Pertanika Journal of

TROPICAL AGRICULTURAL SCIENCE

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Pertanika Journal of Tropical Agricultural Science
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About the Journal

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 is a **quarterly** (*February*, *May*, *August*, and *November*) periodical that considers for publication original articles as per its scope. The journal publishes in **English** and it is open for submission by authors from all over the world.

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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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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.

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Pertanika Journal of Tropical Agricultural Science
Vol. 47 (1) Feb. 2024

Contents

Foreword

Welcome to the first issue of 2024 for the Pertanika Journal of Tropical Agricultural Science (PJTAS)!

PJTAS is an open-access journal for studies in Tropical Agicultural 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 20 articles; two review articles, two short communications, one case study and the rest are regular articles. The authors of these articles come from different countries namely Australia, Bangladesh, India, Indonesia, Malaysia, Nigeria and Thailand.

A regular article entitled "*Bengkuang* (*Pachyrhizus erosus*) Extract Has Significant Antidiabetic Activity in Diabetes Mellitus-induced Rats" evaluated the antidiabetic activity of *bengkuang* extracts administered via oral gavage into rats at 4 days post-induction of streptozotocin-nicotinamide-induced diabetes mellitus. The results showed that *bengkuang* extract has antidiabetic activity, as indicated by a significant decrease in blood sugar levels and recovery of damaged pancreatic cells in treated diabetic rats. The further details of this study are found on page 1.

Bee Lynn Chew and her teammates from Universiti Sains Malaysia assessed the effects of coconut water and banana homogenate in the regeneration of Meyer lemon. The shoots were treated in half-strength Murashige and Skoog media fortified with 2 mg/L 6-benzylaminopurine with varying concentrations of coconut water and banana homogenate without sucrose. This study revealed that the addition of coconut water and banana homogenate has the potential to induce the regeneration of new shoots, indicating that these organic additives exhibited growth-stimulating effects and might be employed as potential carbon sources in the *in vitro* shoot regeneration of Meyer lemon for micropropagation purposes. The detailed information of this article is available on page 147.

A selected article entitled "Survival Rate and Growth Performance of *Holothuria scabra* Towards Different Stocking Densities and Feeding with *Spirulina*" investigated the effects

of stocking densities and *Spirulina* feeding towards the survival of *H. scabra* by divided the juveniles of *H. scabra* into three different stocking densities and fed them with 1 g of dissolved *Spirulina* powder once on alternate days. The outcomes proved that the optimum initial stocking density is between 100 and 200 individuals for a 1-ton fibreglass tank with a 500 L water capacity. In addition, the *Spirulina* can be used as the main protein source as compared to other diets for juvenile *H. scabra*. Full information of this study is presented on page 191.

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.

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Antidiabetic Activity of *Bengkuang* (*Pachyrhizus erosus*) Extracts in Diabetes Mellitus-induced Rats

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ABSTRACT

Bengkuang (*Pachyrhizus erosus*) is in the pea family (Fabaceae) and is a native Mexican vine that produces an edible tuber called jicama or Mexican turnip in English. This study evaluated the antidiabetic activity of *bengkuang* extracts administered via oral gavage into rats at 4 days post-induction of streptozotocin-nicotinamide-induced diabetes mellitus. At 14 days post injection (daily) of extracts at 28 and 56 mg/200 g body weight (BW), blood glucose levels were significantly reduced ($p < 0.05$) from 277 ± 4 milligrams per deciliter (mg/dl) for the Diabetes Control Group to 182 ± 3 and 99 ± 55 mg/dl, respectively. The latter glucose level was comparable to that in the Antidiabetic Control Group rats (111 ± 63 mg/dl) injected with glibenclamide at 0.09 mg/200 g BW. The diabetes-induced rats also showed signs of cell recovery from diabetic-associated pancreatic tissue damage, supporting the efficacy of *bengkuang* treatment. According to phytochemical tests, the *bengkuang* extract contained various metabolites, mainly alkaloids and flavonoids, that may have been responsible for its antidiabetic activity. The results justify further studies on the underlying mechanisms of *bengkuang* antidiabetic activity.

Keywords: Antidiabetic, diabetes mellitus, *Pachyrhizus erosus*

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INTRODUCTION

According to Soelistijo (2015), diabetes mellitus is a group of metabolic disorders brought on by deficiencies in insulin

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secretion and/or function, which results in hyperglycemia. According to Dal Canto et al. (2019) and Majnarić et al. (2020), it is linked to coronary heart, cerebrovascular, and peripheral vascular disease, all of which can cause death or a lower quality of life. Chemical medication side effects in diabetic patients can include tremors, hypoglycemia, nausea, vomiting, and constipation (Putra et al., 2017). Additionally, it necessitates expensive and lengthy treatment. Due to its accessibility, safety, and tolerable side effects, traditional medicine is becoming increasingly popular (Khamees et al., 2020).

Bengkuang (*Pachyrhizus erosus*) is in the pea family (Fabaceae) and is a native Mexican vine that produces an edible tuber called jicama or Mexican turnip in English. Spanish introduced this plant to the Philippines in the 17th century, spreading to the rest of Asia. Nowadays, in Indonesia, *bengkuang* is well-known as a food and source of raw materials for beauty products (Li & Li, 2020). A recent preliminary study showed that administering a *bengkuang* extract dosage of 250 g (150 ml)/day for 7 consecutive days could lower blood glucose levels in diabetic patients (Safitri & Nurhayati, 2018). This initial finding served as the basis for this research. This study aims to evaluate the antidiabetic activity of *bengkuang* extracts in diabetes mellitus-inducible rats to confirm their efficacy as a possible justification for further research on the underlying mechanisms. It could lead to additional practical treatment for diabetes mellitus treatment in humans.

MATERIALS AND METHODS

Ethics Approval

This study obtained animal ethics approval from Dr. Moewardi General Hospital, Surakarta, Indonesia and has been declared to meet the ethical requirements for conducting research with certification number 943/VII/HREC/2019.

Animal Model and Chemicals

This study used a male, 2-month-old Wistar white rats ranging from 150–200 g. The animals were prepared from the laboratory of The Centre for Food and Nutrition Studies, University of Gadjah Mada, Yogyakarta, Indonesia. Rats were kept in small cages with good air circulation and lighting. Cages were differentiated for each experimental group, each containing 5 animals. Each cage had a drinking water container that the rats could easily access. Food was also supplied into the cage, which could be easily accessed. The cage bedding was cleaned and replaced daily so that the living environment of the rats was not mixed with their urine and feces.

Preparation and administered dosage determination of 0.5% sodium carboxymethyl cellulose (Na-CMC, Merck, Germany) was performed following Salma et al.'s (2013) as well as Saputri and Zahara's (2016) research. Streptozotocin-nicotinamide (STZ-NA, Merck, Germany) and glibenclamide (GBC, Merck, Germany) solutions were made according to the instructions provided by Arifin et al. (2011) and Ghasemi et al. (2014).

Preparation of *Bengkuang* Extract and Phytochemical Test

Bengkuang tubers were obtained from Teluk Keramat, Kubu Raya District's local farmer gardens in West Kalimantan, Indonesia. A total of 6,737.82 g of fresh *bengkuang* tubers were peeled, chopped, and blended, followed by macerating with 96% ethanol (Merck, Germany), as described previously (Valentina, 2013). The maceration process was carried out three times within 24 hr. The filtrate was collected at 235.35 g after being condensed in a rotary evaporator at 40°C. The quantitative phytochemical analysis was conducted at the Laboratory of Chemistry, The Faculty of Mathematics and Natural Sciences, Universitas Tanjungpura, Pontianak, Indonesia.

Antidiabetic Activity Test of *Bengkuang* Extract

The number of test animals was determined based on the Fereder formula, namely (t-1) (n-1) ≥15, where t is the number of treatments while n is the number of repetitions in each treatment. A total of 25 experimental rats were randomly divided into 5 groups (i.e., 5 in each): (1) A: Normal Group (without any treatment), (2) B: Diabetes Control (administered with 0.5% Na-CMC (Merck, Germany) with a dosage of 2 ml/200 g BW), (3) C: Antidiabetic Control (GBC (Merck, Germany) administered at 0.09 mg/200 g BW) (10), (4) D: Treatment Group I (administrated with *bengkuang* extract at 28 mg/200 g BW as suggested by Park & Han [2015]), and (5) E: Treatment Group II administrated with *bengkuang* extract at 56 mg/200 g BW.

Blood was withdrawn from the rats via sinus orbitalis. The blood was measured using a glucose oxidase-peroxidase aminoantypirin diagnostic system (GOD-PAP DiaSys). Blood glucose levels of all groups were measured at the start of the experiment (Day 0) following the method of GOD-PAP (Subiyono et al., 2016). On the same day, diabetes mellitus was induced in Groups B, C, D, and E by intraperitoneal injection of STZ (Merck, Germany) (45 mg/kg BW) and NA (Merck, Germany) (110 mg/kg BW). Three days later (Day 3), blood glucose levels were measured to confirm success in diabetes mellitus induction. Subsequently, the animals were injected daily with either GBC (Merck, Germany) or our plant extracts for 14 days, starting from Day 4 and ending on Day 17. On Day 18, their blood was again withdrawn to measure glucose levels.

Histological Examination

All rats were euthanized by cervical dislocation before the organs were collected. On Day 18, the pancreatic organs of all rats were collected and then subject to paraffin methods followed by hematoxylin (Merck, Germany) and eosin (Merck, Germany) (H&E) staining according to the procedures at the Laboratory of Pathology, the Faculty of Veterinary Medicine, University of Gadjah Mada, Yogyakarta, Indonesia. All organs were cut approximately 1 cm × 1 cm in size, followed by histological preparations, including washing, dehydration, clearing, infiltration, embedding, trimming, sectioning, affixing, and staining. The

H&E staining was used to differentiate nuclei from their surroundings (Suvarna et al., 2019). Observations were made under a microscope with 40× magnification to observe the normal cell, necrotic cell, and degenerated Langerhans of insular cells in the Normal Group, Diabetic Control, Antidiabetic Control, and Treated Group (Extract I and II).

Statistical Analysis

The data on blood glucose levels were analyzed using the program Statistical Product and Service Solutions (SPSS, version 24) with one-way analysis of variance (ANOVA) and continued with Tukey's test at a 5% confidence. Histopathological images of the pancreatic tissue were analyzed descriptively.

RESULTS

Phytochemical Test of *Bengkuang* Extract

The *bengkuang* extract tested strongly positive for alkaloids (Dragendroff's reagent, Merck, Germany) and weakly positive for flavonoids (10% sodium hydroxide [NaOH], Merck, Germany), saponins, and steroids (Chloroform [Merck, Germany] and sulfuric acid [H_2SO_4, Merck, Germany]), but negative for alkaloids (Mayer's reagent, Merck, Germany), alkaloids (Wagner's reagent, Ottokemie, India), flavonoids (Magnesium powder [Mg, Laboratorium Discounter, Netherland] and hydrochloric acid [HCl, Merck, Germany]), flavonoids (2N H_2SO_4, Merck, Germany), terpenoids (Salkowski reagent, which was a mixture of 0.5 M of iron (III) chloride [$FeCl_3$, Eisen-Golden Laboratories, Netherland] and 35% perchloric acid solution [$HClO_4$, Merck, Germany]), tannins ($FeCl_3$, Kuhlmann, France), and phenolics (Folin Ciocalteu's phenol reagent, Merck, Germany) (Table 1).

Table 1
Phytochemical test results of bengkuang extract

Parameters	Levels
Alkaloids (Mayer's reagent)	-
Alkaloids (Wagner' reagent)	-
Alkaloids (Dragendroff's reagent)	+++
Flavonoids (Mg + HCl)	-
Flavonoids (2 N H_2SO_4)	-
Flavonoids (10% NaOH)	+
Saponins	+
Terpenoids (Salkowski reagent)	-
Steroids (Chloroform + H_2SO_4)	+
Tannins ($FeCl_3$)	-
Phenolics (Folin Ciocalteu's phenol reagent)	-

Note. - = Not detected; + = Low level; ++ = Sufficient level; +++ = High level; Mg = Magnesium; HCl = Hydrochloric acid; H_2SO_4 = Sulfuric acid; NaOH = Sodium hydroxide; $FeCl_3$ = Iron (III) chloride

Antidiabetic Activity Test of *Bengkuang* Extract

Clearly shown in Table 2, blood glucose levels of all groups were normal on Day 0, then increased to > 250 mg/dl on Day 3 post-STZ-NA injection, indicating that diabetes mellitus had developed in the rats (Ghasemi et al., 2014). However, after administering *bengkuang* extracts, the glucose levels

decreased significantly ($p < 0.05$) to 111 ± 63 mg/dl with 28 mg treatment Group D and 99 ± 55 mg/dl with 56 mg treatment Group E when compared to the Diabetes Control Group B showing elevated glucose levels (Day 18).

Table 2
The average blood glucose levels of streptozotocin-nicotinamide (STZ-NA) injected rats on pre- and post-treatments with glibenclamide (GBC) or plant extracts

Treated group	Average levels of blood glucose in rats (mg/dl)		
	Day 0	Day 3	Day 18
A (Normal Group)	67± 2	69 ± 2	70 ± 2
B (Diabetes Control)	70 ± 2[a]	271 ±13[b]	277 ± 9
C (Antidiabetic Control)	69 ± 3[a]	269 ± 9[c]	111 ± 63
D (Extract I)	70 ± 1[a]	278 ± 4[d]	182 ± 3
E (Extract II)	72 ± 2[a]	277 ± 4[e]	99 ± 55

Note. [a]STZ-NA injected at Day 0 to induce diabetes mellitus; [b,c,d,e] injected with sodium carboxymethyl cellulose, GBC, Extract I (28 mg/200 g BW), and Extract II (56 mg/200 g BW) on Day 4, respectively. Day 0 shows the initial blood glucose levels, while Day 18 shows the blood glucose levels 14 days after the start of treatments. Superscripts indicate it is statistically significant compared to other groups

Histological Examination of the Rat Pancreas

Figure 1 shows the Normal Group (A), the Diabetes Control (B), the Antidiabetic Control (C), the Treated Group with Extract I (D), and the Treated Group with Extract II (E). Histopathological examination of rat pancreatic tissue revealed gross variations in Langerhans pancreatic cells. To illustrate, Normal Group A did not exhibit any specific changes leading to necrosis (i.e., absence of necrotic cells and degeneration of Langerhans islands as indicated by very dense cell nuclei and no cells with edema [swelling]. Islets composition Langerhans appear compact and contain many endocrine cells Figure 1A). In contrast, the Diabetes Control Group B (STZ treatment) displayed damage in the form of necrotic cells on Langerhans islands as indicated by the size of Langerhans islands getting smaller, the

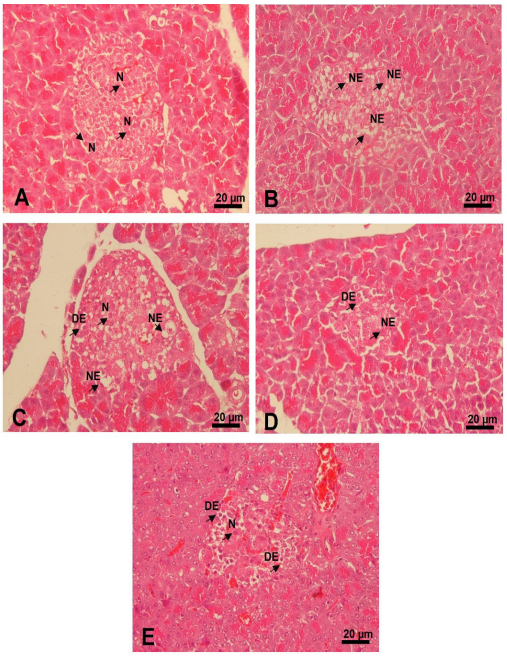

Figure 1. Histopathological images of rat pancreas: (A) Normal Group; (B) Diabetic Control; (C) Antidiabetic Control; (D) Treated Group with Extract I; (E) Treated Group with Extract II

Note. N, NE, and DE indicate the normal cell, necrosis cell, and degenerated Langerhans of insular cells, respectively

number of β-cell masses decreasing, and the empty spaces in the middle of the island Langerhans.

Necrosis is cell death due to fatal damage characterized by damage to the structure and function of the cell followed by cell lysis and tissue inflammation, in Figure 1B, marked with NE in Figure 1B. Interestingly, the pancreatic tissue of the Antidiabetic Group C treated with GBC and Extract Treated Groups D and E showed signs of recovery. These signs included normal starting cells and degeneration of islets of Langerhans as indicated by colonizing cells, characterized by monomorphic β-pancreatic cell shapes, β-pancreatic cells with enlarged nuclei, and visible nucleoli in Figures 1C, 1D, and 1E. Extract II (56 mg/200 g BW) gave the best recovery (Figure 1E). These recoveries were associated with the reduced glucose levels shown in Table 2.

DISCUSSION

This study aims to evaluate the efficiency of *bengkuang* extract in treating diabetes mellitus using a rat model. Both plant extract concentrates (28 and 56 mg/200 g BW) significantly reduced blood sugar levels after 14 days of treatment (Day 18, Table 2), indicating recovery from diabetes as previously reported (Ghasemi et al., 2014). According to Nubatonis et al. (2015), the leading causes of damage to β pancreatic cells are genetics, infections, nutrition, diabetogenesis, and free radicals. In this study, the damage occurring in the Langerhans cells was due to STZ-NA, a cytotoxic hazard for β pancreatic cells (Nubatonis et al., 2015). Histopathology examination of the white rat pancreas shown in Figure 1B, the Diabetes Control Group demonstrated necrotic cells, distinguished by smaller Langerhans islands, fewer β-cell masses, and empty spaces in the middle of Langerhans islands. It demonstrates how STZ-NA administration could cause damage to Langerhans cells. The Normal Group in Figure 1A demonstrates that Langerhans' islets appear normal, characterized by the absence of necrosis or degeneration of the Langerhans islets, a very dense cell nucleus, and the absence of any edema cells (swelling).

The islets of Langerhans have a dense appearance and are dense with endocrine cells. The Antidiabetic Control Group in Figure 1C shows that, despite necrotic cells, the islets of Langerhans have degenerated and are moving toward normal conditions, characterized by the presence of colonizing cells. Meanwhile, the histopathology of the white rat pancreas in Figure 1E showed that the group receiving Extract II experienced degeneration in the islets of Langerhans, and the cells started to appear normal as indicated by the presence of colonizing cells, pancreatic β-cells with enlarged nuclei, and visible nucleoli. Due to the presence of various metabolites, primarily alkaloids, the plant extracts were able to mitigate this damage in a dose-dependent manner (Table 1). This conclusion is supported by earlier research showing that alkaloids, saponins, flavonoids, and steroid groups are effective in treating hyperglycemia because they can stimulate insulin secretion and regenerate β

pancreatic cells (Barky et al., 2017; Kumar et al., 2019; Sandhar et al., 2011; Sediarso et al., 2012).

CONCLUSION

Bengkuang extract has antidiabetic activity, as indicated by a significant decrease in blood sugar levels and recovery of damaged pancreatic cells in treated diabetic rats. Its activity is dose-dependent, with 56 mg/200 g BW showing a comparable efficacy to the GBC (0.09 mg/200 g BW). The results justify further investigations into the underlying antidiabetic mechanisms to assess the potential of using *bengkuang* extracts or an extract component(s) for diabetes treatment.

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Diversity of Wild Gingers (Zingiberaceae) in Southern Peninsular Malaysia: Panti Forest Reserve and Labis Forest Reserve

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ABSTRACT

Family Zingiberaceae is well distributed in Peninsular Malaysia, including the southern region of Johor. A preliminary survey updated the current checklist of wild gingers from Panti Forest Reserve and Labis Forest Reserve. During the brief survey, 28 taxa from 8 genera were recorded. The genera include *Amomum, Alpinia*, *Boesenbergia*, *Conamomum*, *Etlingera*, *Globba*, *Hornstedtia*, *Meistera*, *Scaphochlamys*, *Sundamomum*, and *Zingiber malaysianum*. Three endemic species to Johor were recorded from the study sites: *Scaphochlamys lanceolata*, *Scaphochlamys klossii* var. *glomerata*, and *Zingiber.*

Keywords: Conservation, endemic, Johor, threatened, Zingiberales

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INTRODUCTION

Tropical Asia, as the richest zone for the family Zingiberaceae, harbours a total of 1,449 accepted species across 56 genera, of which 21% are currently classified as threatened (Banaticla-Hilario & Altamirano, 2023). As defined by biogeographic area, Southeast Asia, notably the Sundaland, comprising Peninsular Malaysia, Borneo, and Sumatra, is home to significant numbers of threatened species of wild ginger. With at

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least 150 species from 21 genera, Malaysia recorded the largest number of threatened ginger taxa, followed by Indonesia (113 species; 24 genera), Thailand (30 species; 11 genera), and Vietnam (26 species; 8 genera) (Banaticla-Hilario & Altamirano, 2023). In terms of endemism, the highest number of endemic species are found in Malaysia and Indonesia, where there are 86 species and 66 genera, respectively. With 96 species across 20 genera, Borneo is the most diversified hotspot for vulnerable gingers at high risk of wild extinction. Borneo has 87 species and three endemic genera (*Epiamomum* A. D. Poulsen, *Myxochlamys* A. Takano and Nagam.). Likewise, Peninsular Malaysia is home to the hyperendemic and The International Union for Conservation of Nature (IUCN)-unevaluated genera, particularly *Kedhalia* C. K. Lim, *Perakalia* C. K. Lim, and *Johoralia* C. K. Lim.

Johor is situated at the southern tip of Peninsular Malaysia, with a total area of 19,984 km^2, making it the third largest state after Pahang and Perak. It has 334,292.46 ha of permanent forest reserves consisting of various forest types such as ericaceous forest, montane-oak forest, dipterocarp forest, and peat swamp forest that are rich in flora and fauna diversity (Jabatan Perhutanan Negeri Johor [JPNJ], 2021). Panti Forest Reserve is a dipterocarp forest within Bandar Kota Tinggi with many native flora species and a unique collection of avian diversity (Holttum, 1950; Normaisharah & Norazlimi, 2019). Meanwhile, Labis Forest Reserve is in the district of Segamat. It is known for playing a significant role as a buffer zone between national parks and oil palm plantations and is directly connected to Endau–Rompin National Park (Aqilah et al., 2019).

Wild ginger is one of the important herbs in Malaysia. It is broadly utilised not only for medication and daily spices but also as an ornamental plant (Dissanayake et al., 2020; Kress et al., 2002; Lim, 2002). Being the largest family under order Zingiberales, this aromatic plant is widely distributed from southern to northern Peninsular Malaysia (Larsen et al., 1999; Plants of World Online [POWO], 2023). These rhizomatous herbs are commonly found in a wide range of habitats, such as lowland primary and secondary forests occurring in karsts, stream banks, plateau areas, ravines, as well as roadsides (Mohamad & Kalu, 2019; Setiawan et al., 2022). They prefer shady environments with low-intensity sunlight; nevertheless, some species flourish well in sunny areas (Larsen et al., 1999).

Holttum (1950) documented Zingiberaceae throughout Peninsular Malaysia based on Ridley's drawings, besides Corner's and Valeton's descriptions in his comprehensive account. Twenty-one genera with 156 species of Zingiberaceae in Peninsular Malaysia were documented by Holttum (1950); nevertheless, many of the taxa classifications need revision following the current classification based on molecular evidence (de Boer et al., 2018; Kress et al., 2002). Approximately 160 species of Zingberaceae are dispersed in Peninsular Malaysia (Larsen et al., 1999), but information on the diversity of wild gingers in southern Peninsular Malaysia, especially Johor, is very limited. Thus, this

paper provides data on Zingiberaceae and their endemism in two forest reserves of Johor, namely Panti Forest Reserve and Labis Forest Reserve.

MATERIALS AND METHODS

Study Sites

The study was conducted in two forest reserves in Johor, specifically Panti Forest Reserve (13,150 ha) and Labis Forest Reserve (70,024 ha) (JPNJ, 2021). Panti Forest Reserve is located in the southeast of Johor; meanwhile, Labis Forest Reserve is situated in the northern part of Segamat, Johor. The distance between these two forest reserves is approximately 143 km apart. Both forest reserve study sites were humid (consistent moisture and access to water) and partially to a fully shady environment. A few sites are near the waterfall and full of rocks. Sites with favourable conditions for wild gingers to grow were selected, such as along the stream banks, swampy areas, and lowland forests (200–500 m above sea level [a.s.l.]).

Zingiberaceae Sampling

A series of fieldwork was conducted from August 2022 to June 2023 to document and collect the fertile specimens of ginger at selected trails in the forest reserves. Each ginger locality was tagged with The Global Positioning System (GPS) coordinate. Each specimen was assigned a serial collection number, and duplicates received the same number. Photographs and field notes were taken accordingly.

Plant Description and Identification

Specimens were identified solely based on their morphological characteristics. All plant parts, including floral and vegetative, were examined and measured using a measuring tape and a calliper. In contrast, a USB digital microscope (1000×) (Model MX200-B, T TAKMLY, China) was used to observe their morphology in more detail. The specimens were identified using protologues, online databases, and related published materials (International Plant Names Index [IPNI], 2023; POWO, 2023). Samples were soaked in 70% ethanol (Systerm Chemicals, Malaysia), pressed, and dried in an oven at 50°C for a week. The herbarium specimens were deposited at the herbarium of Universiti Tun Hussein Onn Malaysia. The conservation status of the collected Zingiberaceae was assessed based on the IUCN (2023).

RESULTS AND DISCUSSION

Diversity of Zingiberaceae

A total of 8 genera of Zingiberaceae comprising 28 taxa were found during our recent trip to Panti Forest Reserve (Table 1) and Labis Forest Reserve (Table 2), which updated the current checklist of Zingiberaceae in Panti Forest Reserve and Labis Forest Reserve to 24 taxa (21 species with 3 varieties) and 4 taxa (4 species with 1 variety) correspondingly (Table 3). At present, only three species, namely *Z. malaysianum*, *S. klossii* var. *glomerata*, and *S. lanceolata*, were found to be endemic to Johor (Holttum, 1950; IPNI, 2023; POWO, 2023).

Based on our observation, the distribution pattern of the species collected in both forest reserves overlaps between Sumatra and Thailand. The presence of overlapping species may be because of the impact on ecological conditions since they are under the same biogeographic region. Furthermore, soil nutrition, climate conditions, and elevation ranges contribute to the occurrence of these overlapping species (Ordoñez et al., 2009). Six wild gingers that overlap within the regions are as follows: *Alpinia conchigera*, *Globba leucantha*, *Globba pendula*, *Conamomum xanthophlebium*, *Etlingera megalocheilos*, and *Hornstedtia scyphifera* (Tables 1 and 2). Furthermore, *Boesenbergia prainiana* and *Globba fragilis* only occur in Peninsular Malaysia and Thailand. Meanwhile, two Zingiberaceae species, namely *A. conchigera* and *G. pendula*, are extensively distributed from Peninsular Malaysia, Bangladesh, and Myanmar to Vietnam (Holttum, 1950; Hanh et al., 2014; Phuong et al., 2020; IPNI, 2023; POWO, 2023) (Tables 1 and 2).

The genera *Globba* L. and *Scaphochlamys* Baker were found in four different trails of Panti Forest Reserve with three and two species, respectively, *G. leucantha*, *S. lanceolata*, and *S. klossii* var. *klossii* (Figures 1e, 1g, and 1h). They were abundantly seen during the fieldwork. The *S. lanceolata* is categorised as endangered and endemic to Peninsular Malaysia (particularly

Table 1
List of wild gingers in Panti Forest Reserve

Panti Forest Reserve						
No.	Taxa	Localities	GPS coordinates	IUCN conservation status	Endemism	Occurrence
1	*Alpinia conchigera* Griff.	Roadside of Kg. Temenin near Lukut Trail	1°46′N 103°52′E	LC – Stable	-	Peninsular Malaysia, Andaman Island, Bangladesh, Cambodia, Myanmar, Philippines, Sumatera, Thailand, and Vietnam
2.	*Amomum* sp.	Sungai Segun	1°51′N 103°53′E	-	-	-
3.	*Boesenbergia prainiana* (King ex Baker) Schltr.	Bunker Trail (phenology plot)	1°51′N 103°53′E	LC – Decreasing	-	Peninsular Malaysia, Thailand, and Indochina
		Bunker Trail (D1)	1°51′N 103°53′E			

Table 1 *(Continue)*

Panti Forest Reserve						
No.	Taxa	Localities	GPS coordinates	IUCN conservation status	Endemism	Occurrence
4.	*Conamomum xanthophlebium* (Baker) Škorničk. & A.D.Poulsen	Bunker Trail (D2)	1°52′N 103°55′E	NE	-	Peninsular Malaysia, Borneo, and Sumatra
5.	*Etlingera megalocheilos* (Griff.) A.D.Poulsen	Roadside towards Bunker Trail	1°51′N 103°53′E	LC – Unknown	-	Borneo, Jawa, Peninsular Malaysia, and Sumatera
6.	*Etlingera* aff. *pauciflora* R.M. Sm.	Roadside towards Bunker Trail	1°52′N 103°55′E	LC – Unknown	-	Indonesia, Thailand, and Malaysia
7.	*Hornstedtia scyphifera* (J.Koenig) Steud.	Roadside towards Muntahak Trail	1°45′N 103°51′E	NE	-	Borneo, Peninsular Malaysia, and Sumatera
		Roadside towards Bunker Trail	1°51′N 103°53′E			
8.	*Globba fragilis* S.N.Lim	Bunker Trail (phenology plot)	1°51′N 103°53′E	LC – Decreasing	-	Thailand and Peninsular Malaysia
		Sungai Segun	1°50′N 103°56′E			
9.	*Globba leucantha* Miq.	Roadside towards Lukut Trail	1°51′N 103°53′E	NE	-	Peninsular Malaysia, Thailand, and Sumatera
		Bunker Trail (phenology plot)	1°51′N 103°53′E			
		Lebak Trail	1°48′N 103°48′E			
		Bunker Trail (D2)	1°52′N 103°55′E			

Table 1 *(Continue)*

Panti Forest Reserve						
No.	Taxa	Localities	GPS coordinates	IUCN conservation status	Endemism	Occurrence
10.	*Globba pendula* Roxb.	Bunker Trail (phenology plot)	1°51′N 103°53′E	LC – Stable	-	Peninsular Malaysia, Andaman Island, Assam, Bangladesh, Borneo, Myanmar, Sri Lanka, Sumatera, Thailand, and Vietnam
11.	*Globba* aff. *unifolia*	Lebak Trail	1°48′N 103°48′E	-	-	-
12.	*Scaphochlamys klossii* var. *klossii* (Ridl.) Holttum	Bunker Trail (phenology plot)	1°51′N 103°53′E	NE	Johor	Johor
		Lukut Trail	1°47′N 103°54′E			
		Lebak Trail	1°48′N 103°48′E			
		Batu Tenggek	1°31′N 103°53′E			
13.	*Scaphochlamys lanceolata* (Ridl.) Holttum	Lukut Trail	1°47′N 103°54′E 1°49′N 103°55′E	EN – Category B1 ab (iii) + 2ab (iii)	Panti Forest Reserve	Johor
		Bunker Trail	1°52′N 103°55′E			
		Sungai Padang				
14.	*Sundamomum hastilabium* (Ridl.) A.D. Poulsen & M.F. Newman	Sungai Segun	1°50′N 103°56′E	LC – Unknown	-	Peninsular Malaysia, Thailand, Indonesia, and Brunei

Note. GPS = The Global Positioning System; IUCN = The International Union for Conservation of Nature; - = None; LC = Least concern; NE = Not evaluated; EN = Endangered; D1 = Name of the trail; D2 = Name of the trail

Johor, in Panti Forest Reserve [IUCN, 2023; IPNI, 2023; POWO, 2023]) (Table 1). Ten individuals per locality were recorded throughout the trip. Another interesting finding was *Sundamomum hastilabium* (formerly *Amomum hastilabium*) near Sungai Segun. It is the only species found in Peninsular Malaysia within the genus *Sundamomum*. Unfortunately, the absence of flowers and fruits of *Zingiber* and *Globba* aff. *unifolia* during sample collections poses a significant challenge in accurately describing these species. Another notable rare species under the order Zingiberales was *Phrynium villosulum* at Sungai Segun, which increased the number of species found for Marantaceae to three species in Panti Forest Reserve (Holttum, 1950).

Scaphochlamys klossii var. *glomerata* thrived well at Sungai Bantang in Labis Forest Reserve, where the species was discovered in a shady environment. At first glance, *S. klossii* var. *glomerata* was identified as *Scaphochlamys endauensis* because of the channelled petiole and tomentose at the abaxial leaf, including the midrib (Figures 2a and 2b) (Sam et al., 2015). Nevertheless, *Scaphochlamys klossii* var. *glomerata* is quite different with its loosely elongated bract inflorescence compared to the red compact inflorescence of *S. endauensis*. Moreover, *S. klossii* var. *glomerata* has prominently veined leaves with sparse hairs on the adaxial surface, contrasted to *S. lanceolata*, which possesses glabrous leaves (Figures 2c and 2d). These conspicuous characteristics are further corroborated in detail and verified by comparing them with herbarium specimens, which have fewer leaf blades with 1–2 compared to 2–6 blades in *S. klossii* var. *klossii*.

Commonly known as beehive ginger, *Zingiber spectabile* flourished in its habitat. So far, *Z. spectabile* has the largest inflorescence among Malayan species (Figures 3a and 3b) (Holttum, 1950). A large and spectacular inflorescence with exquisite yellow and purple flecks makes *Z. spectabile* easy to distinguish. Based on observation, the flowers of *Z. spectabile* in Taka Melor Eco Forest opened at about 11 in the morning. This plant reached 3 m in height compared to the species found in Thailand, which was only 2 m tall (Larsen & Larsen, 2006). The leafy shoots of *Z. spectabile* arch downward, a common feature in the *Zingiber* genus. This common, gigantic species is widely distributed in Peninsular Thailand, India, Australia, and Peninsular Malaysia (Holttum, 1950; IPNI, 2023; POWO, 2023) (Table 2). The IUCN categorises *Z. spectabile* as Data Deficient (DD) (IUCN, 2023). Another noticeable wild ginger was *Meistera* aff. *ochrea*. This species was spotted within the same area as *Z. spectabile*. The green thorny fruits of *Meistera* aff. *ochrea* were hidden under thick leaf litter.

Zingiber malaysianum was found at low slopes (300 m a.s.l.) at Sungai Bantang, where the environment was shady and humid (Figures 3f, 3g, and 3h). This species is considered least concerned with a stable

population trend in the IUCN (IUCN, 2023). According to Lim (2002), *Z. malaysianum* can easily be missed due to its dark reddish-brown leaf and small plant. This attractive plant was only found in a small, confined area at Sungai Bantang, with about two to ten individuals per locality. The distinct leaf features of *Z. malaysianum* enable species identification even without flowers and fruits. Some of the *Z. malaysianum* was damaged by the recent flood in Bekok, Johor. Based on our observation, *S. klossii* var. *glomerata* and other *Zingiber* sp. are sympatric with *Z. malaysianum*; they were found in sandy loam with leaf litter. *Zingiber* Mill. species that flourished well on rocks were also observed in Panti Forest Reserve and Labis Forest Reserve. Nonetheless, they could not be identified at the species level since no blooming flowers or fruits were present.

Current work on Zingiberaceae in the Panti Forest Reserve and Labis Forest Reserve in Johor Peninsular Malaysia has yielded some interesting findings and updates to the checklist of these plants. Discovering and documenting plant species in their natural habitats is important for conservation and biodiversity research. The initial checklist of Zingiberaceae in this forest reserve was based on Holttum's collection in 1950, which identified 16 taxa with 2 varieties. These included various species such as *Boesenbergia plicata*, *Conamomum parvula*, *Globba aurantiaca*, *Etlingera pauciflora*, *Etlingera metriocheilos*, *Amomum trilobum*, *Hornstedtia conica*, *Hornstedtia leonurus*, *Plagiostachys albiflora*, *Scaphochlamys lanceolata*, *Scaphochlamys klossii*, *Scaphochlamys klossii* var. *glomerata*, *Scaphochlamys klossii* var. *minor*, *Zingiber puberulum*, *Zingiber puberulum* var. *chryseum*, and *Wurfbainia uliginosa* (Holttum, 1950). Nevertheless, only three species were rediscovered, which are *Etlingera* aff. *pauciflora* encountered in Sungai Padang, besides the other two common species in Panti Forest Reserve, *S. lanceolata* and *S. klossii* var. *klossii*.

Additionally, *S. klossii* var. *glomerata* was found in Labis Forest Reserve rather than Panti Forest Reserve. This sighting suggests that the distribution of certain Zingiberaceae species extends to the northern part of Segamat, Johor, and beyond what was previously known. Another eight notable findings in Panti Forest Reserve are *Alpinia conchigera*, *Boesenbergia prainiana*, *Conamomum xantophlebium*, *Globba fragilis*, *Globba leucantha*, *Globba pendula*, *Globba* aff. *unifolia*, and *Sundamomum hastilabium* were added to the list. The only Zingiberaceae species in Labis Forest Reserve that Holttum revealed was *Boesenbergia clivalis*. The exploration in this forest reserve documented several findings, such as *Z. malaysianum*, *Z. spectabile*, and *Meistera* aff. *ochrea*. The encounters indicate the need for further research and investigation to understand better and conserve the biodiversity in these areas.

Table 2
List of wild gingers in Labis Forest Reserve

Labis Forest Reserve						
No.	Taxa	Localities	GPS coordinates	IUCN conservation status	Endemism	Occurrence
1.	*Meistera* aff. o*chrea*	Pavement towards the waterfall at Taka Melor Eco Forest	2°28′25″N 103°06′25″E	-	-	-
2.	*Scaphochlamys klossii* var. g*lomerata*	Sg. Bantang	2°20′50″N 103°09′24″E	-	Johor	Johor
3.	*Zingiber malaysianum* C.K.Lim	Sg. Bantang	2°20′48″N 103°09′22″E 2°21′06″N 103°09′23″E	LC – Stable	Labis FR	Johor
4.	*Zingiber spectabile* Griff.	Pavement towards the waterfall at Taka Melor Eco Forest	2°28′25″N 103°06′25″E	DD	-	Malaysia, Thailand, India, and Australia
5.	*Zingiber* sp. 4	Sg. Bantang	2°20′51″N 103°09′24″E	-	-	-
6.	*Zingiber* sp. 5	Sg. Bantang	2°20′54″N 103°09′23″E	-	-	-

Note. - = None; LC = Least concern; DD = Data deficient; FR = Forest Reserve

Table 3
Checklist of Zingiberaceae collected by Holttum (1950) and current collections in Panti Forest Reserve and Labis Forest Reserve

Species	Holttum (1950)		Current collections	
	Panti FR	Labis FR	Panti FR	Labis FR
Alpinia conchigera Griff. Synonym: *Languas conchigera* Griff.			/	
Boesenbergia prainiana (King ex Baker) Schltr. Synonym: *Gastrochilus prainianus* (King ex Baker) Ridl., *Kaempferia prainiana* King ex Baker			/	
Boesenbergia clivalis (Ridl.) Schltr. Synonym: *Gastrochilus clivalis* Ridl., *Kaempferia clivalis* (Ridl.) K.Schum.		/		

Table 3 *(Continue)*

Species	Holttum (1950)		Current collections	
	Panti FR	Labis FR	Panti FR	Labis FR
Boesenbergia plicata (Ridl.) Holttum Synonym: *Gastrochilus plicatus* Ridl.	/			
Camptandra parvula (King ex Baker) Ridl. Synonym: *Kaempferia parvula* King ex Baker	/			
Conamomum xanthophlebium (Baker) Škorničk. & A.D.Poulsen Synonym: *Amomum xanthophlebium* Baker			/	
Globba aurantiaca Miq.	/			
Globba fragilis S.N.Lim			/	
Globba leucantha Miq.			/	
Globba pendula Roxb. Synonym: *Ceratanthera pendula* (Roxb.) T.Lestib.			/	
Globba aff. *Unifolia*			/	
Etlingera pauciflora (Ridl.) R.M.Sm. Synonym: *Achasma pauciflorum*	/		/	
Etlingera metriocheilos (Griff.) R.M.Sm. Synonym: *Achasma metriocheilos* Griff., *Amomum metriocheilos* (Griff.) Baker	/			
Amomum trilobum Gagnep. Synonym: *Elettariopsis triloba* (Gagnep.) Loes.	/			
Hornstedtia conica Ridl.	/			
Hornstedtia leonurus (J.Koenig) Retz. Synonym: *Amomum leonurus* J.Koenig, *Cardamomum leonurus* (J.Koenig) Kuntze	/			
Meistera aff. O*chrea*				/
Plagiostachys albiflora Ridl.	/			
Scaphochlamys lanceolata (Ridl.) Holttum Synonym: *Gastrochilus lanceolatus* Ridl.	/		/	
Scaphochlamys klossii var. *klossii* (Ridl.) Holttum Synonym: *Boesenbergia klossii* (Ridl.) Loes.	/		/	
Scaphochlamys klossii var. *glomerata* Holttum	/			/
Scaphochlamys klossii var. *minor* Holttum	/			
Sundamomum hastilabium (Ridl.) A.D.Poulsen & M.F.Newman Synonym: *Amomum hastilabium* Ridl.			/	
Zingiber malaysianum C.K.Lim				/
Zingiber puberulum Ridl.	/			

Table 3 *(Continue)*

Species	Holttum (1950)		Current collections	
	Panti FR	Labis FR	Panti FR	Labis FR
Zingiber puberulum var. *chryseum* (Ridl.) Holttum Synonym: *Zingiber chryseum* Ridl.	/			
Zingiber spectabile Griff.				/
Wurfbainia uliginosa (J.Koenig) Giseke Synonym: *Amomum uliginosum* J.Koenig	/			

Symbol: / = Available species in that forest; FR = Forest Reserve

Figure 1. Selected Zingiberaceae species and notable *Phrynium* (family Marantaceae) in their habitat in Panti Forest Reserve. (a) *Alpinia conchigera*, (b) *Boesenbergia prainiana*, (c) *Conamomum xanthophlebium*, (d) *Etlingera megalocheilos*, (e) *Globba leucantha*, (f) *Hornstedtia scyphifera*, (g) *Scaphochlamys klossii* var. *klossii*, (h) *Scaphochlamys lanceolata*, and (i) *Phrynium villosulum*

(a) (b) (c) (d)

Figure 2. Notable characteristics for *Scaphochlamys klossii* var. *glomerata* and *Scaphochlamys lanceolata*. (a) Channelled petiole of *S. klossii* var. *glomerata*, (b) Tomentose at the abaxial of the leaves of *S. klossii* var. *glomerata*, (c) Prominent veined of *S. klossii* var. *glomerata*, and (d) Glabrous leaves of *S. lanceolata*

(a) (b) (c)

(d) (e) (f)

(g) (h) (i)

Figure 3. Zingiberaceae in Labis Forest Reserve. (a–c) *Zingiber spectabile* (left to right) inflorescence, flower, habitat, (d–e) *Scaphochlamys klossii* var. *glomerata*, (f–h) *Zingiber malaysianum*, and (i) Fruit of *Meistera* aff. *ochrea*

CONCLUSION

The southern region of Peninsular Malaysia is very rich in wild ginger such *S. lanceolata*, *S. klossii* var. *glomerata*, and *Z. malaysianum*. The present study updated the wild ginger checklist for Panti Forest Reserve and Labis Forest Reserve. This valuable information aids in the conservation efforts of Zingiberaceae species to preserve the planet's biodiversity. In the future, systematic study, phytochemical profiling, and tissue culture will be conducted on selected potential Zingiberaceae species.

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Ultrasound and Steam Explosion Treatments on the Quantity and Molecular Size of Soluble Fibre Obtained from Un-purified and Purified Rice Bran

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ABSTRACT

Rice bran (RB) is a major by-product of the rice industry, and the high proportion (~90%) of insoluble fibre (IF) is the main reason limiting its applications in foods. Thus, the objective of this research is to enhance the solubility of rice bran fibre and decrease the molecular weight (MW) of the soluble fibre (SF) fraction through ultrasound (US) and steam explosion (SE) treatments. The main sugars in the RB fibre were xylose and arabinose, with glucose, galactose, and mannose present in the side chains. The ratio of Ara/Xyl was 0.92 for the un-purified and 1.02 for the purified RB, reflecting the high degree of substitution of the xylan backbone. The highest amount of SF was obtained from RB treated at 60% US amplitude, 20 min treatment, where 7.8% (un-purified) and 35.2% (purified), respectively. For SE treatments, the amount of SF in un-purified RB increased as the pressure increased from 0.3 and 0.6 MPa, which were 6.10±0.34 and 8.83±0.56%, respectively. Meanwhile, the highest SF fraction (35.2%) of purified RB was obtained from the SE treatment at 0.6 MPa. The SF produced from both treatments mainly contained oligosaccharides with MW <1 kDa, with those produced by the SE treatment generally smaller than those by the US treatment. Purification of RB significantly enhanced the efficiency of the US and SE treatments in breaking down the IF into the SF.

Keywords: Fibre, purified, rice bran, solubility, steam explosion, ultrasound

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INTRODUCTION

Physical treatments have been well used to modify the native structure of cereal fibre as they offer many advantages over chemical treatments, such as being economical, environmentally safe, and industrially practical (Brodeur et al., 2011). Among the physical treatments, ultrasound and steam explosion are well known for their effectiveness in cutting off the polymeric structure of cereal bran fibre into shorter chains, which led to an increase in fibre solubility by generating soluble oligosaccharides and improving the technological and health properties of the fibre (Daou & Zhang, 2012a; Jiang & Guo, 2016). With regard to the modification of dietary fibre, ultrasound treatment has been applied to a number of cereal bran and fibre materials including corn bran and cob, wheat bran and straw, rice bran, sugarcane bagasse, buckwheat hulls, hazelnut skin, mulberry leaves, cellulose, and citrus pectin (Daou & Zhang, 2012b; Ebringerová & Hromádková, 2002, 2010; Hromádková & Ebringerová, 2003; L. Zhang et al., 2013; Sfalcin et al., 2015; Sun & Tomkinson, 2002; Wang et al., 2014; Yilmaz & Tavman, 2016; Ying et al., 2011; You et al., 2014).

Meanwhile, steam explosion treatment has been applied in many non-food areas, including producing bioenergy, biomass and chemicals, and environmental protection. More recently, it has attracted the attention of food researchers for its capacity to break down lignin (Han et al., 2010; L.-H. Zhang et al., 2008) and fibrous networks such as cellulose and hemicellulose (Jiang & Guo, 2016), which leads to modification of their physicochemical properties. However, the treatment conditions explored in these studies are limited, and, to date, there is no report on the use of this technique to treat rice bran fibre to improve its chemical properties, specifically on the sugar composition and solubility. Therefore, this study aims to determine the effect of ultrasound and steam explosion treatments on the solubility of un-purified and purified rice bran fibre and the molecular weight distribution of the SF fractions, making it more applicable in a variety of food products and potentially be used as a prebiotic.

MATERIALS AND METHODS

Samples, Chemicals, and Materials Used

RB used in this study was donated by SunRice (Australia). Dextran standards with molecular weight (MW) of 2000, 670, 410, 80, 50, 25, 12, 5, and 1 kDa, raffinose pentahydrate ≥99% (high-performance liquid chromatography [HPLC], 594.51 g/mol), dinitro-salicylic acid, 1-methyl-imidazole, allose, rhamnose, fucose, calcium chloride dihydrate, sodium tetra-hydroborate, potassium sodium tartrate, pancreatin, and pullulanase were purchased from Sigma-Aldrich (Australia). Termamyl α-amylase was donated by Novozymes Australia Pty. Ltd. (Australia). Ethanol, acetone, glacial acetic acid, benzoic acids, hydrochloric acid, D-glucose anhydrous, arabinose, xylose, mannose, galactose, ammonium hydroxide, potassium hydrogen phosphate, and potassium hydroxide

were purchased from Chem Supply Pty. Ltd. (Australia). Sulphuric acids, acetic anhydrate, octan-2-ol, bromophenol blue, monobasic sodium phosphate, and dibasic sodium phosphate were purchased from Ajax Chemical Pty. Ltd. (Australia). Phenol and dimethyl sulfoxide (DMSO) were purchased from Ajax Finechem Pty. Ltd. (Australia).

Rice Bran Purification

RB was defatting following the method described by Uraipong and Zhao (2016). The bran samples were dried at 60ºC overnight in an oven and finally stored in sealed polyethene bags at 4°C before purification. RB fibre was purified by removing starch and protein following the procedure described by Hu et al. (2015) with minor modifications. The defatted rice bran 74 was mixed with MiliQ water at 1:10 (w/v) and placed in a shaking water bath with continuous shaking at 250 rpm at 55°C for 2 hr. After that, the pH of the mixture was adjusted to 9.5 by dropwise addition of 5% sodium hydroxide (NaOH) solution. The mixture was then centrifuged at 9,600 ×*g* for 30 min, the supernatant discarded, and the residue washed with MiliQ water several times (each with centrifugation) to remove protein.

Biuret reagent was used to confirm the absence of protein in the supernatant. The protein-free residue was added with MiliQ water at a ratio of 1:5 (w/v), and the pH of the mixture was adjusted to 6.5 with 10% (v/v) acetic acid before adding 1 ml of Termamyl α-amylase. The mixture was digested in a water bath at 100°C with continuous shaking at 250 rpm for 1 hr. After cooling to room temperature, the mixture was centrifuged again at 9,600 ×*g* for 20 min, the supernatant was removed, and the residue was washed with MiliQ water twice (by centrifugation) to remove starch. The absence of starch in the digested rice bran was confirmed using an iodine staining solution.

Ultrasound Treatment

Ultrasound (US) cavitation treatment was applied to defat and purify RB using a sonication immersion probe (20 kHz and 450 W, Branson Sonifier 450, USA). Defatted RB was dispersed in water at a ratio of 1:30 (w/v) in a 250 ml beaker, and the treatment was performed at three different power amplitudes, 60, 80, and 95% and time for 5, 10, 15, and 20 min for each power setting. The beaker containing the sample was partially immersed in an ice water bath to ensure that the samples did not become overheated. The temperature of the slurry during the treatment was maintained at 25±5°C. The sonicated samples were lyophilised (Leybold Lyovac GT2, Germany), sieved to pass 450 µm mesh size, and stored at -18°C until further analysis (Ismail & Zhao, 2022).

Steam Explosion (SE) Treatment

SE treatment (SE) of the bran samples (defatted and purified RB) was performed using a QBS-80 batch SE apparatus (Hebi Steam Explosion Research Center, China). Samples (50 g) were treated at 0.3 MPa

(144°C) and 0.6 MPa (165°C) for 120 and 180 s, respectively. All treated samples were lyophilised (Christ Alpha 1-2LD, Germany), sieved to pass 450 µm mesh size and kept at -18°C for further analysis (Ismail & Zhao, 2022).

Determination of Sugar Composition and Solubility of Rice Bran Fibre

The solubility of RB dietary fibre before and after US and SE treatments was determined by analysing the total amount of neutral sugars (rhamnose, fucose, arabinose, xylose, mannose, galactose, and glucose) in the total fibre and IF fractions of the samples by gas chromatography with flame ionised detector (GC-FID, Agilent 7890A, USA) according to the method introduced by Englyst et al. (1992) and optimised by Ma et al. (2017) with a minor modification. The soluble fraction was derived by subtracting the total and insoluble fractions. The soluble fraction was derived by subtracting the total and insoluble fractions. The samples were subjected to enzymatic digestions to release the simple sugars, which were then derivatised to make them volatile before the gas chromatography (GC) analysis.

Reducing Sugar

Reducing sugars released after US and SE treatments were measured using dinitrosalicylic acid (DNSA) reagent with a spectrophotometric method. The treated RB fibre (1 g) was dispersed in 50 ml deionised water, vortex mixed and centrifuged at 1,000 ×*g* for 20 min. The supernatant was collected and used to analyse the reducing sugar. The supernatant (2 ml) was diluted with 1 ml deionised water, added with 3 ml DNSA reagent, and heated in a boiling water bath for 15 min. After that, the sample was taken out, and 1 ml of 40% (w/v) potassium sodium tartrate solution (Roschell salt) was added immediately and vortex mixed. The mixture was let to cool to room temperature for 30 min, and the intensity of colour produced was read using a spectrophotometer (Spectramax M5e microplate reader, Molecular Device LLC, USA) at 540 nm. A standard curve was plotted by using xylose as a standard at the concentration range of 0.01-0.07 mg/ml in deionised water to determine the concentration of reduced sugar in the supernatant after US and SE treatments.

Determination of Molecular Weight Distribution of Rice Bran Soluble Fibre

The molecular weight distribution of soluble fractions after treated with SE and US was determined using size exclusion high-performance liquid chromatography (SE-HPLC) (LC-20AD, Shimadzu DGU-20A5 Degasser and Shimadzu SIL-20A HT Autosampler, USA) equipped with a refractive index (RI) detector (Shimadzu RID-10A, USA) and a size exclusion column PolySep-GFC-P-Linear column (L:300 mm × i.d: 7.5 mm; Phenomenex, Inc., USA). The elution conditions were as follows: isocratic elution with deionised water as mobile phase, column temperature 35°C, flow rate 0.8 ml/min for 30 min and injection volume 15 µl. Nine dextran standards with molecular weight (MW)

2000, 670, 410, 80, 50, 25, 12, 5, 1 kDa, and raffinose (0.6 kDa), maltose (0.3 kDa), and xylose (0.15 kDa) were used to construct a standard curve. Each standard was dissolved in deionised water at concentrations 0.25, 0.5, 2.0, 3.0, 4.0, and 5.0 mg/ml and left overnight at 4°C to allow it to swell.

For sample preparation, 1 g of RB was dispersed in 30 ml of deionised water, mixed well for 30 min, and centrifuged at 1,400 ×*g* for 20 min. The supernatant was collected and freeze-dried for 72 hr to yield a dry mass of the soluble fraction. After that, the soluble fraction was dissolved in deionised water at a concentration of 1 mg/ml and left overnight at 4°C to allow it to swell. The standards and samples were then filtered through a 0.45 µm membrane filter before being transferred into 1 ml glass vials for injection. A standard curve was built individually (0.25, 0.5, 2.0, 3.0, 4.0, and 5.0 mg/ml), and it was confirmed that the standard concentration is directly proportional to the peak area of the chromatogram ($R^2 = 0.98$). A mixed standard curve (log MW vs retention time) consisting of all dextran standards was also prepared to determine each standard's retention times. The equation from the curve was used to estimate the molecular weight of the SF components in the samples.

Statistical Analysis

The experiments were repeated twice, and all data was collected in triplicate. Data were analysed by one-way analysis of variance (ANOVA) to determine the significant differences, and Tukey pairwise comparisons were used to compare the significant differences between the treatments. The statistical analysis was performed using Minitab version 17 (USA).

RESULTS AND DISCUSSION

Sugar Composition of Rice Bran Fibre

The sugar compositions of un-purified and purified RB are shown in Table 1. Purification led to changes in sugar compositions, particularly the loss of glucose. The loss of glucose could be due to the high heat treatment (100°C) applied during the removal of starch in the purification process, which might have caused the breakdown of some glucose in the side chains of the rice bran fibre. The main sugars found in the rice bran fibre were xylose and arabinose. Rice bran fibre is expected to have a xylan backbone (β-1, 4-D-xylan) with a highly branched structure made mainly of arabinose and xylose (Shibuya & Iwasaki, 1985). Glucose, galactose, and mannose were also present in these samples and suspected to be located at the side chains. The degree of substitution or branching of

Table 1
Sugar composition in the total fibre fraction of un-purified and purified rice bran fibre

Neutral sugars	Amount (%)	
	Un-purified	Purified
Xylose	49.40±3.21	48.07±3.51
Arabinose	45.32±3.42	48.80±2.74
Glucose	3.78±0.05	0.99±0.03
Galactose	1.21±0.01	1.14±0.02
Mannose	0.13±0.02	0.14±0.01
Rhamnose	0.06±0.00	0.06±0.00
Fucose	0.04±0.00	0.05±0.00
Total sugars	99.94±6.71	99.77±6.30

the xylan backbone is reflected by the ratio of arabinose to xylose (Ara/Xyl). A higher ratio indicates a higher degree of substitution on the xylan backbone (Izydorczyk & Biliaderis, 1995). As reported in previous studies (Shibuya & Iwasaki, 1985; Shiiba et al., 1993), the ratio of Ara/Xyl is particularly high in cereal bran (0.98–1.76), which agrees with the finding in this study, where it was 0.92 for the un-purified and 1.02 for the purified RB. The higher degree of branching also suggested higher heterogeneity of rice bran fibre structure, which could influence the hydrolysis of the polysaccharide chain.

Effect of Ultrasound and Steam Explosion Treatment on Chemical Composition of Rice Bran Fibre

The effect of US and SE treatments on the chemical properties of RB fibre was investigated by analysing changes in solubility, molecular weight, and reducing sugar released after the treatments.

Solubility of Rice Bran Fibre After Ultrasound and Steam Explosion Treatments

The effect of US and SE treatments on the solubility of RB fibre was analysed by comparing the amount of IF retained after the treatments for both un-purified and purified RB with the total fibre content before the treatments. The total fibre content of untreated RB (un-purified and purified) was used as a control to obtain the amount of soluble compounds (A) after the treatments. Then, the reducing sugar content (B) in the soluble fraction was determined to obtain the amount of SF by subtracting B from A (A-B), and the data are shown in Tables 2 and 3 for US-treated samples and Table 4 for SE-treated samples, respectively. For the untreated RB (both un-purified and purified), the SF is directly calculated by the difference between the amounts of total fibre and IF in the samples. However, for the US and SE-treated RB, the amount of SF could not be directly calculated by the difference as the treatments had caused changes in the native structure of the fibre, leading to changes or reductions in the total fibre content as some of the SF, which had shorter polysaccharide chains could be broken down into simple sugars (i.e., disaccharides and monosaccharides), and lost during the extraction process.

In un-purified RB (Table 2), the total fibre, IF, and SF were 20.67±0.37, 17.47±0.24, and 3.20±0.6%, respectively. A fluctuating trend of decreases in IF content was observed after US treatment at 60% amplitude for various lengths of time. It could be due to the presence of starch and protein, which hindered the effect of US treatment on the fibre, as discussed in the previous study (Ismail & Zhao, 2022).

However, after treatment at 80 and 95% US amplitudes, the IF content decreased significantly ($p<0.05$) as a function of both US amplitude and time, with corresponding increases in the amount of SF. Reducing sugar content increased after the US treatments compared to the untreated sample. The most soluble fraction was obtained at 60% US amplitude and 20 min treatment. At 95% amplitude of

US treatment, an inconsistent trend in the SF amount produced was observed as the treatment time increased from 5 to 20 min. One probable reason for this could be that the high amplitude of US further broke down the SF into reducing sugars as the amount of reduced sugar increased when the treatment time increased.

The untreated purified RB contained 65.90±4.4% total fibre, 49.27±2.7% IF, and 16.64±0.9% SF (Table 3). It was assumed that the other 34.1% of the material (other than total fibre) was comprised of lignin, which was not made of sugar components and, thus, could not be detected by the GC analysis. After treatment with US or SE, a significant decrease in IF fraction was observed under all conditions with respect to time, amplitude, and pressure. The SF fraction of purified RB increased after US

Table 2
Effects of ultrasound treatment on un-purified rice bran fibre fractions

Ultrasound amplitude (%)	Ultrasound time (min)	Total fibre (%)	Insoluble fibre (%)	Soluble compound (%) A	Reducing sugars (%) B	Soluble fibre (%) A-B
0 (Untreated)	0	20.7±0.4	17.5±0.2$^{A,\ a,\ x}$	5.7±0.1$^{C,d,\ y}$	2.5±0.1$^{C,\ b,\ z}$	*3.2±0.6$^{B,\ c,\ x}$
60	5	n/a	13.7±0.2^{B}	6.9±0.2^{B}	3.1±0.1^{B}	3.8±0.6^{B}
	10	n/a	15.3±2.4^{A}	5.5±0.3^{C}	3.2±0.1^{B}	2.3±0.2^{C}
	15	n/a	14.3±2.2^{B}	6.5±1.2^{B}	3.0±0.1^{B}	3.5±1.1^{B}
	20	n/a	9.1±0.7^{C}	11.7±1.3^{A}	4.0±0.1^{A}	7.8±1.4^{A}
80	5	n/a	13.3±0.5^{b}	7.5±0.5^{c}	4.8±0.1^{a}	2.8±0.3^{d}
	10	n/a	12.0±3.9^{b}	8.9±0.5^{b}	4.8±0.1^{a}	4.1±0.8^{b}
	15	n/a	9.7±1.7^{c}	11.2±0.2^{a}	4.7±0.1^{a}	6.5±0.3^{a}
	20	n/a	9.6±0.8^{c}	11.2±1.6^{a}	4.7±0.1^{a}	6.5±0.8^{a}
95	5	n/a	17.4±0.2^{x}	3.3±0.1^{z}	3.1±0.1^{y}	0.1±0.7^{z}
	10	n/a	12.8±2.5^{y}	7.9±2.3^{x}	3.2±0.1^{y}	4.7±0.4^{w}
	15	n/a	13.3±0.5^{y}	7.4±1.5^{x}	5.5±0.1^{w}	1.9±0.6^{y}
	20	n/a	10.0±0.4^{z}	10.7±1.3^{w}	5.1±0.1^{x}	5.6±0.6^{v}

Note.
For untreated, the soluble fibre* is directly calculated by deducting total fibre from insoluble fibre.
For ultrasound-treated, the soluble compounds (A) are calculated by deducting the total fibre of untreated samples from the insoluble fibre of ultrasound-treated soluble compound (A), which is the sum of reducing sugar and soluble fibre.
For ultrasound treatment, the soluble fibre is calculated by deducting soluble compounds (A) to reduce sugar (B), A-B.
$^{A-C}$ = Means with different superscript letters within the same column of similar US amplitude differ significantly (p<0.05).
$^{a-c:}$ = Means with different superscript letters within the same column of similar US amplitude differ significantly (p<0.05).
$^{w-z}$ = Means with different superscript letters within the same column similar US amplitude differ significantly (p<0.05).
Values are means±S.D.; n = 3; n/a = Not available.

Table 3
Effects of ultrasound treatment on purified rice bran fibre fractions

Ultrasound amplitude (%)	Ultrasound time (min)	Total fibre (%)	Insoluble fibre (%)	Soluble compound (%) A	Reducing sugar (%) B	Soluble fibre (%) A-B
0 (Untreated)	0	65.9±4.4	49.3±2.7$^{A, a, x}$	22.0±1.7$^{C, b, y}$	5.4±0.0$^{C, b, z}$	*16.6±0.9$^{C, c, z}$
60	5	n/a	44.1±4.2^{A}	21.8±3.0^{C}	7.1±0.0^{A}	14.7±2.1^{C}
	10	n/a	26.2±4.1^{B}	39.7±2.1^{B}	6.8±0.1^{B}	32.9±1.0^{B}
	15	n/a	26.9±0.9^{B}	39.0±6.9^{B}	7.0±0.0^{A}	32.0±0.3^{B}
	20	n/a	23.6±1.2^{B}	42.3±2.2^{A}	7.2±0.1^{A}	35.2±0.6^{A}
80	5	n/a	32.8±6.4^{b}	33.1±7.1^{a}	6.8±0.0^{a}	26.3±0.4^{b}
	10	n/a	32.3±3.5^{b}	33.6±3.6^{a}	5.3±0.0^{b}	28.3±1.2^{b}
	15	n/a	29.3±3.2^{b}	36.6±1.3^{a}	5.4±0.0^{b}	31.2±1.3^{a}
	20	n/a	27.1±3.3^{b}	38.3±3.3^{a}	5.6±0.2^{b}	33.2±1.0^{a}
95	5	n/a	33.9±0.8^{y}	32.1±8.5^{x}	7.5±0.1^{w}	24.6±0.3^{y}
	10	n/a	27.9±2.2^{z}	38.0±7.2^{x}	6.8±0.0^{y}	31.3±0.7^{w}
	15	n/a	31.2±1.0^{z}	34.8±8.3^{x}	7.0±0.0^{x}	27.8±0.5^{x}
	20	n/a	34.3±1.5^{y}	31.6±7.8^{x}	6.8±0.2^{y}	24.7±0.8^{y}

Note.
For untreated, the soluble fibre* is directly calculated by deducting total fibre from Insoluble fibre.
For ultrasound treated, the soluble compounds (A) are calculated by deducting the total fibre of untreated samples from the insoluble fibre of ultrasound treated.
For ultrasound treatment, the soluble fibre is calculated by deducting soluble compounds (A) to reduce sugar (B), A-B.
$^{A-C}$ = Means with different supersript letters within the same column of similar US amplitude differ significantly (p<0.05).
$^{a-c}$ = Means with different supersript letters within the same column of similar US amplitude differ significantly (p<0.05).
$^{w-z}$ = Means with different supersript letters within the same column of similar US amplitude differ significantly (p<0.05).
Values are means±S.D.; n = 3; n/a = Not available.

treatment at all amplitudes. The highest amount of SF was achieved at 60% US amplitude and 20 min. However, at 95% US amplitude, a further increment of US treatment time from 10 to 20 min led to an increase in IF content and a corresponding decrease in the SF fraction and reduced sugars compared to 5 min treatment. Furthermore, it was also observed that high levels of reducing sugars were released after 5 minutes of treatment at all US amplitudes, with the highest amount recorded at 95% US amplitude. It demonstrated that, for a shorter treatment time, the US broke down the side chains of the RB fibre, releasing reducing sugars.

For un-purified RB treated with the SE, the amount of reduced sugars decreased compared to untreated samples, as shown in Table 4. It was likely due to the high temperature and pressure (0.3 MPa, 144°C and 0.6 MPa, 165°C) used that had caused

Table 4
Effect of steam explosion treatment on purified and un-purified rice bran fibre fractions

Rice bran	Time (min)	Steam explosion pressure (MPa)	Total fibre (%)	Insoluble fibre (%)	Soluble compounds (%) A	Reducing sugar (%) B	Soluble fibre (%) A-B
Un-purified bran	0	0.0	20.7±0.4	17.5±0.2^{A}	5.8±0.2^{C}	2.5±0.1^{A}	*3.2±0.6^{C}
	2	0.3	n/a	13.9±0.2^{B}	6.9±0.1^{B}	0.8±0.0^{C}	6.1±0.3^{B}
	2	0.6	n/a	10.9±0.3^{C}	9.9±0.4^{A}	1.1±0.0^{B}	8.8±0.6^{A}
Purified bran	0	0.0	65.9±4.4	49.3±2.7^{a}	22.0±1.7^{c}	5.4±0.0^{b}	*16.6±0.9^{c}
	2	0.3	n/a	27.1±2.9^{b}	38.9±0.7^{b}	6.6±0.1^{a}	32.3±0.4^{b}
	2	0.6	n/a	24.3±1.0^{c}	41.6±1.0^{a}	6.4±0.0^{a}	35.2±0.7^{a}

Note.
For untreated, the soluble fibre* is directly calculated by deducting total fibre from insoluble fibre.
For steam explosion treated, the soluble compounds (A) are calculated by deducting the total fibre of untreated samples from the insoluble fibre of steam explosion treated.
For steam explosion treated, the soluble fibre is calculated by deducting soluble compounds (A) to reducing sugar (B), A-B.
$^{A-C}$ = Means with different superscript letters within the same column of un-purified rice bran differ significantly (p<0.05).
$^{a-c}$ = Means with different superscript letters within the same column of purified rice bran differ significantly (p<0.05).
Values are means±S.D.; n = 3.

a further breakdown of the SF and reducing sugars into molecules such as furfural and hydroxymethylfurfural (HMF) (Álvarez et al., 2017), thus making it unable to be detected in the sugar analysis. Furfural and HMF can be formed from the dehydration of pentoses or hexoses due to intense SE treatment (Ramos, 2003).

Similar effects were also observed by Gong et al. (2012), who found that a reduction in soluble carbohydrates occurred after severe SE treatment (temperature range: 210-250°C; time range: 10-120 s) was applied to barley bran. Although the temperatures used in this study were lower, the treatment time was longer, thus making it possible for the formation of furfural and HMF that contribute to the browning effect on the samples, and this is confirmed by the colour analysis as shown in the previous work (Ismail & Zhao, 2022). Nevertheless, the amount of SF in the stream explosion treated samples increased from 6.10±0.34 to 8.83±0.56% as the pressure increased from 0.3 and 0.6 MPa, respectively.

The insoluble fraction of SE-treated purified RB showed the same decrease trend as the un-purified RB (Table 4), where treatment at the higher pressure caused a greater reduction in the amount of IF. In contrast, the level of SF showed a corresponding increase. However, the amount of reducing sugars showed an opposite trend to the un-purified rice bran, i.e., a significant (p<0.05) increase was observed after treatment at both 0.3 and 0.6 MPa. It could be due to the breakdown of side chains of the rice bran fibre, which

mainly consisted of arabinose, as in Table 1 and reported by Truong and Rumpagaporn (2019).

Changes in Molecular Weight Distribution of Rice Bran Soluble Fibre after Ultrasound and Steam Explosion Treatment

The molecular weights (MW) of the saccharides in the soluble fractions of purified RB fibre were measured by size-exclusion high-performance liquid chromatography (SE-HPLC).

Four peaks appeared in the chromatogram of the soluble fraction of untreated purified RB (Table 5), of which peak 4 was dominant while the other three peaks were much smaller in comparison. Peak 4 was eluted at 12.0 min, and the last peak eluded, indicating that it was the smallest oligosaccharide present (0.5 kDa). The second-largest peak was peak 1 (7.7 min), which had a molecular weight between 670 and 410 kDa.

After US treatment, both the number of peaks and their retention times changed from those of the untreated samples, demonstrating that the composition of the oligosaccharides in the soluble fractions had changed as a result of the treatment (Table 6). The soluble fraction produced after US treatment at 60% amplitude for 5–20 min gave an extra peak, eluted at 7.4 min, earlier than the first peak in the untreated sample, indicating a bigger molecular mass than the others. It demonstrated that the US treatment broke down some long-chain polymers (IF) into water-soluble shorter-chain polymers.

However, the area of peak 1' decreased as the treatment time increased. The same trend was also observed for peak 2'. Meanwhile, the area of peak 4' increased with increases in treatment time, demonstrating that some of the high molecular weight polymers were further broken down into lower molecular weight molecules.

After US treatment at 80% amplitude, four peaks appeared at 5.0, 8.3, 12, and 14.5 min, different from those in the untreated sample and those treated with US at 60% amplitudes. A polymer with a large molecular weight (>2,000 kDa) was released after the treatment (peak 1*), and the area of the peak increased as the treatment time increased from 5 to 20 min. Furthermore,

Table 5
Retention time and peak areas of the soluble fraction of untreated purified rice bran

Treatment	Peak	Retention time (min)	Calculated MW (kDa)	Area count of soluble polysaccharides or oligosaccharides ($\times 10^4$)
				Treatment time (min)
				0
Untreated	1	7.7±0.1	~724	1.2±0.1
	2	8.0±0.1	~617	0.2±0.0
	3	8.6±0.1	~537	0.4±0.1
	4	12.0±0.0	~0.5	4.7±0.0
Total area count ($\times 10^4$)				6.5±0.2

Table 6
Retention time and peak areas of soluble fraction of purified rice bran after ultrasound treatment

Ultrasound amplitude (%)	Peak	Retention time (min)	Calculated MW (kDa)	Area count of soluble polysaccharides or oligosaccharides (× 10^4)			
				Treatment time (min)			
				5	10	15	20
60	1'	7.4±0.1	~794	0.3±0.0^{a}	0.2±0.0^{b}	0.2±0.0^{c}	0.1±0.0^{c}
	2'	7.6±0.1	~724	0.2±0.0$^{a'}$	0.2±0.0$^{a'}$	0.1±0.0$^{b'}$	0.1±0.0$^{b'}$
	3'	8.5±0.0	~537	0.2±0.0^{A}	0.2±0.5^{A}	-	-
	4'	11.0±0.1	~6.8	1.0±0.1$^{B'}$	1.4±0.4$^{B'}$	2.9±0.2$^{A'}$	2.9±0.3$^{A'}$
	5'	12.0±0.0	~0.5	11.1±0.5^{A*}	12.7±0.4^{A*}	12.8±2.2^{A*}	13.7±1.3^{A*}
Total area count (× 10^4)				12.8±0.4	14.7±0.6	16.0±1.7	16.8±1.2
80	1*	5.0±0.0	>2,000	0.1±0.0^{c}	0.2±0.0^{b}	0.2±0.0^{b}	0.3±0.0^{a}
	2*	8.3±0.2	~537	0.1±0.0$^{b'}$	0.1±0.0$^{b'}$	0.1±0.0$^{b'}$	0.3±0.0$^{a'}$
	3*	12.0±0.0	~0.5	5.4±0.1^{C}	7.4±0.2^{A}	6.6±0.1^{B}	7.6±0.6^{A}
	4*	14.5±0.1	nd	-	-	0.3±0.0$^{B'}$	0.8±0.1$^{A'}$
Total area count (× 10^4)				5.6±0.1	7.7±0.2	7.2±0.2	9.0±0.7
95	1#	6.0±0.1	>2,000	-	-	-	0.3±0.0
	2#	7.5±0.1	~794	1.8±0.2$^{a'}$	0.9±0.1$^{c'}$	0.5±0.0$^{d'}$	1.5±0.0$^{b'}$
	3#	7.8±0.1	~724	-	0.4±0.0^{A}	0.2±0.0^{B}	-
	4#	8.4±0.2	~537	0.2±0.0$^{A'}$	0.2±0.0$^{A'}$	0.1±0.0$^{B'}$	0.2±0.0$^{A'}$
	5#	10.8±0.2	~10.7	0.2±0.1^{A*}	-	-	0.2±0.^{1A*}
	6#	12.0±0.0	~0.5	3.2±0.2$^{C\#}$	10.4±0.7$^{A\#}$	10.9±0.8$^{B\#}$	1.8±0.1$^{D\#}$
	7#	12.3±0.0	~0.3	-	-	-	0.1±0.0
	8#	13.4±0.0	nd	-	-	-	0.8±0.0
	9#	16.0±0.1	nd	-	0.3±0.1^{x}	0.2±0.0^{x}	-
Total area count (× 10^4)				5.4±0.6	12.2±0.9	11.9±0.8	4.9±0.1

Note.
$^{a\text{-}c}$ = Means with different letters within the same row differ significantly (p<0.05).
$^{A\text{-}C}$ = Means with different letters within the same row differ significantly (p<0.05).
$^{a'\text{-}d'}$ = Means with different letters within the same row differ significantly (p<0.05).
$^{A*\text{-}D*}$ = Means with different letters within the same row differ significantly (p<0.05).
$^{A'\text{-}C'}$ = Means with different letters within the same row differ significantly (p<0.05).
$^{A\#\text{-}D\#}$ = Means with different letters within the same row differ significantly (p<0.05).
$^{x\text{-}y}$ = Means with different letters within the same row differ significantly (p<0.05).
nd = Not determined.
Values are means±S.D.; n=3.

a smaller molecular weight (< 25 kDa) polymer was observed after the treatment for 15 and 20 min (Peak 4*). However, the 80% US amplitude is less effective in producing SF with smaller molecular weight compared to the 60% US amplitude, as the total area of peaks is much lower than the total area of peaks in 60% amplitude samples.

Ultrasound treatment at 95% amplitude produced nine peaks in the chromatogram.

Peak 1#, which was only observed in the sample treated for 20 min, was eluted at 6 min, which means that the polymer had a molecular weight bigger than 2,000 kDa. Peak 2# decreased after 10- and 15-min treatment but increased after 20 min-treatment. This peak was estimated to have a molecular weight of 794 kDa. The area count was increased for peak 6# (<25 kDa) from 5 to 10 min-treatment. However, a further increment in treatment time (15 to 20 min) caused a decrease in area count of peak 6#, indicating that re-polymerisation had occurred, and this result agreed with and supported the GC results in 4.3.1.2, where a decrease in the SF fraction was observed after the same treatment time. Peaks 4# and 6# had similar retention times with peaks 3' and 5' (in 60% amplitude samples) and peaks 2* and 3* (in 80% amplitude samples), eluted at 8.4 and 12.0 min, respectively. The area counts of peak 4# (8.4 min) did not show significant differences among the US amplitudes and treatment times. However, the area count of peak 6# (12.0 min) increased over the treatment time for each US amplitude, except for 95% amplitude, which decreased after 20 min of treatment.

In summary, US treatments caused a breakdown of some of the large polymers of IF into soluble polymers and some of the high molecular weight polymers of SF into lower molecular weight ones. The total area count ($\times 10^4$) increased by 60 and 80% US amplitude as treatment time increased. A contrasting trend was observed for the sample treated with 95% US amplitude, where the total area count ($\times 10^4$) increased after 5- and 10-min treatment and decreased when the treatment time was prolonged to 15- and 20-min. The highest total area count ($\times 10^4$), representing the highest amount of SF, was observed in samples treated at 60% US amplitude at 20 min. It eventually supports the results

Table 7
Retention time and peak areas of soluble fraction of purified rice bran after steam explosion treatment

Peak	Retention time (min)	Calculated MW (kDa)	Area count of soluble polysaccharides or oligosaccharides ($\times 10^4$)	
			Steam explosion pressure (MPa)	
			0.3	0.6
1*	7.3±0.1	~794	-	0.9±0.1
2*	8.2±0.1	~537	1.1±0.1	-
3*	8.7±0.3	~275	1.1±0.1^{b}	1.4±0.1^{a}
4*	13.7±0.1	<1	5.8±0.1$^{b'}$	6.2±0.0$^{a'}$
5*	14.6±0.0	<1	2.7±0.0^{A}	2.8±0.3^{A}
		Total area count ($\times 10^4$)	10.7±0.3	11.3±0.1

Note.
$^{a\text{-}b}$ = Means with different letters within the same row differ significantly (p<0.05);
$^{A\text{-}C}$ = Means with different letters within the same row differ significantly (p<0.05);
$^{a'\text{-}b'}$ = Means with different letters within the same row differ significantly (p<0.05);
Values are means±S.D.; n = 3; - = No peak detected.

in Table 3. The most abundant soluble polymers produced after the US treatment had molecular weights less than 25 kDa for all US amplitudes applied. However, the effect was not always cumulative as the US amplitude and treatment time increased.

