaik, F. N. (2009). The yellow mealworm as a novel source of protein. *American Journal of Agricultural and Biological Sciences*, *4*(4), 319-331. https://doi.org/10.3844/ajabssp.2009.319.331

Grabowski, N. T., & Klein, G. (2017). Microbiology of cooked and dried edible Mediterranean field crickets (*Gryllus bimaculatus*) and superworms (*Zophobas atratus*) submitted to four different heating treatments. *Food Science and Technology International*, *23*(1), 17-23. https://doi.org/10.1177/1082013216652994

Halloran, A., Flore, R., Vantomme, P., & Roos, N. (Eds.). (2018). *Edible insects in sustainable food systems.* Springer. https://doi.org/10.1007/978-3-319-74011-9

Halloran, A., Roos, N., Flore, R., & Hanboonsong, Y. (2016). The development of the edible cricket industry in Thailand. *Journal of Insects as Food and Feed*, *2*(2), 91-100. https://doi.org/10.3920/JIFF2015.0091

Halloran, A., Vantomme, P., Hanboonsong, Y., & Ekesi, S. (2015). Regulating edible insects: The challenge of addressing food security, nature conservation, and the erosion of traditional food culture. *Food Security*, *7*(3), 739-746. https://doi.org/10.1007/s12571-015-0463-8

Hashem, M. Y., Ahmed, S. S., El-Mohandes, M. A., Hussain, A. R. E., & Ghazy, S. M. (2014). Comparative effectiveness of different modified atmospheres enriched with carbon dioxide and nitrogen on larval instars of almond moth *Ephestia cautella* (Walker) (Lepidoptera: Pyralidae). *Journal of Stored Products Research*, *59*, 314-319. https://doi.org/10.1016/j.jspr.2014.09.006

Henderson, B. (2022, November 8). Food safety aspects of edible insects. *Food Safety Magazine*. https://www.food-safety.com/articles/8126-food-safety-aspects-of-edible-insects.

Hernández-Álvarez, A., Mondor, M., Piña-Domínguez, I., Sánchez-Velázquez, O., & Lalanne, G. M. (2021). Drying technologies for edible insects and their derived ingredients. *Drying Technology*, *39*(13), 1991-2009. https://doi.org/10.1080/07373937.2021.1915796

Hewamanage, W. (2016). A critical review of dietary laws in judaism. *International Research Journal of Engineering, IT and Scientific Research*, *2*(3), 58-65. https://doi.org/10.21744/irjeis.v2i3.44

Huang, C., Feng, W., Xiong, J., Wang, T., Wang, W., Wang, C., & Yang, F. (2019). Impact of drying method on the nutritional value of the edible insect protein from black soldier fly (*Hermetia illucens* L.) larvae: Amino acid composition, nutritional value evaluation, in vitro digestibility, and thermal properties. *European Food Research and Technology*, *245*(1), 11-21. https://doi.org/10.1007/s00217-018-3136-y

Jagadeesan, P., Siddiqui, A., Muzahem, S., & Al Shareef, A. J. M. (2024). Would edible insects become part of our gastronomic quest?. *Edelweiss Applied Science and Technology*, *8*(4), 737-745. https://doi.org/10.55214/25768484.v8i4.1456

Jan, A. T., Azam, M., Siddiqui, K., Ali, A., Choi, I., & Haq, Q. M. R. (2015). Heavy metals and human health: Mechanistic insight into toxicity and counter defense system of antioxidants. *International journal of molecular sciences*, *16*(12), 29592-29630.

Janssen, R. H., Canelli, G., Sanders, M. G., Bakx, E. J., Lakemond, C. M. M., Fogliano, V., & Vincken, J. P. (2019). Iron-polyphenol complexes cause blackening upon grinding *Hermetia illucens* (black soldier fly) larvae. *Scientific Reports*, *9*(1), 2967. https://doi.org/10.1038/s41598-019-38923-x

Janssen, R. H., Lakemond, C. M. M., Fogliano, V., Renzone, G., Scaloni, A., & Vincken, J. P. (2017). Involvement of phenoloxidase in browning during grinding of *Tenebrio molitor* larvae. *PloS One*, *12*(12), e0189685. https://doi.org/10.1371/journal.pone.0189685

Ji, K., Chen, J., Li, M., Liu, Z., Wang, C., Zhan, Z., Wu, X., & Xia, Q. (2009). Anaphylactic shock and lethal anaphylaxis caused by food consumption in China. *Trends in Food Science & Technology*, *20*(5), 227-231. https://doi.org/10.1016/j.tifs.2009.02.004

Jongema, Y. (2017, April 1). *List of edible insects of the world.* https://www.wur.nl/en/Research-Results/Chair-groups/Plant-Sciences/Laboratory-of-Entomology/Edible-insects/Worldwide-species-list.htm

Karim, A., Rehman, A., Lianfu, Z., Noreen, A., Ahmad, S., Usman, M., & Jafari, S. M. (2023). Introduction to thermal food processes by steam and hot water. In *Thermal Processing of Food Products by Steam and Hot Water* (pp. 3-26). Woodhead Publishing. https://doi.org/10.1016/B978-0-12-818616-9.00001-8

Keey, R. B. (1992). Drying of loose and particulate materials. *Drying Technology*, *10*(4), 1139-1141. https://doi.org/10.1080/07373939208916507

Kilic, A. (2009). Low temperature and high velocity (LTHV) application in drying: Characteristics and effects on the fish quality. *Journal of Food Engineering*, *91*(1), 173-182. https://doi.org/10.1016/J.JFOODENG.2008.08.023

Kim, S. K., Weaver, C. M., & Choi, M. K. (2016). Proximate composition and mineral content of five edible insects consumed in Korea. *CyTA Journal of Food*, *15*(1), 143-146. https://doi.org/10.1080/19476337.2016.1223172

Kinyuru, J. N., Konyole, S. O., Onyango-Omolo, S. A., Kenji, G. M., Onyango, C. A., Owino, V. O., Owuor, B. O., Estambale, B. B., & Roos, N. (2015). Nutrients, functional properties, storage stability and costing of complementary foods enriched with either termites and fish or commercial micronutrients. *Journal of Insects as Food and Feed*, *1*(2), 149-158. https://doi.org/10.3920/JIFF2014.0011

Koffi, P. V. N., Kouakou, B., Gildas, G. K., Thierry, A., N'golo, K., & Dago, G. (2013). Quality characteristics of biscuits made from sorghum and defatted *Macrotermes subhyalinus*. *International Journal of Biosciences*, *3*(1), 58-69. https://www.cabdirect.org/cabdirect/abstract/20133097507

Kouřimská, L., & Adámková, A. (2016). Nutritional and sensory quality of edible insects. *NFS Journal*, *4*, 22-26. https://doi.org/10.1016/j.nfs.2016.07.001

Kröncke, N., Böschen, V., Woyzichovski, J., Demtröder, S., & Benning, R. (2018). Comparison of suitable drying processes for mealworms (*Tenebrio molitor*). *Innovative Food Science and Emerging Technologies*, *50*, 20-25. https://doi.org/10.1016/j.ifset.2018.10.009

Kröncke, N., Grebenteuch, S., Keil, C., Demtröder, S., Kroh, L., Thünemann, A. F., Benning, R., & Haase, H. (2019). Effect of different drying methods on nutrient quality of the yellow mealworm (*Tenebrio molitor* L.). *Insects*, *10*(4), 84. https://doi.org/10.3390/INSECTS10040084

Larouche, J., Deschamps, M. H., Saucier, L., Lebeuf, Y., Doyen, A., & Vandenberg, G. W. (2019). Effects of killing methods on lipid oxidation, colour and microbial load of black soldier fly (*Hermetia illucens*) larvae. *Animals*, *9*(4), 182. https://doi.org/10.3390/ani9040182

Lautenschläger, T., Neinhuis, C., Kikongo, E., Henle, T., & Förster, A. (2017). Impact of different preparations on the nutritional value of the edible caterpillar *Imbrasia epimethea* from northern Angola. *European Food Research and Technology*, *243*(5), 769-778. https://doi.org/10.1007/s00217-016-2791-0

Ledesma, E., Rendueles, M., & Díaz, M. (2017). Smoked food. In *Current developments in biotechnology and bioengineering* (pp. 201-243). Elsevier. https://doi.org/10.1016/B978-0-444-63666-9.00008-X

Lee, F. A. (1958). The blanching process. In *Advances in food research* (Vol. 8, pp. 63-109). Academic Press. https://doi.org/10.1016/S0065-2628(08)60018-X

Lee, J. H., Kim, T. K., Cha, J. Y., Jang, H. W., Yong, H. I., & Choi, Y. S. (2022). How to develop strategies to use insects as animal feed: Digestibility, functionality, safety, and regulation. *Journal of Animal Science and Technology*, *64*(3), 409. https://doi.org/10.5187/jast.2022.e27

Lee, R. E. (1991). Principles of insect low temperature tolerance. In R. E. Lee & D. L. Denlinger (Eds.), *Insects at low temperature* (pp. 17-46). Springer. https://doi.org/10.1007/978-1-4757-0190-6_2

Lenaerts, S., Van Der Borght, M., Callens, A., & Van Campenhout, L. (2018). Suitability of microwave drying for mealworms (*Tenebrio molitor*) as alternative to freeze drying: Impact on nutritional quality and colour. *Food Chemistry*, *254*, 129-136. https://doi.org/10.1016/j.foodchem.2018.02.006

Leni, G., Caligiani, A., & Sforza, S. (2019). Killing method affects the browning and the quality of the protein fraction of black soldier fly (*Hermetia illucens*) prepupae: A metabolomics and proteomic insight. *Food Research International*, *115*, 116-125. https://doi.org/10.1016/j.foodres.2018.08.021

Li, D., Sihamala, O., Bhulaidok, S., & Shen, L. R. (2009). Changes in the organic compounds following sun drying of edible black ant (*Polyrhachis vicina* Roger). *Acta alimentaria*, *38*(4), 493-501. https://doi.org/10.1556/aalim.38.2009.4.9

Mahnič-Kalamiza, S., Vorobiev, E., & Miklavčič, D. (2014). Electroporation in food processing and biorefinery. *The Journal of Membrane Biology*, *247*(12), 1279-1304. https://doi.org/10.1007/s00232-014-9737-x

Malakar, S., Alam, M., & Arora, V. K. (2022). Evacuated tube solar and sun drying of beetroot slices: Comparative assessment of thermal performance, drying kinetics, and quality analysis. *Solar Energy*, *233*, 246-258. https://doi.org/10.1016/j.solener.2022.01.029

Mancini, S., Fratini, F., Tuccinardi, T., Turchi, B., Nuvoloni, R., & Paci, G. (2019). Effects of different blanching treatments on microbiological profile and quality of the mealworm (*Tenebrio molitor*). *Journal of Insects as Food and Feed*, *5*(3), 225-234. https://doi.org/10.3920/JIFF2018.0034

Mariod, A. A., Saeed Mirghani, M. E., & Hussein, I. (2017). *Acheta domesticus* house cricket. In *Unconventional oilseeds and oil sources* (pp. 323-325). Academic Press. https://doi.org/10.1016/B978-0-12-809435-8.00048-2

Mclaughlin, R. P. (2017). A meatless dominion: Genesis 1 and the ideal of vegetarianism. *Biblical Theology Bulletin*, *47*(3), 144-154.

Melgar-Lalanne, G., Hernández-Álvarez, A. J., & Salinas-Castro, A. (2019). Edible insects processing: Traditional and innovative technologies. *Comprehensive Reviews in Food Science and Food Safety*, *18*(4), 1166-1191. https://doi.org/10.1111/1541-4337.12463

Melis, R., Braca, A., Mulas, G., Sanna, R., Spada, S., Serra, G., Fadda, M.L., Roggio, T., Uzzau, S., & Anedda, R. (2018). Effect of freezing and drying processes on the molecular traits of edible yellow mealworm. *Innovative Food Science and Emerging Technologies*, *48*, 138-149. https://doi.org/10.1016/j.ifset.2018.06.003

Melo, V., Garcia, M., Sandoval, H., Jiménez, H. D., & Calvo, C. (2011). Quality proteins from edible indigenous insect food of Latin America and Asia. *Emirates Journal of Food and Agriculture*, *23*(3), 283-289. https://www.ejmanager.com/fulltextpdf.php?mno=7206-19818-1-PB.pdf

Mlcek, J., Rop, O., Borkovcova, M., & Bednarova, M. (2014). A comprehensive look at the possibilities of edible insects as food in Europe - A review. *Polish Journal of Food and Nutrition Sciences*, *64*, 147-157. https://doi.org/10.2478/v10222-012-0099-8

Mohamad, L., Hasan, H. A., Sudesh, K., & Baidurah, S. (2021). Dual application of black soldier fly (*Hermetia illucens*): Protein-rich animal feed and biological extraction agent for polyhydroxybutyrate. *Malaysian Journal of Microbiology*, *17*(6), 624-634. http://dx.doi.org/10.21161/mjm.211268

Mongkolvai, P., Wingsranoi, A., Sombum, K., & Mongkolvai, J. (2009). *Marketing and culturing of giant water bugs* (Research report, pp. 217-230). Faculty of Natural Resources, Rajamangala University of Technology Isan. Sakonakhon Campus. In Food and Agriculture Organization of the United Nations (FAO), *Six-legged livestock: Edible insect farming, collecting and marketing in Thailand*. https://www.fao.org/4/i3246e/i3246e.pdf

Montevecchi, G., Zanasi, L., Masino, F., Maistrello, L., & Antonelli, A. (2020). Black soldier fly (*Hermetia illucens* L.): Effect on the fat integrity using different approaches to the killing of the prepupae. *Journal of Insects as Food and Feed*, *6*(2), 121-131. https://doi.org/10.3920/jiff2019.0002

Morgan, C., Herman, N., White, P., & Vesey, G. (2006). Preservation of micro-organisms by drying; A review. *Journal of microbiological methods*, *66*(2), 183-93. https://doi.org/10.1016/j.mimet.2006.02.017

Mujumdar, A. S. (2006). Principles, classification, and selection of dryers. In A. S. Mujumdar (Ed.), *Handbook of industrial drying* (3rd ed., pp. 3-32). CRC Press. https://doi.org/10.1201/9781420017618.pt1

Murefu, T. R., Macheka, L., Musundire, R., & Manditsera, F. A. (2019). Safety of wild harvested and reared edible insects: A review. *Food Control*, *101*, 209-224. https://doi.org/10.1016/j.foodcont.2019.03.003

Musundire, R., Osuga, I. M., Cheseto, X., Irungu, J., & Torto, B. (2016). Aflatoxin contamination detected in nutrient and anti-oxidant rich edible stink bug stored in recycled grain containers. *PloS one*, *11*(1), e0145914. https://doi.org/10.1371/journal.pone.0145914

Muzzarelli, R. A., Boudrant, J., Meyer, D., Manno, N., DeMarchis, M., & Paoletti, M. G. (2012). Current views on fungal chitin/chitosan, human chitinases, food preservation, glucans, pectins and inulin: A tribute to Henri Braconnot, precursor of the carbohydrate polymers science, on the chitin bicentennial. *Carbohydrate polymers*, *87*(2), 995-1012. https://doi.org/10.1016/j.carbpol.2011.09.063

Nguyen, T. N. A., & Nguyen, T. N. (2015). Effect of different drying methods on total lipid and fatty acid profiles of dried *Artemia franciscana* biomass. *CTU Journal of Innovation and Sustainable Development*, *1*, 1-9. https://doi.org/10.22144/ctu.jen.2015.015

Nowak, D., & Lewicki, P. P. (2004). Infrared drying of apple slices. *Innovative Food Science and Emerging Technologies*, *5*(3), 353-360. https://doi.org/10.1016/j.ifset.2004.03.003

Nowakowski, A. C., Miller, A. C., Miller, M. E., Xiao, H., & Wu, X. (2021). Potential health benefits of edible insects. *Critical Reviews in Food Science and Nutrition*, *62*(13), 3499-3508. https://doi.org/10.1080/10408398.2020.1867053

Ojha, S., Bußler, S., Psarianos, M., Rossi, G., & Schlüter, O. K. (2021). Edible insect processing pathways and implementation of emerging technologies. *Journal of Insects as Food and Feed*, *7*(5), 877-900. https://doi.org/10.3920/jiff2020.0121

Omari, A., Behroozi-Khazaei, N., & Sharifian, F. (2018). Drying kinetic and artificial neural network modeling of mushroom drying process in microwave-hot air dryer. *Journal of Food Process Engineering*, *41*(7), e12849. https://doi.org/10.1111/JFPE.12849

Osimani, A., Garofalo, C., Milanović, V., Taccari, M., Cardinali, F., Aquilanti, L., Pasquini, M., Mozzon, M., Raffaelli, N., Ruschioni, S., & Riolo, P. (2017). Insight into the proximate composition and microbial diversity of edible insects marketed in the European Union. *European Food Research and Technology*, *243*, 1157-1171. https://doi.org/10.1007/s00217-016-2828-4

Park, Y. S., Choi, Y. S., Hwang, K. E., Kim, T. K., Lee, C. W., Shin, D. M., & Han, S. G. (2017). Physicochemical properties of meat batter added with edible silkworm pupae (*Bombyx mori*) and transglutaminase. *Food Science of Animal Resources*, *37*(3), 351-359. https://doi.org/10.5851/KOSFA.2017.37.3.351

Parniakov, O., Mikhrovska, M., Wiktor, A., Alles, M., Ristic, D., Bogusz, R., Nowacka, M., Devahastin, S., Mujumdar, A., Heinz, V., & Smetana, S. (2021). Insect processing for food and feed: A review of drying methods. *Drying Technology*, *40*(8), 1500-1513. https://doi.org/10.1080/07373937.2021.1962905

Psarianos, M., Dimopoulos, G., Ojha, S., Cavini, A. C. M., Bußler, S., Taoukis, P., & Schlüter, O. K. (2022). Effect of pulsed electric fields on cricket (*Acheta domesticus*) flour: Extraction yield (protein, fat and chitin) and techno-functional properties. *Innovative Food Science and Emerging Technologies*, *76*, 102908. https://doi.org/10.1016/J.IFSET.2021.102908

Purschke, B., Brüggen, H., Scheibelberger, R., & Jäger, H. (2018). Effect of pre-treatment and drying method on physico-chemical properties and dry fractionation behaviour of mealworm larvae (*Tenebrio molitor* L.). *Innovative Food Science and Emerging Technologies*, *244*(2), 269-280. https://doi.org/10.1007/S00217-017-2953-8

Rachmawati, R., Buchori, D., Hidayat, P., Hem, S., & Fahmi, M. R. (2010). Perkembangan dan kandungan nutrisi larva *Hermetia illucens* (linnaeus) (Diptera: Stratiomyidae) pada bungkil kelapa sawit [Development and nutritional content of *Hermetia illucens* (Linnaeus) (Diptera: Stratiomyidae) larvae on palm kernel cake]. *Jurnal Entomologi Indonesia*, *7*(1), 28-41. https://doi.org/10.5994/jei.7.1.28

Raheem, D., Carrascosa, C., Oluwole, O. B., Nieuwland, M., Saraiva, A., Millán, R., & Raposo, A. (2018). Traditional consumption of and rearing edible insects in Africa, Asia and Europe. *Critical Reviews in Food Science and Nutrition*, *59*(14), 2169-2188. https://doi.org/10.1080/10408398.2018.1440191

Ratti, C. (2001). Hot air and freeze-drying of high-value foods: A review. *Journal of Food Engineering*, *49*(4), 311-319. https://doi.org/10.1016/ S0260-8774(00)00228-4

Rumpold, B. A., & Schlüter, O. (2015). Insect-based protein sources and their potential for human consumption: Nutritional composition and processing. *Animal Frontiers*, *5*(2), 20-24. https://doi.org/10.2527/af.2015-0015

Rumpold, B. A., & Schlüter, O. K. (2013). Nutritional composition and safety aspects of edible insects. *Molecular Nutrition and Food Research*, *57*(5), 802-823. https://doi.org/10.1002/mnfr.201200735

Saucier, L., M'ballou, C., Ratti, C., Deschamps, M. H., Lebeuf, Y., & Vandenberg, G. W. (2020). Comparison of black soldier fly larvae pre-treatments and drying techniques on the microbial load and physico-chemical characteristics. *Journal of Insects as Food and Feed*, *8*(1), 45-64. https://doi.org/10.3920/JIFF2021.0002

Ščetar, M., & Galić, K. (2017). A perspective on packaging of fruits and vegetables. In S. Pareek (Ed.), *Novel postharvest treatments of fresh produce* (pp. 481). Taylor and Francis. https://doi.org/10.1201/9781315370149-15

Schlüter, O., Rumpold, B., Holzhauser, T., Roth, A., Vogel, R.F., Quasigroch, W., Vogel, S., Heinz, V., Jäger, H., Bandick, N., & Kulling, S. (2017). Safety aspects of the production of foods and food ingredients from insects. *Molecular Nutrition & Food Research*, *61*(6), 1600520.