Steam explosion-treated purified RB also showed changes in the molecular weight distribution of soluble fractions, as summarised in Table 7. Four peaks appeared in the chromatogram of the soluble fractions of purified RB fibre after the treatments at both 0.3 and 0.6 MPa; however, the retention times of the peaks differed for the samples treated at the two different pressures. The largest peaks were peak 4* for both samples, with the sample treated at 0.6 MPa, giving the larger area. The largest overall peak area of all the peaks was also obtained from the sample treated at 0.6 MPa.

The longer retention time of peak eluted is evidence of steam explosion treatment, which caused a breakdown of some of the polymers of the IF into soluble, smaller MW polymers. The steam explosion treatment also caused a loss of some SF due to the degradation of soluble polymers, which were seen as smaller peak areas compared to ultrasound-treated samples. The smaller MW oligosaccharides and/or by-products (i.e., hydroxymethylfurfural) produced by further breakdown of the SFs were unable to be detected by the SE-HPLC system as the limit of detection of the column used was 1 kDa. This finding agreed with the decrease in reduced sugar content, as discussed previously.

CONCLUSION

Results presented in this study showed that the ultrasound and steam explosion treatments can break down the IF of rice bran into SF with various molecular weights. The treatment intensity affected the SF yield; however, the relationship between ultrasound intensity and SF yield was still unclear. Generally, for US treatment at lower US amplitudes (60 and 80%), the SF yield increased as the treatment time increased. At high ultrasound amplitude (95%), the yield of SF increased initially with time but began to decline with prolonged treatment time (after 10 min). The amount of SF produced for SE treatment was directly proportional to the pressure applied (treatment intensity). The highest SF yield (35.2%) was obtained from US treatment at 60% amplitude for 20 min. The highest yield (35.2%) for SE treatment was obtained at 0.6 MPa SE pressure and 2 min treatment. The SF produced from both treatments mainly contained oligosaccharides with MW smaller than 25 kDa, with those produced by steam explosion treatment generally smaller than those by ultrasound treatment. Purifying rice bran by removing starch and proteins enhanced the efficiency of the ultrasound and steam explosion treatments in breaking down the IF into the SF.

DATA AVAILABILITY

The data used to support the findings of this study are available from the corresponding author upon request.

ETHICAL APPROVAL

No ethical approval is needed.

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Effect of Red Palm Olein and Glutinous Rice Flour Mixture as Fat Replacers on the Physicochemical, Rheological, and Microstructural Properties of Buffalo Meat Emulsion

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ABSTRACT

The present study evaluated the physicochemical, rheological, and microstructural properties of buffalo meat emulsion incorporated with red palm olein (RPO) and glutinous rice flour (GRF) as fat replacers. A total of six different samples were prepared by using combinations of RPO and GRF replacing animal fat viz., control (with animal fat), R1 (100% fat replaced by RPO), R2 (50% fat replaced by RPO), RG (50% fat replaced by 25% RPO and 25% GRF), G1 (100% fat replaced by GRF), and G2 (50% fat replaced by GRF). The samples were analysed for physicochemical parameters, gel strength, rheological and microstructural properties. G1 and G2 samples were recorded with improved emulsion stability, cooking loss, hardness, and chewiness while lowering the fat content. Micrographs of G1 and G2 samples demonstrated a spongy/honeycomb-like structure with roughly spherical crystalline granules. The rheological properties of G1 indicated a more stable emulsion with a higher storage modulus (G'). Using RPO as a fat replacer seemed to produce a lower-quality emulsion. Therefore, replacing the fat with GRF, especially at 100%, is proposed for buffalo meat emulsion.

Keywords: Buffalo meat, fat replacer, glutinous rice flour, meat emulsion, red palm olein

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INTRODUCTION

Fat plays a crucial and fundamental role in sensory and technological attributes such as water-holding capacity (WHC), emulsion stability, shear force, gel strength, and juiciness of the reformulated products. Processed meat products have long been known to contain a high proportion of saturated fat. However, animal fat intake containing high levels of saturated fatty acids and cholesterol has been associated with adverse effects on consumers' health, such as obesity, cardiovascular disease, and coronary heart disease (Asyrul-Izhar et al., 2023). Consumers are becoming more concerned about the amount and composition of daily fat and prefer healthier meat products rich in mono- and polyunsaturated fatty acids. As an alternative, reducing the fat content of meat emulsion and replacing animal fat with plant oils and carbohydrates as fat replacers proved to be an ideal strategy for producing healthier meat products.

Palm olein is a liquid fraction of palm oil containing 39%–45% higher oleic acid and 10%–13% higher linoleic acid than palm oil (Lin, 2002). Read palm olein (RPO) has a balanced ratio of saturated and unsaturated fatty acids and is rich in vitamin E (tocopherol [30%] and tocotrienol [70%]) and β-carotene (Dauqan et al., 2011). Thus, its incorporation in meat during processing could improve the functional value of the developed meat products as well as stabilize the meat emulsion system. Further, carbohydrate-based fat replacers are increasingly applied in producing low-fat meat products. When added to water, these carbohydrate-based fat replacers form hydrogels having quality attributes similar to animal fat used in high-fat meat products in terms of gel-like matrix stability, lubricity, texture, and moisture or water release (Asyrul-Izhar et al., 2023). Glutinuous rice flour (GRF) (*Oryza sativa* L.) has the potential to be used as carbohydrate gels in meat products. The kernels of the glutinous rice will become stickier, softer, and easier to adhere together during cooking/heat treatment (Keeratipibul et al., 2008). GRF has low amylose content but is high in amylopectin content; later, it could be explored to improve the texture of food products. GRF has good binding properties, thereby imparting higher water-retaining properties of foods with GRF. It could improve the stability and technological attributes of the emulsified meat product (Pereira et al., 2016).

Properly selecting fat replacers or a combination of fat replacers is crucial for developing low-fat meat products owing to the wide variation in meat types and formulations (Syan et al., 2022). The excellent water-binding properties of GRF could favour the stability of the emulsified meat product. In contrast, the desirable fatty acid profile in RPO could be used to develop healthier meat products. Thus, a combination of RPO and GRF could be used as fat replacers in a meat emulsion matrix to enhance the functionality of the fat replacer and improve the physicochemical/technological properties of meat emulsion properties, leading to the development of reformulated emulsified meat products.

Therefore, the present study was envisaged to evaluate various physicochemical,

microstructural, and rheological properties of buffalo meat emulsion added with RPO and GRF as animal fat replacers. The outcome of this research is expected to be beneficial in producing reformulated, healthier, low-fat, emulsified buffalo meat-based products.

MATERIALS AND METHODS

Raw Meat

Frozen boneless lean buffalo meat and fat were purchased from the Seri Kembangan wet market in Selangor, Malaysia. The meat and fat were packed in polyethene bags and stored in a freezer at -18°C prior to further processing. Frozen meat was used instead of fresh meat to imitate the current market practice adopted by the manufacturers of buffalo meat-based products.

Preparation of Meat Emulsion

Frozen boneless buffalo lean meat and fat were thawed under refrigeration and minced using a 5-mm plate meat mincer (H.L TJ12-A, Henglian, China). All ingredients (viz., 70% minced meat, 1.0% sugar (Central Sugars Refinery Sdn. Bhd., Malaysia), 1.0% garlic powder (Hexa Food Sdn. Bhd., Malaysia), 4.0% corn flour (THC® Sdn. Bhd., Malaysia), 0%–15% animal fat, 0%–15% red palm olein (CAROTINO, Malaysia), and 0%–15% glutinous rice flour (Erawan, Thailand) and mixed by using a food processor (YM-102, Gourmet Cuisine, China) except salt (1.2%) (Adabi Consumer Industries Sdn. Bhd., Malaysia), ice water (5%, temperature of 5°C), and sodium tripolyphosphate (STPP, 0.3%) (DChemie, Malaysia) and homogenized for 1 min at 220 ×*g*. Then, salt and STPP were added and homogenized for 30 s followed by ice water, and the mixture was homogenized for 30 s at 220 ×*g*. A total of six different formulations were prepared (Table 1) by using combinations of RPO and GRF viz., C (without fat replacers), R1 (100% animal

Table 1
The buffalo meat emulsion formulations with red palm olein (RPO) and glutinous rice flour (GRF) as fat replacers

Ingredients	Formulations (%)					
	C (Control)	R1 (100% RPO)	R2 (50% RPO)	RG (25% RPO and GRF)	G1 (100% GRF)	G2 (50% GRF)
Meat	70	70	70	70	70	70
Fat	15	0	7.5	7.5	0	7.5
RPO	0	15	7.5	3.75	0	0
GRF	0	0	0	3.75	15	7.5
Ice water (5°C)	7.5	7.5	7.5	7.5	7.5	7.5
Salt	1.2	1.2	1.2	1.2	1.2	1.2
Sodium tripolyphosphate	0.3	0.3	0.3	0.3	0.3	0.3
Sugar	1	1	1	1	1	1
Garlic powder	1	1	1	1	1	1
Corn starch	4	4	4	4	4	4
Total	100	100	100	100	100	100

fat replaced by RPO), R2 (50% animal fat replaced by RPO), RG (50% animal fat replaced by 25% RPO and 25% GRF), G1 (100% animal fat replaced by GRF), and G2 (50% animal fat replaced by GRF). The emulsion samples were transferred to the centrifuge tube and centrifuged (KUBOTA 3740, Japan) for 1 min at a speed of 610 ×*g* to eliminate the air bubbles. The samples were then stored at 4°C until subsequent analysis.

Physiochemical and Proximate Parameters

The pH values of the meat emulsion with different concentrations of GRF and RPO are determined according to the method described by M. A. Ismail et al. (2021). A 5 g homogenised buffalo meat emulsion sample was mixed with 45 ml of distilled water using a blender (MX-898M Panasonic, Malaysia), and pH value was recorded by using a pre-calibrated (at pH 4.0, 7.0, and 9.0) pH meter (3505 pH Meter Jenway, United Kingdom).

The percentage of cooking loss was calculated based on recording the emulsion weight before and after cooking. The following formula was used for cooking loss measurement:

$$\text{Cooking loss (\%)} = [(\text{Raw sample weight} - \text{Cooked sample weight}) / (\text{Raw sample weight})] \times 100\% \quad [1]$$

Water holding capacity was determined using a centrifuge as described by Köhn et al. (2015). The empty centrifuge tube was weighed, and 5 g of raw sample with 32 ml distilled water was added and mixed manually for 10 min before being centrifuged (KUBOTA 3740, Japan) at 2,900 × *g* for 25 min. Then, the supernatant in the centrifuge tubes was weighed and discarded. The pellet was dried in the hot air oven for 20 min at a temperature of 50°C. Then, the dried pellet was weighed, and WHC (%) was calculated according to the following equation:

$$\text{WHC (\%)} = \frac{(b-a)-(c-a)}{(b-a)} \times 100\% \quad [2]$$

where a represents the weight of the empty centrifuge, b is the weight of the centrifuge with supernatant, and c is the weight of the dried centrifuge (all weighed in g).

Emulsion stability was evaluated based on the procedure described by N. A. Ismail et al. (2021) with slight modifications to obtain the total fluid released and total fat released. The percentages of total fluid release and the total fat released were calculated as shown in the formulation below:

$$\text{Total fluid release} = \text{Initial weight of the sample} - \text{The weight of the pellet} \quad [3]$$

$$\text{Total fluid release (\%)} = (\text{Total fluid release}/\text{Initial weight of sample}) \times 100\% \quad [4]$$

$$\text{Total fat release (\%)} = (\text{Weight of dried supernatant} - \text{Total fluid release}) \times 100\% \quad [5]$$

The proximate analysis was performed according to the Association of Official

Analytical Chemists (AOAC) standard methods (Association of Official Analytical Chemists [AOAC], 2019). Moisture content was determined using a hot air oven (BINDER, Germany) and total protein (Crude protein, N × 6.25) was determined using the micro-Kjeldahl method.

Colour Determination

The colour of the raw and cooked buffalo meat emulsion samples was determined using a calorimeter (Chroma Meter CR-410, Japan) calibrated with the white tile supplied with the instrument. The samples were recorded for *L** (+lightness/-darkness), *a** (+redness/-greenness), and *b** (+yellowness/-blueness) colour coordinates (Ramle et al., 2021).

Texture Profile Analysis (TPA)

TPA was conducted on the cooked meat emulsion samples using a texture analyzer (TA-XT2i, Stable Micro System, United Kingdom). The hardness, cohesiveness, springiness, chewiness, gumminess, and resilience were determined. The cooked meat emulsion samples were cut into 10 mm length × 20 mm diameter for the analysis. A 75 mm compression platen (P75) was used with the test-speed setting of pre-test: 1.0 mm/s; test: 1.5 mm/s; post-test: 1.5 mm/s, where the sample was compressed twice with a 30-kg load from 75% of the original height (Ming-Min & Ismail-Fitry, 2023).

Gel Strength

The gel strength of the samples was evaluated using a method by Asyrul-Izhar et al. (2021) with slight modifications. The buffalo cooked meat emulsion samples were cut at 15 mm length × 20 mm diameter, and a test speed of 1.5 mm/s by using a Warner-Bratzler (WB) shear blade and connecting with a texture analyser (Stable Micro System, TA-XT2i, United Kingdom) was used to determine the maximum shear force (N).

Rheological Analysis

The dynamic rheological properties of the meat emulsions were continuously monitored and replicated according to Verma et al. (2019) with slide modifications using a rheometer (Rheostress RS600, USA) fitted with a 25 mm diameter stainless steel plate with a 1 mm size gap from the rotating cone. The samples were gently placed onto the plate using a plastic spoon and allowed to set the temperature at 25°C for 5 min. The frequency sweep test was run with a 0.1–10 Hz range at 1 Pa. The changes in storage modulus (G′) and loss modulus (G″) were recorded during the processing.

Scanning Electron Microscope (SEM)

The microstructures of six cooked meat emulsions with different RPO and GRF formulations were analysed using SEM. The preparation of samples was conducted for 24 hr by fixing in 2.5% glutaraldehyde (Chemiz, Malaysia) at 4°C and then in 2% aqueous osmium tetroxide (Systerm Chemicals, Malaysia) for 4 hr. After that, samples were dehydrated in increasing concentrations of ethanol (Chemiz, Malaysia) and dried in tert-butyl alcohol. The sample was applied with a gold coat using a sputter coater and

viewed at 1,000× magnification under the SEM (Model: JOEL-JSM 5600, Japan).

Statistical Analysis

All the data were analysed statistically using MINITAB Statistical Software version 19 (MiniTab Inc., USA). The whole experiment was repeated in triplicate. One-way analysis of variance (ANOVA) with Tukey's tests was performed at the 95% confidence level ($p<0.05$) to assess significant differences between the data collected. The results obtained in the study were expressed as the mean values ± standard deviation.

RESULTS AND DISCUSSION

pH

The pH value of the raw control emulsion was to be comparable ($p>0.05$) to all other treatments. The pH value was recorded highest for R1, which was significantly ($p<0.05$) higher than G2 samples, later showing the lowest pH value (Table 2). It could be due to the higher pH value of vegetable oils (6.9 to 6.7). The processing of RPO caused a further reduction of pH to 6.07 due to the hydrolysis of oil triglyceride, leading to the production of free fatty acids (FFAs) and glycerol (Baig et al., 2022). The pH values for the cooked samples were not significantly different ($p>0.05$) between the treatments and the control, and all samples showed an increased pH compared to the raw samples. The increase in pH value upon cooking might be due to protein denaturation and the production of imidazolium compounds upon cooking (Verma et al., 2022). Similar results were reported by Khalid et al. (2021), where the

Table 2
The physicochemical attributes of buffalo meat emulsions with red palm olein (RPO) and glutinous rice flour (GRF) as fat replacers

Sample	pH		Emulsion stability (%)		Cooking loss (%)	WHC (%)	Gel strength (N)
	Raw	Cooked	Total fluid release	Total fat release			
C	5.74 ± 0.15^{AB}	6.14 ± 0.14^{A}	9.79 ± 1.64^{BC}	5.440 ± 0.612^{BC}	0.21 ± 0.03^{AB}	84.59 ± 0.43^{A}	2.06 ± 0.55^{A}
R1	6.07 ± 0.05^{A}	6.11 ± 0.29^{A}	18.79 ± 8.05^{AB}	19.51 ± 11.17^{A}	0.76 ± 0.42^{AB}	84.44 ± 0.56^{A}	1.78 ± 0.33^{A}
R2	5.90 ± 0.09^{AB}	6.02 ± 0.06^{A}	23.12 ± 2.73^{A}	15.48 ± 2.93^{AB}	1.27 ± 0.92^{A}	84.97 ± 0.31^{A}	1.45 ± 0.25^{A}
RG	5.97 ± 0.15^{AB}	6.19 ± 0.08^{A}	5.24 ± 0.58^{C}	3.690 ±0.669^{BC}	0.47 ± 0.05^{AB}	84.10 ± 0.72^{A}	1.93 ± 0.28^{A}
G1	5.90 ± 0.13^{AB}	6.11 ± 0.21^{A}	2.59 ± 0.65^{C}	0.800 ± 0.674^{C}	0.076 ± 0.008^{B}	83.49 ± 0.49^{A}	3.79 ± 0.40^{A}
G2	5.68 ± 0.17^{B}	5.89 ± 0.14^{A}	3.99 ± 0.44^{C}	2.347 ± 0.617^{C}	0.76 ± 0.22^{AB}	84.98 ± 0.71^{A}	3.01 ± 0.25^{A}

Note. All values are mean ± standard deviation of three replicates. Means that do not share the same letter but are significantly different ($p<0.05$) in the same column. WHC = Water holding capacity; C = Without fat replacer; R1 = 100% animal fat replaced by RPO; R2 = 50% animal fat replaced by RPO; RG = 50% animal fat replaced by 25% RPO and 25% GRF; G1 = 100% animal fat replaced by GRF; G2 = 50% animal fat replaced by GRF

pH values from uncooked to cooked beef patties samples have a significant increase in mixtures of wheat bran fibre and wheat germ oil as fat replacers.

Emulsion Stability

The incorporation of RPO and GRF as fat replacers had a significant impact on total fluid release and total fat release (Table 2). Lower (p<0.05) total fluid release and total fat release values were recorded in G1 and G2 emulsions compared to control and R1 and R2 samples. The RG samples had marginal (p>0.05) lower total fluid release and total fat release values as compared to control samples. It could be due to the formation of a fully gelled matrix of the protein-starch gel (Verma et al., 2015) in the GRF-incorporated buffalo meat emulsion, thereby enhancing water-binding and forming a stable emulsion. Upon heating, further expansion of starch granules increases pressure on the protein matrix, leading to higher emulsion stability. A similar significantly lower total fluid release and total fat release were also reported by Pereira et al. (2019) in low-fat sausage with increasing rice flour levels.

Cooking Loss

The G1 samples (100 % animal fat replaced by GRF) were recorded with the lowest cooking loss as compared to other treatments and significantly (p<0.05) lower than R2 (Table 2). The cooking loss of all remaining samples (C, R1, G2, and RG) was recorded as comparable (p>0.05). Its lower cooking loss in G1 samples could be due to the high content of starch (83-91%) in GRF, leading to more binding with water and swelling/ expansion as well as gelatinisation of starch granules in the embedded protein gel matrix during heating process (Wu et al., 2020). Yi et al. (2012) also noticed the lowest cooking loss in samples with GRF compared to other formulations with corn flour, rice flour, and soy protein isolates.

Water Holding Capacity

WHC is described as the capability of meat to bind internal and external water during the application of any method of operation (Cheng & Sun, 2008). No significant difference (p>0.05) was observed in the WHC of the emulsion prepared using different levels of RPO and GRF mixtures and the control samples (Table 2). Similar findings of a marginal change in WHC were reported in sausage added with a variety of vegetable oil ratios of canola, sunflower, and olive oils by Shin et al. (2020). The fats and oil help improve the water-holding capacity of meat products by acting as binders, holding water molecules in the product, and preventing them from being lost during processing. Similarly, the GRF starch may absorb the excess moisture and maintain the water-holding capacity. Besides, as all buffalo meat emulsion formulations in the present study use the same quantity of salt and STPP, the WHC of the meat emulsion was stabilised.

Gel Strength

Similar to water-holding capacity results, no statistically significant difference (p>0.05)

was observed in the gel strength of the buffalo meat emulsion prepared using different concentrations of RPO and GRF mixtures and the gel strength of the control samples (Table 2). It might be due to the myofibrillar network forming to its maximum extent when cooked treatment is applied, affecting the meat emulsion's gel strength. According to Tang et al. (2019), myofibrillar protein had an impact on the meat product's gel strength. Since all formulations in this study had the same amount of meat protein, no discernible changes in the gel strength of the meat emulsion properties were observed as a result.

Proximate Analysis

The incorporation of RPO and GRF as fat replacers did not affect (p>0.05) proximate parameters viz., moisture, ash content, crude protein, and crude fibre content except fat and carbohydrate (Table 3). It could be attributed to the incorporation of the RPO and GRF having similar content as well as the presence of a similar amount of meat in all samples prepared. A non-significant difference in the protein content was also observed between the control and different formulations, which could be attributed to the similar meat protein level of the formulation in terms of buffalo meat. For fat content, a significantly higher (p<0.05) amount of fat was observed in R1 and R2 samples, which might be attributed to the incorporation of red palm olein having higher fat content. The carbohydrate content in G1 samples was significantly higher compared to other formulations due to 100% replacing animal fat with GRF rich in carbohydrates (83%–91%). Nevertheless, the fat content in G1 reduced significantly (p<0.05) compared to others. In terms of good fat, R1 and R2 might be suitable as fat replacers. However, G1 can be used to produce meat products with a low-fat label because the fat content is below 3%.

Colour Profile

The raw meat emulsion showed significantly (p<0.05) higher lightness (L*) values in the

Table 3
Proximate analysis of buffalo meat emulsions with red palm olein (RPO) and glutinous rice flour (GRF) as fat replacers

Sample	Moisture (%)	Ash (%)	Protein (%)	Fat (%)	Fibre (%)	Carbohydrate (%)
C	61.47 ± 4.37^{A}	2.32 ± 0.12^{A}	14.19 ± 1.12^{A}	5.98 ± 1.07^{BC}	0.45 ± 0.28^{A}	15.59 ± 4.95^{AB}
R1	56.15 ± 1.07^{A}	2.32 ± 0.42^{A}	13.99 ± 0.91^{A}	12.67 ± 1.08^{A}	0.66 ± 0.16^{A}	14.21 ± 1.39^{B}
R2	54.81 ± 4.94^{A}	2.84 ±0.91^{A}	14.66 ± 0.77^{A}	11.27 ± 1.90^{A}	0.58 ± 0.32^{A}	15.84 ± 4.88^{AB}
RG	57.37 ± 5.99^{A}	2.33 ± 0.16^{A}	13.08 ± 2.02^{A}	9.09 ± 1.69^{AB}	0.82 ± 0.09^{A}	17.31 ± 9.24^{AB}
G1	53.89 ± 0.63^{A}	3.48 ± 0.16^{A}	13.22 ± 1.81^{A}	1.72 ± 1.56^{C}	0.56 ± 0.43^{A}	18.79 ± 1.97^{A}
G2	61.46 ± 2.16^{A}	2.44 ± 0.36^{A}	14.38 ± 1.35^{A}	4.82 ± 1.88^{BC}	0.37 ± 0.21^{A}	16.53 ± 1.38^{AB}

Note. All values are mean ± standard deviation of three replicates. Means that do not share the same letter but are significantly different (p<0.05) in the same column. C = Without fat replacer; R1 = 100% animal fat replaced by RPO; R2 = 50% animal fat replaced by RPO; RG = 50% animal fat replaced by 25% RPO and 25% GRF; G1 = 100% animal fat replaced by GRF; G2 = 50% animal fat replaced by GRF

G1 sample as compared to R1 (Table 4). It could be due to the higher flour content in G1 contributing to the white colour. The redness (*a** value) for both raw and cooked samples was observed to be comparable ($p>0.05$) for all samples. However, yellowness (*b** values) was noted significantly ($p<0.05$) higher in the raw and cooked R1 samples as compared to other samples ($p<0.05$). The higher carotene in RPO might be attributed to the higher yellow colour in R1 samples (Ayu et al., 2016). A higher *b** value of ice cream incorporated with higher red palm olein content was also reported by A. H. Ismail et al. (2020). The higher lightness of control samples could be attributed to the presence of animal fat, which had a diluting effect on myoglobin (Serdaroğlu et al., 2016).

Texture Profile Analysis

The higher amounts of GRF in cooked meat emulsion were observed to increase ($p<0.05$) the hardness, chewiness, and gumminess (Table 5). It could be attributed to the higher starch in GRF samples, which led to changes in textural attributes. The high level of amylopectin in GRF caused starch granules to swell and be embedded in the gel matrix, thus leading to a stronger and denser structure (Khalil, 2000). A similar finding was reported by Pereira et al. (2016, 2019); the additional high amount of rice flour in the sample resulted in a significantly increased hardness value of beef sausages compared to the control and with other formulations.

There was no significant ($p>0.05$) difference in springiness, cohesiveness, and resilience properties among all samples. All buffalo meat emulsions were prepared using the same salt, water, and sodium tripolyphosphate, explaining their comparable cohesiveness, springiness, and resilience properties. Similar findings have shown comparable springiness and

Table 4
Colour profile of buffalo meat emulsions with red palm olein (RPO) and glutinous rice flour (GRF) as fat replacers

Sample	Colour profile					
	Raw emulsion			Cooked product		
	*L**	*a**	*b**	*L**	*a**	*b**
C	50.89 ± 0.69^{CD}	14.77 ± 4.67^{A}	17.86 ± 1.00^{C}	50.91 ± 0.73^{A}	9.55 ± 0.20^{A}	17.20 ± 0.97^{BC}
R1	48.48 ± 0.33^{D}	21.07 ± 2.44^{A}	32.89 ± 0.85^{A}	48.05 ± 1.84^{B}	10.66 ± 0.88^{A}	26.14 ± 2.96^{A}
R2	50.05 ± 1.58^{CD}	16.22 ± 4.57^{A}	29.46 ± 0.49^{B}	48.65 ± 0.89^{AB}	10.41 ± 0.53^{A}	25.24 ± 0.89^{A}
RG	52.71 ± 1.25^{BC}	15.44 ± 3.63^{A}	28.05 ± 1.02^{B}	47.04 ± 0.43^{B}	9.44 ± 0.28^{A}	20.69 ± 0.63^{B}
G1	56.97 ± 1.81^{A}	14.63 ± 1.63^{A}	19.98 ± 0.95^{C}	47.40 ± 0.67^{B}	10.34 ± 0.80^{A}	15.06 ± 1.00^{C}
G2	55.90 ± 0.72^{AB}	14.86 ± 1.01^{A}	19.33 ± 0.07^{C}	48.47 ± 0.61^{AB}	9.32 ± 0.59^{A}	15.24 ± 0.94^{C}

Note. All values are mean ± standard deviation of three replicates. Means that do not share the same letter but are significantly different ($p<0.05$) in the same column. C = Without fat replacer; R1 = 100% animal fat replaced by RPO; R2 = 50% animal fat replaced by RPO; RG = 50% animal fat replaced by 25% RPO and 25% GRF; G1 = 100% animal fat replaced by GRF; G2 = 50% animal fat replaced by GRF

Table 5
Texture profile of buffalo meat emulsions with red palm olein (RPO) and glutinous rice flour (GRF) as fat replacers

Sample	Texture Profile Analysis					
	Hardness (g)	Springiness (mm)	Cohesiveness	Gumminess (g)	Chewiness (g)	Resilience
C	14979 ± 2446^{B}	0.75 ± 0.02^{A}	0.43 ± 0.06^{A}	6517 ± 1518^{ABC}	4899 ± 1248^{B}	0.15 ± 0.04^{A}
R1	14736 ± 2498^{B}	0.85 ± 0.01^{A}	0.42 ± 0.05^{A}	6282 ± 1482^{BC}	5380 ± 1345^{B}	0.13 ± 0.02^{A}
R2	11394 ± 1834^{B}	0.79 ± 0.03^{A}	0.43 ± 0.03^{A}	4995 ± 1161^{C}	3969 ± 821^{B}	0.13 ± 0.01^{A}
RG	14878 ± 1809^{B}	0.76 ± 0.04^{A}	0.39 ± 0.02^{A}	5958 ± 952^{BC}	4577 ± 935^{B}	0.13 ± 0.01^{A}
G1	25737 ± 5282^{A}	0.78 ± 0.09^{A}	0.52 ± 0.08^{A}	13572 ± 4036^{AB}	10467 ± 2126^{A}	0.20 ± 0.04^{A}
G2	25413 ± 3057^{A}	0.79 ± 0.06^{A}	0.53 ± 0.13^{A}	13745 ± 4594^{A}	10777 ± 3095^{A}	0.20 ± 0.06^{A}

Note. All values are mean ± standard deviation of three replicates. Means that do not share the same letter but are significantly different ($p<0.05$) in the same column. C = Without fat replacer; R1 = 100% animal fat replaced by RPO; R2 = 50% animal fat replaced by RPO; RG = 50% animal fat replaced by 25% RPO and 25% GRF; G1 = 100% animal fat replaced by GRF; G2 = 50% animal fat replaced by GRF

cohesiveness values upon incorporating rice flour as a fat replacer in sausages (Pereira et al., 2019) and sunflower oil as a replacer to pork backfat in frankfurters (Panagiotopoulou et al., 2016). The gumminess value, a derivative of hardness and cohesiveness, of RPO-incorporated samples, was recorded significantly ($p<0.05$) lower values, with R2 recorded as the lowest value. It could be attributed to the liquid phase of RPO replacing solid animal fat, resulting in droplets acting as "fillers" in the voids of the protein gel matrix (Wu et al., 2009). It indicated that RPO-incorporated meat emulsion was softer and juicier on the palate, allowing it to deform more easily in the mouth. Lower gumminess values in pork grease formulation prepared by partially replacing palm olein and soybean isolate were also reported by Barragan et al. (2018). Therefore, 50 and 100% replacement of animal fat with GRF resulted in a higher value of textural properties of meat emulsion. However, 50% animal fat replacement by RPO could be a good choice in terms of consumers' health as well as the palatability to form a softer buffalo meat emulsion.

Rheological Properties

The rheology properties of the frequency sweep study with storage (G') and loss (G'') modulus against the frequency (Hz) of buffalo meat emulsion are presented in Figure 1. All the formulations showed a similar trend: G' increased with higher frequency values and were higher than G". Kumar et al. (2017) reported that G' and G" refer to the ability of the emulsions and protein gel matrix's intermolecular interactions. The G′ represents a viscoelastic material's solid-like characteristics, whereas G″ indicates its liquid-like characteristics.

Thus, this study showed that incorporating RPO and GRF in the meat emulsion affected their network structure and caused a more elastic characteristic than

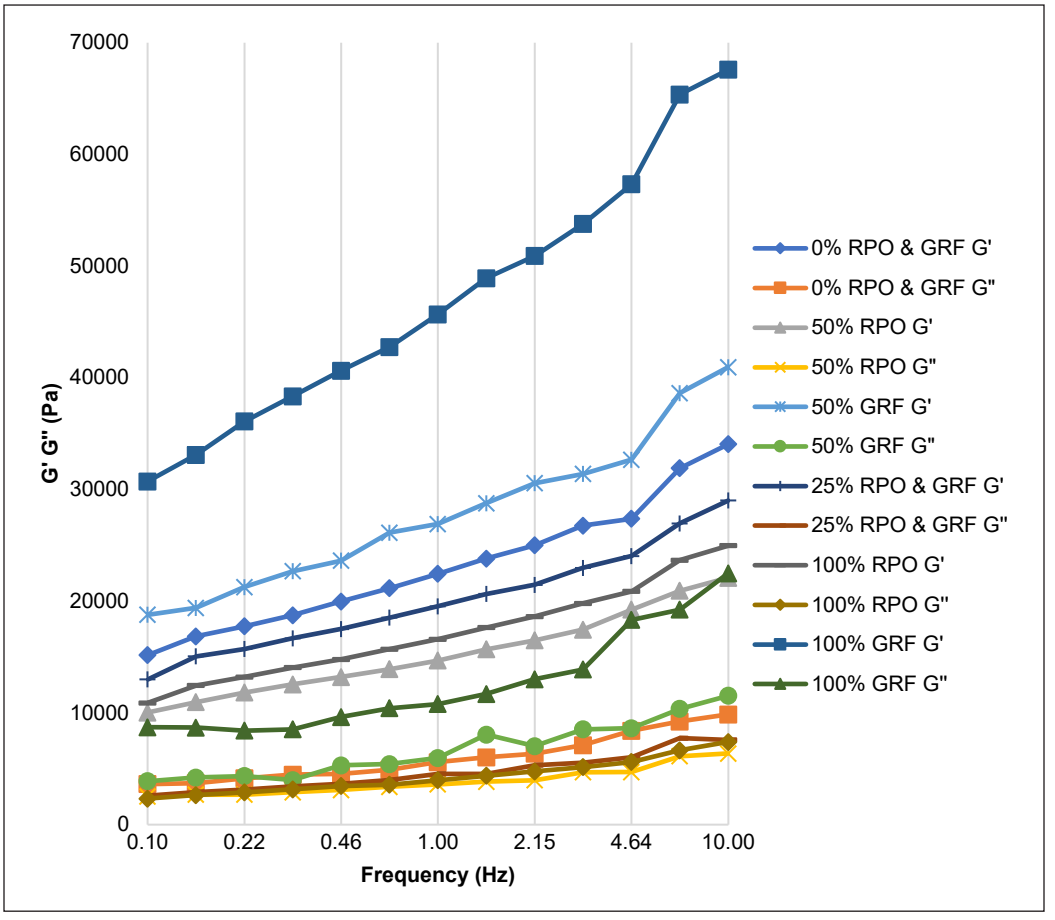

Figure 1. Storage modulus (G') and loss modulus (G") of the buffalo meat emulsion with different levels of red palm olein (RPO) and glutinous rice flour (GRF)

viscous. Apart from that, the value of G' for meat emulsion with 100% GRF (G1) was higher compared to the other formulations, which indicated that the intermolecular connections were stronger and the matrix was more stable. Similar research was stated by Verma et al. (2019), where formulations of 3% quinoa have high values of G′ for the meat emulsion due to the formation of solid-like characteristics. However, the RG sample had the most similarity to the control samples in terms of G' and G" on the rheological properties.

Emulsion Micrograph

The microstructure of buffalo meat emulsion formulated with animal fat is presented in (Figure 2C) and with fat replacers in (Figure 2: R1, R2, RG, G1, and G2). The micrographs demonstrated the microstructural properties of the cooked meat-emulsion systems with a gel-like compact structure of the meat batter. C (control) had the smoothest surface, was porous, and consisted of a coarse structure with numerous smallest cavities, representing very well-emulsified microstructural properties. The cavities

influenced the capillary action, which is responsible for water being held inside while heating the meat emulsion (Liu et al., 2016). Apart from that, G2 and G1, which consist of 50 and 100% animal fat replaced by GRF, showed a spongy (honeycomb-like) structure with roughly spherical crystalline granules. It might be due to the presence of starch content in GRF. The higher starch presence in meat emulsion had a few

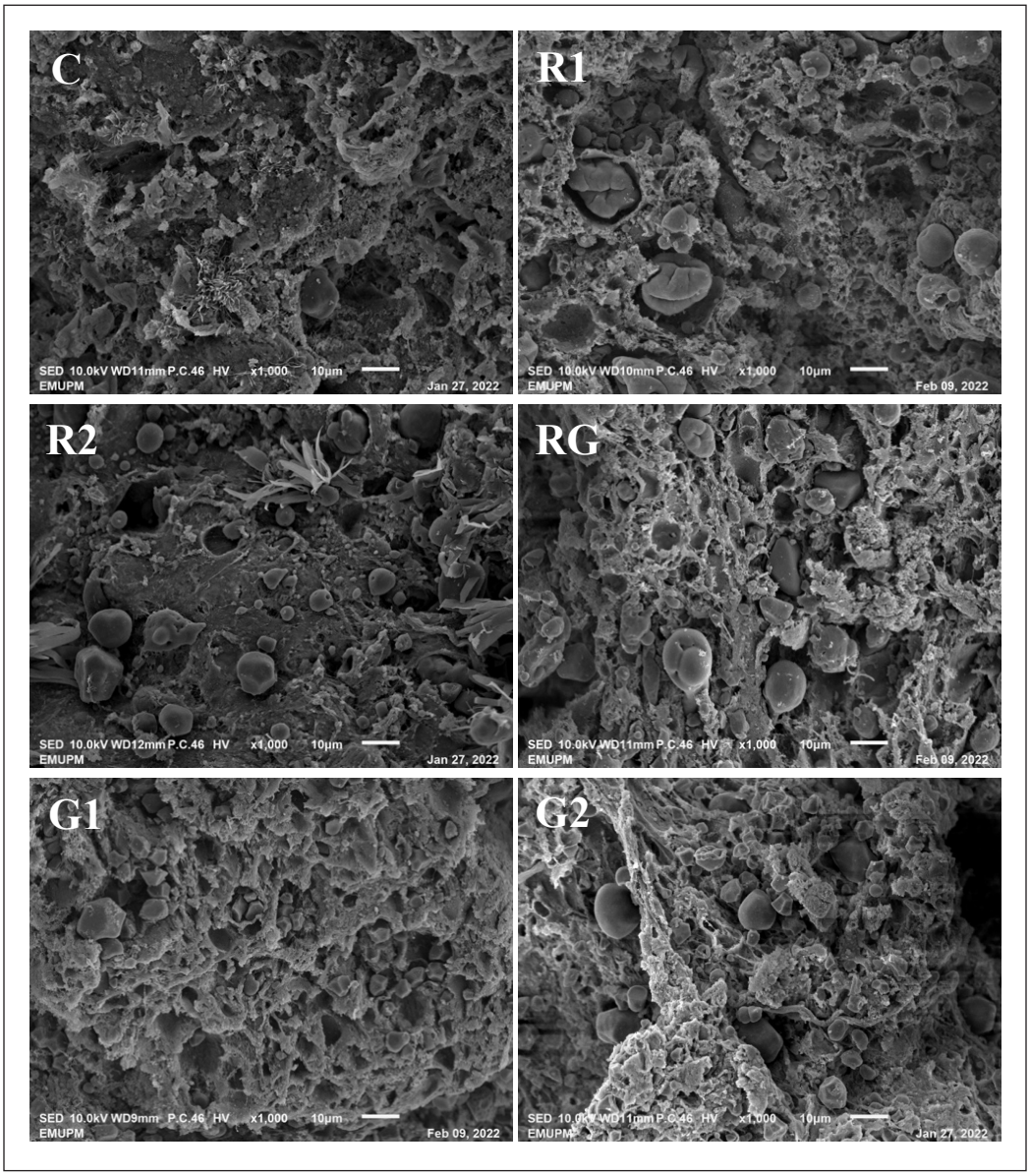

Figure 2. Scanning electron micrograph of buffalo meat emulsions (1,000× magnification). C = Without fat replacer; R1 = 100% animal fat replaced by *red palm olein (RPO)*; R2 = 50% animal fat replaced by RPO; RG = 50% animal fat replaced by 25% RPO and 25% glutinous rice flour (GRF); G1 = 100% animal fat replaced by GRF; G2 = 50% animal fat replaced by GRF

cavities that were smaller in size. As the amount of starch in the protein matrix is high, it becomes significantly more compact due to starch granule expansion and swelling during high-temperature processing.

CONCLUSION

Based on the study, it can be concluded that up to 100% animal fat replacement by GRF (G1) is the best formulation that can be replaced in buffalo meat emulsion, which significantly affected the emulsion stability, cooking loss, hardness, and chewiness of the texture emulsion, lowering the fat content, but produced the most stable meat emulsion, followed by 50% GRF (G2) as the second-best emulsion. However, less stable emulsions were found in the 50% RPO (R2) and 100% RPO (R1), but in terms of health preferences, these formulations could lower cholesterol and provide health benefits, as well as palatability with softer buffalo meat emulsion. The RG samples (mixture of 25% RPO and 25% GRF) had the most similarity to the control in terms of rheological properties. Meanwhile, WHC, gel strength, springiness, cohesiveness, moisture, ash, and fibre content were not significantly different between all the fat replacers and control formulations. Since G1 was the best formulation, it should be known as buffalo meat batter rather than meat emulsion due to the lack of fat. In addition, although meat batter without fat can still have good texture and flavour and can be used for certain applications such as meatballs or meatloaf, the sensory of other meat products with normally high amounts of fat, such as sausages, might be affected. Therefore, the sensory of buffalo meat products produced using the G1 formulation needs to be tested for future work.

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Optimising Okra (*Abelmoschus esculentus* L. Moench.) Fruit Yield and Physiological Responses Through the Integration of Foliar Fertiliser at Different Timings

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ABSTRACT

Okra is an important vegetable crop with high nutritional and economic value. Various approaches have been attempted to increase its production, including foliar fertilisers. This study aims to determine the optimal time to apply foliar fertiliser in a day to improve the final yield in *Abelmoschus esculentus* L. var. Torpedo and evaluate okra's growth and yield responses by integrating foliar fertiliser as supplementary fertiliser with granule fertiliser in a controlled environment. The study was conducted in a rain shelter under a randomised complete block design with 3 blocks comprising 3 replications within each block. The treatments involved a combination of foliar and granule application at different timings: sunrise, midday, and dusk, and solely granule fertiliser at labelled recommended rates. The control group was treated with only granule fertiliser at the same rate as the combined foliar and granule application. The study found that the application of foliar fertiliser during dusk (6–7 p.m.) showed a higher trend of potential yield, which revealed the potential of dusk foliar fertiliser application timing as the optimal

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timing for foliar fertilisation. The study also demonstrated that incorporating liquid foliar fertiliser with granular fertiliser can enhance nutrient uptake and promote plant growth, leading to a more sustainable farming practice by reducing inorganic soil granule fertilisers. The potential yield under the combined fertiliser treatments was 5% to 20% higher when compared to experiment control while comparable to the conventional fertiliser application treatment, despite using 45% less nitrogen. Therefore, the study suggests that farmers integrate foliar and soil fertilisation methods to achieve optimal crop yield production and promote sustainable farming.

Keywords: *Abelmoschus esculentus* L., foliar fertiliser timing, growth, integration fertiliser application, yield responses

INTRODUCTION

Okra (*Albelmoschus esculentus* L. Moench), also known as ladyfinger, is a flowering shrub that belongs to the Malvaceae family. Okra is cultivated globally in tropical and warm temperate regions for the nutritious, fibrous fruit pods (Anwar et al., 2020). Okra pod is one of the most affordable sources of vitamins, minerals, and antioxidants essential for good health (Schreinemachers et al., 2018). According to the United States Department of Agriculture (2019), each 100 g of raw okra pod contains 89.6 g water, 33 kcal energy, 1.93 g protein, 7.45 g carbohydrates, and 0.19 g fat. Also, raw okra is a rich source of dietary fibre, vitamin C, vitamin K, and antioxidants.

Okra is among Malaysia's ten most consumed vegetable crops, with 1.7 kg per capita consumption (Department of Statistic Malaysia [DOSM], 2021). However, Malaysia is still importing okra, and the demand for okra has risen over the past several years. It is reflected in the increment of okra per capita consumption (PCC) (DOSM, 2018, 2020, 2021). Therefore, improving the local production of vegetables is imperative to mitigate this inadequacy. Several factors, such as soil nutrition, seed quality, climate, cultural practices, and the use of plant growth regulators, influence the growth and yield of okra (Shahid et al., 2013), and inadequate soil nitrogen has been identified as a leading cause of low yield, hindering okra production (Ajayi et al., 2017).

Applying foliar fertilisers is one of the many ways to improve crop quality, yield, and metabolism (Fernández & Brown, 2013). Foliar fertiliser or foliar feeding is a technique of feeding plants by applying liquid fertiliser directly to the leaves instead of in the soil. Typically, foliar application has been used to supply major or minor nutrients, plant hormones, and other supplemental matters. Moreover, the integration of foliar fertiliser into a conventional granule fertiliser regimen has been shown to increase the yield of okra crops (Abbasi et al., 2010; Afe & Oluleye, 2017) while simultaneously reducing the need for inorganic soil granule fertilisers and, thus, promotes more sustainable farming practice by minimising soil salinity accumulation (Niu et al., 2020). However,

the penetration and absorption of foliar-applied nutrients into the plant surfaces are affected by various environmental factors such as light, temperature, and relative humidity (Fernández et al., 2013), and these environmental factors vary throughout the day.

Current knowledge on the effects of differential foliar timing application in a day on crop yield is limited. Previous studies on the optimum timing of foliar application for enhancing crop yield have focused mainly on certain plant growth stages, such as early tillering for wheat (Peirce et al., 2019) or a certain number of days after planting for okra (Mehraj et al., 2015). This study aims to determine the optimum time to apply foliar fertiliser in a day to improve the final yield in *A. esculentus* L. var. Torpedo (okra) and evaluate okra's growth and yield responses by integrating foliar fertiliser in a controlled environment. It hypothesises that the leaves' uptake and assimilation to nutrients obtained through foliar application would be more responsive at a certain time during the day, and there is a specific time for the foliar application that would improve okra's yield and harvest quality. Also, it is hypothesising that the combined use of foliar and conventional granule fertiliser regimens has been demonstrated to produce a superior crop yield, even when applied at lower rates than the use of granule fertiliser alone. Understanding the contribution of granular and foliar liquid NPK fertilisers to the growth and final yield of okra is essential to recommend it to farmers for improving crop production.

MATERIALS AND METHODS

Experimental Location and Growing Conditions

The experiment was conducted in a rain shelter located at Field 15, Faculty of Agriculture, UPM, with a latitude of 2.99°N and a longitude of 101.74°E. The seeds were sown and grown under rain shelter conditions with an average temperature and relative humidity of 29.2°C and 75.57%, respectively.

Planting Materials and Media

The okra seeds cv. 551 Torpedo (Sin Seng Huat Seeds, Malaysia) were incubated in a plastic container at 100% relative humidity (RH). After 24 hr, the seeds of okra cv. Torpedo was sown in a 12-hole germination box with a box size of 19 cm (L) × 11 cm (W) × 14.5 cm (H), and each planting hole sized 4 cm (L) × 4 cm (W) × 5.5 cm (D). The medium used in the germination tray and polybag was a mixture of silty clay soil (0.80% sand, 54.20% silt, and 45.10% clay, 1.30% organic carbon, pH 4.74) with local commercial soil (Smart Grow Plant Lover 6 in 1 Organic Soil Potting Mix, Baba, Malaysia) at 1:1 volume ratio. Each germination tray was placed in a reservoir dish containing only tap water. After 7 days after sowing (DAS), the okra seedlings were transplanted from the germination tray into a 40.64 cm × 40.64 cm polybag and maintained until 57 DAS.

Agronomic Practices

The agronomic practices were conducted as growth maintenance to maintain the okra

plants throughout the experiment. These practices included irrigation, pest control, and weeding. The irrigation method is based on a bottom-up concept by placing the plants in a custom-made reservoir about 1 m in diameter the size of the water reservoir. Water in the reservoir was replenished every 1–3 days, depending on the water level. Pest control was carried out using organic pesticides and installing yellow sticky traps. Wood vinegar was sprayed on the plant foliage as an organic pesticide at a concentration of 5% (v/v) with an application frequency of once a week.

Treatment Applications

The experiment involved five different fertiliser treatments, with the first three involving the combination of foliar and granule fertiliser applied at different times: sunrise, midday, and dusk. The foliar spray (Agroniche, Malaysia) was adjusted to an electrical conductivity (EC) of 1.8–2.2 mS/m (Toh et al., 2022) and applied at a rate of 10 s/plant (approximately 40 ml) with a nitrogen rate of 2.4 kg N/ha. The granular NPK 16-10-10 fertiliser (Sin Seng Huat Seeds, Malaysia) was applied at 28 kg N/ha with 0.86 g per polybag, resulting in a total nitrogen rate of 30.4 kg N/ha. The fourth treatment involved the conventional fertiliser method, using only granular NPK 16-10-10 fertiliser (Sin Seng Huat Seeds, Malaysia) at the product-labelled recommended rate of 350 kg/ha (56 kg N/ha), applied at 1.72 g/polybag. The fifth treatment was the experiment control, which used only granular NPK 16-10-10 (Sin Seng Huat Seeds, Malaysia) at 30.4 kg N/ha, applied at 0.93 g/polybag. The fertilisers were applied to okra plants beginning 14 days after sowing and repeated weekly until the plants reached the reproductive stage 35 days after sowing. A 1% (v/v) concentration of dimethyl sulfoxide (DMSO) (Ninelife, Malaysia) was added to the solution as an adjuvant to enhance the effectiveness of the foliar spray. The summary of the different fertiliser treatments used in the experiment is presented in Table 1.

Data Collection

Light Spectrum Profile. The rain shelter's light spectrum readings were recorded using a spectrometer (LI-180; LI-COR Inc., USA) according to the treatment time (sunrise, midday, and dusk). The spectrometer was placed 110 cm above the ground, and three measurements were taken each time at three sections of the rain shelter, namely the top,

Table 1
Summary of treatment application

No.	Treatment	Fertiliser type/combination	Time of application	Rate
1	F:GSun	Combined fertiliser application (foliar + granule)	Sunrise (7–8 a.m.)	30.4 kg N/ha
2	F:GMid		Midday (1–2 p.m.)	
3	F:GDusk		Dusk (6–7 p.m.)	
4	G:Rec	Granules (labelled recommended rate)	-	56 kg N/ha
5	G:Con	Granule (experiment control)	-	30.4 kg N/ha

middle, and bottom sections of the shelter, to obtain average values of the light spectrum

Vegetative Growth Properties. Plant height (cm) was measured from the ground level until the tip of the apical bud using a measuring tape, and the leaf number of a fully expanded leaf was manually counted (Khor, 2022).

Leaf Physiology. The chlorophyll content was measured using the Soil Plant Analysis Development (SPAD) chlorophyll meter (Chlorophyll meter SPAD-502Plus, Konica Minolta Inc., Japan) on the fully expanded leaf of the sample. Readings were taken three times around the midpoints of the leaf, and the average SPAD index was recorded (Khor, 2022; Toh et al., 2022; Yaapar, 2017). Measurements were taken every week from 13 DAS onwards until the eighth week of the experiment. Leaf gas exchange, including assimilation (A_{400}) and stomatal conductance to water vapour (gs_w), were measured using the LI-6800 photosynthesis system (LI-COR Inc., USA) during the vegetative stage (35-38 DAS). The leaf chamber was selected, and one of the top three to five fully expanded leaves were clipped to measure the leaf physiology parameters (Jusoh et al., 2023; Toh et al., 2022). The light intensity was set at 1,500 µmol/m^2/s, composed of 10% blue and 90% red light, temperature at 27–30°C, and humidity at 60%. The flow setpoint was set at 500 µmol/s, valve 0.1 kPa with a fan speed setting of 10,000 rpm, and the CO_2 was injected into the leaf chamber at 400 ppm (Khor, 2022). Intrinsic water use efficiency (iWUE) was calculated as a ratio of assimilation rate over stomatal conductance (Jusoh et al., 2023; Yaapar, 2017).

Fruit Parameters. The okra pods were harvested 45 days after sowing with a standard pod length of more than 10 cm over 2 weeks. Fruit parameters included fruit number, length, circumference, and weight (on a fresh and dry basis). The fresh and dry fruit weight (g) was measured using an electronic weighing balance (FX-1200i, A&D, CO. Ltd., Japan). The fruits were dried at 60°C in an oven (UM 400, MEMMERT GmbH + Co. KG., Germany) for 48 hours to obtain the dry weight (g). The potential yield was calculated from the total fresh fruit weight divided by the harvested area and is expressed in the unit of MT/ha.

Fruit Firmness. The fruit firmness was measured using the Instron texture analyser (Model 5543 load frame, Instron Corp., USA) with a p/5 (5 mm diameter) needle probe at a test speed of 20 mm/s. The okra pod was punched three times at equidistance, and the firmness reading was recorded in Newton (N) using the Instron Merlin Software version M12-13664-EN (Khor, 2022).

Fresh and Dry Weight of Shoot and Root. The fresh and dry matter of the shoot and root were determined by using a destructive method. The plant was cleaned and separated into shoot and root parts. The fresh and dry shoot and root weights were measured separately using an electronic

weighing balance (FX-1200i, A&D, CO. Ltd., Japan). The shoots were then cut into small pieces, and roots were placed in paper bags and dried for 48 hr at 60°C in an oven (UM 400, MEMMERT GmbH + Co. KG., Germany). The shoot-to-root ratio (S:R) was calculated as a ratio of shoot-dry weight over root-dry weight (Reynolds & Thornley, 1982).

Number and Dry Weight of Seeds. The number of seeds was calculated using CountThings version G 3.69.1 software (CountThings, USA). The dried seeds were weighed using the electronic balance (Original Equipment Manufacturer, China).

Experimental Design and Statistical Analysis. Randomised complete block design (RCBD) was conducted with 3 blocks comprising 3 replications within each block. All data were subjected to the normality test of Anderson-Darling and analysis of variance (ANOVA) at $p = 0.05$ using GraphPad Prism version 9 software (GraphPad Software, USA). The data were also subjected to normality and homogeneity of variance testing to ensure that the data followed the assumption for ANOVA. Whenever significant, means were compared using Tukey's honestly significant difference (HSD) test at $p = 0.05$. Pearson's analysis of the correlation between parameters was also performed in Origin(Pro) 2022 software (OriginLab Corporation, USA) at $p = 0.05$ to determine the strength and direction of correlation between the parameters measured.

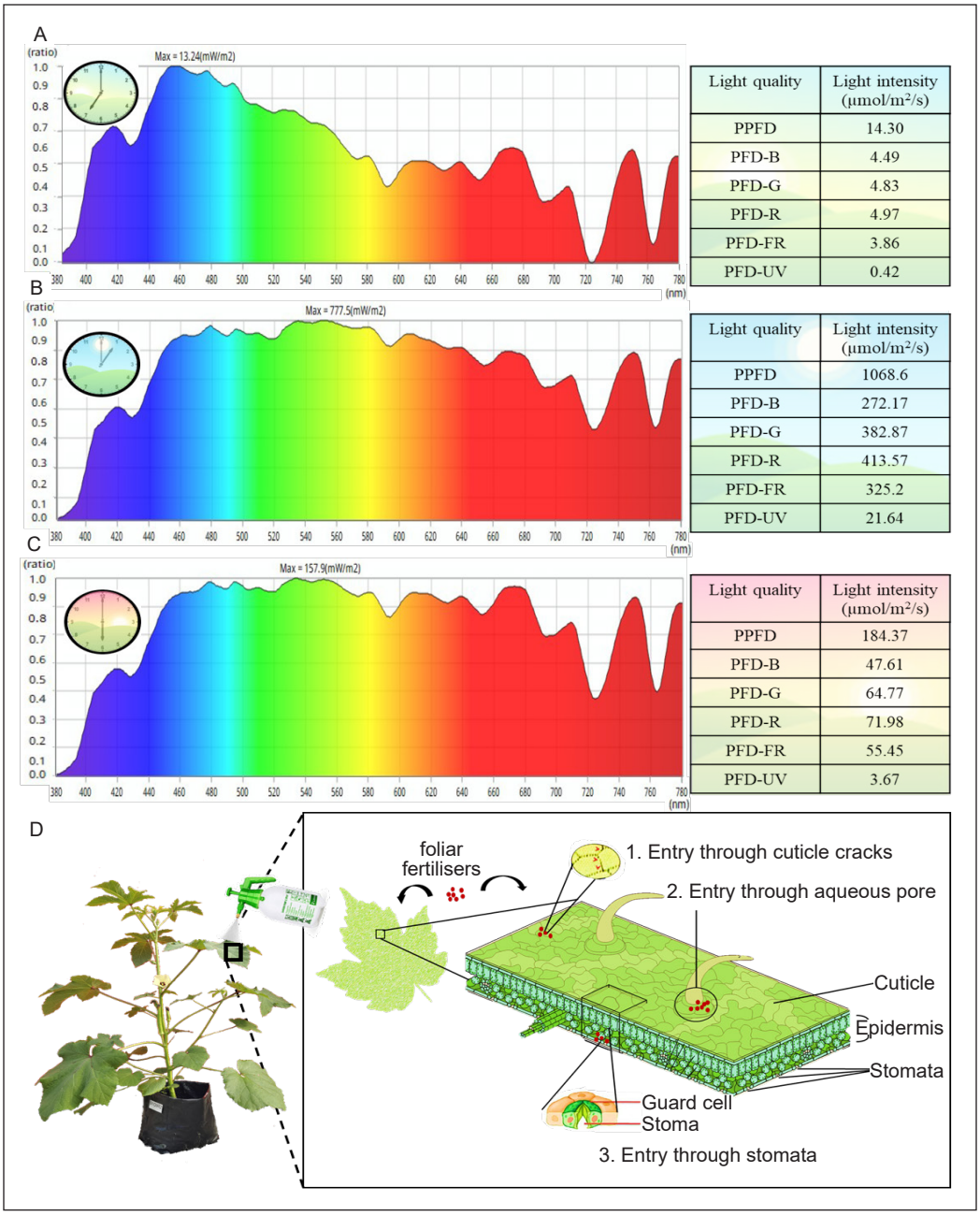
RESULTS

Effects of Periodic Changes of Daytime on Light Spectrum Profile, Temperature, Relative Humidity, and Foliar Nutrient Uptake Pathways

The study recorded light intensity and spectrum at three different times of the day, namely sunrise (7 a.m.), midday (1 p.m.), and dusk (6 p.m.). The highest photosynthetic photon flux density (PPFD) was observed during midday (1,068.6 µmol/m^2/s), followed by dusk (184.37 µmol/m^2/s) and sunrise (14.30 µmol/m^2/s). A similar trend was observed in far-red photon flux density (PFD-FR) and ultra-violet photon flux density (PFD-UV). Temperature and relative humidity were also recorded using CO_2 meters (HT-2000, Hti, Dongguan Xintai Instrument CO. Ltd., China) and tabulated in Table 2. The highest temperature was observed at midday (38.5°C), followed by dusk (27.7°C) and sunrise (25.1°C). Relative humidity was highest at sunrise (88.03%), followed by dusk (75.9%) and midday (52.54%). The foliar nutrients can penetrate the leaf surface through various means, including the cuticle per se, cuticle cracks, or specialised epidermal structures like stomata and aqueous pores at the trichomes' base (Figure 1D).

Table 2
Average temperature and relative humidity corresponding to the foliar application time

Time	Temperature (°C)	Relative humidity (%)
Sunrise (7–8 a.m.)	25.1 ± 2.5	88.03 ± 6.51
Midday (1–2 p.m.)	38.5 ± 3	52.45 ± 7.04
Dusk (6–7 p.m.)	27.7 ± 1.7	75.99 ± 9.81



Light quality	Light intensity (µmol/m²/s)
PPFD	14.30
PFD-B	4.49
PFD-G	4.83
PFD-R	4.97
PFD-FR	3.86
PFD-UV	0.42

Light quality	Light intensity (µmol/m²/s)
PPFD	1068.6
PFD-B	272.17
PFD-G	382.87
PFD-R	413.57
PFD-FR	325.2
PFD-UV	21.64

Light quality	Light intensity (µmol/m²/s)
PPFD	184.37
PFD-B	47.61
PFD-G	64.77
PFD-R	71.98
PFD-FR	55.45
PFD-UV	3.67

Figure 1. (A) The average light spectrum reading of the rain shelter at sunrise. (B) The average light spectrum reading of the rain shelter at midday. (C) The average light spectrum reading of the rain shelter at dusk. The light source value: PFD-R (600–700 nm wavelength range), PFD-G (500–600 nm), PFD-B (400–500 nm), PFD-UV (80–400 nm), and PFD-FR (700–780 nm). (D) The general pathways of foliar nutrient uptake across the leaf surface

Note. PPFD = Photosynthetic Photon Flux Density; PFD-B = Photon Flux Density-Blue; PFD-G = Photon Flux Density-Green; PFD-R = Photon Flux Density-Red; PFD-FR = Photon Flux Density-Far Red; PFD-UV = Photon Flux Density-Ultraviolet

Effects of Differential Foliar Application Timing and Methods on Agronomy Traits

The mean values of agronomy parameters, including plant height, leaf number, total biomass, and shoot-to-root ratio, were presented as a bar graph in Figure 2. The growth appearance corresponding to each treatment is shown in Figure 2A. The highest and lowest plant heights observed in treatment F:GSun (117.42 cm) and REC (105.43 cm), respectively, in Figure 2B. Despite the non-significant difference between treatments, there is a trend of increased plant height under F:GSun treatment by about 6% compared to the regular granule fertiliser method (G:Con). Despite the non-significant difference between treatments, the highest leaf count was observed in treatment G:Con (Figure 2C). It indicated that granule application resulted in higher leaf counts, while the combined application method at midday (F:GMid) tended to lower okra leaf counts.

Regarding biomass accumulation, the G:Rec treatment showed a higher trend of total dry weight, whereas treatment F:GMid resulted in a lower trend of total dry weight (Figure 2D). Among the three application timings, okra plants subjected to dusk application timing (F:GDusk) tended to accumulate more biomass. The shoot-to-root ratio represents the relative biomass allocation between the shoot and the root of a plant. Treatment G:Con has a great tendency to produce a higher shoot biomass partitioning in the okra plant (Figure 2E). Intriguingly, similar agronomic outcomes were observed among the treatments, even though the conventional granule fertilisation treatments (G:Rec) received a higher N rate. Although the differences were not statistically significant, the interpretations of the results were still useful in identifying general tendencies and inclinations in the variables under investigation.

Effects of Differential Foliar Application Timing and Methods on Leaf Physiology

The mean values for leaf physiology parameters, namely total chlorophyll content, assimilation rate (A_{400}), stomata conductance to water vapour (GSW), and iWUE, were presented as bar graphs in Figure 3. There was a significantly highest value of total chlorophyll content observed in treatment G:Rec (Figure 3A). Among the three application timings, the total chlorophyll content was significantly higher in okra plants subjected to dusk application timing (F:GDusk).

Moreover, treatments G:Con and F:GDusk showed a great tendency to result in a higher assimilation rate (Figure 3B). Similarly, treatments G:Con and F:GDusk have the greatest tendency to result in a higher value of gs_w (Figure 3C). Stomatal conductance measures the rate at which water vapour diffuses through the stomatal pores in the leaves of a plant. It reflects the degree of stomatal opening, which is largely controlled by the plant's physiological responses to environmental factors such as light intensity, temperature, humidity, and water availability.

When both A_{400} and gs_w were combined as a ratio to assess iWUE, the okra plants

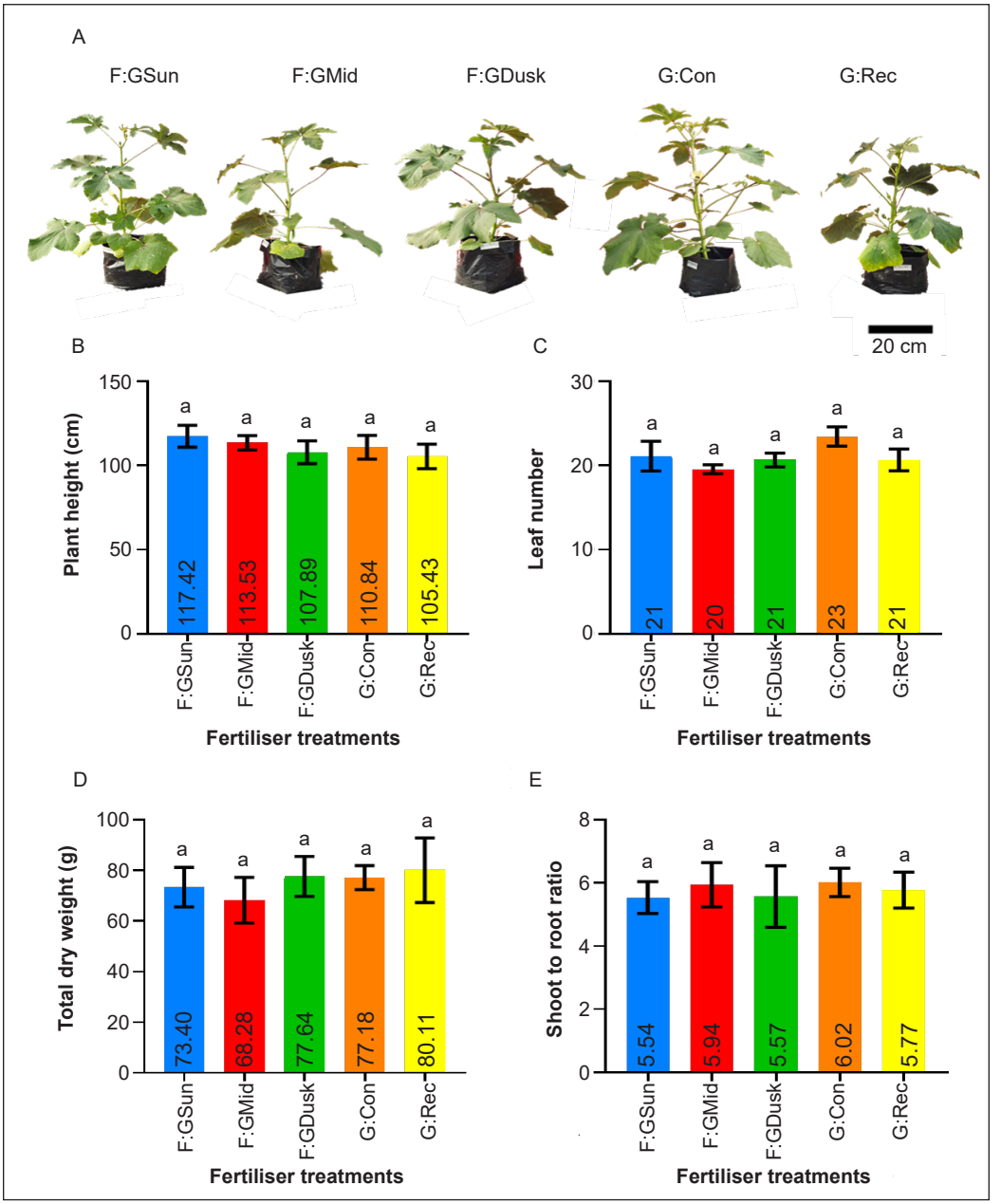


Figure 2. (A) General growth appearance of the okra plant at 42 days after sowing (DAS). (B) Mean comparison of plant height at 57 DAS. (C) Mean comparison of leaf number at 48 DAS. (D) Mean comparison of total dry weight at 57 DAS. (E) Mean comparison of shoot-to-root ratio at 57 DAS. Means with the different alphabets are significantly different using Tukey's honestly significant difference test at $p = 0.05$ (n = 9). Error bars indicate the standard error of means, while the value in the bar indicates the mean value for the respective treatments

Note. F:GSun = Foliar and granule fertiliser at sunrise; F:GMid = Foliar and granule fertiliser at midday; F:GDusk = Foliar and granule fertiliser at dusk; G:Rec = Granule fertiliser at recommendation rate; G:Con = Granule fertiliser at normalise rate as control

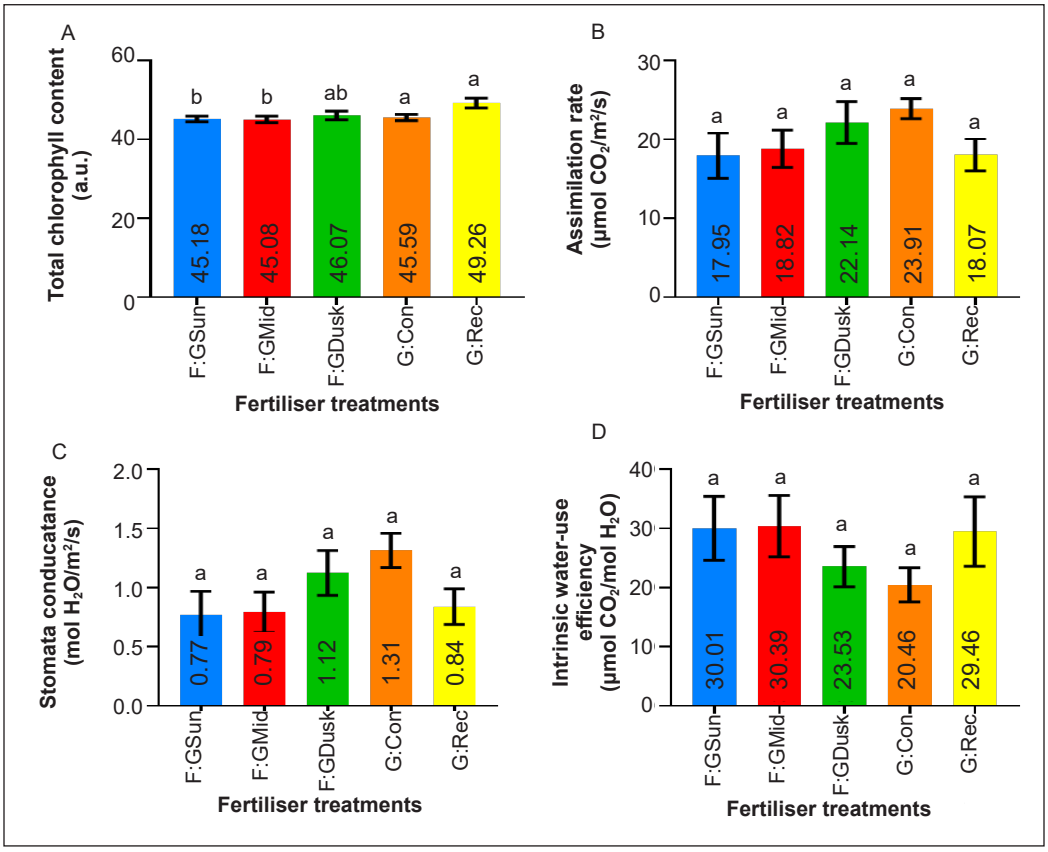


Figure 3. (A) Mean comparison of total chlorophyll at 41 days after sowing (DAS). (B) Mean comparison of assimilation rate. (C) Mean comparison of stomata conductance. (D) The mean comparison of intrinsic water-use efficiency. Means with the different alphabets are significantly different using Tukey's honestly significant difference test at $p = 0.05$ (n = 9). Error bars indicate the standard error of means, while the value in the bar indicates the mean value for the respective treatments

Note. F:GSun = Foliar and granule fertiliser at sunrise; F:GMid = Foliar and granule fertiliser at midday; F:GDusk = Foliar and granule fertiliser at dusk; G:Rec = Granule fertiliser at recommendation rate; G:Con = Granule fertiliser at normalise rate as control

treated with treatments F:GSun, F:GMid, and G:Rec exhibit a relatively high value of iWUE (Figure 3D), and these plants are expected to tolerate drought condition well.

Effects of Differential Foliar Application Timing and Methods on Okra Fruit Parameters

The mean values of okra fruit parameters such as fruit length, fruit middle section circumference, fruit firmness, fruit fresh weight, fruit dry weight, total fruit number, and potential yield were presented as bar graphs in Figure 4. The general morphological characteristics of "Torpedo" okra fruits corresponding to each treatment are shown in Figure 4A.

In terms of fruit length, middle section circumference, and firmness, the treatment G:Con showed significantly lower values

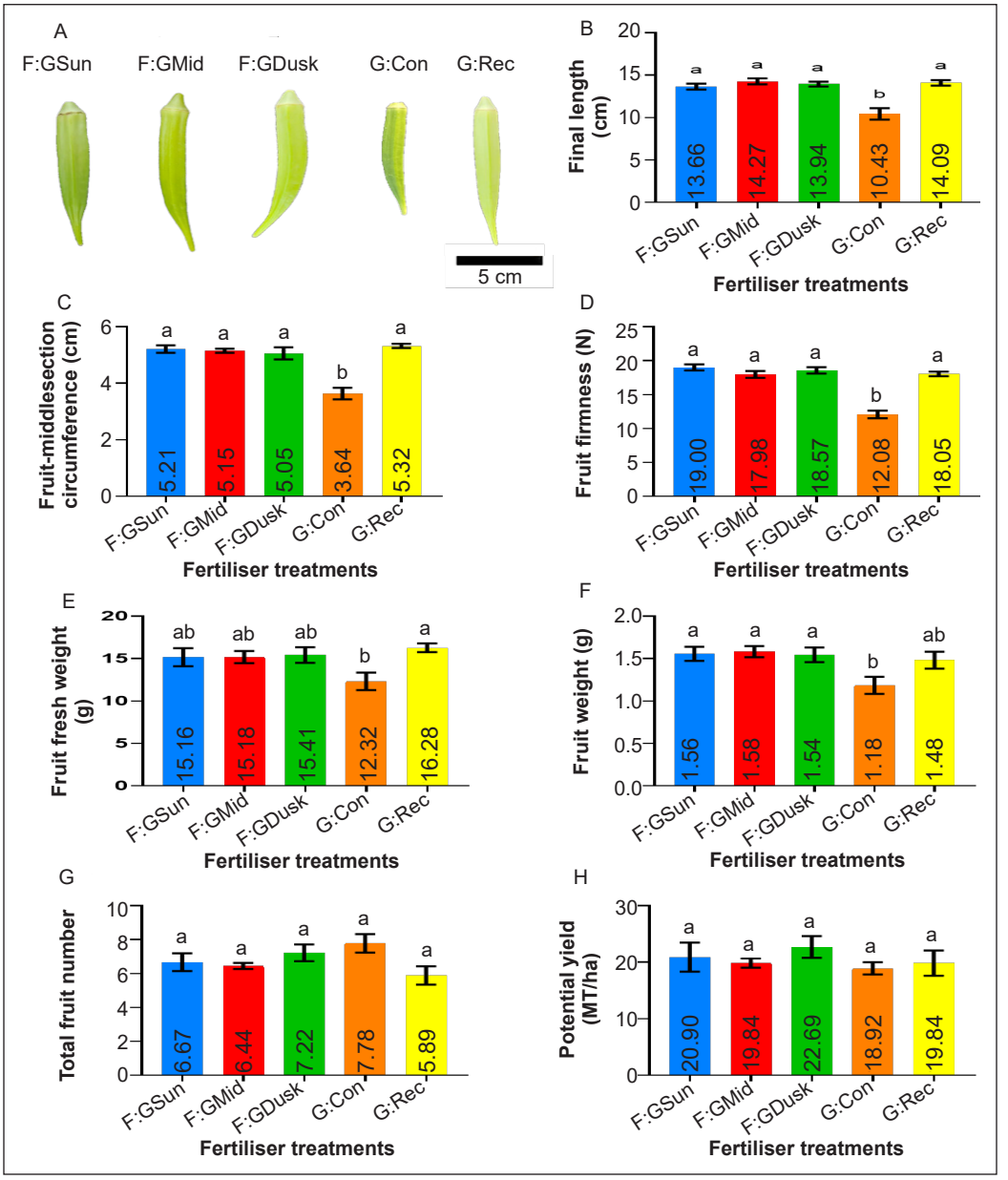


Figure 4. (A) The morphological characteristic of "Torpedo" okra fruits corresponding to each treatment. (B) Mean comparison of average fruit length. (C) Mean comparison of average fruit perimeter. (D) Mean comparison of the average fruit firmness. (E) Mean comparison of average fruit fresh weight. (F) Mean comparison of average fruit dry weight. (G) Mean comparison of total fruit number harvested over 2 weeks of the period. (H) Mean comparison of potential yield calculated. Means with the different alphabets are significantly different using Tukey's honestly significant difference test at $p = 0.05$ (n = 9). Error bars indicate the standard error of means, while the value in the bar shows the mean value for the respective treatments

Note. F:GSun = Foliar and granule fertiliser at sunrise; F:GMid = Foliar and granule fertiliser at midday; F:GDusk = Foliar and granule fertiliser at dusk; G:Rec = Granule fertiliser at recommendation rate; G:Con = Granule fertiliser at normalise rate as control

compared to the other treatments that were tested. In addition, there was a slightly higher tendency for the F:GMid treatment to result in longer fruit length (Figure 4B), while the G:Rec treatment showed a slightly higher tendency to result in larger fruit circumference (Figure 4C). These results suggest that the timing and incorporation of foliar fertiliser applications may affect fruit size, although the effect may be relatively small. Similarly, the fruit firmness was quantified by the force (N) needed to penetrate the fruit surface tissue, and the maximum resistance force (N) was recorded. The higher the value indicates, the harder the fruit pods. F:GSun treatment has a greater tendency to result in harder fruit pods, while treatment G:Con resulted in significantly softer fruit pods (Figure 4D).

Moreover, findings depicted in Figure 4E indicate that treatment G:Rec exhibited a higher propensity to yield greater fresh fruit weight, while the lowest fresh fruit weight was observed in G:Con. Moreover, a higher trend of dry fruit weight was observed in treatment F:GMid, while the lowest dry fruit weight was observed in G:Con (Figure 4F).

Furthermore, more fruits were harvested in the treatments G:Con and F:GDusk (Figure 4G). As a result, treatment F:GDusk showed a greater tendency to higher potential yield by about 20% compared to G:Con.