Seho, R. E. Y., Monteiro, R. L., De Dea Lindner, J., Miotto, M., Carciofi, B. A. M., & Laurindo, J. B. (2021). Effects of vacuum and multiflash drying on the microbiota and colour of dried yellow mealworm (*Tenebrio molitor*). *Journal of Insects as Food and Feed*, *8*(1), 23-33. https://doi.org/10.3920/JIFF2020.0170

Shadung, K. G., Mphosi, M. S., & Mashela, P. W. (2012). Influence of drying method and location on proximate chemical composition of African metallic wood boring beetle, *Sternocera orissa* (Coleoptera: Buprestidae) in Republic of South Africa. *African Journal of Food Science*, *6*(6), 155-158. https://doi.org/10.5897/AJFS11.146

Sharma, G. P., Verma, R. C., & Pathare, P. B. (2005). Thin-layer infrared radiation drying of onion slices. *Journal of Food Engineering*, *67*(3), 361-366. https://doi.org/10.1016/j.jfoodeng.2004.05.002

Shonte, T. T., Duodu, K. G., & De Kock, H. L. (2020). Effect of drying methods on chemical composition and antioxidant activity of underutilized stinging nettle leaves. *Heliyon*, *6*(5), e03938. https://doi.org/10.1016/J.HELIYON.2020.E03938

Shorstkii, I., Comiotto Alles, M., Parniakov, O., Smetana, S., Aganovic, K., Sosnin, M., Toepfl, S., & Heinz, V. (2020). Optimization of pulsed electric field assisted drying process of black soldier fly (*Hermetia illucens*) larvae. *Drying Technology*, *40*(3), 595-603. https://doi.org/10.1080/07373937.2020.1819825

Simpanya, M. F., Allotey, J., & Mpuchane, S. F. (2000). A mycological investigation of phane, an edible caterpillar of an emperor moth, *Imbrasia belina*. *Journal of Food Protection*, *63*(1), 137-140. https://doi.org/10.4315/0362-028x-63.1.137

Sindermann, D., Heidhues, J., Kirchner, S., Stadermann, N., & Kühl, A. (2021). Industrial processing technologies for insect larvae. *Journal of Insects as Food and Feed*, *7*(5), 857-875. https://doi.org/10.3920/jiff2020.0103

Smetana, S., Bhatia, A., Batta, U., Mouhrim, N., & Tonda, A. (2023). Environmental impact potential of insect production chains for food and feed in Europe. *Animal Frontiers*, *13*(4), 112-120. https://doi.org/10.1093/af/vfad033

Smetana, S., Schmitt, E., & Mathys, A. (2019). Sustainable use of *Hermetia illucens* insect biomass for feed and food: Attributional and consequential life cycle assessment. *Resources, Conservation and Recycling*, *144*, 285-296. https://doi.org/10.1016/j.resconrec.2019.01.042

Smith, K., Watson, A.W., Lonnie, M., Peeters, W.M., Oonincx, D., Tsoutsoura, N., Simon-Miquel, G., Szepe, K., Cochetel, N., Pearson, A.G., & Witard, O.C. (2024). Meeting the global protein supply requirements of a growing and ageing population. *European journal of nutrition*, *63*, 1-9. https://doi.org/10.1007/s00394-024-03358-2

Srinroch, C., Srisomsap, C., Chokchaichamnankit, D., Punyarit, P., & Phiriyangkul, P. (2015). Identification of novel allergen in edible insect, *Gryllus bimaculatus* and its cross-reactivity with *Macrobrachium* spp. allergens. *Food Chemistry*, *184*, 160-166. https://doi.org/10.1016/j.foodchem.2015.03.094

Ssepuuya, G., Nakimbugwe, D., De Winne, A., Smets, R., Claes, J., & Van Der Borght, M. (2020). Effect of heat processing on the nutrient composition, colour, and volatile odour compounds of the long-horned grasshopper *Ruspolia differens* servile. *Food Research International*, *129*, 108831. https://doi.org/10.1016/J.FOODRES.2019.108831

Stork, N. E. (2018). How many species of insects and other terrestrial arthropods are there on Earth?. *Annual review of entomology*, *63*(1), 31-45. https://doi.org/10.1146/annurev-ento-020117-043348

Stramarkou, M., Papadaki, S., Kyriakopoulou, K., Tzovenis, I., Chronis, M., & Krokida, M. (2021). Comparative analysis of different drying techniques based on the qualitative characteristics of *Spirulina platensis* biomass. *Journal of Aquatic Food Product Technology*, *30*(5), 498-516. https://doi.org/10.1080/10498850.2021.1900969

Tiencheu, B., Womeni, H. M., Linder, M., Mbiapo, F. T., Villeneuve, P., Fanni, J., & Parmentier, M. (2012). Changes of lipids in insect (*Rhynchophorus phoenicis*) during cooking and storage. *European Journal of Lipid Science and Technology*, *115*(2), 186-195. http://doi.org/10.1002/ejlt.201200284

Turck, D., Castenmiller, J., De Henauw, S., Hirsch-Ernst, K. I., Kearney, J., Maciuk, A., Mangelsdorf, I., McArdle, H. J., Naska, A., Pelaez, C., Petieva, K., Siani, A., Thies, F., Tsabouri, S., Vinceti, M., Cubadda, F., Frenzel, T., Heinonen, M., Marchelli, R. … Knutsen, H. K. (2021). Safety of dried yellow mealworm (*Tenebrio molitor* larva) as a novel food pursuant to Regulation (EU) 2015/2283. *EFSA Journal*, *19*(1), e06343. https://doi.org/10.2903/J.EFSA.2021.6343

United Nations Development Programme. (n.d.). *Decent work and economic growth*. https://www.undp.org/sustainable-development-goals/decent-work-and-economic-growth.

Van der Fels-Klerx, H. J., Camenzuli, L., Van Der Lee, M. K., & Oonincx, D. G. A. B. (2016). Uptake of cadmium, lead and arsenic by *Tenebrio molitor* and *Hermetia illucens* from contaminated substrates. *PloS one*, *11*(11), e0166186. https://doi.org/10.1371/journal.pone.0166186

Van Huis, A. (2016). Edible insects are the future?. *Proceedings of the Nutrition Society*, *75*(3), 294-305. https://doi.org/10.1017/s0029665116000069

Van Huis, A. (2021). Prospects of insects as food and feed. *Organic Agriculture*, *11*(2), 301-308. https://doi.org/10.1007/s13165-020-00290-7

Van Huis, A., & Tomberlin, J. K. (Eds.). (2017). *Insects as food and feed: From production to consumption*. Wageningen Academic Publishers. https://doi.org/10.3920/978-90-8686-849-0

Van Huis, A., Van Itterbeeck, J., Klunder, H., Mertens, E., Halloran, A., Muir, G., & Vantomme, P. (2013). *Edible insects: Future prospects for food and feed security* (No. 171). Food and agriculture organization of the United Nations. https://doi.org/10.1007/s13165-020-00290-7

Van Loey, A., Smout, C., & Hendrickx, M. (2005). Kinetic data for biochemical and microbiological processes during thermal processing. In *Engineering properties of foods* (3rd ed., pp. 611–643). CRC Press.

van Loon, J. J. A., van den Berg, M., & de Boer, I. J. M. (2018). The environmental sustainability of insect production for food and feed: A review of the life cycle assessment literature. *Environmental Research Letters*, *13*(9), 093002.

Vandeweyer, D., Lenaerts, S., Callens, A., & Van Campenhout, L. (2017). Effect of blanching followed by refrigerated storage or industrial microwave drying on the microbial load of yellow mealworm larvae (*Tenebrio molitor*). *Food Control*, *71*, 311-314. https://doi.org/10.1016/j.foodcont.2016.07.011

Verbeke, W. (2015). Profiling consumers who are ready to adopt insects as a meat substitute in a Western society. *Food Quality Preference*, *39*, 147-155. https://doi.org/10.1016/j.foodqual.2014.07.008

Wessels, M. L. J., Azzollini, D., & Fogliano, V. (2020). Frozen storage of lesser mealworm larvae (*Alphitobius diaperinus*) changes chemical properties and functionalities of the derived ingredients. *Food Chemistry*, *320*, 126649. https://doi.org/10.1016/j.foodchem.2020.126649

Wong-Corral, F. J., Castañé, C., & Riudavets, J. (2013). Lethal effects of CO2-modified atmospheres for the control of three Bruchidae species. *Journal of Stored Product Research*, *55*, 62-67. https://doi.org/10.1016/j.jspr.2013.08.005

Wynants, E., Crauwels, S., Lievens, B., Luca, S., Claes, J., Borremans, A., Bruyninckx, L., & Van Campenhout, L. (2017). Effect of post-harvest starvation and rinsing on the microbial numbers and the bacterial community composition of mealworm larvae (*Tenebrio molitor*). *Innovative Food Science & Emerging Technologies*, *42*, 8-15. https://doi.org/10.1016/j.ifset.2017.06.004

Yegrem, L., & Ababele, L. (2022). Pretreatments, dehydration methods and packaging materials: effects on the nutritional quality of tomato powder. *Austin Journal of Nutrition and Food Science*, *10*(2), 1167. https://doi.org/10.26420/austinjnutrifoodsci.2022.1167

Yen, A. L. (2015). Insects as food and feed in the Asia Pacific region: Current perspectives and future directions. *Journal of Insects as Food and Feed*, *1*(1), 33-55. https://doi.org/10.3920/jiff2014.0017

Yi, L., Lakemond, C. M., Sagis, L. M., Eisner-Schadler, V., Van Huis, A., & Van Boekel, M. A. (2013). Extraction and characterisation of protein fractions from five insect species. *Food chemistry*, *141*(4), 3341-3348. https://doi.org/10.1016/j.foodchem.2013.05.115

Yisa, N. K., Osuga, I. M., Subramanian, S., Ekesi, S., Emmambux, M. N., & Duodu, K.G. (2022). Effect of drying methods on the nutrient content, protein and lipid quality of edible insects from East Africa. *Journal of Insects as Food and Feed*, *9*(5), 647-659. https://doi.org/10.3920/JIFF2021.0124

Zhen, Y., Chundang, P., Zhang, Y., Wang, M., Vongsangnak, W., Pruksakorn, C., & Kovitvadhi, A. (2020). Impacts of killing process on the nutrient content, product stability and in vitro digestibility of black soldier fly (*Hermetia illucens*) larvae meals. *Applied Sciences*, *10*(17), 6099. https://doi.org/10.3390/APP10176099

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Biodegradation of Chicken Feather Waste with *Bacillus subtilis* in Vermicomposting

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ABSTRACT

Poultry waste (chicken manure [CD] and chicken feathers [CF]), and agricultural waste, including mushroom media residue (MMR) and banana trunks (BT), were utilised in this vermicomposting with different proportions (15 ratios). This study primarily introduced earthworms (*Eudrilus eugeniae*) and *Bacillus subtilis* (3.9×10^{-5} CFU/mL) as degradation agents. Both agents were added on day 14 into bins, mixed thoroughly, and the subsequent substrate was then retained for the 60-day composting process. T9 treatments demonstrated enhanced biodegradability of chicken feather waste, achieving a biodegradation rate (Kb%) exceeding 80%. The earthworm population increased by 62%, and there was a 53% weight gain in earthworm reproduction at day 60 for the ratio of 6:2:1:1. The maximum pH value was recorded in T1 (7.11±0.06). In contrast, the maximum EC value (ms/cm) was recorded at 3.5±0.40 in T4. The vermicompost contains a nitrogen compound (N) range (1.9%–4.4%), a potassium content (K_2O) between 7.81%–22.9%, and phosphorus (P_2O_5) within 0.91%–3.03%. The investigation revealed that T11 had the highest dehydrogenase enzyme (DHA) activity, whereas T13 showed a greater catalase activity, and T3 exhibited the maximum keratinase enzyme activity. DHA activity showed a correlation with K_2O (r^2=0.507) and also demonstrated a correlation with P_2O_5. Correlation studies have found that enzyme activity and physico-chemical properties influence the vermicomposting process of chicken feathers.

Keywords: *Bacillus subtilis*, biodegradation, chicken feathers, enzyme activity, *Eudrilus eugeniae*

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INTRODUCTION

In order to convert agricultural waste into valuable products, the manipulation of microbes has emerged as a strategy that is environmentally sustainable and cost-effective, and preserves the validity of structure and product efficacy (Daroit & Brandelli, 2014). Utilising earthworms for composting diminishes organic waste, stabilises organic resources, and enhances plant biomass output. Earthworms facilitate the degradation of organic waste by feeding, fragmentation, aeration, turnover, and dispersion, in conjunction with enzymatic digestion aided by bacteria. Thus, vermicomposting is a more effective and safer method for managing agricultural waste compared to traditional composting, which results in nutrient depletion, prolonged processing times, and inferior compost quality.

Keratin has the potential to be utilised in a variety of applications in the agricultural and industrial sectors, such as animal feed, adhesives, organic fertilisers, leather processing chemicals, cosmetics, pharmaceuticals, and biomedical materials (Mengistu et al., 2023), however most chicken feathers are still burned or thrown in the trash (Alahyaribeik & Ullah, 2020). Its keratin may be utilised as a valuable bioresource to produce high-value metabolites and products with additional value. In Malaysia, an estimated 17.745 million tonnes of waste is contributed by chicken poultry (Zayadi, 2021). This keratinase waste is primarily composed of chicken feathers, which are disposed of through land-filling, burning, and open dumping. The gradual degradation of chicken feathers results in their prolonged accumulation of keratinase waste in the environment due to their slow degradation process. Besides being insoluble in water, keratin protein has been shown in the literature to be best enhanced for degradation by heat treatment and somewhat acidic pH conditions (Fagbemi et al., 2020). Concerns about the keratin waste from chicken poultry, an alternative approach by using microbes or maybe further with specific microbial keratinases that are able to catalyse the biodegradation of keratin by using the vermicomposting method.

Bacillus spp. are abundant producers of lignocellulose-degrading enzymes, such as xylanases and cellulases. Nonetheless, an extract from *Bacillus subtilis* exhibited significant cellulase activity in cellulose disintegration, thereby facilitating cellulose degradation (Siu-Rodas et al., 2017). Furthermore, identifying and screening microorganisms capable of degrading feathers, including bacteria and fungi, has been documented (Elhoul et al., 2016; Mini et al., 2017; Zhang et al., 2016). Previous research indicated that two keratinolytic strains, *Bacillus licheniformis* BBE11-1 (Liu et al., 2014) and *Stenotrophomonas maltophilia* BBE11-1 (Fang et al., 2013), can hydrolyse feathers; however, their growth and enzyme production conditions differ significantly, rendering them unsuitable for the degradation of bulky quantities of feathers. Peng et al. (2019) reported that keratinase alone does not hydrolyse feathers. Limited research has investigated the degradation of keratin derived from chicken feathers. A study indicated that *Bacillus* spp. C4 destroys merely 75% of a 5% (w/v) chicken feather suspension over 8 days, employing a laborious and inefficient

method (Patinvoh et al., 2016). Consequently, using earthworms (vermicomposting) and *Bacillus* sp. as agents for the degradation of chicken feathers aims to enhance degradation efficiency and facilitate the conversion into compost products. A suggestion indicated that *Bacillus* sp. may facilitate the degradation of organic materials by generating enzymes that stimulate microbial activities (Peng et al., 2019).

Production of several enzymes, such as dehydrogenase and catalases, can be utilised to determine the maturity and effectiveness of the vermicomposting process. The presence of microbial composition will produce enzymes to decompose the initial substrate. Barrena et al. (2008) indicated that dehydrogenase serves as a useful enzyme for reflecting the biological activity of composting. Simultaneously, catalase activity was assessed to evaluate the breakdown of hydrogen peroxide during degradation. The elevated catalase activity signifies the efficient breakdown of organic materials during degradation. This study examines how changes in enzyme activity during the vermicomposting process are closely related to the presence of physicochemical properties and nutrients produced, as shown in Table 3, using correlation tests.

MATERIALS AND METHODS

Compost Materials and Environmental Site

The substrate comprises mixtures of chicken feathers (CF), mushroom media residue (MMR), banana trunk (BT), and chicken dung (CD). CD and CF have been collected from the poultry farm and the chicken processing centre in the Northern region of Malaysia. CF were dried to ease cutting and placed into smaller pieces using scissors. All substrates were soaked for 14 days using tap water by changing water every day until the electrical conductivity (EC) of the substrate mixtures reached below 2.0 ms/cm (Ahmad, 2020). Pre-composting was adjusted for about 14 days, with a temperature at the environmental site of around 22–35°C, daylight and a moisture content of 55% – 80%, and substrate pH between 7 and 7.6 before composting to keep the earthworms healthy. All substrates were mixed in different (dry volume) amounts of organic matter into bins with proportion ratios (Table 1).

Composting System and Treatment

The composting method was performed with slight modifications based on different proportions of chicken waste. All mixtures were homogenised to obtain 15 different proportions (Table 1). All ratios were made in five replicates. Earthworms were obtained from the Compost Worm Breeding Centre and the Vermicompost Producer, Plant Protection and Plant Quarantine Division, Department of Agriculture Malaysia, Tambun Tulang, Perlis. The earthworms were undergoing an adaptation and breeding using the proportion of 3:2:1 for 2 weeks before being placed in the experimental bins. All treatments were received 10 healthy *E. eugeniae* placed in the experimental bins.

Five replicates of bins were set up with the addition of earthworms, and *B. substilis* was purchased from the website of the American Type Culture Collection (ATCC). The suitability of added agents for vermicomposting immediately after pre-composting of substrate, the EC value reaches below 2 ms/cm approximately for 14 days (Ahmad, 2020). 1 ml of *B. subtilis* (3.9×10^{-5} CFU/mL) was inoculated into each treatment. Treatment without *B. subtilis* was used as a control; These bins were kept undisturbed for 60 days and sprinkled with water daily to keep them moist.

Compost Preparation

The composting process was carried out using only slight modifications to the proportions of chicken waste in bins located at the composting house at Universiti Teknologi MARA (Malaysia). On day 14, the inoculation of the composting agents, *E. eugeniae* and *B. subtilis*, started and was recorded as day 0 of organic matter degradation (initial). Five samples from each replicate were collected from each bin at a depth of 15 cm at 60 days. Approximately 100 g of vermicompost was collected from each bin to determine the enzyme activity and physico-chemical properties. Compost samples were kept under -4°C in the dark storage for a maximum of 4 days before sample preparation for compost determination to reduce the risk of microbial growth. Determining enzyme activity assays were done immediately after the sample was prepared. Sample preparation has potential effects on the enzymes' activity during storage.

Table 1
Different proportions (dry volume) of organic matter (OM) waste and MMR used as the main raw material

Treatment	Degradation agents	Organic matter (OM) waste and proportions			
		MMR	CD	CF	BT
T1	*Bacillus subtilis*	6	3	null	1
T2	*Bacillus subtilis*	6	null	3	1
T3	*Bacillus subtilis*	6	1.5	1.5	1
T4	*Bacillus subtilis*	6	2	1	1
T5	*Bacillus subtilis*	6	1	2	1
T6	*Bacillus subtilis* + *Eudrilus eugeniae*	6	3	null	1
T7	*Bacillus subtilis* + *Eudrilus eugeniae*	6	null	3	1
T8	*Bacillus subtilis* + *Eudrilus eugeniae*	6	1.5	1.5	1
T9	*Bacillus subtilis* + *Eudrilus eugeniae*	6	2	1	1
T10	*Bacillus subtilis* + *Eudrilus eugeniae*	6	1	2	1
T11	*Eudrilus eugeniae*	6	3	null	1
T12	*Eudrilus eugeniae*	6	null	3	1
T13	*Eudrilus eugeniae*	6	1.5	1.5	1
T14	*Eudrilus eugeniae*	6	2	1	1
T15	*Eudrilus eugeniae*	6	1	2	1

Earthworm Population and Reproduction

Population and reproduction of earthworms were determined individually by self-count, and weight was measured using a digital scale. The vermicompost produced has been sieved using a fine 4 mm mesh to separate the compost material from the earthworms. The population and reproduction of earthworms were quantified based on the treatment and replicates, as shown by the formula provided in Equations 1 and 2:

Population: After number of *Eudrilus euginea* - Before number of *Eudrilus eugenia* [1]

Reproduction: After weight of *Eudrilus euginea* - Before weight of *Eudrilus eugenia* [2]

Biodegradable Rate (%)

Organic matter (OM) waste was weighed for all the treatments and replicates at day 0 and day 60 of the vermicomposting period using a weight scale to measure the organic matter conversion during vermicomposting. The biodegradation rate was measured with a slight modification to determine the degradation rate (Manyuchi et al., 2013; Manyuchi et al., 2014).

$$\text{Biodegradation rate (\%)} = 100 \times (B\text{-}A)/B \quad [3]$$

Where, B is the dry weight of the organic matter before degradation, and A is the dry weight of the organic matter after degradation.

Enzyme Activity Assays

The activity of three microbial enzymes (dehydrogenase, catalase and keratinase) was determined on days 0 and 60. For measuring dehydrogenase (DHA), the triphenyl tetrazolium chloride (TTC) method was used based on He et al. (2013), with a slightly modified method and dehydrogenase activity was determined by using the triphenyl tetrazolium chloride method according to the procedure described by Barrena et al. (2008). Values of DHA are expressed as mg of triphenyl formazan (TPF) released g dry matter-1 h-1 and are presented.

Dehydrogenase activity was measured using a calibration curve of dehydrogenase activity with different TTC concentrations as recommended in (Pourakbar et al., 2020). In order to measure the dehydrogenase enzyme activity, a calibration curve was developed using 3 ml of the 3% v/w TTC substrate and 5 ml of methanol as an extractor. Afterwards, the TTC solution was vortexed and filtered through the sample. A UV-visible

spectrophotometer revealed the solution at 485 nm, chloride (TTC). The absorbance of six different concentrations of TTC has been inserted into the standard curve scatter graph. Then, the calibration curve was developed in line according to the standard curve graph's linear equation, with ($r2$= 0.9918), with the equation of a straight line being y = 0.0022x+0.0622. Therefore, the vermicompost sample from each treatment was measured using the procedure given.

Keratinase activity was used on a total of 5 g of the compost sample, as described in the procedure by Kamarudin et al. (2017). Keratinase activity was defined as the amount of enzyme causing a 0.01 absorbance increase between the sample and control at 595 nm under the given conditions. The unit of keratinase was in (µg) in every 5 g of the compost. The keratinase standard curve was created using a concentration in which 5 µg represents 1% of keratin azure. A scatter plot's points are strongly correlated with a linear regression line according to the standard curve graph's linear equation, which ($r2$=0.9901), with the equation of a straight-line being y = 3.18x +0.0349. The value of the keratinase enzyme activities (x) was determined by inserting the absorbance into the formula (Equation 4).

x = Absorbance- Intercept slope [4]

Characterising the Physical and Chemical Properties of Biodegradation

pH value and the electrical conductivity (EC) of the substrate were measured at day-0 and day-60 using Hana Instruments, HI98107. The Oakton EC100 portable conductivity meter (EC) was used to determine the electrical conductivity in the compost by inserting the rod into the compost. All the treatments and replicates were sent to the Global Testing and Consultancy for Rubber (G-TACR) for the measurement of the chemical properties such as nitrogen (N), phosphorus (P_2O_5) and potassium (K_2O).

Data Analysis

The enzyme activities of compost samples were carried out in five replicates and presented as mean ± standard deviation. SPSS 16.0 software was used to perform a correlation analysis of enzyme activities and physico-chemical properties, as well as the biodegradation rate (Kb)% between the composting agents.

RESULTS AND DISCUSSIONS

In the present study, the changes in composting pH were recorded at day 0 and day 60. pH value of the biodegradation process was recorded during pre-composting (initial stage), 7.2–7.5. The peak of pH sharply reduces the value and indicates an acidic pH value when the composting process begins. However, all treatments were indicated in the optimum range of pH value after completing the biodegradation activity and in accordance with the

range of 6.5–7.5 of composting produced, excluding T7, T9 and T12. The three treatments (T7, T9 and T12) indicate that the presence of organic waste leads to an increase in acidic content in the bins, which is a potential danger to the survival of earthworms (Katiyar et al., 2017). However, the dramatic changes in pH recorded during day 60 of composting might be related to the biodegradation of organic materials by a composting agent.

Results also indicate that T7 and T9 were degraded with the combination of both agents, while T12 was found in the earthworm degradation agent only. This finding slightly contradicts the previous finding. The biological oxidation of organic substances during this period may facilitate the movement of hydrogen from the organic substrate to the inorganic acceptor (Aghayani et al., 2018). Hydrogen is an acid cation; particularly, acid cations influence the media or soil pH.

The pH values recorded during the biodegradation process, comprising varying quantities of substrate and either *B. subtilis* or earthworm as the degradation agent, remained within the slightly acidic range of 6.0–7.11. Furthermore, these tests indicated a lower pH of 6.13 in T7, despite the presence of both composting agents. However, this discovery was not parallel with the consistent occurrence of organisms in degradation. Hazardous material waste may contain compounds that are less hazardous or nontoxic. A greater number of earthworms significantly improves the aeration of the compost, hence preventing a decline in pH, whereas aerobic conditions affect ammonium utilisation and pH increase. Furthermore, biochar and earthworms can improve soil structure and boost microbial population and activity (Zhang et al., 2021).

The EC values in all treatments were observed to be under the acceptable limit of 4 ms/cm on the final day of the biodegradation process (day 60). The lowest average EC was obtained in T11 and T6. Meanwhile, the highest was in T1 and T4 (3.5 ms/cm), respectively, at day 60. Electrical conductivity of less than 3.5 µs/cm has been suggested as the optimal value for compost use as an agricultural fertiliser (Chen et al., 2014). In the present study, the changes of EC values between the pre-composting range value of 0.1–0.2 ms/cm in bins were initially recorded, followed by determination on day 60. However, the final EC at day 60, respectively, from T1, T2, T3, T4, and T5, ranged from 3.1 to 3.5 ms/cm, indicating high EC values.

EC reflects the total ion concentration in the compost material, i.e., the concentration of soluble salts. In pre-composting, chicken wastes, mushroom media residue (MMR), banana trunk, and chicken dung mixture were flushed using a volume of water that may eliminate the toxic effect, or soluble ions in the compost material. During composting, the increase in EC may also be due to the biodegradation of organic matter. As small organic molecules decompose and the pH increases, total ions decrease, decreasing EC (Abid & Sayadi, 2006; Geng et al., 2010).

Earthworm Population and *Bacillus subtilis* Population Count

The earthworm population was observed to increase in numbers (population) during the entire biodegradation of chicken feathers at 60 days. The absence of mortality of earthworms during the process demonstrated that a proportion of all treatments provides an optimal environment for earthworms to survive. Treatment T11 showed an 85% increase in the population and a 64% weight gain. This study indicates that the number of earthworms on day 60 for the biodegradation of chicken feathers was not significant in T6 and T9. In contrast, earthworm weight gain reached its highest in T11 but was also not significantly different from T6, T8, T11, T13, T14, and T15. In addition, this study showed a greater number of earthworms and earthworm weight gain in vermicomposting for the proportion ratio without chicken feather (6:3:0:1) and T9 (6:2:1:1). Treatment T7 (6:0:3:1) resulted in a low number of earthworm population, similar to T12 when inoculated with *B. subtilis*. The appropriateness of waste proportion for vermicomposting is evaluated based on the suitability of growth and reproduction of earthworms in compost, which contributes to the nutrient composition of the final product, namely vermicompost.

Li et al. (2023) hypothesised that phosphate-solubilising *Bacillus subtilis* (PSB) may exhibit significant adaptability to the MMR in an aerobic composting environment and improve phosphorus availability through enhanced synergy. Duan et al. (2020) investigated the effects of *B. subtilis* inoculum addition. They observed a subsequent decrease in the C/N ratio resulting from the synthesis of humic compounds in symbiotic association between plant growth-promoting bacteria (PSB) and indigenous microorganisms. Throughout the vermicomposting process, all bins' organic matter (OM) progressively declined until day 60 due to substrate utilisation as energy for microbial metabolism.

In the compost process, earthworm castings (vermicast) offer numerous advantages, including the acceleration of composting, suppression of pathogens, elimination of contaminants, and increased compost quality (Zhang & Sun, 2015). Vermicast promotes microbial growth and activity due to its high cation exchange capacity, crucial for neutralising pH during composting. However, limited knowledge refers to the influence of vermicast from agricultural waste. The population of *B. subtilis* in all treatments within the bins increased on day 60, indicating the compatibility of the degradation agents throughout the biodegradation process.

Biodegradable Rate (Kb)%

The biodegradation rate of vermicompost (Kb)% in a 15-proportion ranged from 66% to 81% (Table 2). The biodegradation rate in T6 and T9 indicated that the maximum (Kb)%, at 81%, was indicated by the action of the earthworm (*E. eugeniae*) and the *B. subtilis* agent during composting (Table 2). In T6, a portion of organic matter was without chicken feathers, but T9 showed a ratio of chicken dung that was double the amount of chicken

Table 2
Earthworm population (number of earthworms), reproduction (earthworm weight gain), Bacillus subtilis *(CFU/mL), and biodegradable rate (Kb)% of chicken feather at day 60*

Treatment	Earthworm (*Eudrilus eugeniae*)		*Bacillus subtilis*	Biodegradable rate (Kb)%
	Number of earthworms	Reproduction (weight gain)	Population X10^6	
T1	n.a	n.a	1.05X10^6	72^{cd}
T2	n.a	n.a	1.98 X10^6	68^{f}
T3	n.a	n.a	1.75 X10^6	71de
T4	n.a	n.a	5.3 X10^6	77^{b}
T5	n.a	n.a	10.5 X10^6	73^{cd}
T6	**79^{a}**	**58^{a}**	1.35X10^6	81^{a}
T7	9.4^{d}	3.9^{bc}	3.98 X10^6	70de
T8	42.9^{bc}	**27^{abd}**	1.75 X10^6	70de
T9	**60.9^{ab}**	**53^{a}**	22.3 X10^6	81^{a}
T10	21.9^{cd}	8^{bc}	5.5 X10^6	76^{b}
T11	**85^{a}**	**64^{a}**	n.a	72de
T12	11^{d}	-15^{c}	n.a	76^{bc}
T13	47^{b}	**33^{ab}**	n.a	66^{f}
T14	**62^{ab}**	**43^{ab}**	n.a	73^{cd}
T15	51^{b}	**25^{abc}**	n.a	73^{cd}

Note. According to Tukey's analysis, the mean value within a column, followed by the same letter, is not significantly different at the $p< 0.05$ level, n.a = not available

feathers, resulting in a biodegradation rate of 81%. In T6, it is not substantial due to the proportion without CF.

All treatments achieved a biodegradation rate exceeding 60% within the 60-day composting procedure. The study involved vermicomposting assessments that were not influenced by the increase in material temperature during composting, since the earthworms (*E. eugeniae*) and *B. subtilis*, as agents, would be affected by temperature elevation and produce heat. While most composting models related heat production to the biodegradation of organic matter or oxygen levels, vermicomposting, in conjunction with bacteria in the compost pile, is more critical for stabilising agents under composting conditions. Composting without *Eudrilus eugeniae* or *B. subtilis* is less successful, as shown by a biodegradation rate below 80%, indicating significant variation when both agents are present. The biodegradable rate was markedly greater in the treatment that combined the degradation agent (*Eudrilus eugeniae*) with *B. subtilis*, followed by the treatment with *B. subtilis* alone.

The dynamics in the biodegradation process were discussed when comparing the population (number of earthworms) and reproduction (weight gain) under different

proportions, revealing significant differences in the *E. eugeniae* population, which contributes to the biodegradation rate compared to the treatments by *B. subtilis* and *E. eugeniae*, respectively. These results show that *E. eugeniae* fed on the substrate mixture other than chicken feather, while adding *B. subtilis* increased the degradation of the chicken feather. Therefore, the effects of proportion without CF in substrate ratios also affected the *B. subtilis* population as well as its microbial metabolic activities.

Dehydrogenase Activity (DHA Activity)

Research has shown that enzyme-catalysed oxidation of simple carbon substrates is the initial step in the biodegradation of organic materials (Pourakbar et al., 2020). At day 60, a maximum dehydrogenase activity was recorded (38.0±1.01) U/mL in T11. The increase in dehydrogenase activity indicated greater availability of complex compounds, which enhanced microbial activity. The DHA activity, however, has been recorded at lower levels in T8 (8.84±0.36) U/mL and T15 (11.7±0.91) U/mL. Treatments T8 was the treatment with both degradation agents, while T15 was the treatment with the earthworm only.

The final biodegradation at day 60 was considerably faster in mature compost, regardless of the proportion of substrate ($p<0.05$). Data were significantly different in the treatment of single degradation agents, *Bacillus* only (19.23±4.31) U/mL introduced, followed by the treatment of earthworm and *Bacillus* (23.02±8.3) U/mL; meanwhile, DHA activity was higher using the earthworm agent only (27.1±9.3) U/mL.

Catalase Activity

The relative catalase activity was enhanced until day 60 of the biodegradation of chicken feathers. High catalase activities indicate the effective biodegradation of organic matter in compost. This study's finding of high catalase resulted in 18.6±0.66 U/mL in T13 and lower in T10 (8.33±2.34) U/mL. However, analysis of correlation in Table 3 showed no significant correlation at the 0.05 level with $r^2= 0.100$ between DHA activity and catalase enzyme activity.

Keratinase Activity

Keratinase activity observed at 60 days showed that T3 indicated the highest keratinase activity (0.15±0.20) U/mL, followed by T6 (0.11±0.10) U/mL. Keratinase activity recorded in this study is slightly different and quantifies keratinase activity higher in treatment with *B. subtilis*, followed by treatment using both agents.

In general, keratinase was able to degrade feathers. He et al. (2018) investigated the enzymes involved in the hydrolysis of various feathers by a particular type of *B. subtilis* and subsequently identified four enzymes related to keratin hydrolysis. Keratin-degrading

microorganisms in this study were derived from chicken feathers, and *Bacillus* was added to the treatment. Based on previous research, the bacteria present are reflected in the optimum pH and temperature of the keratinase activity of these microorganisms. However, the correlation analysis in Table 2 showed no significance at the 0.05 level with other enzymes and physico-chemical properties.

Physico-chemical Properties

In this study, a nitrogen compound in the 15 proportion ratios presented varied between 1.87% and 4.46%. In contrast to this study, treatment without a nitrogen source (ratio 6:0:3:1) recorded higher N% compared to the proportion with chicken dung (T12), while organic matter biodegradable recorded higher in proportion with chicken dung. However, another source of N added, such as chicken dung, in this study showed an opposite effect for N% and Kb% results. In addition, N was determined to be higher in proportion, even without chicken dung. Based on the substrate used in this study, all substrates can be considered as low nitrogen content material (Kumla et al., 2020).

The phosphorus content varied between 0.35% and 3.04% P_2O_5, and the potassium content varied between 7.81% and 22.9% K_2O_5. However, this research slightly different compared with result obtained by Phukan et al. (2013) indicated the total K_2O content varied between 0.69%, 0.73%, and 0.65%, while the P_2O_5 content varied between 1.1%, 1.51%, and 0.82% using slurry method (non-enriched and enriched) and conventional method, for 3.04% P_2O_5 nutrient content was shown in T7 vermicomposting bins, where the proportion without chicken dung recorded the highest 22.9% K_2O_5 nutrient content at T8.

However, this study's findings were concurrent with Bhat et al. (2013) findings, which contained approximately 2.47% P_2O_5 and 2.37% K_2O for both nutrient contents. Based on the trial, the higher potassium content in this research resulted from BT being added as a substrate in each bin. Additionally, Soobhany et al. (2015) also reported a higher nutrient content determined in vermicomposting than in conventional composting processes.

Many researchers concluded that vermicomposting is a recommended solution for the degradation of agricultural waste. In addition, a type of organic waste utilised as substrate in this vermicomposting affects the nutrient content of the finished products. Meanwhile, bacilli in this study affected the degradation of chicken feathers. The nutrient content in several proportions indicated the suitability of the vermicomposting method to convert a bulk of raw chicken feather waste into biofertiliser, enabling more effective agricultural waste management. Soil bacteria, earthworms, and community structure are closely related to waste degradation. The difference in the proportion of substrate also affected the diversity of the population and the nutrient content after degradation occurred. However, this finding shows that the degradation of all substrates into biofertiliser could contribute to soil quality and crop productivity.

Table 3
Correlation between enzyme activity and physico-chemical properties at day 60 of the biodegradation of chicken feather

	Dehydrogenase	**Keratinase**	**Catalase**	**N**	**P_2O_2**	**K_2O**	**pH**	**EC**
Dehydrogenase	1	r^2=0.017 p=0.897	r^2=0.100 p=0.445	r^2=0.207 p=0.112	r^2=0.108 p=0.410	**r^2=-0.504**** p<0.001	r^2=-0.227 P=0.081	r^2=-0.350* P=0.006
Keratinase		1	r^2=0.16 p=0.903	r^2=-0.015 P=0.907	r^2=-0.035 p=0.791	r^2=-0.046 p=0.726	r^2=0.194 p=0.137	r^2=-0.007 p=0.96
Catalase			1	r^2=-0.083 p=0.527	r^2=-0.220 p=0.092	r^2=-0.006 p=0.962	r^2=0.151 p=0.249	r^2=-0.193 p=0.139
N_2				1	**r^2=0.570**** P<0.001	r^2=0.205 P=1.117	**r^2=-0.716**** P<0.001	r^2=-0.017 P=0.899
P_2O_2					1	r^2=0.333** P=0.009	**r^2=-0.550**** P<0.001	r^2=-0.076 P=0.563
K_2O						1	r^2=-0.144 P=2.72	r^2=-0.079 P=0.547
pH							1	r^2=0.308* P=0.017

Note. *Correlation is significant at the 0.05 level (2-tailed)

CONCLUSION

The presence of degradation agents, such as earthworms and *Bacillus*, along with various substrates in composting, contributes to an efficient composting process based on proportion ratios. The biodegradation of chicken feathers was most effectively demonstrated at a ratio 6:2:1:1 in the presence of both earthworms and *Bacillus* degradation agents. The inoculation of *Bacillus* influenced the bacterial population succession by changing the physico-chemical parameters, particularly pH, during the biodegradation process. The nitrogen compounds (N) ranged from 1.9% to 4.4%, while the potassium (K_2O) level ranged from 7.81% to 22.9%, and the phosphorus amount varied from 0.91% to 3.03% P_2O_5 in the compost produced in this study, all of which are considered to be acceptable. The addition of chicken dung affected the N% level in chicken feather degradation. Besides, this work utilised MMR and BT derived from agricultural waste as substrates, contributing to P_2O_5 and K_2O. The optimal biodegradation observed was in the ratio of 6:2:1:1, yielding N (3%), P_2O_5 (1%), and K_2O (17%) nutrient content. Thus, this study suggested that an 80% biodegradation rate using vermicomposting might be considered effective for chicken-feather degradation with *Bacillus*. This study also recommends the addition of *B. subtilis* into chicken feather vermicomposting, when combined with mushroom media residue (MMR), might accelerate the breakdown process and enhance biomass assimilation. A specific keratinolytic microorganism or, preferably, a specific microbial keratinase could be recommended to catalyse the degradation of chicken feathers. For future application in agriculture, an abundance of chicken feathers can be utilised as a value-added ingredient in the biofertiliser industry. Overall, further studies to test compost effectiveness in various soil types or crop requirements could improve soil health and fertility. Furthermore, it will bring about innovative waste management possibilities while improving our understanding of the crop production cycle.

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REFERENCES

Abid, N., & Sayadi, S. (2006). Detrimental effects of olive mill wastewater on the composting process of agricultural wastes. *Waste Management*, *26*(10), 1099–1107. https://doi.org/10.1016/j.wasman.2005.06.015

Aghayani, E., Moussavi, G., & Naddafi, K. (2018). Improved peroxidase-mediated biodegradation of toluene vapors in the moving-bed activated sludge diffusion (MASD) process using biosurfactant-generating

biomass stimulated with H_2O_2. *Journal of Hazardous Materials*, *361*, 259–266. https://doi.org/10.1016/j.jhazmat.2018.08.076

Ahmad, N., Mat Sukri, N., & Samsuddin, N. Z. (2020). The effect of different animal dung in vermicompost production using *Eudrilus eugeniae* worm and spent mushroom substrate as vermicomposting medium. In N. Z. Yahaya, M. R. Yaacob, M. A. Hassan & M. S. Idris (Eds.), *Charting the sustainable future of ASEAN in science and technology: Proceedings from the 3rd International Conference on the Future of ASEAN (ICoFA) 2019* (Vol. 2, pp. 137–145). Springer. https://doi.org/10.1007/978-981-15-3434-8_12

Alahyaribeik, S., & Ullah, A. (2020). Methods of keratin extraction from poultry feathers and their effects on antioxidant activity of extracted keratin. *International Journal of Biological Macromolecules*, *148*, *449–456*. https://doi.org/10.1016/j.ijbiomac.2020.01.144

Barrena, R., Vázquez, F., & Sánchez, A. (2008). Dehydrogenase activity as a method for monitoring the composting process. *Bioresource Technology*, *99*(4), 905–908. https://doi.org/10.1016/j.biortech.2007.01.027

Bhat, S. A., Singh, J., & Vig, A. P. (2013). Vermiremediation of dyeing sludge from textile mill with the help of exotic earthworm *Eisenia fetida* Savigny. *Environmental Science and Pollution Research*, *20*(9), 5975–5982. https://doi.org/10.1007/s11356-013-1612-2

Chen, Y., Yu, F., Liang, S., Wang, Z., Liu, Z., & Xiong, Y. (2014). Utilization of solar energy in sewage sludge composting: Fertilizer effect and application. *Waste Management*, *34*(11), 2014–2021. https://doi.org/10.1016/j.wasman.2014.06.029

Daroit, D. J., & Brandelli, A. (2014). A current assessment on the production of bacterial keratinases. *Critical Reviews in Biotechnology*, *34*(4), 372–384. https://doi.org/10.3109/07388551.2013.794768

Duan, M., Zhang, Y., Zhou, B., Qin, Z., Wu, J., Wang, Q., & Yin, Y. (2020). Effects of *Bacillus subtilis* on carbon components and microbial functional metabolism during cow manure–straw composting. *Bioresource Technology*, *303*, 122868. https://doi.org/10.1016/j.biortech.2020.122868

Elhoul, M. B., Jaouadi, N. Z., Rekik, H., Benmrad, H. O., Mechri, S., Moujehed, E., Kourdali, S., El Hattab, M., Bejar, A. B. S., & Jaouadi, B. (2016). Biochemical and molecular characterization of new keratinoytic protease from *Actinomadura viridilutea* DZ50. *International Journal of Biological Macromolecules*, *92*, 299–315. https://doi.org/10.1016/j.ijbiomac.2016.07.009

Fagbemi, O. D., Sithole, B., & Tesfaye, T. (2020). Optimization of keratin protein extraction from waste chicken feathers using hybrid pre-treatment techniques. *Sustainable Chemistry and Pharmacy*, *17*, *100267*. https://doi.org/10.1016/j.scp.2020.100267

Fang, Z., Zhang, J., Liu, B., Du, G., & Chen, J. (2013). Biodegradation of wool waste and keratinase production in scale-up fermenter with different strategies by *Stenotrophomonas maltophilia* BBE11-1. *Bioresource Technology*, *140*, 286–291. https://doi.org/10.1016/j.biortech.2013.04.091

Geng, Y., Tsuyoshi, F., & Chen, X. (2010). Evaluation of innovative municipal solid waste management through urban symbiosis: A case study of Kawasaki. *Journal of Cleaner Production*, *18*(10-11), 993–1000. https://doi.org/10.1016/j.jclepro.2010.03.003

He, Y., Xie, K., Xu, P., Huang, X., Gu, W., Zhang, F., & Tang, S. (2013). Evolution of microbial community diversity and enzymatic activity during composting. *Research in Microbiology*, *164*(2), 189–198. https://doi.org/10.1016/j.resmic.2012.11.001

He, Z., Sun, R., Tang, Z., Bu, T., Wu, Q., Li, C., & Chen, H. (2018). Biodegradation of feather waste keratin by the keratin-degrading strain *Bacillus subtili*s 8. *Journal of Microbiology Biotechnology*, *28*(2), 314–322. https://doi.org/10.4014/jmb.1708.08077

Kamarudin, N., Sharma, S., Gupta, A., Kee, C. G., Chik, S. M. S. T., & Gupta, R. (2017). Statistical investigation of extraction parameters of keratin from chicken feather using design-expert. *3 Biotech*, *7*(127), 1-9. https://doi.org/10.1007/s13205-017-0767-9

Katiyar, R. B., Suresh, S., & Sharma, A. K. (2017). A review on vermicomposting of different leaf litters. In S. Suresh, A. Kumar, A. Shukla, R. Singh & C. Krishna (Eds.), *Biofuels and bioenergy (BICE2016)* (pp. 305-312). Springer. https://doi.org/10.1007/978-3-319-47257-7_28

Kumla, J., Suwannarach, N., Sujarit, K., Penkhrue, W., Kakumyan, P., Jatuwong, K., Vadthanarat, S., & Lumyong, S. (2020). Cultivation of mushrooms and their Lignocellulolytic enzyme production through the utilization of agro-industrial waste. *Molecules*, *25*(12), 2811. https://doi.org/10.3390/molecules25122811

Li, H., He, Y., Yan, Z., Yang, Z., Tian, F., Liu, X., & Wu, Z. (2023). Insight into the microbial mechanisms for the improvement of spent mushroom substrate composting efficiency driven by phosphate-solubilizing *Bacillus subtilis*. *Journal of Environmental Management*, *336*, 117561–117561. https://doi.org/10.1016/j.jenvman.2023.117561

Liu, B., Zhang, J., Fang, Z., Du, G., Chen, J., & Liao, X. (2014). Functional analysis of the C-terminal propeptide of keratinase from *Bacillus licheniformis* BBE11-1 and its effect on the production of keratinase in *Bacillus subtilis*. *Process Biochemistry*, *49*(9), 1538–1542. https://doi.org/10.1016/j.procbio.2014.04.021

Manyuchi, M. M., & Phiri, A. (2013). Application of the vermifiltration technology in sewage wastewater treatment. *Asian Journal of Engineering and Technology, 1*(4), 108-113. https://www.ajouronline.com/index.php/AJET/article/view/396

Manyuchi, M. M., Whingiri, E., & Mount. (2014). Effect of vermicomposting period, substrate quantity, cow dung composition and their interactions on *Eisenia fetida* during vermicomposting. *International Journal of current Microbiology and Applied Sciences*, *3*(8), 1021-1028. https://api.semanticscholar.org/CorpusID:103093250

Mengistu, A., Angassa, K., & Tessema, I. (2023). Optimization of keratin hydrolysate extraction from tannery sheep hair waste. *International Journal of Chemical Engineering*, *1*, 9293505. https://doi.org/10.1155/2023/9293505

Mini, K. D., Kumar S. S., & Mathew, J. (2017). Dehairing of cattle hide by keratinase enzyme of *Aspergillus flavus* s125. *International Journal of Pharma and Bio Sciences*, *8*(3), 204-209. https://doi.org/10.22376/ijpbs.2017.8.3.b204-209

Patinvoh, R.J., Feuk-Lagerstedt, E., Lundin, M., Sárvári Horváth, I., & Taherzadeh, M. J. (2016). Biological pretreatment of chicken feather and biogas production from total broth. *Applied biochemistry and biotechnology*, *180*, 1401-1415. https://doi.org/10.1007/s12010-016-2175-8

Peng, Z., Mao, X., Zhang, J., Du, G., & Chen, J. (2019). Effective biodegradation of chicken feather waste by co-cultivation of keratinase producing strains. *Microbial Cell Factories*, *18*(84), 1-11. https://doi.org/10.1186/s12934-019-1134-9

Phukan, I. K., Khanikar, L., Ahmed, C.S., Safique, S., Jahan, A., Baruah, A., & Phukan, I. (2013). A novel method for improving the quality of vermicompost. *Two and a Bud*, *60*(2), 24-29. https://api.semanticscholar.org/CorpusID:202872978

Pourakbar, M., Behnami, A., Mahdavianpour, M., Dariyan, F.S., & Aghayani, E. (2020). Developing a method for measurement of dehydrogenase activity in biological wastewater treatment processes applied for toxic compounds degradation. *MethodsX*, *7*, 100970–100970. https://doi.org/10.1016/j.mex.2020.100970

Siu-Rodas, Y., Calixto-Romo, M. de los A., Guillén-Navarro, K., Sánchez, J. E., Zamora-Briseño, J. A., & Amaya-Delgado, L. (2017). *Bacillus subtilis* con actividades de endocelulasa y exocelulasa aislado en la fase termofílica del compostaje con residuos de café. [*Bacillus subtilis* with endocellulase and exocellulase activities isolated in the thermophilic phase from composting with coffee residues]. *Revista Argentina de Microbiología*, *50*(3), 234–243. https://doi.org/10.1016/j.ram.2017.08.005