Effects of Differential Foliar Application Timing and Methods on Okra Seed Parameters

Seed parameters are essential to the seed industry to produce high-quality and consistent seeds as planting material. Seed number (SN) is among the important parameters to indicate the seed yield, while the seedling vigour is significantly

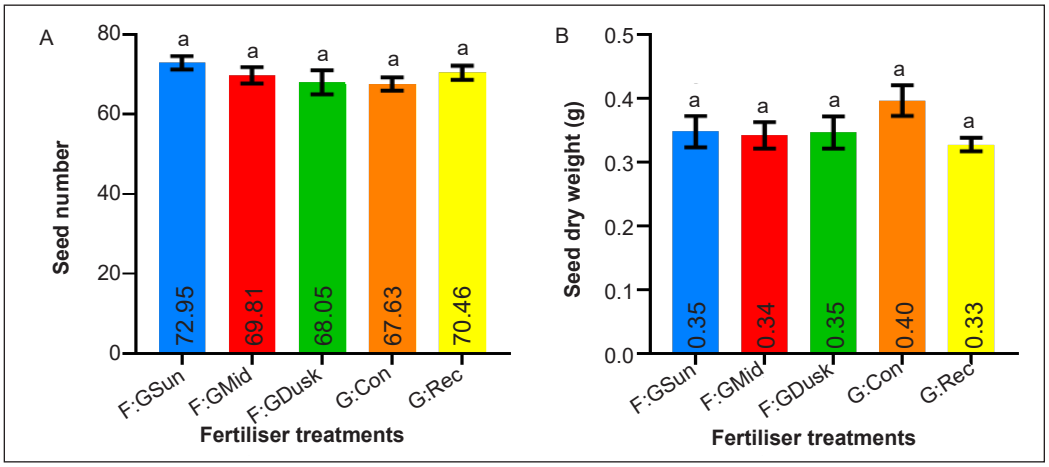


Figure 5. (A) Mean comparison of seed number. (B) Mean comparison of seed dry weight. Means with the different alphabets are significantly different using Tukey's honestly significant difference test at $p = 0.05$ (n = 9). Error bars indicate the standard error of means, while the value in the bar indicates the mean value for the respective treatments

Note. F:GSun = Foliar and granule fertiliser at sunrise; F:GMid = Foliar and granule fertiliser at midday; F:GDusk = Foliar and granule fertiliser at dusk; G:Rec = Granule fertiliser at recommendation rate; G:Con = Granule fertiliser at normalise rate as control

influenced by the seed weight (Kandasamy et al., 2020; Zhang et al., 2017).

Non-significant differences in the SN parameter were observed between treatments; however, combined foliar fertiliser application at sunrise (F:GSun) has a greater tendency to result in higher SN (Figure 5A). The SN was about 7.8% higher when compared to treatment G:Con. On the other hand, treatment G:Con tended to produce a higher dry weight for the seed (Figure 5B).

Heatmap Plot Representing the Correlation Matrix Between the Growth, Physiology, Fruit, and Seed Parameters

Pearson's correlation analysis was carried out to comprehend the strength of the relationship between the growth, physiology, fruit, and seed parameters (Figure 6). The results of the study showed strong to moderate positive correlations between various fruit parameters, including fruit length (FL), fruit circumference (FC), fruit

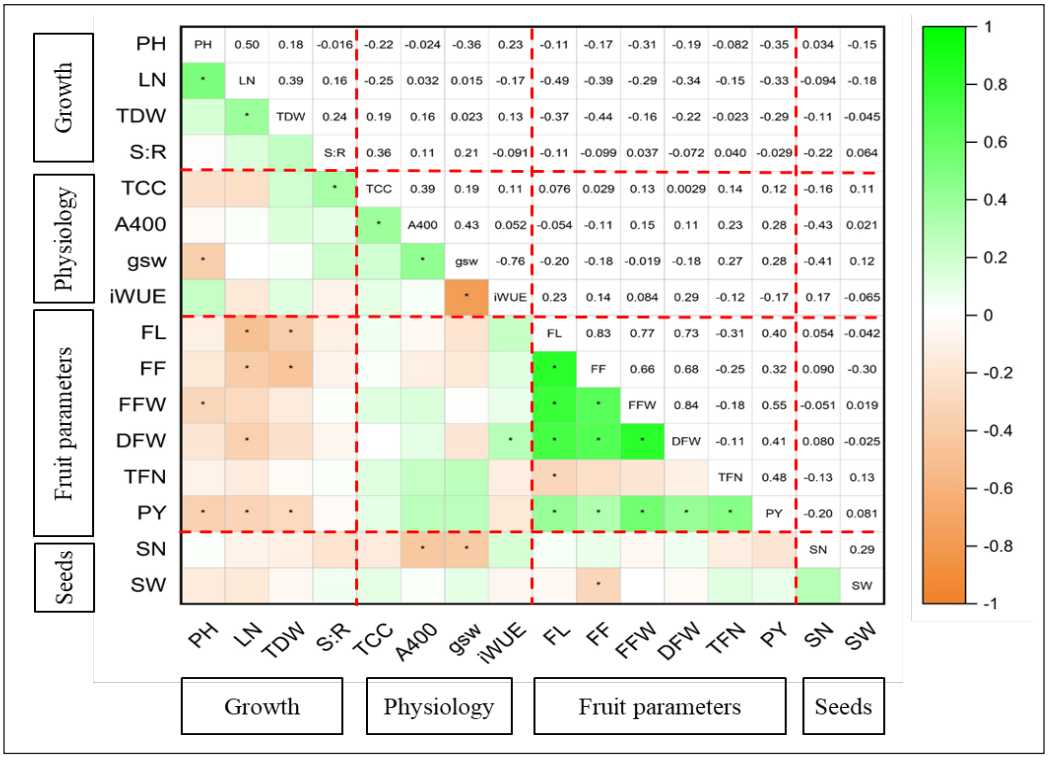


Figure 6. The correlation heatmap analysis represents the strength of correlations between the growth, physiology, fruit, and seed parameters. Pearson's correlation coefficient is used as the correlation descriptor (green and orange for positive and negative correlations, respectively). Symbol * represents a significant difference at $p < 0.05$. Correlation coefficients have a value of between -1 and 1. A "0" means no relationship between the variables, while -1 or 1 means a perfect negative or positive correlation.

Note. PH = Plant height; LN = Leaf number; TDW = Total dry weight; S:R = Shoot to root ratio; TCC = Total chlorophyll content; A_{400} = Assimilation rate; gs_w = Stomata conductance to water vapor; iWUE = Intrinsic water use efficiency; FL = Fruit length; FC = Fruit circumference; FF = Fruit firmness; FFW = Fresh fruit weight; DFW = Dry fruit weight; TFN = Total fruit number; PY = Potential yield; SN = Seed number; SW = Seed dry weight

firmness (FF), fresh fruit weight (FFW), dry fruit weight (DFW), and potential yield (PY). Moreover, high to moderate positive correlations were found between PY and other fruit parameters, such as FL, FC, FF, FFW, DFW, and total fruit number (TFN). Similarly, moderate positive correlations were observed between leaf physiology parameters, such as assimilation rate (A_{400}), total chlorophyll content (TCC), and stomata conductance to water vapour (gs_w), as well as between growth parameters, such as leaf number (LN), plant height (PH), and total dry weight (TDW).

In contrast, moderate negative correlations were observed between certain growth and physiology parameters. LN had moderate to low negative correlations with FL, FC, FF, DFW, and PY, while TDW was negatively correlated with FL, FC, FF, and PY. Additionally, PH was negatively correlated with FFW and PY. Furthermore, the study showed a strong negative correlation between the physiology parameters, namely gs_w and iWUE, while a moderate negative correlation between SN, A_{400}, and gs_w.

DISCUSSION

This experiment studied the effect of foliar application timing and mode on four components of crop performance in okra: plant growth, leaf physiology, fruit, and seed yield. In search of the optimum daytime for the NPK foliar fertiliser application, this study identified the potential of dusk application timing (6–7 p.m.) that tends to result in a better efficiency of nutrient uptake and assimilation, which was reflected in the assimilation rate, total fruit number and potential yield parameters (Figures 3B, 4G, and 4H). This finding is consistent with a previous study on the optimal timing of foliar fertilisation, which reported that the late afternoon period was the most suitable time for foliar fertiliser application (Fageria et al., 2009).

Foliar penetration and assimilation are processes that are influenced by the metabolic status of plants, which, in turn, are regulated by environmental factors such as light, temperature, and humidity (Fernández et al., 2013; Niu et al., 2020). Light signals are particularly dynamic, varying with time, seasons, and the circadian clock (Xu et al., 2021), and have been shown to play a critical role in nutrient uptake and utilisation by plants (Chen et al., 2016; Cui et al., 2019; Lin et al., 2020; Sakuraba & Yanagisawa, 2018). Evidence suggests that light intensity profoundly affects plant ion uptake (Xu et al., 2021), as exemplified in *Brassica campestris*, where the nitrate content decreased significantly under low light intensity (Xin et al., 2009).

Furthermore, light intensity significantly alters the photosynthetic efficiency of plants, resulting in dynamic changes in sugar accumulation in plants (Xu et al., 2021). An earlier study implicated that light increased plant N uptake by enhancing plant photosynthesis and affecting sugar accumulation (Delhon et al., 1996; Xu et al., 2021). Light intensities between 100-800 $\mu mol/m^2/s$ are typically sufficient to drive photosynthesis in most plants (Hofmann et

al., 2016). The light spectrum result (Figure 1C) of this experiment suggests that the optimal time for foliar fertiliser application is during dusk (6–7 p.m.), as this period achieves the necessary light conditions to drive photosynthesis, with an average light intensity of 184.37 µmol/m^2/s.

In addition, the ideal temperature for foliar application in tropical regions varies depending on the specific crop but typically ranges from 18 to 29°C while the optimum relative humidity is above 70% (Alshaal & El-Ramady, 2017). Temperature and relative humidity primarily play an essential role in the drying rate of the sprayed nutrient droplets (Fernández et al., 2013). High relative humidity delays rapid nutrient solution drying (Fernández et al., 2013; Gooding & Davies, 1992) and causes the swelling of the cuticular membrane, which favours the absorption of foliar fertiliser (Fernández et al., 2021; Schönherr & Schreiber, 2004). In combination with the optimal light intensity, temperature (27.7°C), and relative humidity (75.9%) for dusk application (Figure 1C, Table 2), the effectiveness of the foliar fertiliser was further improved, ultimately resulting in a promising yield and plant growth.

Moreover, light intensity was highest at midday, leading to increased photosynthesis and plant sugar accumulation. However, when the light energy absorbed by the photosynthetic apparatus exceeds the utilisation demand, excess light energy causes a decrease in the conversion efficiency of light energy (Bassi & Dall'Osto, 2021). Therefore, excessive light intensity can lead to the generation of destructive singlet oxygen and other active substances in the photosynthetic organs (Patelou et al., 2020), causing oxidative damage to thylakoid membranes and impairing photosynthetic processes (Yin et al., 2010), ultimately attenuating the positive effect on nutrient absorption (Xu et al., 2021). In addition, high light intensity during midday is usually associated with high temperatures and low relative humidity, directly and indirectly reducing foliar uptake and assimilation by plants. High temperature speeds up the drying rate of the foliar nutrients by increasing the rate of evaporation from the spray solutions deposited onto the foliage (Fernández et al., 2013; Niu et al., 2020).

Despite the application time, it is worth mentioning that integrating foliar fertiliser into conventional granule fertiliser application is a promising approach to improving nutrient uptake and utilisation while reducing the usage of inorganic soil granule fertilisers. It promotes more sustainable farming practices by minimising soil salinity accumulation. The potential yield under the combined fertiliser treatments was 5 to 20% higher when compared to the experiment control. Moreover, the overall results presented in Figures 2, 3, 4, and 5 showed that crop performance under combined fertiliser treatments (F:GSun, F:GMid, and F:GDsuk) was statistically similar, with no significant differences, to the conventional fertiliser application treatment (REC), despite the N rate being about 45% lower than that of the G:Rec treatment. Similar results were

reported by Abbasi et al. (2010) as well as Afe and Oluleye (2017), who found that the combination of soil application and foliar fertilisation was promising in enhancing okra's growth and yield traits.

The foliar application of nutrients provides plants with readily available nutrients, promoting faster growth and increased productivity by reducing the distance the nutrients travel within the plant (Simarmata et al., 2021). In addition, the combined fertilisation methods further improve plant growth by providing essential micronutrients that may be lacking in granule fertilisers. Also, micronutrients applied through soil application may be lost to the environment or temporarily accumulate in the soil due to complex soil chemistries, hindering their immediate availability for plant uptake (Bindraban et al., 2015; Sebilo et al., 2013; Thakur & Kumar, 2020). Plant nutrient uptake is a complex process involving multiple transporters, often transporting more than one nutrient type. For instance, the iron-regulated transporter (Irt), which is stimulated by Fe deficiency, facilitates the transportation of Fe, Mn, Cu, Zn, and possibly other divalent cations into the plant (Quintana et al., 2022; Rai et al., 2021; Sinclair & Krämer, 2012), leading to competition and antagonism due to the shared uptake and transport systems of these ions (Bindraban et al., 2015). Thus, applying the combined fertilisation method allowed the plant to absorb nutrients through various pathways, including the root and shoot, while reducing the likelihood of antagonism.

An additional advantage of combining foliar and granule application can be achieved by exploiting synergistic effects (Bindraban et al., 2015; Niu et al., 2020). Many studies have suggested that the nutrient elements and other constituents of foliar fertiliser formulations may stimulate the uptake of soil-applied fertilisers (Niu et al., 2020). Oprica et al. (2014) demonstrated that supplementing a combination of nitrogen, phosphorus, potassium, iron, copper, and manganese via foliar application resulted in improved nutrient levels in the leaves and seeds of maise and sunflower, along with a 50% increase in yield compared to using only NPK as the basal application. The N isotopic tracer technique demonstrated that cotton plants had a higher nitrogen uptake efficiency of 28% and accumulated 11.35 mg of nitrogen via root uptake with ammonium N treatment after foliar application compared to the water control treatment (Cangsong et al., 2018). The increased uptake of nitrogen was a result of the positive impact of foliar nutrients on root proliferation, growth, and development, resulting in better anchorage and deep penetration of root into the soil, which led to a greater amount of nutrient uptake from the rhizosphere (Prajwal Kumar et al., 2018). Foliar nutrients can be absorbed and transported through the stem to the roots, thus enhancing root activity and preventing premature senescence of the roots, thereby enhancing the absorptive capacity of the roots; this interaction makes the combination of soil and foliar fertilisation a relevant practice (Niu et al., 2020).

CONCLUSION

It is ascertained that integrating liquid foliar fertiliser with the basal applied granular fertiliser could be a promising approach for enhancing nutrient uptake and promoting plant growth. Hence, it is recommended that farmers integrate foliar and soil fertilisation practices for optimal okra yield production. Furthermore, it is plausible to infer that the optimal timing for foliar fertilisation is during dusk (6–7 p.m.) when environmental factors such as ideal levels of light, temperature, and relative humidity converge to create favourable conditions for efficient absorption of nutrients applied through the foliage. This observation holds significant scientific significance as it underscores the importance of timing in achieving optimal foliar fertilisation efficacy, which can be instrumental in promoting healthy plant growth and enhancing okra yields. However, it is worth noting that the study results only account for the optimal timing of foliar fertilisation during dusk, and further research is required to evaluate the efficacy of other application timings. Notwithstanding, it is hypothesised that morning application between 8 and 9 a.m. could potentially result in superior outcomes compared to an earlier application window of 7 to 8 a.m., although additional research would be necessary to confirm this hypothesis. Therefore, by carefully timing foliar fertilisation and combining it with appropriate soil fertilisation techniques, farmers can enhance the nutrient uptake of their crops, promote healthy plant growth, and ultimately increase their yields.

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Accumulation and Phytotoxicity of Cypermethrin and Deltamethrin to Aquatic Plants

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ABSTRACT

Synthetic pyrethroid contamination in water is a serious environmental concern as this pesticide is highly toxic to aquatic animals. Phytoremediation using aquatic plants that can tolerate and accumulate pyrethroid pesticides is an interesting alternative. In this study, the phytotoxicity of cypermethrin and deltamethrin, alone or in combination, to three aquatic plants, *Azolla microphylla*, *Salvinia cucullata*, and *Spirodela polyrrhiza* were tested. The results show that *S. cucullata* was the most sensitive species because the pigment content in the fronds significantly decreased when exposed to pyrethroid in water. *Azolla microphylla* was the most tolerant species because the pigment content in their fronds significantly increased when exposed to pyrethroid and cypermethrin, which could also significantly increase the plant fresh weight of *A. microphylla*. Both species could accumulate synthetic pyrethroid pesticides in their tissue. The bioconcentration factors of cypermethrin and deltamethrin in *A. microphylla* were 3,508.8 and 2,323.5, respectively, while the bioconcentration factors of cypermethrin and deltamethrin in *S. cucullata* were 453.0 and 381.7, respectively. *Azolla microphylla* is appropriate for use in pyrethroid phytoremediation in water.

Keywords: Aquatic fern, *Azolla*, pyrethroid pesticide, *Salvinia*, *Spirodela*

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INTRODUCTION

The increasing use of insecticides worldwide has increased the contamination and toxicity in the ecosystem. A synthetic pyrethroid is one group of popular insecticides used

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for agricultural purposes in rice farms in Thailand and soybean farms in Argentina (Maneepitak & Cochard, 2014; Mugni et al., 2011). Contamination with synthetic pyrethroid in water and sediment is a serious environmental problem due to its high toxicity to fish and aquatic animals. For example, the 50% lethal concentration (LC_{50}) of cypermethrin in fish is 2.8 µg/L (Sangchan et al., 2014). Cypermethrin has been reported to be toxic to the valve activity of marine mussels (*Mytilus galloprovincialis*), with the lowest effect concentration at 100 µg/L (Ayad et al., 2011). Contamination with synthetic pyrethroid pesticides in water and sediment has been reported. Pyrethroid concentrations in water from California's San Joaquin River, Orestimba and Del Puerto Creeks ranged from 0.005 to 0.021 µg/L (Ensminger et al., 2011). Cypermethrin has been found in surface water, riverbed sediment, and suspended sediment in the Mae Sa River, northern Thailand, at 0.01 µg/L, 10.5, and 82.8 µg/kg, respectively (Sangchan et al., 2014). The removal of synthetic pyrethroid contamination in water should be done immediately.

Phytoremediation is an environmentally friendly method to remove synthetic pyrethroid contamination in water. Rhizofiltration, using hyperaccumulating aquatic plants to adsorb and absorb pollutants from aquatic environments (Rahman & Hasegawa, 2011), is a phytoremediation process appropriate for pollutant removal in water. Many aquatic plants have been reported to remove pollutants via this process. For example, *Spirodela polyrrhiza* has been reported to accumulate cadmium (Cd) at 54.45 mg/kg fresh weight when cultured in 10 µM Cd contaminated water for four days with 55% of the Cd accumulated in the cell wall (Su et al., 2017). *Spirodela polyrrhiza* also accumulates fluoride when grown in water contaminated with fluoride at 3–20 mg/L (Karmakar et al., 2016). Plants in the genus *Salvinia* were reported to accumulate arsenic (Rahman & Hasegawa, 2011), chromium (Prado et al., 2020), cadmium, nickel, lead, and zinc (Iha & Bianchini Jr., 2015). In addition, plants in the genus *Azolla* have been reported to accumulate arsenic (Rahman & Hasegawa, 2011), mercury, cadmium (Rai, 2008), and methyl violet 2B dye (Kooh et al., 2018). These plant species are naturally found in the aquatic environment in central Thailand, which is the main rice growing area and pyrethroid pesticides are used normally (Maneepitak & Cochard, 2014). However, reports on the phytotoxicity and phytoaccumulation of pyrethroid pesticides are rarely found. This study selected *Azolla microphylla*, *Salvinia cucullate*, and *Spirodela polyrrhiza* to test their tolerance to synthetic pyrethroids, cypermethrin and deltamethrin, alone or in combination. The most tolerant and sensitive species were selected to assess the accumulation capacity for cypermethrin and deltamethrin co-contaminated water. If these aquatic plants could tolerate and accumulate synthetic pyrethroids in water, it would be useful for phytoremediation in agricultural aquatic sites in central Thailand.

MATERIALS AND METHODS

Plant and Water Preparation

Two aquatic ferns, *A. microphylla* and *S. cucullate*, and one species of aquatic angiosperm plant, *S. polyrrhiza*, were used in this study. *Azolla microphylla* and *S. polyrrhiza* were collected from Thung Pueng Sub-district, Nnong Kha Yang District, Uthai Thani Province, Thailand, and *S. cucullata* was collected from Khwae Yai Sub-district, Mueang Nakhon Sawan District, Nakhon Sawan Province, Thailand. The water used in this experiment was tap water with chlorinevolatiled for 3–5 days before use. This water was contaminated with commercial pyrethroid pesticides (Good Knock©, Thailand) that contained 10% (w/v) cypermethrin and 3% (w/v) deltamethrin (Delta 3%©, Thailand) to final concentrations of cypermethrin and deltamethrin in water at 2.5, 5, 7.5, and 10 mg/L.

Experimental Design

For each plant species, the experimental design for each compound, cypermethrin, deltamethrin, and cypermethrin and deltamethrin (1:1), was a completely randomized design (CRD) with one factor, five treatments per experiment and three replicates per treatment. The pots used for *A. microphylla* and *S. polyrrhiza* were 10.61 cm in diameter and each contained 300 ml of water. For *S. cucullata*, the pots were 16.24 cm in diameter and 600 ml of water. There were 6, 6, and 10 g of *A. microphylla*, *S. polyrrhiza*, and *S. cucullata* per pot. All plants were cultured in a nursery that received natural sunlight and was maintained at room temperature for seven days.

Plant Growth Analysis

Plants from each treatment were collected on day seven after planting to determine the plant's fresh weight and dry weight. The moisture in each plant was calculated by (Fresh weight – Dry weight)/Fresh weight (Aveek et al., 2019). The chlorophyll and carotenoid contents in the leaves or fronds were determined according to the method described in Arnon (1949). Briefly, 200 mg of fresh leaves or fronds were crushed with 80% acetone (Merck, Germany), and the volume was adjusted to 10 ml. The absorbances were measured at 663, 645, and 470 nm, and the concentrations of each pigment (mg per g tissue) were calculated as below:

Chlorophyll *a* content = $[12.7(A_{663}) - 2.69(A_{645})] *V/(1000*W)$

Chlorophyll *b* content = $[22.9(A_{645}) - 4.68(A_{663})] * V/(1000*W)$

Total chlorophyll content = Chl.*a* + Chl.*b*

Carotenoid content = $[1{,}000(A_{470}) + 3.27$(chlorophyll *a* – chlorophyll *b*)$] * V/(W *229)$

where A = absorbance at the wavelength mentioned, V = final volume of chlorophyll extract in 80% acetone, and W = fresh weight of tissue extracted.

In addition, the proline contents in the leaves were analyzed by spectrophotometry by measuring the absorbance of the leaf

solution at 520 nm. The leaf or frond solutions were extracted by sulphosalicylic, which was then reacted with acid ninhydrin and extracted with toluene before being measured (John et al., 2008).

Phytoaccumulation Experiment and Pyrethroid Analysis

Based on the dried weight of each plant, *A. microphylla* and *S. cucullata* were selected to study the phytoaccumulation capacity. For the phytoaccumulation experiment, *A. microphylla* and *S. cucullata* were cultured in cypermethrin and deltamethrin co-contaminated water for seven days in the same environment described above. The pots used for *A. microphylla* and *S. cucullata* were 44.5 cm in diameter, each containing 6 L of water. There were 500 and 250 g of *A. microphylla* and *S. cucullata* per pot, respectively. For each replicate, 2 L of pyrethroid-contaminated water was collected and sent for analysis of the pyrethroid pesticide concentration at the Central Laboratory Thailand, Ltd., Bangkok branch, using an in-house method based on the TE-CH-207 method using a gas chromatograph with μ-electron capture detector (GC-μECD) with a limit of detection at 0.50 mg/L. The starting concentrations of cypermethrin and deltamethrin were 9.6 and 8.4 mg/L, respectively. In addition, dried plant tissue of *A. microphylla* and *S. cucullata* was collected and sent for analysis of the pyrethroid pesticide accumulation at the Central Laboratory Thailand, Ltd., Bangkok branch, using an in-house method with TE-CH-030 based on Steinwandter (1985) with the limit of detection at 0.01 mg/kg. Each synthetic pyrethroid's bioconcentration factor (BCF) was calculated from each synthetic pyrethroid concentration in the plant tissue/each synthetic pyrethroid concentration in the water (Somtrakoon & Chouychai, 2023).

Statistical Analysis

One-way analysis of variance (ANOVA) and Duncan tests were used for variance analysis and pairwise comparison. The *t*-test was used to compare the bioconcentration factors of the two plant species.

RESULTS AND DISCUSSION

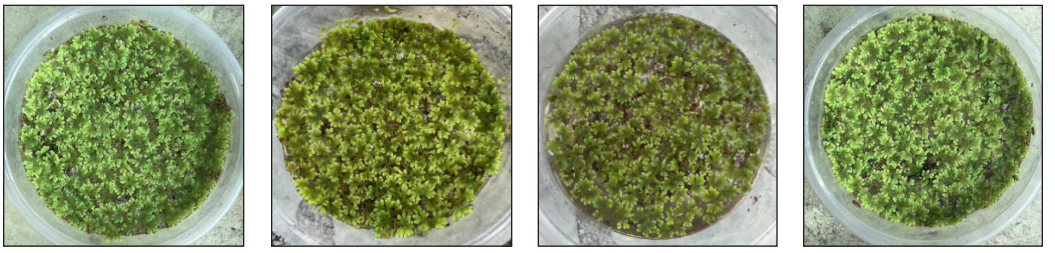
Toxicity of Synthetic Pyrethroid on Weight and Moisture of Aquatic Plants

The toxicity of synthetic pyrethroid on plant weight differed depending on the type of pyrethroid compound and plant species. Cypermethrin or deltamethrin, alone or in combination, did not affect the moisture content of *A. microphylla* and *S. cucullata*. In addition, cypermethrin significantly increased the plant fresh weight of *A. microphylla* but did not affect the fresh weight of *S. cucullata* and the dry weight of both species. Deltamethrin decreased the plant fresh weight of both *A. microphylla* and *S. cucullata* significantly, at 10.0 and 2.5 mg/L, respectively (Table 1). Deltamethrin did not affect the plant dry weight of *S. cucullata* but decreased the plant dry weight of *A. microphylla* at 10.0 mg/L. The combination of cypermethrin and deltamethrin (1:1) only decreased the plant

fresh weight of *S. cucullata* significantly at 5.0-10.0 mg/L (Table 1).

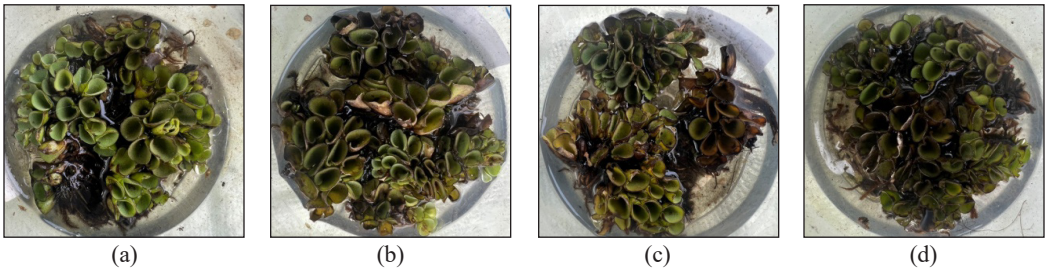
The characteristics in fronds of *A. microphylla* were exposed to different concentrations of deltamethrin, cypermethrin, or the combination of deltamethrin and cypermethrin (1:1) were not different when compared with control (Figure 1), but *S. cucullata* sensitive as the color of fronds were slightly different from control (Figure 2). Even though the combination of cypermethrin and deltamethrin (1:1) did not affect the weight and moisture of *S. polyrrhiza*, just cypermethrin decreased the plant dry weight and increased the moisture of *S. polyrrhiza* significantly. Deltamethrin also decreased the fresh and dry weight of *S. polyrrhiza* at 7.5 and 2.5 mg/L, respectively, but it did not affect the plant's moisture (Table 1). The characteristics in leaves of *S. polyrrhiza* were exposed to different concentrations of deltamethrin, cypermethrin, or the combination of deltamethrin and cypermethrin (1:1) not different from the control (Figure 3).

The concentration of synthetic pyrethroid pesticides in this experiment (2.5–10.0 mg/L) was not toxic to the weight and moisture of aquatic plants. Cypermethrin tended to increase the fresh weight of *A. microphylla*. It was the same with the fresh weights of *Eichornia crassipes*, *Pista stratiotes*, and algae grown in 1 mg/L pyrethroid pesticide for seven days that increased dramatically from 15.70, 15.09, and 15.08 mg on day 0 to 17.35,



(a) (b) (c) (d)

Figure 1. Azolla microphylla grew in cypermethrin, deltamethrin, cypermethrin, and deltamethrin (1:1) for 7 days. (a) Non-contaminated water, (b) cypermethrin 10 mg/L, (c) deltamethrin 10 mg/L, and (d) cypermethrin and deltamethrin (1:1) 10 mg/L



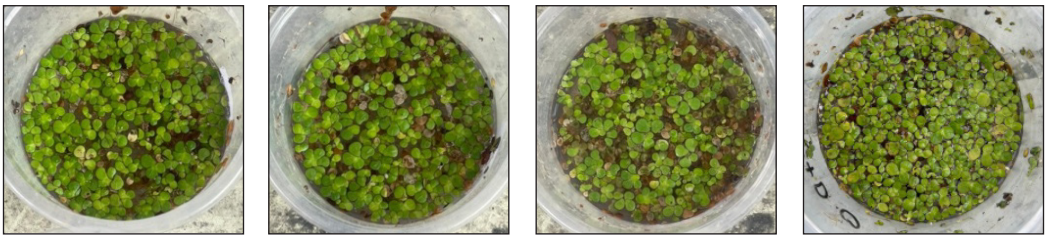
(a) (b) (c) (d)

Figure 2. Salvinia cucullata grew in cypermethrin, deltamethrin, cypermethrin, and deltamethrin (1:1) for 7 days. (a) Non-contaminated water, (b) cypermethrin 10 mg/L, (c) deltamethrin 10 mg/L, and (d) cypermethrin and deltamethrin (1:1) 10 mg/L

Table 1
Weight and moisture of three aquatic plants growing in pyrethroid-contaminated water for 7 days

Pyrethroid concentration (mg/L)	*Azolla microphylla*			*Salvinia cucullata*			*Spirodela polyrrhiza*		
	Plant fresh weight (g)	Plant dry weight (g)	% Moisture	Plant fresh weight (g)	Plant dry weight (g)	% Moisture	Plant fresh weight (g)	Plant dry weight (g)	% Moisture
Deltamethrin									
0	3.8±0.11a	0.15±0.011a	96.0±0.24a	18.6±0.71a	0.58±0.03a	96.9±0.28a	8.5±0.15a	0.45±0.01a	94.8±0.20a
2.5	4.1±0.32a	0.17±0.008a	95.1±0.76a	14.0±1.07b	0.59±0.02a	95.8±0.32a	8.1±0.22a	0.36±0.02b	95.3±0.30a
5.0	3.6±0.32a	0.13±0.008b	96.4±0.29a	13.4±0.31b	0.67±0.07a	95.0±0.45a	7.9±0.30a	0.36±0.01b	96.4±1.25a
7.5	3.6±0.26a	0.15±0.007ab	95.8±0.23a	14.4±1.37b	0.64±0.03a	95.4±0.54a	7.0±0.12b	0.35±0.01b	95.0±0.14a
10.0	2.8±0.03b	0.10±0.002c	96.3±0.05a	15.0±0.36b	0.72±0.04a	95.2±0.36a	7.0±0.26b	0.35±0.01b	94.9±0.09a
Cypermethrin									
0	2.9±0.09b	0.13±0.013a	95.6±0.56a	18.6±0.71a	0.58±0.03a	96.9±0.28a	7.9±0.80a	0.49±0.04a	93.8±0.17c
2.5	4.5±0.29a	0.16±0.012a	96.4±0.06a	20.2±0.59a	0.70±0.06a	96.5±0.40a	6.1±0.68a	0.35±0.02b	94.2±0.24b
5.0	4.8±0.23a	0.16±0.021a	96.8±0.35a	19.8±2.05a	0.61±0.07a	96.9±0.12a	6.2±0.30a	0.35±0.01b	94.4±0.13b
7.5	4.7±0.22a	0.14±0.005a	96.9±0.03a	16.7±0.32a	0.73±0.06a	95.6±0.37a	5.4±0.14a	0.29±0.01b	94.6±0.20ab
10.0	4.4±0.30a	0.15±0.009a	96.6±0.05a	16.6±0.97a	0.66±0.08a	96.0±0.37a	6.5±0.31a	0.32±0.01b	95.0±0.13a
Deltamethrin + Cypermethrin (1:1)									
0	3.7±0.41a	0.12±0.016a	96.8±0.12a	18.6±0.71ab	0.58±0.03a	96.9±0.28a	6.4±0.18a	2.27±0.09a	64.5±1.52a
2.5	4.7±0.77a	0.23±0.085a	95.5±0.85a	22.0±2.20a	0.66±0.04a	97.0±0.27a	6.3±0.32a	1.61±0.15a	74.2±2.45a
5.0	3.8±0.40a	0.14±0.018a	96.1±0.23a	16.6±1.31b	0.59±0.04a	96.4±0.06a	6.8±0.30a	1.67±0.27a	75.3±4.13a
7.5	3.2±0.16a	0.12±0.004a	96.3±0.24a	14.9±1.01b	0.61±0.07a	95.8±0.68a	5.8±0.18a	1.65±0.15a	71.6±2.57a
10.0	2.8±0.25a	0.12±0.008a	95.8±0.08a	14.7±0.14b	0.72±0.08a	95.1±0.52a	6.3±0.34a	1.62±0.12a	74.1±2.39a

Note. Different small case letters showed a significant difference ($P<0.05$) at different concentrations of each compound



(a) (b) (c) (d)

Figure 3. *Spirodela polyrrhiza* grew in cypermethrin, deltamethrin, and cypermethrin and deltamethrin (1:1) for 7 days. (a) Non-contaminated water, (b) cypermethrin 10 mg/L, (c) deltamethrin 10 mg/L, and (d) cypermethrin and deltamethrin (1:1) 10 mg/L

17.51, and 17.56 mg on day seven (Riaz et al., 2017). However, higher concentrations of cypermethrin have been reported to be more toxic. The biomass of *Azolla pinnata* significantly decreased when grown in 30 mg/L cypermethrin for 96 hr (Prasad et al., 2015). Increases in the dry weight and decreases in the moisture content were found in three plant seedlings, *Zea mays*, *Allium cepa*, and *Lathyrus sativus*, exposed to 0.2–0.8 g/L cypermethrin before germination (Aveek et al., 2019).

Toxicity of Synthetic Pyrethroid on Pigment and Proline Content in Leaves/ Fronds of Aquatic Plants

The pigment response to pyrethroid-contaminated water in each aquatic plant's leaves differed. The chlorophyll content in the fronds of *A. microphylla* exposed to 7.5–10.0 mg/L deltamethrin increased compared to that grown in non-contaminated water. The chlorophyll *a* content in the fronds of *A. microphylla* in non-contaminated water was 0.020 mg/g FW, while the chlorophyll *a* content in the fronds of *A. microphylla* exposed to 7.5–10.0 mg/L deltamethrin was 0.024 mg/g FW (Table 2). However, increases in the total chlorophyll and carotenoid in the fronds of *A. microphylla* were seen when exposed to 2.5 mg/L deltamethrin. Surprisingly, the chlorophyll *a* and total chlorophyll contents in the fronds of *A. microphylla* were highest when exposed to 5.0 mg/L cypermethrin, and they dramatically decreased when the cypermethrin concentration was increased. The chlorophyll *b* content in the fronds of *A. microphylla* was not affected by any pyrethroid pesticide, and the combination of deltamethrin and cypermethrin did not affect any pigment content in the fronds of *A. microphylla* (Table 2). The proline content in the fronds of *A. microphylla* increased significantly from the control when exposed to 7.5 mg/L deltamethrin, while the proline content decreased significantly from the control when exposed to 10.0 mg/L. The combination of deltamethrin and cypermethrin (1:1) at 2.5–10.0 mg/L decreased the proline content in the fronds significantly (Table 2).

The all-pigment content in the leaves of *S. polyrrhiza* was not significantly different when exposed to different concentrations of deltamethrin, cypermethrin, or the

Table 2
Chlorophyll, carotenoid, and proline content in fronds of Azolla microphylla *growing in pyrethroid-contaminated water for 7 days*

Pyrethroid concentration (mg/L)	Chlorophyll *a* (mg/g FW)	Chlorophyll *b* (mg/g FW)	Total Chlorophyll (mg/g FW)	Carotenoid (mg/g FW)	Proline (mg/g FW)
Deltamethrin					
0	0.020±0.001b	0.012±0.002a	0.032±0.002b	8.02±0.27b	0.65±0.04ab
2.5	0.021±0.001b	0.015±0.001a	0.036±0.001a	10.69±0.91a	0.53±0.06bc
5.0	0.022±0.001b	0.017±0.001a	0.038±0.000a	11.94±0.48a	0.51±0.04c
7.5	0.024±0.000a	0.013±0.002a	0.037±0.002a	12.26±0.63a	0.77±0.02a
10.0	0.024±0.000a	0.015±0.000a	0.039±0.000a	11.68±0.61a	0.73±0.03a
Cypermethrin					
0	0.017±0.000b	0.016±0.001a	0.033±0.001b	11.57±0.70a	0.75±0.02ab
2.5	0.018±0.001b	0.013±0.001a	0.031±0.001b	10.63±0.42a	0.67±0.02b
5.0	0.028±0.001a	0.016±0.003a	0.044±0.003a	13.68±1.18a	0.70±0.04ab
7.5	0.018±0.000b	0.017±0.004a	0.036±0.004ab	12.45±1.44a	0.79±0.03a
10.0	0.018±0.001b	0.014±0.003a	0.033±0.003b	11.69±1.55a	0.57±0.02c
Deltamethrin + Cypermethrin (1:1)					
0	0.021±0.000a	0.015±0.001a	0.036±0.000a	9.58±0.54a	1.00±0.02a
2.5	0.024±0.002a	0.013±0.001a	0.036±0.001a	9.82±0.54a	0.55±0.10b
5.0	0.024±0.001a	0.012±0.000a	0.036±0.001a	10.61±0.81a	0.49±0.19b
7.5	0.026±0.004a	0.031±0.020a	0.058±0.017a	9.62±0.48a	0.41±0.08b
10.0	0.022±0.001a	0.012±0.000a	0.035±0.000a	10.54±0.61a	0.55±0.08b

Note. Different small case letters showed a significant difference ($P<0.05$) at different concentrations of each compound

combination of deltamethrin and cypermethrin (1:1). The proline content in the leaves of *S. polyrrhiza* increased from the control (1.0 mg/g FW) to be 1.8–3.0 mg/g FW when exposed to 2.5–10.0 mg/L deltamethrin while cypermethrin and the combination of deltamethrin and cypermethrin (1:1) did not affect the proline content in the leaves of this plant (Table 3).

The response of the pigment contents in the fronds of *S. cucullata* was sensitive when exposed to pyrethroid pesticides. The chlorophyll *a*, *b*, and total chlorophyll contents in the fronds of *S. cucullata* exposed to 5.0–10.0 mg/L deltamethrin (0.10–0.12 mg/g FW) significantly decreased when compared to the chlorophyll content in the fronds of *S. cucullata* grown in non-contaminated water (0.19 mg/g FW). However, 7.5–10.0 mg/L cypermethrin only significantly decreased the chlorophyll *a* and total chlorophyll contents but did not affect the chlorophyll *b* content in the fronds of *S. cucullate*. The combination of deltamethrin and cypermethrin (1:1) decreased the chlorophyll *a*, *b,* and total chlorophyll contents in the fronds of *S. cucullata* significantly by 10.0, 2.5, and 5.0

Table 3
Chlorophyll, carotenoid, and proline content in leaves of Spirodela polyrrhiza *growing in pyrethroid-contaminated water for 7 days*

Pyrethroid concentration (mg/L)	Chlorophyll *a* (mg/g FW)	Chlorophyll *b* (mg/g FW)	Total chlorophyll (mg/g FW)	Carotenoid (mg/g FW)	Proline (mg/g FW)
Deltamethrin					
0	0.10±0.003a	0.05±0.002a	0.15±0.01a	46.6±0.33a	1.0±0.02c
2.5	0.10±0.005a	0.05±0.002a	0.15±0.01a	47.8±1.23a	3.0±0.26a
5.0	0.10±0.006a	0.06±0.005a	0.16±0.01a	50.0±2.72a	2.1±0.19b
7.5	0.10±0.012a	0.06±0.005a	0.15±0.02a	47.7±4.34a	1.8±0.15b
10.0	0.08±0.008a	0.05±0.004a	0.13±0.01a	42.6±3.33a	2.0±0.27b
Cypermethrin					
0	0.10±0.003a	0.05±0.002a	0.15±0.01a	46.6±0.33a	1.0±0.02a
2.5	0.11±0.002a	0.06±0.004a	0.17±0.01a	54.0±2.36a	1.0±0.22a
5.0	0.11±0.007a	0.06±0.003a	0.17±0.01a	54.0±1.70a	0.9±0.05a
7.5	0.11±0.005a	0.06±0.001a	0.17±0.01a	55.2±2.10a	1.0±0.13a
10.0	0.11±0.011a	0.06±0.007a	0.18±0.02a	54.5±4.00a	0.8±0.08a
Deltamethrin + Cypermethrin (1:1)					
0	0.10±0.003a	0.05±0.002a	0.15±0.01a	46.6±0.33a	1.0±0.02a
2.5	0.10±0.003a	0.04±0.002a	0.14±0.01a	43.8±1.43a	0.7±0.11a
5.0	0.09±0.004a	0.04±0.003a	0.14±0.01a	43.4±1.71a	0.8±0.26a
7.5	0.10±0.010a	0.04±0.006a	0.14±0.02a	43.8±3.95a	1.1±0.13a
10.0	0.10±0.009a	0.04±0.005a	0.14±0.01a	44.9±4.47a	0.9±0.30a

Note. Different small case letters showed a significant difference ($P<0.05$) at different concentrations of each compound

mg/L, respectively. The carotenoid content in the fronds of *S. cucullata* significantly decreased when exposed to 2.5–10.0, 5.0–10.0, and 5.0–10.0 mg/L of deltamethrin, cypermethrin, and a combination of deltamethrin and cypermethrin (1:1), respectively. The proline content in the fronds of *S. cucullata* was not affected by cypermethrin, and the combination of deltamethrin and cypermethrin (1:1), while 2.5–10.0 mg/L deltamethrin decreased the proline content significantly (Table 4).

A decrease in the chlorophyll content was seen clearly in the fronds of *S. cucullata*. All synthetic pyrethroid pesticides could decrease their chlorophyll content significantly. On the other hand, synthetic pyrethroid pesticides did not affect the chlorophyll content in the leaves of *S. polyrrhiza* and tended to increase some pigments in the fronds of *A. microphylla*. Decreasing pigment content and increasing antioxidant enzyme activity have been reported in plants exposed to cypermethrin. For example, a decrease in the chlorophyll content in the leaves was found in three plant seedlings, *Z. mays*, *A. cepa*, and *L. sativus*, exposed to 0.2–0.8 g/L

cypermethrin before germination (Aveek et al., 2019). Exposure to 5–15 mg/L cypermethrin was reported to increase antioxidant enzymes in A. pinnata. (Prasad et al., 2015). However, in this study, exposure to 2.5–10 mg/L cypermethrin or the combination of cypermethrin and deltamethrin decreased the proline content in the fronds of *A. microphylla*. The response of the carotenoid content in the fronds of *A. microphylla* to deltamethrin was the same as the response of *A. pinnata* to cypermethrin and increased when pyrethroid was present in the water (Prasad et al., 2015).

Accumulation of Synthetic Pyrethroid in Aquatic Plant Tissue

After seven days, *S. cucullata* and *A. microphylla* could accumulate deltamethrin and cypermethrin. The concentrations of deltamethrin within the biomass of *A. microphylla* and *S. cucullata* were 988.2±64.3 and 623.0±28.7 mg/kg, respectively, while cypermethrin was found in the biomass of *A. microphylla* and *S. cucullata* at 593.7±43.4 and 316.7±45.0 mg/kg, respectively (Table 5). The bioconcentration factor of both species shows the capacity to accumulate deltamethrin and cypermethrin, and *A. microphylla* could significantly accumulate

Table 4
Chlorophyll, carotenoid, and proline content in fronds of Salvinia cucullata *growing in pyrethroid-contaminated water for 7 days*

Pyrethroid concentration (mg/L)	Chlorophyll *a* (mg/g FW)	Chlorophyll *b* (mg/g FW)	Total Chlorophyll (mg/g FW)	Carotenoid (mg/g FW)	Proline (mg/g FW)
Deltamethrin					
0	0.19±0.008a	0.12±0.029a	0.32±0.04a	81.2±4.23a	1.0±0.07a
2.5	0.18±0.016a	0.10±0.018ab	0.27±0.03a	65.9±1.43b	0.1±0.10c
5.0	0.12±0.005b	0.04±0.004b	0.16±0.01b	65.4±5.04b	0.4±0.21bc
7.5	0.10±0.004b	0.04±0.013b	0.14±0.01b	66.7±4.19b	0.7±0.20ab
10.0	0.10±0.020b	0.05±0.009b	0.14±0.03b	41.2±3.16c	0.4±0.05bc
Cypermethrin					
0	0.19±0.008a	0.12±0.029a	0.32±0.04a	81.2±4.23a	1.0±0.07a
2.5	0.17±0.014ab	0.07±0.004a	0.24±0.01ab	73.4±7.28a	0.5±0.25a
5.0	0.16±0.014ab	0.08±0.011a	0.24±0.02ab	46.8±2.11b	0.6±0.18a
7.5	0.12±0.026b	0.06±0.013a	0.18±0.04b	41.9±0.39bc	0.7±0.15a
10.0	0.12±0.016b	0.06±0.008a	0.18±0.02b	33.0±1.53c	0.8±0.33a
Deltamethrin + Cypermethrin (1:1)					
0	0.19±0.008a	0.12±0.029a	0.32±0.04a	81.2±4.23a	1.0±0.07a
2.5	0.18±0.019ab	0.06±0.006b	0.24±0.02ab	79.6±1.50a	0.6±0.51a
5.0	0.14±0.010bc	0.06±0.003b	0.20±0.01b	65.2±0.80b	0.3±0.26a
7.5	0.15±0.002abc	0.06±0.004b	0.22±0.01b	63.0±0.37b	0.8±0.15a
10.0	0.12±0.022c	0.06±0.010b	0.18±0.03b	59.4±2.18b	0.9±0.20a

Note. Different small case letters showed a significant difference (P<0.05) at different concentrations of each compound

Table 5
Pyrethroid remaining in water and plant biomass after growing with aquatic plant for 7 days

Treatment	Cypermethrin	Deltamethrin
Water concentration (mg/L)		
Starting concentration	9.6±0.65	8.4±0.31
Azolla – Day 7	0.3±0.01	0.2±0.02
Salvinia – Day 7	1.6±0.38	1.2±0.47
Plant concentration (mg/kg)		
Azolla – day 0	0.03±0.00	B.D.
Salvinia – day 0	0.03±0.00	0.2±0.04
Azolla – Day 7	988.2±64.30	593.7±43.40
Salvinia – Day 7	623.0±28.70	316.7±45.00
Bioconcentration factor		
Azolla	3508.8±172.90a	2323.5±199.60a
Salvinia	453.0±115.30b	381.7±176.10b

Note. Different small case letters showed a significant difference (P<0.05) at different treatments of each compound; B.D. = Below detection limit

both pyrethroid compounds more than *S. cucullata*.

Aquatic plants have been reported to remove pyrethroid contamination in water. For example, *Eichornia crassipes*, *Pista stratiotes*, and algae could remove pyrethroid (permethrine, cypermethrine, deltamethrine, and bifenthrine) at 76, 68, and 70%, respectively, within seven days, but the mechanism of pollutant removal was not indicated (Riaz et al., 2017). *Lemna* sp. was also reported to decrease cypermethrin in water by 99.1% (initiation concentration = 10 µg/L), while in the *Lemna*-free treatment, cypermethrin was decreased by 98% within 12 days. The adsorption of the pesticide by the biological surface was assumed to be the main mechanism of *Lemna* sp., but the cypermethrin amount in the plant tissue was not reported (Mugni et al., 2011). In our study, both plant species could accumulate cypermethrin at 94.6 and 90.8%, while deltamethrin was accumulated at 65.0 and 52.8% for *A. microphylla* and *S. cucullata*, respectively, within 7 days. However, all the dried tissue of the plants was sent for analysis of the pyrethroid content, and the adsorption and absorption mechanisms could not be separated. These results show that *A. microphylla* is an effective aquatic plant for pyrethroid phytoremediation compared to other species.

CONCLUSION

Synthetic pyrethroids were more toxic to the pigment content in the leaves of aquatic plants than the plant's fresh and dry weight. Among the three species, *S. cucullata* was the most sensitive, and *A. microphylla* was the most tolerant to synthetic pyrethroids when considered with pigment content. Based on plant dry weight, *S. polyrrhiza* was the most sensitive, and *S. cucullata* was the most tolerant to synthetic pyrethroids.

However, both species have the capacity to accumulate deltamethrin and cypermethrin within the plant biomass. It is interesting to use both plant species for phytoremediation of pyrethroid-contaminated water, but using *A. microphylla* biomass from pyrethroid-contaminated sites as green manure in agricultural situations may be a concern.

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Effect of Effective Microorganisms in Fermentation of Rice Husk and Anchovy Head Using Lab-scale Treatment

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ABSTRACT

The rice husk and anchovy filet industry is one of the world's most successful industries and generates many biowastes with valuable bioproducts. Combining effective microorganisms (EM) with anchovy head (AH) and rice husk (RH) will produce a beneficial fertilizer and make nutrients more valuable to the plant. Thus, this study aims to determine the most effective anchovy head and rice husk treatment ratio for agricultural applications to reduce waste disposal from anchovy heads and rice husks worldwide, which may cause environmental problems. In this study, rice husk and anchovy head were fermented at five different ratios of treatment weight for 25 days: Treatment A with 100% AH, treatment B with 100% RH, treatment C with 50% AH:50% RH, treatment D with 70% AH:30% RH, and treatment E with 30% AH:70% RH. The pH, temperature, and number of colonies of every treatment were measured every 0, 5, 10, 15, 20, and 25 days. This study shows that treatment D with a ratio of 70% AH: 30% RH is the most suitable based on pH range within 6–8.5, consistently increasing temperature and the highest number of colonies to be applied to plants. The ratio of rice husk and anchovy head of treatment D in this study can be used further by other researchers to discover their potential, especially for the agricultural industry.

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INTRODUCTION

Rice is the principal carbohydrate source in Malaysia, with a total paddy cropping area of about 0.70 million ha, and generates significant waste, including rice husk,

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straw, and bran (Firdaus et al., 2020). It is also a standard waste product in rice countries such as China, India, Bangladesh, Cambodia, Vietnam, and Southeast Asia. About 20%–33% of the paddy weight comprises rice husk, and 1,000 kg of paddy rice produces 200 kg of the husk (Pode, 2016). Rice husk is a cellulose-based fiber and contains approximately 40% cellulose, 30% lignin group, and 20% silica (Chindaprasirt et al., 2007). The high silica (Si) in rice husk makes it valuable in the agricultural industry because it can increase the soil's moisture content (Ramli et al., 2023). Furthermore, it showed that *Brassica oleracea* var. *alboglabra* L. (Chinese kale) uptake nitrogen (N) and increases growth by rice husk addition to palm oil-based compost (Ramli et al., 2023). The same is valid with anchovy heads. Paone et al. (2021) stated that the anchovy filet industry produces a large amount of biowaste, such as fish heads, bones, and tails, which can extract many potentially valuable bioproducts. Therefore, it is necessary to evaluate the nutritional makeup of fish waste to establish whether it can offer plant nutrients like nitrogen, a combination of nitrogen and phosphorus, or enrich a compost. According to Unggang et al. (2023), fish-based fertilizers typically contain macronutrients with recorded high elements of nitrogen (N), potassium (K), and phosphorus (P).

However, fish industry by-products such as heads, viscera, skin, bones, and scales (Paone et al., 2021) were previously considered waste and a cause of environmental damage. However, when both industries grow, the waste will pollute the ecosystem since there will be dumping of abandoned waste that does not benefit the earth. The rice husk and anchovy head waste must be treated and converted into fertilizer to avoid this problem.

Effective microorganisms (EM) are microbial inoculants containing numerous microorganism species that can stimulate plant growth and improve soil fertility (Talaat, 2019). Combining EM with organic waste will produce a beneficial fertilizer because it can make nutrients more beneficial to plants and increase their life expectancy. After all, many beneficial microorganisms in EM solution, such as lactic acid bacteria and yeast, will help improve plant production—biofertilizers from organic waste benefit nutrition management through their considerable potential to improve the efficiency of nutrient usage. Compared to ordinary fertilizers, the nutrients are released very slowly because they are bound to the nano-dimensional adsorbents (Zulfiqar et al., 2019). Plants receive air nitrogen directly from microbes in the biofertilizers (Karki, 2020). In addition, the product produced is eco-friendly, cost-effective, and can increase crop yield by 20%–30%. Furthermore, the most important is that it can help to reduce the waste produced by the food and fish industries. It is also more efficient for agricultural purposes. When biofertilizers are made from organic waste, waste is reduced, while nutrients in organic waste will boost plant product development and bring advantages to customers and the globe.

Utilizing agricultural by-products, like anchovy heads and rice husk, has enormous potential for value-added product creation and sustainable resource management. However, applying lab-scale treatment techniques to ferment these by-products effectively is still difficult. Furthermore, the utilization of these by-products is restricted due to the lack of a systematic strategy to improve the fermentation process, resulting in waste and lost chances for value development. In order to improve the procedure and maximize resource usage, it is necessary to look at how EM affects the fermentation of rice husks and anchovy heads at a lab-scale level. Therefore, the first objective of this study was to evaluate the effect of EM on the fermentation of rice husk and anchovy head by monitoring key parameters, including pH, temperature, and microbial activity. The second objective was to determine the most effective anchovy head and rice husk treatment ratio to be used as biofertilizers for agricultural applications.

MATERIALS AND METHODS

Effective Microorganism Activated Solution (EMAS) Preparation

The authentic EM, which contains yeast, lactic acid, and phototrophic bacteria, was obtained from EMRO Malaysia Sdn. Bhd. (Malaysia). The EM solution was required to produce an Effective Microorganism Activated Solution (EMAS) in the fermentation sample. The concentrated EM solution diluted with chlorine-free water and molasses (EMRO Malaysia Sdn. Bhd., Malaysia), which serves as a food supply for the microbe, was added to activate the process further. One part of the EM microbial inoculants and one part of the molasses were combined with 20 parts of chlorine-free water to activate the EM and kept that solution for three to five days in an airtight ferment container (Jusoh et al., 2013).

Fermentation Treatment Process

The sample materials were weighed and mixed into a different closed container (500 ml). The fermentation containers were labeled treatments A to E and their control with a different ratio (Table 1). Each treatment was done in triplicates. Then, water was added until the moisture content of each fermentation mixture reached 60% (wet basis). The fermentation containers were then stored in dark conditions to prevent excessive heat loss and to preserve moisture. The moisture content was maintained at 50%–60% throughout the twenty-five days of the fermentation period by adding water.

Table 1
Different ratios of sample anchovy head (AH) and rice husk (RH) supplemented with effective microorganisms (EM)

Treatment	% Ratio (w/v)	% EM
A	100% AH	5
B	100% RH	5
C	50% AH: 50% RH	5
D	70% AH: 30% RH	5
E	30% AH: 70% RH	5
Control A	100% AH	-
Control B	100% RH	-
Control C	50% AH: 50% RH	-
Control D	70% AH: 30% RH	-
Control E	30% AH: 70% RH	-

The mixtures were turned every five days to maintain porosity. The pH and temperature were measured and recorded.

A 5-ml EM solution was added to a given mixture for treatments A, B, C, D, and E. An amount of 350 ml of sterile distilled water was added to each fermenting mixture. The samples were mixed well. All the samples were placed in a dark place at room temperature for 25 days.

Measurement of pH and Temperature

A pH meter (No. H1 8915 ATC, Hanna Instruments Sdn. Bhd., Malaysia) and a digital thermometer (RS PRO RS40, Wired Digital Thermometer, Malaysia) were used to record pH and temperature of samples at 0, 5, 10, 15, 20 and 25 days. A graph was plotted to observe the ratio treatment.

Measurement of the Total Number of Bacteria

For a total number of bacterial analyses, a sample was serially diluted by adding 1 ml of the sample to 9 ml of distilled water in a test tube. Each dilution sample was distributed on a nutrient agar plate and incubated for 24 hr. After 24 hr, the number of colonies on each agar plate was determined. The number of bacteria in each treatment was calculated using the following formula (Sieuwerts et al., 2008):

$$\text{Total number of bacteria [Colony Forming Unit (CFL)/ml]} = \frac{\text{Number of colonies} \times \text{total dilution factor}}{\text{Volume of culture plate (ml)}}$$

RESULTS

Changes of pH

According to Figure 1, all treatments except for treatments B and E are within the range of pH 6.00–7.00, while for treatment E, the pH range is 5.00–7.00. At the same time, for treatment B, the pH drastically dropped from pH 5.35 on day 0 to 4.31 on day 25.

Changes of Temperature

Figure 2 depicts the temperature profiles of five fermentation treatments (treatment A – treatment E). Immediately after fermentation begins, all treatments show an increase in temperature. On day 5, the temperature for treatment rose to 25.60°C from 23.23°C for treatment A, 25.23°C from 23.83°C for treatment B, 25.40°C from 24.00°C for treatment C, 25.70°C from 23.30°C for treatment D, and 25.53°C from 23.30°C for treatment E. Treatment D reached the highest values of 25.70°C on day 25 compared to other treatments. All treatments with EM have a temperature higher than the control treatment except for day 0 treatments D and E. However, all treatments increased in temperature along 25 days of fermentation.

Total Number of Bacteria

The number of colonies of each fermentation process of making biofertilizer from rice husk and anchovy head based on treatment ratio is shown in Figure 3. It shows that the total number of bacteria proves the existence of microorganisms

sourced from rice husks and anchovy heads. The highest number of bacterial colonies was in treatment D, with a total bacterial presence of 37.87 × 10^5 CFU/ml, followed by treatment A, 35.3 × 10^5 CFU/ml. For treatments A and D, the

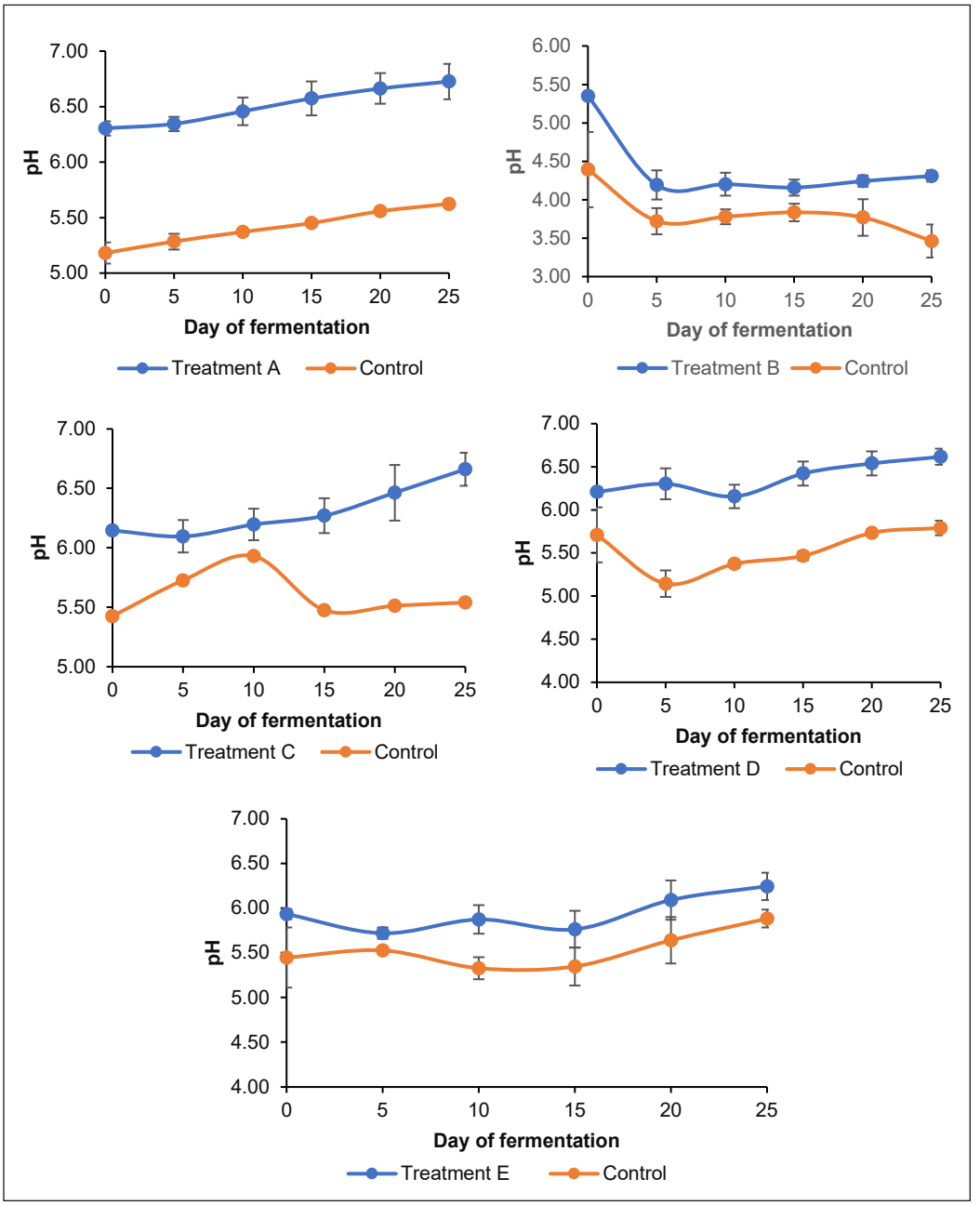

Figure 1. The pH of treatments A-E with it controls during the fermentation process
Note. Treatment A = 100% AH; Treatment B = 100% RH; Treatment C = 50% AH : 50% RH; Treatment D = 70% AH: 30% RH; Treatment E = 30% AH: 70% RH. Error bars = Standard deviation

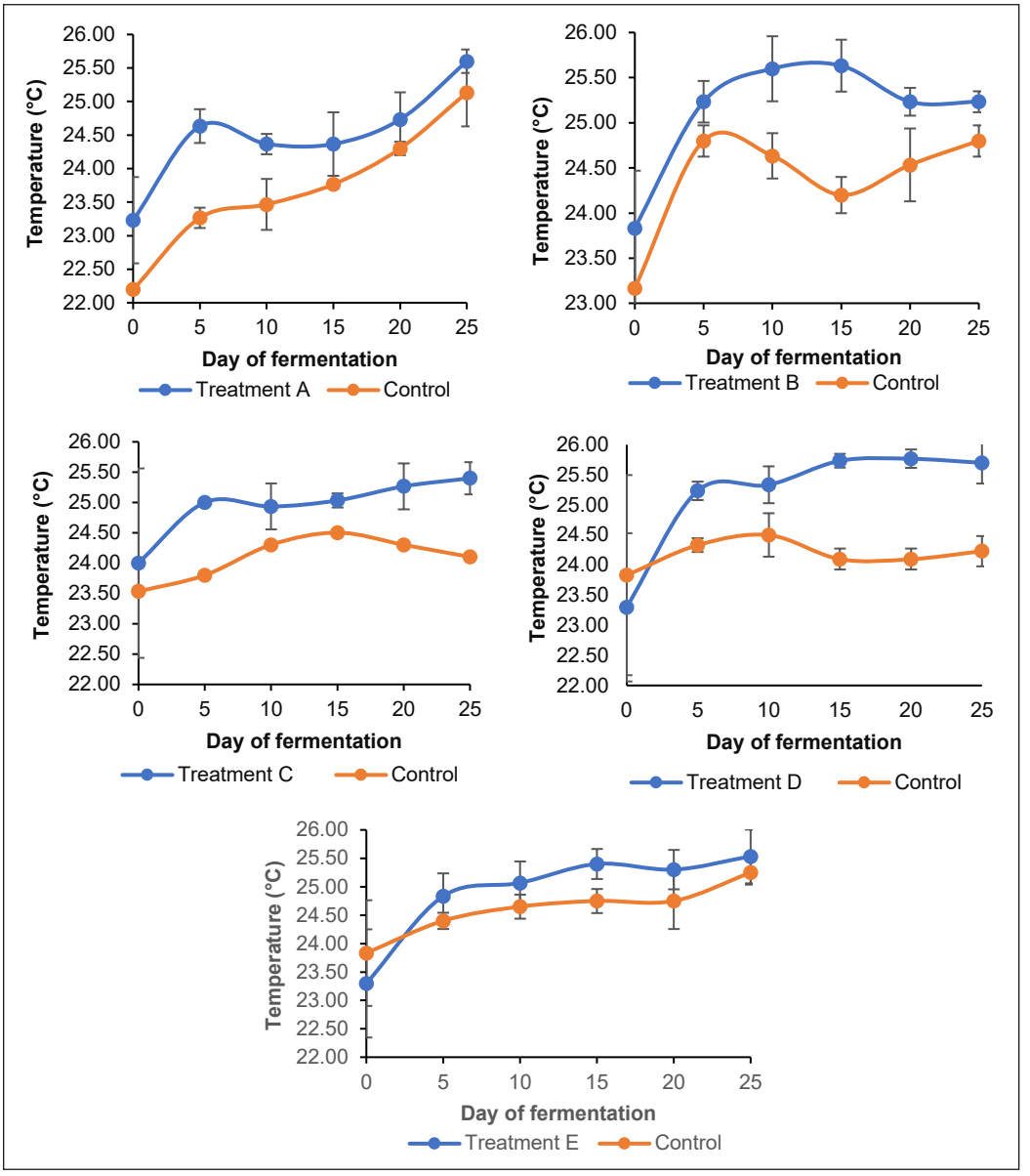

Figure 2. The temperature of treatments A-E with it controls during the fermentation process
Note. Treatment A = 100% AH; Treatment B = 100% RH; Treatment C = 50% AH : 50% RH; Treatment D = 70% AH: 30% RH; Treatment E = 30% AH: 70% RH. Error bars = Standard deviation

number of colonies increased until day 15 and started to drop on day 20. The graph trend for all treatments showed that the number of colonies decreased at the end of fermentation.

DISCUSSION

pH and Temperature During Fermentation

Based on the result, microbial activity of treatment with EM is higher than control

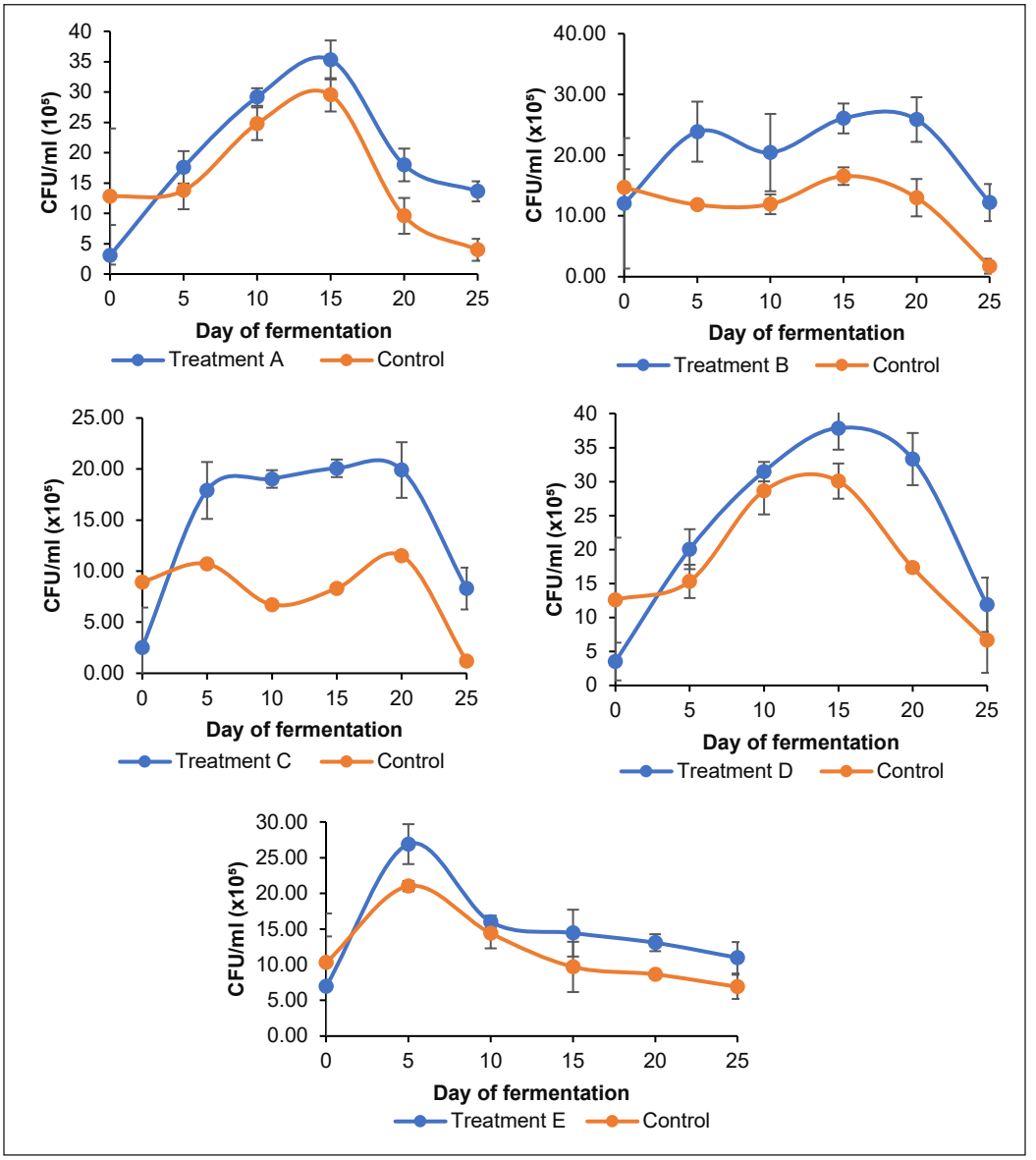

Figure 3. The total number of bacteria (CFU/ml) of treatments A-E with its controls during the fermentation process

Note. Treatment A = 100% AH; Treatment B = 100% RH; Treatment C = 50% AH : 50% RH; Treatment D = 70% AH: 30% RH; Treatment E = 30% AH: 70% RH. Error bars = Standard deviation. CFU = Colony Forming Unit

treatment, resulting in higher pH and temperature at the end of fermentation. It shows that EM plays a role in increasing microbial activity during the fermentation process. Changes in pH indicate that incubation works well. Based on the result obtained, the pH of all treatments was increasing, except for treatment B. Based on a previous study, this indicates a good quality compost within the suggested range

of pH 6–8.5 (Jusoh et al., 2013; Romero-Perdomo et al., 2015). This range is optimal for promoting microbial growth and activity.

Heat is released during the fermentation treatment process. According to Kornievskaya et al. (2020), microorganisms that decompose organic matter produce energy from heat, carbon dioxide, and water vapor, causing temperature rises. The heat generated by the population of microorganisms' respiration and decomposition of sugar, starch, and protein also contributes to the increase in temperature during the fermentation process (Jusoh et al., 2013). The rising temperature is a good indicator of microbial activity in the fermentation process, as higher temperatures indicate more microbial activity. Even though no microbial tests were performed on the sample, based on the high temperature achieved, it is reasonable to conclude that the fermentation treatment with EM has higher microbial activity than the control treatment without EM. A similar study by Molina-Favero et al. (2008) found that temperatures between 29 and 32ºC produce biomass, auxins, and nitrous oxide in bacteria belonging to the genus *Azospirillum* during the fermentation process.

Total Microbial Colonies During Fermentation

As the day of fermentation increases, then the number of colonies increases. The increased colony count could be attributed to adequate microbial nutrient intake in EMAS, allowing microbes to reproduce actively during fermentation. The fact that there were more colonies in the EM treatment than in control indicates that EM affected the growth of many microbial colonies because they still had access to sufficient energy from molasses for growth.

Microorganisms derived from organic waste materials can produce fertilizer in the same way organic solid waste, which contains a high concentration of nutrients like N, P, K, and other organic materials (Bamdad et al., 2022). Some microorganisms, such as plant growth-promoting rhizobacteria (PGPR), turned into biofertilizers because they release phytohormones, particularly cytokinins (Wong et al., 2015). The biological functions of phytohormones, such as auxins, cytokinins, and gibberellins, are useful in contributing to the observed physiological traits and crop yield of plants. Furthermore, using microorganisms derived from organic waste can improve soil structure and quality. However, with the addition of EM to organic waste materials, the number of microorganisms is greater than that of organic waste alone. As a result, using EM will improve the efficiency of organic waste fermentation and shorten the time required to convert organic waste to biofertilizer. However, several environmental factors, such as temperature, pH, the presence of oxygen, and the availability of food for these microbes, impact microbial growth, leading to unstable growth and a decline in the number of bacteria (Febria & Rahayu, 2021).

Identification of Best Treatments Ratio

The pH is gradually increasing for treatment A, as is the temperature. However, the number of colonies formed increased until day 15 and decreased until day 25. The pH of treatment B is only higher on day 0, but it tends to decrease to around 4.0–5.0. However, when the temperature and number of colonies are analyzed, the graph shows that the number of colonies decreased when the temperature was high. The number of colonies increased when the temperature was slightly reduced. In treatment C, the pH and temperature rise concerning the fermentation day. However, the trend of the number of colonies may be unstable because the number of colonies increased for a few days, then decreased, and this cycle continued. In treatment D, the pH and temperature rise with the fermentation day. The trend of graphs of colony number for treatment D is nearly identical to treatment A's. When day 20 arrived, the number of colonies decreased. The pH differences in treatment E were not particularly noticeable, and the differences from the beginning to the end of fermentation were also minor. However, the temperature rises during the fermentation process. Nonetheless, when the number of colonies was examined, the number of colonies of treatment E decreased.

So, after considering the pH, temperature, and number of colonies, treatments A and D are suitable for biofertilizers. Treatment D, on the other hand, has the best treatment ratio. It is because, when comparing the number of colonies, both treatments begin to decline after day 15. The number of colonies, however, varies significantly between days 15 and 20. The number of colonies in treatment A decreases from 35.3×10^5 to 18×10^5 CFU/ml. On the other hand, the number of colonies in treatment D has been reduced from 37.87×10^5 to 29.23×10^5 CFU/ml. The number of colonies in treatment A is higher than in treatment D. According to Stoffella and Kahn (2001), longer fermentation times result in fewer microbes and less substrate for growth. Based on that statement and the results obtained, it can be concluded that treatment D with a ratio of 70% AH:30% RH can be applied to the plant around day 15 of fermentation because the microbial activity is active at that time. If microbial activity declines, sugar energy sources, such as molasses used in this study, must be added to allow microbes to redevelop.

Comparison of AH and RH Volume Ratio

Another factor can be investigated to determine the best treatment ratio. This study concludes that anchovy head and rice husk volume influence microbial activity during fermentation based on the volume ratio of anchovy head and rice husk. When the volume of AH is high, microbial activity rises, resulting in the highest number of colonies. It occurs in treatments A and D. However, when the volume of the rice husk is greater than the volume of the anchovy head, the treatment becomes unstable because the number of colonies fluctuates based on the trend of the number

of colonies in treatments B and E. It could happen because both anchovy head and rice husk contain nutrients. Although no nutrient content analysis was performed on the sample in this study, based on the findings, it can be assumed that the nutrient content of the anchovy head and rice husk influenced microbial activity during the fermentation process. Yusof et al. (2022) supported that anchovy heads have high nutritional quality such as carbohydrates, protein, fat, calcium, iron, fiber, zinc, vitamins A, B12, D, E, DHA, EPA, and trans-fat. Meanwhile, rice husk has a lot of silicon and potassium, which are nutrients that can be utilized by indigenous microorganisms and can be used to improve soil quality (Milla et al., 2013).

CONCLUSION

All fermentation parameters revealed in this study show a similar pattern for both treatments, with and without EM. The measured parameters show that the decomposition of AH and RH occurs in both treatments. The increasing pH, temperature, and number of colonies indicate that organic matter decomposition occurs over 25 days. All treatments are suitable to be used on plants. Nevertheless, treatment D is the best treatment ratio, with a 70% AH:30% RH. Even though treatments A and D have the same graph pattern, there is a difference in the number of colonies between the treatments, which indicates that treatment D is the best treatment ratio. The amount of anchovy head and rice husk also affected microbial activity during fermentation. Using EM in fermentation increases microbial activity, which will shorten the time for organic waste to become biofertilizers. Plants benefit from EM as well because it increases crop yield. Finally, the ratio of rice husk to anchovy head in treatment D in this study should be studied further by other researchers to discover its potential, particularly for the agricultural industry.

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DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported herein.