Soobhany, N., Mohee, R., & Garg, V. K. (2015). Recovery of nutrient from Municipal Solid Waste by composting and vermicomposting using earthworm *Eudrilus eugeniae*. *Journal of Environmental Chemical Engineering*, *3*(4), 2931–2942. https://doi.org/10.1016/j.jece.2015.10.025

Zayadi, R. A. (2021). Current outlook of livestock industry in Malaysia and ways towards sustainability. *Journal of Sustainable Natural Resources*, *2*(2), 1–11. https://doi.org/10.30880/jsunr.2021.12.02.001

Zhang, L., & Sun, X. (2015). Effects of earthworm casts and zeolite on the two-stage composting of green waste. *Waste management*, *39*, 119-129. https://doi.org/10.1016/j.wasman.2015.02.037

Zhang, R. -X., Gong, J. -S., Su, C., Zhang, D. -D., Tian, H., Dou, W. -F., Li, H., Shi, J. -S., & Xu, Z.-H. (2016). Biochemical characterization of a novel surfactant-stable serine keratinase with no collagenase activity from *Brevibacillus parabrevis* CGMCC 10798. *International Journal of Biological Macromolecules*, *93*, 843–851. https://doi.org/10.1016/j.ijbiomac.2016.09.063

Zhang, Q. M, Li, S. Z., Saleem, M, Ali, M. Y., & Xiang, J. H. (2021). Biochar and earthworms synergistically improve soil structure, microbial abundance, activities and pyraclostrobin degradation. *Applied Soil Ecology*, *168*, 104-154. https://doi.org/10.1016/j.apsoil.2021.104154

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Evaluation of Soybean (*Glycine max* (L.) Merr.) Varieties for Tolerance to Relay Intercropping with Chili (*Capsicum annuum* L.) in Coastal Sandy Land

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ABSTRACT

The relay intercropping of soybean (*Glycine max* (L.) Merr.) planted between rows of chili (*Capsicum annuum* L.) in coastal sandy soils presents a potential strategy for increasing soybean production in Indonesia. This study aimed to evaluate the performance of soybean varieties for tolerance to relay intercropping with chili in coastal sandy lands based on yield, yield index, and plant growth. The research was conducted at Samas Beach, Bantul Regency, Yogyakarta, from January to May 2023, using a randomized complete block design with two factors and three blocks. Five soybean varieties ('Anjasmoro', 'Dena 1', 'Demas 1', 'Grobogan', and 'Malika'), and two cropping systems (monoculture and relay intercropping) were evaluated. Water was irrigated when rainfall was insufficient to maintain adequate soil moisture levels. Data were analyzed using *t*-test, ANOVA, Principal Component Analysis, Cluster Analysis, and Pearson's correlation. The results showed that relay intercropping significantly enhanced the seed yield of all soybean varieties compared to the monoculture, with 'Anjasmoro' showing the highest increase of 443.39%. Differences among the varieties under the relay intercropping system showed that 'Anjasmoro', 'Malika', 'Demas 1', and 'Dena 1' consistently outperformed 'Grobogan' in terms of yield and growth. The varieties were grouped into high-yielding ('Anjasmoro', 'Malika', 'Demas 1', and 'Dena 1') and low-yielding ('Grobogan'). High-yielding varieties had high positive correlations with the yield index (r=0.98), harvest index (r=0.61), and 100-SW (r=0.64). These findings

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suggest that utilizing high-yielding soybean varieties in relay intercropping with chili could significantly enhance soybean yields in coastal sandy lands.

Keywords: Coastal sandy land, monocropping, relay intercropping, soybean, yield

INTRODUCTION

Soybean (*Glycine max* (L.) Merrill) is one of the world's most important food crops and is consumed as a source of vegetable protein and oil (Wu et al., 2017). In Indonesia, soybeans are highly valued as the main side dish. According to the Agricultural Data and Information Center (2021), approximately 90% of soybeans are used for the industrial production of tempeh (50%) and tofu (39%), and 11% of soybeans are used as raw materials in the food industry. Therefore, soybean cultivation must be developed through the extensification of suboptimal land, such as coastal sandy land. Planting soybeans on fertile land is challenging because they are less competitive than other strategic crops, such as rice and corn.

Yogyakarta, a province in Indonesia, offers a potential solution to its coastal sandy land, accounting for approximately 3.300 ha or 4% of the total area, extending along 110 km of the southern coast of Indonesia (Yuwono, 2009). This land has considerable potential for soybean crop development because of its abundant, relatively shallow groundwater and high sunlight. However, coastal sandy lands are marginal and have low productivity. The low productivity of coastal sandy lands is caused by several limiting factors, such as low water-holding capacity, high infiltration, low amounts of organic matter, and low water-use efficiency (Lipiec & Usowicz, 2021; Wu et al., 2019). Technological innovations can improve productivity in coastal sandy lands, including soil conditioners and fertilizers, irrigation, waterproofing, mulching, windbreaks, and biofertilizer inoculation (Indradewa, 2021).

Farmers in Yogyakarta's coastal sandy area grow chili as a valuable commodity crop because of its economic value (Istiyanti et al., 2015; Nugroho et al., 2018). Chili has adapted well to these regions as an essential commodity and provides high profitability. Therefore, relay intercropping offers a promising approach to increasing land use efficiency by introducing soybean as a second crop alongside chili, which allows for more effective use of resources such as water, sunlight, and nutrients. Relay intercropping of soybeans has been widely practiced in various regions around the world, including Asia (Hussain et al., 2020; Suntari et al., 2023; Zhou et al., 2019), Africa (Kermah et al., 2019; Namatsheve et al., 2020), America (Cecchin et al., 2021; Shrestha et al., 2021), and Europe (Koskey et al., 2022; Leoni et al., 2022).

Relay intercropping involves planting two or more crops in the same field at different times during different parts of the life cycle of each crop. This method ensures that the second crop is introduced after the first crop has reached its reproductive growth stage

but before it is ready for harvest (Andrews & Kassam, 1976). This staggered planting strategy minimizes competition for essential resources. It helps optimize crop growth and productivity, making it particularly valuable in resource-limited soils, such as those in coastal sandy areas. Relay intercropping has the advantages of enhanced resource-use efficiency (Ahmed et al., 2020; Chen et al., 2017; Rahman et al., 2017) and reduced pest and disease infestations (Biszczak et al., 2020; Chang et al., 2020), making it a viable option for improving agricultural productivity in coastal sandy lands.

The selection of soybean cultivars plays an important role in determining the success of soybean cultivation via relay intercropping in coastal sandy lands. Several studies have been conducted to select soybean varieties tolerant to intercropping under various conditions. Permanasari et al. (2023) and Harsono et al. (2020) examined several soybean varieties' growth response and yield under dry conditions. Sundari et al. (2020) evaluated the tolerance of various soybean varieties to shading in intercropping with cassava. However, little information is available on soybean varieties effectively intercropped with chili in coastal sandy soils, highlighting a gap in the current research. By exploring the adaptability and yield potential of various soybean varieties, this study addresses this critical gap and seeks to enhance productivity through optimized soybean-chili intercropping practices.

Several soybean cultivars have been released and tested in Indonesia for adaptation to coastal sandy lands. These varieties include 'Anjasmoro', 'Dena 1', and 'Grobogan', with 2.23, 2.24, and 2.27 tons per hectare, respectively (Handriawan et al., 2017), 'Demas 1' with a seed yield of 59.03 g per plant (Faozi et al., 2018), and 'Malika' with a seed yield of 1.18 tons per hectare (Purnamasari et al., 2016). Therefore, this study evaluates and identifies high-yielding soybean varieties compatible with relay intercropping with chili in coastal sandy lands, specifically focusing on their adaptability and performance in terms of yield and growth parameters. This study aims to provide practical insights into sustainable agricultural practices in marginal coastal regions, providing farmers with evidence-based recommendations for soybean-chili intercropping.

MATERIALS AND METHODS

Materials and Experimental Site

The five varieties adapted to sandy soil were 'Anjasmoro', 'Dena 1', 'Demas 1', Grobogan', and 'Malika'. This study was conducted on Samas beach sand farmland, Bantul Regency, Yogyakarta (8°00′06.7 ″S, 110°15'32.7’ E), at an altitude of 10 m above sea level and a distance of ±100 m from the shoreline. This study was conducted during the rainy season from January to May 2023. The total rainfall during the study was 1244.12 mm, according to the National Aeronautics and Space Administration (NASA) (2024). The soil at the research site had a sandy texture (80%) with a pH (H_2O) of 7.63, organic C content of 0.12%, total N content of 0.35%, available P content of 129.90 ppm, available K content of 7.52 ppm, and cation exchange capacity (CEC) of 5.53 cmol (+) kg^{-1} (Table 1).

Table 1
Characteristics of the soil in the study area

No.	Soil characteristics	Value	Criteria*	Methods
1	Texture		loamy sand class	Oxidation H_2O_2+HCl, Gravimetry
	Clay (%)	1		
	Sand (%)	80		
	Dust (%)	19		
2.	pH (H_2O)	7.63	Slightly alkaline	H_2O Extract 1:5, pH Meter
3.	CEC cmol ((+) kg^{-1})	5.53	Low	Percolation of Ammonium Acetate, Titrimetry
4.	Organic C(%)	0.12	Very low	Walkey-Black, Spectrophotometry
5.	Total N %	0.35	Moderate	Kjeldahl
6.	Available P (ppm)	129.90	Very high	Olsen, Spectrophotometry
7.	Available K (ppm)	7.52	Very low	Morgan-Wolf, AAS

Note. Soil samples were analyzed at the Integrated Laboratory of the Indonesian Agricultural Environment Standardization Institute, Central Java, Indonesia. *Indonesian Soil Research Institute (2005)

Experimental Design

A soybean-chili relay intercropping system was used in the field experiments. The experiments were conducted using a two-factor randomized complete block design with three blocks as replicates. The first factor was five varieties: 'Anjasmoro', 'Dena 1', 'Demas 1', 'Grobogan', and 'Malika'. The second factor was the cropping system: monoculture and relay intercropping. The experimental plots measured 2.5 m × 4 m, and each treatment plot contained two beds. Each bed measured 1 m × 4 m with a height of 20–25 cm. Soybeans were planted 30 days before the transplantation of chili seedlings (Figure 1a). In relay intercropping, soybeans were planted between two rows of chili plants (Figure 1b). The spacing between the chili plants was 50 cm × 40 cm. Soybeans were planted between rows of chili plants with a spacing of 40 cm between soybean plants and 25 cm between chili plants. Conversely, in the soybean monoculture, soybeans were planted with a 20 × 40 cm spacing. Each monoculture and relay intercropping plot contained 200 (two seeds × 100 planting holes) and 40 (two seeds × 20 planting holes) plants, respectively.

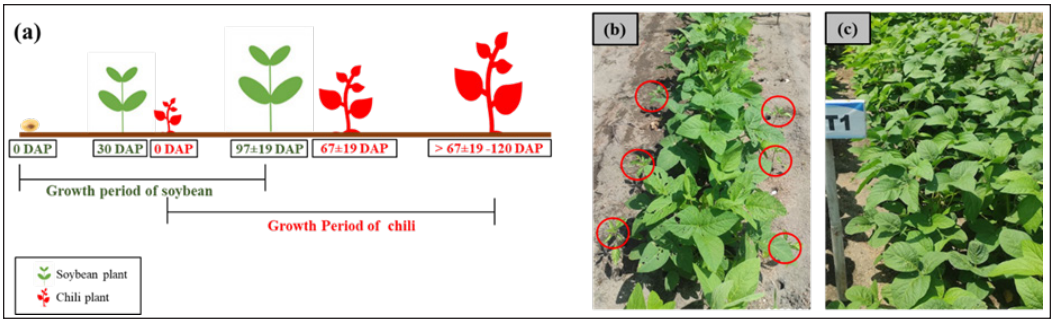

Figure 1. Illustration of the growth period of soybean-chili in relay intercropping systems (a), soybean-chili relay intercropping (b), and monoculture soybean (c)
Note. The circle in red indicates the chili plant

Experimental Procedure

The soil was plowed and fertilized with cow manure (20 t.ha^{-1}). Before planting, soybean seeds were mixed with Leguminosae inoculum (Legin®) at a rate of 30 g per 10 kg of seeds. Four soybean seeds were sown per planting hole, and thinning was conducted two weeks after germination to ensure only two plants per hole. Thinning was performed by cutting the stems at the base of the plants to avoid disturbing the root systems of the remaining plants. Soybeans were fertilized with urea (50 kg.ha^{-1}), SP36 (100 kg.ha^{-1}), and KCl (100 kg.ha^{-1}) at two weeks after planting (WAP) and NPK 15:15:15 (50 kg.ha^{-1}) at four WAP. Chili plants were fertilized at 1 WAP with NPK 15:15:15 (200 kg.ha^{-1}) and ZA (200 kg.ha^{-1}). The second supplementary fertilizer was given every week starting at 2–4 WAP in NPK 15:15:15 (125 kg.ha^{-1}) and ZA (125 kg.ha^{-1}). The third supplementary fertilizer was given every week starting at 5–8 WAP in the form of NPK 15:15:15 (150 kg.ha^{-1}) and ZA (150 kg. ha^{-1}). Applying fertilizer by pouring involves dissolving the fertilizer in water and then watering the plants with this solution during regular watering times. Watering was performed daily with a *pantek* well irrigation system, which pumps water from the ground and then flows it to the land using a pipe. Watering was applied using a pantek well irrigation system that pumped water from the ground and channeled it to the field through pipes. During rainy days or when soil moisture was adequate, irrigation was not performed to avoid overwatering. However, without rainfall, daily irrigation ensured the crops were not under water stress. Harvesting was performed after the plants had reached physiological maturity. The leaves turned brown and fell off, the pods and stems turned brown, and the seeds rattled when fully mature.

Growth, Yield Components, and Yield

Plant growth was determined by assessing plant height (cm), stem diameter (mm), number of branches, and leaf area (dm^2) at 8 weeks after planting (WAP), whereas plant dry biomass (g) was measured at harvest. Leaf area was measured using a leaf area meter (MK2, Delta-T Device Ltd., Serial No. CB380495, 220 V, 50 Hz, UK) using WinDIAS software (Delta-T Devices Ltd., UK). Yield components and yield variables were obtained at harvesting, including the number of pods, number of seeds, 100-seed weight (g), seed weight per plant (g), seed weight per plot (kg) and harvest index.

Soybeans were harvested from a 4 m² harvest area (subplot) in each plot. From this area, six plants were randomly selected as representative samples for each treatment at harvest to determine seed yield and dry weights of shoots and roots. These samples were carefully uprooted to prevent root loss and maintain the belowground biomass's integrity. After uprooting, the samples were sun-dried until they reached approximately 12% moisture content. Once dry, the seeds were manually threshed from the pods to determine the seed yield per plant. After the seeds were removed, the remaining whole plant biomass was

oven-dried at 90 °C until a constant weight was achieved, and then the dry weights of the roots and shoots were measured separately. Seed weight per plot was determined for all seeds harvested from the 4 m² harvest area.

Harvest Index

The Harvest index was calculated as the ratio of seed weight and total aboveground dry biomass of six plants (Wang et al., 2020)

Leaf Nutrient Uptake

The uptake of nitrogen by the leaves was assessed at 8 WAP. Leaf nitrogen content was measured following the Kjeldahl method (Bremmer, 1996), and leaf nitrogen uptake was calculated using the following formula: leaf nitrogen uptake = leaf dry weight ($g.plant^{-1}$) × leaf N content ($g.g^{-1}$) (Chen et al., 2017).

The Yield Index (YI)

The yield index (YI) was calculated using YI = YR/mean YR (Gavuzzi et al., 1997), where YR is the soybean intercropping relay system yield.

Soil Analysis and Microclimate Research Site

At the beginning of the study, the soil was analyzed for texture (%clay, %sand, and %dust), pH (H_2O), CEC, organic C, total N, and nutrient availability (P and K). Soil moisture content was measured three times during the study: at 4 WAP (soybean-only phase), 8 WAP (soybean and chili co-growth phase), and harvest. Microclimate observations were acquired daily in the form of temperature and humidity. Rainfall data were obtained from the National Aeronautics and Space Administration (NASA) (2024).

Statistical Analysis

All observational data on monoculture and relay intercropping were compared using the Student's t-test in Excel. Analysis of variance (ANOVA) was used to determine the significance of varieties in the intercropping system using the Microsoft Excel version 2021 VBA add-in computer application (DSAASTAT ver 1.514) (Onofri & Pannacci, 2014). Duncan's mean separation test was also used. Principal component analysis (PCA) was performed to identify and highlight the key variables that significantly influenced the growth and yield characteristics of tolerant soybean varieties using Originpro 2024b. The grouping of varieties was based on the similarity of characters using a cluster analysis in Minitab. Pearson's correlation was used to determine the correlation between seed weight per plant and other variables using OriginPro 2024b. All data analyses were statistically significant at $p < 0.05$ and marginally significant at $p < 0.10$.

RESULTS AND DISCUSSION

Research Field Condition

The research site was on a coastal sandy land (Figure 2). The figure shows a landscape characterized by flat topography and sand-dominated soil, serving as the primary growing medium.

Figure 2. Coastal sandy land

The soil type at the research site was a sandy loam class, with 80% of the texture being sand. The soil sample was characterized by a slightly alkaline pH (H_2O) of 7.63; very low content of organic carbon (C), equal to 0.12%; moderate content of total nitrogen (N), equal to 0.35%; very high content of available phosphorus (P), equal to 129.90 ppm; very low content of available potassium (K), equal to 7.52 ppm; and low value of cation exchange capacity (CEC), equal to 5.53 cmol [+] kg^{-1} (Table 1). These properties limit sustainable soybean growth in the study area. The very low organic carbon content limits microbial activity and nutrient availability; the low CEC indicates a limited ability of soils to hold and supply essential nutrients, and the sandy texture is likely to result in rapid nutrient leaching. Very high phosphorus content limits plant nutrient availability at a slightly alkaline pH, causing the precipitation of calcium into insoluble compounds (Baccari & Krouma, 2023). Additionally, very low K content restricts plant growth because of its high mobility and leaching in sandy soils (Degryse & McLaughlin, 2014). Therefore, it is crucial to incorporate organic nutrients to mitigate these issues. Weil and Brady (2017) indicated that incorporating sufficient organic material, such as manure, may produce a healthy soil quality, which increases nutrient holding and availability. Approximately 15–20 tons per hectare should be applied to sandy soil to make it fertile (Indradewa, 2021).

Figures 3a and 3 b show the climatic conditions during the study period. As shown in Figure 3a, the monthly rainfall from January to mid–May ranged from 101.07 mm to 388.26 mm, with a relatively even distribution throughout the rainy season. Sumarno and Manshuri (2013) noted that soybeans require monthly rainfall between 120 mm and 135 mm for optimal growth. Despite the ample rainfall during the study period, good drainage of coastal sandy land prevented flooding and minimized the risk of plant root rot. As shown in Figure 3b, average temperature and relative humidity were recorded during the study period. The daytime temperatures are high on sandy land near the coast, having an average temperature variation ranging from 35.05 °C to 38.83 °C, while the air humidity fluctuates between 54.20% and 62.45%. These conditions are typical for coastal areas that receive intense solar radiation.

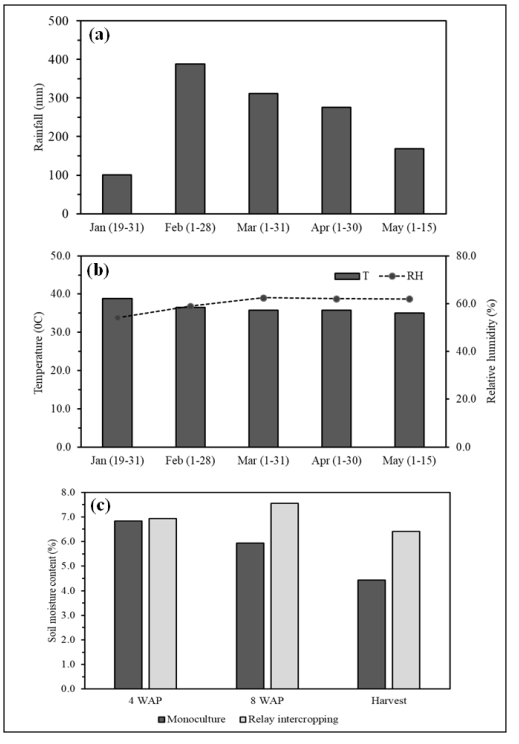

Figure 3. Monthly rainfall totals (a), average temperatures and relative humidity (b), and soil moisture content during the study (c)

Soil moisture levels varied between monoculture and relay intercropping systems (Figure 3c). At 4 WAP, soil moisture levels were similar between the two systems, with 6.83% in monoculture and 6.94% in relay intercropping. However, at 8 WAP and harvest, relay intercropping maintained higher soil moisture levels (7.56% and 6.42%, respectively) compared to monoculture (5.93% and 4.43%, respectively). This variation can likely be attributed to reduced intraspecific competition in the relay intercropping system, which enabled more efficient water usage and resource sharing between the soybean and chili crops. Additionally, water was irrigated when there was no rainy day or insufficient soil moisture, preventing drought stress and supporting the growth of soybeans and chilies in sandy soils with low water retention.

Yield and Yield Components

The weights of the soybean seeds in the monoculture and relay intercropping with chili are shown in Figure 4.

The cropping system significantly affected the weight of soybean seeds per plant (Figure 4a). The *t*-test results showed significant differences ($p < 0.05$) between monoculture

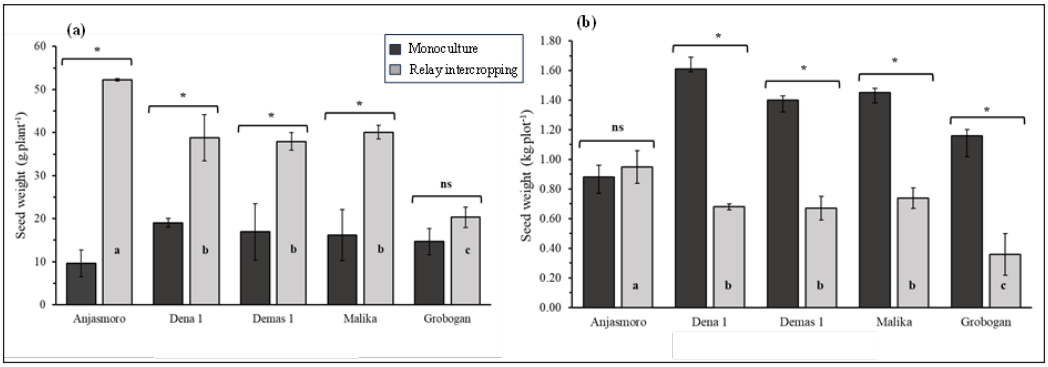

Figure 4. Seed weights per plant (g) (a) and plot (b) of five soybean varieties in monoculture and relay intercropping systems

Note. For each soybean variety in each cropping system, */ns denotes a significant/non-significant difference between monoculture and relay intercropping using a *t*-test at $p < 0.05$. Different letters within varieties in relay intercropping indicate significant differences using Duncan's Multiple Range test at $p < 0.05$. Means are averages over three replicates ± standard deviation

and relay intercropping for all varieties except 'Grobogan', which showed no significant difference. Relay intercropping increases seed production per plant, with all varieties, except 'Grobogan', producing significantly higher seed weights under relay intercropping than in monoculture. 'Anjasmoro' exhibited the greatest increase (443.39.87%), followed by 'Malika' (147.36%), 'Demas 1' (124.22%), 'Dena 1' (103.89%), and 'Grobogan' (38.71%). In relay intercropping, ANOVA revealed significant differences ($p < 0.05$) among varieties, with 'Anjasmoro' producing the highest seed weight per plant (52.21 g), followed by 'Malika' (40.08 g), 'Dena 1' (38.77 g), and 'Demas 1' (37.93 g), while 'Grobogan' produced the lowest (20.34 g).

For seed weight per plot (Figure 4b), the *t*-test results indicated significant differences ($p < 0.05$) between monoculture and relay intercropping for all varieties except 'Anjasmoro', which showed no significant difference. Relay intercropping resulted in significantly lower seed weights than monoculture due to the reduced soybean population, with only one row of soybeans planted between chili rows. This reduced population lowered total plot yields but minimized intraspecific competition, improving resource use efficiency per plant. However, 'Anjasmoro' showed no significant difference in seed weight per plot between the two systems, reflecting its superior adaptability to relay intercropping. ANOVA in relay intercropping revealed significant differences ($p < 0.05$) among varieties. 'Anjasmoro' had the highest seed weight (0.95 kg.plot^{-1}), followed by 'Malika' (0.74 kg.plot^{-1}), 'Dena 1' (0.68 kg.plot^{-1}), 'Demas 1' (0.67 kg.plot^{-1}), and 'Grobogan' (0.36 kg.plot^{-1}). The significant reduction in seed weight per plot under relay intercropping compared to monoculture underscores the trade-off between population density and individual plant performance. The yield and plant growth components further demonstrate the efficiency of soybean relay intercropping, as shown in Figures 5, 6, and Table 2.

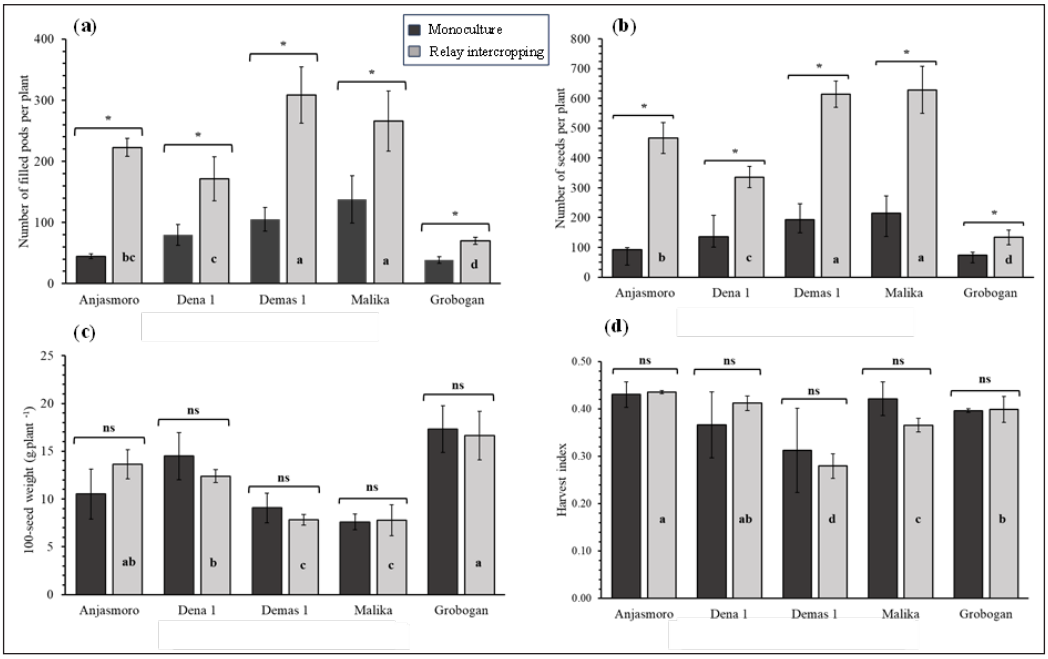

Figure 5. Number of filled pod plants (a), number of seeds per plant (b), 100-seed weight (c), and harvest index (d) of the five soybean varieties in the monoculture and relay intercropping systems *Note*. For each soybean variety in each cropping system, */ns denotes a significant/non-significant difference between monoculture and relay intercropping using *a t*-test at $p < 0.05$. Different letters within varieties in relay intercropping indicate significant differences using Duncan's Multiple Range test at $p < 0.05$. Means are averages over three replicates ± standard deviation

The number of filled pods differed significantly ($p < 0.05$) between monoculture and relay intercropping for all varieties (Figure 5a). Relay intercropping produced more filled pods than monoculture. Among the varieties in the relay intercropping system, significant differences ($p < 0.05$) were observed, with 'Demas 1' and 'Malika' producing the highest number of filled pods, while 'Grobogan' produced the fewest.

Significant differences ($p < 0.05$) were found in the number of seeds per plant between monoculture and relay intercropping across all varieties (Figure 5b). Relay intercropping significantly increased the number of seeds per plant, with the greatest increase observed in 'Anjasmoro' (407%), followed by 'Demas 1' (217.80%), 'Malika' (193.54%), 'Dena 1' (146.91%), and 'Grobogan' (80.63%). Within the relay intercropping system, significant differences $p < 0.05$) were noted among the varieties, where 'Demas 1' and 'Malika' produced the highest number of seeds per plant, while 'Grobogan' had the fewest.

The 100-seed weight did not differ significantly ($p > 0.05$) between monoculture and relay intercropping (Figure 5c). However, significant differences ($p < 0.05$) were observed among the varieties in the relay intercropping system. Based on the standard classification

of soybean seed size (Krisnawati & Adie, 2015), soybean seeds are categorized into three groups: small (<10 g/100 seeds), medium (10-14 g/100 seeds), and large (>14 g/100 seeds). In the relay intercropping system, 'Grobogan' had the largest seed size, averaging a weight of 16.26 g/100 seeds, classifying it as large. 'Anjasmoro' and 'Dena 1' were classified as medium-sized, with seed weights of 13.64 g and 12.39 g/100 seeds, respectively. In contrast, 'Demas 1' and 'Malika' were categorized as small-seeded varieties, weighing 7.82 g and 7.77 g/100 seeds, respectively. Although the maximum seed size is primarily determined by genetic traits, environmental conditions during the seed-filling stage also play a critical role in influencing final seed size (Suwitono et al., 2021)

The cropping system did not significantly affect the harvest index ($p > 0.05$) for any of the varieties, indicating that the efficiency of biomass partitioning between seed yield and vegetative growth was consistent across monoculture and relay intercropping systems (Figure 5d). However, significant differences ($p < 0.05$) were observed among the varieties in the relay intercropping system. 'Anjasmoro' recorded the highest harvest index (0.44), followed by 'Dena 1' (0.41), 'Grobogan' (0.40), 'Malika' (0.37), and 'Demas 1' with the lowest (0.28). A higher harvest index generally reflects a plant's ability to allocate more resources to seed production, which is crucial for maximizing the economic yield (Liu et al., 2020). Variations in the harvest index among varieties may be attributed to inherent genetic traits that influence biomass allocation efficiency (Wang et al., 2020).

Crop Growth Parameters

These measurements supported the quantitative determination of changes in crop growth parameters between the monoculture and relay intercropping systems. Plant height, stem diameter, and number of branches are crop growth parameters that determine soybean yield (Fattah et al., 2024; Xu et al., 2021; Zhang et al., 2021). Soybeans grown in relay intercropping had lower plant height, thicker stem diameter, and more branches than monoculture ones (Table 2).

The *t*-test results indicated that plant height differed significantly ($p < 0.05$) between monoculture and relay intercropping for 'Dena 1' and 'Demas 1', where monoculture consistently resulted in taller plants. However, for 'Anjasmoro', 'Malika' and 'Grobogan,' no significant differences ($p > 0.05$) were observed between the cropping systems. On average, plant height in monoculture (62.83 cm) was 17.69% higher than in relay intercropping (51.72 cm) (Table 2). The taller plants in monocultures are attributed to intraspecific competition, which induces stem elongation due to reduced light availability, as Klimek-Kopyra et al. (2020) reported. In the relay intercropping, ANOVA revealed significant differences ($p < 0.05$) among varieties. 'Malika' (59.28 cm), 'Anjasmoro' (56.00 cm), and 'Demas 1' (55.75 cm) had taller plant heights compared to 'Dena 1' (44.49 cm) and 'Grobogan' (43.08 cm), reflecting varietal differences in adaptation to the intercropping system.

Table 2
Plant height, stem diameter, and number of branches per plant at 8 WAP for five soybean varieties in monoculture and relay intercropping systems

Varieties	Plant height (cm)			Stem diameter (mm)			Number of branches		
	MC	RC	D (%)	MC	RC	D (%)	MC	RC	D (%)
'Anjasmoro'	61.21^{a}	56.00^{a} A	−8.51	0.58^{a}	0.97^{b} AB	66.67	3.33^{b}	7.39^{a} B	121.67
'Dena 1'	59.60^{a}	44.49^{b} B	−25.35	0.54^{a}	0.84^{b} AB	53.52	6.08^{a}	7.25^{a} B	19.18
'Demas 1'	75.33^{a}	55.75^{b} A	−26.00	0.76^{a}	1.13^{b} A	48.03	4.42^{b}	9.50^{a} A	115.09
'Malika'	64.19^{a}	59.28^{a} A	−7.66	0.67^{a}	0.95^{b} AB	40.15	5.50^{b}	7.62^{a} B	47.83
'Grobogan'	53.83^{a}	43.08^{a} B	−19.97	0.64^{a}	0.72^{a} B	11.55	3.83^{b}	5.67^{a} C	43.94
Average	62.83	51.72		0.64	0.92		4.63	7.54	

Note. Average values followed by the same lowercase letters in the horizontal direction and the same variable are not significantly different according to the *t*-test at $p < 0.05$, and those followed by uppercase letters in the vertical direction and the same variable are not significantly different according to Duncan's multiple range test at $p < 0.05$. M: Monoculture, RC: Relay intercropping, D: The difference between monoculture and relay intercropping

The *t*-test results indicated that stem diameter was significantly greater ($p < 0.05$) in relay intercropping compared to monoculture for all soybean varieties except 'Grobogan'. (Table 2). On average, stem diameter increased by 43.34% under relay intercropping. Among the varieties in relay intercropping, significant differences ($p < 0.05$) were observed. 'Demas 1' exhibited the largest stem diameter (1.13 mm), followed by 'Anjasmoro' (0.97 mm), 'Malika' (0.95 mm), and 'Dena 1' (0.84 mm), while 'Grobogan' had the smallest diameter (0.72 mm).

The number of branches per plant was significantly higher ($p < 0.05$) in relay intercropping, with an average increase of 62.83% compared to monoculture (Table 2). All varieties, except 'Grobogan', showed significant increases in branch number under relay intercropping. Significant differences ($p < 0.05$) were observed among varieties within the relay intercropping. 'Demas 1' produced the highest number of branches (9.50), followed by 'Malika' (7.62), 'Anjasmoro' (7.39), and 'Dena 1' (7.25), while 'Grobogan' had the fewest (5.67). These findings align with Rosso et al. (2021), who reported a positive correlation between soybean yield and the number of productive branches, underscoring their importance for pod development. Enhancing branch development in relay intercropping systems thus plays a critical role in improving soybean yields.

The improved growth and yield of soybeans in the relay intercropping system can be attributed to several synergistic factors that optimize resource utilization. Planting soybeans first allows early establishment, reducing direct competition for light, water, and nutrients

during critical growth stages and promoting complementary rather than competitive resource use (Ahmed et al., 2020; Raza et al., 2023). Observed soil moisture data (Figure 3c) indicate that relay intercropping consistently maintains higher moisture levels than monoculture, particularly at 8 WAP and harvest, likely due to more efficient water use and reduced competition. Additionally, nutrient sharing within the relay intercropping system allows the fertilizers applied to chili to indirectly benefit soybeans, enhancing resource availability and yield (Chen et al., 2017; Wang et al., 2014). Relay intercropping strategically combines the timing of crop establishment and resource sharing, creating a synergistic effect that supports the observed higher yield of soybeans, highlighting its potential as a sustainable agricultural practice.

In relay intercropping, the leaf area across all tested varieties was significantly greater ($p < 0.05$) than that observed in the monoculture, exhibiting an average increase of 126.18%, as shown in Figure 6a. Among the varieties, 'Anjasmoro' showed the most pronounced increase in leaf area, reaching 216.15%. This enhanced growth in relay intercropping systems is attributed to the more efficient utilization of resources than monocultures. However, ANOVA within the relay intercropping system revealed no significant differences ($p > 0.05$) among the varieties, indicating that all varieties performed similarly in terms of leaf area under this system. Factors such as genotype and environmental conditions significantly influence leaf area, which in turn supports plant growth, biomass accumulation, and vigor owing to the role of photosynthesis in producing essential assimilates for plant survival (Du et al., 2022; Fattah et al., 2024).

All soybean varieties grown in relay intercropping showed significantly higher N uptake ($p < 0.05$) compared to those grown in monoculture, with N uptake ranging from 0.10 to 0.16 g of leaf dry weight^{-1} (Figure 6b). However, ANOVA revealed no significant differences ($p > 0.05$) in N uptake among the varieties within the relay intercropping system, indicating that nitrogen absorption efficiency was consistent across varieties in this planting system. The amount of N uptake aligns with shoot dry weight and seed yield. According to Permanasari et al. (2023), improved N content enhances leaf development because nitrogen participates in the formation and development of plant cell structures. The higher nutrient uptake in soybean relay intercropping was likely due to the additional fertilizer applied for chili plant growth during the same period. Compared with monocultures, crops grown in relay intercropping can use resources more efficiently to produce higher yields. Previous research has revealed that the superiority of relay intercropping over other systems results from using various complementary resources such as land and nutrients (Jensen et al., 2020; Raza et al., 2019).

Soybean varieties grown in monoculture and relay intercropping showed significant differences ($p < 0.05$) in root dry weight, except for the 'Demas 1' (Figure 6c). In relay intercropping, the root dry weight increased for 'Anjasmoro' by 148.78%, 'Dena 1'

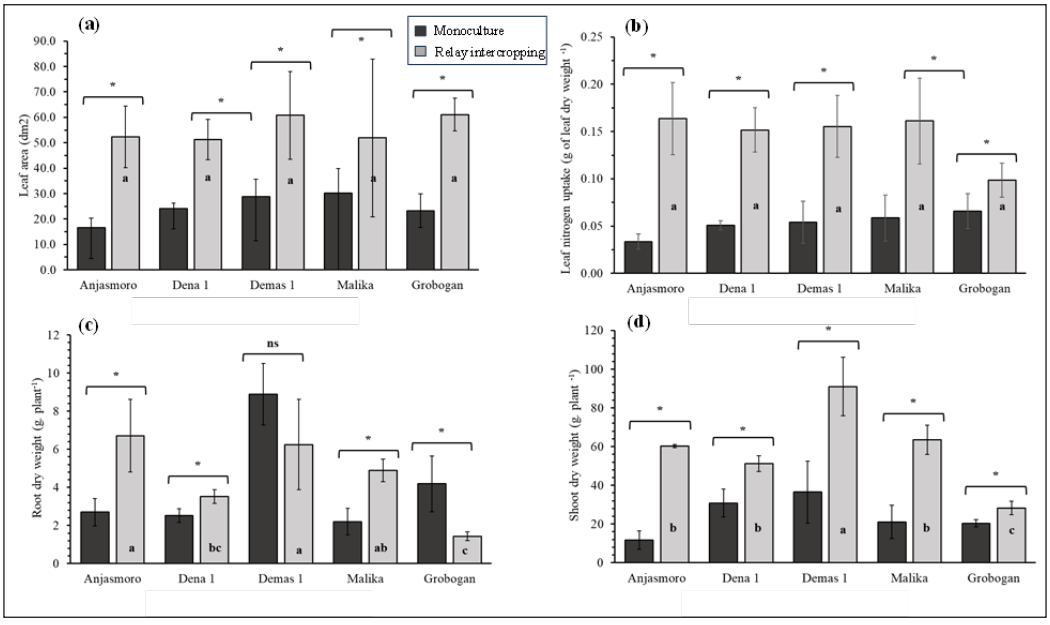

Figure 6. Leaf area (a) and leaf nitrogen uptake (b) at 8 WAP, and root dry weight (c) and shoot dry weight (d) at harvest of five soybean varieties in monoculture and relay intercropping *Note*. For each soybean variety in each cropping system, */ns denotes a significant/non-significant difference between monoculture and relay intercropping using a *t*-test at $p < 0.05$. Different letters within varieties in relay intercropping indicate significant differences using Duncan's Multiple Range test at $p < 0.05$. Means are averages over three replicates ± standard deviation

by 39.80%, and 'Malika' by 121.79%. This indicates their adaptability to competitive intercropping conditions, likely enhancing their ability to absorb water and nutrients. Conversely, 'Demas 1' and 'Grobogan' exhibited decreased root dry weight by 29.70% and 65.66%, respectively, indicating a lower tolerance to interspecific competition for belowground resources. Despite this, 'Demas 1' maintained a relatively high root biomass (6.25 g), second only to 'Anjasmoro' (6.71 g), and achieved the highest shoot dry weight (91.03 g) (Figure 6d), reflecting a compensatory growth strategy that prioritizes aboveground productivity (Poorter et al., 2012; Sarto et al., 2021). This adaptive biomass allocation enables 'Demas 1' to sustain productivity under intercropping stress, unlike 'Grobogan,' which consistently exhibited lower biomass across all variables, highlighting its reduced competitiveness in such systems. (Lv et al., 2014; Sarto et al., 2021). Meanwhile, 'Malika,' 'Dena 1,' and 'Grobogan' recorded lower root dry weights at 4.89 g, 3.53 g, and 2.14 g, respectively, underscoring the variability in adaptability among the soybean varieties.

For shoot dry weight, all soybean varieties grown in monoculture and relay intercropping showed significant differences ($p < 0.05$) (Figure 6d). Relay intercropping significantly increased shoot dry weight, with the highest increase in the 'Anjasmoro' (416.65%), followed by 'Malika' (202.37%), 'Demas 1' (149%), 'Dena 1' (65%), and

'Grobogan' (39.21%). ANOVA within the relay intercropping system revealed significant differences ($p < 0.05$) among varieties. 'Demas 1' had the highest shoot dry weight (91.03 g), significantly exceeding the other varieties. 'Anjasmoro', 'Dena 1', and 'Malika' had similar shoot dry weights of 60.35 g, 51.14 g, and 63.58 g, respectively. The lowest shoot dry weight was found in the 'Grobogan' at 28.14 g. Shoot dry matter is related to the availability of resources such as adequate water and nutrients. Sufficient nutrients during soybean growth produce assimilates for pod formation. Raza et al. (2023) stated that the accumulation of dry matter increases the number and weight of soybean seeds, which are crucial for increasing soybean yields.

The Seed Weight, Yield Index, Principal Component Analysis, and Cluster Analysis of Five Soybean Varieties in Relay Intercropping

The average seed weight (YR) of the five varieties in the soybean relay intercropping was 37.86 g. 'Anjasmoro', 'Dena 1', 'Demas 1', and 'Malika' had above-average yields of 52.21 g, 38.71 g, 37.93 g, and 40.08 g, respectively (Figure 7a). In contrast, 'Grobogan' had a lower yield (20.34 g), compared to the other four varieties and below the average yield of 37.86 g. The YR values were consistent with the yield index (YI), where a high YI value indicated tolerance to relay intercropping. Figure 7b shows that 'Anjasmoro', 'Dena 1', 'Demas 1', and 'Malika' had a high YI value of ≥ 1, while 'Grobogan' had the lowest YI value of ≤ 1. The high YR and Y1 values of 'Anjasmoro', 'Dena 1', 'Malika 1', and 'Dena 1' indicate that the four varieties were high-yielding soybean varieties, whereas 'Grobogan' was a low-yielding variety.

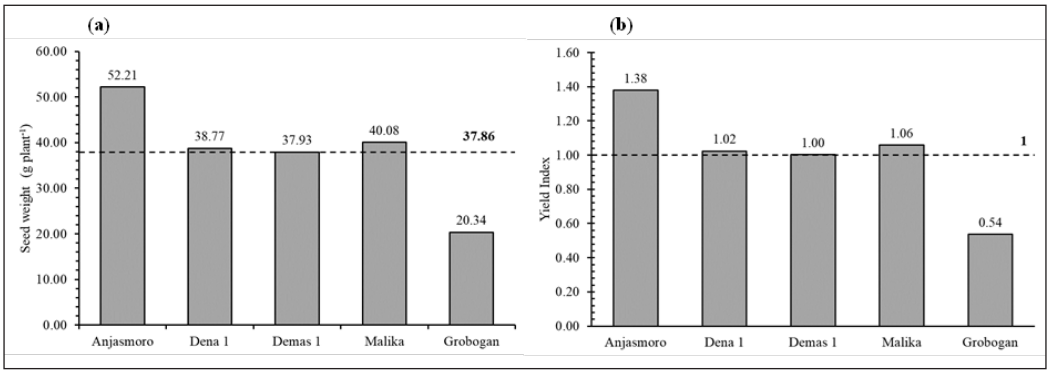

Figure 7. Average seed weight (a) and yield index (b) of five soybean varieties in relay intercropping

Principal Component Analysis (PCA) was conducted to identify and highlight the key variables that significantly influenced the growth and yield characteristics of tolerant soybean varieties. The two main components (PC1 and PC2) explained 88.87% of the total data variability (Figure 8a). PC1 explained 63.64%, and PC2 explained 25.23% of

the variability, respectively. The number of pods, seeds, branches, and stem diameters significantly contributed to PC1, with values of 0.35, 0.34, 0.33, and 0.33, respectively. These variables indicate that PC1 was primarily associated with growth and yield components, emphasizing vegetative growth and reproductive output. The seed weight per plant, harvest index, shoot dry weight, root dry weight, and yield index contributed significantly to PC2, with values of 0.41, 0.39, -0.41, -0.41, and -0.41, respectively. The positive contributions of seed weight and yield index suggest that PC2 reflects the yield efficiency. In contrast, the negative contributions of root and shoot dry weights indicate an inverse relationship between vegetative biomass and yield efficiency.

'Anjasmoro' had a high PC2 value, indicating superiority in seed weight and yield index variables. 'Demas 1' had a high value on PC1, showing superiority in the number of pods and seeds and strong vegetative growth components like the number of branches and stem diameter. Conversely, 'Grobogan' had a high negative value for both components, indicating significant differences in the measured variables, and tended to have low yields. 'Dena 1' and 'Malika' had values closer to the average on both components, indicating more balanced growth and yield characteristics without extreme dominance in either direction.