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Growth and Yield of Shallot (*Allium cepa* L. Aggregatum Group) with Application of Amino Acid Biostimulant Dosages

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ABSTRACT

Intensive shallot cultivation needs high application rates of inorganic fertilizers that can cause environmental problems. Therefore, it is essential to lessen the rate of inorganic fertilizers by environmentally friendlier approaches, such as the application of biostimulants like amino acids. The present study determined the most effective dosage and application method of amino acid biostimulant to increase shallot yield and growth while using only half the amount of inorganic fertilizers. The research was arranged in a randomized complete block design with two factors and four blocks as replications. The first factor was the dose of amino acids biostimulant (0, 0.5, 1, and 2 L/ha), and the second factor was the application method (through leaves and soil). Data were observed on nitrogen (N), phosphorus (P), potassium (K), manganese (Mn), boron (B), indole acetic acid (IAA), gibberellin, zeatin, kinetin, nitrate reductase activity (NRA), chlorophyll as well as the growth and yield of shallot. The results showed that the application of amino acids biostimulants increased IAA, gibberellin, and kinetin content in both application methods. Amino acids biostimulants increased N, P, K, B, Mn, and chlorophyll. Amino acids biostimulant 1 L/ha was the best dosage to increase leaf diameter, leaf dry weight, total dry weight, number of bulbs (5.63 per plant; 44%), and productivity (16.46 tons/ha; 33.77%). The application through the leaves improved NRA, leaf area, and crop growth rate. It was indicated that amino acid biostimulant through leaves provides a useful instrument for plant growth, allowing the reduction of inorganic fertilizer without compromising crop yields.

Keywords: Amino acids, macro and micronutrients, phytohormones, supplement

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INTRODUCTION

Shallot has high economic and strategic values with consequently intensive cultivation, which generally needs a high inorganic fertilizer application. The required inorganic shallot fertilizers are nitrogen: phosphorus: potassium (NPK) 15:9:20 300 kg/ha, zwavelzure ammonium (ZA) 200 kg/ha, and potassium chloride (KCl) 75 kg/ha, as recommended based on research by Pangestuti et al. (2022) in tropical lowland and regosol soil. Intensive farming with high levels of inorganic fertilizers negatively affects the environment, including air, water, and soil pollution, which is followed by the phenomenon's production of greenhouse gases, disease resistance, nutritional deficiencies, and toxicity to organisms above and below the soil (Ning et al., 2017; Tyagi et al., 2022). Therefore, reducing the amount of inorganic fertilizers and replacing them with friendlier organic materials seems necessary (Souri et al., 2019; Souri & Sooraki, 2019). Amino acids represent the most common and effective compounds integrated in fertilizer manufacturing as well as one of the most known biostimulants in cropping systems (Mohammadipour & Souri, 2019; Souri, 2016; Souri et al., 2018; Souri & Hatamian, 2019).

Biostimulants are substances that contain one or more compounds or microorganisms formulated to stimulate plant growth, as well as mechanisms for regulating plant resistance to stress conditions from both biotic and abiotic sources, improving microbiological rhizosphere activity and soil enzymes, and optimizing the photosynthesis process to improve crop production with low application doses (du Jardin, 2015; Sharma et al., 2014; Yakhin et al., 2017). The use of biostimulants has been reported can increase the activity of vital enzymes involved in the metabolism of carbon and nitrogen, hormone activity as well as physiological and biochemical changes in plant tissues (Alfosea-Simón et al., 2020; Sheng et al., 2020). In horticultural crops, the use of biostimulants enables the reduction of fertilizer without a significant reduction in yield or quality (Noroozlo et al., 2019; Souri & Bakhtiarizade, 2019), as well as increasing essential micronutrients (Fe, Cu, and Zn) for human health (Mannino et al., 2020).

Amino acids are the fundamental components of proteins, play important roles in metabolic processes and transport, also serve as precursors for active substances, and influence physiological activity in plant growth (Moormann et al., 2022; Popko et al., 2018). Amino acid-based biostimulants can regulate the absorption and assimilation of nitrogen in plants as mediated by enzyme activity (Noroozlo et al., 2020). Applying amino acids increased nitrate, amino acid, and total nitrogen contents in leaves and yield of soybeans by up to 21% (Teixeira et al., 2018). Several studies have suggested that amino acid-based biostimulants applied through leaves increased antioxidant activity, pigment content, and production in pepper; chlorophyll, ion transport, photosystem II activity, assimilation of vital nutrients like N, P, Ca, Mg, S, and stress response at different levels of nitrogen

application in spinach and lettuce (Carillo et al., 2019; Mola et al., 2020; Parađiković et al., 2011; Tsouvaltzis et al., 2020); they also increased the number of pods, seeds, phenol, flavonoids, and yields of soybean (Kocira, 2019). Meanwhile, amino acids from plants applied through the roots can increase reductase activity, chlorophyll and iron (Fe) content, leaf area, number of fruits, and yield of tomato (Cerdán et al., 2013; Souri et al., 2017).

Amino acid-based biostimulants (Amiboost®) are made from plant-based organic materials (sugar cane and corn) and fermented using microbes to produce L-amino acids that plants can absorb. The nutritional content of the amino acids biostimulant consists of 6.5% nitrogen, 2.0% phosphorus, 1.5% potassium, 0.1% boron, 0.1% manganese, and 10% total amino acids. Considering the problems associated with inorganic fertilizers, using amino acids-based biostimulants can be a promising method for reducing the use of inorganic fertilizers, which can support sustainable agriculture. Shallot has never been exposed to amino acids-based biostimulants. Therefore, studies regarding the impact of the application of amino acids-based biostimulants (Amiboost®) must be conducted to determine the ideal dosage and efficient application method on the growth and yield of shallot.

MATERIALS AND METHODS

Material Preparation

The research was conducted from March to June 2022 at the Tri Dharma Fields Laboratory, Faculty of Agriculture, Universitas Gadjah Mada, Bantul, Special Region of Yogyakarta Province, Indonesia (07°48'17"S and 110°24'45"E) at 107 m a.s.l. Throughout the experiment, the average temperature was 30.5°C, the humidity was 34%, the soil temperature was 34.57°C, and the amount of sunlight intensity was 38,259 lux. The shallot variety was Gamaba, and the biostimulant product for amino acids was Amiboost® (Korea). The amino acids biostimulants used contain macronutrients, micronutrients, and total amino acids, and the elemental content was 6.5% nitrogen, 2.0% phosphorus, 1.5% potassium, 0.1% boron, 0.1% manganese, and 10% total amino acids.

Experimental Design

The experiment was arranged in a randomized complete block design with two factors and four blocks as replications. The first factor was the dose of amino acid biostimulants (0, 0.5, 1, and 2 L/ha), and the second factor was the application method (through leaves and soil). There are eight treatment combinations. In a 95 cm × 80 cm field plot, 15 bulbs were planted with 20 cm × 15 cm spacing. The planting medium was regosol soil.

In the present study, amino acids biostimulants were applied twice, 14 and 28 days after planting (DAP), with a volume of 1,000 L/ha. The amino acids biostimulant application methods involved applying through the leaves by directly spraying it on the leaves and through the soil by watering it directly into the soil around

the plants. Fertilization was applied three times, the time before planting, at 28 and 42 DAP, by giving half the recommended rate of inorganic fertilizer based on research by Pangestuti et al. (2022). The fertilizer rate before planting was manure 20 ton/ha (Dharma Jaya, Indonesia), SP36 130 kg/ha (Petro, Indonesia), NPK 16:16:16 100 kg/ha (Mutiara®, Indonesia), and ZA 85 kg/ha (Petro, Indonesia); at 28 DAP, it was NPK 15:9:20 75 kg/ha (Mutiara®, Indonesia), ZA 100 kg/ha ha (Petro, Indonesia); and at 42 DAP, it was NPK 15:9:20 75 kg/ha ha (Petro, Indonesia), KCl 75 kg/ha ha (Petro, Indonesia).

The Analysis of the Plant Tissue, Phytohormones, Nitrate Reductase, and Chlorophyll

The plant tissue, phytohormones, nitrate reductase, and chlorophyll were analyzed 35 DAP. Plant tissue analysis included N, P, K, B, and Mn. Tissue N content was examined utilizing wet destruction and semi-automatic distillation equipment Kjeldahl UDK 139 (Velp Scientifica, Italy). The P contents were examined utilizing wet destruction and a GENESYS™ 10 UV-Visible spectrophotometer (Thermo Fisher Scientific, USA). The K contents were examined utilizing wet destruction and a PFP7C flame photometer (Rose Scientific Ltd., Canada). Tissue B content was analyzed using the dry destruction and a GENESYS™ 10 UV-Vis spectrophotometer. The Mn content was analyzed using dry destruction and atomic absorption spectrophotometry (AAS) (Thermo Fisher Scientific, USA). Phytohormone analysis measurements, including IAA, gibberellin, zeatin, and kinetin, were analyzed using the Linskens and Jackson (1987) method and high-performance liquid chromatography (HPLC, Thermo Fisher Scientific, USA). Nitrate reductase was analyzed spectrophotometrically at a wavelength of 540 nm using the method described by Jaworski (1971). Nitrate reductase activity (NRA; in μmol/NO_2/hr) is measured using the following equation:

$$\text{NRA} = \frac{\text{Sample absorbance}}{\text{Standard absorbance}} \times \frac{1{,}000}{\text{Leaf fresh weight (mg)}} \times \frac{1}{\text{Incubation time (hour)}} \times \frac{50}{1{,}000}$$

Chlorophyll contents were analyzed spectrophotometrically at 645 nm and 663 nm wavelengths using the Coombs et al. (1985) method. The formula was used to determine chlorophyll *a* (mg/g), chlorophyll *b* (mg/g), and total chlorophyll (mg/g):

$$\text{Chlorophyll } a = (12.7 \times A_{663}) - (2.69 \times A_{645})$$
$$\text{Chlorophyll } b = (22.9 \times A_{645}) - (4.68 \times A_{663})$$
$$\text{Total chlorophyll} = (20.2 \times A_{645}) + (8.02 \times A_{663})$$

Growth and Yield

Plant growth and yield variables, including plant height (cm), number of leaves, leaf diameter (mm), leaf area (cm²), net assimilation rate (mg/cm²/week), crop growth rate (mg/cm²/week), root dry weight (g), leaf dry weight (g), total dry weight (g), harvest index, number of bulbs per plant, and productivity (ton/ha) were analyzed. The leaf surface area was measured using

leaf area meters software WInDIAS 3 (United Kingdom). The dry weight of roots, leaves, and bulbs was measured during a 48-hr period in an oven set to 80°C.

Data Analysis

All observational data were examined utilizing analysis of variance followed by Tukey's test with a significance level of 95%. The analysis used version 3.2.2 of the R statistical computing platform (The Austrian R foundation).

RESULTS AND DISCUSSION

Plant Tissue Analysis

Tissue analysis was carried out on the macro and micronutrients in shallot leaves. The highest accumulation of N content was 1.02% in the leaf tissue at a dose of 1 L/ha through the leaves (Figure 1). Plants need N for metabolism in preparing proteins, making up leaf chlorophyll, cell division, and stimulating vegetative plant growth (Zhang et al., 2013). In shallot, N can increase the number of leaves, plant height, stem diameter, bulbs, and yield (Biru, 2015; Kemal, 2013). Furthermore, N deficiency in plants can occur due to the application of inorganic fertilizer (NPK) at half the recommended rate. Navarro-León et al. (2022) stated that the total N content accumulated in lettuce plant tissue increased by up to 30% when L-amino acid-based biostimulants were applied with N fertilizer only 30% and 60%. Amino acids play roles as different physiological signaling factors in plant processes. In *Arabidopsis thaliana*, the glutamate receptor can bind to other amino acids. Amino acids activate the receptor and trigger physiological processes to regulate nitrogen uptake (Miller et al., 2007). Moreover, based on the application method, the N content accumulated in higher concentrations in the application through the leaves. Biostimulants containing amino acids could become nutrients for microbes when applied through the soil; hence, it is possible for biostimulants that are used through the soil to be easily captured and decomposed by bacteria in the soil. Jones et al. (2009) reported that soil microorganisms consume 30–40% of amino acids for respiration and the remaining amino acids-C are used to produce and maintain cell biomass.

P content accumulated was the highest at 0.3% at a dosage of 0.5 L/ha through the leaves (Figure 1). P is a necessary nutrient that plays an aspect in the preparation of adenosine diphosphate (ADP), adenosine triphosphate (ATP), deoxyribonucleic acid (DNA), and ribonucleic acid (RNA). Furthermore, P can stimulate root growth, strengthen stems, and increase onion bulbs' weight and size (Anbes et al., 2018). According to Khan et al. (2019), spraying individual amino acids improved the uptake of N (67.44%), P (66.57%), and K (50.55%) and the yield of lettuce.

The highest accumulation of K content was 3.5% at an amino acid biostimulant dose of 1 L/ha through leaves (Figure 1). K is involved in several biochemical and physiological processes. Tränkner et al. (2018) stated that K regulates stomata for photosynthetic carbon dioxide fixation

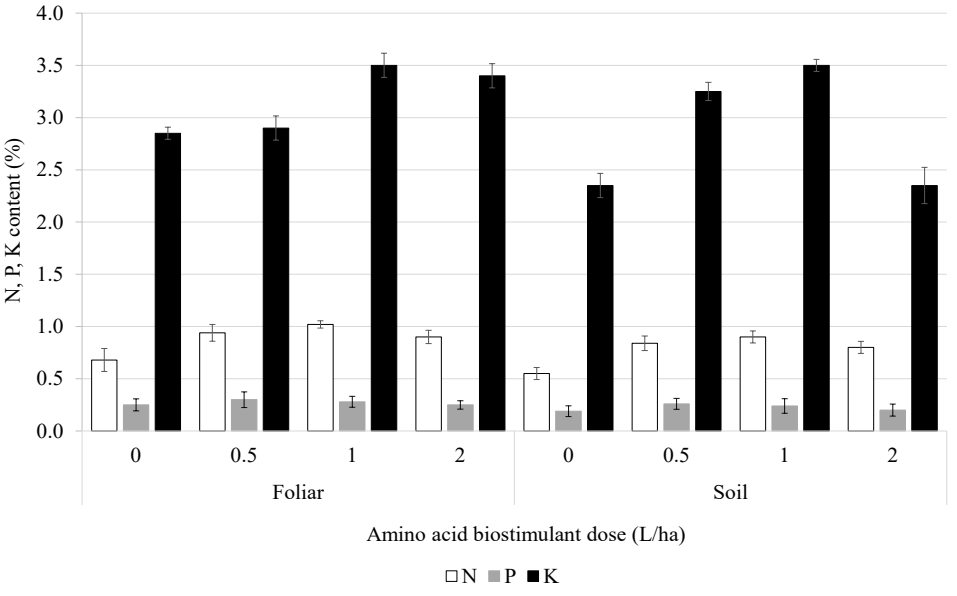

Figure 1. Tissue analysis of N, P, and K content in shallot leaves 35 days after planting

as transport as well as photoassimilates consumption. K also activates the enzyme adenosine triphosphate synthase (ATP), which affects the plasma membrane (Juhaszova et al., 2019). Jiku et al. (2020) stated that K can increase garlic's bulb weight, size, and yield. Al-Karaki and Othman (2023) reported that biostimulants containing amino acids enhance N, P, K, and Mg content in lettuce leaves by 20.5%, 25.1%, 19.8%, and 58.5%.

Figure 2 shows that the highest accumulated Mn content was 17.51 ppm at a dosage of 0.5 L/ha through the leaves. Meanwhile, the Mn content decreased in plants without amino acid biostimulants. Mn is an element that plants require for photosynthesis, increasing the nitrate reductase enzyme's activity and the production of photosynthetic pigments, carotenoid content, and membrane stability index (Shahi & Srivastava, 2018).

The highest accumulation of B content was 134.63 ppm at an amino acid biostimulant dose of 1 L/ha through the leaves (Figure 2). In the absence of amino acids biostimulant dose, the boron content was merely 2.02 and 8.02 ppm. B plays a role in developing and growing new cells in merismatic tissue and could influence root cell elongation and division, phenol metabolism, carbohydrate metabolism, cell wall structure, and auxin synthesis (González-Fontes et al., 2016; Li et al., 2016). In addition, amino acids influence other micronutrients' availability and soil absorption by acting as chelating agents in the soil, translocating micronutrients in the phloem, and affecting root morphology (Halpern et al., 2015). In tomatoes, the

application of biostimulants that contain amino acids and microelements (boron) could increase plant growth by up to 48.5% and the number of fruits by up to 105.3% (Francesca et al., 2020).

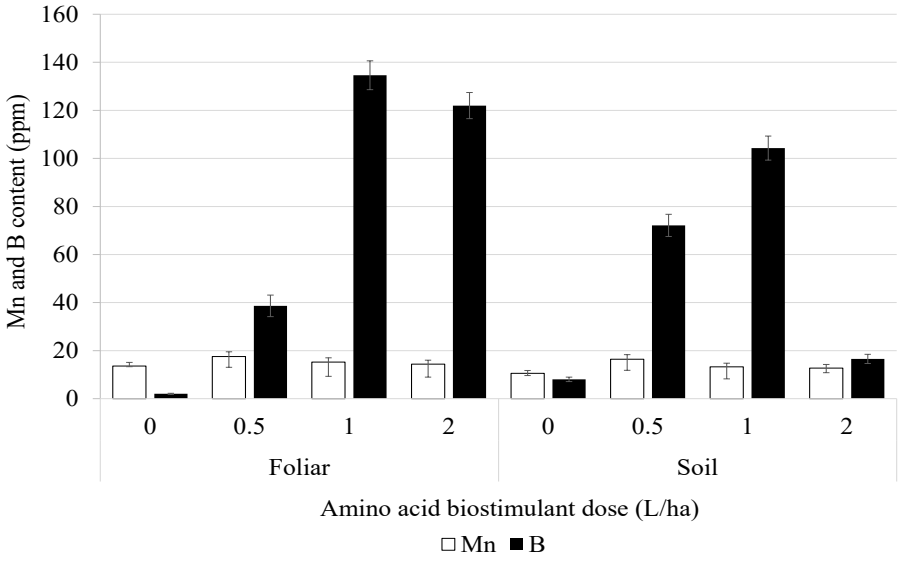

Figure 2. Tissue analysis of manganese (Mn) and boron (B) content in shallot leaves 35 days after planting

Phytohormone Analysis

The phytohormone content in shallot was observed in the leaf organs 35 DAP. Table 1 shows an interaction between the dosage of the amino acids biostimulant and the application method for IAA, gibberellin, and kinetin, whereas zeatin showed no interaction.

Amino acids biostimulant dose of 0.5–2 L/ha through leaves and soil had significantly higher IAA and gibberellin content than without amino acids biostimulants through leaves or soil. The highest IAA content was found at amino acids biostimulant dose of 2 L/ha through the soil (5.81 mg/g) but was not significantly different with amino acids biostimulant dose of 0.5–2 L/ha through leaves. Meanwhile, the highest gibberellin content was found at an amino acids biostimulant dose of 1 L/ha through the leaves (5.25 mg/g). Auxin content has increased in plants due to amino acids in biostimulants. Amino acids are considered precursors for auxin (IAA) synthesis, essential in regulating cell division and elongation processes (Yue et al., 2021). L-tryptophan promotes auxin production in plants, enhancing plant growth and productivity when foliar and seed are applied (Mustafa et al., 2018). Auxin promotes the formation of root and lateral fibers, thereby optimizing the absorption of water and minerals in plants. Gibberellins can increase cell division and growth, which will cause stem elongation and increase the number of internodes (Liu et al., 2019).

Table 1
IAA, gibberellin, zeatin, and kinetin content of shallot 35 days after planting

		Application method		
Indole acetic acid (mg/g)	Amino acids biostimulant dose (L/ha)	Foliar	Soil	Mean
	0	2.43 ± 0.10 c	2.38 ± 0.06 c	2.41 ± 0.05
	0.5	5.49 ± 0.26 a	4.46 ± 0.11 b	4.98 ± 0.24
	1	5.01 ± 0.12 ab	5.17 ± 0.16 ab	5.09 ± 0.10
	2	5.76 ± 0.18 a	5.81 ± 0.27 a	5.78 ± 0.15
	Mean	4.67 ± 0.35	4.45 ± 0.34	(+)
	CV (%)			7.67
Gibberellin (mg/g)	Amino acids biostimulant dose (L/ha)	Foliar	Soil	Mean
	0	2.31 ± 0.09 d	2.22 ± 0.02 d	2.26 ± 0.04
	0.5	3.68 ± 0.10 b	3.04 ± 0.15 c	3.36 ± 0.15
	1	5.25 ± 0.13 a	3.11 ± 0.04 c	4.18 ± 0.41
	2	3.27 ± 0.02 bc	3.67 ± 0.06 b	3.47 ± 0.08
	Mean	3.63 ± 0.28	3.01 ± 0.14	(+)
	CV (%)			5.56
Zeatin (mg/g)	Amino acids biostimulant dose (L/ha)	Foliar	Soil	Mean
	0	1.28 ± 0.07	1.27 ± 0.05	1.28 ± 0.04 q
	0.5	1.44 ± 0.05	1.63 ± 0.10	1.53 ± 0.06 p
	1	1.19 ± 0.02	1.39 ± 0.06	1.29 ± 0.05 q
	2	1.25 ± 0.02	1.39 ± 0.03	1.32 ± 0.03 q
	Mean	1.29 ± 0.03y	1.42 ± 0.04x	(-)
	CV (%)			8.29
Kinetin (mg/g)	Amino acids biostimulant dose (L/ha)	Foliar	Soil	Mean
	0	0.49 ± 0.01 c	0.48 ± 0.01 c	0.49 ± 0.00
	0.5	1.05 ± 0.03 b	1.35 ± 0.00 a	1.20 ± 0.06
	1	0.93 ± 0.02 b	1.29 ± 0.06 a	1.11 ± 0.08
	2	1.05 ± 0.05 b	1.07 ± 0.08 b	1.06 ± 0.04
	Mean	0.88 ± 0.06	1.05 ± 0.09	(+)
	CV (%)			9.10

Note. The Tukey's test at 5% revealed no significant differences between means that were followed by the same letters in a row or columns; (-) = No interaction between treatment; (+) = There was an interaction between treatment

Kinetin and zeatin are hormones belonging to the group of natural cytokinins that can process cell division. The dosage of amino acids biostimulants and the application method significantly affected the zeatin content in shallot plants. Applying an amino acids biostimulant dose of 0.5 L/ha had significantly higher zeatin content (1.53 mg/g) than other amino acids biostimulant doses. Meanwhile, based on the application method, the zeatin content was significantly higher in the application through the soil. For the kinetin hormone, applying amino acids biostimulant doses of 0.5–2 L/ha through the leaves and soil had a significantly higher kinetin content than without amino acids biostimulants. The highest kinetin contents were 1.35 and 1.29 mg/g at amino acids biostimulant doses of 0.5 and 1 L/ha, respectively, through the soil (Table 1). The high content of zeatin and kinetin in the application of amino acids biostimulants through the soil could be due to the synthesis of cytokinins in the root tips.

Cytokinin is synthesized in the roots and translocated to the shoots via the xylem vessels. The occurrence of cytokinin accumulation in leaves results from transportation through the xylem. Cytokinin has the function of encouraging cell division and differentiation in cooperation with auxins. Cytokinin can stimulate development by modulating plants' morphologies, physiology, and biochemistry. Furthermore, cytokinin causes the expression of phototropin involved in the opening of the stomata to increase stomatal conductance, regulates the synthesis of chlorophyll pigments, and regulates the production of crucial proteins necessary for the assembling and enzyme activation of the Rubisco (Gujjar et al., 2020). Hormones can also be synthesized through rhizobacteria in the soil, producing phytohormones. Rhizobacteria can use biostimulants containing amino acids through the soil to synthesize phytohormones.

The Analysis of Nitrate Reductase and Chlorophyll

Figure 3 shows that the highest NRA was 1.97 μmol/NO_2/hr at amino acids biostimulant dose of 1 L/ha. Meanwhile, based on the application method, NRA was highest in applications through the leaves (Figure 3). Nitrate reductase is the initial enzyme in the process of nitrate assimilation. Nitrate reductase analysis illustrates the high uptake of N in the plant body. There is a tendency to increase nitrate reductase at amino acids biostimulant doses. Ertani et al. (2009) stated that applying a hydrolysate protein-based fertilizer enhanced corn's glutamine synthetase and nitrate reductase activity, transforming nitrate to organic nitrogen. Kunicki et al. (2010) studied the impact of the biostimulant (Aminoplant), which contains an amino acid in spinach, which increased NRA—the improvement in nitrate reductase related to the chlorophyll content of plants. The highest chlorophyll *a*, *b*, and total content were found at amino acids biostimulant dose of 1 L/ha. Meanwhile, based on the application method, application through the leaves produced the highest total chlorophyll content (Figure 4). Similar

studies showed that chlorophyll *a*, *b*, and total increased with the application of amino acid biostimulants in *Achillea millefolium* (Shafie et al., 2021), faba bean (Desoky et al., 2021), tomato (Alfosea-Simón et al., 2020), and lettuce (Tsouvaltzis et al., 2020). Furthermore, the application of protein hydrolysate from legumes at different N fertilization levels can also increase chlorophyll content in spinach and lettuce (Mola et al., 2020).

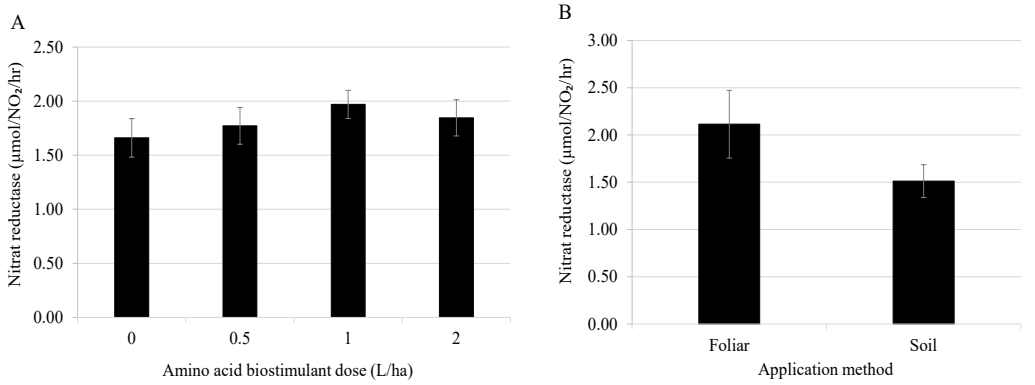

Figure 3. Nitrate reductase activity of shallot plants 35 days after planting based on (A) amino acids biostimulant dose and (B) application method

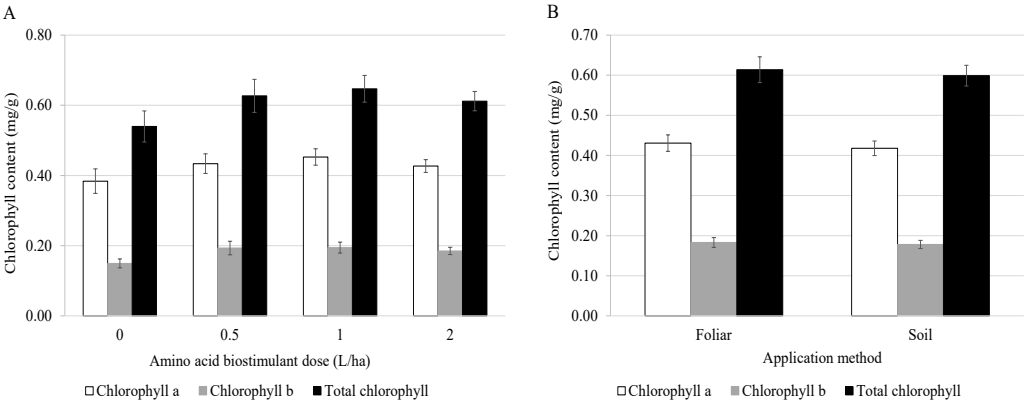

Figure 4. The chlorophyll content of shallot plants 35 days after planting based on (A) amino acids biostimulant dose and (B) application method

Growth and Yield

Growth indicators, including plant height, the number of leaves, and leaf diameter, were observed at 35 DAP. Table 2 shows no interaction between the dose of amino acids biostimulants and the application method. Individually, the dosage of amino acids biostimulants and the application method had the same effect on plant height and number of leaves. Meanwhile, in leaf diameter, amino acids biostimulant dosage of 1 L/ha was significantly higher than without amino

acids biostimulant. The growth-stimulating effects of amino acids linked to an increase in the content of phytohormones (IAA and gibberellins) are responsible for the increase in plant leaf diameter. Auxin cooperates with gibberellins to promote cell division, growth, and elongation by activating cell wall structural proteins (Kou et al., 2021), positively affecting plant growth, including leaf diameter. Furthermore, providing amino acid biostimulants can increase the availability and absorption of nutrients, especially nutrient N, which plants require to stimulate their vegetative growth.

Table 2
Plant height, the number of leaves, and leaf diameter of shallot 35 days after planting

Amino acids biostimulant dose (L/ha)	Plant height (cm)	Number of leaves	The leaf diameter (mm)
0	38.04 ± 1.78 a	20.38 ± 1.60 a	3.93 ± 0.10 b
0.5	38.85 ± 1.46 a	22.42 ± 1.63 a	4.05 ± 0.14 ab
1	43.88 ± 1.19 a	25.17 ± 2.19 a	4.62 ± 0.22 a
2	39.88 ± 1.95 a	20.42 ± 2.23 a	4.37 ± 0.18 ab
Application method			
Foliar	41.25 ± 1.21 p	21.88 ± 1.37 p	4.38 ± 0.15 p
Soil	39.07 ± 1.22 p	22.31 ± 1.46 p	4.10 ± 0.11 p
Interaction	(-)	(-)	(-)
CV (%)	12.20	21.84	11.25

Note. The Tukey's test at 5% revealed no significant differences between means that were followed by the same letter in columns; (-) = No interaction between treatment

The highest leaf area was found on the application of a 1 L/ha amino acids biostimulant dose. Meanwhile, in the application method, the highest leaf area was found through the leaves (Figure 5). Leaves are plant organs that play an essential role as a place for photosynthetic activity and transpiration and as light receptors. Studies revealed that the nature of the biostimulants that induced plant growth was related to the expansion of the leaf surface (Calvo et al., 2014) and improved biochemical, thus raising metabolism in plant tissues (Sadak et al., 2014).

The highest net assimilation and crop growth rates were found at amino acids biostimulant dosage of 1 L/ha. Meanwhile, in the application method, the highest net assimilation and crop growth rates were found through the leaves (Figure 6). According to Khan et al. (2019), applying amino acids to leaves increased photosynthesis efficiency and lettuce growth. The higher crop growth rate in plants can be due to the biostimulant composition, which contains various stimulants such as amino acids that can act as signaling molecules to improve fertilizer assimilation (Sheng et

al., 2020). Amino acids are crucial for the growth and development of horticultural crops (du Jardin, 2015). Amino acids and proteins are the main components of living cells and play a crucial part in many cells' metabolic processes. Amino acid biostimulants greatly affect morphology, physiology, and biochemistry (Tadros et al., 2019).

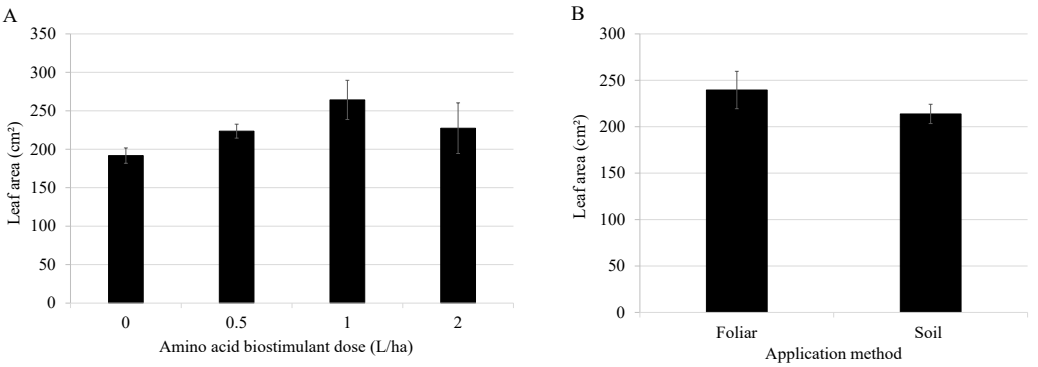

Figure 5. The leaf area of shallot 63 days after planting based on (A) amino acids biostimulant dose and (B) application method

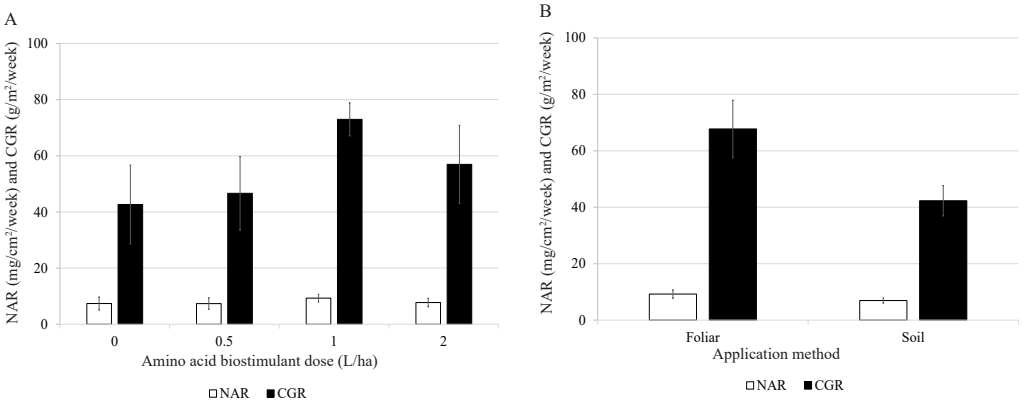

Figure 6. Net assimilation rate (NAR, mg/cm²/week) and crop growth rate (CGR, g/m²/week) of shallot at 5–9 weeks after planting based on (A) amino acids biostimulant dose and (B) application method

The growth parameters show no interaction between the dose of amino acid biostimulants and the application method on root dry weight, leaf dry weight, bulb dry weight, and total dry weight (Table 3). The dosage of amino acids biostimulants and the application method individually had the same effect on root and bulb dry weights. Meanwhile, the dose of amino acids biostimulants significantly affected leaf dry weight and total dry weight. Amino acids biostimulant dose of 1 L/ha resulted in significantly higher leaf dry weight and total dry weight compared to without the amino acids biostimulant. The increased dry weight of a plant occurs due to the formation

of photosynthates into biomass stored in the plant body. This outcome is relevant to Navarro-León et al. (2022), where the application of 60 and 30% N fertilizer reduced the value of leaf biomass of lettuce, whereas adding amino acids biostimulants was able to improve both the fresh and dry weight of the leaves.

Table 3
Root dry weight, leaf dry weight, bulbs dry weight, and the total dry weight of shallot 63 days after planting

Amino acids biostimulant dose (L/ha)	Root dry weight (g)	Leaf dry weight (g)	Bulbs dry weight (g)	Total dry weight (g)
0	0.14 ± 0.01 a	1.41 ± 0.11 b	5.29 ± 1.46 a	7.01 ± 1.53 b
0.5	0.15 ± 0.02 a	1.83 ± 0.09 ab	6.72 ± 2.27 a	8.82 ± 2.40 ab
1	0.31 ± 0.13 a	2.13 ± 0.16 a	7.46 ± 0.85 a	10.23 ± 0.94 a
2	0.14 ± 0.03 a	1.86 ± 0.25 ab	6.76 ± 1.56 a	9.10 ± 1.55 ab
Application method				
Foliar	0.23 ± 0.07 p	1.88 ± 0.16 p	7.01 ± 1.13 p	9.35 ± 1.20 p
Soil	0.14 ± 0.01 p	1.73 ± 0.09 p	6.11 ± 1.10 p	8.22 ± 1.16 p
Interaction	(-)	(-)	(-)	(-)
CV (%)	10.82	15.70	14.81	18.06

Note. The Tukey's test at 5% revealed no significant differences between means that were followed by the same letter in columns; (-) = No interaction between treatment

The yield parameter indicates no interaction between the dosage of amino acids biostimulant and the application method on the number of bulbs per plant, harvest index, and the productivity of shallot. The dosage of amino acids biostimulants and the application method individually had the same effect on the harvest index. Applying an amino acids biostimulant dose of 1 L/ha was significantly higher in the number of bulbs and productivity than without an amino acids biostimulant. Meanwhile, it has the same effect based on the application method, both through leaves and soil (Table 4). Several studies reported that applying amino acids biostimulant had positive effects on the yield of vegetable crops such as lettuce (Bulgari et al., 2019), basil (Noroozlo et al., 2020), and mint (Tarasevičienė et al., 2021).

Inorganic fertilizer is ideal for plant cultivation because it provides nutrients for plants in the early stages of growth. The application of amino acid-based biostimulants (Amiboost®) could provide nutrition to the end stages of growth and positively affect yield with only 50% inorganic fertilizer. This result related to Klokić et al. (2020) that the effects of amino acid-based biostimulants increased

Table 4
The number of bulbs per plant, harvest index, and productivity of shallot

Amino acids biostimulant dose (L/ha)	The number of bulbs per plant (cm)	Harvest index	Productivity (ton/ha)
0	3.13 ± 0.35 b	0.65 ± 0.09 a	10.90 ± 1.12 b
0.5	4.88 ± 0.44 ab	0.67 ± 0.06 a	11.55 ± 0.86 b
1	5.63 ± 0.73 a	0.72 ± 0.03 a	16.46 ± 1.02 a
2	4.50 ± 0.82 ab	0.70 ± 0.06 a	11.87 ± 0.74 b
Application method			
Foliar	4.56 ± 0.56 p	0.67 ± 0.05 p	13.01 ± 0.83 p
Soil	4.50 ± 0.39 p	0.70 ± 0.03 p	12.38 ± 0.88 p
Interaction	(-)	(-)	(-)
CV (%)	27.14	27.46	22.01

Note. The Tukey's test at 5% revealed no significant differences between means that were followed by the same letter in columns; (-) = No interaction between treatment

plant growth and yield in conventional nutrition and prevented yield loss in low-input nutrition. Furthermore, Koleška et al. (2017) stated that the yield of tomatoes in two varieties improved by applying biostimulants containing amino acids up to 14 and 13% by reducing the amount of NPK nutrition. Kocira (2019) stated that biostimulants with free amino acids and *Ascophyllum nodosum* extracts increased soybean yields by up to 25%. Applying amino acids biostimulant (Perfectose) through the leaves enhanced fresh yield by up to 39%, dry yield by up to 55.3%, and the number of leaves by up to 22.8% of lettuce (Al-Karaki & Othman, 2023).

CONCLUSION

Application of amino acids-based biostimulants (Amiboost®) 0.5−2 L/ha to shallot plants with 50% reduced NPK fertilizer has positive effects on enhancing nutrient acquisition and assimilation (high N, P, K, B, and Mn accumulation), as well as an increased IAA, gibberellin, kinetin, zeatin, NRA, and chlorophyll. Amino acid-based biostimulant 1 L/ha was the best dosage to increase leaf diameter by 18%, plant growth rate by 42%, leaf dry weight by 56%, total dry weight by 30.88%, number of bulbs by 5.63 per plant (44%), and productivity by 16.46 ton/ha (33.77%). The application method through leaves increases NRA, leaf area, and crop growth rate. It was indicated that using amino acid-based biostimulant 1 L/ha through leaves makes it possible to reduce inorganic fertilizer with high growth and productivity of shallot.

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Case Study

Chromobacterium violaceum Infection in a Domestic Shorthaired Cat with Dog Bite Wounds

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ABSTRACT

Chromobacterium violaceum is a motile, facultative anaerobe and Gram-negative *Bacillus*, a common inhabitant of soil and water in tropical and subtropical regions. *Chromobacterium violaceum* is recognised as an opportunistic pathogen of humans and animals. Although rare, a few chromobacteriosis cases were reported in humans and several species of animals. However, there is no published report of *Chromobacterium* infection in cats. This report describes a *C. violaceum* infection in a cat with a history of non-healing dog bite wounds. The bacterial culture of the wounds revealed pure growth of *C. violaceum* on both aerobic and anaerobic cultures. The infection in this cat was successfully treated after changing the antibiotic to enrofloxacin based on an antibiotic sensitivity test (AST). This case demonstrates the importance of culture and AST tests in non-healing wounds. Chromobacteriosis can be included in the differentials, especially if wounds are contaminated by soil or stagnant water.

Keywords: Cat, chromobacteriosis, *Chromobacterium violaceum*, dog bite wound, Malaysia, zoonosis

INTRODUCTION

Chromobacterium violaceum is a motile, facultative anaerobe and Gram-negative *Bacillus*, which can grow readily on simple nutrient media, including MacConkey agar, at 35–37°C. This organism is a common inhabitant of soil and water in tropical and subtropical regions (Saigin et al., 1994; Teoh et al., 2006). *Chromobacterium violaceum* is recognised as an opportunistic pathogen of humans and animals (Ciprandi et al., 2013). Its transmission route is through the exposure of wounds and traumatic lesions to soil and water containing *C. violaceum* (Ciprandi et

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al., 2013). The main clinical presentations are fever, abdominal pain, skin lesions, and the formation of metastatic abscesses (Chen et al., 2003; Manjunath, 2007). Although rare, a few chromobacteriosis cases were reported in humans, for example, in Malaysia (Ang, 2004; dan Lin et al., 2016; Saigin et al., 1994; Sharmin et al., 2019), Brazil (de Siqueira et al., 2005), Argentina (Kaufman et al., 1986), China (Teoh et al., 2006), India (Parajuli et al., 2016), and Bangladesh (Mazumder et al., 2020).

There were also a few reports on several species of animals, such as horses (Hammerschmitt et al., 2017), calves (Soares et al., 2019), dogs (Crosse et al., 2006), turtles (Scheelings et al., 2012), cougar (*Puma concolor*) (Mesquita et al., 2021), pig (Liu et al., 1989), and non-human primate in Malaysia and Costa Rica, e.g., gibbon (Donny et al., 2018) and monkey (Baldi et al., 2010). There is no published report on chromobacteriosis in a cat with wounds. Therefore, this report describes the first reported case of *C. violaceum* in a domestic shorthaired (DSH) cat in Malaysia, diagnosed based on clinical presentation, cytology, and bacterial culture.

CASE REPORT

A 6-month-old male DSH cat was presented with a history of non-healing dog bite wounds sustained two weeks prior. According to the owner, it had fallen into a drain containing stagnant water after escaping from the dog and has been inappetent since then. Details of initial treatment by a private practice could not be obtained. Physical examination revealed that the cat was alert and responsive; all vitals were within normal limits.

The right periorbital and nasal bridge were mildly swollen (Figure 1). Multiple punctured and lacerated wounds were found on the cat's dorsum, the base of the tail, and the left perianal region.

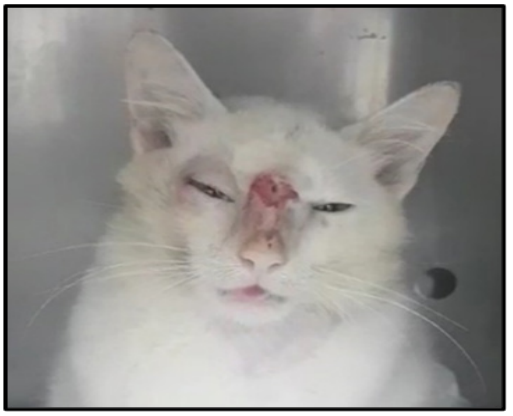

Figure 1. Swelling at the right periorbital and nasal bridge

The following diagnostic tests were conducted: haematology and serum biochemistry, impression smear for cytological analysis, and wound swab sample for bacterial (i.e., aerobic and anaerobic) and fungal culture and AST. The haematological analysis was normocytic normochromic nonregenerative anaemia (packed cell volume [PCV] = 0.19, normal range = 0.24–0.45, reticulocyte index [RI] = 0.6).

The leukogram result was moderate neutrophilia (21.79, normal range = 2.5–12.5) with a left shift and monocytosis (1.09, normal range = 0.2–0.8), which

indicates infection or inflammation. Serum biochemistry revealed low normal sodium (145, normal range = 146–156) with slight hypoalbuminemia (24.2, normal range = 25–40) could be due to inappetence and hyperglobulinemia (50.6, normal range = 25–45) due to infection or inflammation.

Impression smears of the wounds only showed red blood cells with intact and degenerated neutrophils.

For bacterial culture, there was a pure growth of *C. violaceum* isolated on equine blood agar and MacConkey agar. The cultures were incubated aerobically at 37°C for 24 hr. After 24 hr incubation, smooth, round, convex, butyrous, and violet-coloured colonies were noticed (Figure 2). The colonies were characterised as Gram-negative *Bacillus*, sulphate (negative), indole (negative), motility (positive), and oxidase positive (more details in Table 1). There is no microorganism isolated from the fungal culture.

The *in vitro* AST was performed using the Kirby-Bauer Disc diffusion method. The *C. violaceum* was sensitive to enrofloxacin (5 µg), gentamicin (10 µg), norfloxacin (10 µg), polymyxin B (300 iU), and tetracycline (30 µg). The *C. violaceum*, however, was found resistant to ceftriaxone (30 µg).

The cat was initially treated empirically with metronidazole (5 mg/ml) (Metogryl®, Unique Pharmaceutical Laboratories, India) at 10 mg/kg, intravenous, given slowly twice a day for 4 days only. The antibiotic was changed to enrofloxacin (50 mg, KVP Pharma + Veterinär Produkte GmbH, Germany), 5 mg/kg, orally, once a day based on AST results. Concurrently, the cat was given tramadol (50 mg/ml, Duopharma (M) Sdn. Bhd, Malaysia) at a dosage of 3 mg/kg, subcutaneously, twice daily for 5 days as pain management. Wound cleaning once a day was also performed using chlorhexidine gluconate 4% (Pahang Pharmacy Sdn.

Table 1
Biochemical characteristics of Chromobacterium violaceum *isolated from cat wounds*

Test	Results
Gram-staining	-
Growth on MacConkey agar	+
Oxidase	+
β-haemolysis	+
Motility	+
Indole production	-
Growth on TSI agar	-
Glucose	O/F
Gas glucose	-
H_2S production	-
Citrate	-
Urease production	-

Note. - = Negative; + = Positive; TSI = Triple Sugar Iron; O/F = Oxidative/Fermentative; H_2S = Hydrogen sulfide

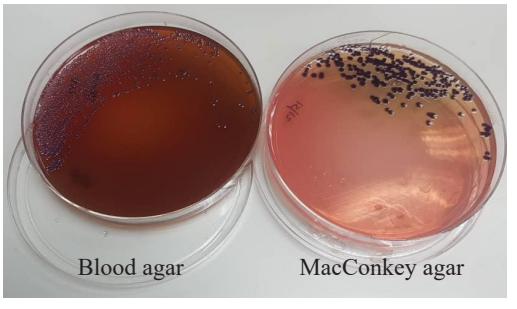

Figure 2. Colonies of *Chromobacterium violaceum* on blood (equine) and MacConkey agar

Bhd., Malaysia), followed by flushing with normal saline (sodium chloride [NaCl] 0.9%) and dabbing the wound surface with povidone-iodine 10% (Pahang Pharmacy Sdn. Bhd., Malaysia). Then, the wounds were left open for aeration. The wounds improved and healed completely with this combination of treatments and wound management for 14 days (Figures 3 and 4). The second bacterial culture and AST were performed after 14 days of treatment, and the results revealed negative results for *C. violaceum*; instead, there were opportunistic bacteria from the isolates, such as *Bacillus* sp., *Staphylococcus pseudintermedius*, and *Aggregatibacter actinomycetemcomitans*. The cat was treated for another two weeks with enrofloxacin (50 mg, KVP Pharma + Veterinär Produkte GmbH, Germany) beyond resolution.

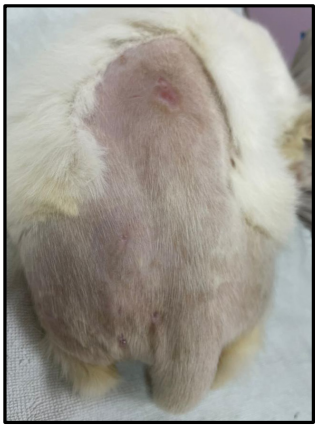

Figure 3. Mild erythema and dried scab at tail base, left perianal, and dorsum region

Figure 4. A dried wound on the right supraorbital region

DISCUSSION

As described, *C. violaceum* is a facultative pathogenic opportunistic saprophyte (inhabitant of soil and stagnant water), which can be transmitted through exposure to wound contamination or any traumatic lesions (Ansari et al., 2015; Baker et al., 2008; Teoh et al., 2006). For a case such as a chronic non-healing animal bite wound with a history of wounds contaminated with contaminated soil and water, *C. violaceum* infection should be considered as one of the potential differential diagnoses. Based on physical examination findings, a few differential diagnoses were also listed for the non-healing dog bite wound (i.e., multiple punctured and lacerated wounds at the dorsal body, tail-based, and perianal region). For example, this condition would be caused by a bacterial infection (e.g., *Pasteurella multocida*, *Staphylococcus* spp., *Streptococcus* sp., and *Corynebacterium* sp.) (Presutti, 2001). Fungal infection can also be suspected in the non-healing wound in cats, for example, *Cryptococcus* spp., where there is a possibility that spores from the contaminated environment can infect the open wound (Duncan et al., 2006).

In mild chromobacteriosis cases, the infected patients will experience inappetence with a non-healing wound similar to clinical manifestations in the current case. However, in severe cases, where the patient is immunocompromised (Hammerschmitt et al., 2017) or with concurrent illness, for instance, due to feline leukaemia virus (FeLV) or feline immunodeficiency virus (FIV) infection, the patient might experience poor wound healing and possible promote the infection of *Chromobacterium* spp. As a result, this organism could lead to bacterial sepsis or septicaemia. For example, the previous case report on two black-handed gibbons showed several clinical signs, such as diarrhoea, inappetence, and pyrexia (Donny et al., 2018). The gibbons, however, died after 48 hr of treatment with antipyrexia and multivitamins. Post-mortem of the two gibbons revealed isolation of *C. violaceum* via bacterial isolation and identification from the lung, liver, spleen, and kidney (Donny et al., 2018). Since early treatment with antimicrobials was not performed, systemic infection would have occurred, causing septicaemia resulting in organ failure and death (Donny et al., 2018).

In human chromobacteriosis cases, better diagnostics and appropriate choices of antibiotics aid in the reduction of the rate of fatality (Batista & da Silva Neto, 2017). Because of the rapidly progressive nature of this infection, empirical antibiotic administration should be considered pending AST results (Crosse et al., 2006). In humans, a few antibiotics are effective against chromobacteriosis, such as fluoroquinolones and carbapenems (de Siqueira et al., 2005; Teoh et al., 2006). Besides that, fluoroquinolone, tetracycline, and gentamicin are demonstrated to be effective in animals such as calves (Soares et al., 2019), sheep (Carrasco et al., 1996), and dogs (Crosse et al., 2006). Therefore, the antibiotics can be used empirically prior to receiving AST results.

However, precautions should be taken in treating and managing chromobacteriosis as *C. violaceum* is recognised to develop antibiotic resistance easily in mammals such as sheep (Carrasco et al., 1996) and horses (Hammerschmitt et al., 2017). Due to that, treatment failure may occur, leading to death (de Siqueira et al., 2005). These can be seen in a few reported cases, for example, in a calf (Ajithdoss et al., 2009) and human (de Siqueira et al., 2005), where these patients were reported to die within three days of presentation due to lacked diagnostic tests: bacterial culture and AST (Ajithdoss et al., 2009; de Siqueira et al., 2005). A study showed that the isolation and identification of *Chromobacterium* spp. can take up to 48 hr (Vishnu & Palaniswamy, 2016) and in general, the AST results can be retrieved by day four or five from a bacteriology laboratory. When bacterial culture and AST are performed, the recovery chance is high.

For instance, chromobacteriosis was diagnosed in two critically ill dogs from Florida. Evidence showed that the bacterial culture and AST were done before prescribing enrofloxacin to the infected dogs (Crosse et al., 2006). As a result, the prescribed antibiotic helped in the complete

resolution of chromobacteriosis within two weeks of treatment in one of the infected dogs, but the other dog did not survive (Crosse et al., 2006). The use of empiric therapy, thus, helps to reduce the chance of a flare-up of the infection, and it is highly recommended to prescribe fluoroquinolones such as enrofloxacin in future cases if there is a possibility of bite wounds in cats with possible soil or water contamination with the organism, *Chromobacterium* spp. and if the animal is systematically ill.

Chromobacterium violaceum is a potential zoonotic disease (Scheelings et al., 2012). Therefore, precautions should be taken seriously, especially for those handling chromobacteriosis cases in animals (i.e., veterinarian, veterinary assistant, staff, and client). For example, there is a safety guideline published by the University of South Carolina on how to manage *C. violaceum*, and one of them is the Animal Biosafety Level 2 (ABSL-2), which will be applied to activities that involve experimentally infected animals (more details in the guideline, University of South Carolina, 2022). Apart from that, the need to use personal protective equipment (Donny et al., 2018), such as wearing proper gloves, an apron or laboratory coat, closed-toed shoes, and eye protection (i.e., for potential splashes, sprays, or droplets), are required. It is also recommended to disinfect the contaminated area or cages (e.g., *C. violaceum* is susceptible to 10% bleach [i.e., sodium hypochlorite] and 70% ethanol) (University of South Carolina, 2022).

CONCLUSION

It is the first reported case of chromobacteriosis in a cat in Malaysia. Although rare, it is recommended to include it in the list of differential diagnoses, especially for a case with a history of chronic non-healing animal bite wounds exposed to contaminated soil and water. Bacterial culture and AST are highly recommended to provide accurate information on the type of organism and reduce the chance of treatment failure. In this case, the *C. violaceum* in the cat was isolated and successfully treated with enrofloxacin. Therefore, in future cases, empirical therapy with fluoroquinolones, such as enrofloxacin, can be considered highly suspected of chromobacteriosis.

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Supplementation of *Chlorella vulgaris* Ameliorates the Stress-induced Hematological Alterations in Wistar Rats

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ABSTRACT

Stress has been associated with various diseases and physiological disruptions. *Chlorella vulgaris* is known for its antioxidant properties. This study examined the effects of *C. vulgaris* on the hematological profile of stress-induced Wistar rats. Stress significantly impacted various hematological parameters, including erythrocyte count, hemoglobin concentration, hematocrit levels, white blood cell count, and platelet count. The supplementation of cultivated *C. vulgaris* effectively restored erythrocyte count and suppressed elevated lymphocyte levels, while commercially available *C. vulgaris* and amitriptyline drugs had no significant effects on these parameters. Stress caused smaller-sized erythrocytes but did not significantly affect mean corpuscular volume. Both amitriptyline and *C. vulgaris* restored platelet levels, while mean platelet volume remained unaffected. Overall, *C. vulgaris* showed promise as a therapeutic intervention for countering stress-induced inhibition of erythropoiesis and restoring erythrocyte count, but more research is needed to understand the underlying mechanisms and develop effective strategies for managing stress-related changes in hematological parameters.

Keywords: *Chlorella vulgaris*, hematological profile, rats, stress

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INTRODUCTION

In recent years, there has been a growing global awareness of the importance of overall health, encompassing physical and mental well-being (Saraceno, 2020). Mental health, in particular, has gained significant attention due to its impact on individuals' quality of life. As a pervasive aspect of modern life, stress has been recognized

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as a significant risk factor for various illnesses and serious health conditions (Kessler & Bromet, 2013; Moussavi et al., 2007). The implications of stress extend beyond mental health, as it has been linked to disruptions in the immune system, elevated cholesterol levels, and increased susceptibility to cancer, cardiovascular diseases, and diabetes (Abate et al., 2020; Balkan et al., 2004; Dar et al., 2019; Harris et al., 2017; Wattoo et al., 2008). Chronic stress also impacts neurotransmitter levels in the brain, specifically serotonin and noradrenaline, which play crucial roles in mood regulation. A decline in these neurotransmitter levels can contribute to the development of depression (Goddard et al., 2010; Hammen, 2005; Natarajan et al., 2015). Moreover, stress-induced reductions in antioxidant levels occur within the body, further exacerbating the detrimental effects of stress (Srivastava & Kumar, 2015). These findings underscore the far-reaching impact of stress on the physiological functioning of the human body.

According to data from the Institute of Health Metrics and Evaluation (IHME), an alarming 970 million people worldwide suffered from mental disorder-related issues in 2019 (IHME, 2022). The symptoms associated with stress are wide-ranging and can manifest as anhedonia (loss of pleasure), sleep difficulties, reduced appetite, lack of energy, and other physical and emotional disturbances (Schneiderman et al., 2005; Yaribeygi et al., 2017). Despite the prevalence and severity of stress-related disorders, there persists a misconception among certain segments of the population that stress is not a disease (Willenberg et al., 2020). This misguided belief hampers efforts to raise awareness, promote early intervention, and provide appropriate support for individuals affected by stress and depression.

A range of treatment options are available for managing stress, yet in Indonesia, the utilization of stress treatment remains relatively uncommon. In 2018, it was estimated that approximately 19 million Indonesians, accounting for approximately 9.8% of the overall population, were affected by emotional disorders (Ministry of Health Indonesia, 2022). However, due to various limitations, only a mere 9% of individuals suffering from mental illnesses received professional treatment or had access to mental health facilities (Ministry of Health Indonesia, 2022).

Among the commonly prescribed interventions for individuals with depression or stress are antidepressant medications, which unfortunately come with unpleasant side effects on the body (Almohammed et al., 2022; Carvalho et al., 2016; Faquih et al., 2019). Antidepressant drugs, being non-selective, can lead to significant side effects by affecting neuronal receptors in various parts of the body. These side effects may manifest as heart palpitations, constipation, insomnia, restlessness, migraines, excessive sweating, vomiting, and long-lasting health risks (Ferguson, 2001). The long-term effects include withdrawal symptoms, sexual impairments, and weight increase (Cartwright et al., 2016). Understanding the

limitations in stress treatment accessibility and the potential side effects of conventional antidepressant medications highlights the need for alternative approaches that can effectively alleviate stress and its associated symptoms.

Chlorella vulgaris is a species of unicellular green alga characterized by its non-motile nature, eukaryotic cellular structure, and circular shape. It possesses a high chlorophyll concentration, rendering it an exceptional photosynthetic organism. Extensive research has revealed that *C. vulgaris* exhibits notable antioxidant properties (Kumar & Singh, 2019) and is capable of synthesizing various carotenoids, which have been shown to enhance immune function and protect against degenerative diseases (Azlan et al., 2020; Bito et al., 2020; Kwak et al., 2012). Due to its established safety profile and beneficial qualities, *C. vulgaris* has long been utilized as a safe and health-promoting dietary supplement for individuals (Bito et al., 2020). Consequently, considering these attributes, *C. vulgaris* holds potential as an alternative treatment for depression. In light of these characteristics, this study aimed to explore the effects and potential of commercially available and cultivated *C. vulgaris* in modulating the hematological profile of Wistar rats subjected to stress. By examining the effects of *C. vulgaris* on the hematological profile of stressed rats, this study contributes to the understanding of *C. vulgaris*'s potential as an alternative approach to alleviate the physiological effects of stress.

METHODS

The study design received approval from the Animal Ethics Commission Team from the Faculty of Veterinary Medicine, Universitas Gadjah Mada, Indonesia, under Ethical Clearance Certificate No: 00042/04/LPPT/VI/2018. Wistar Rats (*Rattus novergicus*), sourced from Laboratorium Penelitian dan Pengujian Terpadu (LPPT) Universitas Gadjah Mada, Indonesia, were divided into 5 groups according to the previously described protocol (Karima & Mulyati, 2019; Soetantyo & Sarto, 2019). The groups for treatment included a no-stress control group, a stress-induced placebo group, and stress-induced groups treated with the antidepressant drug amitriptyline (Indofarma, Indonesia; 2.25 mg/kg of body weight), cultivated *C. vulgaris* (Blue Green Microalga Technology, Indonesia; 153 mg/kg of body weight), or commercially available *C. vulgaris* (CNI Sun Chlorella, Indonesia; 153 mg/kg of body weight). Each group consisted of 5 individuals.

The stress-induced treatments were performed as previously described (Hu et al., 2017; Karima & Mulyati, 2019; Soetantyo & Sarto, 2019; Zhang et al., 2014). Throughout the 42-day experimental period, rats were subjected to seven types of stress treatments (Table 1).

All experimental groups, except the control group, were exposed to the stress treatments. In total, each stress type was applied for six days. The sequential administration of stress types was predetermined, with each type being reapplied after six days from the previous

Table 1
Description of stress treatments

No.	Stress type	Treatment
1	Cold water	Rats were placed in a water tank (25 cm in depth), 5°C (cold) or 45°C (warm) for 3 min, followed by drying up in a lamp-illuminated cage with fresh bedding
2	Warm water	
3	Wet cages	Exposure to 5 cm wet beddings for 24 hr
4	Reversal of dark-light cycle	Rats were subjected to light from 18:00 to 06:00 and covered in dark from 06:00-18:00
5	Ultrasonic exposure	Rats were treated with ultrasonic exposure for 12 hr
6	Tilted cages	The rat cages were tilted at 45° for 24 hr
7	Fasting	Food and water removal for 24-hr

application of the same type to maintain uniformity. Following the completion of the stress treatment phase, from day 43 to day 56, rats were provided with drug supplementation and *C. vulgaris*. The main parameters observed in this study were hematological profiles. These parameters were measured at day 56, at the end of the drug or *C. vulgaris* supplementation. Blood samples were obtained by collecting venous blood from the orbital vein using a micro hematocrit capillary. The hematological parameters of the blood samples were analyzed using a hematology analyzer (Sysmex KX-21, China). The data were analyzed using Microsoft Excel and SPSS 16.0 software through one-way analysis of variance (ANOVA) followed by the post hoc test.

RESULTS AND DISCUSSION

As previously reported (Karima & Mulyati, 2019; Soetantyo & Sarto, 2019), the stress-induced rats exhibited reduced body weight, a significant decrease in sucrose preference, and an increased cholesterol level. The primary focus of this study was to examine the impact of stress and *Chlorella* supplementation on the hematological profile of rats. Figure 1 demonstrates a notable decrease in the number of erythrocytes in the stress-treated group compared to the control group. The data indicates that the stress condition may impair the process of erythrocyte formation in rats. It suggests that stress acts as a disruptive factor, negatively influencing the generation of new erythrocytes. A noteworthy finding of this study is that supplementation with cultivated

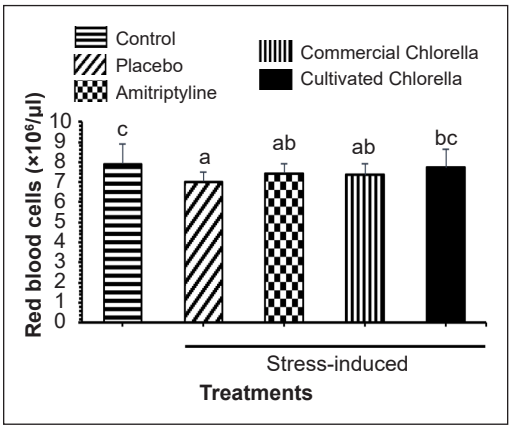

Figure 1. Supplementation of cultivated *Chlorella vulgaris* counteracts the suppressive impact of stress on erythropoiesis
Note. Groups with different letters represent statistically significant differences

C. vulgaris effectively restored the number of erythrocytes in rats experiencing stress.

Intriguingly, this restorative effect was even more pronounced than that observed in the positive control group, which received treatment with amitriptyline. These results strongly suggest that the supplementation of cultivated *C. vulgaris* effectively mitigates the detrimental impact of stress on erythrocyte production. However, it is important to highlight that commercially available *C. vulgaris* did not demonstrate the same ability to rescue the decreased number of erythrocytes induced by stress. This discrepancy could be attributed to several factors. Firstly, variations in the cultivation and processing methods of commercially available *C. vulgaris* products may result in differences in their bioactive composition or nutrient content. The specific combination or concentration of bioactive compounds responsible for stimulating erythrocyte production in cultivated *C. vulgaris* may be absent or present at lower levels in commercially available variants.

Moreover, the quality and purity of commercially available *C. vulgaris* products can vary, and contaminants or impurities introduced during the production or packaging process may interfere with their biological activity. These contaminants could potentially hinder the erythropoietic effects of *C. vulgaris* or introduce other substances that counteract its beneficial properties. Additionally, the timing of harvesting and processing the commercially available *C. vulgaris* may influence its efficacy. The optimal stage of growth or maturation at which *C. vulgaris* exhibits maximum bioactivity for erythropoiesis stimulation may not have been captured during the commercial production process. Further investigations into the specific factors influencing the efficacy of commercially available *C. vulgaris* are warranted to fully understand and address these limitations. Taken together, the results of this study strongly suggest that *C. vulgaris* possesses notable potential as a therapeutic intervention to mitigate the inhibitory influence of stress on erythropoiesis. The ability of *C. vulgaris* to restore the erythrocyte count in stress-induced rats highlights its efficacy in promoting the formation of new erythrocytes, thus aiding in maintaining a healthy erythrocyte population.

Hemoglobin, a respiratory pigment found in red blood cells, plays a crucial role in oxygen transport from the lungs to various tissues in the body (Ahmed et al., 2020). Its iron (Fe)-containing structure facilitates the binding and release of oxygen. Normal hemoglobin levels are typically associated with the overall erythrocyte count and hematocrit values, ensuring efficient oxygen delivery (Ahmed et al., 2020; Gell, 2018). Interestingly, *C. vulgaris* possesses a high concentration of Fe at 749 mg/kg, as Widiyanto et al. (2018) reported. Consequently, *C. vulgaris* shows promise in increasing hemoglobin levels, particularly in individuals experiencing stress.

Figure 2 displays the impact of stress on hemoglobin concentration, which is evident through the significant difference between the control and stress-induced

groups. The data illustrates a decrease in hemoglobin concentration under stressful conditions. However, the administration of amitriptyline and the supplementation of *C. vulgaris* showed minimal effect on the recovery of hemoglobin concentration in the stress-induced groups. The decrease in hemoglobin concentration during stress could be attributed to several scientific factors. Stress triggers the release of stress hormones such as cortisol, which can lead to changes in blood volume and hematocrit levels. Additionally, stress-related alterations in erythropoiesis, the red blood cell production process, could contribute to a decrease in hemoglobin concentration. These combined mechanisms may explain the observed decrease in hemoglobin levels in the stress-induced groups despite supplementing amitriptyline or *C. vulgaris*. Further research is necessary to fully elucidate the underlying mechanisms and potential strategies to mitigate stress-induced changes in hemoglobin levels.

Hematocrit, the percentage of red blood cells in the total blood volume, indicates the number of erythrocytes present (Kishimoto et al., 2020). Figure 3 presents the average hematocrit values obtained in our study. Interestingly, our findings reveal a significant decrease in hematocrit levels within the stress-induced group. The decrease in hematocrit observed in the stress-induced group can be closely linked to the decrease in erythrocyte count, as demonstrated in Figure 1. Erythrocytes are the primary component contributing to hematocrit values, and any changes in their quantity can consequently influence hematocrit levels. Thus, the significant decrease in hematocrit levels observed in the stress-induced group may be attributed to the diminished number of erythrocytes. No significant changes in hematocrit levels were observed in the

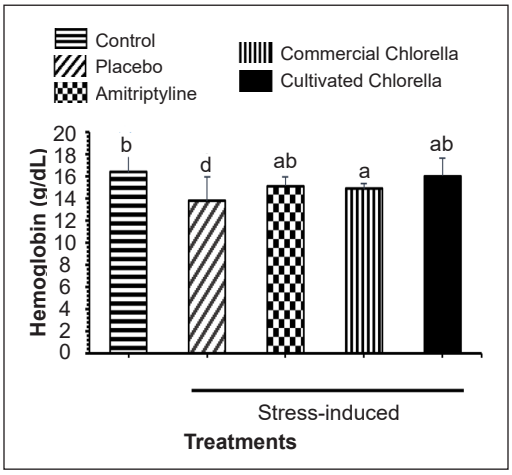

Figure 2. Negative impact of stress treatments on hemoglobin concentration and the minimal effect of *Chlorella vulgaris* supplementation
Note. Groups with different letters represent statistically significant differences

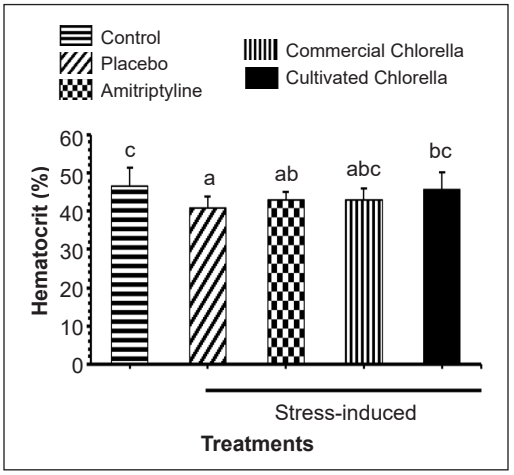

Figure 3. Supplementation of cultivated *Chlorella vulgaris* effectively restores the diminished hematocrit values in stress-induced rats
Note. Groups with different letters represent statistically significant differences

stress group following supplementation with amitriptyline or commercial *C. vulgaris*. However, in contrast, the supplementation of cultivated *C. vulgaris* demonstrated a notable restorative effect on hematocrit values in stressed rats. Similar trends were also observed in Figure 1, showing the positive effect of cultivated *C. vulgaris* on the decreased erythrocyte numbers upon stress exposure. Therefore, these findings provide strong evidence supporting the effectiveness of cultivated *C. vulgaris* supplementation in mitigating the adverse effects of stress on erythrocyte production.

Mean corpuscular volume (MCV) is a widely utilized parameter to assess the average size of erythrocytes (Yavorkovsky, 2021). In our study, depicted in Figure 4, we observed a significant decrease in MCV values of the stress-induced group compared to the control. Surprisingly,

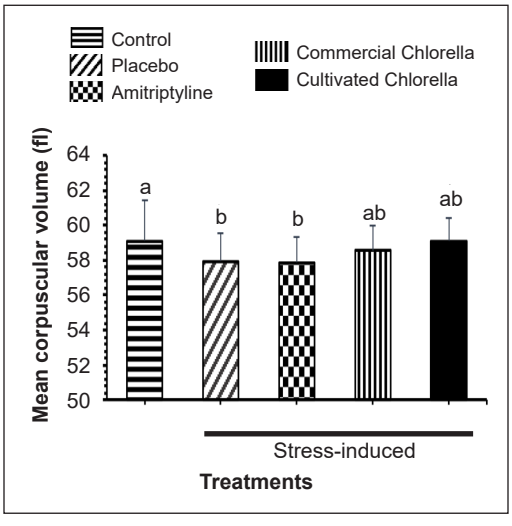

Figure 4. Stress induces a reduction in mean corpuscular volume values in rats
Note. Groups with different letters represent statistically significant differences

supplementation with amitriptyline or *C. vulgaris* exhibited no significant effect on the MCV values of the stress-induced groups. The decrease in MCV observed in the stress-induced groups may be attributed to several plausible reasons. Stress is known to stimulate the release of stress hormones, such as cortisol, which can lead to alterations in erythropoiesis (Lodish et al., 2010). Disturbances in erythropoiesis can result in the generation of smaller-sized erythrocytes, leading to a decrease in MCV. Furthermore, stress-induced oxidative stress and inflammation can influence erythrocyte characteristics, including their size (Maiese et al., 2008; Paulson et al., 2020). Oxidative stress can lead to cellular damage, affecting erythrocyte morphology and potentially leading to smaller-sized erythrocytes, thereby contributing to the decrease in MCV (Ghaffari, 2008; Maiese et al., 2008). Further investigations are warranted to comprehensively understand the underlying mechanisms and explore potential interventions to counteract the stress-induced decrease in MCV.

The mean corpuscular hemoglobin (MCH), which represents the average amount of hemoglobin per red blood cell, was evaluated in our study. Interestingly, despite observing a decrease in the number of erythrocytes and a decrease in hemoglobin concentration in the stress-induced groups, the MCH values did not differ significantly compared to the control group. The lack of significant changes in MCH values between the control and stress-induced groups suggests that stress may not substantially

impact the hemoglobin content within individual red blood cells. This observation also demonstrates that supplementation with amitriptyline or *C. vulgaris* did not show any notable effect on MCH values in the stress-induced groups.

The mean corpuscular hemoglobin concentration (MCHC) represents the average hemoglobin concentration within individual red blood cells and is an important parameter in assessing the hemoglobin content (Ahmed et al., 2020; Gell, 2018). The MCHC values for each group are presented in Figure 6. Notably, the supplementation of amitriptyline and *C. vulgaris* in the stress-induced groups exhibited minimal effect on MCHC values. Furthermore, our findings from Figure 5, which illustrates the MCH values, align with the results observed for MCHC, indicating that stress does not induce significant changes in MCHC.

Individuals who experience stress often face a higher susceptibility to pathogen infection due to disruptions in their immune system (Morey et al., 2015; Segerstrom & Miller, 2004). Monitoring the total blood leukocyte count serves as a simple and accessible indicator of immune system activity. In our study, the stress-induced group exhibited elevated white blood cell (WBC) levels compared to the control group (Figure 7). The observed increase in WBC count in the stress group can be attributed to the body's response to stress-related immune dysregulation. Stress promotes the release of stress hormones such as cortisol (Lodish et al., 2010). These hormones play a role in mobilizing immune cells, including white blood cells, to combat potential threats. Therefore, the elevated WBC count in the stress group can be seen as an adaptive immune system response to potential infections.

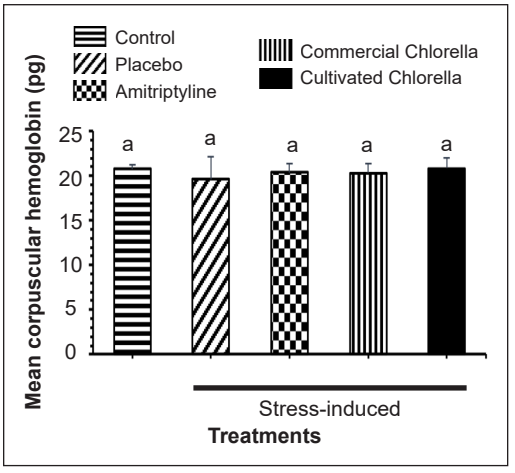

Figure 5. Stress and supplementation of *Chlorella vulgaris* yield no significant impact on mean corpuscular hemoglobin in rats
Note. Groups with different letters represent statistically significant differences

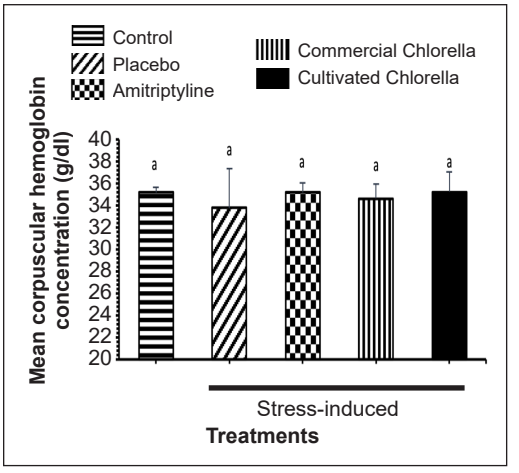

Figure 6. Stress exposure and *Chlorella vulgaris* supplementation exhibit no discernable impact on mean corpuscular hemoglobin concentration in rats
Note. Groups with different letters represent statistically significant differences

However, despite the potential benefits of amitriptyline and *C. vulgaris* supplementation in stress management, our study revealed that neither intervention could effectively reduce the increased WBC levels observed in the stress group. One possible explanation for this lack of effect could be the complex nature of stress-induced immune dysregulation. Stress can influence various immune mechanisms, including cytokine production, lymphocyte function, and cellular communication, which may not be easily modulated solely by amitriptyline or *C. vulgaris* supplementation. Additionally, it is important to consider that the duration and severity of stress exposure, as well as individual variations in stress response, can impact the effectiveness of interventions in modulating immune responses. Further research is needed to explore alternative strategies or combination therapies that may have a more pronounced impact on reducing WBC levels in individuals experiencing stress.

Lymphocytes, a subpopulation of white blood cells, play a crucial role in mounting immune responses against invading antigens (Gasteiger & Rudensky, 2014). As a result, monitoring the number of lymphocytes can provide insights into the ongoing immune responses within the body. Our study demonstrated that the stress-inducing treatments resulted in an elevation of lymphocyte count (Figure 8).

The observed increase in lymphocyte count in response to stress can be attributed to activating the body's immune system. The increase in lymphocyte count indicates the immune system's heightened responsiveness to potential antigens or pathogens in the body. Significantly, our study revealed that the supplementation of *C. vulgaris* was effective in suppressing the elevated levels of lymphocytes, comparable to the effects of the amitriptyline drug. The immunomodulatory properties of *C. vulgaris*, characterized by its bioactive compounds

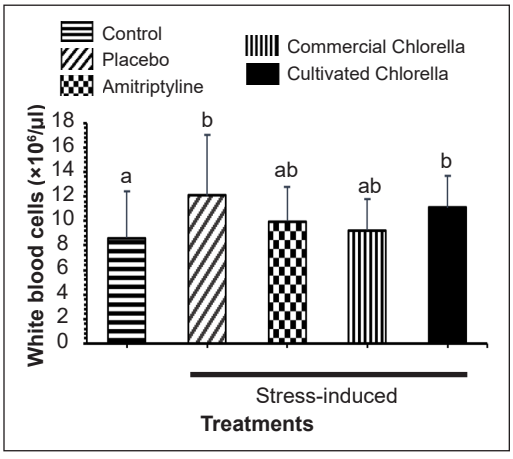

Figure 7. Stress induces an elevation in the white blood cell count in rats

Note. Groups with different letters represent statistically significant differences

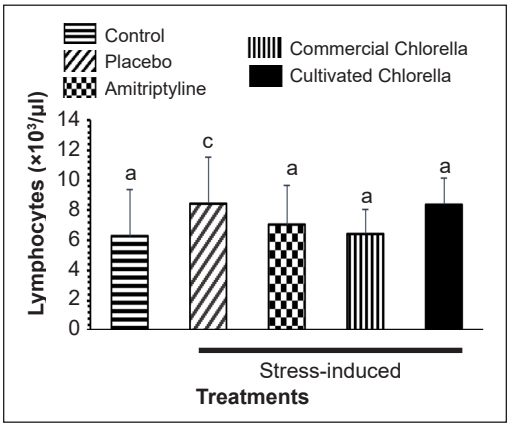

Figure 8. Supplementation of *Chlorella vulgaris* alleviates the elevated lymphocyte numbers in rats subjected to stress

Note. Groups with different letters represent statistically significant differences

and nutritional components, may contribute to its ability to regulate lymphocyte levels. Regueiras et al. (2021) demonstrated that certain components present in *C. vulgaris*, such as polysaccharides and antioxidants, possess anti-inflammatory properties and can modulate immune responses. These properties may help to counterbalance the stress-induced immune activation, resulting in the normalization of lymphocyte levels. However, the specific mechanisms through which *C. vulgaris* exerts its immunomodulatory effects on lymphocytes require further investigation. It is possible that *C. vulgaris* acts by regulating the production and activity of pro-inflammatory cytokines, influencing lymphocyte proliferation and differentiation, or modulating cellular signaling pathways involved in immune responses.