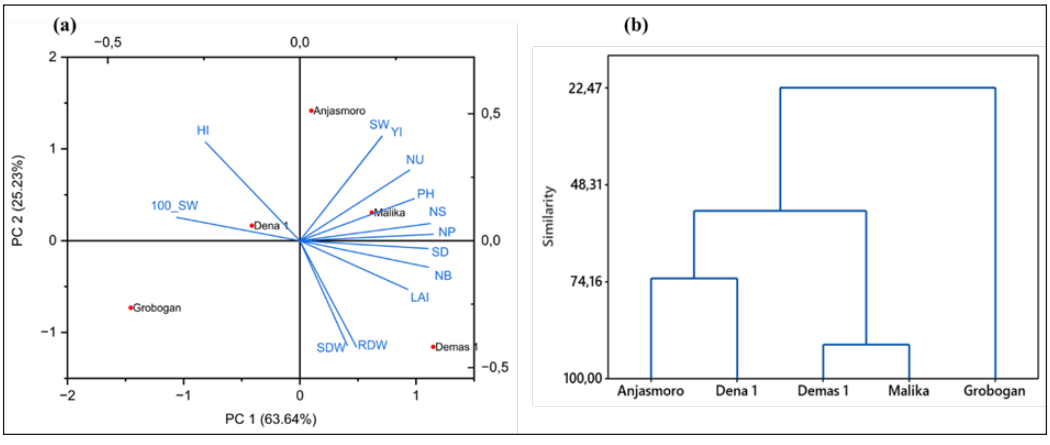

Figure 8. Principal component analysis (a) and cluster analysis (b) were based on the growth, yield components, yield, and yield index of soybean plants in relay intercropping
Note. PH: plant height, NB: number of branches, SD: stem diameter, SDW: shoot dry weight, RDW: root dry weight, LA: leaf area, NU: leaf nutrient uptake, NP: number of pods, NS: number of seeds, HI: harvest index, SW: seed weight, YI: yield index

Cluster analysis based on the PCA results showed that the two groups of tolerant soybean varieties had high and low yields (Figure 8b). 'Anjasmoro,' 'Dena 1,' 'Demas 1,' and 'Malika' are grouped as high-yielding varieties at a 55.35% similarity level. The PCA biplot supported this grouping, indicating that these varieties share similar characteristics, such as above-average seed weight and yield index (YI) ≥ 1, and strong similarities in

plant growth variables, showing good tolerance in the intercropping system. In contrast, the 'Grobogan' is identified as low-yielding, with the lowest similarity level of 22.47% and significant differences from the other varieties. This finding is consistent with the PCA results, where 'Grobogan' is distinctly separated from other varieties in the biplot due to its low seed weight (20.34 g) and yield index of ≤ 1 (0.54). The integration of these analyses provides a comprehensive classification of soybean varieties based on their yield performance and growth characteristics.

Pearson's Correlation in High and Low-yielding Tolerant Soybean Varieties

The correlation between the high- and low-yielding soybean groups reveals key differences in how these varieties allocate resources and manage growth to influence yield outcomes. The correlation of the high- and low-yielding soybean groups is shown in Figure 9.

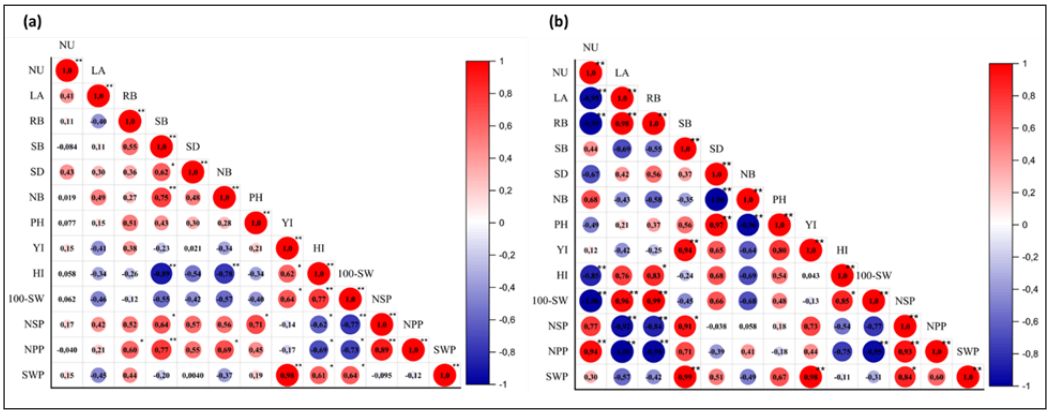

Figure 9. (a) Pearson correlation analysis of the high-yielding ('Anjasmoro', 'Dena1', 'Demas 1' and 'Malika') and (b) low-yielding ('Grobogan') tolerant soybean varieties

Note. PH: plant height, NB: number of branches, SD: stem diameter, SDW: shoot dry biomass, RDW: root dry biomass, LA: leaf area, NU: leaf nutrient uptake, NP: number of pods, NS: number of seeds, HI: harvest index, SWP: seed weight, YI: yield index. * and ** indicate statistically significant correlations at p <0.05 and p <0.01, respectively

Significant correlations were observed in high-yielding tolerant soybean varieties ('Anjasmoro', 'Dena 1', 'Demas 1', and 'Malika') (Figure 9a). In the high-yielding group, significant positive correlations were observed between seed weight per plant (SWP) and 100-seed weight (100-SW) (r = 0.64*), yield index (YI) (r = 0.98 **), and harvest index (HI) (r = 0.61*). These correlations suggest that these varieties efficiently convert growth resources into higher seed yields by balancing seed size and quantity without compromising overall productivity. Furthermore, the number of pods per plant (NPP) and number of seeds per plant (NSP) were strongly positively correlated with shoot dry weight (SDW) (NPP: r = 0.77**; NSP: r = 0.64*) and root dry weight (RDW) (NPP: r = 0.60*). This indicates

that robust vegetative growth contributes significantly to the increase in pod and seed numbers, which supports the higher yields observed in these varieties.

In contrast, the low-yielding variety 'Grobogan' exhibits weaker and often non-significant correlations between SWP and similar variables. While a positive correlation exists between SWP and NPP (r = 0.61) and NSP (r = 0.84), these relationships were not statistically significant, indicating that they were not strong or consistent enough to support significant yield improvements. Moreover, the HI, which indicates the efficiency of converting biomass into seed yield, does not show a significant correlation in 'Grobogan'. This suggests inefficiencies in the utilization of biomass for seed production. The negative correlation between SWP and 100-SW (r = -0.32) in 'Grobogan' further indicates a trade-off where increasing seed size reduces the total seed weight per plant, exacerbating the inefficiency in resource allocation.

Overall, the primary differences between the high- and low-yielding tolerant soybean varieties are their efficiency in using resources to produce seeds, their ability to balance seed size with the number of seeds, and the consistency with which these factors contribute to overall yield. The high-yielding group was more effective at converting growth resources into seed production, maintaining a good balance between producing a large number of seeds and a large number of seeds, leading to greater productivity. In contrast, the low-yielding group struggles to manage these resources efficiently, often increasing seed size at the expense of total seed number, thereby limiting their overall yield potential.

CONCLUSION

Five soybean varieties ('Anjasmoro', 'Dena 1', 'Demas 1', 'Malika', and 'Grobogan') were adapted to coastal sandy lands. Relay intercropping between soybeans and chili on coastal sandy lands has significantly improved soybean yield and growth components compared to monoculture systems, demonstrating its effectiveness in optimizing land use and resource utilization. Water was irrigated when there was no rainy day or insufficient soil moisture, which was critical in maintaining adequate soil moisture levels, preventing drought stress, and supporting the growth of soybeans and chilies throughout their life cycle. The five soybean varieties were classified into high-yielding ('Anjasmoro', 'Dena 1', 'Demas 1', and 'Malika') and low-yielding ('Grobogan') groups based on yield, yield components, and plant growth. These findings suggest that selecting high-yielding soybean varieties can maximize productivity in relay intercropping systems.

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REFERENCES

Agricultural Data and Information Center. (2021). *Buletin konsumsi pangan tahun 2021* [Food Consumption Bulletin 2021]. Kementerian Pertanian.

Ahmed, S., Raza, M. A., Yuan, X., Du, Y., Iqbal, N., Chachar, Q., Soomro, A. A., Ibrahim, F., Hussain, S., Wang, X., Liu, W., & Yang, W. (2020). Optimized planting time and co-growth duration reduce the yield difference between intercropped and sole soybean by enhancing soybean resilience toward size-asymmetric competition. *Food and Energy Security*, *9*, e226. https://doi.org/10.1002/fes3.226

Andrews, D. J., & Kassam, A. H. (1976). The importance of multiple cropping in increasing world food supplies. In R. I. Papendick, P. A. Sanchez & G. B. Triplett (Eds.), *Multiple cropping* (pp. 1–10). ASA Special Publications. https://doi.org/10.2134/asaspecpub27.c1

Baccari, B., & Krouma, A. (2023). Rhizosphere acidification determines phosphorus availability in calcareous soil and influences faba bean (*Vicia faba*) tolerance to P deficiency. *Sustainability*, *15*(7), 6203. https://doi.org/10.3390/su15076203

Biszczak, W., Różyło, K., & Kraska, P. (2020). Yielding parameters, nutritional value of soybean seed and weed infestation in relay-strip intercropping system with buckwheat. *Acta Agriculturae Scandinavica, Section B — Soil & Plant Science*, *70*(8), 640–647. https://doi.org/10.1080/09064710.2020.1831586

Bremmer, J. M. (1996). Nitrogen-total. In D. L. Sparks, A. L. Page, P. A. Helmke, R. H. Loeppert, P. N. Soltanpour, M. A. Tabatabai, C. T. Johnston & M. E. Sumner (Eds.), *Methods of soil analysis: Part 3—Chemical methods* (pp. 1085–1121). Soil Science Society of America.

Cecchin, A., Pourhashem, G., Gesch, R. W., Lenssen, A. W., Mohammed, Y. A., Patel, S., & Berti, M. T. (2021). Environmental trade-offs of relay-cropping winter cover crops with soybean in a maize-soybean cropping system. *Agricultural Systems*, *189*, 103062. https://doi.org/10.1016/j.agsy.2021.103062

Chang, X., Yan, L., Naeem, M., Khaskheli, M. I., Zhang, H., Gong, G., Zhang, M., Song, C., Yang, W., Liu, T., & Chen, W. (2020). Maize/soybean relay strip intercropping reduces the occurrence of fusarium root rot and changes the diversity of the pathogenic fusarium species. *Pathogens*, *9*(3), 211. https://doi.org/10.3390/pathogens9030211

Chen, P., Du, Q., Liu, X., Zhou, L., Hussain, S., Lei, L., Song, C., Wang, X., Liu, W., Yang, F., Shu, K., Liu, J., Du, J., Yang, W., & Yong, T. (2017). Effects of reduced nitrogen inputs on crop yield and nitrogen use efficiency in a long-term maize-soybean relay strip intercropping system. *Plos One*, *12*(9), e0184503. https://doi.org/10.1371/journal.pone.0184503

Degryse, F., & McLaughlin, M. J. (2014). Phosphorus diffusion from fertilizer: visualization, chemical measurements, and modeling. *Soil Science Society of America Journal*, *78*(3), 832–842. https://doi.org/10.2136/sssaj2013.07.0293

Du, Q., Chen, P., Zheng, B., Hu, Y., Yang, W., & Yong, T. (2022). Screening soybean for adaptation to relay intercropping systems: Associations between reproductive organ abscission and yield. *Agronomy*, *12*(10), 2379. https://doi.org/10.3390/agronomy12102379

Faozi, K., Yudono, P., Indradewa, D., & Maas, A. (2018). Banana stem bokashi and its effect to increase soybean yield (*Glycine max* L. Merrill) in coastal sands area. *Agrotechnology*, *7*(2), 1–7. https://doi.org/10.4172/2168-9881.1000184

Fattah, A., Idaryani, Herniwati, Yasin, M., Suriani, S., Salim, Nappu, M. B., Mulia, S., Irawan Hannan, M. F., Wulanningtyas, H. S., Saenong, S., Dewayani, W., Suriany, Winanda, E., Manwan, S. W., Asaad, M., Warda, Nurjanani, Nurhafsah, … Ella, A. (2024). Performance and morphology of several soybean varieties and responses to pests and diseases in South Sulawesi. *Heliyon, 10*(5), e25507. https://doi.org/10.1016/j.heliyon.2024.e25507024.e25507

Gavuzzi, P., Rizza, F., Palumbo, M., Campanile, R. G., Ricciardi, G. L., & Borghi, B. (1997). Evaluation of field and laboratory predictors of drought and heat tolerance in winter cereals. *Canadian Journal of Plant Science*, *77*(4), 523–531. https://doi.org/10.4141/P96-130

Handriawan, A., Respatie, D. W., & Tohari. (2017). Pengaruh intensitas naungan terhadap pertumbuhan dan hasil tiga kultivar kedelai (*Glycine max* (L.) Merrill) di lahan pasir pantai Bugel, Kulon Progo [The effect of shade intensity to the growth and result of three soybean (Glycine max (L.) Merrill) cultiv. *Vegetalika*, *5*(3), 1–14. https://doi.org/10.22146/veg.25346

Harsono, A., Elisabeth, D. A. A., Muzaiyanah, S., & Rianto, S. A. (2020). Soybean-maize intercropping feasibility under drought-prone area in East Java, Indonesia. *Biodiversitas Journal of Biological Diversity*, *21*(8), 3744–3754. https://doi.org/10.13057/biodiv/d210842

Hussain, S., Liu, T., Iqbal, N., Brestic, M., Pang, T., Mumtaz, M., Shafiq, I., Li, S., Wang, L., Gao, Y., Khan, A., Ahmad, I., Allakhverdiev, S. I., Liu, W., & Yang, W. (2020). Effects of lignin, cellulose, hemicellulose, sucrose and monosaccharide carbohydrates on soybean physical stem strength and yield in intercropping. *Photochemical & Photobiological Sciences*, *19*(4), 462–472. https://doi.org/10.1039/c9pp00369j

Indonesian Soil Research Institute. (2005). *Petunjuk teknis: Analisis kimia tanah, tanaman, air dan pupuk* [Technical guidelines: Chemical analysis of soil, plants, water, and fertilizers]. Balai Penelitian Tanah. https://repository.pertanian.go.id/bitstream/handle/123456789/14959/juknis_kimia.pdf

Indradewa, D. (2021). Potensi lahan pasir pantai sebagai lahan pertanian produktif [Potential of coastal sandy land as productive agricultural land]. In D. Indradewa, S. N. H. Utami, V. D. S. Handayani & S. N. R. Irwan (Eds.), *Inovasi teknologi agronomi di lahan pasir pantai* [Innovations in agronomic technology on coastal sandy land] (pp. 1–30). Deepublish. https://kubuku.id/detail/inovasi-teknologi-agronomi-di-lahan-pasir-pantai/23359

Istiyanti, E., Khasanah, U., & Anjarwati, A. (2015). Pengembangan usahatani cabai merah di lahan pasir pantai kecamatan Temon kabupaten Kulonprogo [Development of red chilli farming in coastal land of Temon, Kulonprogo]. *Agraris: Journal of Agribusiness and Rural Development Research*, *1*(1), 6–11. https://doi.org/10.18196/agr.112

Jensen, E. S., Carlsson, G., & Hauggaard-Nielsen, H. (2020). Intercropping of grain legumes and cereals improves the use of soil N resources and reduces the requirement for synthetic fertilizer N: A global-scale analysis. *Agronomy for Sustainable Development*, *40*, 5. https://doi.org/10.1007/s13593-020-0607-x

Kermah, M., Franke, A. C., Ahiabor, B. D. K., Adjei-Nsiah, S., Abaido, R. C., & Giller, K. E. (2019). Legume–maize rotation or relay? Options for ecological intensification of smallholder farms in the guinea savanna of Northern Ghana. *Experimental Agriculture*, *55*(5), 673–691. https://doi.org/10.1017/S0014479718000273

Klimek-Kopyra, A., Bacior, M., Lorenc-Kozik, A., Neugschwandtner, R. W., & Zając, T. (2020). The Intraspecific competition as a driver for true production potential of soybean. *Italian Journal of Agronomy*, *16*(1), 1709. https://doi.org/10.4081/ija.2020.1709

Koskey, G., Leoni, F., Carlesi, S., Avio, L., & Bàrberi, P. (2022). Exploiting plant functional diversity in Durum wheat–lentil relay intercropping to stabilize crop yields under contrasting climatic conditions. *Agronomy*, *12*(1), 210. https://doi.org/10.3390/agronomy12010210

Krisnawati, A., & Adie, M. M. (2015). Selection of soybean genotypes by seed size and its prospects for industrial raw material in Indonesia. *Procedia Food Science*, *3*, 355–363. https://doi.org/10.1016/j.profoo.2015.01.039

Leoni, F., Lazzaro, M., Ruggeri, M., Carlesi, S., Meriggi, P., & Moonen, A. C. (2022). Relay intercropping can efficiently support weed management in cereal-based cropping systems when appropriate legume species are chosen. *Agronomy for Sustainable Development*, *42*, 75. https://doi.org/10.1007/s13593-022-00787-3

Lipiec, J., & Usowicz, B. (2021). Quantifying cereal productivity on sandy soil in response to some soil-improving cropping systems. *Land*, *10*(11), 1199. https://doi.org/10.3390/land10111199

Liu, W., Hou, P., Liu, G., Yang, Y., Guo, X., Ming, B., Xie, R., Wang, K., Liu, Y., & Li, S. (2020). Contribution of total dry matter and harvest index to maize grain yield—A multisource data analysis. *Food and Energy Security*, *9*(4), 1–12. https://doi.org/10.1002/fes3.256

Lv, Y., Francis, C., Wu, P., Chen, X., & Zhao, X. (2014). Maize-soybean intercropping interactions above and below ground. *Crop Science*, *54*(3), 914–922. https://doi.org/10.2135/cropsci2013.06.0403

Namatsheve, T., Cardinael, R., Corbeels, M., & Chikowo, R. (2020). Productivity and biological N2-fixation in cereal-cowpea intercropping systems in sub-Saharan Africa. A review. *Agronomy for Sustainable Development*, *40*, 30. https://doi.org/10.1007/s13593-020-00629-0

National Aeronautics and Space Administration. (2024). *Rainfall data*. https://power.larc.nasa.gov/data-access-viewer/

Nugroho, A. D., Prasada, I. M. Y., Putri, S. K., Anggrasari, H., & Sari, P. N. (2018). Rantai nilai cabai di lahan pasir pantai kabupaten Kulon Progo [Chili value chain on coastal sandy land in Kulon Progo Regency]. *Economics Development Analysis Journal*, *7*(4), 458–467. https://doi.org/10.15294/edaj.v7i4.25013

Onofri, A., & Pannacci, E. (2014). Spreadsheet tools for biometry classes in crop science programmes. *Communications in Biometry and Crop Science*, *9*(2), 43–53.

Permanasari, I., Sulistyaningsih, E., Kurniasih, B., & Indradewa, D. (2023). Morphophysiological and yield traits of soybean varieties tolerant of intercropping with maize. *Biodiversitas*, *24*(7), 3872–3880. https://doi.org/10.13057/biodiv/d240726

Poorter, H., Niklas, K. J., Reich, P. B., Oleksyn, J., Poot, P., & Mommer, L. (2012). Biomass allocation to leaves, stems and roots: Meta-analyses of interspecific variation and environmental control. *New Phytologist*, *193*(1), 30–50. https://doi.org/10.1111/j.1469-8137.2011.03952.x

Purnamasari, E. T., Yudono, P., & Rogomulyo, R. (2016). Pertumbuhan gulma dan hasil kedelai hitam (*Glycine max* (L.) Merrill) 'Mallika' terhadap pengolahan tanah dan jarak tanam di lahan pasir pantai [The weeds growth and yield of 'Mallika' black soybean (*Glycine max* (L .) Merrill) against to tillage and spac. *Vegetalika*, *5*(1), 23–31. https://doi.org/10.22146/VEG.24652

Rahman, T., Ye, L., Liu, X., Iqbal, N., Du, J., Gao, R., Liu, W., Yang, F., & Yang, W. (2017). Water use efficiency and water distribution response to different planting patterns in maize–soybean relay strip intercropping systems. *Experimental Agriculture*, *53*(2), 159–177. https://doi.org/10.1017/S0014479716000260

Raza, M. A., Bin Khalid, M. H., Zhang, X., Feng, L. Y., Khan, I., Hassan, M. J., Ahmed, M., Ansar, M., Chen, Y. K., Fan, Y. F., Yang, F., & Yang, W. (2019). Effect of planting patterns on yield, nutrient accumulation and distribution in maize and soybean under relay intercropping systems. *Scientific Reports*, *9*, 4947. https://doi.org/10.1038/s41598-019-41364-1

Raza, M. A., Din, A. M. U., Zhiqi, W., Gul, H., Ur Rehman, S., Bukhari, B., Haider, I., Rahman, M. H. U., Liang, X., Luo, S., El Sabagh, A., Qin, R., & Zhongming, M. (2023). Spatial differences influence nitrogen uptake, grain yield, and land-use advantage of wheat/soybean relay intercropping systems. *Scientific Reports*, *13*, 16916. https://doi.org/10.1038/s41598-023-43288-3

Rosso, L. H. M., Reis, A. F. de B., & Ciampitti, I. A. (2021). Vertical canopy profile and the impact of branches on soybean seed composition. *Frontiers in Plant Science*, *12*, 725767. https://doi.org/10.3389/fpls.2021.725767

Sarto, M. V. M., Borges, W. L. B., Bassegio, D., Rice, C., & Rosolem, C. A. (2021). Maize and sorghum root growth and yield when intercropped with forage grasses. *Agronomy Journal*, *113*(6), 4900–4915. https://doi.org/10.1002/agj2.20920

Shrestha, R. K., Richer, E., Clevenger, W. B., Davis, M., & Lindsey, L. E. (2021). Effect of mono-, relay-, and double-crop systems on yield and profitability. *Agronomy Journal*, *113*, 1747–1757. https://doi.org/10.1002/agj2.20598

Sumarno., & Manshuri, A. G. (2013). Persyaratan tumbah dan wilayah produksi kedelai di Indonesia [Requirements for soybean growth and production areas in Indonesia]. In Sumarno, Suyamto, A. Widjono, Hermanto & H. Kasim (Eds.), *Kedelai: Teknik produksi dan pengembangan* [Soybeans: Production techniques and development] (pp. 74–103). Puslitbangtan.