Furthermore, our study observed no significant difference between the effects of cultivated *C. vulgaris* and commercially available *C. vulgaris* in reducing the number of lymphocytes. This finding suggests that both forms of *C. vulgaris*, whether cultivated or commercially sourced, possess comparable immunomodulatory properties. The lack of significant difference between the two forms indicates that the beneficial effects on lymphocyte count reduction are likely attributed to the inherent properties of *C. vulgaris* itself rather than variations in cultivation or processing methods. However, it is important to note that further comparative studies investigating the specific composition and bioactivity profiles of cultivated and commercially available *C. vulgaris* would be beneficial to gain a deeper understanding of any potential differences in their immunomodulatory effects.

Platelets, known as thrombocytes, play a crucial role in blood coagulation following injury to the body, facilitating the formation of blood clots and the cessation of bleeding (Franco et al., 2015). The count of platelets in the bloodstream is a key determinant of the coagulation process and the ability to control bleeding. In the stress group of our study, a significant decrease in platelet count was observed when compared to the control group (Figure 9). The decrease in platelet count observed in the stress group can be attributed to the impact of stress on hematopoiesis, the process of blood cell formation. Stress can disrupt the production and regulation of platelets,

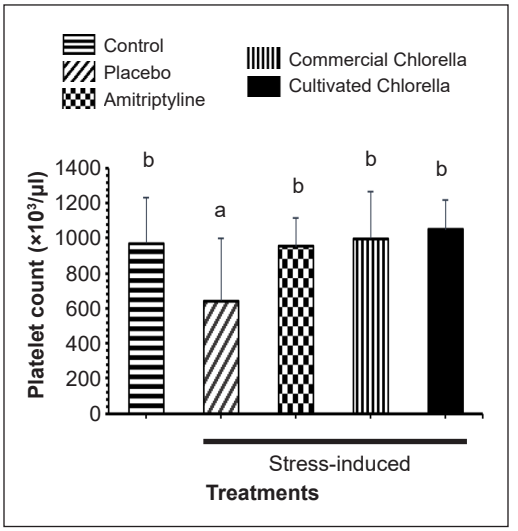

Figure 9. Supplementation with *Chlorella vulgaris* rescues the decreased platelet count in stress-exposed rats

Note. Groups with different letters represent statistically significant differences

potentially affecting their production rate and lifespan. Hormonal changes induced by stress and alterations in the bone marrow microenvironment may contribute to the decrease in platelet count (Cognasse et al., 2019; Koudouovoh-Tripp & Sperner-Unterweger, 2012).

Both *C. vulgaris* supplementation and amitriptyline administration effectively increased platelet levels, restoring them to the normal range. Furthermore, no significant difference was observed between cultivated and commercially available *C. vulgaris* in their capacity to restore platelet levels. It might suggest that the efficacy of *C. vulgaris* in platelet recovery is not influenced by variations in cultivation or processing methods. Both forms of *C. vulgaris* demonstrate comparable effectiveness, indicating that commercially available *C. vulgaris* products can be a reliable option for individuals seeking to restore platelet levels.

Mean platelet volume (MPV), a parameter reflecting the average size of platelets, was included in the study to provide insights into platelet morphology and potential alterations induced by stress (Korniluk et al., 2019). Measurement of MPV allows for assessing platelet size variation, which can indicate platelet activation and function. Interestingly, our findings revealed that stress did not have a significant effect on MPV, as evidenced by the comparison between the stress group and the control group in Figure 10. It suggests that stress-induced changes in platelet count, as observed in Figure 9, were not accompanied by alterations in platelet size. The lack of significant change in MPV may imply that stress predominantly affects platelet production and activation pathways rather than platelet size. Furthermore, despite the increase in platelet count shown in Figure 9, no corresponding changes in platelet volume were observed. This discrepancy may be attributed to the complex regulatory mechanisms involved in platelet production and maturation. It is possible that stress-induced alterations primarily influence platelet formation and release from megakaryocytes rather than affecting their size or volume.

Regarding the supplementation of amitriptyline and *C. vulgaris*, our results indicate that they had no significant effect on MPV. It suggests that these interventions do not directly influence platelet size or

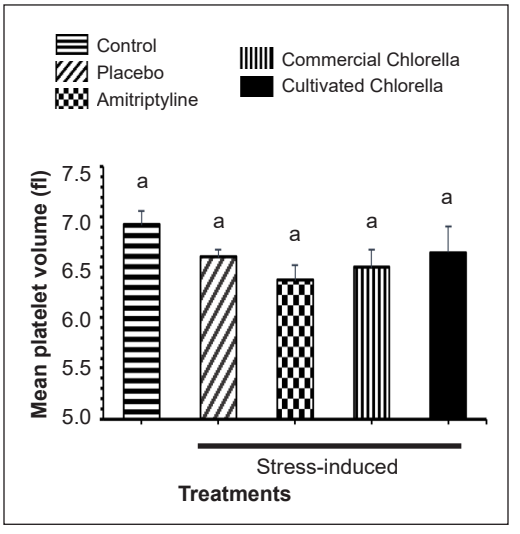

Figure 10. Exposure to stress and supplementation with *Chlorella vulgaris* in rats show no notable impact on mean platelet volume

Note. Groups with different letters represent statistically significant differences

volume. The lack of impact on MPV could be attributed to the specific mechanisms of action of amitriptyline and *C. vulgaris*, which may primarily target platelet function, activation, or other pathways associated with platelet biology rather than altering platelet size. It is important to note that MPV represents an average value, and individual platelets within the population may still exhibit size variations. The lack of significant changes in MPV suggests that stress, as well as the interventions of amitriptyline and *C. vulgaris*, did not induce substantial shifts in platelet size distribution.

CONCLUSION

This study provides compelling evidence of the significant impact of stress on various hematological parameters in rats. Stress leads to decreased erythrocyte count and hematocrit values, which can be effectively restored by supplementation with cultivated *C. vulgaris*, surpassing the positive control group treated with amitriptyline. However, commercially available *C. vulgaris* does not exhibit the same restorative effect. Stress negatively affects hemoglobin concentration, which is likely influenced by stress hormones and altered erythropoiesis. However, neither amitriptyline nor *C. vulgaris* supplementation significantly rescues the negative impact of stress on hemoglobin concentration. In addition, stress induces smaller-sized erythrocytes but does not significantly affect MCH or MCHC.

Notably, WBC count increases under stress, accompanied by elevated lymphocyte levels. *Chlorella vulgaris* effectively suppresses elevated lymphocyte levels, similar to amitriptyline, but does not reduce WBC count. Stress also decreases platelet count, which can be restored by amitriptyline and *C. vulgaris* supplementation. Meanwhile, MPV remains unaffected by stress or interventions, suggesting that stress primarily impacts platelet production and activation. Our findings highlight the potential of *C. vulgaris* as a therapeutic intervention to counteract stress-induced inhibition of erythropoiesis and restore erythrocyte count, although further research is necessary to elucidate underlying mechanisms and develop strategies for managing stress-related hematological changes.

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Effects of Coconut Water and Banana Homogenate on Shoot Regeneration of Meyer Lemon (*Citrus* × *meyeri*)

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ABSTRACT

Meyer lemon (*Citrus* × *meyeri*) is a hybrid citrus fruit from the Rutaceae family, originating from China. It is well-known for its distinctive appearance and flavor, as well as its health-nourishing nutrients. Micropropagation is an efficient alternative in the multiplication of plant stocks suitable for the commercial scale. The inclusion of organic additives in culture media has been found to provide a cost-effective option as a plant growth stimulant for *in vitro* plant development. The current study intends to assess the effects of coconut water and banana homogenate in the regeneration of Meyer lemon. *In vitro,* shoots were treated in half-strength Murashige and Skoog media fortified with 2 mg/L 6-benzylaminopurine with varying concentrations of coconut water and banana homogenate without sucrose. Results revealed that the treatment of 30% coconut water and 40 g/L banana homogenate resulted in the greatest proliferation of new shoots (3.00 ± 0.873 and 1.57 ± 0.297, respectively), whereas treatment of 40% coconut water resulted in the greatest shoot elongation of 0.239 ± 0.026 cm. The current study suggested the incorporation of coconut water and banana homogenate as potential substitutes for carbon sources and growth stimulants in the regeneration of Meyer lemon.

Keywords: Banana homogenate, *Citrus* × *meyeri*, coconut water, Meyer lemon, organic additive, shoot regeneration

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INTRODUCTION

Citrus fruits, belonging to the Rutaceae family, hold a prominent position as one of the most valuable and widely sought-after agricultural products. According to Tanaka and Tanaka (1954), *Citrus* is believed to

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originate from Southeast Asia, including countries like Myanmar and India. However, Gmitter and Hu (1990) refuted their claim, providing evidence that modern *Citrus* is native to the Yunnan province of China and has spread to other regions through human civilization. According to Peña et al. (2009), citron (*Citrus medica*), mandarin (*Citrus reticulata*), and pummelo (*Citrus grandis*) are the true species of *Citrus*. The hybridization between the true *Citrus* species contributed to the commercially important varieties such as lemon, lime, sweet orange, and sour orange. *Citrus* fruits are cultivated in over 140 countries worldwide as they strongly demand consumption, processing, and exports. Based on the records from the United States Department of Agriculture (USDA) (2021), Brazil, China, and the USA are the major producers of citrus oranges and mandarins account for 80% of the global citrus production, followed by tangerines, lemons, and grapefruits. Commercially cultivated lemon varieties include Meyer, Eureka, Lisbon, Verna, and Fino (Ladaniya, 2008). Among them is Meyer lemon (*Citrus* × *meyeri*), which is of Chinese origin and is a hybrid cross between true lemon (*Citrus limon*) and sweet orange (*Citrus sinesis*). It has a spherical shape with delicate skin, is exceptionally juicy and has low acidity, making it a key ingredient for culinary purposes (Hardy, 2004). However, this hybrid lemon species has yet to be widely cultivated in Malaysia.

Citrus fruit is usually consumed fresh or squeezed into juice and plays an important role in daily diets, improving the health of individuals through its nutritional benefits. It was reported that vitamin C in lemon fruits is an effective antioxidant, boosting the immune system while protecting human skin from harmful free radicals (Chambial, 2013; Ye, 2018). Furthermore, lemons have a high proportion of dietary fiber, predominantly pectin, which promotes digestive health as well as reduces blood cholesterol (Rao et al., 2016). It also contains a good percentage of essential minerals, such as iron, calcium, and potassium, which help reduce the risk of cardiovascular disease (D'Elia et al., 2011). Lemon extracts contain citric acid, which has proven qualities of preventing kidney stones, whereas lemon fruits possess a variety of phytochemicals, including phenolic compounds such as flavonoids, which are essential components of a healthy diet in the prevention of cardiovascular disease, hypertension, and cancer (González-Molina et al., 2010; Penniston et al., 2007).

Citrus propagation is conventionally accomplished by grafting and cutting, which is relatively slow and may have sanitary concerns when propagated using vegetative materials. Furthermore, most *Citrus* have a long juvenile phase that takes 4 to 7 years before flowering occurs (Carimi & De Pasquale, 2003). Besides, when growing in their natural settings, *Citrus* species are vulnerable to citrus pests such as the false codling moth and *Ceratitis* sp. of fruit flies, including the Mediterranean and Natal fruit flies. These citrus pests could contribute to direct fruit loss caused by pre- and post-harvest fruit drops. The pests could feed on the fruits, producing inedible fruits due

to fungal infections caused by the puncture wounds of ovipositing females. It could also result in major revenue losses associated with the pests (Goble et al., 2011). Therefore, the micropropagation technique has been widely used to overcome these issues as it allows rapid mass propagation of selected plant species in an aseptic environment while ensuring the genetic purity of the mother plant by yielding identical clones.

Previous studies on the micropropagation of Meyer lemon were reported by Haradzi et al. (2021), who successfully established an efficient micropropagation protocol for this hybrid species from shoot tip and epicotyl explants. The optimal shoot regeneration effect was observed at half-strength Murashige and Skoog supplemented with a combination of 6-benzylaminopurine (BAP) and indole-3-acetic acid (IAA). Haradzi et al. (2021) reported that rooting of Meyer lemon was optimal in Woody Plant Medium (WPM), and the authors successfully achieved an 82 % survival rate following acclimatization. Micropropagation, on the other hand, can incur higher costs compared to conventional propagation methods due to its capital-intensive nature, necessitating the continuous use of chemicals, culture media, equipment, and trained labor to ensure the production of superior tissue culture (Ahloowalia et al., 2002).

Hence, utilizing and incorporating organic additives could be a cost-effective option for plant growth stimulants in plant culture media due to their role in supplying vitamins, amino acids, fatty acids, carbohydrates, and different growth regulators (George et al., 2008). Organic additives, such as malt extract and coconut, banana, and papaya fruit juices can effectively provide indeterminate blends of organic nutrients and growth factors. From the previous studies, malt extract at various concentrations shows enhanced growth of *Citrus* callus and shoot culture (Badrelden, 2017; Rattanpal et al., 2011). Furthermore, the utilization of coconut water and banana homogenate has been widely documented, especially in inducing different types of plant cell regeneration, as they are rich in carbohydrates, vitamins, minerals, as well as endogenous cytokinin and auxin (Al-Khayri, 2010; Daud et al., 2011). However, the effects of coconut water and banana homogenate have not been reported in the *in vitro* regeneration of *Citrus*. Thus, the present study aims to assess the regeneration effects of organic additives such as coconut water and banana homogenate on *in vitro* nodal explants of Meyer lemon.

MATERIALS AND METHODS

Source of Explants

Nodal explants were collected from 4 weeks old *in vitro* cultures of Meyer lemon (*Citrus* × *meyeri*) maintained in half-strength Murashige and Skoog (MS) media (Duchefa Biochemie, The Netherlands) (Murashige & Skoog, 1962) fortified with 2 mg/L of BAP (Duchefa Biochemie, The Netherlands) with 15 g of sucrose (Duchefa Biochemie, The Netherlands), and 8 g of plant agar (Duchefa Biochemie, The Netherlands). The cultures were maintained at 25 ± 2°C, following a 16-hr light and 8-hr dark

photoperiod. Illumination was provided by a cool white fluorescent lamp (Philip TLD, 36 W, Malaysia) with an intensity of 150 μmol/m^2/s.

Preparation of Media with Organic Additives

Pandan coconut (*Cocos nucifera*) and Cavendish banana (*Musa acuminata* 'Dwarf Cavendish') were used in this experiment. The coconut was broken open using a knife, and the coconut water was filtered through a sieve to separate the kernel from the coconut water. Cavendish banana was peeled open and sliced into small cubes, followed by pureeing in a blender to obtain homogeneity. Precise measurements of coconut water volume and banana homogenate weight were taken before adding them to the culture media. The prepared media were adjusted to pH 5.80 prior to autoclaving at 121°C and 105 kPa for 15 min.

Shoot Regeneration in ½ MS Media with BAP and Organic Additives

Sterile *in vitro* shoot explants approximately 0.5 to 1.0 cm in length were inserted into half-strength MS media fortified with 2 mg/L BAP. The media was devoid of sucrose and instead enriched with different concentrations of organic additives such as coconut water (10, 20, 30, and 40%) and banana homogenate (10, 20, 30, and 40 g/L). A positive control of half-strength MS media with sucrose as the carbon source, as well as a negative control without sucrose, along with 2 mg/L BAP, respectively, was prepared. Each treatment consisted of 8 explants, with an explant per culture vessel. The cultures were maintained at a temperature of 25 ± 2°C, following a 16-hr light and 8-hr dark photoperiod and were illuminated using a cool white fluorescent lamp (Philip TLD, 36 W, Malaysia) with an intensity of 150 μmol/m^2/s. Parameters such as the average number and length of induced shoots were recorded after 6 weeks of culture.

Data Analysis

The data analysis was conducted using IBM SPSS Statistics 27 software. Differences among the treatments were assessed through one-way analysis of variance (ANOVA), followed by Duncan's multiple range test at a significance level of $p \leq 0.05$. Additionally, the treatment with the highest average induced shoot number and shoot length was compared to the control treatments using an independent samples *t*-test at a 95% confidence interval.

RESULTS AND DISCUSSION

Effect of Organic Additives on Shoot Regeneration

In the present study, the organic additives were supplemented into the culture media without adding sucrose to evaluate its effect as a replacement of carbon source and on shoot regeneration for the *in vitro* shoot explants. Following a 6-week culture period, 30% coconut water treatment was optimal to promote the greatest proliferation of new shoots, generating 3.00 ± 0.873 new shoots (Table 1 and Figure 1b). However, this value is not significant to the average number of

induced shoots across all concentrations. Furthermore, the average shoot length produced was directly proportional to coconut water concentration, producing the greatest length at 40%, with an average shoot elongation of 0.239 ± 0.026 cm. This value was significantly higher than the positive control (0.146 ± 0.016 cm), indicating the stimulatory effect of coconut water on shoot regeneration of Meyer lemon shoot explants.

Whereas with reference to Table 2 and Figure 1e, the maximum concentration of 40 g/L banana homogenate resulted in the highest shoot number of 1.57 ± 0.297, in comparison to the positive control treatment (1.33 ± 0.211). Both negative control and 10 g/L banana homogenate produced the minimum average number of 1.00 ± 0.000 new shoots per explant. However, no significant difference was observed across all banana homogenate concentrations in terms of the average number of induced shoots. Nonetheless, the greatest shoot length was observed at the positive control, generating an average shoot elongation of 0.200 ± 0.026 cm, in contrast to the maximum concentration of 40 g/L banana homogenate supplemented (0.167 ± 0.018 cm). The current study found that the addition of coconut water and banana homogenate into half-strength MS media without sucrose increased the average number and length of new shoots formed in Meyer lemon shoot explant, in contrast to their respective control treatments, indicating that both additives had growth-stimulating effects and are viable options of carbon source in the shoot regeneration of the Meyer lemon.

In this study, the highest number of shoots produced for the treatments of coconut water was observed at 30% coconut water instead of the highest concentration of 40%. Daud et al. (2011) also observed a comparable pattern in their study on *Celosia* sp., where the addition of 50 ml/L of coconut water produced the optimal shoot number (13.14 ± 10.33), whereas further increasing coconut water concentration to

Table 1
The average number of induced shoots and average length of induced shoots of Meyer lemon shoot explants in coconut water treatment after six weeks of culture

Treatments (%)	Average number of induced shoots ± S. E.	Average length of induced shoots ± S. E. (cm)
0 (Negative control)	1.80 ± 0.374[a]	0.140 ± 0.040[a]
10	2.83 ± 0.703[a]	0.134 ± 0.129[a]
20	2.43 ± 0.528[a]	0.188 ± 0.023[ab]
30	3.00 ± 0.873[a]	0.174 ± 0.025[ab]
40	2.71 ± 0.918[a]	0.239 ± 0.026[b*]
Positive control	2.50 ± 0.563[a]	0.146 ± 0.016[a]

Note. Means with the same letter within columns are not significantly different according to Duncan's multiple range test at $p \leq 0.05$. Means with * are significantly different from the control treatments according to independent samples *t*-test

Table 2
The average number of induced shoots and the average length of induced shoots of Meyer lemon shoot explants in banana homogenate treatment after six weeks of culture

Treatments (g/L)	Average number of induced shoots ± S. E.	Average length of induced shoots ± S. E. (cm)
0 (Negative control)	1.00 ± 0.000[a]	0.125 ± 0.025[a]
10	1.00 ± 0.000[a]	0.133 ± 0.021[a]
20	1.38 ± 0.183[a]	0.156 ± 0.018[ab]
30	1.43 ± 0.202[a]	0.143 ± 0.020[a]
40	1.57 ± 0.297[a]	0.167 ± 0.018[ab]
Positive control	1.33 ± 0.211[a]	0.200 ± 0.026[b]

Note. Means with the same letter within columns are not significantly different according to Duncan's multiple range test at $p \leq 0.05$

(a) (b) (c) (d) (e) (f) (g)

Figure 1. Shoot regeneration of Meyer lemon treated in half-strength Murashige and Skoog media with 2 mg/L 6-benzylaminopurine in different concentrations of coconut water and banana homogenate after six weeks of culture. (a) 0% (negative control), (b) 30% coconut water, (c) 40% coconut water, (d) 30 g/L banana homogenate, (e) 40 g/L banana homogenate, (f) positive control, and (g) positive control after 8 weeks of culture
Note. Bars = 1 cm

70 ml/L demonstrated a suppressive effect on the formation of new shoots (11.14 ± 5.61). Furthermore, a similar observation was also demonstrated in the *in vitro* shoot regeneration of carnation, where the culture supplemented with 10% coconut water exhibited the highest number of shoots, producing 113.83 ± 0.4 shoots on nodal explant and 93.33 ± 0.43 shoots in shoot tip explant.

Further, an increase in coconut water concentration to 20% has indicated a repressive effect on the formation of new shoots (Khatun et al., 2018). This inhibitory effect was also parallel to the study on somatic embryogenesis of date plum with coconut water as a media additive (Al-Khayri, 2010). In addition, this study demonstrated that the highest concentration of 40% coconut water encouraged shoot elongation, aligning with a prior study that observed a gradual increase in shoot length from 7.5 to 12.8 mm as the concentration rose from 0 to 30% during the *in vitro* shoot regeneration of common hazel (*Corylus avellana*) (Sandoval Prando et al., 2014).

It is also consistent with plant regeneration of sweet passion fruit, where increasing the concentration of coconut water improved shoot elongation from 3.29 ± 0.4 cm at 0% to 6.08 ± 0.7 cm at the maximum concentration of coconut water supplemented (Pacheco et al., 2012).

Similarly, in nodal explants of Spanish jasmine (*Jasminum grandiflorum*), the mean shoot length steadily rises as the concentration of coconut water increases (Rahman et al., 2018). Coconut (*Cocos nucifera*) is one of Malaysia's primary industrial crops and is extensively distributed locally at a low cost. The stimulatory effect of coconut water was due to its biochemical composition, which included an abundance of sugars, amino acids, proteins, minerals, and phytohormones. The presence of natural cytokinins such as trans-zeatin and kinetin as a major phytohormone in coconuts was found to positively influence cell division and differentiation as well as in seed germination (Prades et al., 2012; Yong et al., 2009). Furthermore, different cytokinin types were present in coconuts, including kinetin, kinetin riboside, isoprenoid, and aromatic cytokinin (Ge et al., 2005). Moreover, several types of sugar alcohol were identified in coconut water, such as sorbitol, myo-inositol, and scyllo-inositol, which are commonly supplemented in culture media as plant vitamins to regulate the biosynthetic pathway for cell wall formation and ion uptake (George et al., 2008). Furthermore, Yong et al. (2009) reported the presence of gibberellin in coconut water, which aids in shoot elongation and regulates cambial activity in plant cells.

This study observed that shoot number increases with increased concentration, where 40 g/L banana homogenate was optimal in inducing the greatest number of shoots. It is in accordance with the study in the shoot regeneration of *Physalis angulate*, where the gradual increase of concentration of banana homogenate to 5% generated the best effect on the number of new shoots (3.33 ± 1.23) in contrast to the control (1.75 ± 0.50) (Apensa & Mastuti, 2018). Furthermore, a similar observation was observed, where the increase of banana homogenate to 50 g/L produced the maximum number of new shoots per explant (7.75 ± 0.2) on the protocorm-like bodies (PLBs) of orchid (*Cymbidium pendulum*) (Kaur & Bhutani, 2012). Daud et al. (2011) also demonstrated that the 50 ml/L banana homogenate supplementation produced the highest shoot regeneration of 9.57 ± 4.68 new shoots per explant in contrast to the lower concentrations tested.

In the current study, the treatment of banana homogenate at the concentration of 40 g/L resulted in the highest shoot elongation for all banana homogenate treatments, with a 25% increase in shoot length elongation observed as the concentration of banana homogenate in the culture medium increased from 10 to 40 g/L, despite being not significant to the control treatments. According to Islam et al. (2015), the shoot length in PLB-derived *Dendrobium* orchids plantlets was improved by the addition of 25 ml/L banana homogenate

(2.8 cm) in contrast to the control (1.8 cm). Furthermore, Vilcherrez-Atoche et al. (2020) demonstrated the optimal shoot regeneration effect in the PLBs of *Cattleya maxima* with the addition of 30 g/L banana powder in which the average new shoots and shoot length produced were 2.37 ± 0.10 and 1.17 ± 0.02 cm, respectively, in contrast to the control, which produced 1.38 ± 0.10 new shoots and 0.69 ± 002 cm shoot length.

Banana from the genus *Musa* ranks among the foremost global fruit crops renowned for raw and processed consumption, primarily due to their high nutritional value. The chemical composition of bananas at various physiological phases, such as fresh, dried, or flour, varies and is especially high in carbohydrate content (Aurore et al., 2009). The high concentration of carbohydrates in its composition may indeed be responsible for its growth-stimulating properties, operating as a carbon source and supplying energy to heterotrophic plants during the early phases of *in vitro* cultivation (Al-Khateeb, 2008). According to Aurore et al. (2009), ripe bananas are rich in vitamins A, B, and C and relatively rich in pyridoxine (B6). Vitamin B, such as thiamine, niacin, and pyridoxine, is essential to culture cells in plant tissue culture (George et al., 2008). Furthermore, bananas have been recognized to have a high potassium content. Potassium, a crucial macronutrient necessary for plant tissue, assumes a fundamental function in diverse physiological processes of plants, such as protein-synthesizing enzyme activation, stomatal function, and ATP generation in addition to osmoregulation in plant tissue and cells (Xu et al., 2020). The supply of osmotically active potassium ions enhances cell elongation in shoots, leaves, and roots as it accumulates in the vacuole, providing adequate turgor pressure for cell expansion (Takahashi & Kinoshita, 2016).

In addition to that, banana homogenate contains organic acids such as citric acid, malic acid, and oxalic acid, along with essential amino acids like glutamine and asparagine, that can potentially release hydrogen ions and ammonia, respectively, acting as buffers and thereby helping to maintain a stable pH in the culture medium (Lee et al., 2019; Wyman & Palmer, 1964). Furthermore, a significant amount of minerals, including manganese, calcium, and sodium, was found in banana content (Adubofuor et al., 2016). According to Zaffari et al. (2000), IAA and 6-(y,y-dimethylallylamino)purine (2iP) were discovered as endogenous auxin and cytokinin in banana homogenate and were demonstrated to have a beneficial effect on lateral bud growth when supplied to the plant.

Despite the fact that the use of organic additives such as coconut water and banana homogenate in the current study brought a beneficial effect by supplying carbohydrates, vitamins, amino acids, fatty acids, minerals, and growth regulators essential for the plant *in vitro* development, organic additives, after all, have undefined composition and nutritional content that varies between batches. As the composition of the growth medium holds significant influence over

the growth and development of plant tissues, this could lead to discrepancies in the results. Nonetheless, as indicated by the enhancing effect in the current study, organic additives can potentially substitute the carbon source in culture media.

CONCLUSION

Of all the coconut water treatments evaluated, 30 and 40% coconut water concentrations incorporated into half-strength MS media supplemented with 2 mg/L BAP resulted in the highest number of induced shoots and length, respectively. At the same time, the treatment of banana homogenate at a concentration of 40 g/L effectively generated the highest number and length of induced shoots of all the banana homogenate treatments. This study revealed that the addition of coconut water and banana homogenate has the potential to induce the regeneration of new shoots, indicating that these organic additives exhibited growth-stimulating effects and might be employed as potential carbon sources in the *in vitro* shoot regeneration of Meyer lemon for micropropagation purposes.

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Pathogenicity Evaluation of Low Pathogenic Avian Influenza (H9N2) Virus Isolated from Layer Flocks in Malaysia in Specific-pathogen-free Chickens

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ABSTRACT

Infection with the low pathogenic avian influenza (LPAI) H9N2 virus has been reported worldwide and poses a health risk to poultry as well as to global health due to its ability to re-assort with other avian influenza viruses. Besides, the silent spread of the H9N2 infection causes significant economic damage to the poultry industry. Recently, Malaysia reported major outbreaks of LPAI H9N2 in commercial layer chicken flocks. Genome sequence analysis indicated that the predominant LPAI H9N2 viruses are of the Y280/BJ94-like lineages. However, the pathogenicity of the virus has not been evaluated. This study determines the pathogenicity of LPAI H9N2 strain UPM994/2018, previously isolated from commercial layer chickens, in one-week-old specific-pathogen-free (SPF) chickens. Clinical signs such as ruffled feathers, mild tracheal rales, facial edema, sero-nasal discharge, and diarrhea were observed from days 6 to 10 post-inoculation (PI). However, no mortality was recorded. Based on a real-time polymerase chain reaction assay, the viruses can be detected in the lungs, trachea, and kidneys of the inoculated chickens on the second day,

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increased until day 10, then declined at day 16 PI. However, swab samples collected from the oropharyngeal and cloacal regions remain positive from day 2 to day 14 PI, with the highest viral load detected at day 10 PI. In conclusion, although the virus is an LPAI, it is pathogenic in SPF chickens, causing respiratory, gastrointestinal, and renal-associated illnesses.

Keywords: Clinical sign, LPAI H9N2 virus, pathogenicity, SPF chicken, viral load

INTRODUCTION

The disease caused by avian influenza viruses (AIVs) may vary depending on the host species, virus subtypes, and lineage. Based on the requirements of the World Organization for Animal Health (WOAH) and the amino acid sequences of the HA gene cleavage site, AIVs can be divided into low pathogenic avian influenza (LPAI) and highly pathogenic avian influenza (HPAI) (Umar et al., 2017). The increase in the virulence and pathogenicity of AIV is associated with polygenic traits. However, hemagglutinin (HA) is the dominant gene in determining the virus pathogenicity (Stech & Mettenleiter, 2013). Nevertheless, molecular determinants of all eight segments, especially mutations of the polymerase genes complex, are associated with high virulence of AIV (Sun et al., 2020). The HPAI viruses encode polybasic cleavage sites at the HA gene that are cleaved by a family of subcellular-like proteases, such as furin and proprotein convertase, which are expressed abundantly in different tissues (Matrosovich et al., 2001).

Hence, infection with HPAIV is associated with systemic disease with high mortality in experimental inoculation of chickens (Mo et al., 2021). All HPAIVs discovered so far are H5 and H7 subtypes; however, not all H5 or H7 viruses are highly pathogenic. However, due to antigenic shift and drift, LPAI viruses can be transformed into HPAIV (Su et al., 2015). On the other hand, LPAIV, with a monobasic cleavage site at the HA gene, can activate soluble trypsin-like proteases, which are present only in cells of the mucosae of the respiratory and intestinal tracts. Hence, experimental infection with the LPAI virus in SPF chickens is associated with mild or asymptomatic disease (Umar et al., 2017). In addition, in an experimental infection study by Gharaibeh (2008), SPF chickens show milder pathogenic lesions compared to broiler chickens. However, the reasons for this were not specified clearly.

Field H9N2 infections in poultry have been reported to show varying degrees of clinical manifestations depending on the presence of host species, virus strains, secondary respiratory pathogens, and immunological factors (Aamir et al., 2007; Wang et al., 2015). Sinuses inflammation, nasal and ocular secretions, severe respiratory symptoms, and low mortality were observed in most reported cases. However, mortality may vary depending on the pathogenicity of the virus and conditions in the field. Experimental studies after H9N2 infection reported mortality

rates of 5, 20, and 65% in commercial broiler chickens (Gharaibeh, 2008; Nili & Asasi, 2003) and, in some cases, of up to 80% in commercial broiler chickens due to concurrent bacterial infections (Guo et al., 2000). A review study by Umar et al. (2017) reported that experimental infection of SPF chickens with LPAI H9N2 is normally associated with mild clinical manifestations and low mortality. However, concurrent experimental infections of H9N2 with other pathogens such as Newcastle disease virus, Infectious bronchitis virus, *Mycoplasma*, and *Escherichia coli* may increase the severity of H9N2 infection (Umar et al., 2017).

H9N2 is also a primary pathogen in field outbreaks in layer and breeder chickens. Severe respiratory signs, drop in egg production by 10 to 27%, and mortality has been observed (Awuni et al., 2019). In most cases, the infected layer and breeder flocks were prone to secondary bacterial infections that may aggravate the severity of the infection (Awuni et al., 2019; Lai et al., 2021). In another study, Hassan and Abdul-Careem (2020) reported that the H9N2 virus could infect the infundibulum of chickens, which eventually affects the proper function of oviducts in layers and breeder chickens. Furthermore, virus binding ability depends upon the virus inoculation route (Pantin-Jackwood et al., 2012; Umar et al., 2017). Thus, all these factors may influence the outcome of H9N2 infection in chickens, including the molecular determinants that have been found to govern the pathogenicity of AIV in chickens.

In 2018, major outbreaks of LPAI H9N2 were reported in commercial chickens in Peninsular Malaysia, leading to massive economic burdens (Syamsiah et al., 2019). Infection with LPAI H9N2 in long-lived birds, namely layers and breeder, is associated with a drop in egg production and quantity and quality of fertile eggs. In addition, the infected birds may die due to secondary bacterial infection. Sequence analysis of the HA gene indicated that the predominant virus isolated during the outbreaks is of the Y280/BJ94 lineage (Gunasekara, 2021; Syamsiah et al., 2019). The complete genome sequence of two Malaysian LPAI H9N2 strains isolated from 2018 to 2019 indicates the virus is a novel reassorted virus of the reassort lineage originating from China (Gunasekara, 2021). Sequence analysis of the HA cleavage site of H9N2 strain UPM994/2018 revealed the presence of a dibasic cleavage site at position 333-PSRSSR-GLF-341 (data not shown). This study determined the pathogenicity of one of the genome-sequenced LPAI H9N2 viruses, A/chicken/Malaysia/UPM994/2018, in SPF chickens.

MATERIALS AND METHODS

Ethical Statement

The live animal trial of this study was conducted according to the guidelines of the institutional animal care and use committee (IACUC), which Universiti Putra Malaysia (UPM) approved under UPM/IACUC/AUP-R055/2020.

Virus Preparation and EID_{50} Measurements

The virus used in this study, A/chicken/Malaysia/UPM994/2018, was isolated from a breeder farm in Negeri Sembilan in 2018 (Gunasekara, 2021). The infected flocks had a history of depressed birds, with mortality ranging from 2 to 4 % per week. Upon post-mortem examination, the affected birds had pulmonary edema with fibrinonecrotic exudate deposition in the trachea and bronchi. The reproductive organs were congested with the presence of egg-yolk peritonitis. According to the WOAH Terrestrial Manual, the virus was propagated in embryonated SPF chicken eggs (WOAH, 2023). Briefly, the allantoic fluid of the inoculated eggs was harvested and centrifuged for 30 min at 1,200 × *g*, and the supernatant was collected and stored at -80°C.

The virus stock was thawed and diluted from 10^{-1} to 10^{-10} in sterile phosphate-buffered saline (PBS, Invitrogen, USA). Five eggs were inoculated with 100 µl of each dilution via the allantoic route and incubated at 37°C for 4 days. Another 5 eggs were inoculated with sterile PBS as the negative control. All the eggs were monitored daily for embryo mortality. Eggs demonstrating embryonic mortality in less than 24 hr were discarded and not considered. The virus titer in embryo infectious dose 50 (EID_{50}) was calculated using the Reed and Muench (1938) method as the reciprocal of the highest dilution that gave HA-positive results for 50% of the inoculated eggs. The HA assay was done in V-bottom 96-well plates with 1% chicken red blood cells.

SPF Chicken

SPF embryonated chicken eggs (Malaysian Vaccines and Pharmaceuticals, Malaysia) were hatched in the Laboratory of Vaccine and Biomolecules, Institute of Bioscience (IBS), UPM. The hatched chicks were transferred to the experimental animal house facility at Biologics Laboratory, IBS, UPM, where they were housed in clean stainless steel bird cages and fed pelleted chicken feed. Water was provided ad libitum, and the drinkers were cleaned daily.

Pathogenicity Study

Seven-day-old SPF birds were randomly allocated into two groups: Group 1 consisted of 48 birds for the infected, and Group 2 consisted of 24 as the control. Before virus inoculation, a blood sample from eight birds was collected randomly to confirm their freedom from H9N2. Each chicken in the infected group was inoculated via the intraocular route with 0.1 ml of 10^7 EID_{50} of H9N2 strain UPM994/2018. For the control group, 0.1 ml of sterilized PBS was inoculated. After inoculation, the chickens in each group were observed daily, and body weight gain was recorded. Three chickens in the control group and six in the infected group were randomly selected and euthanized on days 2, 3, 5, 7, 10, 12, 14, and 16 PI accordingly.

At post-mortem examination, tissue samples of the trachea, lung, gastrointestinal tract, spleen, kidneys, and brain were collected and divided into two parts. Oropharyngeal and cloacal swabs were also collected from these birds prior to

the necropsy. For each tissue sample, a portion was kept in 10% neutral buffered formalin (Sigma-Aldrich, Singapore) for histopathological examination and a portion in 3 ml of PBS for molecular detection.

The body weight of the chickens was measured, and the following equation was used to calculate the percentage of growth retardation rate of the SPF chickens.

$$\text{Retardation rate (\%)} = \frac{\text{Mean weight gain (Control, g)} - \text{Mean weight gain (Infected, g)}}{\text{Mean weight gain (Control, g)}} \times 100\%$$

RNA Extraction

Tissue samples from the kidneys, lungs, and trachea were homogenized with a sterile 1x PBS solution. After centrifugation for 1,200 x *g* (Eppendorf 5810R Centrifuge, Germany) for 15 min at 4°C, the supernatant was passed through a 0.45 µM Minisart® syringe filter (Sartorius AG, Germany) for the RNA extraction. Total RNA was extracted from the supernatant using TRIzol™ Reagent (Invitrogen, USA) according to the manufacturer's instructions. Viral RNA was extracted from the oropharyngeal and cloacal swabs using the innuPREP Virus RNA Kit (Analytik Jena, Germany). The extracted RNA was subjected to a quantitative real-time-polymerase chain reaction (qRT-PCR) to detect virus copy numbers (VCNs).

Clinical Signs, Gross, and Histopathologic Examination

Infected chickens were monitored daily for the presence of respiratory and gastrointestinal distress. Necropsy was performed on chickens on days 2, 3, 5, 7, 10, 12, 14, and 16 PI. Tissues from the respiratory system and gastrointestinal tracts, spleen, kidneys, and brain were collected and kept in 10% buffered formalin for 24 hr. The tissues were processed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) (Feldman & Wolfe, 2014). The prepared slides were viewed under the microscope (Leica® Biosystems, USA) to evaluate lesions.

Quantitative Real-time PCR

A TaqMan real-time RT-PCR assay was conducted using a designed and optimized forward primer, 5'-TGCAGCGTAGACGTTTTGTC-3', reverse primer 5'-CAAGCGCACCAGTTGAGTAA-3', and probe 5'-(6-FAM)-TAAATGGGAATGGAGACCCA-(MGB)-3', which could amplify the 153 bp fragment of the M gene. The TaqMan real-time PCR reaction mixture was prepared according to the manufacturer's instructions using a one-step SensiFAST™ Probe No-ROX Kit (Bioline, Germany). The cycling condition was programmed in a CFX96 real-time system (Bio-Rad, USA) at 45°C for 10 min for cDNA synthesis, 95°C for 2 min, followed by 40 cycles at 95°C for 5 s and 60°C for 20 s.

Virus stock RNA amplification was performed by 10-fold serial dilution starting

from 100 to 0.01 ng/µl per reaction to create a standard curve as a positive control. All dilutions were performed in triplicate. The amount of viral shedding was expressed as VCN, where the absolute quantification of the number of viral RNA copies in the swabs was based on quantification cycle (Cq) values for each sample and the qPCR standard curve. The number of viral RNA copies was calculated with the following formula:

$$\text{Number of copies} = \frac{(\text{Concentration in g}) \times (6.023 \times 10^{23})}{330 \times \text{Total amplicon length}}$$

Statistical Analyses

The results of the VCNs of the swab and tissue samples were analyzed using one-way analysis of variance (ANOVA) with Turkey as the post hoc test ($p<0.05$) using SAS 9.1 software. Statistically significant differences in the VCNs were determined by comparing the groups at the given time ($p<0.05$). All quantitative data were expressed as mean ± standard error.

RESULTS

Clinical Signs

The H9N2-infected chickens showed varying clinical signs, from moderate to mild, or the absence of clinical signs in some infected birds. No mortality was recorded in the infected group. Meanwhile, the control group remained healthy throughout the study period. Generally, clinical signs were first recorded at day 6 PI, where two of the six infected birds presented mild diarrhea and ruffled feathers (Table 1). Clinical signs such as depression, sneezing, gasping, ocular discharge, and a slightly swollen face were observed in 2 out of the 6 chickens on days 7 and 10 PI. After day 10 PI, the clinical signs subsided and disappeared on day 12 PI.

On days 3, 5, 7, 10, 12, 14, and 16 PI, birds in the infected group exhibited 7.68, 12.57, 13.3, 11.5, 9.6, 6.7, and 7.5% retardation of growth, respectively, with the highest retardation rate on day 7 PI (Table 2). The body weights of the chickens in the infected and control groups were significantly different ($p<0.01$) from day 5 to day 16 PI onwards.

Table 1
Clinical signs in specific-pathogen-free chickens following inoculation with H9N2 at different days post-inoculation

Clinical signs	Number of chickens showing clinical signs on different days post inoculation													
	2	3	4	5	6	7	8	9	10	11	12	13	14	16
RFD	0/6	0/6	0/6	0/6	1/6	2/6	2/6	2/6	2/6	0/6	0/6	0/6	0/6	0/6
G	0/6	0/6	0/6	0/6	0/6	1/6	1/6	2/6	2/6	0/6	0/6	0/6	0/6	0/6
SN	0/6	0/6	0/6	0/6	0/6	1/6	2/6	2/6	2/6	0/6	0/6	0/6	0/6	0/6
SF	0/6	0/6	0/6	0/6	0/6	0/6	0/6	1/6	1/6	0/6	0/6	0/6	0/6	0/6
WF	0/6	0/6	0/6	0/6	0/6	0/6	1/6	1/6	1/6	0/6	0/6	0/6	0/6	0/6

Note. RED = Ruffled feathers and depression; G = Gasping; SN = Sneezing; ND = Nasal discharge and lacrimation; SF = Swollen face; WF = Watery faeces

Table 2
Growth retardation of specific-pathogen-free chickens after H9N2 inoculation

Days post inoculation (dpi)	Mean weight (g)±SD		Retardation (%)
	Group 1	Group 2	
3	41±3.34	44.33±2.16	7.68
5	45.17±1.16	51.67±3.20	12.57*
7	52.83±1.94	61±3.34	13.3*
10	71.33±3.5	80.67±3.66	11.5*
12	82.33±3.38	91.17±2.78	9.6*
14	94.67±2.25	101.5±2.88	6.7*
16	101.83±1.60	110.17±3.97	7.5*

Note. Groups 1 = Infected; Group 2 = Control; * =The body weight of the chickens in the infected and control groups were significantly different (p<0.01) from day 5 to day 16 post-inoculation onwards

Macroscopic Lesions

Some infected birds, especially cachexic birds, had prominent keel bones at necropsy. Generally, mild gross lesions were observed in H9N2-infected SPF chickens between days 7 to 10 PI. The kidneys were enlarged in one out of six chickens on day 7 PI and two out of six chickens on day 10 PI, respectively. The intestinal and trachea mucosae were mildly hyperemic in H9N2-infected birds. Mild congestion in the lungs was exhibited on days 7 and 10 PI. No gross lesions were observed on day 10 PI afterward (data not shown).

Microscopic Lesions

The tracheas revealed an increased number of goblet cells on day 3 PI. Lymphoid cell infiltration of the lamina propria, epithelial cell desquamation, hyperplasia of epithelial and congested blood vessels was observed at days 7 and 10 PI (Figure 1). In addition, detachment of the trachea's cilia lining with fibrin-heterophilic exudates inside the tracheal lumen was observed on day 10 PI. The tracheal lesions subsided on day 14 PI.

The histopathological changes in the lungs were seen between days 2 to 16 PI, with varying degrees of (mild to moderate) congestion, hemorrhages, edema, and infiltration of lymphocytes into the submucosa of secondary bronchi (Figure 2). Fibrin exudates and focal infiltration of lymphocytes was observed at days 5 to 10 PI, and congestion and hemorrhages of the parabronchi were seen at day 16 PI (Figure 2).

In the case of the kidney, varying degrees of (mild to moderate) congestion, hemorrhage, degeneration, and multifocal necrosis of the renal tubules were seen from day 2 to day 16 PI (Figure 3). Focal infiltration of lymphocytes was also noticed in the renal interstitial tissue. By day 10 PI, cystic tubules containing cellular and hyaline casts and foci of mononuclear leukocyte and macrophage infiltration were prominent, mostly in the medullary region. Mild edema fluid accumulation was detected in the renal interstitial tissue (Figure 3).

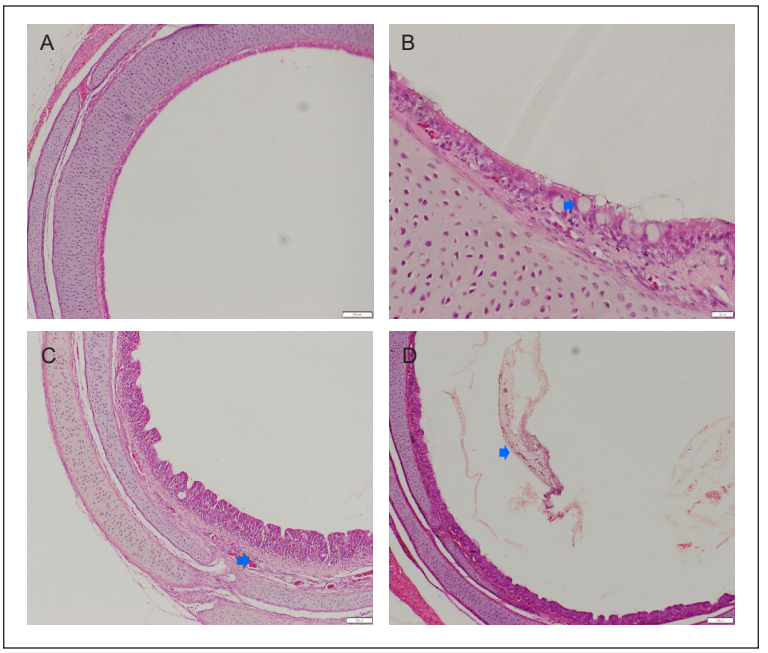

Figure 1. Microscopic lesions in tracheas of H9N2 infected chickens with hematoxylin and eosin staining
Note. A = Normal trachea; B = Increased number of goblet cells on day 3 post-inoculation (PI) (arrows); C = Hyperplasia of epithelium with diffuse loss of cilia, lymphocytic infiltration on day 7 PI; D = Total detachment of the cilia lining on day 10 PI; Scale bars A, C, and D = 100 µm, B = 20 µm

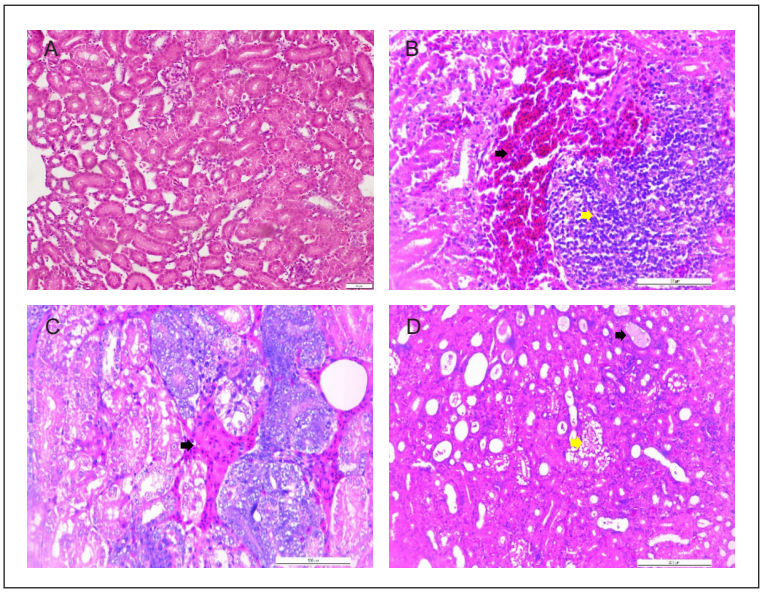

Figure 2. Microscopic lesions in kidneys of H9N2 infected chickens with hematoxylin and eosin staining
Note. A = Normal kidney control; B = Hemorrhages (black arrows) and granulocytic leukocytes dense infiltration and disruption of the tubulointerstitial tissue on day 3 PI (yellow arrow); C = Oedema fluid and cell infiltration (black arrow); D = Mild hyaline cast deposit in the tubular lumen (black arrow), and vacuolation of the cell due to degeneration (10 PI) (yellow arrow); Scale bars B = 20 µm, A and D =50 µm, C = 100 µm

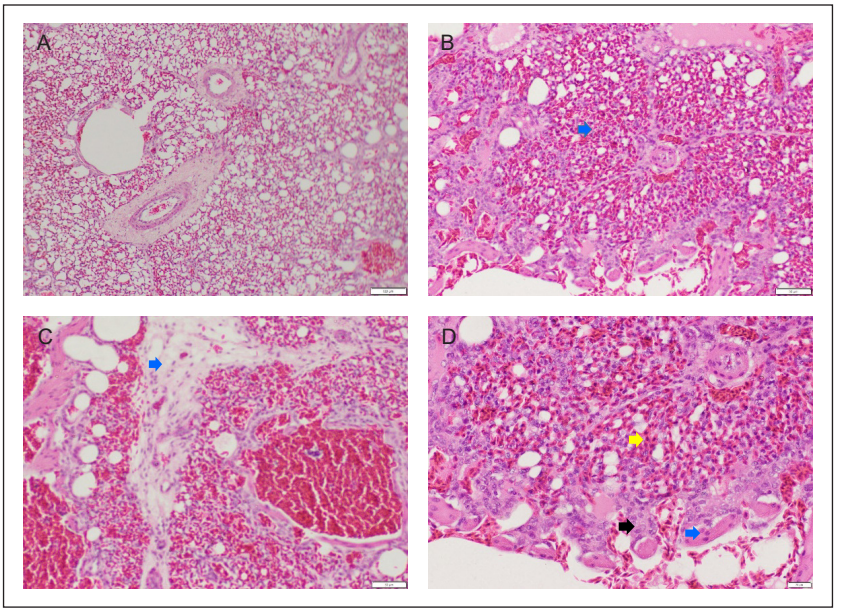

Figure 3. Microscopic lesions in lungs of H9N2 infected chickens with hematoxylin and eosin staining
Note. A = Normal lung control; B = Congested lung on day 5 PI (arrow); C = Edematous lung with accumulation of edema fluid on day 7 PI (arrow); D = Mononuclear cell infiltration (black arrow), hemorrhages (yellow arrow), and presence of edema fluid on 10 PI (blue arrow); Scale bars A = 100 µm, B, C, and D = 50 µm

Mild depletion of lymphoid cells was seen in the spleen at day 14 PI. Mild mucus cell hyperplasia was observed in the intestine, but most changes were carefully observed to differentiate from the control group chickens. No pathological lesions were observed in brain tissue. As expected, no detectable lesions were observed in the control group of chickens. All the microscopic lesions observed in the lung and trachea are summarized in Table 3.

Table 3
Microscopic lesions observed in H9N2 infected specific-pathogen-free chickens

Histopathological changes	2	5	7	10	14	16
Congestion[A]	3/3	3/3	1/3	2/3	2/3	2/3
Haemorrages[AB]	3/3	3/3	2/3	3/3	1/3	1/3
Edema[AB]	0/3	0/3	3/3	3/3	0/3	0/3
Mono nuclear cell infiltration[AB]	0/3	3/3	3/3	3/3	2/3	2/3
Necrosis[B]	0/3	2/3	3/3	2/3	1/3	1/3
Tubular degeneration[B]	0/3	0/3	3/3	3/3	1/3	1/3
Eosinophilic materials inside the lumen[B]	0/3	0/3	0/3	3/3	0/3	0/3
Perirenal haemorrages[B]	0/3	0/3	2/3	0/3	1/3	1/3

Note. A = Lung; B = Kidney

Standard Curve

The standard curve was constructed based on a 10-fold dilution of a known RNA concentration of H9N2 UPM944/2018 using the Bio-Rad CFX manager 3.1 software (Bio-Rad, USA). The standard curve y-intercept is 58.298, and the slope of the standard curve is -3.334. The calculated coefficient of the standard curve is 99.6%, and the real-time PCR efficiency is 99.5% (Figure 4). The graph shows the linear relationship of quantification threshold (ct) versus virus $\log_{10}$ virus copy.

Viral Copy Number

The virus shedding of the H9N2-infected chickens from the cloacal and oropharyngeal swabs was measured using TaqMan-based real-time PCR. The produced viral copy number was calculated as $\log_{10}$ at each time point, and the mean significant difference between the groups was analyzed using one-way ANOVA and Tukey's post hoc test. As shown in Table 4, all the infected chickens from day 2 to 14 PI were detected positive for H9N2. However, the amount of viral load varies according to different time points.

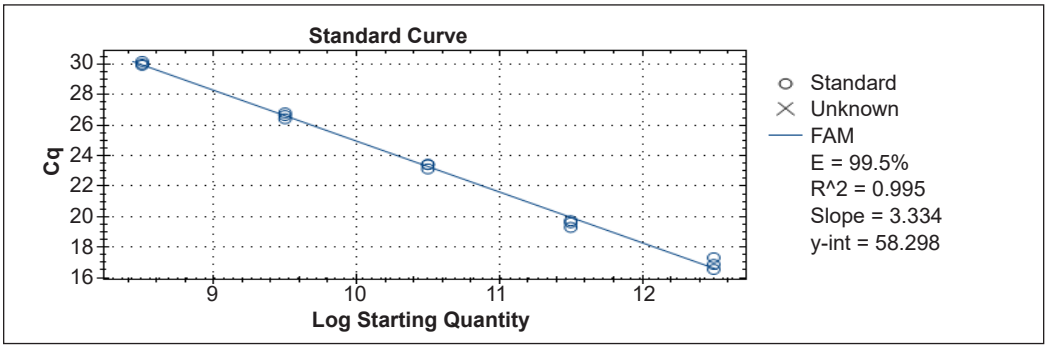

Figure 4. Standard curve of real-time polymerase chain reaction detection of H9N2 virus

Table 4
Virus shedding from oropharyngeal and cloacal swabs of H9N2 infected specific-pathogen-free chickens at different time points

Time points (dpi)	Oropharyngeal swab		Cloacal swab	
	Number of positive/ Total samples	Virus copy number, $\log_{10}$ (means ± SE)	Number of positive/ Total samples	Virus copy number, $\log_{10}$ (means ± SE)
2	6/6	11.00 ± 0.01^{aA}	6/6	10.05 ± 0.12^{bB}
3	6/6	11.10 ± 0.07^{aA}	6/6	9.55 ± 0.06^{cB}
5	6/6	9.39 ± 0.04^{cB}	6/6	$9.71\pm0.11b^{cA}$
7	6/6	9.99 ± 0.04^{b}	6/6	9.97 ± 0.05^{b}
10	6/6	11.24 ± 0.09^{aA}	6/6	10.96 ± 0.09^{aB}
12	6/6	9.99 ± 0.14^{b}	6/6	10.05 ± 0.06^{b}
14	6/6	9.36 ± 0.08^{c}	6/6	9.16 ± 0.06^{d}
16	0/6	ND	0/6	ND

Note. * The values with different superscripts (a, b, c, and d) within a column in each group are significantly different ($p<0.05$). The values with different superscripts (A, B) compared the effect between oropharyngeal and cloacal swabs, and each row is significantly different ($p<0.05$); ND = None detected

The viral load in cloacal and oropharyngeal swab samples showed a declining pattern with low detection at day 3 and day 5 PI, respectively, while the highest viral loads in both samples were detected at day 10 PI. On the last day of the sampling at day 16 PI, no virus load was detected from the oropharyngeal and cloacal swab samples (Table 4). The virus copy number in the oropharyngeal of challenged SPF chickens is significantly higher (p<0.05) than in the cloacal of challenged SPF chickens at days 2, 3, and 10 PI. In contrast, the value in the oropharyngeal of SPF chickens was lower than in the cloacal of challenged SPF at day 5 (p<0.05).

The virus copy numbers in the oropharyngeal were significantly reduced on days 5 and 14 compared to those on other days in the challenged study (p<0.05). The highest virus copy number (p<0.05) was recorded in the oropharyngeal of H9N2-challenged SPF chickens at days 2, 3, and 10 PI. However, no significant changes were shown among virus copy numbers of oropharyngeal of challenged SPF chickens at day 2, 3, and 10 PI. Meanwhile, the highest virus copy number (p<0.05) was recorded in the cloacal route at day 10 PI.

Viral load detection was detected in both renal and respiratory tissues of the H9N2-infected chickens throughout the study. However, on day 5 PI, the mean viral load was significantly higher (p<0.05) in respiratory tissue compared to kidney tissue. In contrast, the viral load detection was significantly (p<0.05) elevated in renal tissue compared to respiratory tissues at day 7, 10, and 14 PI (Table 5).

DISCUSSION

The H9N2 virus is widespread in selected Asian, European, and African countries (Peacock et al., 2019). H9N2 viruses detected in Eurasian poultry can be classified into three well-defined poultry lineages: G1, Y280/BJ94, and Y439 (Korean-like) (Guan et al., 2000; Matrosovich et al., 2001; Peacock et al., 2019). Among the three lineages, the Y280/BJ94-like viruses are

Table 5
Detection of virus copy numbers from the respiratory tract and kidneys of H9N2 infected specific-pathogen-free chickens at different time points

Time points (dpi)	Lung/Trachea		Kidney	
	Number of positive/ Total samples	Virus copy number, $\log_{10}$ (means ± SE)	Number of positive/ Total samples	Virus copy number, $\log_{10}$ (means ± SE)
2	6/6	10.20±0.06^{b}	6/6	9.94±0.06^{b}
5	6/6	10.35±0.09^{a}	6/6	10.01±0.04^{b}
7	6/6	10.29±0.03^{b}	6/6	11.05±0.34^{a}
10	6/6	10.62±0.08^{c}	6/6	11.48±0.11^{a}
14	6/6	10.28±0.15^{b}	6/6	10.74±0.06^{a}
16	6/6	10.27±0.06	6/6	10.44±0.06

Note. The values with different superscripts (a, b, and c) compared the effect between respiratory and kidneys in each column are significantly different (p<0.05)

predominantly found in Asian countries, resulting in severe respiratory signs, drops in egg production, and mortality. In addition, the H9N2 viruses from G1 and Y280/BJ94 can also infect humans (Lai et al., 2021).

Several Malaysian H9N2 viruses have been characterized by sequencing analysis (Syamsiah et al., 2019). Gunasekara (2021) sequenced the genomes of two Malaysian H9N2 viruses of the Y280/BJ94 lineage, UPM994/2018 and UPM2033/2019, isolated from breeder and layer flocks, respectively. In this study, the pathogenicity of one of the isolates (UPM994/2018) were carried out in SPF chickens. In addition, the ability of this virus to replicate in the respiratory tract and the kidneys was evaluated using a TaqMan real-time quantitative PCR assay designed against the H9N2 M gene. Previous studies have used real-time PCR based on the M gene to detect and quantify H9N2 viral load (Cattoli et al., 2004; Spackman et al., 2005; Yang et al., 2018).

One-week-old SPF chickens inoculated intraocularly with the Malaysian LPAI H9N2 UPM994/2018 exhibited mild to moderate clinical signs starting from day 6 PI that subsided gradually, and by day 12 PI, most of the chickens were healthy. The infected chickens exhibited signs of depression, ruffled feathers, sneezing, gasping, ocular discharge, a swollen face, and mild watery diarrhea. Bijanzad et al. (2013) reported similar observations of a generalized clinical illness associated with respiratory distress in SPF chickens infected with H9N2. The post-mortem examination also recorded swollen and pale kidneys and mild hemorrhages in the lungs of the infected chickens. These gross lesions are in agreement with the results of Hablolvarid et al. (2004).

In addition, at day 7 PI, the infected chickens demonstrated a 13% growth retardation that is statistically significant ($p<0.01$) compared to the control group. Bijanzad et al. (2013) reported a similar observation of significant weight loss in SPF chickens after infection with the H9N2 virus. The incubation period of LPAI H9N2 may vary from a few days to 2 weeks (3 to 14 days) depending on various factors associated with the different strains of the H9N2 virus, host, age, and environment (Bóna et al., 2023).

Histopathology lesions in H9N2-infected chickens in this study include tracheitis, pneumonia, and tubulointerstitial nephritis. The qPCR approach detected the virus in the respiratory tissues and kidneys. Previous findings have shown that the Y280/BJ94 and G1 lineages of H9N2 viruses replicate efficiently in the lungs and multiple organs, including the kidneys of SPF chickens, following intranasal inoculation (Song et al., 2019). Gu et al. (2021) reported the mechanism underlying the cytokine-storm network and how it causes pathological damage to the host after infection with the influenza A virus. Therefore, in this study, histopathological changes can be associated with the release of cytokines in response to inflammation (Mahana et al., 2019) and appear beyond the incubation period of LPAI H9N2 viruses.

Although the SPF chickens did not display any clinical manifestations by day 12 PI, the persistence of the virus in kidney and lung tissues was detected throughout the study period. Mosleh et al. (2009) observed that virus persistence is longer in the kidney tissues of broilers experimentally infected via intranasal inoculation. Therefore, it seems that it would be necessary to collect samples from the kidneys to detect the persistence of the virus in this organ. Furthermore, virus shedding from the cloaca and oropharyngeal persisted throughout the study period in all infected chickens from day 2 to day 14. A similar finding was reported by Iqbal et al. (2013), where the presence of the virus in both the oropharyngeal and cloaca began on day 3 PI and resolved by day 11 or 15 PI following intranasal inoculation of Pakistan isolated H9N2 viruses in both broiler and layer chickens experimentally.

The detection of viruses in organs other than the respiratory tracts, such as the kidney, is probably associated with molecular characteristics of the virus, including a dibasic cleavage site at position 333-PSRSSR-GLF-341 of HA gene cleavage locations. Previous studies have indicated that dibasic cleavage motif like RSSR is associated with systemic infections in poultry (Baron et al., 2013; Thuy et al., 2016). In addition, LPAI H9N2 viruses with dibasic HA1/HA2 cleavage sites have been isolated from Japan, Vietnam, Indonesia, and China (Mase et al., 2007; Thuy et al., 2016).

Our experiment detected the highest viral load in oropharyngeal swabs compared to cloacal swabs on days 2, 3, and 10 PI. However, the highest viral load from cloacal swabs was detected on day 5 PI, suggesting that the Malaysian isolate could be shed via the oropharyngeal and cloacal routes. These findings corroborate those of the Chinese isolate belonging to the Y280 (Song et al., 2019). In addition, since the virus replicates in the kidneys, this may contribute significantly to virus shedding in the cloacal. Previous studies have shown that the gastrointestinal route and the urinary tract are involved in both virus replication and excretion (Slemons & Swayne, 1990).

Although clinical signs were observed in a few H9N2 infected birds from day 6 to day 10 PI, all the infected chickens continuously shed the virus via the oropharyngeal and cloacal routes until day 14 and stopped at day 16 PI, suggesting the silent shedding of the virus from the infected birds. Song et al. (2019) also reported a similar finding during the H9N2 pathogenicity study on SPF chickens in China. The real-time PCR measurement based on viral copy is a measurement that quantifies the virus copy numbers. Based on the virus copy numbers, we cannot determine the spread of infection, which can only be addressed by a virus transmission sentinel study. Future research is, therefore, required to measure the virus transmission sentinel study to determine the spread of infection. Although, a pathogenicity study is not a routine step in a virus characterization study. However, it is an important step in identifying suitable challenge viruses for vaccine efficacy study

since some layer and breeder farms in Malaysia vaccinate against H9N2.

The isolation of novel reassorted AIVs has been reported in terrestrial birds such as chickens and ducks as well as in non-terrestrial birds such as migratory birds and waterfowl (Li et al., 2020). In addition, Youk et al. (2020) showed that the live bird market (LBM) serves as an evolutionary epicenter for the emergence of the reassorted novel H9N2 viruses. Mixed infections of low and highly pathogenic subtypes of H9 and other pathogenic subtypes, such as H5 and H7 viruses in live birds, may result in genetic reassortments of genes from different influenza subtypes, which can lead to a complete change in the antigenic structure and emergence of new viruses. Hence, detecting this virus and its spread across the poultry population in Malaysia requires continuous monitoring to identify the circulating H9N2 strains. In addition, surveillance for the H9N2 viruses in other poultry birds, such as ducks and quails, and non-poultry birds located in hotspot areas, such as live bird markets and bird sanctuaries, is essential to determining the genetic profiles of the viruses in these avian species. Rapid detection and continuous virus monitoring are crucial for implementing warning systems to prevent and control the disease effectively.

CONCLUSION

Detecting and characterizing H9N2 viruses are paramount for developing proper control and prevention measures. The characterized Malaysian LPAI H9N2 isolate is pathogenic to SPF chickens, generally causing a mild to moderate illness and lesions associated with the respiratory and gastrointestinal tracts and the kidneys, with a gradual recovery, and the clinical signs subsided and disappeared by day 12 PI. However, virus shedding persisted until the last sampling day, day 16 PI.

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The Nutritional, Physico-chemical, and Antioxidant Changes during the Production of Soursop Vinegar Influenced by Yeast and Aeration

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ABSTRACT

This study aims to produce soursop vinegar and evaluate how its nutritional composition changes during production. This process offers a solution to convert acidic fruits like soursop into a nutritious product with a longer shelf life. Fresh soursop juice was extracted using a mechanical press, followed by alcoholic and acetous fermentation under different aerobic and anaerobic conditions, with some samples aerated. The nutritional analysis included proximate composition, vitamin C content, acetic acid, pH, and soluble solids (TSS or ºBrix). Results during fermentation showed increased moisture content (90.73–93.99%) compared to the control (78.56%) and a decrease in protein content (0.5–0.61%) when *Saccharomyces cerevisiae* was present (1.53–1.84% without it). Acetic acid and vitamin C levels remained relatively stable, while ºBrix values significantly decreased (4.83–7.00 ºBrix) compared to the initial 15 ºBrix of the control during fermentation. Aeration during production improved the vinegar's antioxidative capacity, with the highest enhancement observed when *Acetobacter* sp. was added. Overall, this study highlights the potential of soursop vinegar to retain the nutritional composition of the fruit, resulting in a healthier, natural product with an extended shelf life.

Keywords: Antioxidant, fermented food, food waste, functional food, postharvest, soursop, vitamin

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INTRODUCTION

Vinegar, known for its sour taste due to the presence of acetic acid, has a long history dating back to ancient times. Babylonians produced wine vinegar from palm as early as 5000 BC (Jamaludin et al., 2017), while cereal vinegar was found in China around 3000 BC (Wu et al., 2012). Today, vinegar comes in various types, each with its colors, tastes, and smells, derived from different ingredients, resulting in unique flavors. It comes from a plant-based source and is rich in flavonoids, phenolic acids, and aldehydes, making it a functional food (Bakir et al., 2016; Shin Yee et al., 2021).

Although synthetic methods exist for acetic acid production, bacterial fermentation is the preferred process for food intended for human consumption due to its safety and suitability for food products (Faizal et al., 2023; Hajar-Azhari, Wan-Mohtar et al., 2018; Khudair et al., 2023; Wan-Mohtar et al., 2019). Additionally, The Malaysia Food Regulation 1985 (Regulation 334) stated that the minimum acetic acid content in vinegar should be not less than 4% by weight. It ensures the vinegar has the characteristic acidic taste and properties of this product (Jamaludin et al., 2017).

Commercial vinegar production employs both slow and fast fermentation processes. Slow fermentation, a traditional method, takes several months to years, resulting in a rich taste and aroma (Abd Rahim et al., 2023). Fast fermentation, on the other hand, is more commonly used and involves a double fermentation process. Yeast converts sugar to alcohol, which is then transformed into acetic acid by acetic acid bacteria, known as the "mother" of vinegar (*Acetobacter* sp.) (Ho et al., 2017). This faster process can be completed within a week, making it ideal for commercialization.

Soursop fruit, scientifically known as *Annona muricata*, is a popular local fruit in Malaysia. It has a green outer skin and a creamy white pulp with a sour taste. Locals believe it has high nutritional value and benefits health (Badrie & Schauss, 2010). However, soursop has a short shelf life and reduced production due to climate change, making it prone to rapid spoilage and chilling injury (Badrie & Schauss, 2010). Diseases like black canker (*Phomopsis anonnaccarum*) and diplodia rot (*Botryodiplodia theobramae*) further contribute to fruit spoilage (Sanusi & Abu Bakar, 2018).

This study focuses on producing soursop vinegar using a double fermentation method, with or without aeration, to address the rapid spoilage and preserve its nutritional content. The double fermentation method involves variations in the presence or absence of yeast *Saccharomyces* sp. Thus, a "double" fermentation as the subsequent fermentation will be performed by *Acetobacter* sp. The objective is to investigate the nutritional changes during the vinegar production process and compare them to the nutritional profiles of fresh soursop juice (negative control 1, SJ) and Bragg's organic raw and unfiltered Apple Cider vinegar (positive control 2, AC).

By utilizing the double fermentation process, the study aims to create soursop vinegar that retains its nutrients and enhances its shelf stability. Overall, this research highlights the potential of soursop vinegar as a value-added product, offering a practical and sustainable solution to prevent rapid spoilage while maximizing its nutritional benefits.

MATERIALS AND METHODS

Materials

The soursop fruit used in this study was purchased from Mawai, Kota Tinggi, Johor, Malaysia. Instant yeast powder (*Saccharomyces cerevisiae*) of Mauri-Pan (Malaysia) was obtained from Rakanda Enterprise Sdn. Bhd. in Seri Kembangan, Selangor, Malaysia. The "mother" of vinegar (*Acetobacter* sp.) was obtained directly from Bragg (USA)'s organic raw and unfiltered Apple Cider (AC) vinegar. The experiment used fresh soursop juice (SJ) and commercial AC vinegar as controls.

Preparation of Soursop Vinegar

The soursop pulp was collected and stored in a freezer at -20°C to prepare soursop vinegar. Thawed pulp was mechanically pressed to obtain the juice. Two variables were considered: the presence of yeast and the aerated environment (Figure 1). The first stage involved alcoholic fermentation for 7 days at 30°C. Subsequently, acetous fermentation was initiated by adding the "mother" of vinegar (*Acetobacter* sp.), and the samples were subjected to aeration in an incubator shaker at 30°C and 60 rpm for 1 day. Samples without aeration were fermented for 15 days at 30°C to reach an adequate level of acetic acid. All experiments were performed in triplicate.

Analytical Procedures

Total soluble solid (TSS) was measured by using the Brix value. The Brix value is expressed in degrees Brix (ºBx), with 1 degree Brix being 1 g of soluble solids content in 100 g of solution. The Brix value of samples was determined using a hand refractometer. A pH meter determined the pH value (Abd Rahim et al., 2022). The acetic acid content was determined by adding 2 ml of sample with 20 ml of distilled water to ensure compliance with the Malaysia Food Regulation 1985 (Regulation 334). After proper mixing, 3 to 5 drops of phenolphthalein (R&D Chemicals, Malaysia) were added as an indicator. The solution was then titrated with standard 0.1 M sodium hydroxide (NaOH, Sigma-Aldrich, USA). The burette reading was measured and used to calculate acetic acid content.

For vitamin C determination, 2 cm^3 of 1% vitamin C solution (Sigma-Aldrich, USA) was prepared in a conical flask. The 1% 2,6-dichlorophenolindophenol solution (DCPIP, Sigma-Aldrich, USA) was added drop by drop into the conical flask and gently shaken until the blue color disappeared. The amount used for the DCPIP solution was recorded. Then, the procedure was repeated by substituting the vitamin C solution with the soursop vinegar samples. The vitamin C

content is calculated in the standard solution in mg/cm^3.

The antioxidant properties of the samples were assessed using modified versions of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) and ferric-reducing antioxidant power (FRAP) methods. The antioxidant assays were conducted following the procedure suggested by Jasmi et al. (2020) and Mansor et al. (2020). For the DPPH assay, 100 mM Tris-hydrochloric acid buffer (Tris-HCl, pH 7.4, R&D Chemicals, Malaysia) was combined with 400 µl of the samples or blank. This mixture was added with 2 ml of 500 µM DPPH solution (Sigma-Aldrich, USA) in absolute ethanol. The resulting mixture was vigorously mixed and then incubated at room temperature for 20 min. The absorbance of the solution was measured at 517 nm using a UV-VIS spectrophotometer.

The FRAP assay involved the preparation of the FRAP solution by combining three solutions in a 10:1:1 ratio. These solutions included 10 mM 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ, Sigma-Aldrich, USA), 300 mM acetate buffer (pH 3.6, Sigma-Aldrich, USA), and 20 mM iron(III) chloride ($FeCl_3$, Sigma-Aldrich, USA). Subsequently, 3 ml of the FRAP reagent (Sigma-Aldrich, USA) was mixed with the samples and heated at 37°C for 10 min. The FRAP value was determined by measuring the difference in absorbance between the samples and the blank solution.

Proximate Analysis

The moisture content of the samples was determined according to the Association of Official Agricultural Chemists (AOAC) method (AOAC, 2005). The crucible, along with its lid, was cleaned and dried in an oven for 30 min, followed by cooling in a desiccator. The weight of the crucible and lid was repeatedly measured until a constant reading was obtained. Next, 1–2 g of the sample was weighed into the crucible, and with the lid covered, it was heated at 105°C until a constant reading was achieved.

About 3–5 g of the sample was placed in a cleaned and dried crucible, which was then covered with its lid to determine the ash content. The crucible was heated in a muffle furnace at 550°C for 3 hr or until no black particles were present and a constant weight was obtained. Subsequently, the crucible with the ash was cooled in the desiccator and weighed until a constant reading was achieved.

The protein content was measured using the Kjeldahl method. A 0.5 g sample was mixed with 0.8 g of Kjeldahl catalyst (Merck, USA), and 2.5 ml of concentrated sulfuric acid (H_2SO_4, Merck, USA) was added. The mixture was slowly heated for 10 min under a fume hood until it became clear. The temperature was then gradually reduced to 40°C, and 10 ml of distilled water was added to the distillation tube. Next, 10 ml of 45% 0.313N NaOH solution (Sigma-Aldrich, USA) was slowly added to the distillation tube to form a two-layered solution. Prior to distillation, 10 ml of 2% boric acid (Sigma-Aldrich, USA) with a few drops of indicator (Merck, USA) was added to a 50 ml conical flask. The resulting

green-colored product was gently swirled, and the unreacted boric acid was titrated with 0.05 N H_2SO_4 (Merck, USA) until a purple color appeared, indicating the determination of Kjeldahl nitrogen (Hajar-Azhari, Shahruddin, et al., 2018).

The different methods determined the carbohydrate content, assuming the fat and crude fiber content to be 0 (Okokon & Okokon, 2019).

Data Analysis and Interpretation

Data analysis was performed using one-way analysis of variance (ANOVA), with a confidence interval of 95%. Mean differences were tested, and the nutritional values of soursop vinegar were compared between treatments (A, B, C, and D) and the control (SJ and commercialized AC vinegar).

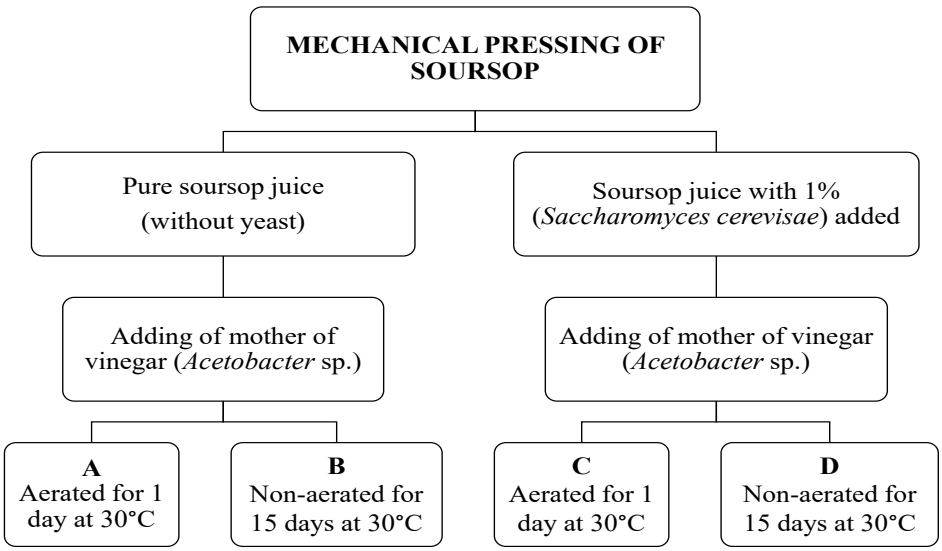

Figure 1. The experimental design to produce soursop vinegar involved four different conditions: A = Soursop vinegar with yeast under aerobic conditions; B = Soursop vinegar with yeast under anaerobic conditions; C = Soursop vinegar without yeast under aerobic conditions; and D = Soursop vinegar without yeast under anaerobic conditions

RESULTS AND DISCUSSION

Table 1 illustrates the results and the impact of yeast and aeration conditions on the nutritional composition of soursop vinegar, soursop juice, and AC vinegar. Significant changes (p<0.05) were observed for all nutritional components except vitamin C. The preservation of vitamin C from soursop juice is a promising finding, indicating that the production of soursop vinegar results in a product with a higher nutritional value compared to commercially available synthetic vinegar. Fruits are known for their vitamin C content because they naturally synthesize and accumulate this nutrient for their growth and development. Vitamin C

is synthesized in the fruit's tissues through enzymatic processes, primarily ascorbic acid.

Among the samples, the soursop juice (control) exhibited the highest TSS content at 15.0 ºBrix, while AC vinegar displayed the lowest TSS content at 3.90 ºBrix. Notably, a significant reduction in the percentage of carbohydrates was observed in all treatments, with values ranging from 4.62 to 7.61% (and AC vinegar at 1.06%), in contrast to the soursop juice (control), which had a carbohydrate content of 18.89%. This reduction can be attributed to fermentation, which converts the sugar substrate into alcohol (Hajar-Azhari, Wan-Mohtar, et al., 2018), subsequently transforming it into acetic acid. While fruit sugars are generally preferable to refined sugars, a decrease in sugar content is desirable, especially for reducing the risk of human metabolic diseases (Hajar-Azhari et al., 2020, 2021).

Table 1
Nutritional value of soursop juice (SJ), Apple Cider commercial control (AC), and soursop vinegar produced from fermentations (denominated from A–D)

Parameters	SJ	AC	A	B	C	D
TSS (º Brix)	15.00 ± 0.00^{a}	3.90 ± 0.00^{c}	6.33 ± 0.17^{bc}	5.89 ± 0.38^{bc}	7.00 ± 2.55^{b}	4.83 ± 1.43^{bc}
pH	3.67 ± 0.26^{b}	2.98 ± 0.13^{c}	4.27 ± 0.06^{a}	4.59 ± 0.05^{a}	4.34 ± 0.07^{a}	4.33 ± 0.09^{a}
Moisture (%)	78.56 ± 0.36^{c}	98.46 ± 0.10^{a}	92.99 ± 1.07^{ab}	91.92 ± 2.65^{b}	90.73 ± 4.73^{b}	93.99 ± 1.46^{ab}
Ash (%)	1.45 ± 0.63^{a}	0.32 ± 0.04^{b}	0.69 ± 0.16^{ab}	0.65 ± 0.13^{b}	1.06 ± 0.07^{ab}	0.90 ± 0.20^{ab}
Protein (%)	1.09 ± 0.32^{b}	0.16 ± 0.08^{d}	0.61 ± 0.10^{c}	0.50 ± 0.01^{cd}	1.53 ± 0.06^{a}	1.84 ± 0.11^{a}
Carbohydrate (%)	18.89 ± 0.38^{a}	1.06 ± 0.08^{c}	4.79 ± 1.13^{bc}	5.59 ± 2.77^{bc}	7.61 ± 4.74^{b}	4.62 ± 1.31^{bc}
Vitamin C (mg/cm^{3})	0.61 ± 0.00^{a}	0.40 ± 0.18^{a}	0.48 ± 0.06^{a}	0.22 ± 0.08^{a}	0.61 ± 0.17^{a}	0.51 ± 0.29^{a}
Acetic Acid (%)	NT	4.59 ± 1.75^{a}	3.98 ± 1.85^{b}	2.96 ± 0.67^{b}	4.90 ± 0.66^{a}	3.39 ± 0.32^{b}

Note. A = Aerated for 1 day at 30°C without yeast; B = Non-aerated for 15 days at 30°C without yeast; C = Aerated for 1 day at 30°C with yeast; D = Non-aerated for 15 days at 30°C with yeast. Values are expressed as mean ± standard deviation of triplicate measurement. Superscripts with different letters significantly differ at $p<0.05$ in the same row. NT = Not tested

TSS and moisture content generally have an inverse relationship, which can be attributed to the conversion of sugar to alcohol and acetic acid during fermentation (Deshmukh et al., 2014). This relationship is demonstrated in Table 1, where higher Brix values correspond to lower moisture content. The AC vinegar sample displayed the highest moisture content of 98.46%, attributed to the proper filtering process that

removes most residues from the solution. While adding external yeast is beneficial, the absence of yeast does not significantly impact alcoholic fermentation, as yeast naturally exists on the surface of fruits (Aneja et al., 2014). However, the absence of yeast may reduce fermentation efficiency, requiring more days to achieve the desired alcohol level.

It is important to note that there were no significant differences in pH among the different treatments, except for the control samples, which demonstrated significantly lower pH. According to the Malaysia Food Regulation 1985 (Regulation 334), vinegar must contain a minimum of 4% weight per volume of acetic acid, excluding other mineral acids. In the case of treatments A, B, and D, this requirement was not fully met, indicating that a longer fermentation period may be necessary to achieve the desired concentration of acetic acid. Interestingly, the non-aerated treatments (B and D) showed lower levels of acetic acid than aerated treatment, suggesting that oxygen may play a crucial role in vinegar production. The absence of oxygen in these treatments may have restricted the growth and activity of acetic acid bacteria, as previously demonstrated (Vikas et al., 2014). The combination of aeration (hence, higher dissolved oxygen) and yeast has shown satisfactory acetic acid content (4.9%) in sample C, comparable to commercial control.

It is also interesting to note that the pH values do not correspond to the acetic acid levels produced by the soursop vinegar. For instance, although treatment B showed a low percentage of acetic acid, its pH was not significantly different from treatments A, C, and D. This observation suggests that there may be other acids present in the soursop juice, either naturally or as a result of fermentation processes (Bhat & Paliyath, 2016). Furthermore, the increased pH observed in the treatments compared to the soursop juice control indicates that yeast might have caused the breakdown of citric acid during fermentation, which can be beneficial during the acetous stage of vinegar production (Schwan & Wheals, 2004).

The presence of yeast had a minimal (not significant) impact on the ash content and protein content of the samples. Treatments C and D (with yeast) exhibited slightly higher ash content, with 1.06 and 0.90%, respectively, but not significant to treatments A and B (without yeast). Notably, the ash content of the treatments with yeast is closer to that of AC control, likely because yeast is utilized in both treatments. The higher ash content in treatments C and D could be attributed to the presence of yeast during fermentation. Yeast is a rich source of macro- and micro-minerals, such as sodium, potassium, and magnesium, providing more than 80% of the daily recommended intake per 100 g (Jaeger et al., 2020).

The significantly higher protein content observed in treatments C and D can also be attributed to the higher presence of yeast. Yeast is known to be a rich source of protein. During fermentation, the nitrogen values can increase due to the presence of microbial

biomass or the concentration of protein as sugars are consumed (Day & Morawicki, 2018), which resulted in the higher protein content observed in those samples.

Antioxidant Capacities of Soursop Vinegar

In addition to the essential nutrients, antioxidants in functional foods are widely recognized for their importance. While the human body possesses its internal antioxidative enzymes, the supplementation of antioxidants from external sources, particularly through dietary intake, is highly beneficial (Amirah et al., 2020; Ramli et al., 2016; Salleh et al., 2022; Tan et al., 2021). Antioxidants play a crucial role in counteracting the harmful effects of free radicals, both internally and externally, thus preventing chain reactions that can lead to cellular damage (Abadl et al., 2022; Mediani et al., 2014; Nawawi et al., 2023). Plant-based juices, such as sugarcane, often contain abundant beneficial antioxidants (Abd Rahim et al., 2022; Jasmi et al., 2020; Mohd Zaini et al., 2023).

Natural antioxidants in soursop vinegar, derived from the fruit's inherent compounds, enhance its potential health benefits. By incorporating soursop vinegar into their diet, individuals can obtain an additional supply of antioxidants, which aids in the neutralization of free radicals and offers protection against oxidative stress-related harm in the body (Halim et al., 2023; Kadum et al., 2019). The assessment of FRAP value serves as an indicator of the antioxidant content, with higher values representing a greater concentration of antioxidants. Conversely, a lower DPPH value signifies enhanced antioxidant activity.

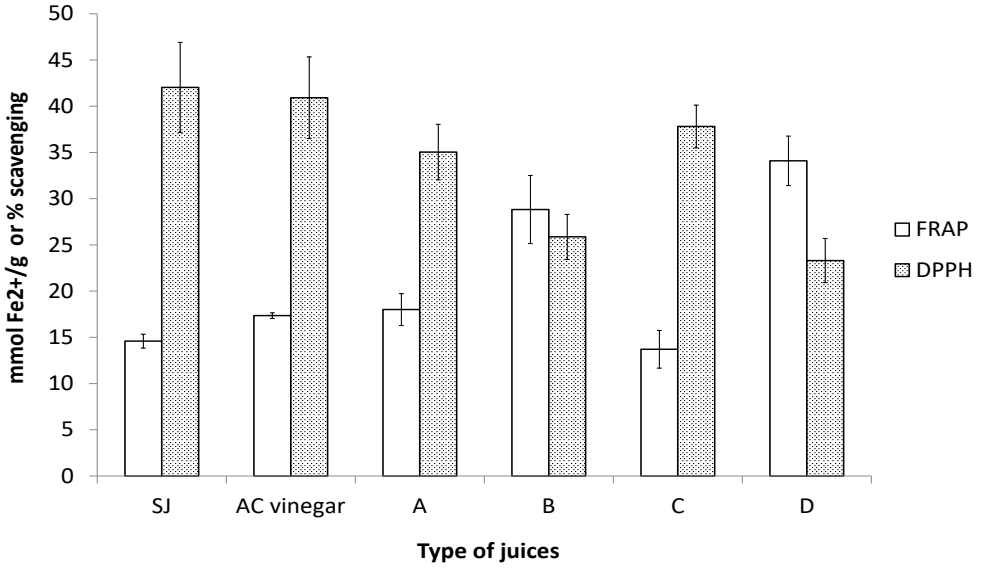

Figure 2. The antioxidant activities of SJ (soursop juice), AC (Apple Cider) vinegar, and soursop vinegar

Note. A = Aerated for 1 day at 30°C without yeast; B = Non aerated for 15 days at 30°C without yeast; C = Aerated for 1 day at 30°C with yeast; D = Non aerated for 15 days at 30°C with yeast

Figure 2 visually represents the antioxidant content and activity, as assessed by FRAP and DPPH measurements, respectively. Based on the figure, converting soursop juice into vinegar successfully retained or enhanced its antioxidant properties. Notably, treatments B and D, produced under anaerobic/non-aerated conditions, exhibited a significant increase in antioxidant content, surpassing both control samples. Treatment B demonstrated an impressive 97.60% higher FRAP content and a significantly lower DPPH value of 38.46% compared to the SJ control. Similarly, treatment D exhibited significantly higher FRAP and DPPH values, with increases of 133.65 and 44.58%, respectively.

The findings from this study suggest that the anaerobic/non-aerated vinegar production process, exemplified by treatments B and D, resulted in a noteworthy enhancement in antioxidant content compared to the soursop juice control. It indicates the potential for these vinegars to effectively neutralize free radicals and combat oxidative stress, positioning them as functional food products with higher antioxidant properties. It is worth noting that acetic acid bacteria such as *Acetobacter* sp. typically require oxygen to convert ethanol to acetic acid. Hence, the lower acetic acid content observed in vinegar B and D can be attributed to the absence of oxygen in the anaerobic conditions. However, the mechanisms underlying the observed increase in antioxidants under oxygen-limited conditions remain partially understood.

One possibility is that the anaerobic pathway of acetic acid production, which involves the recycling of nicotinamide adenine dinucleotide (NAD^+) through pyruvate reduction, may also lead to the formation of other derivatives that contribute to the heightened antioxidant capacity. Similar phenomena have been observed in anaerobic fermentation processes involving Baker's yeast and lactic acid bacteria, where the conversion of ferulic and caffeic acids resulted in increased amounts of their derivatives, such as sinapic acid (Verni et al., 2019). These findings highlight the promising potential of anaerobic vinegar production in yielding vinegar with enhanced antioxidant properties, further enhancing its value as a functional food with potential health benefits.

CONCLUSION

The production of soursop vinegar presents an excellent opportunity to add value to overripe soursop fruit, reducing waste while preserving or enhancing its nutritional content. Soursop vinegar stands out due to its elevated levels of vitamin C and protein and its reduced sugar content compared to the original fruit. Additionally, including non-nutritive components, such as antioxidants, has shown promising outcomes in soursop vinegar.

Utilizing aeration or adding specific microorganisms during the production process has proven to be effective in significantly boosting the vinegar's antioxidant content. It emphasizes the significance of selecting appropriate

processing methods, including aeration, to optimize the production of nutritious soursop vinegar. Ultimately, the transformation of soursop fruit into vinegar allows for the utilization of excess or overripe fruit and results in a vinegar product with improved nutritional properties.

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Short Communication

Survival Rate and Growth Performance of *Holothuria scabra* Towards Different Stocking Densities and Feeding with *Spirulina*

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ABSTRACT

Holothuria scabra is widely used in traditional medicine or consumed as a healthy or exotic food, causing wild stocks to decrease. Therefore, aquaculture is the best solution to address this issue and support the growing market demand. However, up to now, *H. scabra* production is facing problems related to the nursery phase, survival rate of larvae and juveniles, production cost, and broodstock for mass production. In this study, the juveniles of *H. scabra* were divided into three different stocking densities and fed with 1 g of dissolved *Spirulina* powder once on alternate days. Their length was recorded every two weeks. After six weeks, the juveniles reared with 100 individual densities showed the highest survival rate at 80% compared to 200 and 400 stocking densities. Meanwhile, every tank showed a positive growth rate, indicating that *Spirulina* powder could potentially promote the growth of juvenile *H. scabra*. The specific growth rate for stocking density at 100, 200, and 400 were 1.2, 0.86, and 1.37%/day, respectively. In conclusion, the optimum initial stocking density is between 100 and 200 individuals for a 1-ton fibreglass tank with a 500 L water capacity. The *Spirulina* can be used as the main protein source as compared to other diets for juvenile *H. scabra*.

Keywords: *Holothuria scabra*, sandfish, sea cucumber, *Spirulina*, stocking density

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INTRODUCTION

Holothuria scabra Jaeger, also known as sandfish and locally known as 'trepang' and 'gamat pasir' in Malaysia, is a species of sea cucumber from the family Holothuriidae (Kamarudin et al., 2019). Holothurians are invertebrate species without a backbone and inhabit coastal areas with sandy bottoms. People believe this sea cucumber is highly medicinal and widely used in traditional medicine or consumed as a healthy or exotic food. However, the demand for it has increased over time, and until today, the current stock of sandfish cannot support the market demand, neither locally nor internationally. Uncontrolled harvesting of wild sandfish has led to overexploitation, resulting in this species being listed as endangered (EN) under the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (Barclay et al., 2017; Battaglene & Bell, 2004; Han et al., 2016).

Aquaculture is considered the best solution to address sandfish's declining wild stock issue and support market demand. The technique for producing *H. scabra* in hatcheries was developed in early 2000 and has now become one of the new target species in aquaculture (Battaglene & Bell, 2004). However, up to date, *H. scabra* production has faced problems related to the nursery phase, the survival rate of larvae and juveniles, production cost, and broodstock for mass production. A study on the biological life cycle of *H. scabra* has been done, and new technology has been developed to cope with the life cycle stage in aquaculture. The early stages of culturing *H. scabra* (larvae and juveniles) are the most critical because of the low survival rate (Indriana et al., 2017). When the larvae of *H. scabra* develop into the second stage (aurikularia to doliolaria), they need more space for attachment or settlement on substrates like tank walls. The technology for site attachment was developed by using stacked plates and sand in rearing tanks (Altamirano et al., 2017; Sitoresmi & Pursetyo, 2020). However, the survival rate of juvenile *H. scabra* at the grow-out phase (3–15 g) is still low, with only 10–30% after being released in sea pens (Purcell, 2004).

Stocking density is the most important factor for the survival rate and growth performance of *H. scabra* at all rearing levels, from larval, juvenile, and grown-up levels. The survival rates are inversely related to the stocking density. A study showed that the environment or nature of the hatchery did not affect its growth, but the stocking density influenced its growth (Lavitra et al., 2010). At the larval level, many studies suggest the stocking density of the larvae is between 1 and 1.5 per ml (Abidin et al., 2019; Asha & Diwakar, 2013). At the juvenile level, studies showed significantly faster growth and higher survival in low densities compared to high densities (Altamirano & Noran-Baylon, 2020; Cantero et al., 2016; Gorospe et al., 2017). Besides, the growth rate of the juveniles was stunted after being held at higher densities (Battaglene et al., 1999).

In the sea pen and earthen pond, the food for *H. scabra* depends on the naturally occurring microorganisms and algae. However, in the hatcheries, artificial feed is provided for *H. scabra*. The study on the effect of *Sargassum* sp. on juvenile growth performance has been explored. The result showed positive growth performance, but other issues related to the exploitation of seaweed as a source of artificial feed will affect sustainable aquaculture development (Magcanta et al., 2021). To face this issue, *Sargassum* sp. was replaced with a mixture of seagrass (*Enhalus acoroides*), Napier (*Pennisetum purpureum*), and cow manure as artificial feed for juveniles, which showed a positive result in growth performance (Indriana et al., 2017).

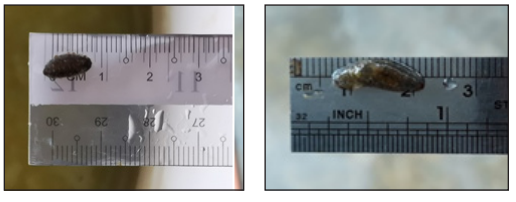
Another study using corn leaf in the diet of *H. scabra* as a substitute for seaweed also showed positive effects on the specific growth rate without any negative effects on weight gain or body composition (Wu et al., 2015). *Spirulina* has been recognised as one of the commercial diets for sea cucumbers. Some successful large-scale nurseries used *Spirulina* to feed the juveniles and broodstock of *H. scabra* (Militz et al., 2018; Simoes & Knauer, 2012). However, some claimed that no significant difference was observed in the growth in length and weight of the juveniles fed with varying proportions of *Spirulina* (Asha et al., 2004). Therefore, this study aims to obtain the best survival rate of juvenile *H. scabra* reared at three different stocking densities and to determine the potential of *Spirulina*'s effect on the growth of juvenile sandfish.

MATERIALS AND METHODS

Survival Rate at Different Stocking Densities

The origin of the *H. scabra* brood stocks was Johor, Malaysia. These mature stocks were induced using the algae bath method (Abdelaty et al., 2021). The juveniles from the same batch with an initial mean length of 1 cm (± 0.19 cm) aged 42 days were used as in Figure 1(a). One-ton fibreglass tanks were used to rear the juveniles, and 500 L of salt water were filled into each tank. Then, 1 g of *Spirulina* powder (Ocean Star International, USA) was dissolved into each tank and allowed to settle completely at the bottom. After that, the juveniles were reared for six weeks under three different stocking densities, namely 100, 200, and 400 individuals per tank. Each treatment was conducted in triplicate. The total number and initial length of juvenile sandfish were recorded every 2 weeks from the first week until the sixth week. The survival rate (SR) of juvenile *H. scabra* was measured after 42 days of culture based on the equation below:

$$SR = \left(\frac{Nt}{No}\right) \times 100\%$$



(a) (b)

Figure 1. The size of juvenile *Holothuria scabra* : (a) Before; and (b) After 42 days rearing in the tank

where SR calculated the percentage survival of juvenile *H. scabra* (Nt), which is the final number of individuals left in the experiment tank after 42 days, and 'No' represents the initial number of individuals stocked at the beginning of the experiment. Statistical analysis was calculated using a one-way analysis of variance (ANOVA, SPSS 27.0). The results were considered significant if the *p*-value < 0.05.

Specific Growth Rate with *Spirulina* Feeding

The cultured juveniles in each tank were fed 1 g of *Spirulina* powder (Ocean Star International, USA) dissolved in 1 L of seawater once every two days. A 50% (250 L) seawater exchange was conducted before the feeding sessions. For control, the juveniles were not fed any algae. The specific growth rate (SGR) of juvenile *H. scabra* was estimated after 42 days of culture based on the equation:

$$SGR = \frac{100(\ln Lt - \ln Lo)}{t}$$

where SGR calculated the percent growth of juvenile *H. scabra* per day, 'Lt' and 'Lo' represent the final length (cm) and initial length (cm), and t is the rearing period throughout the experiment period.

Each juvenile was reared under ambient conditions, and four aeration points were set up for every tank. Water quality monitoring was conducted weekly. Statistical analysis was calculated using one-way ANOVA (SPSS 27.0). The results were considered significant if the *p*-value < 0.05.

RESULTS AND DISCUSSION

Survival Rates

The survival rates of juvenile *H. scabra* for all the stocking densities at 100, 200, and 400 individuals per tank showed decreased per cent survival during the rearing period. There was a more than 50% survival rate of juvenile *H. scabra* in all the treatment tanks starting from the first day of the experiment until the end (Figure 2). Treatment A (100 individuals) showed the best survival rate after the 42-day rearing period, with 80 (± 1.15%) survival rate and 80 individuals surviving on average. As for Treatment B (200 individuals), 143 individuals, on average, survived up to the end of the rearing period with 71.5 (± 0.87%) survival. Treatment C (400 individuals) showed a 53.75 (± 8.22%) survival rate, with an average of 215 individuals left from the initial stocking density. A comparison of these three treatments showed that

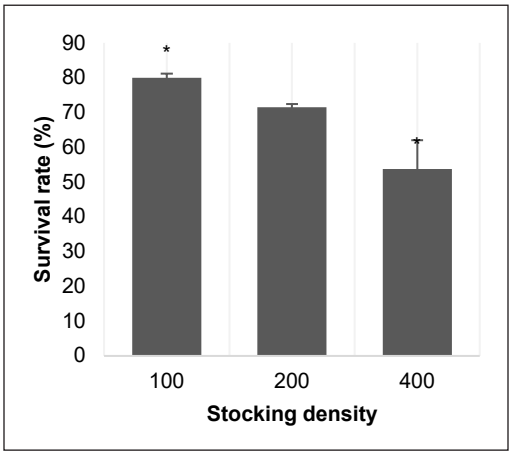


Figure 2. Survival rates ± standard deviation for the different stocking densities of juvenile *Holothuria scabra* after 42 days of culture
Note. * = The results were considered significant if the *p*-value < 0.05

juvenile *H. scabra* had the highest survival rates at a stocking density of 100. In contrast, a stocking density of 400 showed the lowest survival rate. The survival rate for Treatment A was significantly higher than that of Treatment C, with a *p*-value of 0.006.

The juvenile stage of *H. scabra* can attach to the walls of the tank, which act as substrate. They fed on the food attached to the tank wall and bottom (Ridwanudin et al., 2018). Competition for space and food occurred, resulting in decreased juvenile survival rates due to increased juvenile *H. scabra* in the nursing tanks (Cantero et al., 2016). Previous studies on the stocking density of juvenile *H. scabra* showed that when three different stocking densities (100, 200, and 400) were used for rearing in the ocean-based floating hapa, the lowest stocking density exhibited the highest survival rate (Yussuf & Yahya, 2021). The total area of the tank covered with water was 2.5 m². For Treatment A (100 individuals), every juvenile had 0.025 m² space, while Treatment B (200 individuals) was 0.0125 m²/individual, and Treatment C was 0.00625 m²/individual. The movement of *H. scabra* is quite slow, but there is high competition for space and food. The monitored water quality showed normal levels for salinity, pH, temperature, ammonia, nitrate, and dissolved oxygen without drastic changes for the duration of the experiment, as recorded in Table 1.

Growth Performance

Generally, juveniles reared at all three different stocking densities showed positive growth in all the treatment tanks. According to Table 2, the specific growth rate for Treatment A was 1.19% growth/day, while Treatments B and C showed SGR of 0.86 and 1.36%/day, respectively. The initial mean length of juvenile *H. scabra* for Treatment A was 1.1 (± 0.27) cm, and at the

Table 1
Average water parameter levels for all the treatment tanks

Parameter	Range
Salinity (ppt)	28.00 ± 0.51
pH	8.05 ± 0.09
Temperature (°C)	29.0 ± 1.1
Ammonia (ppm)	0.05 ± 0.01
Nitrate (ppm)	0.01 ± 0.01
Dissolve oxygen (ppm)	4.82 ± 0.20

Table 2
Specific growth rates calculated for each treatment

Treatment (Individual)	Mean length (cm)				Specific growth rate (%/day)
Days	0	14	28	42	
A (100)	1.1 ± 0.27	1.34 ± 0.17	1.75 ± 0.43	1.82 ± 0.38	1.19*
B (200)	0.96 ± 0.54	1.18 ± 0.53	1.31 ± 0.51	1.38 ± 0.38	0.86
C (400)	0.71 ± 0.26	1.06 ± 0.61	1.22 ± 0.50	1.26 ± 0.51	1.36*
Control	1.43 ± 0.46	1.18 ± 0.65	1.09 ± 0.77	0.84 ± 0.56	-1.26

Note. The experimental data represented as mean length ± standard deviation; * = The results were considered significant if the *p*-value < 0.05

end of the experiment, it was 1.82 (± 0.38) cm, respectively. While the initial mean lengths of Treatments B and C were 0.96 (± 0.54) and 0.71 (± 0.26) cm, respectively. After the cultured day, 1.38 (± 0.38) and 1.26 (± 0.51) cm were recorded at the end of the experiment for B and C, respectively. It means *Spirulina* powder showed positive results in feeding the juvenile *H. scabra* in all treatments. The SGR for Treatments A and C were significantly higher than the control, with *p*-values of 0.049 and 0.040, respectively.

Several types of food were used as alternative foods to feed the post-metamorphic juvenile *H. scabra*, such as *Nannochloropsis* sp., *Chaetoceros calcitrans*, *Chaetoceros gracilis*, *Isochrysis galbana*, and *Tetraselmis chuii* (Abdelaty et al., 2021; Abidin et al., 2019). *Sargassum latifolium* gave the best growth when the juveniles attained 73 mm in 8 weeks from an initial size below 10 mm (Magcanta et al., 2021). However, the issue of using *Sargassum* sp. is the exploitation of seaweed sources as food for sea cucumbers, and artificial feed is a good substitute for the sustainability of aquaculture development at a commercial level (Wu et al., 2015). Besides, Dabbagh (2012) has compared the growth performance of *H. scabra* treated with four commercially available feeds (such as Algamac 2000, Algamac protein plus, *Spirulina*, and *Dunaliella* gold). Although the growth rate of *H. scabra* treated with *Spirulina* powder was slower compared to others, a lower mortality rate was recorded.

The dry *Spirulina* powder on the market has a high protein content, with 60% crude protein present, and is the best source to complement the main diet for sandfish (Abdelaty et al., 2021). In addition, 1 g of *Spirulina* contains 130 mg of phycocyanin, 9 mg of chlorophyll, 8 mg of phosphorus, and 7 mg of calcium, which are beneficial for the growth of *H. scabra*. Phycocyanin was reported to have potential medicinal benefits for animals (Yuniati & Sulardiono, 2020). Besides protein, *H. scabra* also consumes calcium to produce a thin protective layer as they grow up. Calcium and phosphorus were two of the main mineral compositions in *H. scabra* (Ardiansyah et al., 2020). In addition, *Spirulina* crude formed the settlement faster and with a higher settlement compared with live *Navicula* sp. and *C. calcitrans* (Sibonga et al., 2022). Moreover, *Spirulina* provided a higher volume of epibenthic or seabed biomass that indirectly increased the optimum rearing density, survival, and growth rate of juveniles of *H. scabra* (Lavitra et al., 2009).

CONCLUSION

Based on the results, a high stocking rate resulted in high mortality in juvenile *H. scabra*. Competition for space and food was a major problem, causing high mortality or disappearance on day 42. The optimum initial stocking density is between 100 and 200 individuals for a 1-ton fibreglass tank with a 500 L water capacity. Grading activities must be conducted every 2 weeks, depending on the growth performance of juvenile *H. scabra*, to reduce competition.

Due to the positive growth rates, *Spirulina* can be used as the main protein source compared to other diets for juvenile *H. scabra*. The results of this study can be used as a preliminary reference for future studies to improve the production of *H. scabra* for the aquaculture industry.

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Short Communication

Zebrafish Housing: The Recirculating and Cost-effective Open Design Aquaria System

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ABSTRACT

The zebrafish's ease of care and high reproductive rate have made them a popular animal model. It is routinely kept and maintained in commercial aquariums. However, the expense of a particular system was prohibitive for researchers with limited budgets or who worked in universities without zebrafish-rearing facilities. Thus, a simple custom-made aquaria system was developed. The recirculating open-design aquaria system was cost-effective and may frequently be improved. This study has developed a distinctive aquaria system that can tackle the issues related to expanding research based on zebrafish.

Keywords: Aquaria system, cost-effective, custom-made, open-design, recirculating, zebrafish

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INTRODUCTION

According to Bhargava (2018), the custom-made system has been termed an open design to distinguish it from a commercial system. Open design was different, especially in its design and holding capacity. The available open designs were simple, modular, and cost-effective, providing zebrafish with chemical-free water. However, the varieties of open designs raised several maintenance issues, thus complicating mass reproducibility.

This study demonstrates the technical development of a manageable and cost-efficient recirculating open design system, showcasing its feasibility and practicality. The system (Figure 1) was constructed in a well-ventilated animal retention room and positioned in a closed area next to the tap water resource at the back of the room. The system was maintained (half an hour daily) by an end user under the Department of Basic Medical Sciences, Kulliyyah of Pharmacy, International Islamic University Malaysia (IIUM), Kuantan, Pahang, Malaysia. It took nearly six months for the system to be developed, starting from July 2019 and ending in January 2020. It has proven effective in safely housing zebrafish during a series of experiments by Raduan et al. (2023).

THE RECIRCULATING OPEN-DESIGN SYSTEM

An effective recirculating water system should incorporate a filter system, chemical monitoring and regulation, ultraviolet (UV) irradiation, and a light and temperature control unit (Aleström et al., 2020; Lawrence & Mason, 2012; Varga, 2016). This study designated the recirculating open-design system with a built-in filtration and sterilisation unit embedded with single commercial light-emitting diodes (LEDs) (18 W) lamp on the top level of the rack

(a) (b)

Figure 1. Recirculating open-design (custom-made) zebrafish system: (a) Front view of the system; and (b) Back view of the system

row to meet the entire rack's light need using a 14/10 light-dark photoperiod timer. The essential water quality parameters were regularly monitored and maintained. Total ammonia (using AMMONIA TEST KIT, API®, USA), temperature (using thermometer Model SKU: LAB010, Brannan, United Kingdom), pH (using a portable pH meter), and salinity (using a refractometer, Cold-Palmer, USA) were checked daily, whereas dissolved oxygen was checked monthly using a dissolved oxygen meter (Model HD 3030, Trans Instrument, Singapore). The desired total ammonia should be maintained at 0 mg/L, the temperature should be kept within the range of 24–30°C, the pH within 6.8–8.5, and the salinity at 0–5 g/L. The dissolved oxygen level should not be less than 4 mg/L, following the recommendations of Harper and Lawrence (2011).

Figure 2 illustrates the schematic diagram of our recirculating open-design zebrafish system. In concept, the return

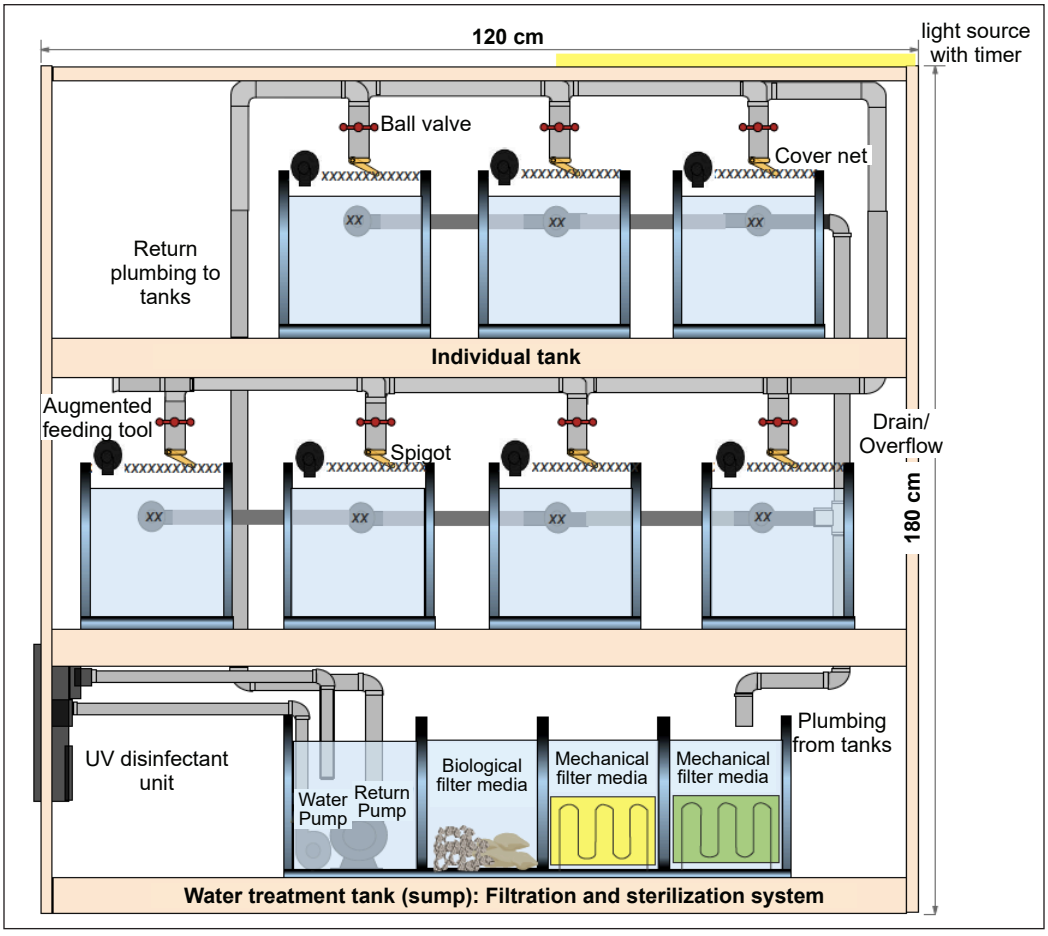


Figure 2. Schematic diagram of recirculating open-design (custom-made) zebrafish system. Before recirculating the clean water (blue arrow) back to the individual tanks, the wastewater (red arrow) that enters the sump must undergo filtration and ultraviolet irradiation

pump pumps water into the various tanks through the regulated water outflow of the manifold, which runs the length of the shelf. Following the principle of an overflow system, water exits the tank through an overflow outlet and drains water (wastewater) to undergo filtration systems before being recirculated. The water treatment tank's water level is checked regularly, and fresh dechlorinated (tap) water is refilled as needed. The water level in the system will typically decrease when residual solid waste from the back of each tank and collected solid waste from the water treatment tank are manually discarded regularly. In addition, natural evaporation occurs and contributes to the drop in water levels in the system. Regular exchanges of fresh (dechlorinated) water are also required to maintain water quality and reduce chemical accumulation (especially nitrogen). Compared to flow-through systems, recirculating systems use much less water overall. Thus, a significant advantage of the recirculating system is that it maintains water quality while minimising water loss (Bhargava, 2018).

CONSIDERATIONS OF COMPONENTS FOR THE RECIRCULATING OPEN-DESIGN SYSTEM

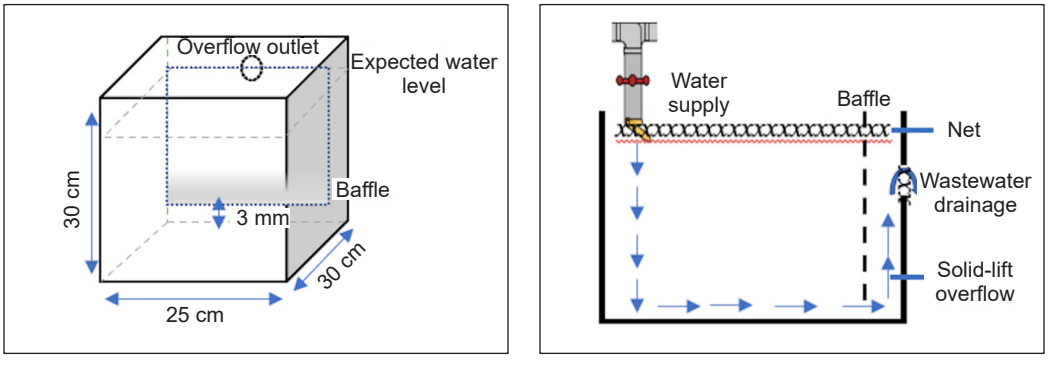
Individual Active Overflow Tank

The tanks are the principal housing enclosures of an aquaculture operation. The design, dimensions, and materials used to construct the tanks will vary based on the scope and scale of the projects. In addition, the approaches to water exchange and specimen confinement will vary (Cockington, 2020). Lawrence and Mason (2012) observed that all these aspects influence the fish's well-being, the system's functionality, and the pace and capability of research. Consequently, selecting a particular system should involve carefully assessing its associated tank types and their contribution to achieving research and husbandry objectives.

Standard zebrafish tanks are often made of polycarbonate, polysulfone, high-quality glass, or acrylic (Matthews et al., 2002). One of the downsides of polycarbonate and polysulfone is their susceptibility to release bisphenol-A (BPA), an acknowledged oestrogen mimic (Howdeshell et al., 2003). That flaw is particularly characteristic in standard tanks. Additionally, the glass is relatively affordable, does not scratch easily, does not produce biofilms or other types of tank fouling, and is chemically inert, so there is no chance of chemicals leaking into the inhabitants (Cockington, 2020). As a result, this study's aquaria system is outfitted with premium transparent glass tanks (using standard glass grade [5 mm thickness]) sealed with SN-503 RTV neutral silicone sealant by Soon Lee Frame & Glass Works, Terengganu) that are net-covered. Each tank is designed to be flexible and can be easily reassembled from the water pipelines. Since most pet stores carry inexpensive rectangular (box) glass aquariums, glass tanks are the most popular type of housing for small-scale operations (Cockington, 2020).

Researchers who wish to conduct small-scale experiments but do not have access to a large-scale production facility still rely heavily on the traditional box tank despite its decreased prevalence in modern times. These tanks range in size from 20 to 50 L and offer a comprehensive approach to fish care. The considerable water volume provides sufficient capacity to buffer against variations in water quality and permits fish to display their natural schooling behaviour (Cockington, 2020). The schematic diagram of the rectangular individual tank is shown in Figure 3. With a total capacity of 17 L and dimensions of 30 × 25 × 30 cm^3 (height × width × depth), each tank can hold around 85 adult zebrafish. The standard recommendation for animal density was five fish per litre (Liu & Zhong, 2017; Vargesson, 2007). Following the desired fish capacity and research requirement, the tank may be set with varying sizes and shapes. As Lawrence and Mason (2012) mentioned, there are no established specifications for the size and form of zebrafish aquariums. Depending on requirements, the fitted glass tanks can be altered, disconnected, and reconnected to the main water supply.

The passive overflow within the individual tanks hinders the capacity to maintain tank hygiene, needing periodic cleaning interventions to keep the tanks functioning as intended (Cockington, 2020). In assisting the removal of waste or solids, tanks should be equipped with specific rear baffles or siphons (Hammer, 2020). A tank's "self-cleaning" capability to remove waste solids is essential when selecting a tank (Timmons & Ebeling, 2013). Typically, the commercial tank manufacturer has a particular design that employs the solids-lift (active) overflow principle while simultaneously preventing fish from fleeing the specific tank (Hammer, 2020). In this study, the individual tank was custom-designed with a partitioning glass baffle (Figure 3). The 5 mm baffle thickness (25 × 25 cm^2; width × length) was positioned so that there was a 3 mm gap at the tank's base. It aims to change the tank's



(a) (b)

Figure 3. The primary hydrodynamic design of an active (solids-lift) tank. A spigot on the front of the tank pumps water into the tank. (a) The ingenious design of the separating baffle at the rear of the tank creates a pressure differential between the holding space and the overflow; and (b) Particulate waste is drawn to the back of the tank by a strong undertow current (blue arrows) and lifted to the tank overflow

hydrodynamics so that an undertow current can develop, producing an active draw across the tank base (Cockington, 2020). In the meantime, with continuous water circulation in the system, it was determined that the overflow outlet of the tank would be closed with a net (200 µm mesh size) to prevent small zebrafish (2-3 mm) from escaping. The net segregates fish to prevent mixing with other treatment or study groups in a specific experimental design.

Combination of Water Treatment Tank with Filtration and Sterilization System

The water treatment tank (sump), or the reservoir, is positioned at the bottom of a self-contained recirculating system. It provides a place to store water circulated throughout the system. The sump was configured to a total capacity of 70 L and dimensions of 38 × 38 × 76.5 cm^3 for our recirculating system (width, height, and length) (Figure 4). The sump's material composition is comparable to the individual tank's grade. According to Bhargava (2018), the sump may have a single large chamber or numerous small chambers to hold the pump, aeration assembly, charcoal bag, heating and cooling coils, and other components. As for this study, the sump was split into four interconnected compartments by baffles, as shown in Figure 4.

Technically, when the water level in the sump decreased (below the ideal capacity) due to natural evaporation, the fresh dechlorinated (tap) water was manually refilled (using a syphon) into the sump. The tap water directly from the public water supply system was initially kept (without filtration) in the 5 L polypropylene (PP) bucket for two days to allow the chlorine to evaporate naturally before being into the system. The bucket was placed near the tap water source. Any rust-prone or leaching

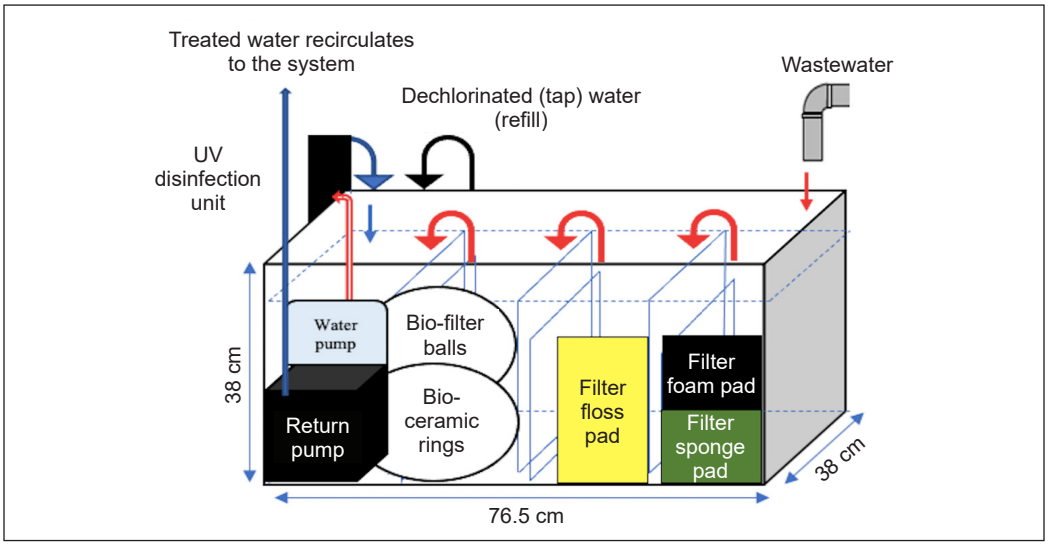


Figure 4. Wastewater (red arrow) undergoes filtration and UV treatment in the sump before recirculating as clean water (blue arrow) to individual tanks. The water pump transfers water to the sterilisation unit, while the return pump brings clean water back to the system.

material, i.e., iron hooks, should not come into direct contact with the sump water, as even trace levels of copper and zinc are toxic (Hernandez et al., 2011; Ribeyre et al., 1995). The sump should be frequently cleaned. A separate syphon eliminates debris from filtration media, uneaten food, and fish waste from the sump as needed. Excessive cloudiness indicates an accumulation of waste.

Mechanical filtration is often the initial step in the filtration assembly of a recirculating system, and its function is to separate and remove suspended solids from the effluent water. Solid waste, such as uneaten food and faeces pellets, must be removed from the circulating water as quickly as possible. It will then disintegrate, providing food for heterotrophic microorganisms. Subsequently, dissolved oxygen concentrations may decline drastically, followed by increased ammonia concentrations. Particle filters for mechanical filtration may be depth or surface filters that collect solids as water passes (Cockington, 2020). As referred to in Figure 4, the coarse and fine mechanical filters were used to fill the sump's first two compartments, consisting of filter foam, sponge, and floss pad (commercial grade supplied by Perniagaan Moon Lai, Pahang). According to Bhargava (2018), scientists rely on foam, sponge or polyester fibres (beginning at 10 microns) for mechanical filtering.

The individual tanks and biological filters of recirculating systems are home to various microorganisms (Rurangwa & Verdegem, 2015). All recirculating systems contain and sustain populations of various microorganisms, i.e., bacteria and viruses. Many species are safe or valuable for fish (e.g., nitrifying bacteria), but some can be harmful, especially in huge numbers. Recirculating systems are designed to preserve water by exchanging a negligible portion of the overall system volume to enable the removal of nitrates. This conservation strategy will accumulate many of these co-occurring organisms in a system over time. In order to maintain low nitrate levels in recirculating systems, it is recommended to utilise a biological filter (commercial grade biofilter balls and bio-ceramic rings/granules supplied by Perniagaan Moon Lai, Pahang) with regular fresh (dechlorinated) water exchange (5-10%/day) (Lawrence & Mason, 2012). Without this filtering, any recirculating zebrafish system would be incomplete (Bhargava, 2018). This process uses fluidised bed biofilters to reduce the total nitrogen content in the system water by bacterial action (Leveque et al., 2016). It is essential because zebrafish are exceedingly harmful to high nitrogen loading, particularly ammonia and nitrite (Harper & Lawrence, 2011; Kroupová et al., 2018). Biological filtration is a process that harnesses the capabilities of chemolithotrophic bacteria, including *Nitrosomonas* and *Nitrobacter*, to cleanse nitrogenous waste by oxidising ammonia to nitrite and nitrate. An effective open-design system should have recirculating water with ammonia levels of 0 mg/L, nitrite levels of 10 mg/L, and nitrate levels of 75 mg/L (McNabb et al., 2012).

The aquaria system employs a disinfection procedure in which water passes through a disinfection unit after solids removal (mechanical filtration), biological filtration, and chemical filtration (optional) to control the populations of the particular organisms (Lawrence & Mason, 2012). UV disinfection unit, also known as an ultraviolet steriliser, use light to produce ultraviolet C (UV-C) ultraviolet radiation with a short wavelength (250-280 nm) that has disinfectant qualities, destroying organisms' DNA and killing or rendering them inactive. There is no established standard for the level of irradiation required for disinfection (Lawrence & Mason, 2012). Following Emperor Aquatics Inc.'s operating and service instructions, an 18-W UV lamp can kill algae, bacteria, and protozoa in an aquarium with 200–250 L of water (Bhargava, 2018). Our system employed 36 W of UV-C power and a maximum flow rate of 4,500 L/hr (model CUV-136, Sunsun, China) to cover a 189 L water storage capacity. Installing the UV assembly in the water inlet line of recirculating systems is typically recommended rather than in the line collecting the waste (Harper & Lawrence, 2011). Figure 4 demonstrates that the disinfection step was performed immediately after mechanical and biological filtration, ensuring that the disinfection unit was supplied with nearly clean water before recirculating throughout the system. Since a UV lamp's lifespan is limited, regularly inspecting its output is recommended (Avdesh et al., 2012).

Pumps and Circulation

All recirculating systems require a route to transport water to higher elevations to serve individual tanks, enhance system pressure, and move water for filtering, disinfection, or other treatment (Timmons & Ebeling, 2013). Water pumps are essential to provide fish with oxygenated, clean water. It aids in the elimination of solid and nitrogenous wastes from individual tanks. The oxygenated water and nitrogenous wastes are transferred to aerobic nitrifying bacteria in the biological filter (Hammer, 2020).

Due to many small-diameter valves and connections that deliver water to several separate tanks in the recirculating system, pumps designed for high water pressures are required. Our system utilised a commercial submersible pond pump (model SOBO WP-12,000 DW, China, with the specification of 175 W and 12,000 L/hr), in line with other commercial systems, as a return pump installed in the sump's last chamber (Figure 4). In addition, a water pump (model Dophin P-2000, China, with specifications of 14.8 W and 700 L/hr) was installed in the last chamber, which connected to the UV sterilisation unit. It helps to move post-filtered water through the UV sterilisation process. The submersible pond pump's motor (model SOBO WP-12,000 DW, China) is a powerful magnetic drive in a waterproof plastic housing that rotates a magnetised impeller blade in the pump head (Malone, 2013). Tiny air leaks on the pump head close to the impeller housing are the most common cause of gas bubble disease (GBD), which leads to a significant

mortality rate among zebrafish. GBD can also occur if insufficient water is in the sump near the pump's intake, forcing it to draw in air and water, thus compressing the gases. To prevent GBD, the sump's water level must be regularly maintained. GBD symptoms include tiny bubbles in the water spigots and on the tank walls and fish remaining at the tank's bottom (Hammer, 2020).

The Rack

The rack aims to hold the entire system in place securely (Figures 1 and 2). The zebrafish's housing requirements will define the tank's dimensions, typically housed in groups whose numbers are set by the study. The size and shape of the tank determine the requirements for the rack. Its designation should consider the safety of humans, the benefits of fish housing, and the appropriateness of fish housing. The rack's appropriate design allows for the three-dimensional grid organisation of the tanks and integrates critical aquaria infrastructures. The infrastructure consists of water pipelines for the influent and effluent streams, aeration, lighting, and monitoring capabilities (Lawrence & Mason, 2012).

The single-sided commercial rack was employed in this study. It was measured at $180 \times 120 \times 45$ cm^3: height × width × depth. The rack can be as tiny as a single tabletop unit or as vast as the available space in the room. It is typically designed with four to six regularly spaced shelves to provide easy access to the individual tanks for personnel (Lawrence & Mason, 2012). This rack was configured with three evenly spaced shelves to store seven 17 L tanks (up to a maximum of eight), including the 70 L sump tank.

This rack system adopted a standalone design with a water treatment tank at the bottom, following Lawrence and Mason (2012), who favoured a rack design that utilised space beneath the lowest rack row to house filters, pumps and UV sterilisers. Above each tank, water delivery polyvinyl chloride (PVC) pipes (supplied by ZNE Hardware, Terengganu and sealed using SS100 PVC pipe adhesive) were built into the rack to transport water from a spigot (metal brass was used and supplied by Dynamic Expansion Sdn. Bhd., Pahang) into the tank. Water was collected from each rack, sent to a joint water return pipe, filtered in a separate unit, and recirculated back to the racks (Figures 1 and 2). Aquaneering Incorporated commercial aquatic system (Aquaneering, n.d.) was comparable to our water delivery system. A physically isolated water treatment section considerably reduces noise and vibration in the fish holding or culture area, which is the actual value of this technique (Hammer, 2020).

The water in the zebrafish system contains salts and minerals capable of corroding a range of metals (Lawrence & Mason, 2012). The high salinity of the water (> 300 ppm) could corrode the rack if water spillage or leakage happens during zebrafish handling; consequently, the rack material must be rustproof (Bhargava, 2018). The materials chosen by top commercial providers, such as stainless-steel racks, plastic-coated metal racks, and PVC-coated corrosion-resistant racks, have resolved this

issue (Aquaneering, n.d.; Iwaki Aquatic, n.d.; Tecniplast, 2022). In addition, the rack material selected for open-design systems must sustain the whole weight of filled zebrafish tanks and withstand seismic waves (Bhargava, 2018). In the current setup, the rack is made of metal that underwent the most advanced epoxy coating technique during its manufacturing process. The decking material is composed of durable high-density fibreboard that can support up to 200 kg/level (TTF Group, n.d.).

CONCLUSION

Open-design systems are gaining popularity among zebrafish researchers because they are inexpensive, flexible, scalable, and effective. These configurations can also create opportunities for independent work for technical specialists who wish to construct and sell them to regional or national academic institutions or laboratories. In our system, water exchange is currently done manually. Consequently, introducing a programmable and automated water exchange system could be the subject of future research. It would be intriguing to have automation in open-design systems, particularly for cost-effectively measuring oxygen and pH levels and nitrogen content. However, skilled, well-trained, knowledgeable, and experienced human labour remains essential.

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Identification of Phytochemicals in *Cleome rutidosperma* DC. Methanol Extract and Evaluate its Efficacy on Some Common Rice Field Weeds

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ABSTRACT

Screening different plant species for herbicidal activity and identifying new allelochemicals with novel structures and phytochemical activity could be promising candidates for reducing the negative consequences of chemical herbicides. Our study aims to investigate the allelopathic substance(s) and herbicidal efficacy of *Cleome rutidosperma* DC. on rice filed weeds in the lab and glasshouse. The phytochemical constituents of the methanol extract of *Cleome rutidosperma* were analyzed by high-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry (HPLC-ESI-QTOF-MS). The allelopathic effect of *C. rutidosperma* has been further studied on the germination and early development of five common rice field weeds: *Echinochloa crus-galli* (L.) P. Beauv., *Fimbristylis miliacea* (L.) Vahl, *Oryza sativa* f. *spontanea* Roshev., *Leptochloa chinensis* (L.) Nees, and *Cyperus iria* L. The seed germination and growth of tested weeds under lab and glasshouse conditions were compared to three concentrations of *C. rutidosperma* methanol extract at 2.5, 5, and 10% with the control (only distilled water). The results indicated the presence of 64 and 10 known chemicals using positive and negative ionization techniques, the majority of which were toxic. The inhibitory effect of *C. rutidosperma* was stronger in the lab than

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in the glasshouse. No seed germination of the tested species was observed when 10% *C. rutidosperma* extract was applied. The photosynthesis rate of *C. iria* exhibited a higher reduction (70.56%) compared to other species at higher doses (10%) of *C. rutidosperma*. These findings demonstrated that *C. rutidosperma* is a significant source of phytotoxic components and can be used to develop future bio-herbicides. The outcome of this study can be employed in the organic management of weeds and reduce our heavy reliance on synthetic herbicides.

Keywords: Allelopathy, *Cleome rutidosperma*, germination, growth, physiology, phytochemicals

INTRODUCTION

Rice (*Oryza sativa* L.), a primary food for more than half of the world's population, is cultivated in >100 countries, with Asia accounting for 90% of global production (Fukagawa & Ziska, 2019). Ray et al. (2013) predicted that world rice demand would more than double by 2050. In lowland and highland environments, weed infestations are a significant biological restriction to rice production at all seasons (Reynolds et al., 2015).

The most threatened weed is *Echinochloa crus-galli* (L.) P. Beauv. (barnyard grass), which can decrease rice production by up to 64%, depending on the rice variety (Yang et al., 2021). *Echinochloa crus-galli* possesses the adaptive characteristics and competitive qualities required for effective competition and survival in various geographical and environmental situations (Clements & Ditommaso, 2011). In rice fields, *Fimbristylis miliacea* (L.) Vahl, sometimes known as hoorahgrass, is an invasive sedge with an emergence density ranging from 54 to 3,074 plants per square meter in Southeast Asia (Siddique & Ismail, *2013).* According to Siddique and Ismail (2013), *F. miliacea* is ranked third and fifth among the most troublesome weeds in Malaysia. *Oryza sativa* f. *spontanea* Roshev. (weedy rice), popularly known as "red rice", is now one of the major weeds in many places of the world that produce rice (Juliano et al., 2020; Mispan et al., 2019; Ziska et al., 2015). Countries switching to direct seeding rice instead of transplanting are facing a serious problem (Nadir et al., 2017). *Leptochloa chinensis* (L.) Nees (Chinese sprangletop) is a major global agricultural grass weed spread in rice fields and has developed resistance to cyhalofop-butyl herbicide (Yu et al., 2017). *Leptochloa chinensis* can grow in flooded and upland environments, making it a common weed in rice and other crops (Wang et al., 2022). According to Jiang et al. (2018), *Cyperus iria* L. has successfully adapted to habitats and is a problematic weed in rice cultivation. Approximately 5,000 seeds can be produced by one *C. iria* plant, and the first rice seedlings often emerge within a few weeks after planting (Awan et al., 2022).

Weeds are a growing issue than diseases and pests when it comes to reducing rice yields in tropical Asian countries (Motmainna et al., 2021a; Juraimi et al., 2013). In rice production, the use of herbicides and other types of chemical

control is the most common practice (Hasan, Ahmad-Hamdani, et al., 2021; Motmainna, Juraimi, Uddin, Asib, Islam, Ahmad-Hamdani, & Hasan, 2021; Sherwani et al., 2015). However, by 2020, global pesticide usage has been estimated to increase to 3.5 million tons (Sharma et al., 2019). Herbicide-resistant weeds are developed when similar herbicides are used repeatedly at the same field site. Eight of the 16 weed species detected in Malaysia that have been documented to be resistant to various herbicides were found in rice fields (Ruzmi et al., 2017). The global interest in inorganic farming supports alternative methods that prevent herbicide-resistant weed development (Motmainna et al., 2021b). Such circumstances have promoted using other alternatives, such as bioherbicide, to control the population of weeds.

Screening different plant species for herbicidal activity and identifying new allelochemicals with novel structures and phytochemical activity could be promising candidates for reducing the negative consequences of chemical herbicides. Taking an example, WeedLock is a commercial bioherbicide obtained from *Solanum habrochaites* S. Knapp & D. M. Spooner (wild tomato) extract and showed promising weed control efficacy in both glasshouse and field conditions (Hasan, Mokhtar, et al., 2021). Verdeguer et al. (2020) reported that, by 2020, six commercial bioherbicides, i.e., Matratec, GreenMatch, GreenMatchEX, WeedZap, Weed Slayer, and Avenger Weed Killer, derived from essential oils and/or their compounds were registered and available in the USA. Bioherbicides such as BioWeed, Avenger Weed Killer, and Weed Slayer successfully controlled *Ochna serrulata* Walp., *Digitaria sanguinalis* (L.) Scop., and *Echinochloa crus-galli* (L.) P. Beauv., respectively (Travlos et al., 2020).

Invasive weeds may release chemicals into the environment that suppress nearby plants trying to compete with them (Kato-Noguchi et al., 2014; Lorenzo et al., 2012). Phytochemicals are plant-based substances and non-nutritional secondary metabolites that are omnipresent in plants and can be beneficial to human health and reduce the risk of major chronic diseases (Mendoza & Silva, 2018). Phytochemicals can be found in many parts of plants, such as leaves, roots, and seeds, and have the potential as bioherbicides (Mushtaq et al., 2020). Seeds, leaves, and roots of *Cleome* plants have been used medicinally for several purposes, including as an antiscorbutic, stimulant, anthelmintic, vesicant, carminative, and rubefacient (Prabha et al., 2017; Singh et al., 2016). Some species of *Cleome* have the potential to serve as an alternative pesticide due to the presence of chemical pesticide components responsible for poisonous and insecticidal activities (Upadhyay, 2015). *Cleome*'s crude extracts were extremely poisonous to egg-masses of *Meloidogyne javanica* root-knot nematode (Krishnappa & Elumalai, 2013; Stephan et al., 2001). However, antioxidant activity was observed in methanolic (MeOH) extracts of leaves from five different *Cleome* species. *Cleome viscosa* L. had strong insecticidal action, suggesting it could replace pesticides

against *Spodoptera litura* (Lakshmanan et al., 2018; Mali, 2010). It has been previously reported that *C. rutidosperma* possesses antiplasmodial action (Bose et al., 2007). Many scientists have reported the healing effects of *C. rutidosperma*, but nowadays, the phytochemicals of *C. rutidosperma* can be used in environmentally friendly weed control. The objective of the present study is to identify the phytochemicals profiling using HPLC-ESI-QTOF-MS and evaluate the phytotoxic effect on *E. crus-galli*, *F. miliacea, O. sativa*, *L. chinensis*, and *C. iria*.

MATERIALS AND METHODS

Test Plants

This experiment used five different weed species as the control group: *E. crus-galli*, *F. miliacea*, *O. sativa*, *L. chinensis*, and *C. iria*. The weed seeds were taken from Farm 15, Faculty of Agriculture, Universiti Putra Malaysia.

Extraction

Previously, many scientists revealed the insecticidal and medicinal effects of *C. rutidosperma* (Prabha et al., 2017; Singh et al., 2016; Upadhyay, 2015), but little information is available regarding its herbicidal effect. Therefore, it was selected for use in this study to determine the herbicidal effect. *Cleome rutidosperma* was harvested at its most vegetative and matured stage in a natural environment of weed infestation from Universiti Putra Malaysia. The whole plant was harvested, hand-cleaned running water was used to eliminate dirt or debris, and ten were air-dried for three weeks. Then, the grinder was used to grind the gathered plant material to a powder. In a paraffin-wrapped conical flask, 100 g of *C. rutidosperma* were soaked in 1,000 ml of methanol (80%, Merck, Germany). The flask was agitated with an orbital shaker for 48 hr at room temperature (24–26°C). The solution was centrifuged for 1 hr at 1,107 ×*g* after being filtered using four layers of cheesecloth and then re-filtered using a 0.2-µm, 15-mm syringe filter (Phenex, non-sterile, luer/slip, LT Resources, Malaysia). The collected supernatant was evaporated using a rotary evaporator set to 40°C. The extraction percentage is calculated as follows:

$$\text{Extraction percentage} = \frac{\text{Extract weight (g)}}{\text{Powder weight (g)}} \times 100\%$$

For bioassay purposes, various concentrations of extracts were prepared by diluting the stock extracts with sterile distilled water. Before being used, all extracts were stored in the fridge at 4°C in the dark. The methanol extracts were obtained following the procedure described by Aslani et al. (2016).

HPLC-ESI-QTOF-MS Analysis

For HPLC-ESI-QTOF-MS analysis, the crude sample (20 mg) was diluted in 100% high-performance liquid chromatography (HPLC) Grade methanol (20 ml, Merck, Germany) and filtered through 0.2-µm, 15-mm syringe filters (Phenex, non-sterile, luer/slip, LT Resources, Malaysia). The chemical contents of the *C. rutidosperma* sample obtained from the methanol extract

were examined following the approach described in Tamsir et al. (2020), with a few minor changes. A dual electrospray ionization (ESI) source Agilent 6520 Accurate-Mass Q-TOF mass spectrometer (Germany) and an Agilent 1290 Infinity LC system (Germany) were used to analyze the chemical substances. To perform a more accurate analysis of the chemical profile, the settings of the mass spectrometry (MS), as well as the type of column used, were all subjected to optimization.

With the goal of achieving rapid and efficient separations at lower column pressures (Guiochon & Beaver, 2011), an ACQUITY UPLC BEH C18 column (150 mm × 2.1 mm × 3.5 m, Germany) was used and maintained at 50°C with a constant flow rate of 0.4 ml/min during the entire liquid chromatography (LC) run time of 26 min. In this study, a mobile phase was used for sample elution. It consisted of water liquid chromatography–mass spectrometry (LC-MS Grade) containing 0.1% formic acid (solvent A, Merck, Germany) and acetonitrile (LC-MS Grade, Merck, Germany) containing 0.1% formic acid (solvent B, Merck, Germany). The MS/MS investigations were conducted at 325°C with a drying gas flow of 10 L/min and a nebulizer pressure of 40 psi. Analysis of positive and negative ion modes at varying collision energy (CE) was performed to optimize signals and extract maximum structural information from ions in the mass range of 100 to 3,200 m/z, achieving the most sensitive ionization effect for analytes. MassHunter Qualitative Analysis software (version B.07.00) was used to process the data, and peaks were identified by comparing them with values from the literature and an online database (Abu Bakar et al., 2020).

Laboratory Bioassay

A bioassay was performed in a growth chamber at the Seed Technology Laboratory, Department of Crop Science, Universiti Putra Malaysia. The most consistent and healthy seeds were selected and soaked in potassium nitrate (KNO_3, Merck, Germany) at a concentration of 0.2%. They were soaked in water for 24 hr, then cleaned, and placed in an incubator (between 24 and 26°C) until a radicle measuring 1 mm in length appeared. Twenty-five seeds of *E. crus-galli*, *F. miliacea*, weedy rice, *L. chinensis*, and *C. iria* that had already sprouted were put in Petri dishes with two sheets of Whatman No. 1 filter paper. After that, 10 ml of methanol extracts from *C. rutidosperma* were applied to the filter paper in concentrations of 0 (distilled water), 2.5, 5, and 10%, respectively. The experiment was carried out using a completely random design with four replicates. The Petri dishes were placed in a growth chamber with fluorescent light (8,500 lux) at 30°C (day) and 20°C (night) on a 12-hr day, 12-hr night schedule. The relative humidity ranged between 30 and 50%. Due to the need to prevent anaerobic conditions and allow for gas exchange, the covers of the Petri dishes were not attached. At 7 days following treatment, the survival rate, hypocotyl, and radicle length were measured.

Glasshouse Experiment

Experimental Site, Treatments, and Design. The efficacy experiment was carried out between June and July of 2021 at the Faculty of Agriculture, Universiti Putra Malaysia, Selangor, Malaysia. Before being placed in germination trays, seeds were soaked in a solution of 0.2% KNO_3 (Merck, Germany) for 24 hr. One healthy, pre-germinated seedling was successfully transplanted into each soil-filled plastic pot (9 cm diameter). River sand, peat growth, and topsoil at a 3:2:1 ratio were used to prepare the soil of each pot. The weeds were treated with methanol extract of *C. rutidosperma* at three rates (2.5, 5, and 10%) and left untreated (control) when they reached the 2-3 leaf stage. A 1 L multipurpose sprayer (Deluxe pressure sprayer, Malaysia) was used to spray where the spray volume is 100 ml/m^2. The experiment was set up with a randomized complete block design (RCBD) and four replications.

Data Collection.

Plant Injury. Injury to plants was visually evaluated 21 days after spraying using a scale established by Burrill et al. (1976), where 0 indicates no effect (all foliage is still green and healthy), >70% indicates acceptable control, and 100% indicates complete kill (dead).

Photosynthetic Rate, Transpiration, and Stomatal Conductance. From 9 a.m. to 11 a.m., the LI-COR-6400XT Portable Photosynthetic System (USA) was used to measure photosynthetic rate, transpiration, and stomatal conductance. The observations were performed at a carbon dioxide (CO_2) flow rate of 400 µmol/m^2/s on the abaxial surface, with the saturating photosynthetic photon flux density (PPFD) set at 1,000 mmol/m^2/s.

Plant Height, Fresh and Dry Weight. At 21 days after spray (DAS), the height of all plant species was measured using a measuring tape from the top of the soil. At 21 DAS, weeds were picked 1 cm above the ground. The samples' fresh weight was measured using a digital balance, and dry weight was measured after drying them in an oven at 65°C for 72 hr. Weed control efficiency was determined using the following equation:

$$\text{Weed control efficiency (\%)} = \frac{\text{Dry weight of untreated pot} - \text{Dry weight of treated pot}}{\text{Dry weight of untreated pot}} \times 100\%$$

Statistical Analysis

A two-way analysis of variance (ANOVA) was carried out to determine any significant differences between each treatment and the control; the differences among the treatment's means were grouped using Tukey's test with a 0.05 probability level. Statistical analysis system (SAS, version 9.4, USA) software was used to conduct the analysis.

RESULTS

Identification of Phytotoxic Components in *C. rutidosperma*

The methanol extract of *C. rutidosperma* was analyzed and profiled by LC-MS analysis in positive and negative ionization modes to characterize chemical constituents qualitatively. To our knowledge, this is the first validated method for detecting active compounds in the whole plant of *C. rutidosperma* using LC-MS analysis. The results obtained from the LC-MS analysis allowed 64 and 10 proposed known compounds to use positive and negative ionization modes between 1 and 20 min, respectively (Table 1). There are six different phenolic compounds (anthranilic acid, quercitrin, irisolidone 7-O-glucuronide, 1,6-hexanediol dimethacrylate, auraptene, and ferujol), three alkaloids (indoline, quinoline, and indole-3-acrylic acid), four amino acids (thyroliberin N-ethylamide, hexadecasphinganine, 15-methylhexadecasphinganine, and eicosasphinganine) and some amines, benzofurans, terpenoid, and fatty acids were detected. Trichothecine ($C_{19}H_{24}O_5$), a terpene, was identified and exhibited the $[M+H]^+$ ion at 12.183 min with 332.1618 m/z. The $[M-H]^-$ ion at 448.0614 m/z was proposed at 2.834 retention time for glucobrassicin ($C_{16}H_{20}N_2O_9S_2$). Quercitrin ($C_{21}H_{20}O_{11}$), a well-known flavonoid, was exhibited at 7.24 min at 449.108 m/z. Our study found that in positive-ion mode, two indole-type alkaloids were tentatively named indoline (RT 2.626 with 119.0739 m/z) and indole-3-acrylic acid (RT 3.674 with 187.0632 m/z). Five amines (hercynine, phytosphingosine, dioctylnitrosamine, laurixamine, and sphinganine) were identified and all exhibited a $[M+H]^+$ ion. Hercynine was identified at 1.321 min, and its fragment ion is 197.1167 m/z.

Table 1
Chemical composition of a Cleome rutidosperma *methanol extract as determined by liquid chromatography–mass spectrometry*

Sl no	RT (min)	Determined compound	Molecular formula	Mass fragment (m/z)	Polarity	Error (ppm)
1	1.321	Hercynine	$C_9H_{15}N_3O_2$	197.1167	Positive	-1.3
2	1.44	Anthranilic acid	$C_7H_7NO_2$	137.0473	Positive	2.8
3	1.441	W-5 hydrochloride	$C_{16}H_{23}ClN_2O_2S$	342.1168	Negative	0.25
4	1.442	Pyroglutamic acid	$C_5H_7NO_3$	129.0422	Positive	2.92
5	1.445	3-Diazo-1-[(4-methylphenyl) sulfonylamino]-1-methylsulfonylurea	$C_9H_{11}N_5O_5S_2$	333.0202	Negative	-0.16
6	1.448	Diethadione	$C_8H_{13}NO_3$	171.0892	Positive	2
7	1.764	2-Coumaranone	C_8H_6O	118.0408	Positive	9.01
8	2.626	Indoline	C_8H_9N	119.0739	Positive	-3.65
9	2.834	Glucobrassicin	$C_{16}H_{20}N_2O_9S_2$	448.0614	Negative	-0.85
10	2.86	Quinoline	C_9H_7N	129.0578	Positive	0.1
11	3.674	Indole-3-acrylic acid	$C_{11}H_9NO_2$	187.0632	Positive	0.89

Table 1 (*continue*)

Sl no	RT (min)	Determined compound	Molecular formula	Mass fragment (m/z)	Polarity	Error (ppm)
12	3.675	Benzoylacetonitrile	C_9H_7NO	145.0528	Positive	-0.3
13	8.81	[4-[[N-(4-Acetyloxybutanoyl)-C-[4-(azidomethyl)piperidin-1-yl]carbonimidoyl]amino]-4-oxobutyl] acetate	$C_{19}H_{30}N_6O_6$	438.2232	Positive	-1.08
14	9.005	4-[4-(4-Azidophenyl)-6-morpholin-4-yl-1,3,5-triazin-2-yl]-N,N-dimethylpiperazine-1-carboxamide	$C_{20}H_{26}N_{10}O_2$	438.2234	Positive	1.49
15	9.286	Quercetin	$C_{15}H_{10}O_7$	302.0431	Positive	-1.38
16	9.288	Irisolidone 7-O-glucuronide	$C_{23}H_{22}O_{12}$	490.1115	Positive	-0.8
17	9.971	Biochanin A 7-(6-malonylglucoside) (Isoflavonoids)	$C_{25}H_{24}O_{13}$	532.122	Positive	0.71
18	10.51	2-Amino-7-methyl-3,7-dihydropyrrolo[3,2-d]pyrimidin-4-one;ethane;9-methylpurine-2,6-diamine	$C_{17}H_{28}N_{10}O$	388.2442	Positive	1.34
19	11.271	1,6-Hexanediol dimethacrylate	$C_{14}H_{22}O_4$	254.1524	Positive	-2.18
20	11.659	Eudesmin	$C_{22}H_{26}O_6$	386.173	Positive	-0.29
21	11.739	2-[2-Amino-4-[2-(methylideneamino) ethyl]pyrimidin-5-yl]-9-(cyclopropylmethyl)-6-morpholin-4-ylpurin-8-amine	$C_{20}H_{26}N_{10}O$	422.2285	Positive	1.51
22	11.856	Thyroliberin N-ethylamide	$C_{18}H_{26}N_6O_4$	390.2012	Positive	0.94
23	11.998	Hexadecasphinganine	$C_{16}H_{35}NO_2$	273.2665	Positive	1.14
24	12.037	Phytosphingosine	$C_{18}H_{39}NO_3$	317.2931	Positive	-0.41
25	12.179	Dihydroxyethyl lauramine oxide	$C_{16}H_{35}NO_3$	289.2618	Positive	-0.45
26	12.183	Trichothecine	$C_{19}H_{24}O_5$	332.1618	Positive	1.66
27	12.195	Dodecyldimethylamine oxide	$C_{14}H_{31}NO$	229.2407	Positive	-0.46
28	12.29	Kobusone	$C_{14}H_{22}O_2$	222.1611	Negative	4.16
29	12.315	Dioctylnitrosamine	$C_{16}H_{34}N_2O$	270.2669	Positive	0.61
30	12.321	15-Methylhexadecasphinganine	$C_{17}H_{37}NO_2$	287.2829	Positive	-1.75
31	12.328	N(3)-Benzylthymidine	$C_{17}H_{20}N_2O_5$	332.1359	Positive	4.05
32	12.344	Dodecylacrylamide	$C_{15}H_{29}NO$	239.2249	Positive	0.13
33	12.349	Tetrabutylurea	$C_{17}H_{36}N_2O$	284.2832	Positive	-1.54
34	12.355	Lauryl aminopropylglycine	$C_{17}H_{36}N_2O_2$	300.2783	Positive	-2.15
35	12.553	Laurixamine	$C_{15}H_{33}NO$	243.2561	Positive	0.39
36	12.701	Aminopregnane	$C_{21}H_{37}N$	303.2937	Positive	-3.62
37	13.067	s-Triazine, 2-amino-4-(morpholinomethyl)-6-piperidino-	$C_{13}H_{22}N_6O$	278.1863	Positive	-2.81

Table 1 (*continue*)

Sl no	RT (min)	Determined compound	Molecular formula	Mass fragment (m/z)	Polarity	Error (ppm)
38	13.157	4-Methyl-6-{[3-(Piperidin-4-Ylmethoxy)phenoxy]methyl} yridine-2-Amine	$C_{19}H_{25}N_3O_2$	327.1948	Negative	-0.37
39	13.213	Auraptene	$C_{19}H_{22}O_3$	298.1558	Positive	3.76
40	13.239	6-(Cyclopentylamino)-2-[(3-hydroxypropyl)amino]-9-isopropylpurine	$C_{16}H_{26}N_6O$	318.2175	Positive	-2.03
41	13.273	Sphinganine	$C_{18}H_{39}NO_2$	301.2989	Positive	-2.81
42	13.313	Calanone	$C_{27}H_{20}O_5$	424.1308	Positive	0.56
43	13.315	Ferujol	$C_{19}H_{24}O_4$	316.1663	Positive	3.72
44	13.337	Estriol	$C_{18}H_{24}O_3$	288.1741	Positive	-5.47
45	13.35	Kinetensin 1-3	$C_{15}H_{30}N_6O_4$	358.2327	Positive	0.34
46	13.701	Olomoucine	$C_{15}H_{18}N_6O$	298.1551	Positive	-3.08
47	13.71	Stearic acid hydrazide	$C_{18}H_{38}N_2O$	298.2982	Positive	0.71
48	13.733	Hexadecyl isocyanate	$C_{17}H_{33}NO$	267.2561	Positive	0.3
49	13.754	Myristamidopropylamine oxide	$C_{19}H_{40}N_2O_2$	328.3103	Positive	-4.15
50	13.81	N',N'-Bis(carbamoyl) ethylenediamine-N,N-diacetic acid	$C_{10}H_{26}N_6O_6$	326.1915	Negative	-0.27
51	13.95	4-Dodecylbenzenesulfonic acid	$C_{18}H_{30}O_3S$	326.1914	Negative	0.61
52	14.045	Decylcarnitine	$C_{17}H_{35}NO_3$	301.2625	Positive	-2.73
53	14.179	Piptamine	$C_{23}H_{41}N$	331.3244	Positive	-1.41
54	14.271	Angoletin	$C_{18}H_{20}O_4$	300.1348	Positive	4.48
55	15.044	Rubrenolide	$C_{17}H_{30}O_4$	298.2135	Positive	0.2
56	15.195	Dodecanamide	$C_{12}H_{25}NO$	199.194	Positive	-2.15
57	15.403	Eicosasphinganine	$C_{20}H_{43}NO_2$	329.3301	Positive	-2.16
58	15.642	2-Decoxysulfanyl-7H-purine	$C_{15}H_{24}N_4OS$	308.1683	Positive	-4.05
59	15.644	[1-(2-Aminoethyl)triazol-4-yl]-(4-cyclopentylpiperazin-1-yl) methanone	$C_{14}H_{24}N_6O$	292.2017	Positive	-1.79
60	15.896	Nitrosostromelin	$C_{15}H_{32}N_2O_5$	320.231	Positive	0.49
61	16.528	10-Oxo-13-hydroxy-11-octadecenoic acid	$C_{18}H_{32}O_4$	312.2305	Positive	-1.32
62	16.825	Dodecylsuccinic anhydride	$C_{16}H_{28}O_3$	268.2043	Positive	-1.79
63	16.931	Lauryl sulfate	$C_{12}H_{26}O_4S$	266.1551	Negative	0.12
64	17.00	1-Azido-2-tridecylpyrrole	$C_{17}H_{30}N_4$	290.2475	Positive	-1.52
65	17.125	Lagochilin	$C_{20}H_{36}O_5$	356.2554	Positive	2.56
66	17.262	3-[2-(Dimethylamino)propyl]-1-({4-[(1H-1,2,4-triazol-1-yl) methyl]phenyl}methyl)urea	$C_{16}H_{24}N_6O$	316.2004	Positive	2.56
67	19.04	Acridorex	$C_{24}H_{24}N_2$	340.1936	Positive	1.14
68	19.224	1,2-Dinaphthalen-1-ylhydrazine	$C_{20}H_{16}N_2$	284.1299	Positive	5.04

Table 1 (*continue*)

Sl no	RT (min)	Determined compound	Molecular formula	Mass fragment (m/z)	Polarity	Error (ppm)
69	19.224	Cyclododecanone tritylhydrazone	$C_{31}H_{38}N_2$	438.3025	Positive	2.34
70	19.589	Methyl dodecylbenzenesulphonate	$C_{19}H_{32}O_3S$	340.2072	Negative	0.18
71	19.852	2,2-Bis(azidomethyl)-3-decoxypropan-1-ol	$C_{15}H_{30}N_6O_2$	326.2435	Positive	-1.33
72	19.935	Stearyldiethanolamine	$C_{22}H_{47}NO_2$	357.3612	Positive	-1.55
73	20.292	Hexadecanamide	$C_{16}H_{33}NO$	255.263	Positive	-0.14
74	20.54	Benzenesulfonic acid, undecyl-	$C_{17}H_{28}O_3S$	312.1758	Negative	0.5

Note. RT = Retention time

The Survival Rate and Initial Growth of Weed Seeds

Cleome rutidosperma extract was found to have a notable effect on the survival rate, hypocotyl, and radicle length of the examined weed species (Table 2). The inhibitory magnitude of all species was enhanced by increasing the extract concentration from 2.5

Table 2
Effect of Cleome rutidosperma *on seed survival, hypocotyl length and root length of test weeds*

Test species	Dose (%)	Survival rate (%)	Hypocotyl length (cm)	Root length (cm)
Weedy rice	0	100.00a	5.01a	2.29a
	2.5	32.00b	1.02b	0.44b
	5	14.00c	0.77c	0.25c
	10	0.00d	0.00d	0.00d
Cyperus iria	0	100.00a	1.70a	1.57a
	2.5	31.00b	0.72b	0.44b
	5	10.00c	0.45c	0.27c
	10	0.00d	0.00c	0.00d
Fimbristylis miliacea	0	100.00a	2.07a	2.50a
	2.5	42.00b	0.88b	0.88b
	5	18.00c	0.51c	0.41c
	10	1.00d	0.00d	0.00d
Leptochloa chinensis	0	100.00a	1.80a	3.65a
	2.5	61.00b	0.86b	0.87b
	5	26.00c	0.45c	0.23c
	10	0.00d	0.00d	0.00c
Echinochloa crus-galli	0	100.00a	3.61a	6.47a
	2.5	60.00b	2.07b	3.11b
	5	32.00c	1.38c	2.09c
	10	12.00d	0.82d	0.65d

Note. Mean values sharing similar letters for each weed species in the column are considered not significant at $p<0.05$

to 10% in a concentration-response bioassay. Weedy rice, *C. iria*, and *L. chinensis* did not survive at 10%. Meanwhile, weed survival was significantly reduced by different extract concentrations of *C. rutidosperma*. The extracts were more effective against *C. iria* and weedy rice than against *F. miliacea*, *L. chinensis*, and *E. crus-galli*.