Sundari, T., Purwantoro, P., Artari, R., & Baliadi, Y. (2020). Respons genotipe kedelai sebagai tanaman sela pada tumpang sari dengan ubi kayu [Response of soybean genotypes in intercropping with cassava]. *Jurnal Ilmu Pertanian Indonesia*, *25*(1), 129–137. https://doi.org/10.18343/jipi.25.1.129

Suntari, Ghulamahdi, M., & Melati, M. (2023). Relay-cropping soybean-maize in saturated soil culture increases efficiency of land use and nitrogen fertilizer. *Indonesian Journal of Agronomy*, *51*(1), 91–100. https://doi.org/10.24831/ija.v51i1.44905

Suwitono, B., Aji, H. B., Hidayat, Y., Cahyaningrum, H., Lala, F., & Habehaan, K. B. (2021). Pertumbuhan dan produktivitas beberapa varietas kedelai di bawah tegakan kelapa [Growth and productivity of soybean varieties under coconut plantation]. *Buletin Palawija*, *19*(1), 31–40. https://doi.org/10.21082/bulpa.v19n1.2021.p31-40

Wang, M., Wu, C., Cheng, Z., Meng, H., Zhang, M., & Zhang, H. (2014). Soil chemical property changes in eggplant/garlic relay intercropping systems under continuous cropping. *PLoS ONE*, *9*(10). https://doi.org/10.1371/journal.pone.0111040

Wang, X., Wu, X., Ding, G., Yang, F., Yong, T., Wang, X., & Yang, W. (2020). Analysis of grain yield differences among soybean cultivars under maize–soybean intercropping. *Agronomy*, *10*(1), 110. https://doi.org/10.3390/agronomy10010110

Weil, R. R., & Brady, N. C. (2017). *The nature and properties of soils* (15th ed.). Pearson. http://lccn.loc.gov/2016008568

Wu, H., Lü, L., Zhang, Y., Xu, C., Yang, H., Zhou, W., Wang, W., Zhao, L., He, N., Smith, M. D., Han, X., Hartley, I. P., & Yu, Q. (2019). Sediment addition and legume cultivation result in sustainable, long-term increases in ecosystem functions of sandy grasslands. *Land Degradation & Development*, *30*(14), 1667–1676. https://doi.org/10.1002/ldr.3348

Wu, Y., Yang, F., Gong, W., Ahmed, S., Fan, Y., Wu, X., Yong, T., Liu, W., Shu, K., Liu, J., Du, J., & Yang, W. (2017). Shade adaptive response and yield analysis of different soybean genotypes in relay intercropping systems. *Journal of Integrative Agriculture*, *16*(6), 1331–1340. https://doi.org/10.1016/S2095-3119(16)61525-3

Xu, C., Li, R., Song, W., Wu, T., Sun, S., Hu, S., Han, T., & Wu, C. (2021). Responses of branch number and yield component of soybean cultivars tested in different planting densities. *Agriculture*, *11*, 69. https://doi.org/10.3390/agriculture11010069

Yuwono, N. W. (2009). Membangun kesuburan tanah di lahan marginal [Building soil fertility on marginal land]. *Jurnal Ilmu Tanah Dan Lingkungan*, *9*(2), 137–141.

Zhang, X., Ding, W., Xue, D., Li, X., Zhou, Y., Shen, J., Feng, J., Guo, N., Qiu, L., Xing, H., & Zhao, J. (2021). Genome-wide association studies of plant architecture-related traits and 100-seed weight in soybean landraces. *BMC Genomic Data*, *22*, 10. https://doi.org/10.1186/s12863-021-00964-5

Zhou, T., Wang, L., Yang, H., Gao, Y., Liu, W., & Yang, W. (2019). Ameliorated light conditions increase the P uptake capability of soybean in a relay-strip intercropping system by altering root morphology and physiology in the areas with low solar radiation. *Science of The Total Environment*, *688*, 1069–1080. https://doi.org/10.1016/j.scitotenv.2019.06.344

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Morphology and Nutritional Composition of *Gracilaria changii* at Different Maturation Stages

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ABSTRACT

Gracilaria changii is a red seaweed macroalgae that holds economic potential for the Malaysian economy, as it has now started to be cultivated commercially. Establishing the harvesting standards for seaweed is important for its marketing purposes. Thus, this study evaluated the effect of different growth stages on its postharvest quality. The seaweed was studied at three different maturation stages, which were Stage I (30–40 days), Stage II (40–50 days) and Stage III (60 days and above). Morphology, physicochemical properties, and bioactive compounds were evaluated to establish the postharvest quality. There was a significant difference in postharvest quality in terms of morphology and physicochemical properties at different maturation stages. Morphologically, the thallus of seaweed was straight and cylindrical. The secondary and tertiary branches were longer than the primary branches during stages II and III. During the whole development stage, *G. changii* was found in a pH range of 6.23–7.04, ash content (28.53–38.93%), (3.70–23.45 µg/g/100 ml) ascorbic acid, and (0.21–0.23 %) titratable acidity. Total phenolic content (TPC) was significantly highest at Stage III, whereas total flavonoid content (TFC) and the antioxidant activities measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays remained unaffected by the maturation stage. This study revealed that different maturation stages affect the postharvest quality of seaweed. This information is valuable to guide the establishment of the harvesting standard for local seaweed production.

Keywords: Antioxidant activity, *Gracilaria changii*, growth stages, morphology, nutritional composition

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INTRODUCTION

Gracilaria changii, also known as red seaweed, is an agarophyte that contains abundant nutrients. It can be consumed fresh or utilised in the phycocolloids industry. The succulent thallus contains a high amount of polysaccharides, minerals, vitamins, and some bioactive substances such as proteins, lipids, and polyphenols that demonstrate cytotoxic, antioxidant, anticancer, antibacterial, anti-viral, antifungal, antidiabetic, and anti-inflammatory activities (Bouzenad et al., 2024). These characteristics have enabled seaweeds to be employed as ingredients in functional food and nutraceutical applications (Chan & Matanjun, 2017). The chemical and nutritional profiles of seaweeds are subject to variation depending on species, habitats, growth stages, and environmental conditions. For instance, *Ulva lactuca* cultivated in the Israeli seashore has the maximum amount of lipid during summer compared to winter (Gnayem et al., 2024). The composition and nutritional content of *Sargassum fusiforme* vary according to different maturation stages, in which the seedling and early growth stages are recommended for preparing high-protein foods and health supplements. The chemical profile and antioxidant activity of *Caulerpa lentillifera* differ accordingly to different sites and environmental conditions in Thailand (Koodkaew et al., 2024).

Previously, the chemical composition of wild-grown *G. changii* at Santubong, Sarawak, Malaysia, was investigated by Chan & Matanjun (2017). This species was high in dietary fibre (64.74 ± 0.82%), low in fat (0.30 ± 0.02%) and a total amino acid of 91.90 ± 7.70%. Nevertheless, the composition at different maturation stages has not yet been investigated. The genus Gracilaria follows a triphasic life cycle known as the polysiphonia (Pereira & Yarish, 2008). Post fertilisation, a distinct cystocarp that evolved in a hemispherical shape will swell throughout the surface of the female gametophyte's thallus (Baweja et al., 2016). The cystocarp releases the diploid carpospores (2n), forming the tetrasporophyte (2n). The tetrasporophyte (2n) generates haploid tetraspores (n) through meiosis within cortical sporangia. These tetrasporangia eventually produce tetraspores, germinating into male and female gametophytes, forming into mature thallus, thus completing their whole life cycle (Baweja et al., 2016).

The growing thallus is harvested at different maturation stages. For the current cultivation practices, the farmers use the vegetative thalli to obtain rejuvenated juveniles that grow into a mature thallus (Pereira & Yarish, 2008). The cultivation date is counted once the process of putting thalli onto the ropes and lines is completed. Currently, there are three main stages used to refer to the maturity level of seaweed, involving: Stage I (30–40 days), Stage II (40–50 days) and Stage III (60 days above) (Laman Alam Jaya Sdn Bhd, 2024). The current agricultural practice of the maturation stage is the reference for the seaweed harvesting stage. It is believed that different maturity stages will produce different chemical and nutritional compositions that affect the final quality of seaweed used in the industry.

Seaweed was acknowledged as an important marine crop in Malaysia under the 10th Malaysia Development Plan through the National Key Economic Areas (NKEA) and Entry Point Project (EPP 3). The plan aimed to boost seaweed production from 13,500 metric tonnes in 2010 to a projected 150,000 metric tonnes by 2020 (Chan & Matanjun, 2017). In 2023, it was reported to increase to 225,048 metric tonnes valued at RM100 million. Recently, *G. changii* has gained popularity in Peninsular Malaysia, where it has been cultivated commercially in Muar, Johor. Since *G. changii* is a new commercialised species, knowledge of composition and nutritional contents at different maturation stages is still limited. This knowledge gap necessitates the current study to be undertaken. Knowing the composition difference between different maturity stages will lead to establishing a harvesting standard that can guide producers and marketers in deciding the market price by providing a common language for marketers and producers. This harvesting standard is expected to ensure that seaweed is harvested at the right time, providing optimal nutritional content and quality for industrial applications.

MATERIALS AND METHODS

Reagents and Apparatus

The chemicals used in this study were sodium hydroxide (QRec, Malaysia), phenolphthalein (Sigma Aldrich, Malaysia), malic acid (Sigma Aldrich, Malaysia), 2,6-dichloroindophenol (Sigma Aldrich, Malaysia), metaphosphoric acid (Sigma Aldrich, Malaysia), acetic acid (Sigma Aldrich, Malaysia), ascorbic acid (Sigma Aldrich, Malaysia), Folin-ciocalteu reagent (Merck, Germany), gallic acid (Merck, Germany), sodium carbonate (Merck, Germany), methanol (QRec, Malaysia), aluminium trichloride (QRec, Malaysia), ethanol (Sigma Aldrich, Malaysia), potassium acetate (Merck, Germany), quercetin (Sigma Aldrich, Malaysia), 2,2-diphenyl-1-picryhydrazyl (DPPH) (Merck, Germany), 2-2'-azino-bis (3-ethylbenzothiozoline-6-sulfonic acid) (ABTS) (Merck, Germany), 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (Merck, Germany), and potassium persulfate (Sigma Aldrich, Malaysia). They were analytical grades from procured sources.

The apparatus and instruments used in this study were a scanning electron microscope (SEM) (Coxem, South Korea), quorum sputter coater (QuorumTech, UK), high duty blender (XUETAO, China), muffle furnace (Carbolite, UK), calorimeter (PCE Instruments, Germany), texture analyser (Stable Micro System, UK), UV-VIS spectrophotometer (PG Instruments, UK), water bath (Memmert, Germany), pH meter (Trans instruments, Singapore), vortex mixer (Labmart, Malaysia), pipette (Eppendorf, Germany) and drying oven (Memmert, U40, Germany).

Collection of *G. changii* and Sample Preparation

The *G. changii* specimens were freshly collected from Laman Alam Jaya, a local Parit Bulat, Muar, Johor farm. The seaweed was raised using filtered seaweed water in a man-

made pond using standard seaweed cultivation practices. Seaweed at three harvesting stages: Stage I (30–40 days), Stage II (40–50 days), and Stage III (60 days above), was harvested randomly from nine different plots, with three biological replications representing one maturity level. Upon harvesting, the seaweed is thoroughly washed using filtered seawater to eliminate salt, debris, epiphytes, and foreign matter. The freshly cleansed seaweed samples were immediately subjected to morphological examination, including physical, colour assessment and texture analysis. The cellular structure of seaweed was also included in the morphological assessment. Post morphological assessment, the seaweed was subjected to dehydrator drying at 50 °C until constant weight was achieved. Small portions of dried thallus were subjected to cellular study using a scanning electron microscope, while the rest was processed into a powdered form. The powdered samples were subjected to physicochemical and antioxidant analysis. The dried thallus was pulverised into the powdered form using a Waring blender, passed through a sieve with a mesh size of 0.85 mm (pore size) and kept in an airtight bag in a freezer (-40 °C) until further analysis.

Morphology Studies

Physical Features

All individuals' thalli were photographed at the time of collection. The photographs were used to quantify the number of adventitious branch patterns.

Colour

The colour of the thallus was measured by using a handheld portable digital calorimeter (PCE-CSM 1, Germany). Three random spots at each sample were taken for the readings of L* (lightness), a* (green/red) and b* (blue/yellow) parameters. The C* (chroma) and h° (hue) parameters were calculated using a formula mentioned by Temocico et al. (2019).

Texture Analysis

A single-arm texture analyser (Stable Micro Systems Ltd., Godalming, UK) was used to evaluate the tensile strength (TS) and elongation at break (EAB) of the fresh thallus. The data were analysed using Texture Exponent software (Godalming, UK). Seaweed samples were uniformly cut to a length of 5 cm and loaded with a 30 kg load cell, following the ASTM D882-02 procedure. The tensile test was conducted with an initial grip separation (Lo) of 60 mm and a crosshead speed of 100 mm/min. The TS and EAB values were calculated from the tensile force and the length of the specimen after fracture, following a modified method of Hamdan et al. (2021).

Scanning Electron Microscope (SEM)

The surface morphology of the dried thallus was observed using an SEM. The sample was placed onto the SEM holder with double-sided electrically conductive carbon adhesive tape, and the specimens were then coated with a thin gold-palladium layer. The samples were observed using 5000 x magnifications (Khalil et al., 2016).

Physicochemical Properties Evaluation

Titratable Acidity (TA) and pH

TA was measured by acid-base titration, and pH using a pH electrode meter. Titration was performed using 10 mL of seaweed filtrate pre-added with two drops of phenolphthalein. Then, the solution was titrated against sodium hydroxide until a light pink solution appeared for 15 s. The result was expressed as a percentage of malic acid (Nor et al., 2023).

Ascorbic Acid (AA)

The ascorbic acid was determined using titration with 2,6-dichloroindophenol as an indicator. In a 50 mL Erlenmeyer flask, around 5 mL of metaphosphoric acid-acetic acid solution and 2 mL of the sample were carefully pipetted. The burette was loaded with 2,6-dichloroindophenol dye solution and titrated against each sample until a light rose-pink colour appeared within 5 s. Throughout the titration, the flask was swirled continuously. The initial and final readings of the burette were recorded, and the result was quantified as a µg/100 ml ascorbic acid (Nor et al., 2023).

Ash Content

The ash content was calculated using the muffle furnace method. The empty crucibles were dried at 110 °C for 3 h, followed by cooling in a desiccator before weighing. About 1 g of the sample was transferred to a silica crucible and heated in a muffle furnace at 550 °C for 3 h. Following incineration, the remaining content was cooled and weighed. The result was expressed as a percentage (Zakaria et al., 2018).

Antioxidant Activity Analysis

Sample Preparation and Extraction of Bioactive Compound

Approximately 1 g of powdered seaweed was dispersed in 5 mL of 95 % ethanol in a conical flask and heated in a water bath at 35 °C for 30 min. Centrifugation of the sample was done at 4200 × *g* for 10 min. Finally, the supernatant was filtered and collected for quantification (Nor et al., 2023).

Total Phenolic Content (TPC)

A 0.5 mL aliquot of the extracts was added to 2.5 mL of Folin-Ciocalteu reagent in a test tube. The mixture was then diluted tenfold with distilled water, and 2 mL of sodium carbonate was added. The sample was incubated for 30 min in the dark, with the test tube wrapped in aluminium foil. Following incubation, the absorbance was recorded at 765 nm using a UV-vis spectrophotometer. The total phenolic content (TPC) was quantified and reported as milligrams of gallic acid equivalents (GAE) per gram of sample (mg GAE/g) (Nor et al., 2023).

Total Flavonoid Content (TFC)

A 0.5 mL sample was combined with 2.8 mL of distilled water in a test tube wrapped with aluminium foil. Following that, 0.1 mL of 10 % aluminium trichloride and 0.1 mL of potassium acetate were added and thoroughly mixed. The mixture was incubated for 30 min and measured at 415 nm using a UV-vis spectrophotometer. The result was expressed as milligrams of quercetin equivalent (QE) per gram of sample (mg QE/g) (Nor et al., 2023).

2-2-diphenyl-1-picrylhydrazyl Radical Scavenging (DPPH) Assay

A 0.3 mM DPPH stock solution was freshly prepared on the analysis day. The analysis was conducted in the dark, with the test tubes wrapped in aluminium foil. Approximately 1 mL of the sample was added to each test tube and mixed with 3 mL of DPPH. The mixture was thoroughly vortexed before being incubated for 30 min. Absorbance was then measured at 515 nm using a UV-vis spectrophotometer. The DPPH stock solution was the negative control, while ethanol was the blank. Both the sample and the negative control were analysed in triplicate. Standard curve was done using a Trolox solution, and the result was expressed as µmol Trolox equivalent per 100 g of dried sample (µmol TE/100 g) (Nor et al., 2023).

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) Assay

ABTS stock solution, consisting of 7 mM ABTS in water and 2.45 mM potassium persulfate, was prepared to perform the ABTS assay. This solution was allowed to stand in the dark at room temperature for 16 h. After the incubation period, the ABTS solution was diluted with ethanol to achieve an absorbance of 760 nm. Once the desired absorbance was reached, 150 µL of the extract was mixed with 3,850 µL of the ABTS solution. The mixture was immediately analysed by measuring absorbance at 734 nm. The results were compared to the Trolox standard curve and expressed as µmol of Trolox equivalent (TE) per 100 g of dried sample (µmol TE/100 g) (Nor et al., 2023).

Statistical Analysis

All analyses were conducted in triplicate, and the results were reported as mean ± standard deviation. One-way analysis of variance (ANOVA) with Minitab Software version 21, at a significance level of α=0.05, was used to evaluate significant differences between means. Tukey's test was employed to compare all treatments.

RESULTS AND DISCUSSION

Morphology Studies

Physical Features

The *G. changii*'s main body is known as the thallus. The thallus exhibited a cylindrical and straight shape, with constrictions at the base of the branches, swelling in the middle and tapering towards the ends (Figure 1). Branches formed sporadically, with the tips of

Figure 1. The branching pattern of freshly harvested seaweed from three random ponds at three different maturation stages. Stage followed by different letter and different number indicates different stages and different cultivation plots; (a1–a3) Stage I at 30–40 days; (b1-b3) Stage II at 40–50 days; and (c1–c3) Stage III at 60 days and above

secondary branches dividing into two short branchlets. The tertiary branches emerge along the secondary branch. During Stage I, it shows that the branching pattern of the thallus was shorter within a 5 cm length than secondary branches at 10–15 cm (Figure a1–a3). At stage 2 (Figure b1–b3) and stage 3 (Figure c1–c3), the primary thallus of the seaweed grew to 10 cm, while the secondary branches extended to 20–25 cm. The changes in seaweed morphology positively show that the seaweed is undergoing a growth and development process due to photosynthesis and nutrient accumulation responses (Roleda & Hurd, 2019).

Colour

Colour can be denoted as a prominent postharvest standard in rating the quality of fresh produce (Nor et al., 2023). This study shows that the harvesting stage impacts the colour of fresh *G. changii* (Table 1). The lightness (L*) values of stages 1 and II were similar. Then, it became significantly lighter ($p<0.05$) when the seaweed reached Stage III. For the a* value, stages 1 and II had negative values, indicating that the colour tends towards greenness. Later, when it reached Stage III, it changed significantly to 0.39, indicating the seaweed becoming redder in colour. The colour thallus was more likely to be brown-red as all stages possessed negative b*. The blueness (-b*) of *G. changii* decreased ($p>0.05$) along with the increase in the maturation stage. Rating on the colour intensity, the chroma (c*) value decreased progressively ($p>0.05$) from Stages I to III. The hue angle shifted towards brownness during Stage III. Rating on the colour quality, *G. changii* in Stage III, produced a more intense red brown colour during its late maturation stages. This finding aligns with studies of other seaweeds, such as Ulva fasciata, that shift towards red hues in a response to age development, which have been attributed to carotenoid accumulation and degeneration of chlorophyll (Beer et al., 2000). Research conducted by Chan and Matanjun (2017) has quantified that *G. changii* contains multiple tetrapyrrole pigments such as chlorophyll, carotenoid, xanthophylls, zeaxanthin, lutein and antheraxanthin. These pigments may change at various stages of maturation, as indicated by the corresponding shifts in colour.

Table 1
The effect of different maturation stages of G. changii *on colour (L*, a*, b*, c* and h°)*

Factors	L*	a*	b*	c*	h°
Stage I	24.68±0.28^{a}	-1.30±0.12^{a}	-5.30±0.49^{a}	5.46±0.50^{a}	256.13±0.32^{a}
Stage II	24.77±0.25^{a}	-1.07±0.21^{a}	-4.91±0.32^{ab}	5.03±0.35^{ab}	257.74±1.77^{ab}
Stage III	27.97±0.85^{b}	0.39±0.98^{b}	-3.56±0.88^{b}	3.76±0.70^{b}	278.12±14.64^{b}

Note. The result was expressed in means ± standard deviation ($n = 27$), followed by the same letter in the same column, which was not significantly different at ($p > 0.05$) according to Tukey's post-hoc tests

Texture Properties

Texture is a crucial factor influencing the acceptability of fresh and processed seaweeds in the industry. The tensile strength values of fresh *G. changii* were within the range of 10.00–33.85 MPa (Table 1). These values were lower than those of other Gracilaria species, such as *G. salicornia* (68.24 MPa) and *G. edulis* (85.05 MPa) (Phang, 2006). The difference in tensile strength could be due to the polysaccharide composition that builds up the main thallus structure (Rhein-Knudsen et al., 2017). For instance, *G. salicornia* contain 18%–30% agar (Buriyo & Kivaisi, 2004), *G. edulis* contain 23%–28% agar and *G. changii* contain 18%–22% agar (Lee et al., 2016). A thallus with a higher agar content, particularly rich in agarose, builds a stronger and more rigid thallus to resist mechanical stress from water currents and waves (Pica et al., 2024). Matured seaweed during stages II and III exhibited significantly higher tensile strength (29.79±2.85 MPa and 33.85±10.12 MPa) compared to Stage I (10.05±5.20 MPa). Additionally, the elongation (%) of the seaweed correlated with the tensile strength (MPa), showing that increased maturation time results in greater elasticity. This is likely due to the maturation process, during which the seaweed strengthens with age as its cell walls thicken by accumulating hydrocolloids such as agar, carrageenan, and alginates (Shao & Duan, 2022). Integrating these hydrocolloids into the cell walls of the thallus may form a composite material that enhances the seaweed's strength and elasticity (Norziah & Karim, 2006). In the global market, the fresh seaweed that is fibrous, sticky and tactile is highly appreciated by Western and Japanese consumers (Figueroa et al., 2022). From the rating on the consumer perspective, when the seaweeds are consumed fresh, consumers would appreciate Stage II and III more due to greater tensile strength and higher elongation (%), making the texture springier and chewier.

Scanning Electron Microscope (SEM)

The SEM micrograph in Figure 2 strengthens the texture analysis conducted in this study. Clearly, the thallus surface from all stages was dominated by tear ridges and irregular round pores known as dimples. This surface would cause the thallus to have a brittle and elastic in texture (Wang et al., 2022). In Stage I, I had a greater number of dimples on its surface than in the other stages, explaining its low ductility and low stretchability (Table 2). In addition, this study also displays that the dimple size of Stage I (Figure 2a) seaweed was smaller compared to the Stages II (Figure 2b) and III (Figure 2c). Wichard et al. (2015) explained that mitotic spots distributed across the thallus are responsible for active cell division, resulting in cell size and number variations during different developmental phases. During Stage I, the cells of the thallus could actively undergo mitosis. The small and high number of dimples in Stage I could be attributable to active cell division, the mitotic process (Figure 2a).