The hypocotyls of the selected weeds were considerably reduced ($p<0.05$) by *C. rutidosperma* methanol extract. The hypocotyl growth of weedy rice, *F. miliacea*, *C. iria*, *L. chinensis*, and *E. crus-galli* was reduced by 84.57%, 75.44%, 71.71%, 74.76%, and 61.80% when treated with 5% of *C. rutidosperma* extract. No hypocotyl growth was recorded at the highest concentration (10%) for weedy rice, *F. miliacea*, *C. iria*, and *L. chinensis*. All tested species showed a decrease in root elongation by *C. rutidosperma*. The radicle growth inhibition ranged by 80%–100%, 72%–100%, 64%–100%, 76%–100%, and 51%–90% for weedy rice, *F. miliacea C. iria*, *L. chinensis*, and *E. crus-galli*, respectively. As a result, weedy rice showed the greatest degree of inhibition among the species examined.

The Effect of *C. rutidosperma* on the Growth and Physiology of Weeds

Table 3 represents the effect of *C. rutidosperma* methanol extract on the growth parameter of the tested plants. Additionally, a dose-dependent inhibition effect was identified. The efficacy of *C. rutidosperma* methanol extract on weedy rice, *F. miliacea C. iria*, *L. chinensis*, and *E. crus-galli* was assessed visually. At the highest concentration (10%), *C. rutidosperma* efficacy was significantly higher in all tested species. There was a statistically significant ($p<0.05$) decrease in photosynthesis, stomatal conductance, and transpiration rate when compared to the untreated (control) condition across all species. A higher dose of *C. rutidosperma* (10%) showed a 49.76% photosynthesis reduction in weedy rice, 70.56% in *C. iria*, 31.82% in *F. miliacea*, 57.95% in *L. chinensis*, and 64.72% in *E. crus-galli*. The methanol extract of *C. rutidosperma* inhibited the stomatal conductance of more than 50% for all tested weeds except weedy rice (43.59%) and *F. miliacea* (27.57%). All the weeds evaluated showed a dose-dependent response to *C. rutidosperma* extract on their transpiration rate, and this effect was statistically significant ($p<0.05$). However, transpiration rate reduction varied among the tested species. At a lower concentration of *C. rutidosperma* (2.5%), *C. iria* had the highest reduction in transpiration rate at 73.27%, followed by *E. crus-galli* at 69.57%, weedy rice at 60.08%, *L. chinensis* at 58.36%, and *F. miliacea* at 36.09%.

Each weed studied had a unique response to the methanol extract of *C. rutidosperma* on its plant height. However, the highest plant height was observed in untreated (control). *Cleome rutidosperma* extract reduced plant height from 7.72% to 31.04% in weedy rice, 15.50% to 44.56% in *C. iria*, 1.52% to 18.35% in *F. miliacea*, 7.16% to 37.36% in *L. chinensis*, and

Table 3
Effect of Cleome rutidosperma *on the growth and physiological parameters of weeds*

Test plants	Dose (%)	Injury scale	Photosynthesis (μmol/m²/s)	Stomatal conductance (mol/m²/s)	Transpiration (mmol/m²/s)	Plant height (cm)	Fresh weight (g)	Dry weight (g)
Weedy rice	0	1.00d	47.40a	0.59a	15.29a	77.50a	31.93a	1.44a
	2.5	2.00c	40.61b	0.51b	12.63b	71.50b	27.79b	1.17b
	5	3.25b	34.60c	0.45c	10.66c	68.00b	24.70c	1.00c
	10	4.75a	23.79d	0.33d	6.09d	53.42c	18.64d	0.74d
Cyperus iria	0	1.00c	41.21a	0.42a	12.30a	61.00a	40.20a	2.14a
	2.5	2.50b	33.71b	0.35b	9.15b	51.50b	31.03b	1.68ab
	5	3.50b	24.53c	0.28c	6.19c	43.37c	25.26c	1.22bc
	10	5.50a	12.13d	0.18d	3.29d	33.75d	19.67d	0.76c
Fimbristylis miliacea	0	1.00c	36.48a	0.36a	10.66a	72.45a	36.67a	0.83a
	2.5	1.50bc	32.56b	0.33b	9.16ab	71.32a	35.10b	0.78a
	5	2.50b	28.47c	0.30c	8.02bc	66.82ab	32.10c	0.70b
	10	3.75a	24.87d	0.26d	6.76c	59.20b	28.04d	0.57c
Leptochloa chinensis	0	1.00c	39.16a	0.45a	11.70a	92.00a	50.63a	2.08a
	2.5	2.75b	33.66b	0.39b	9.94b	85.42a	45.27b	1.79b
	5	3.50b	24.80c	0.31c	7.11c	74.19b	39.58c	1.50c
	10	5.50a	16.46d	0.21d	4.85d	57.58c	29.83d	0.98d
Echinochloa crus-galli	0	1.00d	43.82a	0.51a	13.20a	39.03a	30.04a	0.85a
	2.5	3.50c	33.22b	0.41b	9.36b	33.78ab	25.46b	0.67b
	5	5.00b	22.47c	0.27c	6.11c	28.65b	19.20c	0.51c
	10	6.50a	15.46d	0.20d	4.02d	22.47c	14.05d	0.30d

Note. Mean values sharing similar letters for each weed species in the column are considered not significant at $p<0.05$

13.42% to 42.37% in *E. crus-galli* compared to untreated (control). The dry weight of the examined weeds was also decreased with an increase in *C. rutidosperma* concentration.

The methanol extract of *C. rutidosperma* resulted in a significant ($p<0.05$) reduction in the fresh and dry weights of all the evaluated species. Fresh weight loss was most pronounced in *C. iria* (37.17%) after being treated with a 5% solution of a *C. rutidosperma* extract, followed by *E. crus-galli* (36.01%), weedy rice (22.61%), *L. chinensis* (21.83%), and *F. miliacea* (12.46%). The foliar application of *C. rutidosperma* at the higher dose (10%) reduced the dry weight by 48.40% in weedy rice, 64.13% in *C. iria*, 30.89% in *F. miliacea*, 52.76% in *L. chinensis*, and 64.20% in *E. crus-galli*.

Weed Control Efficacy of *C. rutidosperma*

The efficiency of weed control was considerably ($p<0.05$) impacted by *C. rutidosperma* methanol extract (Figure 1). However, the control efficacy was

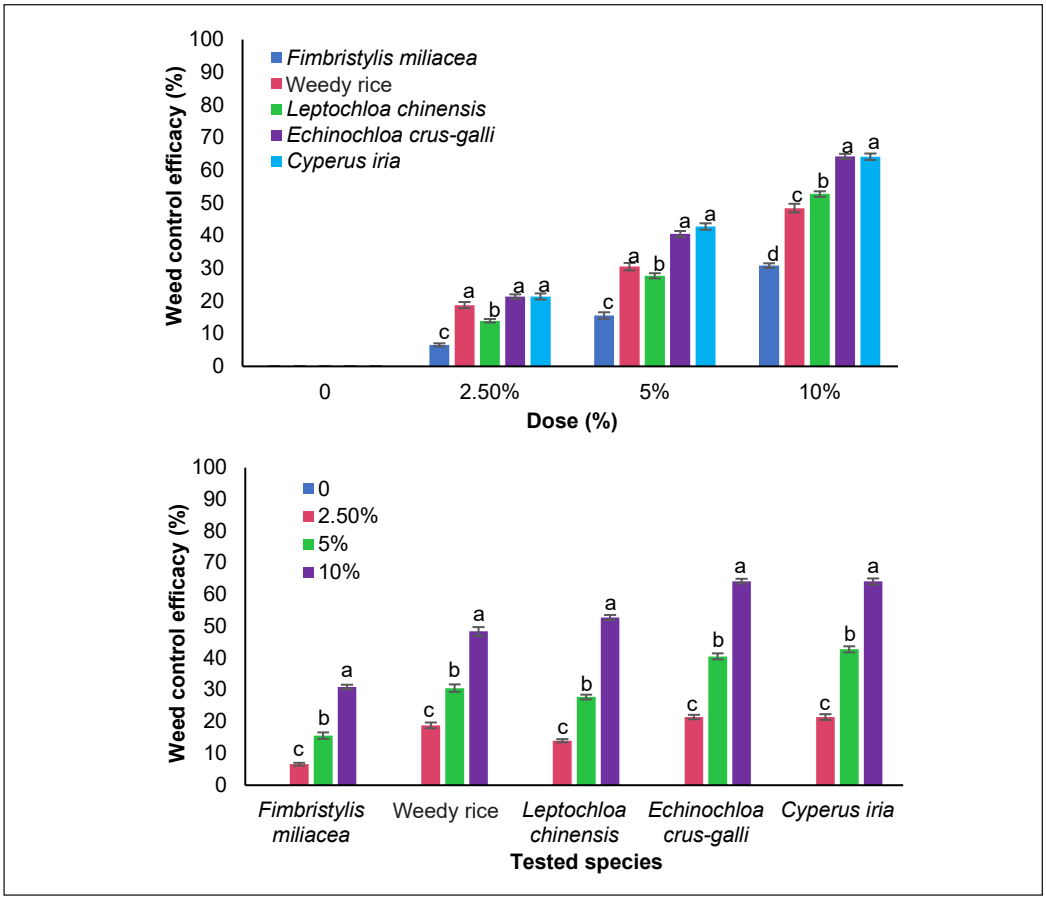

Figure 1. Weed control efficacy of *Cleome rutidosperma*. Mean values sharing similar letters are considered not significant at $p<0.05$

measured at 21 DAS and varied among the *C. rutidosperma* application rates compared to the control (untreated). The efficacy of *C. rutidosperma* was highest in *C. iria*, ranging from 21.41% to 64.13%, while *F. miliacea* showed the lowest inhibition, ranging from 6.59% to 30.89% compared to untreated (control). The highest application rate of *C. rutidosperma* (10%) showed the highest weed control efficacy for *E. crus-galli*, 64.20%, followed by 64.13%, 52.76%, 48.40%, and 30.89% for *C. iria*, *L. chinensis*, weedy rice, and *F. miliacea*, respectively. Overall, 5% and 10% application rates exhibited excellent efficacy compared to 2.5% for all tested weed species.

DISCUSSION

Weed management using agrochemicals in agricultural systems has increased dramatically in recent years. The increased public interest in safe "green" herbicides has resulted in the development of several new bioherbicides for weed management. For example, *C. rutidosperma*, a plant-based

bioherbicide, showed a promising weed control efficacy.

Our study detected important fatty acids, indole, amines, amino acids, flavonoids, terpenes, coumarins, carboxylic acids, benzoic acids, benzofuran, and several unknown compounds. Quercetin is a flavonoid of *C. rutidosperma,* which inhibits the shoot growth of *Arabidopsis thaliana* (Weston et al., 2013). Several modes of action of exogenously applied flavonoids on plants have been demonstrated by scientific research. Changes in membrane permeability and inhibition of plant nutrient absorption, suppression of cell division, elongation, and submicroscopic structure, effects on photosynthesis and respiration of the plant, impact on photosynthesis and respiration, enzymatic functions and activities, hormone and protein synthesis, and ATP generation (Shah & Smith, 2020).

Quinoline, indoline, and indole-3-acrylic acid are identified alkaloids of *C. rutidosperma*. Quinoline inhibited the growth of aquatic duckweed and reduced cell division in nion (Shang et al., 2018). Alkaloids caused strong inhibition of coleoptile development and full suppression of protein synthesis, exhibiting antimitotic activity (Hu et al., 2015). Five amines (hercynine, phytosphingosine, dioctylnitrosamine, laurixamine and sphinganine) were detected from *C. rutidosperma*. Phytosphingosine (amines), also found in wheat root exudates, inhibited *Fusarium oxysporum* f. sp. *niveum* (Fusarium wilt of watermelon) (C. Li et al., 2020). Like many other terpenes, trichothecine has allelopathic effects on the seed germination of *A. thaliana* (Malmierca et al., 2015). Terpenes inhibited weed germination and respiratory metabolism. Their strong phytotoxic effects suggest they could be used as a main basis for developing bioherbicides (Z. Li et al., 2019).

Auraptene, a coumarin, was identified in *C. rutidosperma,* which displayed allelopathic effects and stunted seed germination, shoot, and root growth of lettuce (Razavi, 2010). Coumarin decreased gibberellic acid 3 in the hormone system, reducing amylase activity and starch consumption during germination. In addition, coumarin caused oxidative stress by reducing catalase activity, which manifested as an increase in the production of reactive oxygen species such as hydrogen peroxide and malondialdehyde (Yang et al., 2023).

Allelochemicals are not normally released into the environment as a single substance, and the amount of allelochemicals that get released varies depending on the situation. When studying their allelopathic potential, it is important to consider the variety and quantity of allelochemicals produced by plants. Although some allelochemicals may not exhibit allelopathic activity when used alone, they may enhance the allelopathy of other allelochemicals in specific conditions (Cheng & Cheng, 2015). Synergy, antagonism, and additive effects are only some of the interactions between different allelochemicals that need to be explored. The synergistic effects of multiple polyphenol allelochemicals against *Microcystis aeruginosa* were stronger than

those of a single polyphenol therapy, as Huang et al. (2020) reported.

Cleome rutidosperma methanol extract has modest efficiency against weeds by producing severe damage. Injury symptoms such as chlorosis, stunted development, and burn-down, all of which eventually led to death, were visible. In addition, *C. rutidosperma*, at a higher rate, exhibited mild to moderate damage symptoms. The effectiveness of *C. rutidosperma* increased as the rate of application increased. Similarly, increasing the extract concentration of *Parthenium hysterophorus L.*, *Borreria alata* (Aubl.) DC., and *Cleome rutidosperma* DC. showed excellent efficacy on *Ageratum conyzoides* and *Euphorbia hirta* L. (Motmainna et al., 2021c).

At 10% growth reduction of inhibition of *E. crus-galli*, *C. iria*, *L. chinensis*, weedy rice, and *F. miliacea* was measured at 64.20%, 64.13%, 52.76%, 48.40%, and 30.89%, respectively. Growth reduction occurred due to *C. rutidosperma* methanol extract stress. It is a result of damage to the leaves, specifically necrosis, leaf fire, and wrinkled leaves, all of which inhibit photosynthesis and hinder plant development. The present study agrees with Hasan, Mokhtar, et al. (2021) that foliar application of wild tomato plant extract bioherbicide WeedLock at high concentration hindered the morphological characters of *Euphorbia hirta* L., *A. conyzoides*, *Axonopus compressus* (Sw.) P. Beauv, *F. miliacea* (L.) Vahl, *Eleusine indica* (L.) Gaertn., *C. iria*, *Abelmoschus esculentus* (L.) Moench, *Zea mays* L., *Amaranthus gangeticus* L., and *O. sativa* L.

In our study, the highest reduction in photosynthesis rate was observed when *C. rutidosperma* was applied to *C. iria*. Oxidative stress increased intracellular reactive oxygen species (ROS) production, damaged macromolecules, and reduced plant defense levels, all resulting from a decline in photosynthesis (Hasan et al., 2022; Motmainna, Juraimi, Uddin, Asib, Islam, Ahmad-Hamdani, Berahim, et al., 2021). Photosynthesis is the principal cause of oxidative stress, which considerably impacts plant growth under stressful environmental conditions. Applying *C. rutidosperma* extract reduced the stomatal conductance in the weeds significantly. The stomatal mechanism is a crucial property of plants that minimizes water loss, affecting gas exchanges. Our research demonstrates that methanol extract of *C. rutidosperma* significantly impacted the transpiration rate of tested weeds. Reduced photosynthesis and transpiration rate, regulated by stomatal conductance, are an undeniable result of stress. As the stomata of a plant open, water is lost by evaporation via transpiration, and CO_2 is absorbed through photosynthesis (Motmainna, Juraimi, Uddin, Asib, Islam, Ahmad-Hamdani, Berahim, et al., 2021). The result demonstrated in our study was similar to Hasan et al. (2022), who found that WeedLock (a plant-based bioherbicide) inhibited the photosynthetic mechanism in *E. indica* (L.) Gaertn, *A. conyzoides* L., *A. gangeticus* L, and *Z. mays* L.

CONCLUSION

The current research confirms the herbicidal potential of *C. rutidosperma* extract and shows that it can prevent the germination and growth of test weeds. The *C. rutidosperma* extract was also found to have 74 compounds. Some of these compounds are toxic in different studies. Because of its high potency and selectivity, this weed might be classified as a natural weed control product. This study will promote research toward sustainable weed management programs, especially in the fields of rice and plantation, that could reduce weed infestation and competition over time and less dependence on chemical herbicides.

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The Role of Floral Morphology and Epidermal Outgrowths in *Etlingera elatior* (Jack) R. M. Smith (Zingiberaceae) True Flower

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ABSTRACT

The inflorescence of *Etlingera elatior* forms tubular, true flowers in an acropetal sequence that blooms ostentatiously. The aromatic plant also produces excellent flavour and fragrance afforded by the presence of secretory structures capable of accumulating, storing, and releasing volatile compounds. However, there is a lack of botanical description of the flowering morphology and the type of secretory structures involved in plant-pollinator interactions. This study aims to describe the floral morphology, characterise the diverse micromorphology of the epidermal outgrowths in the true flower, and analyse their histochemical contents by scanning electron and light microscopes. Labellum defines the anthesis stage of the true flower: unveiling by petals at anthesis and furling inwards that closes the top part of the flower at post-anthesis. In addition to the floral advertisement accessory function demonstrated by the labellum, the closure provides additional exaptation of post-pollination protection to the flower. Both glandular trichomes and osmophores are involved in biochemical functions that release heterogeneous substances (mucilage, terpenes, and phenolic compounds) to help secure anthesis. Non-glandular trichomes, on the other hand, are structurally involved in the floral development by providing physical and mechanical protection to the flower by acting as glue to maintain the closed structure of the flower, connecting floral accessories, and forming a formidable barrier surrounding the ovary, the most important reproductive organ of the flower. Findings from the present study demonstrate that the presence of secretory structures coordinated with the flower's functional traits enhances the pollination mechanism. It is the maiden report for *E. elatior* on

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epidermal outgrowths and their participation in floral structure and development besides plant-pollinator interaction.

Keywords: Capitate glandular trichomes, corolla tube, epigynous glands, labellum, non-glandular trichomes, osmophores

INTRODUCTION

Flowers are sophisticated, primary organs demonstrating luxuriant display with specialised functions to ensure plant fitness (de Santiago-Hernández et al., 2019). The form and function of flowers often correlate to plant-pollinator interactions designed to maximise the chances of reproductive success (van der Kooi et al., 2021). Floral rewards and secretory structures such as nectary glands, osmophores, and trichomes are often enlisted as accessories to secure pollination. Their presence is associated with olfactory cues that, along with a visual display by the floral ornaments, aim to entice pollinators into visiting the flowers.

Trichomes are epidermal outgrowths on plant surfaces that act as the primary line of defence by providing chemical, mechanical, and physical protection against ecological adversities (Wang et al., 2021). Trichomes are composed of single-cell or multicellular structures. These structures are divided into two general categories: glandular (presence of glandular head) or non-glandular (absence of glandular head), depending on their morphology and secretion ability. Glandular trichomes are the metabolically active type of secretory structures capable of synthesising olfactory or gustatory-repellent compounds (Chalvin et al., 2020). The significance of glandular trichomes is often highlighted as a natural factory of compounds with high biotechnological interest, such as artemisinin, the antimalarial drug produced in *Artemisia annua* (Huchelmann et al., 2017). The non-glandular type of trichomes, on the other hand, usually protect the plant against excessive light, water loss and extreme temperature by decreasing light absorbance and trapping layers of air that conserve heat and moisture (Karabourniotis et al., 2019; Lusa et al., 2015).

In flowers, trichomes are not only responsible for releasing volatiles with distinctive secondary metabolites to help secure anthesis by glistening the floral surface to appeal to pollinators and facilitate pollen capture and redistribution (Tissier et al., 2017), but they are also structurally important in maintaining floral shape (Tan et al., 2016). According to Tan et al. (2016), trichomes act as glue, especially in young petals, that ultimately help shape the floral bud of cotton (*Gossypium hirsutum*). Reports of trichomes participating in the connection of floral organs are not new; in fact, El Ottra et al. (2013) described the terms 'coherent' and 'adherent' for trichomes involved in joining the same whorl and different whorls of organs, respectively. Furthermore, the description of trichomes also provides characters for phylogenetic and comparative morphological studies.

Etlingera elatior is a perennial, herbaceous plant indigenous to Indonesia and Malaysia. The plant consists of a leafy and inflorescence shoot system emerging from the rhizome. The leafy shoot can

grow up to ≈ 3-4 m in height, whereas the inflorescence shoot can reach up to 1.5 m (Choon & Ding, 2017a). The inflorescence of *E. elatior* is a raceme with 90 to 120 flowers (Choon & Ding, 2016) initiated in an acropetal sequence. *E. elatior* is one of the most popular and most studied species in the family of Zingiberaceae, especially in phytochemistry (Juwita et al., 2018; Sungthong & Srichaikul, 2018). Ornamentally, the perennial plant demonstrates distinct inflorescence borne on a peduncle that blooms ostentatiously like a torch, hence its common name, torch ginger (Choon et al., 2016). The aromatic plant also produces excellent flavouring and fragrance that has attracted scientific communities to investigate the chemical composition of its essential oil (Anzian et al., 2020; Bezerra-Silva et al., 2016; Sangthong et al., 2022). However, despite a great deal of attention on the chemical aspects of the plant, there is a lack of botanical characterisation of the floral morphology and their epidermal outgrowths. More importantly, the type of secretory structures responsible for the floral volatiles in torch ginger has yet to be reported, undermining the plant's potential as a source of pharmacological feedstock. Thus, this study was undertaken to provide data on the role of floral morphology and epidermal outgrowths in the true flower of *E. elatior*.

MATERIALS AND METHOD

Plant Materials

The true flowers of *Etlingera elatior* were collected randomly from Field 2, Faculty of Agriculture, Universiti Putra Malaysia (3° 00'28' N, 101° 42'10' E). True flowers at pre-, during, and post-anthesis were removed from inflorescence and dissected for examination using a digital colour camera Leica DFC310 FX (Leica Microsystems, Germany) equipped with Leica software application suite (LAS, version 3.8) (Leica Microsystems, 2013).

Surface Analysis

The true flowers were subjected to surface analysis using a scanning electron microscope (SEM). Samples were fixed in FAA (10% formaldehyde, 5% acetic acid, 50% ethanol, which were purchased from Sigma Aldrich®, Germany) for 24 hr and placed in a vacuum pump to remove air from tissue (Choon & Ding, 2017b). Samples were rinsed thoroughly with distilled water and then post-fixed in 1% osmium tetraoxide (Sigma Aldrich®, Germany) for 24 hr. After a series of dehydration in graded ethanol (50, 60, 70, 80, 90, and 100%, Sigma Aldrich®, Germany), samples were subjected to critical point drying (Leica EM CPD030, Germany) and then sputter coated (Baltec SC030, Switzerland) with gold. Observations were carried out using JEOL JSM-5610V SEM (JEOL Ltd., Japan) at an accelerating voltage of 15 kV.

Histochemistry Analysis

The chemical content in the secretory structures was investigated in fresh hand-section materials using the following histochemical tests: Nadi reagent (Sigma Aldrich®, Germany) for essential oils

and terpenes (Caissard et al., 2004), ruthenium red (Sigma Aldrich®, Germany) for mucilage and pectin (Johansen, 1940), Sudan IV (Sigma Aldrich®, Germany) for lipids (Jensen, 1962), and toluidine blue O (Sigma Aldrich®, Germany) for phenolic compounds (Uzelac et al., 2015). Distilled water and lipid removal solution of methanol (Sigma Aldrich®, Germany), chloroform (Sigma Aldrich®, Germany), water, and chloride acid (Sigma Aldrich®, Germany) mixture (66:33:4:1) (Machado et al., 2006) were performed as blank and negative control procedure, respectively. All sections were mounted on a glass slide with a cover slip and then examined with a light microscope (Meiji Techno, Japan) equipped with a digital single-lens reflex (DSLR) camera (Olympus E-420, Japan).

Quantitative Measurements and Analyses

Digitally recorded micrographs were subjected to quantitative measurements and estimation using Image J software (National Institute of Health, Bethesda, USA). The morphological characteristics of the branched, non-glandular trichomes were measured according to Zhang et al. (2005). The secretory structure distribution was quantified in a 10 mm^2 area and then averaged from ten samples of each plant part and developmental stages. In addition, the dimensions of stalk length, basal cell, and head diameter; shortest, second, and longest branch length of trichomes were measured and expressed in mean ± standard deviation.

RESULTS

Floral Morphology

The true flowers of *E. elatior* are distended by floral bracts (Figure 1a). The floral symmetry of a true flower exhibits zygomorphic (Figure 1b)—a floral accessory comprised of a tubular bracteole with a deep slit on one side. The perianth consisted of a calyx of three-toothed dentate with the apex pubescent and three petals of tubular or oblong shaped with entire margin (Figures 1c–e). The anthesis stage of the *E. elatior* true flower is indicated by the emergence of the labellum, where the pink-coloured petals conceal the labellum at the pre-anthesis stage. During anthesis, deep red coloured with yellow margin labellum emerged and then wilted at post-anthesis by the end of the day, enclosing the top part of the flower (Figure 1f). The pistil is comprised of a long, pubescent style with the upper part wrapped by an anther crest, and the stigma is simple, capitate shaped and is positioned on top of the other (Figure 1g). The stamen of the true flower comprised an anther and a short, arching filament (Figure 1h). The anther is adnate, extrorse, and pubescent around the opening, with the tetrasporangiate pollen dehisced longitudinally prior to anthesis (Figure 1i)—the labellum and the short filament connate to an elongated tube that extends to median-superior of the ovary. The corolla tube of *E. elatior* true flower is reflexed. The lower part of the true flower is comprised of an epigynous gland wrapping the basal style and sits above the closure of the ovary (Figure 1j). The ovary placentation of *E. elatior*'s true flower is the trilocular axile.

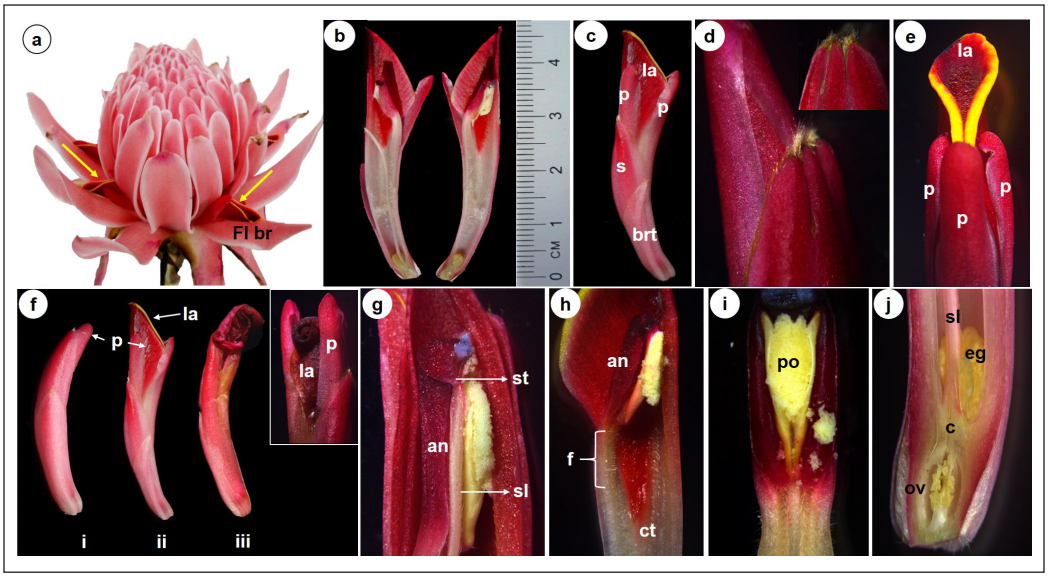

Figure 1. (a) Inflorescence of *Etlingera elatior* at full bloom stage indicated by the emergence of red-coloured true flower (arrow) distended by a floral bract (fl br); (b) Longitudinal cut of true flower showing the floral symmetry as zygomorphic; (c) Lateral view of the whitish pink colour of *E. elatior* true flower comprising a one-sided, deep slit bracteole (brt), sepal (s), and two of the three petals (p); (d) Three-toothed dentate and hairy apex of the sepal; (e) Ventral view of the folded, deep red, and yellow margin labellum (la) surrounded by three petals; (f) True flower at three anthesis stages indicated by the emergence of the labellum, where (i) at pre-anthesis, labellum is concealed by petal, (ii) petals unfurl and labellum emerged during anthesis, and (iii) at post-anthesis, labellum wilted, and enclosed the top part of flower (inset); (g) Lateral view of the upper part of the true flower. Stigma (st) positioned on top of anther (an) that embraces the long style (sl); (h) Arching stamen with short filament (f) and anther is adnate and extrorse. Labellum is connate to the basal filament (fi) that elongates to form corolla tube (ct); (i) Ventral view of the anther with tetrasporangiate dehisced longitudinally; (j) Lower part of the true flower. Epigynous gland (e.g.) surrounding the basal style that sits above the closure (c) of ovary (ov)

Epidermal Outgrowths

Trichomes and osmophores were the epidermal outgrowths of *E. elatior* true flower revealed in this study. There are three floral trichomes in the true flowers of *E. elatior*, i.e., capitate glandular, unbranched, and branched non-glandular.

Capitate Glandular Trichomes. The basal anther and filament of *E. elatior* true flower is covered by a dense capitate type of glandular trichomes (Figure 2a) constituted by basal cell, unicellular stalk, and glandular head. Two morphotypes are observed as characterised by the stalk length: short and long, undulating stalk (Figure 2b). Short- and long-stalked capitate glandular stalk lengths were 43.1 and 104.0 µm, respectively (Table 1). Short-stalked capitate glandular basal cell and head diameters were 19.0 and 21.9 µm, respectively (Table 1). At the same time, the basal cell diameter and head diameter of long-stalked capitate glandular were 21.8 and 23.9 µm, respectively (Table 1). Three secretory stages are observed,

where, at pre-secretory, a copious number of secretory products accumulate and occupy the entire glandular head (Figure 2c). Secretions are released during the secretory stage, as seen on the glandular head, presumably by means of cuticular exudation since no cuticle ruptures are observed (Figures 2d-e). Following the release of secretion, an empty glandular head is observed at the post-secretory while maintaining its round/oval shape without shrinking (Figures 2f-g). The glandular head was stained positively by Nadi reagent and Sudan IV, indicating the presence of terpenes and lipophilic contents, respectively (Figures 2h-k).

Branched Non-glandular Trichomes. Three morphotypes of branched non-glandular trichomes were found specifically on the protruding region of *E. elatior* true flower corolla tube at exceptionally high density (Figures 3a-b). The morphology of branched non-glandular trichomes is characterised according to Zhang et al. (2005). Type A is characterised by two branches with distinctively unequal lengths (Figure 3c), where the stalk length, basal cell diameter, and shortest and longest branch length were 903.2, 41.3, 125.4, and 307.4 µm, respectively (Table 1). Type B is characterised by two branches, where the shortest branch seemingly appeared defective as if branch

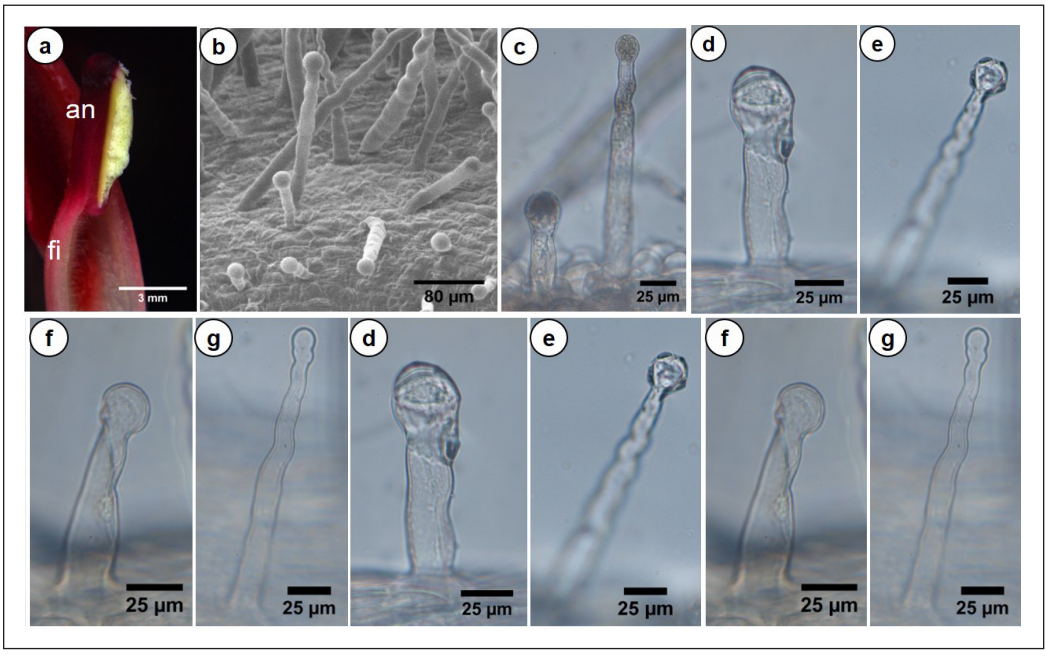

Figure 2. (a) Dense pubescence on basal anther (an) and filament (fi) of *Etlingera elatior* true flower; (b) Scanning electron microscope micrograph of short- and long-undulating stalk of capitate glandular trichomes; (c) Capitate trichomes at pre-secretory stage with secretion materials accumulated in the glandular head; (d–e) Trichomes at secretory stage with exudates secreted from the glandular head: (d) Short-stalked; (e) Long-stalked trichome; (f–g) Empty glandular head at post-secretory stage; (h–i) Positive histochemical tests by Nadi reagent indicating the presence of terpenes; (j–k) Secretion materials tested positive by Sudan IV indicating the presence of lipophilic materials

Table 1
Morphological characteristics of floral trichomes in true flowers of Etlingera elatior

Type of trichomes	Stalk length (µm)	Basal cell diameter (µm)	Head diameter (µm)	Shortest branch length (µm)	Second branch length (µm)	Longest branch length (µm)
Capitate glandular						
Short-stalked	43.1 ± 15.7	19.0 ± 4.4	21.9 ± 2.5	N. A.	N. A.	N. A.
Long-stalked	104.0 ± 43.9	21.8 ± 4.0	23.9 ± 4.2	N. A.	N. A.	N. A.
Branched, non-glandular						
Type A	903.2 ± 175.4	41.3 ± 15.5	N. A.	125.4 ± 41.2	N. A	307.4 ± 106.4
Type B	513.7 ± 118.3	29.2 ± 12.6	N. A.	29.8 ± 9.4	N. A	220.6 ± 66.2
Type C	489.7 ± 61.1	31.2 ± 7.6	N. A	26.3 ± 8.9	181.3 ± 30.8	272.0 ± 56.8

Note. Data are mean ± standard deviations of $n = 10$; N. A. = Not applicable

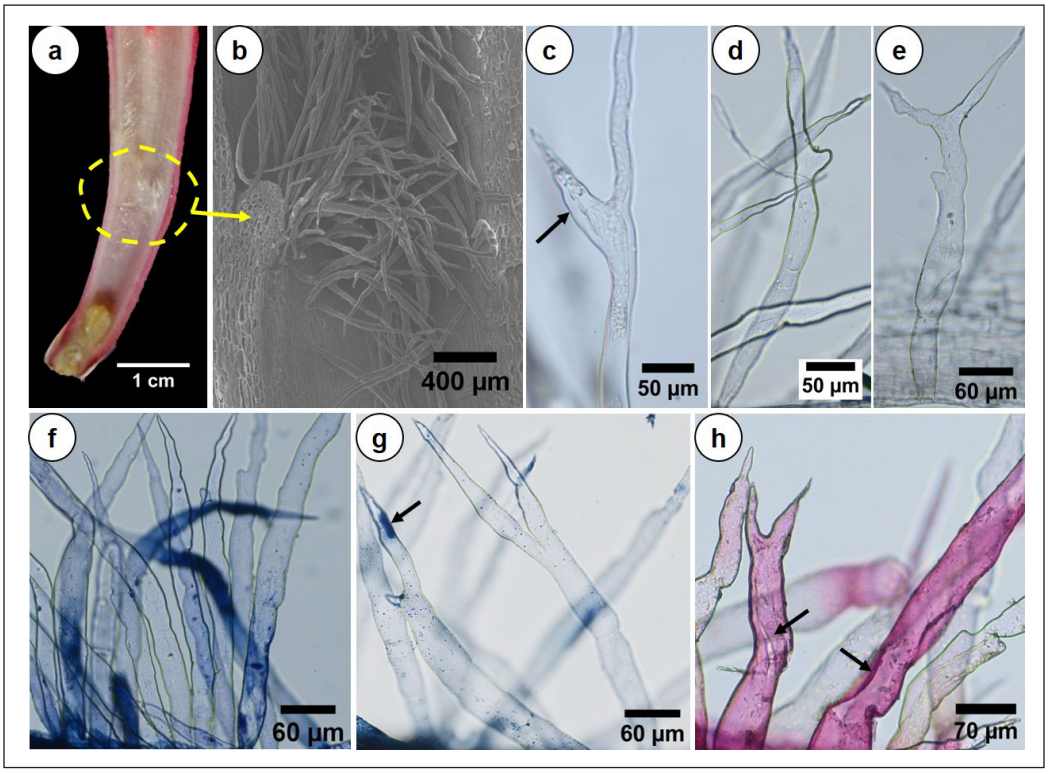

Figure 3. (a) Corolla tube of *Etlingera elatior* true flower. Protruding region (circle), where branched non-glandular trichomes occurred at high density; (b) Close-up view of the protruding region viewed under scanning electron microscope; (c–e) Branched non-glandular trichomes: (c) Type A with one of the branches shorter than the other. Note the thick cuticle of the trichomes indicated by arrow; (d) Type B with the shortest branch seemingly appeared as defect due to aberrant expansion of the branch; (e) Type C with three branches and the third, shortest branch on the lower stalk; (f–g) Nadi reagent staining of the trichomes indicating the presence of terpenes; (g–h) Arrows indicate the slit cuts resulted during the transverse hand sectioning of corolla tube that provided leeway for the staining of the internal content of the trichomes; (h) Staining by ruthenium red for mucilage content

elongation stopped abruptly during trichome morphogenesis (Figure 3d). The stalk length, basal cell diameter, and shortest and longest branch of *E. elatior* type B branched non-glandular trichomes were 513.7, 29.2, 29.8, and 220 µm, respectively (Table 1). Type C appeared as a morphed combination of types A and B, with three branches total (Figure 3e). Two branches are either similar in length or slightly shorter than the other one, whereas the third branch appeared as a defect. The stalk length, basal cell diameter, shortest, second, and longest branch of *E. elatior* type C branched non-glandular trichomes were 489.7, 31.2, 26.3, 181.3, and 272.0 µm, respectively (Table 1).

All three types of branched trichomes are constituted by thick cuticles, as evidenced by the histochemical tests. Staining was proven difficult to permeate the hydrophobic cuticle unless the cut trichomes (indicated by arrows in Figures 3g–h) resulted from the hand sectioning provided leeway for the reagents to react with the internal content of the trichomes. Nonetheless, positive staining by Nadi reagent and ruthenium red indicated the presence of terpenes and mucilage in the trichomes (Figures 3f–h).

Unbranched Non-glandular Trichomes. The true flowers of E. elatior bear three morphotypes of non-glandular trichomes. The first type is undulating, unicellular with thinly cutinised lateral walls and pointed apex, mostly found on the filament (Figures 4a–b). The second type is uni-, bi-, or multicellular, with a thickly cutinised lateral wall and a rounded apex (Figures 4c–d). These structures are found on the protruding regions of the corolla tube. The third type is a simple, unicellular structure with a thinly cutinised lateral wall and a pointed

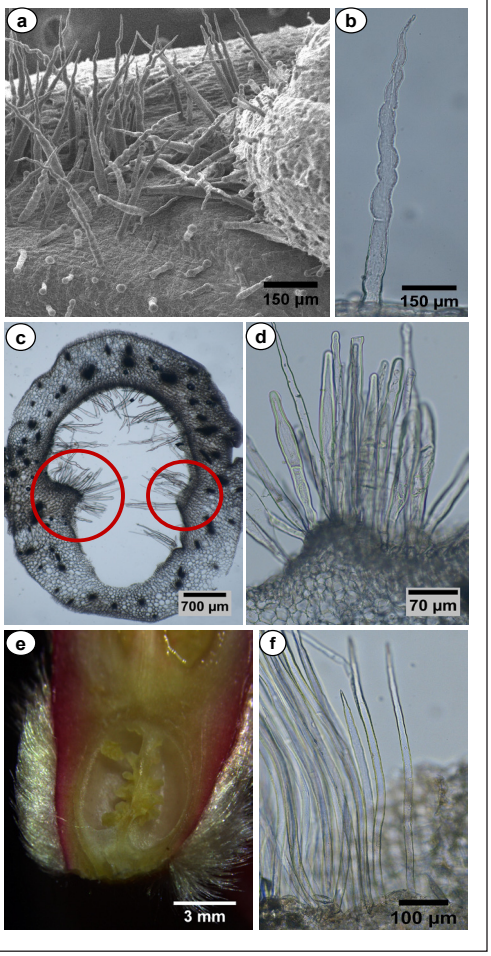

Figure 4. (a) Scanning electron microscope micrograph of dense non-glandular trichomes on the filament of *Etlingera elatior* true flower; (b) Undulating stalk with thinly cutinised lateral wall and pointed apex; (c) A transverse section of the true flower, viewed using a light micrograph, shows the protruding regions (red circled) of the corolla tube where non-glandular trichomes occur abundantly; (d) Uni- to multicellular non-glandular trichomes with thickly cutinised lateral wall and globose apex; (e) A thick layer of trichomes at the base of the true flower that surrounds the ovary; (f) Long, unicellular trichomes with pointed apex

apex (Figures 4e–f). These structures occur abundantly on the basal part of the true flower, creating a thick layer that surrounds the ovary.

Osmophores. In addition to trichomes, osmophores that consist of an epidermis of specialised secretory cells and/or secretory parenchyma (Tölke et al., 2018) were also observed in *E. elatior* true flower. Unicellular, rounded and sometimes irregular protuberances are observed on the basal anther (Figures 5a–b) and are distinct from all other neighbouring cells. Light micrographs of fresh, hand-cut sections revealed that the structures appeared as layers of translucent convex with smooth cuticles (Figure 5c). The epidermal cells exhibited polarity, composed of thick cuticles, and stained positively by ruthenium red for mucilaginous polysaccharides (Figures 5d–e). The presence of mucilage, terpenes, and phenolic compounds by Nadi reagent, ruthenium red, and toluidine blue O, respectively, indicated that the structures may be osmophores (Figures 5f–h) because *E. elatior* true flowers engaged in scent production (Lee, 2019). The floral fragrance of *Anacardium humile* and *Mangifera indica* have been related to the presence of osmophores (Tölke et al., 2018). However, further investigation using a transmission electron microscope is needed to confirm this structure.

DISCUSSION

Floral Traits and Structure

The floral characteristics of *E. elatior* reflect the general features of Zingiberaceae, being pentacyclic and zygomorphic (PIñeyro-Nelson et al., 2017). Both labellum and epigynous glands are prominent features of the family, especially in terms of formation (Song et al., 2007). In this study, labellum

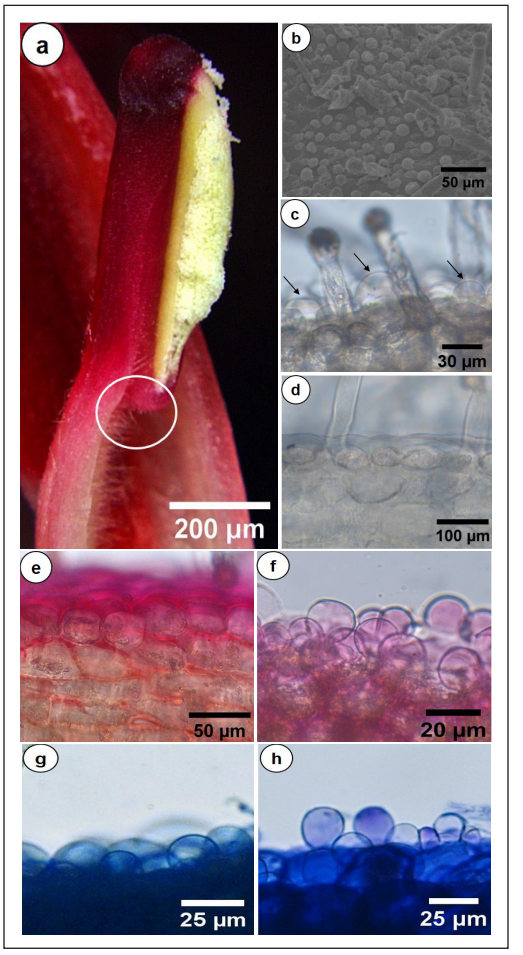

Figure 5. (a) Dense pubescence on basal anther (circle) of *Etlingera elatior* true flower; (b) SEM micrograph of the osmophores appeared as globular trichomes; (c) Light micrograph of the osmophores as translucent convex (arrows) layers with smooth cuticle on the epidermal surface; (d) The epidermal cells comprised of single layer exhibiting polarity; (e) Positive staining by ruthenium red indicates the presence of mucilaginous polysaccharides on the epidermal surface; (f–h) Positive histochemical tests of the osmophores for ruthenium red (f), Nadi reagent (g), and toluidine blue O (h)

demonstrates an important function as an additional exaptation with the striking combination of red-coloured and yellow margins, serves primarily as a visual attraction (Armbruster, 1997). The classic floral advertisement system indicates an open invitation and an announcement that the pollen dehisced prior to the anthesis of *E. elatior* is ready for pollination, or rewards are available for collection. By the end of the day, the labellum wilted, and the top part of the flower was marked as post-anthesis. Given the acropetal architecture of the inflorescence, where flowers open sequentially by the ring, the labellum closure suggests protection, especially for successfully pollinated flowers and ensures limited contamination or disruption from constant visitation by pollinators.

Structurally, the labellum contained within the petals provides a tubular rigidity of the flower (Bull-Hereñu et al., 2022), presumably assumed by the folding of lateral lobes that enclose the stamen and pistil. The connotation of the labellum and the basal filament that elongates to the base of petals and extends to the median-superior of the ovary forms the corolla tube of *E. elatior*. According to Pedersen (2004), the corolla tube formation and the labellum furling inwards that encloses the top part of the flower at post-anthesis are automorphy characteristics of *Etlingera*.

The tubular flowers of *E. elatior* is associated with nectar-feeding birds such as hummingbirds, sunbirds, and spiderhunters as the main pollinators of *E. elatior* (Kittipanangkul & Ngamriabsakul, 2006; Sakai et al., 2013). Lesser banded hornets (*Vespa affinis*), spiders, and ants are also commonly observed visiting the flowers at the research plot. Accordingly, the long needle-like bills of the avian are adapted to nectar exploration long enough to prod through the corolla tube (Rico-Guevara et al., 2019; Riegert et al., 2011). The main source of rewards in *E. elatior* is epigynous glands located at the basal corolla tube surrounding the style (distance from stigma was 3.7 ± 0.7 cm). In Zingiberaceae, nectaries are transformed into epigynous glands that are also known as "supragynopleural" nectaries (Box & Rudall, 2006) or stylodes (Khaw, 2001). Closure of the locules that separate the epigynous nectary provides protection, especially the ovule, from damage inflicted by the rigorous movements of pollinators (Kirchoff, 1998).

Floral Trichomes and Osmophores as Secretory Structures

The true flowers of *E. elatior* produce sweet, scented fragrance as non-food rewards and nectar, protein-rich pollen, and oil as food rewards. Osmophores have been associated with producing heterogeneous substances such as mucilage, terpenes, and phenolic compounds (Tölke et al., 2018). On the other hand, capitate glandular trichomes secrete terpenes and lipophilic compounds (Guo et al., 2020). Generally, the chemicals produced by the secretory structures on the stamen indicate floral cues that attract pollinators and signalling pathways to locate primary rewards (Essenberg, 2021). The abundance of mucilaginous polysaccharides

suggests the significance of the secretion as glistening the epidermal surface of anther to attract pollinators and help pollen get glued on the bodies of visitors (Koptur et al., 2020), as well as a carbohydrate source for metabolic processes (Pansarin et al., 2014).

Terpenes and lipophilic compounds constitute floral volatiles that enable long-distance attraction of pollinators by perfuming their immediate environment (Guo et al., 2020). Essential oils analysis of the inflorescence head at full bloom stage revealed the presence of *(E)*-caryophyllene and *(E, E)*-*α*-farnesene (Lee, 2019), which are commonly emitted in floral organs (Chakira et al., 2022; Qiao et al., 2021; Yang et al., 2022). The secreted materials observed on the glandular heads suggest oil rewards that pollinators can collect, especially bees, although there is no direct evidence to support this. Alternatively, the exudates could play dual roles as repellents to herbivores or illegitimate, non-pollinating nectar collectors (Johnson et al., 2017; Mesquita-Neto et al., 2020).

The occurrence of osmophores and glandular trichomes indicates the strategic pollination mechanism exhibited by *E. elatior*. The olfactory cues that led visitors to the flower increased the possibility of pollen distribution from the anther to the body parts of pollinators by the sticky mucilage as they foraged deeper into the corolla tube for nectars. Subsequent pollen redistribution onto the stigma is possible as the visitors retreat from the corolla tube. On the other hand, the presence of phenolic compounds suggests deterrent chemicals that make plants hostile to herbivores, especially predatory insects, from acquiring protein-rich pollen (Wang et al., 2021; War et al., 2018).

Floral Trichomes Participate in Floral Development

Trichomes are abundant in several locations of the *E. elatior* true flower: the apex of three-toothed dentate margins of sepal, filament and style, corolla tube, and basal flower surrounding the ovary. The apical trichomes on the sepal suggest mechanical protection, especially during the early ontogeny prior to the petal emergence and anthesis. The trichomes acted as a seal by interlocking the apex of the true flower, thereby maintaining a closed structure that would allow the development of internal floral organs with minimal interruption from external forces (Takeda et al., 2013) (Supplementary data Figure S1). This finding appeared to substantiate Zhao and Chen's (2016) on the function of trichomes in cotton (*Gossypium hirsutum*), which maintains the correct flower shape while protecting the young tissues.

Similarly, the non-glandular trichomes on the basal true flower of *E. elatior* enveloped the sepal act as 'Velcro' to the bracteole (Supplementary data Figure S2). In addition, the thick layers of trichomes surrounding the ovary create a formidable barrier that enhances greater protection of the most important reproductive organ by providing insulation from desiccation and, particularly, the seeds against predators during the later fruiting stage (Armbruster, 1997).

The clusters of trichomes on the protrusion regions of the *E. elatior* corolla tube coincided with the fusion of petals (Supplementary data Figure S3). Observation in the early ontogeny of the true flower showed that the clusters of trichomes are present during the simultaneous deepening of the corolla tube and petal elongation, as well as separation of common primordia (Supplementary data Figure S4), indicating their presence as anchor and protection to the developing reproductive organ. In addition, the clusters also indicate another hurdle of deterrents for predatory microorganisms such as parasitoids from accessing the source

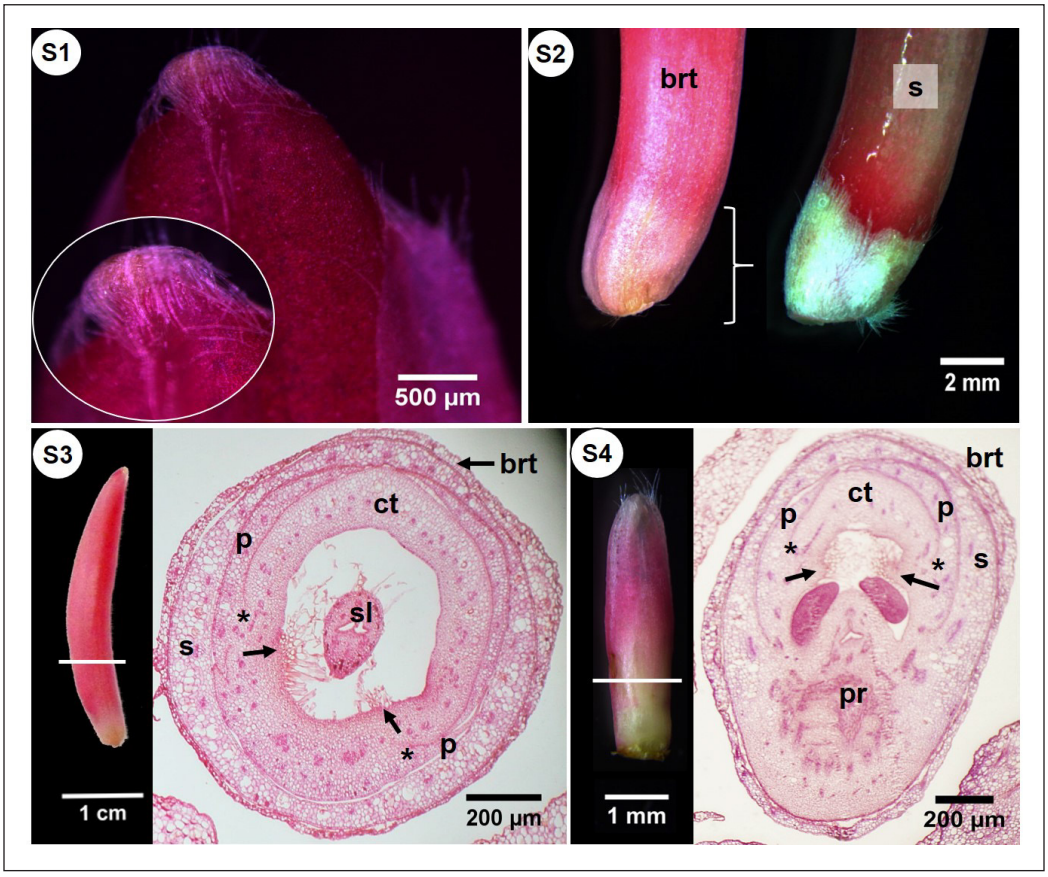

Figure S1. The apex of the sepal covered by interlocking non-glandular trichomes helps to maintain a closed structure of the developing true flower of *Etlingera elatior*; (*S2*) Basal true flower showing the pink-coloured bracteole with the curly brace indicates the non-glandular trichomes enveloping the red-coloured sepal and acting as 'Velcro' to the bracteole; (*S3–S4*) Transverse section of true flower stained in astra blue where the occurrence of branch non-glandular trichomes (arrows) coincided with the growth of petals on the corolla tube (asterisk). The solid line on the left figure of the true flower reflects the transverse cut of the anatomy: (*S3*) True flower development at stage completed sepal development; (*S4*) Early ontogeny of the true flower where petal primordia elongate, corolla tube deepens, and common primordia separates
Note. The clusters of trichomes (arrows) on the corolla tube present prior to the separation of common primordia; brt = Bracteole; ct = Corolla tube; p = Petal; pr = Primordia; s = Sepal; sl = Style

of rewards located at the basal flower, especially during the anthesis.

Interestingly, the morphology of branched trichomes in *E. elatior* also appeared similar to the mutational phenotype of *IRREGULAR TRICHOME BRANCH$_1$* (*ITB$_1$*), which controls trichome morphogenesis in *Arabidopsis*. According to Zhang et al. (2005), the *ITB$_1$* genes regulate anisotropic expansion of the stalk and branches that produce bloated and misshapen branched trichomes. The fact that both branch and unbranched non-glandular trichomes with diverse morphotypes occurred in two specific clusters suggests a complex interplay of cellular interactions during the expansion of the corolla tube and petal elongation. Trichome formation in the corolla tube is regulated by the *AmMYBML1* gene in *Antirrhinum majus* (Serna & Martin, 2006).

The developmental processes of flowering organs such as sepals, petals, stamens, and carpels involve specific control by floral organ identity genes that are able to repress trichome proliferation (Matías-Hernández et al., 2016). Given that the clusters of trichomes occurred at the fusion point of petals to the corolla tube, it is, therefore, curious if the branched trichomes in *E. elatior* are associated with any mutational consequences of cell expansion derived from the ontogeny of floral organs in the true flower. Genetic analysis of the trichomes will provide insights into the differential regulation of cell expansion within the floral tissues and characters for future taxonomic delimitation and phylogenetic relationships currently lacking in Zingiberaceae.

CONCLUSION

The floral traits of *E. elatior,* mainly the tubular characteristic, demonstrate how nectar-feeding birds are associated as the main pollinators in the flower-pollinator relationships. The labellum proved to be more than just an advertisement accessory as it is commonly regarded; it was also an additional exaptation to protect the flower. The present study also demonstrates the presence of secretory structures coordinated with the functional traits of the flower to enhance the pollination mechanism. Both glandular trichomes and osmophores signified their importance in securing anthesis by releasing secretion materials as indicated by the histochemistry, providing a signalling pathway that attracts pollinators and deters herbivores from robbing the floral rewards. The diversity of trichome structures, possibly with different concoctions of chemical signals, contributes to the survival strategies of the plant in mediating the environment during the anthesis period. Specifically, the non-glandular trichomes continually provide physical and mechanical protection and are involved structurally throughout flower development. Although further studies are required to confirm the underlying mechanism of the occurrence of trichomes in clusters at the corolla tube, this is the maiden report for *E. elatior* on trichomes diversity and their participation in floral structure and development besides plant-pollinator interaction.

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A Preliminary Checklist of Fish Species in the Peat Swamp Forest of Ayer Hitam Utara State Park Forest, Johor, Malaysia

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ABSTRACT

Ayer Hitam Utara State Park Forest (AHUSPF) is an invaluable sanctuary in Johor, the last remaining intact peat swamp forest reserve that hosts many species unique to this distinctive habitat. Some rapid surveys conducted from 2019 to 2022 recorded 40 species of freshwater fish within AHUSPF. Notably, 11 are stenotopic to acid blackwater, and 12 are stenotopic to acid water, most belonging to the family Osphronemidae and Danionidae. The International Union for Conservation of Nature Red List has designated two species, *Encheloclarias curtisoma* and

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Betta persephone, as Endangered, while *Betta omega* is classified as Critically Endangered. The checklist presented herein was first produced from an inventory study in AHUSPF. The preservation of peat swamp forests in the area is under imminent threat due to the alarming rate of land conversion, forest fires, and the recent potential threat from the red-claw crayfish invasion in the aquatic environment. Hence, continuous monitoring is vital for documenting and updating the conservation status of the fish species. These endeavours will facilitate the development of practical conservation approaches to ensure the long-term sustainability of AHUSPF and its associated species.

Keywords: Acid blackwater, alien species, biodiversity inventory, conservation, endemic species, sustainable practices, threatened fish species

INTRODUCTION

Ayer Hitam Utara State Park Forest (AHUSPF) stands as the largest and the last remaining peat swamp forest reserve in Johor, Peninsular Malaysia, making it a crucial ecological stronghold (Ahmat & Norazlimi, 2022). Other forests in Johor have suffered significant fragmentation, leaving behind mere patches of the once-expansive peat swamp landscape (P. K. L. Ng et al., 1994; Wetlands International, 2010). Due to its gloomy appearance, characterised by a soft, spongy bottom, an abundance of decaying woods, and a dense understory of spiny vegetation, the biodiversity of the peat swamp forest has received limited attention (Posa et al., 2011). The unfavourable water conditions, including dark colouration, high acidity, and low dissolved oxygen levels, have perpetuated the fallacy of low aquatic biodiversity in peat swamps (P. K. L. Ng et al., 1994).

However, a paradigm shift occurred with a comprehensive study conducted by P. K. L. Ng et al. (1992) in the North Selangor Peat Swamp Forest, which recorded 47 species of freshwater fishes from the blackwater habitat, with 14 species regarded as stenotopic taxa (P. K. L. Ng et al., 1994). Subsequent studies conducted post-1992 revelation, such as in Ulu Sedili, Johor (Zakaria et al., 1999), Pondok Tanjung, Perak (Mansor et al., 1999), Sungai Bebar, Southeast Pahang (Amirrudin et al., 2005), Jambu Bongkok, Terengganu (Amirrudin et al., 2011), Kuala Langat, Selangor (Fahmi-Ahmad & Samat, 2015), Tasek Bera, Pahang (Fahmi-Ahmad et al., 2015), and North Selangor Peat Swamp Forest (Azmai et al., 2020), have further contributed to the understanding of aquatic biodiversity, specifically freshwater fishes, within peat swamp ecosystems.

Given the constant threats of climate change and urbanisation, which have led to substantial degradation and conversion of peatlands for industry, human settlements, and agriculture (Giam et al., 2012; Girkin et al., 2022; Roucoux et al., 2017), the fate of freshwater fish species dependent on peatland habitats has become a growing concern. The conversion of peatlands, particularly for oil palm plantations, has contributed to Malaysia's demise of peat swamp ecosystems (Manzo et al., 2020).

In Peninsular Malaysia, approximately 281,652 ha of peatland is dedicated to agriculture, with 72% utilised for oil palm plantations. Among the states, Johor holds the highest area of converted peatland into oil palm plantations, with 68,468 ha (Wetlands International, 2010). The once-extended peat swamp forests in southwest Johor have now been fragmented due to the increasing global demand for palm oil (Shevade & Loboda, 2019; Wetlands International, 2010).

AHUSPF, situated in North-West Johor, remains an intact and protected forest amidst the encroachment of small oil palm plantations, agriculture, and residential areas. The surrounding land-use changes could significantly impact the main forest and its unique freshwater fish biodiversity if not closely monitored. Despite its ecological importance, the diversity of fishes within AHUSPF has not been adequately documented. Thus, this study aims to present the first checklist of freshwater fish thriving in AHUSPF and illuminate the potential threats the fish and its habitat might encounter. By providing essential baseline data, this study could contribute to future conservation efforts to preserve the ecological integrity of AHUSPF and its valuable diversity of freshwater fishes.

MATERIALS AND METHODS

The AHUSPF is in the northwestern part of Johor, specifically in the Muar district, within the designated Ayer Hitam Utara Forest Reserve area (Figure 1). Encompassing 3,797 ha with a terrain elevation of approximately 31 m a.s.l., this area receives an average annual rainfall of 2,060 mm (Shamsuddin et al., 2021). The local economy in the area is predominantly reliant on agricultural activities. The land surrounding AHUSPF is mainly utilised for small-scale oil palm estates, alongside cultivating vegetables and fruit orchards. Fish collections were conducted during the Scientific Expedition on Biological Diversity in AHUSPF from April 15th to April 16th, 2019, followed by biannual visits in 2021 and 2022. During each biannual visit, fish collection activities spanned three days. The first visit for each year occurred in April, the dry period, while the second visit took place in November, during the wet period.

This study utilised the purposive sampling method, wherein the sampling sites were chosen based on expert judgment, particularly when targeting specific habitats for certain species. This approach is suitable for preliminary studies to gain a broader understanding of the fish community in the area. The study site represents a typical peat swamp forest environment with loose soil and substrate consisting of wood branches, submerged vegetation, and dead leaves from surrounding trees (Ahmad & Samat, 2015).

The pH for the blackwater area ranged from 3.6 to 3.8, while for the freshwater swamp, it ranged from 4.8 to 5.4. The blackwater area is in the main forest area, characterised by isolated pools of water among forested hummocks during the dry period. These pools become interconnected during the rainy season when the forest

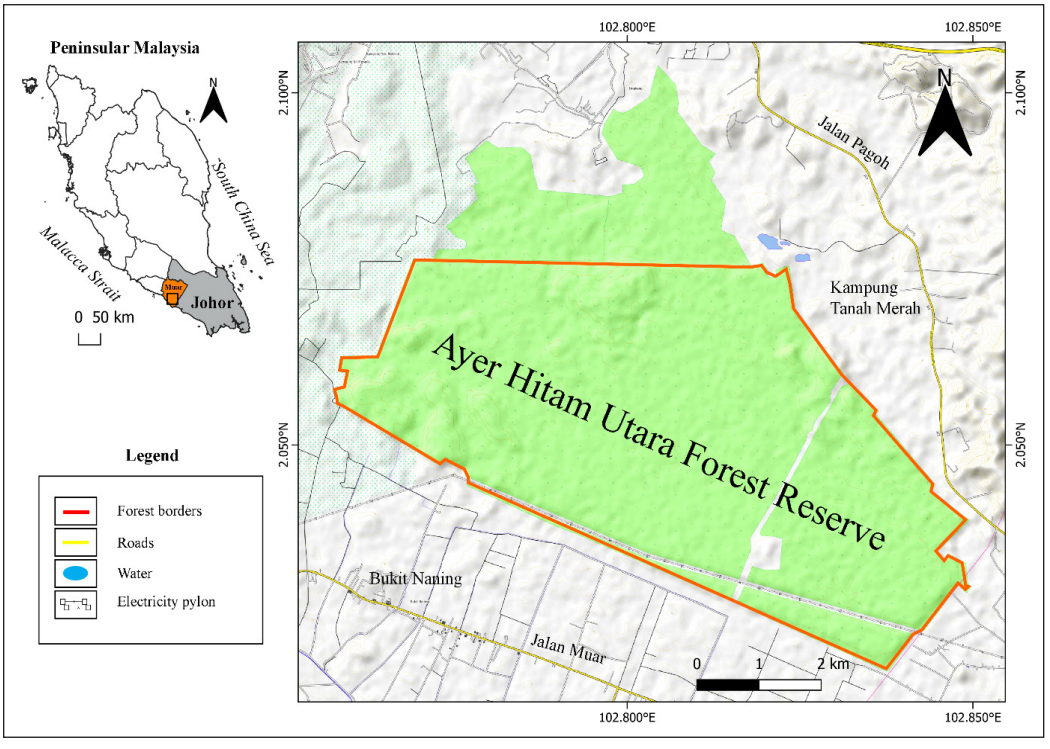

Figure 1. Map of the study area in Ayer Hitam Utara State Park Forest, Johor, Peninsular Malaysia

floor becomes flooded. Adjacent to the main peat swamp forest, in the peripheral area of AHUSPF is a flooded forest housing a freshwater swamp stream. The water in this stream is slow-moving and appears murky, resembling the colour of milk tea. Despite originating from the nearby peat swamp forest, the water in the stream is less acidic compared to the main forest (Figure 2).

Scoop net (1 mm mesh size) was the primary method gear throughout this study. The lightweight and easy-to-handle features in tight space made the scoop net the most effective gear. Cast nets (19.05 mm mesh size) and fish traps (25.4 mm mesh size) were alternative gears used in the spacious canals or streams. Fish were identified in the field and released back to the swamp whenever possible. A small representative sample was fixed in 10% formalin solution (diluted from formaldehyde solution 37%, Systerm Chemicals, Malaysia) and later preserved in 70% alcohol (diluted from ethyl alcohol 95% denatured, Systerm Chemicals, Malaysia) for long-term preservation. Voucher specimens were deposited in the Makmal Rujukan Zoologi at Universiti Sains Malaysia (USMFC) and Muzium Zoologi Universiti Malaya (UMKL).

The historical records of fishes from Ayer Hitam Forest Reserve, which are in the collection of local institutions, were also included. All caught fish were identified based on the general diagnostic characteristics following Zakaria-Ismail et al. (2019) and the taxonomic key of

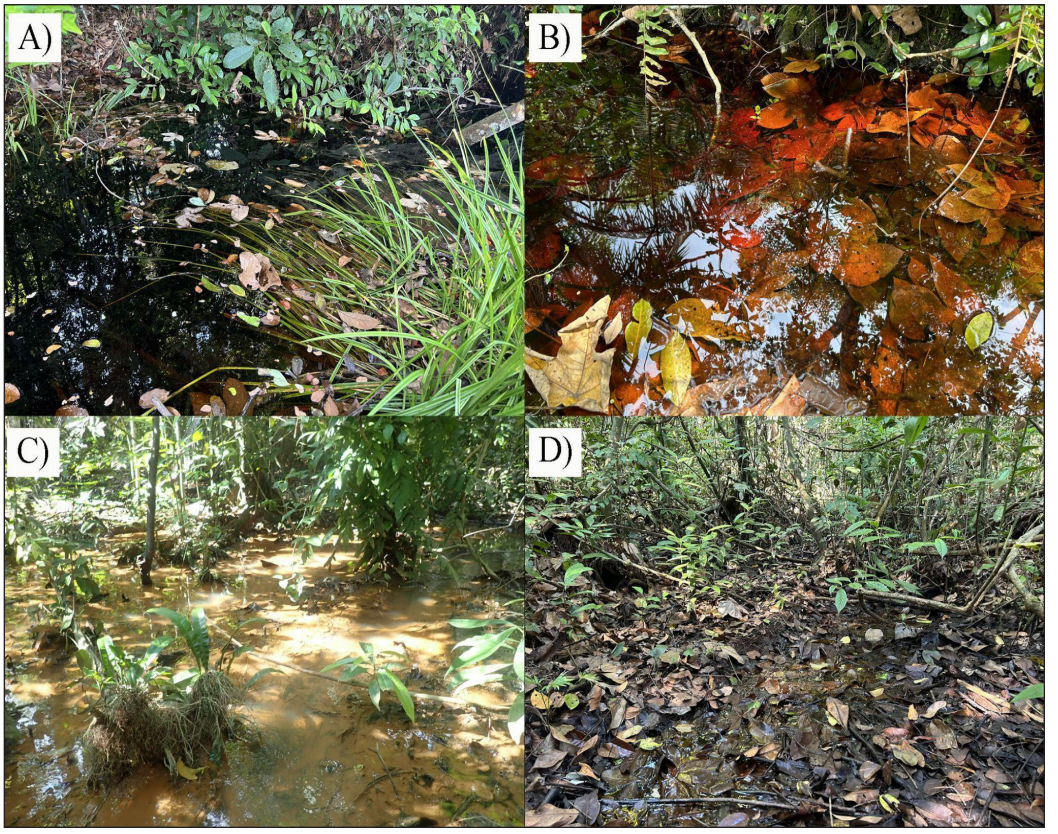

Figure 2. Overview of the habitats in the Ayer Hitam Utara State Park Forest: A) Blackwater habitat; B) Water in the forest darkened by tannins leached from fallen leaves; C) Freshwater swamp in the flooded forest; D) Puddles on the forest floor serving as fish refuges during the dry period

Kottelat et al. (1993) and Rainboth (1996). The fish systematic arrangement follows California Academy of Sciences (CAS) (n.d.) and Kottelat (2013). The abbreviation 'ex.' next to the number of materials examined means the exemplar or specimen. Habitat preferences were determined using recommendations by P. K. L. Ng et al. (1994). Stenotopic acid blackwater species are specifically adapted to highly acidic (pH 3 to 4) and dark-coloured blackwaters. Stenotopic acid water species thrive in acidic waters with a pH below 6; some may tolerate blackwaters. Eurytopic species are highly adaptable, commonly found, and thrive across a wide range of habitats, from slightly acidic to alkaline conditions. It is important to note that these habitat preferences are not solely based on water pH but on observations and expert verdicts. Species not classified by P. K. L. Ng et al. (1994) were assigned categories based on our experience and current knowledge about the fish. The International Union for Conservation of Nature (IUCN) status for all fish species recorded was based on The IUCN Red List of Threatened Species version 2022-2 (IUCN, 2022).

RESULTS

A total of 40 species representing 15 families of freshwater fishes were recorded in AHUSPF (Table 1 and Figure 3). The list of fish species in AHUSPF was dominated by the family Osphronemidae (10 species), followed by Danionidae (six species) and Channidae (five species). Only one species was encountered in each of the following families: Bagridae, Akysidae, Mastacembelidae, Chaudhuriidae, Synbranchidae, Anabantidae, Helostomatidae, and Zenarchopteridae. One species, *Betta persephone* from the family Osphronemidae, was reported as endemic to AHUSPF. Most of the fishes were caught in the blackwater habitats. In the freshwater swamp stream adjacent to the peat swamp forest within AHUSPF, the cyprinids (e.g., *Barbodes sellifer* and *Osteochilus vittatus*) and the rasborines (e.g., *Rasbora elegans* and *Rasbora myersi*) were commonly found thriving in the less acidic conditions of this habitat.