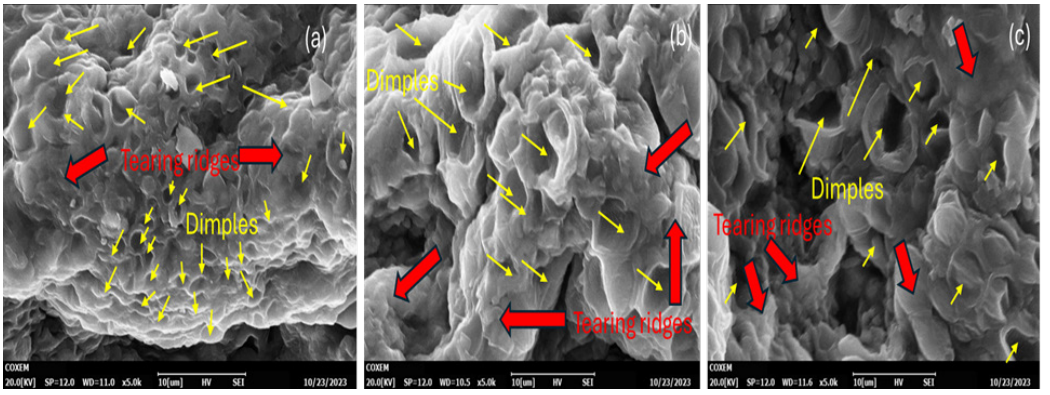

Figure 2. The surface of thallus G. changii at different stages of maturation: (a) Stage I at 30–40 days; (b) Stage II at 40–50 days; (c) Stage III at 60 days and above in 5000x magnification under SEM. The yellow arrows show the visible pores known as dimples, while the red arrows show tearing ridges on the surface of the thallus

However, cell division may cease upon reaching stages II and III, allowing the expansion process to occur. A few dimples characterise this, but their size increases (Figure 2b, 2c). Tear ridges is known as fold that running along thallus (Wang et al., 2022). Tear ridges are functional in adding mechanical strength to the thallus by preventing it from tearing under high water pressure (Pica et al., 2024). Tear ridges were more noticeable in stages II and III (Figure 2b, 2c). Tear ridges caused greater stretchability and higher tensile strength, as confirmed by the texture analysis, which showed increased tensile strength values in stages II and III (Table 2).

Table 2
The effect of different maturation stages of G. changii *on the texture profile*

Factors	Tensile strength (MPa)	Elongation (%)
Stage I	10.00±5.20[a]	39.11±8.57[a]
Stage II	29.79±2.85[b]	51.17±2.95[b]
Stage III	33.85±10.12[b]	50.79±2.32[b]

Note. The result was expressed in means ± standard deviation ($n = 27$), followed by the same letter in the same column, which was not significantly different at ($p > 0.05$) according to Tukey's post-hoc tests

Physicochemical Properties

Table 3 presents the physicochemical data of *G. changii* seaweed at different maturation stages, including pH, ascorbic acid (AA), titratable acidity (TA), and ash content. The pH of *G. changii* varied during the maturation stages, with the lowest pH (6.23) in Stage I and the highest pH (7.04) in Stage II, while the pH of Stage III was 6.94. Meanwhile, the titratable acidity (TA) showed no significant difference across different maturation stages.

Table 3
The content of ash, pH, ascorbic acid and titratable acidity of G. changii *at different maturation stages*

Factor	pH	Ascorbic acid (µg/100ml)	Titratable acidity (% malic acid)	Ash content (%)
Stage I	6.23 ±0.48[a]	23.45±5.31[a]	0.20±0.06[a]	38.93±3.10[a]
Stage II	7.04 ± 0.22[b]	13.69±10.25[ab]	0.23±0.08[a]	34.50±1.32[ab]
Stage III	6.94 ± 0.31[b]	3.70±3.90[b]	0.21±0.07[a]	28.53±4.15[b]

Note. The result was expressed in means ± standard deviation (n=27), followed by the same letter in the same column, which was not significantly different (p>0.05) according to Tukey's post-hoc tests

The ascorbic acid (AA) content of *G. changii* was found to be lower than that of wild *G. changii* grown in Santubong, Sarawak (2.51 mg/100 g) and higher than that of other brown and green seaweed species cultivated in Indonesia (Chan & Matanjun, 2017; Bocanegra et al., 2009). The current study reveals that the maturation stage influences antioxidant activity (AA), with values significantly decreasing from Stage I (23.45 µg/100 ml) to Stage II (13.69±10.25 µg/100 ml), and further to Stage III (3.70 µg/100 ml). Ascorbic acid is normally present in seaweed in all cell compartments, including the cell wall. It normally acts as an enzyme cofactor for controlling cell growth by aiding in the biosynthesis of hydrocolloid (Smirnoff et al., 2000). In Stage I, the ascorbic acid could be abundant because of the need to aid in the biosynthesis of hydrocolloid in the cell wall of the thallus (Smirnoff & Wheeler, 2024).

When the seaweed reached Stage II and III, the seaweed could focus more on structure development as more tear ridges were on the thallus surface (Figure 2b, 2c). Deposition of hydrocolloid could happen during this process, as evidenced by the increase in tensile strength and elongation percentage (Table 2). As a result, the demand for ascorbic acid in metabolic pathways reduced, leading to its gradual decline (Table 3). In industry, seaweed is normally added to food products to develop new products and nutraceuticals (Chandrasekhar et al., 2023). The pH, TA, and AA values indicated that seaweed had a natural pH and low acidity, making it suitable for incorporation into a wide range of food products. The characteristics would allow it to blend effortlessly with other ingredients without affecting the overall taste or stability of the final product.

The ash content is a key indicator of seaweed's nutritional quality, reflecting the presence of minerals and trace elements. The ash content of *G. domingensis* was 23.8%, while *G. birdiae* was 22.5% (Gressler et al., 2010). The lower amount of ash content was found in Gracilaria, ranging from 5% to 6% (Purwaningsih et al., 2024). This study observed that the ash content of *G. changii* decreased (p>0.05) progressively from Stage I to III, dropping from 38.93% in Stage I to 34.50% in Stage II and further to 28.53% in Stage III. Seaweed harvested during Stage I could have more mineral and trace elements due to a significant ash content (%). Chan and Matanjun (2017) reported that K and Na are

the most abundant minerals in *G. changii*, with K being eight times higher than Na. The presence of minerals in seaweed may result from the ability of its surface to absorb elements from the surrounding seawater (Kumar et al., 2011). The thallus surface can undergo ion exchange, facilitating the uptake of minerals from the marine environment into the cell cytoplasm (Roleda & Hurd, 2019). The decline in mineral content is likely due to a reduced ability of the thallus surface to absorb minerals from its surroundings, as reflected by the distinct changes in thallus morphology (Figure 1) and its surface characteristics observed from stages I to III (Figure 2).

Antioxidant Activity

Phenols and flavonoids are the most common bioactive compounds of land plants and seaweed, responsible for antioxidant activity (Sadeghi et al., 2024). The bioactive compounds present in seaweed can be exploited for functional ingredients to enhance the nutritional, textural, and sensory attributes of food products. TPC and TFC quantified phenolic and flavonoid compounds, respectively. The TPC measures the total amount of phenolic compounds in a sample, while TFC measures the total amount of flavonoids, a subclass of phenolic compounds (Nor et al., 2023). Parallelly, the antioxidant capacity of seaweed extract was assessed using DPPH radical scavenging activity and ABTS radical scavenging methods (Table 4). DPPH and ABTS assays are common techniques used to measure the antioxidant potential of a sample. The DPPH assay involves the generation of the DPPH• radical, a stable nitrogen-centred free radical characterised by its deep purple colour and maximum absorbance at 517 nm. In contrast, the ABTS assay produces the ABTS•+ radical cation, a blue-green chromophore with a strong absorbance peak at 734 nm. Flavonoids and phenols containing hydrogen-donating groups reduce DPPH and ABTS solutions by forming non-radical species, demonstrating their antioxidant potential (Ramli et al., 2023).

Table 4
Antioxidant properties of extracts of G. changii *quantified by TPC, TFC, ABTS and DPPH at different maturation stages*

Stages	TPC (mg GAE/g)	TFC (mg QCE/g)	ABTS (µmol TE/100g)	DPPH (µmol TE/100g)
Stage I	1.16±0.00016[a]	0.279±0.00417[a]	26.86±7.10[a]	11.84±0.086[a]
Stage II	1.139±0.000047[a]	0.264±0.00236[a]	24.53±5.11[a]	11.87±0.078[a]
Stage III	11.015±0.0000597[b]	0.296±0.00171[a]	24.63±3.83[a]	11.78±0.188[a]

Note. The result was expressed in means ± standard deviation (n=27), followed by the same letter in the same column, and was not significantly different (p>0.05) according to Tukey's post hoc tests. GAE=Gallic acid equivalent, QCE=Quercetin equivalent, DPPH =2,2-diphenyl-1-picryl-hydrazyl, ABTS = 2-2'-azino-bis (3-ethylbenzothiozoline-6-sulfonic acid)

The TPC values of the seaweed extracts ranged from 1.16 to 11.015 mg GAE/g, with Stage III exhibiting the highest TPC value. Meanwhile, the TFC contents were not significantly (p>0.005) different across different maturation stages, with values ranging from 0.264 to 0.279 mg QCE/g. The value of cultivated *G. changii* obtained from this study was higher than the TPC (0.1848–0.2365 mg GAE/g) and TFC (0.003–0.0032 mg CE/g) values of selected seaweed species grown in Sabah, including *Sargassum polycystum, Eucheuma denticulatum* and *Kappaphycus alvarezii* (Fu et al., 2016). Similarly, the ABTS (24.63–26.86 µmol TE/100g) and DPPH (11.84–11.87 µmol TE/100g) values for *G. changii* were higher than those of the species cultivated in Sabah, where DPPH and ABTS were reported to range from 0.3–3.0 µmol TEAC/100 g dried sample and 0.01–0.4 µmol TEAC/100 g, respectively (Fu et al., 2016). In the present study, TPC did not seem to significantly contribute to antioxidant activity, as TPC increased drastically (p<0.05) from Stage II to Stage III, while DPPH and ABTS activities remained stable (p>0.05). This trend parallels the antioxidant activity of the stem, peel and flesh of dragon fruit, which the TPC has not contributed to (Fidrianny et al., 2018; Nurliyana et al., 2010).

Flavonoids could contribute to antioxidant activity, as the similar trends observed in TFC, ABTS, and DPPH assays suggested. Hydrocolloids that build the seaweed thallus could potentially be the primary contributors to its antioxidant activity, which should be determined in the future. Several studies have demonstrated that hydrocolloids exhibit antioxidant properties due to their carbohydrate content, which contains hydroxyl groups capable of donating electrons or hydrogen to neutralise free radicals (Luo et al., 2024). Furthermore, sulphated polysaccharides enhance this antioxidant activity by increasing electron density and improving the molecule's capacity to donate electrons or hydrogen for free radical neutralisation (Hu et al., 2024). Since the antioxidant activity in all stages is not significantly different, Stage II could be pointed out as the optimal Stage for seaweed harvesting, considering other physicochemical and textural profiles.

CONCLUSION

The physical and chemical properties of *G. changii* have been shown to be influenced by the stages of maturation. The thallus of *G. changii* seaweed was cylindrical, straight and branched in all stages of development. The physical feature of young seaweed l (Stage I) produced light brown, possessed weak tensile strength, and was less elastic than stages II and III. This study suggests that Stage II is the optimal harvesting stage for fresh use and processing for various applications in the food and nutraceutical industries. In Stage II, the *G. changii* was red-brown as indicated by CIELAB colour. The texture of the seaweed stabilised during Stage II, resulting in strong mechanical properties that give it springy and tactile properties. The physicochemical properties of stages II *G. changii* can be described as having a natural pH, low acidity and an acceptable amount of ascorbic acid and ash

content. In addition, the contents of bioactive compounds were at a considerable amount as indicated by TPC, TFC, ABTS and DPPH, which could work as free radical neutralisers and reduce oxidative stress. In developing nutraceutical products, this antioxidant activity is a major criterion required for food ingredient incorporation. This study provides fundamental knowledge for establishing a postharvest standard for fresh quality seaweed to be used as food and functional ingredients in the industry.

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REFERENCES

Baweja, P., Kumar, S., Sahoo, D., & Levine, I. (2016). Biology of seaweeds. In J. Fleurence & I. Levine (Eds.), *Seaweed in health and disease prevention* (pp. 41–106). Elsevier. https://doi.org/10.1016/B978-0-12-802772-1.00003-8

Beer, S., Larsson, C., Poryan, O., & Axelsson, L. (2000). Photosynthetic rates of Ulva (Chlorophyta) measured by pulse amplitude modulated (PAM) fluorometry. *European Journal of Phycology*, *35*(1), 69–74. https://doi.org/10.1080/09670260010001735641

Bocanegra, A., Bastida, S., Benedí, J., Ródenas, S., & Sánchez-Muniz, F. J. (2009). Characteristics and nutritional and cardiovascular-health properties of seaweeds. *Journal of Medicinal Food*, *12*(2), 236–258. https://doi.org/10.1089/jmf.2008.0151

Bouzenad, N., Ammouchi, N., Chaib, N., Messaoudi, M., Bousabaa, W., Bensouici, C., Sawicka, B., Atanassova, M., Ahmad, S. F., & Zahnit, W. (2024). Exploring bioactive components and assessing antioxidant and antibacterial activities in five seaweed extracts from the Northeastern coast of Algeria. *Marine Drugs*, *22*, 1-26. https://doi.org/10.3390/md22060273

Buriyo, A., & Kivaisi, A. (2004). Standing stock, agar yield and properties of *Gracilaria salicornia* harvested along the Tanzanian Coast. *Western Indian Ocean Journal of Marine Science*, *2*(2), 171-178. https://doi.org/10.4314/wiojms.v2i2.28433

Chan, P. T., & Matanjun, P. (2017). Chemical composition and physicochemical properties of tropical red seaweed, *Gracilaria changii. Food Chemistry*, *221*, 302–310. https://doi.org/10.1016/j.foodchem.2016.10.066

Chandrasekhar, T., Riazunnisa, K., Vijaya Lakshmi, D., Anu Prasanna, V., & Veera Bramhachari, P. (2023). Exploration of bioactive functional molecules from marine algae: Challenges and applications in nutraceuticals. In P. V. Bramhachari & C. V. Berde (Eds.), *Marine bioactive molecules for biomedical and pharmacotherapeutic applications* (pp. 187–196). Springer. https://doi.org/10.1007/978-981-99-6770-4_10

Fidrianny, I., Suhendy, H., & Insanu, M. (2018). Correlation of phytochemical content with antioxidant potential of various sweet potato (*Ipomoea batatas*) in West Java, Indonesia. *Asian Pacific Journal of Tropical Biomedicine*, *7*(1), 25–30. https://doi.org/10.4103/2221-1691.221131

Figueroa, V., Bunger, A., Ortiz, J., & Aguilera, J. M. (2022). Sensory descriptors for three edible Chilean seaweeds and their relations to umami components and instrumental texture. *Journal of Applied Phycology*, *34*(6), 3141–3156. https://doi.org/10.1007/s10811-022-02848-2

Fu, C. W. F., Ho, C. W., Yong, W. T. L., Abas, F., Tan, T. B., & Tan, C. P. (2016). Extraction of phenolic antioxidants from four selected seaweeds obtained from Sabah. *International Food Research Journal*, *23*(6), 2363–2369. https://doi.org/10.7287/peerj.preprints.1249v1

Gnayem, N., Unis, R., Gnaim, R., Chemodanov, A., Israel, Á., Gnaim, J. M., & Golberg, A. (2024). Seasonal and culture period variations in the lipid and fatty acid content of *Ulva lactuca* cultivated in Mikhmoret onshore (Israel). *Botanica Marina*, *67*(2), 101-114. https://doi.org/10.1515/bot-2023-0027

Gressler, V., Yokoya, N. S., Fujii, M. T., Colepicolo, P., Mancini Filho, J., Torres, R. P., & Pinto, E. (2010). Lipid, fatty acid, protein, amino acid and ash contents in four Brazilian red algae species. *Food Chemistry*, *120*(2), 585-590. https://doi.org/10.1016/j.foodchem.2009.10.028

Hamdan, M. A., Khairatun Najwa, M. A., Jose, R., Martin, D., & Adam, F. (2021). Tuning mechanical properties of seaweeds for hard capsules: A step forward for a sustainable drug delivery medium. *Food Hydrocolloids for Health*, *1*, 1-10. https://doi.org/10.1016/j.fhfh.2021.100023

Hu, Y., Zheng, Z., Zhai, D., Liang, P., Wang, Z., Jiang, C. P., Guo, Y., Chen, H., Shen, C., Wu, Y., Liu, L., Yi, Y., Zhu, H., & Liu, Q. (2024). A mini-review: Exploring the application prospects of the three major rules in the field of antioxidants. *Journal of Molecular Structure*, *1304*, 137746. https://doi.org/10.1016/j.molstruc.2024.137746

Khalil, A. H. P. S., Tye, Y. Y., Chow, S. T., Saurabh, C. K., Paridah, Md. T., Dungani, R., & Syakir, M. I. (2016). Cellulosic pulp fiber as reinforcement materials in seaweed-based film. *BioResources*, *12*(1), 29-42. https://doi.org/10.15376/biores.12.1.29-42

Koodkaew, I., Pitakwongsaporn, S., Jarussophon, N., & Wichachucherd, B. (2024). Chemical profile, antioxidant activity and α-glucosidase inhibition of sea grape *Caulerpa lentillifera* collected from different sites in Thailand. *Trends in Sciences*, *21*(4), 1-15. https://doi.org/10.48048/tis.2024.7520

Kumar, M., Kumari, P., Trivedi, N., Shukla, M. K., Gupta, V., Reddy, C. R. K., & Jha, B. (2011). Minerals, PUFAs and antioxidant properties of some tropical seaweeds from Saurashtra coast of India. *Journal of Applied Phycology*, *23*(5), 797–810. https://doi.org/10.1007/s10811-010-9578-7

Laman Alam Jaya Sdn Bhd. (2023, December 5). *Welcome to Laman Alam Jaya Sdn Bhd | First Gracilaria seaweed farm in Peninsular Malaysia* [Video]. YouTube. https://www.youtube.com/watch?v=L3uBPVr3wWI

Lee, W. K., Lim, P. E., Phang, S. M., Namasivayam, P., & Ho, C. L. (2016). Agar properties of *Gracilaria* species (Gracilariaceae, Rhodophyta) collected from different natural habitats in Malaysia. *Regional Studies in Marine Science*, *7*, 123–128. https://doi.org/10.1016/j.rsma.2016.06.001

Luo, X., Wu, Y., Wang, S., Xiong, Z., Du, X., Zheng, M., & Zhu, Y. (2024). Frontier review of key reduction technologies and resource utilization of waste during the seaweed gel production process. *Blue Biotechnology*, *1*(12),1–14. https://doi.org/10.1186/s44315-024-00013-7

Nor, S. M., Ding, P., & Chun, T. J. (2023). Locule position and thawing duration affect postharvest quality of freshly cryo-frozen Musang King durian fruit. *Pertanika Journal of Tropical Agricultural Science*, *46*(2), 517–528. https://doi.org/10.47836/pjtas.46.2.09

Norziah, M. H., Foo, S. L., & Karim, A. A. (2006). Rheological studies on mixtures of agar *(Gracilaria changii*) and κ-carrageenan. *Food Hydrocolloids*, *20*(2-3), 204–217. https://doi.org/10.1016/j.foodhyd.2005.03.020

Nurliyana, R., Syed Zahir, I., Mustapha Suleiman, K., Aisyah, M. R., & Kamarul Rahim, K. (2010). Antioxidant study of pulps and peels of dragon fruits: A comparative study. *International Food Research Journal*, *17*(2), 367–375.

Pereira, R., & Yarish, C. (2008). Mass production of marine macroalgae. In S. E. Jørgensen & B. D. Fath (Eds.), *Encyclopedia of ecology* (pp. 2236–2247). Elsevier. https://doi.org/10.1016/B978-008045405-4.00066-5

Phang, S. M. (2006). Seaweed resources in Malaysia: Current status and future prospects. *Aquatic Ecosystem Health & Management*, *9*(2), 185–202. https://doi.org/10.1080/14634980600710576

Pica, M. L., Vitale, E., Donadio, R., Costanzo, G., Munari, M., Fabbrizzi, E., & Arena, C. (2024). Functional ecological traits in young and adult thalli of canopy-forming brown macroalga *Gongolaria barbata* (Phaeophyta) from a transitional water system. *PeerJ, 12*, e17959. https://doi.org/10.7717/peerj.17959

Purwaningsih, S., Ramadhan, W., Nabila, W. T., Deskawati, E., & Baabud, H. M. (2024). Dataset on the chemical composition and bioactive compound of estuarine seaweed Gracilaria from four different cultivation area in Java and Lombok Island, Indonesia. *Data in Brief*, *56*, 11085. https://doi.org/10.1016/j.dib.2024.110825

Ramli, N. I. anah, Abas, F., Ismail, I. S., Rukayadi, Y., & Nor, S. M. (2023). Determination of antioxidant activity, phenolic compounds, and toxicity of methanolic and ethanolic extracts of pink pigmented facultative methylotrophs (PPFM) bacteria pigment. *Pertanika Journal of Tropical Agricultural Science*, *46*(4), 1407–1421. https://doi.org/10.47836/pjtas.46.4.21

Rhein-Knudsen, N., Ale, M. T., Ajalloueian, F., Yu, L., & Meyer, A. S. (2017). Rheological properties of agar and carrageenan from Ghanaian red seaweeds. *Food Hydrocolloids, 63*, 50-58.

Roleda, M. Y., & Hurd, C. L. (2019). Seaweed nutrient physiology: Application of concepts to aquaculture and bioremediation. *Phycologia, 58*(5), 552–562. https://doi.org/10.1080/00318884.2019.1622920

Sadeghi, A., Rajabiyan, A., Nabizade, N., Meygoli Nezhad, N., & Zarei-Ahmady, A. (2024). Seaweed-derived phenolic compounds as diverse bioactive molecules: A review on identification, application, extraction and purification strategies. *International Journal of Biological Macromolecules*, *266*, 131147. https://doi.org/10.1016/j.ijbiomac.2024.131147

Shao, Z., & Duan, D. (2022). The cell wall polysaccharides biosynthesis in seaweeds: A molecular perspective. *Frontiers in Plant Science*, *13*, 1-7. https://doi.org/10.3389/fpls.2022.902823

Smirnoff, N., & Wheeler, G. L. (2000). Ascorbic acid in plants: Biosynthesis and function. *Critical Reviews in Plant Sciences*, *19*(4), 267-290. https://doi.org/10.1080/10409230008984166

Smirnoff, N., & Wheeler, G. L. (2024). The ascorbate biosynthesis pathway in plants is known, but there is a way to go with understanding control and functions. *Journal of Experimental Botany*, *75*(9), 2604–2630. https://doi.org/10.1093/jxb/erad505

Temocico, G., Sturzeanu, M., Ion, V., & Cristea, S. (2019). Evaluation of strawberry fruit quality for new selections and cultivars. *Romanian Biotechnological Letters*, *24*(4), 742-748. https://doi.org/10.25083/rbl/24.4/742.748

Wang, Y., Yang, Y., Qiao, L., Liu, Z., Xing, H., & Li, S. (2022). Enhanced mechanical properties of Al-4.5 wt.% Cu single crystals with seaweed morphology. *Journal of Materials Research and Technology*, *17*(2022), 2410–2418. https://doi.org/10.1016/j.jmrt.2022.02.011

Wichard, T., Charrier, B., Mineur, F., Bothwell, J. H., De Clerck, O., & Coates, J. C. (2015). The green seaweed Ulva: A model system to study morphogenesis. *Frontiers in Plant Science*, *6*, 1–8. https://doi.org/10.3389/fpls.2015.00072

Zakaria, Z., Farahin Mohamod, N., Mohd Noor Nuriah, S., Hussin, N., & Shahidan, N. (2018). Development and physicochemical properties of breadfruit (*Artocarpus altilis*) resistant starch bread. *Journal of Agrobiotechnology, 9*, 182–193.

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