Table 1 shows 11 species of fish that are stenotopic to acid blackwater, 12 that are stenotopic to acid water, and 17 that are eurytopic. The high number of stenotopic

Table 1
Checklist of fishes from Ayer Hitam Utara State Park Forest with habitat preference and International Union for Conservation of Nature (IUCN) Red List status (2022) for each species

No.	Family	Species	Habitat preference	IUCN Red List status
1	Cobitidae	*Lepidocephalichthys tomaculum*	A	LC
2		*Kottelatlimia katik*	S	LC
3	Cyprinidae	*Barbodes sellifer*	E	NE
4		*Desmopuntius hexazona*	A	LC
5		*Osteochilus vittatus*	E	LC
6	Danionidae	*Boraras maculatus*	A	LC
7		*Esomus metallicus*	E	LC
8		*Rasbora einthovenii*	A	LC
9		*Rasbora elegans*	E	LC
10		*Rasbora kalochroma*	S	LC
11		*Rasbora myersi*	E	LC
12	Bagridae	*Mystus bimaculatus*	S	NT
13	Akysidae	*Parakysis verrucosus*	A	VU
14	Siluridae	*Ompok fumidus*	S	NT
15		*Silurichthys indragiriensis*	A	NT
16	Clariidae	*Clarias leiacanthus*	E	LC
17		*Clarias macrocephalus*	E	DD
18		*Clarias nieuhofii*	A	LC
19		*Encheloclarias curtisoma*	S	EN
20	Mastacembelidae	*Macrognathus circumcinctus*	A	LC
21	Chaudhuriidae	*Bihunichthys monopteroides*	A	VU
22	Synbranchidae	*Monopterus javanensis*	E	LC
23	Anabantidae	*Anabas testudineus*	E	LC

Table 1 *(continue)*

No.	Family	Species	Habitat preference	IUCN Red List status
24	Helostomatidae	*Helostoma temminckii*	A	LC
25	Osphronemidae	*Belontia hasselti*	S	LC
26		*Betta bellica*	A	LC
27		*Betta coccina*	S	VU
28		*Betta omega*	S	CR
29		*Betta persephone*	S	EN
30		*Betta pugnax*	E	LC
31		*Luciocephalus pulcher*	A	LC
32		*Sphaerichthys osphromenoides*	S	DD
33		*Trichopodus trichopterus*	E	LC
34		*Trichopsis vittata*	E	LC
35	Channidae	*Channa bankanensis*	S	NT
36		*Channa limbata*	E	NE
37		*Channa lucius*	E	LC
38		*Channa melasoma*	E	LC
39		*Channa striata*	E	LC
40	Zenarchopteridae	*Hemirhamphodon pogonognathus*	E	LC

Note. S = Stenotopic to acid blackwaters; A = Stenotopic to acid waters; E = Eurytopic (acidic to neutral or slightly alkaline); NE = Not Evaluated; DD = Data Deficient; LC = Least Concern; NT = Near Threatened; VU = Vulnerable; EN = Endangered

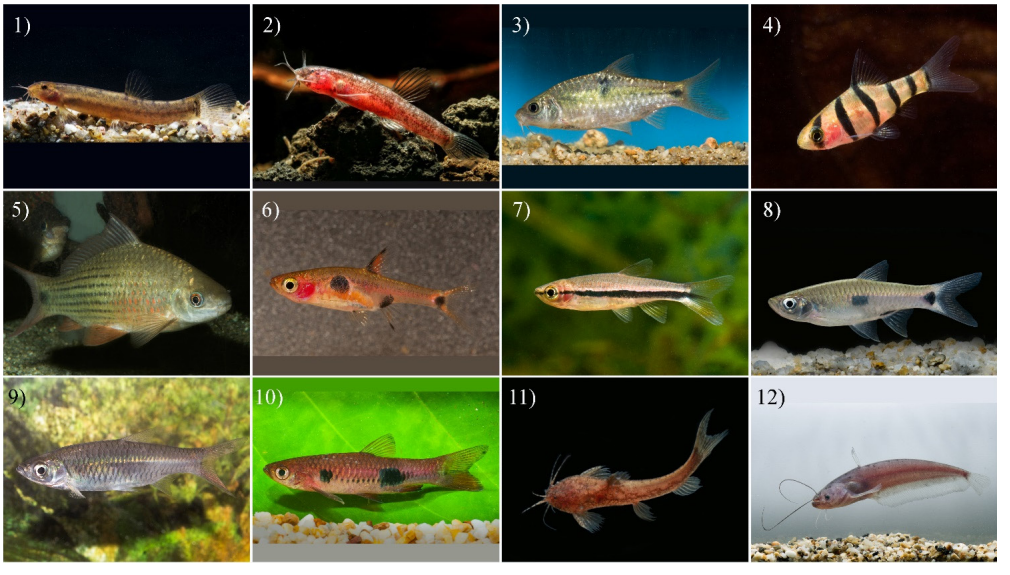

Figure 3. Freshwater fishes recorded in Ayer Hitam Utara State Park Forest: 1) *Lepidocephalichthys tomaculum*; 2) *Kottelatlimia katik*; 3) *Barbodes sellifer*; 4) *Desmopuntius hexazona*; 5) *Osteochilus vittatus*; 6) *Boraras maculatus*; 7) *Rasbora einthovenii*; 8) *Rasbora elegans*; 9) *Rasbora myersi*; 10) *Rasbora kalochroma*; 11) *Parakysis verrucosus*; and 12) *Ompok fumidus*

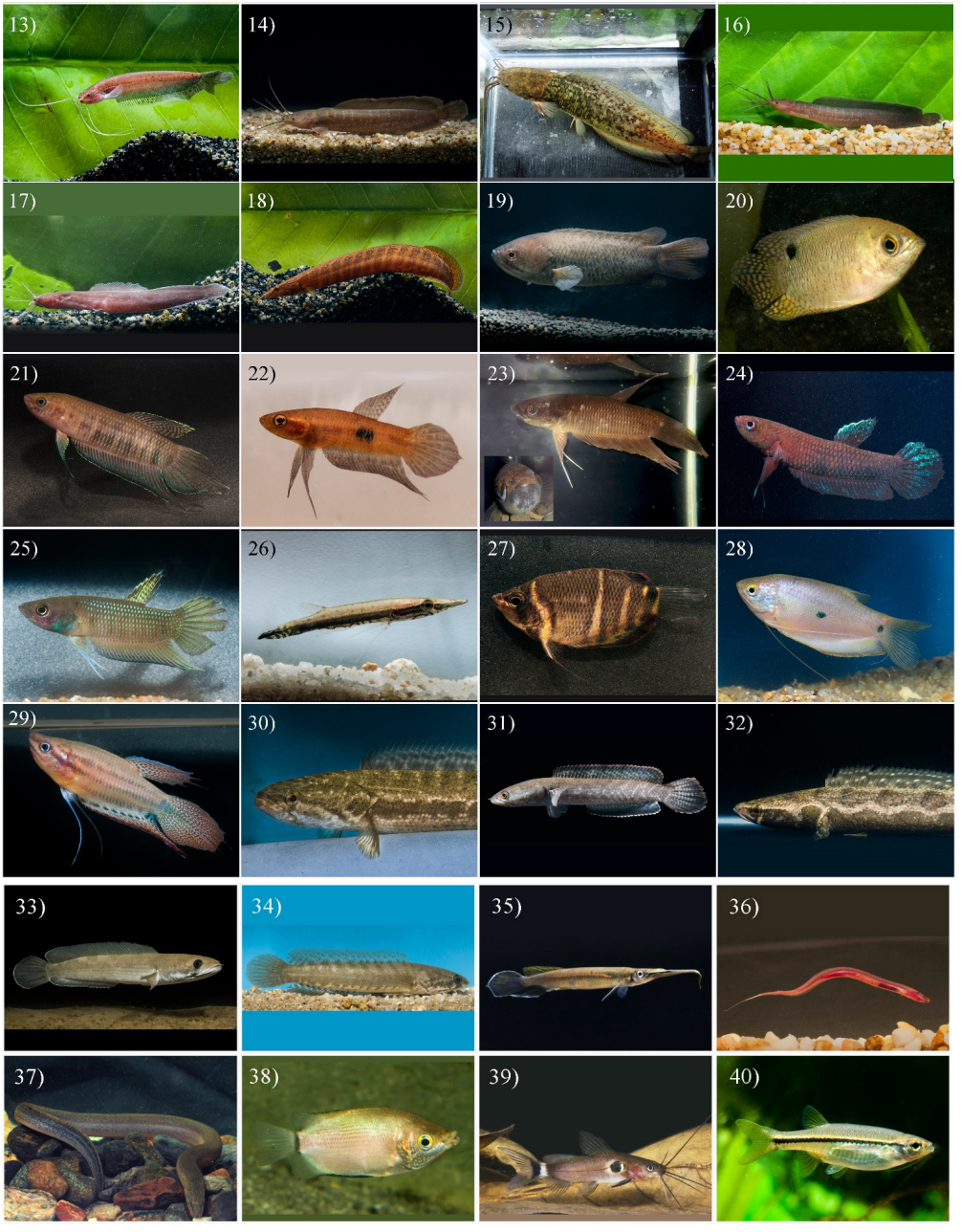

Figure 3 (continue). 13) *Silurichthys indragiriensis*; 14) *Clarias leiacanthus*; 15) *Clarias macrocephalus*; 16) *Clarias nieuhofii*; 17) *Encheloclarias curtisoma*; 18) *Macrognathus circumcinctus*; 19) *Anabas testudineus*; 20) *Belontia hasselti*; 21) *Betta bellica*; 22) *Betta coccina*; 23) *Betta omega*; 24) *Betta persephone*; 25) *Betta pugnax*; 26) *Luciocephalus pulcher*; 27) *Sphaerichthys osphromenoides*; 28) *Trichopodus trichopterus*; 29) *Trichopsis vittata*; 30) *Channa bankanensis*; 31) *Channa limbata*; 32) *Channa lucius*; 33) *Channa melasoma*; 34) *Channa striata*; 35) *Hemirhamphodon pogonognathus*; 36) *Bihunichthys monopteroides*; 37) *Monopterus javanensis*; 38) *Helostoma temminckii*; 39) *Mystus bimaculatus*; and 40) *Esomus metallicus*

to blackwater (five species) came from the family Osphronemidae. A total of six species belong to the threatened categories: Critically Endangered (CR), Endangered (EN), and Vulnerable (VU). *Betta omega* has been listed as CR, meanwhile *Betta persephone* and *Encheloclarias curtisoma* have been included as EN, and *Parakysis verrucosus*, *Bihunichthys monopteroides*, and *Betta coccina* have been listed as VU. Most of the fish species that occurred in AHUSPF are listed as Least Concern (LC), comprising 26 species. Additionally, four species are listed as Near Threatened (NT), two species as Not Evaluated (NE), and two species fall within the Data Deficient (DD) category, lacking information for further IUCN assessments (Table 1).

Annotated Checklist of Freshwater Fishes of Ayer Hitam Utara State Park Forest

Order Cypriniformes

Family Cobitidae

Lepidocephalichthys tomaculum Kottelat & Lim

Material Examined. 1 ex., UMKL 12739; 8 ex., UMKL 12782.

Kottelatlimia katik (Kottelat & Lim)

Material Examined. 1 ex., UMKL 12760; 1 ex., USMFC (10) 00019.

Remarks. This species is small in size. The size of an adult is about 13.5 mm in standard length (Kottelat & Lim, 1992). AHUSPF is the second locality in Peninsular Malaysia to record the occurrence of this species after Mawai, Kota Tinggi, Johor.

Family Cyprinidae

Barbodes sellifer Kottelat & Lim

Material Examined. 11 ex., UMKL 12778.

Remarks. Previously known as *Barbodes banksi*. However, morphological and molecular studies indicate that the populations in Peninsular Malaysia and Singapore are distinct from Borneo *B. banksi*, and this population has not yet been described (C. K.-C. Ng & Tan, 2021; Fahmi-Ahmad et al., 2020; Sobri et al., 2021). Recently, Kottelat and Lim (2021) described a new species, namely *Barbodes sellifer,* for the Peninsular Malaysia and Singapore population, while *B. banksi* is restricted to western Borneo.

Desmopustius hexazona (Weber & de Beaufort)

Material Examined. 8 ex., UMKL 12413; 4 ex., UMKL 12738; 2 ex., UMKL 12750; 2 ex., UMKL 12787; 3 ex., USMFC (7) 00195.

Osteochilus vittatus (Valenciennes, in Cuvier & Valenciennes)

Material Examined. 1 ex., UMKL 12864

Remarks. This species was observed in the streams in the AHUSPF.

Family Danionidae

Boraras maculatus (Duncker)

Material Examined. 16 ex., UMKL 12414; 7 ex., UMKL 12736; 12 ex., UMKL 12752.

Remarks. This species has been described from Bukit Treh, Muar. However, the population from the type locality no longer exist, and the population in AHUSPF is the closest to the type locality. The body colour

is bright red; this species is famous as an ornamental fish.

Esomus metallicus Ahl

Material Examined. 2 ex., UMKL 12865.

Remarks. Found abundantly in the drainage system adjacent to AHUSPF.

Rasbora einthovenii (Bleeker)

Material Examined. 3 ex., UMKL 12866.

Remarks. This species is commonly found in the flowing blackwater in the AHUSPF.

Rasbora elegans Volz

Material Examined. 11 ex., UMKL 12779.

Rasbora kalochroma (Bleeker)

Material Examined. 1 ex., UMKL 12415; 5 ex., UMKL 12742; 1 ex., UMKL 12759; 1 ex., UMKL 12777.

Remarks. This rasborine species is only found in the acid blackwater habitat. Has a reddish body colour. This species is popular as an ornamental fish.

Rasbora myersi Brittan

Material Examined. 3 ex., UMKL 12867.

Remarks. This species was observed in the slow-moving, murky water streams in the AHUSPF.

Order Siluriformes

Family Bagridae

Mystus bimaculatus (Volz)

Material Examined. 1 ex., UMKL 12863.

Remarks. The occurrence of this species in this area recorded the southernmost distribution of this species in Peninsular Malaysia.

Family Akysidae

Parakysis verrucosus Herre

Material Examined. 2 ex., UMKL 12776.

Family Siluridae

Ompok fumidus Tan & Ng

Material Examined. 1 ex., UMKL 12743.

Silurichthys indragiriensis Volz

Material Examined. 7 ex., UMKL 12746.

Family Clariidae

Clarias leiacanthus Bleeker

Material Examined. 1 ex., UMKL 12788.

Clarias macrocephalus Günther

Material Examined. 1 ex., UMKL 12868.

Clarias nieuhofii Valenciennes, in Cuvier & Valenciennes

Material Examined. 3 ex., UMKL 12745.

Encheloclarias curtisoma Ng & Lim

Material Examined. 2 ex., UMKL 12753; 3 ex., USMFC (19) 00012.

Remarks. Specimens examined have a total number of dorsal fins 24–26; the total number of anal fins 47–48; pectoral spine serrae 6–7; and a total number of gills rakers 9. Tentatively, it is identified as *Encheloclarias curtisoma*, a species previously recorded only in North Selangor Peat Swamp Forest, Selangor (Azmai et al., 2020; P. K. L. Ng & Lim, 1993).

Order Synbranchiformes

Family Mastacembelidae

Macrognathus circumcinctus (Hora)

Material Examined. 2 ex., UMKL 12747.

Family Chaudhuriidae

Bihunichthys monopteroides Kottelat & Lim

Material Examined. Uncatalogued USMFC

Remarks. This species has a small adult size (40 mm) and may be considered

miniaturised (Britz & Kottelat, 2003). Typically found in the bottom substrate with the leaf litter. This species seems restricted to the highly acidic black waters in peat swamp forests. Interestingly, it is named for its resemblance to rice vermicelli, a variety of very slender noodles served in many Asian countries, known as 'bihun' in Malay or 'bee hoon' in Hokkien Chinese (Kottelat & Lim, 1994).

Family Synbranchidae

Monopterus javanensis La Cepède

Material Examined. 2 ex., UMKL 12869.

Remarks. This species is commonly found in ditches near the AHUSPF. Locals usually catch this fish using a hook and line baited with an earthworm.

Order Anabantiformes

Family Anabantidae

Anabas testudineus (Bloch)

Material Examined. 1 ex., UMKL 12870.

Family Helostomatidae

Helostoma temminckii (Cuvier)

Material Examined. 2 ex., UMKL 12871.

Remarks. The only species from the family Helostomatidae.

Family Osphronemidae

Belontia hasselti (Cuvier, in Cuvier & Valenciennes)

Material Examined. 3 ex., UMKL 12872.

Betta bellica Sauvage

Material Examined. 9 ex., UMKL 12412; 7 ex., UMKL 12748; 4 ex., UMKL 12785; 2 ex., USMFC (37) 00009.

Remarks. It is the largest bubble-nesting *Betta* and is only found in the blackwater stream.

Betta coccina Vierke

Material Examined. 3 ex., UMKL 12751; 1 ex., UMKL 12775; 7 ex., UMKL 12737.

Remarks. In Malaysia, this species can only be found in AHUSPF.

Betta omega Tan & Ahmad

Material Examined. 2 ex., UMKL 12862.

Remarks. The fish has been described from a small area in the southern part of Johor. The occurrence of this species in AHUSPF has expanded its distribution.

Betta persephone Schaller

Material Examined. 4 ex., UMKL 12741; 10 ex., UMKL 12749; 28 ex., UMKL 12783; 3 ex., USMFC (37) 00020; 2 ex., USMFC (37) 00051.

Remarks. This species is endemic to AHUSPF.

Betta pugnax (Cantor)

Material Examined. 6 ex., UMKL 12786.

Luciocephalus pulcher (Gray)

Material Examined. 1 ex., UMKL 12740; 1 ex., UMKL 12758.

Sphaerichthys osphromenoides Canestrini

Material Examined. 7 ex., UMKL 12755; 1 ex., UMKL 12784; 2 ex., USMFC (37) 00008.

Remarks. Commonly known as Chocolate Gourami, this fish has a chocolate colour with golden bands running down their bodies. It is one of the two maternal mouthbrooders in the genus, including *Sphaerichthys selatanensis*. The female carefully tends to the eggs in her mouth until they hatch.

Trichopodus trichopterus (Pallas)

Material Examined. 1 ex., UMKL 12781; 2 ex., UMKL 12744.

Trichopsis vittata (Cuvier, in Cuvier & Valenciennes)

Material Examined. 7 ex., UMKL 12754.

Family Channidae

Channa bankanensis (Bleeker)

Material Examined. 4 ex., UMKL 12756.

Remarks. Stenotopic to the acid blackwater habitat.

Channa limbata (Cuvier)

Material Examined. 1 ex., UMKL 12757.

Channa lucius (Cuvier)

Material Examined. 1 ex., USMFC (38) 00045.

Channa melasoma (Bleeker)

Material Examined. 1 ex., USMFC (38) 00025; 1 ex., USMFC (38) 00026.

Channa striata (Bloch)

Material Examined. 2 ex., UMKL 12873.

Order Beloniformes

Family Zenarchopteridae

Hemirhamphodon pogonognathus (Bleeker)

Material Examined. 3 ex., UMKL 12874.

DISCUSSION

The declining peat swamp forest area in Peninsular Malaysia is a pressing concern despite some areas being protected. However, the knowledge of fish inhabiting these forests, especially AHUSPF, remains limited. This study recorded 40 species of freshwater fishes in AHUSPF, with over half being stenotopic to acid blackwater and acid water categories. These findings underscore the rich diversity and ecological significance of AHUSPF's freshwater fish community, laying the foundation for conservation and ecological arguments.

Johor and Selangor are among the states with the largest peatland areas in Peninsular Malaysia (Wetlands International, 2010). Comparing AHUSPF to the extensively studied North Selangor Peat Swamp Forest (NSPSF) reveals several intriguing insights. While AHUSPF recorded a lower number of freshwater fish species (40) compared to NSPSF (124) (Azmai et al., 2020), it is notable that the number of stenotopic acid blackwater and acid water species (23) remains relatively high, as observed in NSPSF (65)—accounting for over half of the total number of species in each respective site. It features the importance of peat swamp forests in upholding fish specialists and endemic species (Posa et al., 2011). Additionally, AHUSPF has documented range extensions for certain species in Peninsular Malaysia, such as *Mystus bimaculatus* and *Encheloclarias curtisoma*, which were previously reported only in NSPSF (P. K. L. Ng et al., 1992; P. K. L. Ng & Lim, 1993). Despite being protected forests, AHUSPF and NSPSF face ongoing pressure from surrounding agricultural activities. In NSPSF, evidence of illegal encroachment involving oil palm plantations has been observed, leading to an expansion of oil palm areas along the forest fringes (Charters et al., 2019). Without sustainable management, the protected areas will end up becoming isolated and consequently affected by changes in their properties and function (Anamulai et al.,

2019; Word et al., 2022). Emphasising good practices in oil palm monoculture, such as protecting riparian buffer zones, can contribute to sustaining freshwater fish biodiversity (Aqmal-Naser et al., 2022) and preserving the peat swamp forest as a vital habitat for fish species. Acknowledging the potential impact of similar agricultural developments on AHUSPF in the future, given the current trends observed in NSPSF and the country's economic growth, is crucial.

Freshwater fish assemblage within the blackwater of AHUSPF remains highly vulnerable to various threats despite their unique adaptation to this specific habitat. Studies have shown a consensus that anthropogenic factors, including overexploitation, habitat change, fragmentation, biological invasions, and climate change, are key factors contributing to species vulnerability (Albert et al., 2021; Pouteau et al., 2022). Deforestation and drainage for agricultural purposes are prominent threats to the peat swamp forest ecosystem (Austin et al., 2019; Miettinen et al., 2016). Local communities resorting to quick land-clearing methods involving fire could hoist peatland degradation (Cole et al., 2019). Uncontrolled fires can rapidly spread, encroaching into adjacent areas and posing a significant risk to the freshwater fish assemblage and overall ecosystem. Figure 4A depicts the forest fire that occurred in 2020 within AHUSPF. The phenomenon is believed to be associated with land exploitation activity in the forest reserve's peripheral area. The fire, initially lit for land clearing purposes, promptly spread to the nearby forest during drought, causing devastating effects on the ecosystem. The peatland fires affect not only the adjacent forest reserve's well-being but also the health of local people, as they can cause air pollution and, in turn, respiratory disease (Hein et al., 2022). Despite these threats, effective conservation planning and efforts can help combat the adverse impact of land conversion and forest fire on peat swamp forests (Charters et al., 2019).

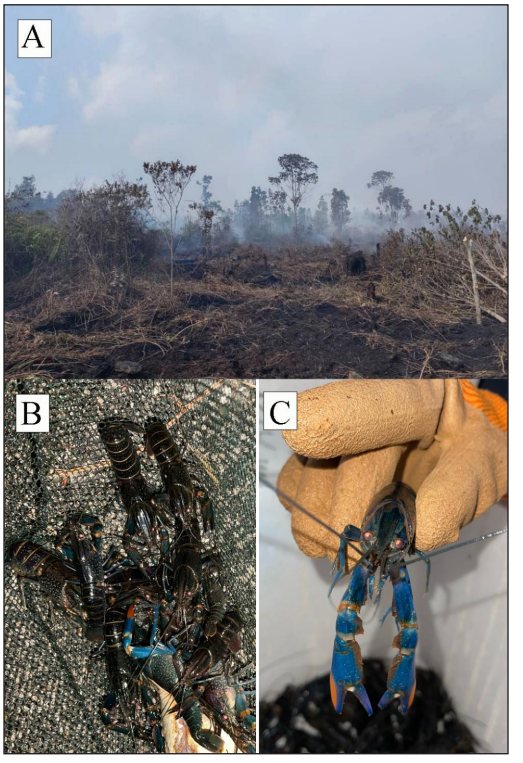

Figure 4. A) The 2020 forest fire in Ayer Hitam Utara State Park Forest resulted in the catastrophic destruction of vegetation and peat soil. This devastation is linked to agricultural activities, including land clearing and the drainage of peatlands; B) and C) The red-claw crayfish, *Cherax quadricarinatus,* from the adjacent area of AHUSPF (Photo by Norhidayah Haris)

Apart from land opening for plantation purposes, the potential threat of alien species also warrants consideration. While this study did not record the presence of alien species within the AHUSPF area, there have been reports of the red-claw crayfish, *Cherax quadricarinatus* von Martens, in AHUSPF peripheral areas, likely resulting from accidental or deliberate release from aquaculture facilities (Figures 4B and 4C). Originally native to freshwater habitats of Australia and Papua New Guinea, *C. quadricarinatus* has already established feral populations in many parts of the world, including Malaysia and Singapore (Ahyong & Yeo, 2007; Naqiuddin et al., 2016). This species was first introduced in Malaysia, specifically in Johor states, for the aquarium and aquaculture industry in the 1980s (Naqiuddin et al., 2016).

Cherax quadricarinatus is recognised as an invasive species due to its burrowing behaviour, which causes damage to riverbanks and generates public concern (Jabatan Pertanian Malaysia, 2021). Its high tolerance to low dissolved oxygen and pH environments (Haubrock et al., 2021; Reynolds et al., 2013) has enabled its appearance in the blackwater canal at the AHUSPF border. Although not currently found within AHUSPF, the connected drainages between the reservoir and the forest streams and swamps could serve as potential dispersal corridors, posing a threat to the native species of AHUSPF. Its adults' ecological roles are similar to benthic fishes, potentially leading to competition and disadvantages for the native fishes (Reynolds et al., 2013). Raising awareness of the possible impact of this alien species on native fishes and fostering collaboration between the local community and the Fishery Department is essential to combat the expansion of the red-claw crayfish population.

Six species from AHUSPF require significant attention from all stakeholders based on their IUCN Red List status. *Parakysis verrucosus*, *Bihunichthys monopteroides*, and *Betta coccina* have a VU status. *Encheloclarias curtisoma* and *Betta persephone* are classified as EN. The only species in CR status is *Betta omega*. These six species are all classified as stenotopic to acid blackwater and acid water, which rely heavily on peat swamp forests for their survival to maintain their already distressed population. The findings of *Encheloclarias curtisoma* in this study have increased its distribution range to the southwest of Peninsular Malaysia and lessened the pressure of this species to become extinct regionally. Its congener *Encheloclarias kelioides*, reported from Pahang and the east of Johor, has been assessed as CR and ostensibly extinct (Ahmad, 2019). *Betta persephone* is another species with a narrow distribution, i.e., endemic to the western part of Johor (Tan & Ng, 2005).

Given the recent rapid land utilisation for development, AHUSPF has become the last extensive haven for this species. The risk of endemic species extinction due to human socio-economic disturbances is a pressing concern in conservation biology. The increase in human population and socio-economic hardships have contributed to the erosion of biological diversity much

faster than historical extinction rates (Fonzo et al., 2013; Semmelmayer & Hackländer, 2020). The high rate of discovering fish species that are new to science and high endemicity has highlighted the importance of the peat swamp forest ecosystem (Azmai et al., 2020; Kottelat et al., 2006; Tan & Ng, 2005). For instance, *Betta omega*, a recently described species found in the remnants of the peat swamp (i.e., oil palm plantation ditches) in Johor's southwestern region, faces challenges due to the lack of connection to healthy forests, resulting in its CR status (Ahmad & Low, 2019; Tan & Ahmad, 2018). Endemic species face an even higher extinction risk due to expected habitat loss, particularly in regions where these remaining endemic species are largely unprotected (Gonçalves-Souza et al., 2020). Nonetheless, this study has provided some relief as the population of *Betta omega* in AHUSPF is extended and in better ecological condition compared to its type locality.

Protected areas like AHUSPF play a crucial role in conserving biodiversity; however, they may not be sufficient to negate the impact of anthropogenic species extinction (Ceballos et al., 2020). To effectively mitigate the risk of endemic species extinction in AHUSPF, conservation strategies should implement a comprehensive approach that considers the intricate interplay between human activities and societal response (Lee & Jetz, 2011). It calls for collaborative efforts involving various stakeholders at local and national levels. Integrating technological advancements alongside economic and regulatory stimuli could encourage local communities, farmers, consumers, and companies to adopt sustainable practices (Albert et al., 2021; Barbier, 2017).

Besides, documenting and assessing the local extinction threat faced by endemic species is of utmost importance. The state and national species red list needs to be meticulously developed and consistently updated to identify and address local threats effectively. The most essential and ongoing efforts are to strengthen public education and raise awareness about the conservation of AHUSPF and its endemic species through awareness campaigns, educational programs, and active community engagement. While in species-specific conservation priorities, it is crucial not to overlook miniature species, as the conventional approach tends to prioritise larger and charismatic species, inadvertently risking the extinction of small endemic fishes that inhabit the peat swamp forest (Collares-Pereira & Cowx, 2004). Here *Betta persephone* is proposed as the prime candidate and theme for targeted conservation efforts within AHUSPF.

Continuous and comprehensive monitoring of the fish assemblage in AHUSPF is vital to detect species and ecosystem health changes. Early detection enables prompt and appropriate action by stakeholders to prevent biodiversity decline. Future studies should investigate the link between fish species abundance and composition with AHUSPF's ecosystem to understand how the changes in fish diversity affect the ecosystem. More information is

needed on the past and current condition of AHUSPF and its freshwater fish diversity to predict their future response to changes. Additionally, evaluating AHUSPF efficacy as a secure haven for freshwater fish through comparisons with agriculture areas, monoculture sites, and other degraded ecosystems will yield valuable insights to bolster conservation efforts and promote sustainable practices. AHUSPF can strengthen its role in safeguarding endemic species and contribute significantly to global conservation efforts.

CONCLUSION

The preliminary checklist of fish species in this study underlines the significance of embracing sustainable practices to protect the rich fish diversity within AHUSPF. These measures and the proposed conservation initiatives serve as invaluable tools in advocating biodiversity preservation and sustainable management, especially in the face of mounting anthropogenic pressures. Despite being a protected area, the AHUSPF faces challenges from surrounding land use and invasive alien species, which should not be underestimated. Collaborative initiatives, public engagement, and continuous monitoring are imperative to realise the proposed conservation efforts and guarantee this vital ecosystem's long-term health and resilience. By aligning conservation efforts with sustainable practices, a harmonious coexistence between human activities and nature will be fostered, ensuring the lasting protection of AHUSPF's precious biodiversity for generations to come.

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Review Article

The Jewel Orchids of Crocker Range Park, Sabah, Malaysia: A Review

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ABSTRACT

Jewel orchids are usually recognised by their distinctive foliar and can be found in undisturbed habitats, such as Crocker Range Park, Sabah. The pristine habitat in the park provided a suitable condition for jewel orchids to thrive in their habitat. In Malaysia, jewel orchids are appreciated for their ornamental value due to their uniqueness. At the same time, in other countries, it was reported that jewel orchids are used as medicinal plants due to their medicinal properties. Although jewel orchids have been recorded in Sabah, specific studies and species checklists have yet to be made for the Crocker Range Park due to insufficient data. Moreover, the current conservation status of all recorded jewel orchid species in Sabah remains unknown. Therefore, this study comprises a review of information on jewel orchid species in the park, including other general or worldwide diversity, economic importance, conservation status, and action plans. This review used secondary data, including fieldwork collections, checklists, published journals, and available online data. From the review, eight genera and ten species of jewel orchids have been recorded in the park. Three endemic species were found from all recorded species: *Anoectochilus monicae* J. J. Wood, *Goodyera condensata* Ormerod & J. J. Wood, and *Goodyera rostellata* Ames & C. Schweinf. The outcome of this study can be utilised in the upcoming diversity study on jewel orchids in the park while providing the baseline for a management plan for the conservation of jewel orchids in the park.

Keywords: Borneo, checklist, conservation, endemic, Goodyerinae, IUCN, Orchidaceae

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INTRODUCTION

Borneo is notable for its richness of orchid diversity, which includes 2,500–3,000 species, or 10% of all orchid species worldwide and 75% of the Malesian orchid

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flora (Lamb, 1991). Crocker Range Park (Figure 1), close to Kinabalu National Park, is renowned for having high biodiversity within its forest ecosystem. Crocker Range Park, situated at the northern edge of Mount Kinabalu, has largely intact vegetation. Crocker Range Park is one of the largest protected areas in East Malaysia, covering around 139,919 ha. The park was denoted as a state park in 1984 to preserve the region's ecosystem and natural resources. The highest peak of this park is 1,670 m a.s.l, and it also consists of mountains, hills and small basins resulting from a dissected deep river valley.

The highest point of Crocker Range Park is Mount Alab (1,951 m), which extends to Tenom town. Crocker Range Park has become one of the interesting spots for wild orchid study in Sabah because it still has an undisturbed mixed Dipterocarp Forest and stretches of montane mossy forest near the summit. The higher slopes of the park are dominated by moss forests, rhododendrons, and a variety of wild orchids (Das, 2006).

Orchid belongs to the family Orchidaceae and is notably known for its remarkable floral diversity and its ability to adapt to attract pollinators. Amongst all flowering plants, Orchidaceae is considered one of the largest and most diverse families, which comprises 736 genera with around an estimated 27,000 species overall (Chase et al., 2015). It has various vegetative and floral morphology, which confers a high taxonomic and ornamental importance. The presence of jewel orchids in Crocker Range Park has been recorded in various

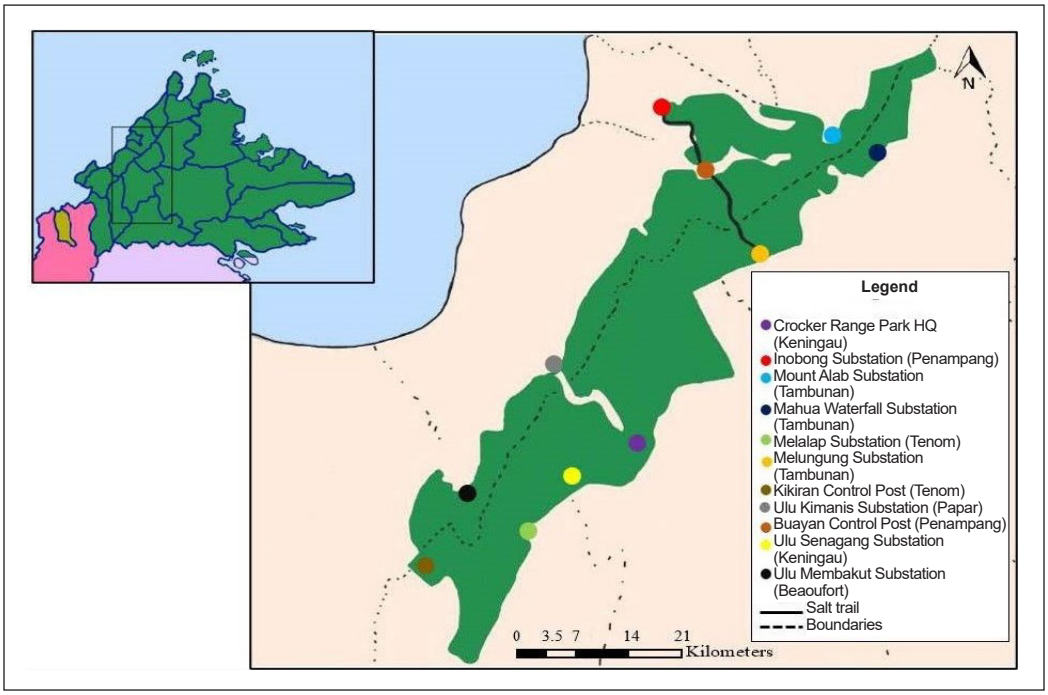

Figure 1. Map of Crocker Range Park in Sabah
Note. This map was adapted from Suleiman et al. (2017)

documentation, as included in Majit et al. (2014), Wood (2004), Wood and Cribb (1994), Wood et al. (1993) as well as Seidenfaden and Wood (1992). However, no comprehensive and exclusive checklist for jewel orchids has been made from Crocker Range Park.

Therefore, this study intends to compile all known data on jewel orchids in Crocker Range Park, including fieldwork collections by various authors over several years, accompanied by additional general information such as their economic importance, conservation status, and conservation action plan. The outcome of this study can be utilised in the upcoming diversity study on jewel orchids in Crocker Range Park while also providing baseline data for the conservation of jewel orchids in the park to preserve the biodiversity of jewel orchids. The data used for this study was gathered from secondary data. The data was extracted from various documents such as literature, checklists, journals, and available online data. The validity of species names and distribution of genera was referred according to the World Flora Online Plant List (WFO), Global Biodiversity Information Facility (GBIF), and Plants of The World Online (POWO), while the status of species is referred to the International Union for Conservation of Nature (IUCN).

SPECIES RICHNESS OF WILD ORCHIDS IN CROCKER RANGE PARK

Ridley published the first-ever Bornean orchids list in 1896, in which he described 49 new species and recorded 224 species in 62 different genera, followed by Masamune in 1942. The number of species found increased to 1,203 in 99 genera. In 1994, Wood and Cribb (1994) listed over 1,400 species of orchids in 149 genera. The most recent compilation of Mount Kinabalu orchids was by Wood et al. (2011), which listed 866 taxa of orchids in 134 different genera. In Crocker Range Park, the first orchid was reported by Cribb and Vermeulen (1991), followed by Chan et al. (1994), which reported 16 species of orchids in 15 genera and was continued by Wood (2001).

The first scientific expedition at Crocker Range Park was dated back in 1999, as reported by Latiff et al., where the authors reported three species of orchids from Mount Alab (Latiff et al., 2001). The second scientific expedition was done in 2002 by Rimi et al., who reported 12 species of orchids from Ulu Kimanis (Rimi et al., 2004). The next expedition was in 2004 by Miadin et al. called the Melalap Scientific Expedition. The expedition recorded 37 orchid species in 23 genera (Miadin et al., 2005). The most recent expedition in Crocker Range Park in the past ten years was reported by Majit et al. (2011), where 149 species in 33 genera were recorded, while in 2014, the authors updated the checklist and currently, a total of 342 species in 102 genera of wild orchids were documented in Crocker Range Park. One of the documented orchids subtribes is Goodyerinae, commonly known as the jewel orchids. Jewel orchids have been documented in various documentation, and some species are considered endemic species for Borneo.

Checklist of Jewel Orchids in Crocker Range Park

The most recent study on wild orchids in the Crocker Range Park was conducted by Majit et al. (2014). Up until recently, species of jewel orchids in the park were included in several publications or checklists such as in Majit et al. (2014), Seidenfaden and Wood (1992), Wood (2004), Wood and Cribb (1994) as well as Wood et al. (1993). Hence, based on the record of wild orchids in Crocker Range Park, eight different genera and ten species of jewel orchids are recorded in the park area. The recorded genera are *Anoectochilus* Blume, *Cystorchis* Blume, *Goodyera* R. Br. in W. T. Aiton, *Hetaeria* Blume, *Hylophila* Lindl., *Lepidogyne* Blume, *Vrydagzynea* Blume, and *Zeuxine* Lindl. Some species recorded are endemic to Borneo and Sabah (Table 1).

Table 1
Genus and species recorded in Crocker Range Park

Genus	Species	General distribution	IUCN (2022)	References
Anoectochilus	*Anoectochilus monicae* J. J. Wood*	Sabah	NE	Majit et al. (2014); Wood (2004)
Cystorchis	*Cystorchis variegata* Blume	Sabah, Peninsular Malaysia, Sumatra, Java, and Vanuatu	NE	Majit et al. (2014); Wood et al. (1993)
Goodyera	*Goodyera condensata* Ormerod & J. J. Wood*	Sabah	NE	Wood et al. (1994)
	Goodyera rostellata Ames & C. Schweinf*	Sabah	NE	
	Goodyera procera Hook.	Borneo, India, Sri Lanka, Myanmar (Burma), Thailand, Indonesia, China, Taiwan, Japan, and Philippines	NE	
Hetaeria	*Hetaeria alta* Rindl.	Borneo (Sabah), Peninsular Malaysia, and Peninsular Thailand	NE	Majit et al. (2014); Seidenfaden and Wood (1992)
Hylophila	*Hylophila lanceolata* (Blume) Miq.	Sabah, Kalimatan, Thailand, Sumatra, Java, Flores, and the Philippines, and is newly recorded in Peninsular Malaysia	NE	Majit et al. (2014); Wood and Cribb (1994)
Lepidogyne	*Lepidogyne longifolia* (Blume) Blume	Sabah, Peninsular Malaysia, east to Philippines, and New Guinea	NE	
Vrydagzynea	*Vrydagzynea albida* (Blume) Blume	Borneo, India, Thailand, Vietnam, Peninsular Malaysia to the Phillipines, and New Guinea	NE	Majit et al. (2014); Seidenfaden and Wood (1992)
Zeuxine	*Zeuxine gracilis* (Breda) Blume	Sumatra, Java, and Krakatau, as well as in Borneo and Peninsular Malaysia	NE	Majit et al. (2014); Seidenfaden and Wood (1992); Wood and Cribb (1994)

Note. * = Endemic species to Sabah; NE = Not Evaluated

Genus *Anoectochilus* Blume. Genus *Anoectochilus* is a terrestrial herb and can be spotted by its green leaves with colourful silvery or red nerves. Their flower is usually white or pink flushed and has a short peduncle inflorescence with few flowers. Only one species from this genus has been recorded in the park. *Anoectochilus monicae* J. J. Wood is a Sabah endemic terrestrial orchid found in hill and lower montane forests within the altitudinal range of 200–900 m (Wood, 2013). This species has very dark green leaves. The nerve on the leaf is gold, sometimes red or pink, reticulate on the above of the leaf, while it can be pinkish purple on the underside of the leaf. In addition, *A. monicae* also has hairy, showy flowers with pink or white sepals and white petals.

Genus *Cystorchis* Blume. Another genus recorded is genus *Cystorchis*. This genus is also a terrestrial herb with a few leaves on its stems. They have green, purplish and sometimes variegated leaves. In contrast, in a saprophytic species, the leaves on the stem are brown scale leaves. The inflorescence is dense with few to many flowers and has small flowers. *Cystorchis variegata* Blume is the only species in this genus recorded in the Crocker Range Park. This species of orchid has a vast distribution. It is a terrestrial orchid found on hills or lower montane forests with a 600–1,500 m elevation range. The leaves of *C. variegata* are light green with a dark green network and have a slightly undulating margin. The erected inflorescence is covered with short hairs, while their flower is white to off-white (Lok et al., 2011).

Genus *Goodyera* R. Br. in W. T. Aiton. Among all recorded genera in the Crocker Range Park, *Goodyera* is the only one that recorded more species than other genera. Orchids in the genus *Goodyera* are terrestrial and rarely epiphytic. Their leaves are ovate, asymmetric, and sometimes variegated, while their flower colour can be white, pale green or pink to purplish. *Goodyera condensata* Ormerod & J. J. Wood is a species from this genus that was recorded in the park. This species inhabits rocky terrain covered in alluvial soil close to waterfalls in deep mixed lower montane *Castanopsis*/*Lithocarpus* forests, which range in elevation from 1,400 to 2,700 m (Hu et al., 2016). They have green leaves with fine white reticulate nerves on the upper surface of the leaf with white coloured flowers and have a musky scent. Another species that was found is *Goodyera rostellata* Ames & C. Schwein. It is a terrestrial orchid in the lower montane forest about 900-2,700 m elevational range. Both *G. condensata* and *G. rostellata* is endemic to Sabah. Apart from these two species, another recorded species from this genus is *Goodyera procera* Hook. Like the other species in this genus, *G. procera* is also a terrestrial orchid. It can be found in the upper montane forest with a 2,200–2,400 m elevation range along the forest trails or on a moss-covered rock.

Genus *Hetaeria* Blume. Genus *Hetaeria* is another genus recorded in the park. They are usually terrestrial herbs and have broad, ovate to elliptic, asymmetric green leaves

with small flowers and raceme inflorescence (Wood & Cribb, 1994). Only one species in this genus was recorded. *Hetaeria alta* Rindl. lives in humus and can be found in lowland and hill forests (limestone) with a 100-500 m elevation range. They have ovate to elliptic leaves and have short, hairy yellow flowers.

Genus *Hylophila* Lindl. Genus *Hylophila* is another additional genus to all recorded genera in the park. Species in this genus are terrestrial with fleshy and creeping rhizomes. Their leaves are dark green, ovate to elliptic, and often asymmetric with small, pale greenish or reddish orange, softly hairy flowers, and dense terminal flower inflorescence. Only one species in this genus was recorded. *Hylophila lanceolata* (Blume) Miq. can be found in a damp and shady area on a mossy rock in the hilly or lower montane forest with 900–1,500 m of elevation range (Wood et al., 1993).

Genus *Lepidogyne* Blume. Another recorded genus is the *Lepidogyne*. They are tall terrestrial orchids. Their leaves are grouped at the base and elliptic with reddish-brown flowers and dense inflorescence (Niissalo & Choo, 2021; Wood et al., 1993). *Lepidogyne longifolia* (Blume) Blume is the only species in this genus that has ever been recorded in the park. Their natural habitat is in hill and lower montane forests (oak-laurel) with an elevation of 900-1,500 m and can be found on the ultramafic substrate.

Genus *Vrydagzynea* Blume. Species in the genus *Vrydagzynea* are usually terrestrial orchids with decumbent rhizomes and root nodes. They have weak, fleshy steam with few green and median white strip leaves. Their flower is small, resupinate, and has a short, dense inflorescence (Schuiteman et al., 2015). Only one species of this genus has ever been recorded in the park. *Vrydagzynea albida* (Blume) Blume can be found on limestone soils along stream banks in hill and montane forests with an elevational range of 300-1,500 m. Flowers of *V. albida* are pale greenish and white.

Genus *Zeuxine* Lindl. Last but not least, the recorded genus in Crocker Range Park is the 'grass-like' herbs genus *Zeuxine*. Like the genus *Vrydagzynea*, *Zeuxine* is a terrestrial orchid with a decumbent rhizome and root nodes. They also have a similar weak, fleshy stem. However, the general characteristics of the leaves and flowers of these two genera are different. Leaves of the genus *Zeuxine* are green or purplish, ovate, linear, or elliptic with the coloured median nerve, while their flowers are white or green, small, and hardly open with dense, terminal hairy inflorescence. The only recorded species from this genus is *Zeuxine gracilis* (Breda) Blume. This species inhabits low-elevation oak-laurel forests, mixed-dipterocarp forests, secondary forests, and occasionally ultramafic substrates (Ormerod, 2018).

GENERAL CHARACTERISTICS AND DISTRIBUTION OF JEWEL ORCHIDS

The most recent phylogenetic position of the subtribe Goodyerinae results from the

persistent study on this subtribe for over 40 years. The first ever hypothesis about this subtribe was made by Dressler (1982), which proposed that Goodyerinae were related to the Tropidieae because of their long rhizomes and scattered roots. However, these plants have since been demonstrated to be in the family Epidendroideae (Cameron et al., 1999; Chase et al., 2015). It was then discovered that Goodyerinae is related to Cranichidae due to the similarity of their floral characteristics (Chen et al., 2019). The members of the jewel orchids are closely related to each other as they are all members of the subfamily Orchidoideae, tribe Cranichideae, and Subtribe Goodyerinae (Hayden, 2016). However, despite their prominent criteria, it does not represent a specific genus in the group (Indan et al., 2021).

Commonly, orchids are noticeable because of their appealing, showy flower, but some are appreciated for their alluring foliage. Jewel orchids are usually distinguished by their distinct leaf shape, colour, and venation (Bhattacharjee & Chowdhery, 2012). The distinctive feature of reticulate venation combined with plain green or various colours of its leaves contributes to the name 'jewel orchid' (Smidt et al., 2021), sometimes known as the 'marbled jewel orchid'. They are mostly terrestrial and lithophytic, which explains why they can be found on rocks and are seldom epiphytes (Juswara, 2010). They are usually easy to be noticed due to their distinctive habit. They have a prostrate rhizome that produces moniliform and hairy roots from their node, which appears as a creeping rhizome, which allows them to attach to the media (Dressler, 1993). However, some species may only possess rhizoids and have no roots. The rhizome also produces a relatively thinner stem compared to the rhizome. Depending on the species, the stem will produce leaves in a spiral arrangement, either sessile or with the petiole. The flower of the jewel orchid is small (about 0.5–150 mm long) and has a variety of colours, such as white, pale green, yellowish, brownish, and pinkish flowers. Nevertheless, most of the time, the colour of the flower is white to pale green.

Genera of Goodyerinae subtribe can be found across the continent due to their vast distribution in Asia, South America and North America (Smidt et al., 2021), as shown in Table 2. Asia has the most significant distribution of jewel orchids, with 29 genera and 510 species (Ormerod, 2008), followed by Nearctic and Neotropical America, with nine native genera and 280 species (Ormerod, 2013). Based on Table 2, 16 genera of jewel orchids can be found in Malaysia.

The distribution of the genera is highly influenced by geography and local climate. Based on the Global Biodiversity Information Facility (GBIF) database, 16 genera of Goodyerinae subtribe have been recorded in Malaysia in recent years. Eight genera have been recorded in Peninsular Malaysia, while 14 are in Sabah and Sarawak. From the record, the genus *Dossinia* C. Morren has the most abundant distribution in Malaysia compared to other

countries that have recorded the genus, such as Vietnam, Indonesia, India, and Chinese Taipei. Most species recorded in Malaysia are also recorded in other countries. In contrast, some species can only be found in Malaysia or are endemic to Malaysia—for example, *Anoetochilus monicae* J. J. Wood and *Dossinia marmorata* C. Morren.

Table 2
Genus of subtribe Goodyerinae listed based on Chase et al. (2015) as well as Wood and Chan (2003) and their general distribution according to Royal Botanic Kew Gardens, Plants of The World Online

Genus	General distribution
Aenhenrya Gopalan	India
Anoectochilus Blume*	Ranging from Tropical and Subtropical Asia to the Pacific
Aspidogyne Garay	Ranging from Tropical and Subtropical America
Chamaegastrodia Makino & F. Maek.	Ranging from Assam to Japan
Cheirostylis Blume*	Ranging from Tropical & South Africa to the West Pacific
Cystorchis Blume*	Ranging from Thailand to the West Pacific.
Danhatchia Garay & Christenson	New South Wales, New Zealand North, New Zealand South
Dossinia C. Morren*	Borneo
Erythrodes Blume*	Ranging from Tropical and Subtropical Asia to the Southwest Pacific
Eurycentrum Schltr.	Ranging from Papuasia to Vanuatu
Gonatostylis Schltr.	New Caledonia
Goodyera R.Br. in W.T.Aiton*	Ranging from Europe, Madeira, Mozambique, and the West Indian Ocean to the Pacific, North and Central America, and Hispaniola.
Halleorchis Szlach. & Olszewski	Cameroon, Gabon
Herpysma Lindl.	Ranging from the Himalayas to South Central China and Sumatera
Hetaeria Blume*	Ranging from West Tropical Africa to Tanzania, the West Indian Ocean, Tropical and Subtropical Asia to the West Pacific
Hylophila Lindl.*	Ranging from Taiwan and Thailand to Papuasia
Kreodanthus Garay	Ranging from Tropical and Subtropical America
Lepidogyne Blume*	Ranging from Malesia to New Guinea
Ludisia A.Rich.*	Ranging from South China to West and Central Malesia
Macodes Lindl.*	Ranging from South Nansei-shoto, Vietnam, to Vanuatu
Microchilus C.Presl	Ranging from Mexico to Tropical America
Odontochilus Blume*	Ranging from the Himalayas to the Kuril Islands and Southwest Pacific
Orchipedum Breda*	Ranging from Indochina to West and Central Malesia
Pachyplectron Schltr.	New Caledonia
Papuaea Schltr.	New Guinea
Rhomboda Lindl.*	Ranging from Tropical and Subtropical Asia to the Southwest Pacific
Schuitemania Omerod	Philippines
Stephanothelys Garay	Ranging from Tropical and Subtropical America
Vrydagzynea Blume*	Ranging from Tropical and Subtropical Asia to the West Pacific
Zeuxine Lindl.*	Ranging from Tropical Africa to Central Asia and the West Pacific

Note. * = Found in Malaysia

ECOLOGY OF JEWEL ORCHIDS

The jewel orchid is a terrestrial plant that grows from the ground. Naturally, they grow well in damp conditions with low-medium-intensity sunlight. They are keen on the tropical environment and are mostly found in Indochina and Pacific Islands jungle forests, including Malaysia (Krumov et al., 2022).

Due to the natural mountainous landscape in Crocker Range Park, the forest zoning in Crocker Range Park can be divided into their respective vegetational zone according to their elevational zone above sea level (Lowland dipterocarp forest, below 300 m a.s.l; Hill dipterocarp forest, 300–800 m a.s.l; Upper dipterocarp forest, 800–1,300 m a.s.l; Lower montane forest, 900–1,800 m; Upper montane forest, above 1,800 m) (Majit et al., 2014). Even though the exact localities of jewel orchids distribution in Crocker Range Park are unspecified, the recent evidence of jewel orchids' presence in the area has been documented in a preliminary checklist by Majit et al. (2014). Due to their ideal environment and habitat, jewel orchids live well in the park area. They typically reside on the forest floor in shaded, cool, humid, wet, but well-drained forests. The thick forest canopy provides enough shade for the jewel orchids. Additionally, the build-up of green-black mosses and dead tree leaves creates a thick layer of decomposing humus favourable for terrestrial orchids like jewel orchids (Besi et al., 2021). Correspondingly, the constant humidity in the tropics continuously adds water from the air to the forests.

The undisturbed ecology in the park also provides an excellent environment for jewel orchids. Since Crocker Range Park was gazetted as a forest reserve through the Sabah Park Enactment 1984, the park's governance has been authorised by Sabah Park. As the gazettement of forest reserves, the rights of communities living within the boundaries of national parks are extinguished (Toh & Grace, 2005). It means there is no external intervention or exclusive rights for an outsider to do illegal activities such as logging, cultivation, extracting forest products, and others without permission. However, specifically for the management of Crocker Range Park, communities are permitted to stay in their traditional areas and collaborate with Sabah Park to establish traditional use zones within the parking area, which will be covered in the park's management plan. It is because several local villages are located inside the park boundaries to create a 'win-win' situation between the authorities and villagers (Ioki et al., 2019). Nevertheless, the strategy worked very well in creating the ideal ecosystem for conserving flora and fauna in the region. As a result, the presence of jewel and terrestrial orchid species in a forest area signifies an ideal environment.

CONSERVATION STATUS OF JEWEL ORCHIDS

Based on the IUCN Red List status for 2022 in Table 1, all recorded jewel orchid species in the Crocker Range Park are rated as Not Evaluated (NE). As a result, the

current status of the recorded jewel orchids is still being determined. This matter raised concerns because some species may be endangered and extinct in the wild without a proper conservation plan. Nowadays, numerous species are rapidly going extinct globally due to increasing anthropogenic activities such as rising human populations, urbanisation, habitat fragmentation, and greater reliance of the global population on finite natural resources (Woodruff, 2001). Along with the current trend of forest deforestation, overharvesting and changes in the Earth's surface, immediate action must be taken to ensure the livelihood of jewel orchids in the country. Globally, jewel orchids are categorised as an endangered species. For example, in Vietnam, jewel orchids have been included in the Vietnam Red Book of endangered species (Tran et al., 2022), while *Anoectochilus koshunensis* is categorised as an endangered species by CITES checklist (https://checklist.cites.org). This situation is alarming as it may lead to the global extinction of jewel orchids, not only in Malaysia.

The main factor that hinders the conservation of jewel orchids in Malaysia is the need for more available data. It happened due to insufficient attention given to oversee this matter. The negligence of authorities in providing enough data regarding the status and well-being of jewel orchids may be damaging the situation more since no measures were made to minimise the impact of the threat (Wraith et al., 2020). With the unknown current status of the jewel orchids and lack of data on the exact locality, it is hard to determine the population of jewel orchids in the Crocker Range Park.

ECONOMIC IMPORTANCE OF JEWEL ORCHIDS

Although jewel orchids are uncommon in Malaysia, globally, jewel orchids have a variety of functions with great potential to generate economic growth. For instance, in Asia countries like China, Sri Lanka, Taiwan, and Vietnam, certain species of *Anoectochilus* have been used as local medicine widely for a very long time (Pridgeon et al., 2004), as shown in Table 3. The most famous medicinal *Anoectochilus* species is *Anoectochilus formosanus* Hayata. The tuber of *A. formosanus* has anti-cancer, anti-inflammatory, anti-diabetic, and antioxidant properties to prevent hepatitis, hypertension, and cancer due to kinsenode (De et al., 2013; Kayalvizhi et al., 2020). Apart from that, the whole part of *Goodyera schlechtendaliana* Rchb. f. can be used as a tonic to cure internal injuries, improve blood circulation to clear heat, and remove toxins in Traditional Chinese Medicine (TCM) (Dai et al., 2021; Kayalvizhi et al., 2020). Therefore, due to their importance in the medicinal field, they are considered expensive folk medicine (Tseng et al., 2006). Besides their medicinal value, certain species of *Anoectochilus* leaves are also used as vegetables, particularly in Indonesia and Malaysia (De et al., 2013).

Although not all jewel orchids have medicinal properties, most species are appreciated for their beauty and aesthetic

Table 3
Species of jewel orchid and their medicinal properties

Species	Medicinal properties
Anoectochilus koshunensis Hayata	Anti-inflammatory
Anoectochilus formosanus Hayata	Anti-cancer/tumour, convulsive disease, anti-inflammatory, antioxidant, anti-diabetic, high endurance capacity, ameliorative, maturation
Anoectochilus roxburghii (Wall.) Lindl.	Antioxidant
Goodyera schlechtendaliana Rchb.f.	Prevent convulsive disease, remove toxins, prevent fever

value. An example is *Macodes petola* (Blume) Lindl., widely cultivated as a potted and indoor plant (de Loubresse, 2021). With the development of technology and a better understanding of each species, hybrid breeding for jewel orchids is common among jewel orchid breeders. The hybrid's uniqueness will capture the orchid collectors' attention and have its market value.

POTENTIAL THREATS AND CONSERVATION ACTION PLANS

The current situation regarding the country's declining population of orchids has become more concerning as time passes. Sabah, the richest state with orchids, has an estimated 1,300 orchid species with 250 endemic species (Sabah Forestry Department, 2023). Meanwhile, Sabah has lost 39.5% of its forest cover during the past 40 years (Gaveau et al., 2014) and is threatening the population of orchids in Sabah. The main threats that may lead to the extinction of orchid species are logging, land clearance and illegal collection.

Logging

Logging activities, whether legal or illegal, are not a rare scene in Malaysia. Malaysia harvests 128,000 acres and produces 350 million cubic feet of wood annually, making it Malaysia's second highest-earning export after petroleum (Teoh, 2019). The survival of the ground layer's shade-loving plants is directly impacted by the detrimental consequences of opening up the forest canopy, erosion, and stream silting. This act destroys the habitat of orchids and alters the local microclimate and wild orchids' micro-temperature. Wild orchids, like jewel orchids, are susceptible to environmental changes and may decrease their chances of survival. Currently, due to extensive logging, many unique orchid species, such as *Phalaenopsis gigantea* J. J. Sm, *Paraphalaenopsis denevei* (J. J. Sm) A.D. Hawkes, and *Paphiopedilum rothschildianum* (Rchb. f.) Stein, although they are not jewel orchid species, disappeared from the East Malaysia forest (Teoh, 2019).

Land Clearance

The growth of the human population demands a more extensive development area. In developing countries, land areas are cleared for agriculture, traditional farming, and infrastructure. However, timber extraction led to the loss of host trees

and terrestrial orchids' habitats (Teoh, 2019). For example, in some areas of Sabah, local farmers still practice shifting cultivation and clearing land yearly to cultivate cash crops. Additionally, fire is used while clearing land to remove nearby vegetation, which affects the survival of epiphytes and terrestrial orchids (Swarts & Dixon, 2009).

Illegal Collections

Orchid is widely known for its unique features and has an established fan base for its collectors. Therefore, it is unsurprising that orchids are often smuggled or over-collected to meet market demand. Some species of orchids, including jewel orchids, were commonly sold at the roadside and nurseries from Kundasang to Ranau. Also, some foreign researchers and travellers were apprehended while attempting to transport some rare wild orchids from Malaysia into another country (Go et al., 2020). With the convenience of e-commerce such as Shopee and Lazada, the orchid market has grown even more significantly, advertising various orchid species for sale and ready to be shipped to their customer. For endemic species, the orchids are collected by locals and sold to nurseries before being exported to other countries. In the long run, the number of orchid species, especially the endemic species, will decrease and eventually become extinct in their natural habitat.

The degrees of these threats may eventually lead to the extinction of jewel orchids. Hence, an efficient conservation action plan is crucial to ensure the survival of wild jewel orchids in their natural habitat. It includes proper conservation management, aiding with political intervention for total enforcement. The conservation of jewel orchids can be carried out either through *in-situ* conservation or *ex-situ* conservation.

***In-situ* Conservation.** An *in-situ* conservation or habitat conservation plan involves intensive management of the selected area. Habitat conservation is sometimes complicated and calls for common sense, keen observation, and in-depth study of management measures' timing, intensity, and interrelation (Pacicco et al., 2018). This area must not be prone to flooding and must be cleared of any form or disturbance, such as agricultural activities, construction, logging, land clearance, trespasses, orchid poachers, and other potential threats. The area's ecology, namely its temperature range and relative humidity, must be optimum and suitable for jewel orchids' vegetation. Several initiatives can be done in the conservation area, such as reintroducing jewel orchid species in their original habitat or relocating the species if the area is threatened by other means. It can be done by replanting wild orchid seedlings with their symbiotic mycorrhiza. Orchid seeds usually need a specific mycorrhizal fungus to germinate because fungal elicitors from mycorrhiza promote cell proliferation, seedling development, and metabolite synthesis, while phytoalexins from mycorrhiza protect orchids from pathogens (Teoh, 2019).

In addition, wild orchids also need a particular insect pollinator and a specific

group of plants to complete their life cycle (Seaton et al., 2013). Another initiative is to design an allotted harvesting area or collect forest resources. Rural communities often rely on forest resources for food, crafts, medicine, and more to sustain their living. However, the total restriction within the conservation area may hinder their norms. Therefore, by allocating a specific area for these people to collect forest resources, they can obtain them without disturbing the whole area.

***Ex-situ* Conservation**. *Ex-situ* conservation refers to the conservation mode that is not taken in the original habitat. It can be done in other places, such as in botanical gardens and nurseries, with means to preserve the species. This method must be done thoroughly, considering the site's ecology. An altered and unsuitable ecology may destroy the orchids as they may not adapt to the new place. An estimated one-third of botanic gardens maintain orchid collections or scientific programmes, making them traditional focal points for orchid research and presentation (Swarts & Dixon, 2009). For example, the Royal Botanic Garden Kew and New York Botanical Garden have collected a large collection of living orchid specimens for display and research. Therefore, all these botanical gardens and nurseries act as the primary organisations participating in programmes for the conservation and reintroduction of orchids and are a global conservation resource with a long history of excellence in horticultural and taxonomic science.

Another conventional method in *ex-situ* conservation is by creating a germplasm gene bank. Gene banks play a significant role in orchid conservation by preserving orchids' genetic material, especially threatened orchid species (Merritt et al., 2014). These germplasms are mostly collected either in or outside their natural habitat. In India, ICAR-NRC for Orchids, Pakyong focuses solely on orchid breeding and the development of high-quality planting materials, both of which are thought to be important bottlenecks in the growth of the orchid industry in the country (Meitei et al., 2019). This institute is essential for the orchid study conducted to collect, characterise, evaluate, conserve, and utilise the genetic resources accessible in the nation, particularly in the northeastern region of India (De & Pathak, 2018).

Moreover, all these genetic materials can also be used to clone orchid species *in vitro* and reintroduce them to their original habitat to ensure their longevity (Brundrett et al., 2003). Also, orchids produced via *in-vitro* can be used as stock plants, decreasing the need to collect orchids in the wild, especially for medicinal orchids such as *A. formosanus*. Most of the time, after determining the targeted orchid species' natural habitats, their seeds are gathered, and the plants may be reintroduced to the specified habitat after being multiplied in large numbers by tissue or seed culture to establish the population and allow multiplication under natural conditions (Medhi et al., 2012). However, due to the heterozygous and diverse genetic structure

of orchids, along with a very high degree of cross-fertilisation, the genetic structure of distinct accession of a species cannot be preserved (Davies, 2009). Therefore, this technique is likely suitable for preserving orchid species' biodiversity.

CONCLUSION

Eight genera and ten species of jewel orchids have been recorded in Crocker Range Park. Among all recorded species, three species are endemic to Sabah. They are *Anoectochilus monicae*, *Goodyera condensata*, and *Goodyera rostellata*. It highlighted the importance of law enforcement in Crocker Range Park by Sabah Parks and Sabah authorities.

Extensive studies in Crocker Range Park regarding orchid diversity, especially for jewel orchids, must be done in the future, considering that the latest study of orchids in the park was almost ten years ago. Apart from that, until this date, there has yet to be a complete checklist of jewel orchids in the park or Sabah generally. It raises concern about the status of these documented and undocumented orchids as they need to be updated. They may be endangered due to illegal logging, overharvesting, or other internal or external factors. Thus, systematic studies about orchids must be done to conserve the flora.

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Review Article

Hexanal Treatment for Improving the Shelf-life and Quality of Fruits: A Review

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ABSTRACT

Fruits are rich sources of bioactive compounds such as lycopene, tannins, β-carotene, resveratrol, and lignan. These bioactive compounds' antioxidative, antimicrobial, and antidiabetic properties are important in the human diet. Since fruits are one of the major sources of health-promoting nutrients for human consumption, they have high economic value. Ripening is a developmental process which involves changes in the colour, texture, taste, and metabolite composition of fruits, thus affecting their quality. In the market, the good quality of fruits depends on the ripening stage. Rapid ripening could shorten the shelf-life and quality of fruits. Shortened shelf-life causes fruit spoilage during post-harvest, transport, storage, and distribution. In turn, it will cause economic losses in the fruit market. Low-temperature storage is one of the techniques to prolong the shelf-life of fruits. However, this technique requires an expensive facility to achieve and maintain the low temperature. On the other hand, it has been reported that hexanal treatment could prolong the shelf-life and quality of fruits. It is also cheaper and easier to apply. However, the ripening inhibition mechanism of hexanal is not yet fully understood. The effectiveness of hexanal treatment on different fruits is also unclear. Numerous publications on hexanal

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treatment reported different formulations, techniques, and effectiveness rates on different fruits. Optimised formulation and technique are important to develop an efficient hexanal treatment strategy. Therefore, the mechanism, effectiveness, formulation, technique, and development of hexanal-based products to prolong the shelf-life of fruits are discussed in this review.

Keywords: Antimicrobial, biosafety, hexanal, phospholipase D, post-harvest, ripening, shelf-life

INTRODUCTION

Fruits are major nutrient sources for human consumption. Thousands of bioactive compounds, such as lycopene, tannins, β-carotene, resveratrol, and lignan, are found in fruits (Dhalaria et al., 2020). Studies have shown that the bioactive compounds have antioxidant, antimicrobial, and antidiabetic properties (Karasawa & Mohan, 2018). The consumption of bioactive compounds has a beneficial impact on human health. In addition, fruits have high economic value as fruit production contributes to the economic development of a country. The production and demand for fruits have increased worldwide. According to the Food and Agriculture Organization of the United Nations (FAO) (2023), about 933 million tonnes of fruits were produced in 2022. The major fruits, such as watermelons, bananas, grapes, oranges, and apples, contributed to 52% of total production worldwide in 2022. Bananas, a tropical fruit, are one of the major traded fruits globally, with an estimated value of up to USD11 billion in 2017 (Voora et al., 2020). Therefore, studying fruit ripening and extending its shelf-life is crucial to improving fruit production to meet the growing demand for fruit.

Ripening is a fruit developmental process. As the fruit ripens, coordinated and complex metabolic pathways are involved. The texture, taste, colour, and metabolite content of fruits change due to ripening. Ethylene plays an important role in fruit ripening. The ripening molecular mechanisms mediated by ethylene were reported in apples (*Malus domestica*) (Lv et al., 2023) and bananas (*Musa acuminata*) (Chang & Brecht, 2023). In durian (*Durio zibethinus*), a gene related to ethylene perception, *DzETR2*, was up-regulated during ethylene-induced ripening (Thongkum et al., 2018). Fruit softening is an important event during ripening that affects the quality of fruits, which is associated with membrane deterioration (El Kayal et al., 2017). Phospholipase D (PLD) is a key enzyme in the membrane degradation pathway (Jincy et al., 2017). The inhibition of PLD has resulted in the enhanced shelf-life and quality of bananas (L. Li et al., 2022). Therefore, studying the ripening mechanism is important for developing post-harvest techniques to improve the shelf-life and quality of fruits.

Rapid ripening shortens fruits' shelf-life. Fruit spoilage can occur at any point during harvest, transport, storage, and distribution, which leads to profit losses in the fruit market (dos Santos et al., 2020;

Gustavsson & Stage, 2011). For example, durian has a limited shelf-life of three to four days at room temperature (Tan et al., 2019). During the durian season, typically from May to August, durians are spoiled in abundance before reaching consumers due to their limited shelf-life. Therefore, an efficient method to improve or prolong the shelf-life of fruit is important. Low or chilling temperature storage is one of the most used methods to prolong the shelf-life of fruits. However, it requires expensive facilities to achieve and maintain a low or chilling temperature for storage. Furthermore, certain fruits are prone to chilling injury due to chilling temperature (Razali et al., 2022). In addition, low or chilling temperatures could also alter the taste, aroma, and quality of fruits.

Hexanal is a naturally produced compound from plants. It is generally recognised as safe (GRAS) material. Hexanal treatment could prolong the shelf-life and quality of several fruits. Delayed ripening of fruits treated with hexanal was observed in many fruits, such as cherry (*Prunus avium*) (Sharma et al., 2010), blueberry (*Vaccinium cyanococcus*) (Songe et al., 2010), and mango (*Mangifera indica*) (Silué et al., 2022). Hexanal treatment inhibits the expression and activity of the enzyme PLD (El Kayal et al., 2017). However, the complete mechanism of delayed ripening by hexanal treatment remains unclear. The formulation and effectiveness of hexanal treatment on different types of fruits also vary. Therefore, these are discussed in the present review.

HEXANAL

Hexanal is an organic compound with a molecular formula of $C_6H_{12}O$. Hexanal exists as a colourless, highly volatile liquid. Hexanal is naturally produced by plants with high concentrations found in maise (*Zea mays*) (K. Zhang et al., 2022), tea (*Camellia sinensis*) (Ho et al., 2015), and black walnut (*Juglans nigra*) (Lee et al., 2011), while low concentrations found in thornless blackberry (*Rubus ulmifolius*) (Du et al., 2010) and grapes (*Vitis vinifera*) (Kaya et al., 2022). The United States Food and Drug Administration (USFDA) has approved hexanal as a food additive with a lethal dose (LD_{50}) of 3,700 mg/kg (Khan & Ali, 2018). Figure 1 shows the molecular structure of hexanal.

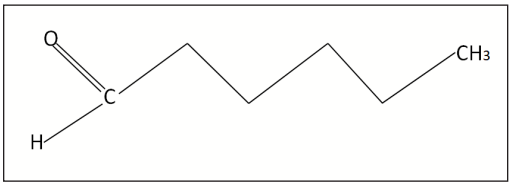

Figure 1. 2D molecular structure of hexanal (National Library of Medicine [NIH], n.d.)

Hexanal Biosynthesis

Hexanal is naturally biosynthesised in trace amounts by certain species of plants. Linoleic and linolenic acids are the biological precursors of hexanal biosynthesis (Pérez et al., 1999). Lipoxygenase (LOX) and hydroperoxide lyase (HPL) metabolic pathways are involved in hexanal biosynthesis (Pérez et al., 1999). Enzymes responsible for hexanal biosynthesis include LOX, HPL, lipolytic acyl hydrolase, and (E,Z)-2,3-enal isomerase (Pérez et al.,

1999). The metabolic pathway of hexanal biosynthesis is shown in Figure 2.

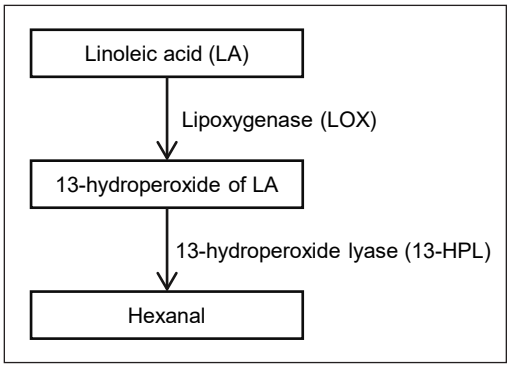

Figure 2. Metabolic pathway of hexanal biosynthesis (Pérez et al., 1999)

Mechanism of Shelf-life Enhancement by Hexanal Treatment

Fruit softening is an important event that occurs during ripening. The decrease in texture and firmness of fruits due to the loss of membrane integrity leads to senescence, which causes post-harvest losses in the fruit market (Padmanabhan et al., 2020). The mechanism of fruit softening involves coordinated and complex enzymatic reactions and pathways (Marangoni et al., 1996). Cell wall degradation is a major cause of fruit softening (Payasi et al., 2009). Cell wall degradation is a complex process that involves diverse protein families such as expansins and cellulase (Payasi et al., 2009; Whitney et al., 2000). In plant cell walls, cellulose microfibrils and xyloglucans are bound by a hydrogen bond (Payasi et al., 2009). Expansins disrupt the hydrogen bond between cellulose microfibrils and xyloglucans (Whitney et al., 2000).

Cellulase hydrolyses β-1,4 glucan linkages in cellulose and xyloglucan (Payasi et al., 2009). These enzyme activities cause cell wall degradation and play an important role in fruit softening. The structure of cellulose and xyloglucan are shown in Figure 3. Understanding ripening mechanisms, including ethylene biosynthesis cell wall, chlorophyll, and phospholipid membrane degradation, is important to develop pre- or post-

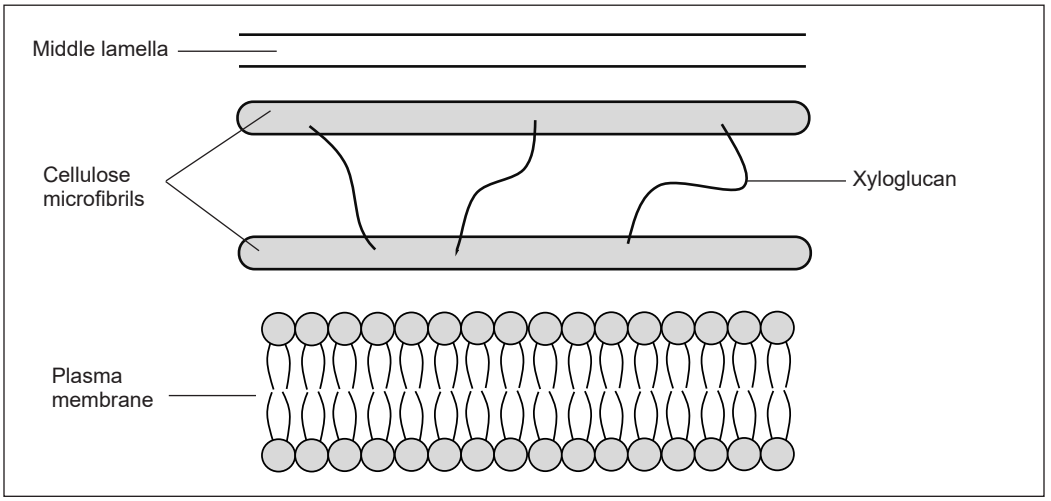

Figure 3. The structure of cellulose, xyloglucan and plasma membrane (Payasi et al., 2009)

harvest techniques to enhance the shelf-life and quality of fruits. For example, 1-methylcyclopropene (1-MCP), which is commercially available as Ethylbloc™ or SmartFresh™, is used to inhibit the ethylene binding to the ethylene receptors in fruits (Thongkum et al., 2018), thus delaying the ripening process, such as in durian (*Durio zibethinus*) and apple (*Malus domestica*) (Lv et al., 2023; Thongkum et al., 2018).

Hexanal treatment is another effective method to enhance the shelf-life and quality of fruits by inhibiting PLD, which is the key enzyme in phospholipid membrane degradation associated with fruit softening during ripening and decay (Jincy et al., 2017; Marangoni et al., 1996). The pre- or post-harvest techniques used to improve the shelf-life of fruits affect the ripening mechanism. Numerous studies have revealed the mechanism of fruit ripening at the genomic, transcriptomic, proteomic, and metabolomic levels in response to hexanal treatment as a promising method to enhance the shelf-life and quality of fruits.

PHOSPHOLIPASE D (PLD)

PLD is a key enzyme in phospholipid membrane degradation during fruit ripening, leading to the loss of membrane integrity, fruit softening, and decay (El Kayal et al., 2017). PLD belongs to the phospholipase enzyme superfamily along with phospholipase A (PLA), phospholipase B (PLB), and phospholipase C (PLC). PLD activity was first described in carrots (*Daucus carota*) (Hanahan & Chaikoff, 1947). The choline content in phospholipids extracted from carrots exposed to steam was higher than in untreated carrots. Therefore, it was suggested that the presence of an enzyme later known as phospholipase D (PLD) in the carrot could split choline from phospholipid in untreated carrot. In addition, the enzyme was inactive due to the steam treatment (Hanahan & Chaikoff, 1947).

The amount of PLD enzyme which can be extracted from plants is limited. The cloning and expression of PLD genes are important to provide an adequate amount of PLD protein to study the biochemical properties of PLD. PLD was cloned from castor bean (*Ricinus communis*) heterologously expressed in *E. coli* (Wang et al., 1994). Recombinant PLD with the size of 2,834 bp cDNA encoding 808 amino acids exhibited PLD activity, catalysing transphosphatidylation reaction (Wang et al., 1994). PLD catalyses the hydrolysis of phosphodiester bonds of glycerophospholipids such as phosphatidylcholine (PC) as a substrate to generate phosphatidic acid (PA) and choline. PC is a major substrate of PLD. However, PLD also exhibits activity on other substrates such as phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS), which are the constituents of phospholipid membrane in many cellular processes, including membrane deterioration in plant cells associated with ripening (J. Li et al., 2020). The advancement of genomic study has paved the way for discovering PLD in other

organisms. PLD genes can be found in plants (Qin & Wang, 2002), animals (Frohman et al., 1999), and bacteria (Zambonelli & Roberts, 2005). In plants, PLD is encoded by a multigene family. There are 12 copies of PLDs reported in *Arabidopsis thaliana* (Qin & Wang, 2002).

In eukaryotes, PLD has two domains, an N-terminal PX/PH or C2 domain and a C-terminal catalytic domain comprising two histidine (H), lysine (K), and aspartic acid (D) motifs in their gene structure (Hammond et al., 1997; J. Li et al., 2020). Therefore, PLDs are classified as PX/PH-PLDs and C2-PLDs based on their N-terminal domains. *Arabidopsis* has two PX/PH-PLDs and ten C2-PLDs. Moreover, plant PLD comprises α, β, γ, δ, ε, and ζ subtypes. The specific function of each isoenzyme remains unclear. Numerous studies have suggested that each isoform of PLD in the plant genome is differentially regulated in response to different types of stresses on plants. Different PLD genes have different expression patterns in tissues, organs, and developmental stages. It has been reported that PLDα1 in *Arabidopsis* was highly expressed in root cells and suggested to be involved in membrane assembly and vesicle trafficking (Novák et al., 2018).

A previous study showed that PLDα in *Arabidopsis* (AtPLDα) is involved in abscisic acid (ABA) and ethylene-induced senescence (Fan et al., 1997). Meanwhile, PLDδ was expressed near the plasma membrane binding to the cortical microtubule in hypocotyl cells (Novák et al., 2018; Q. Zhang et al., 2017). PLD is activated by calcium ions (Ca^{2+}) to perform its catalytic function. The Ca^{2+} binding site is present in the structure of PLD (Hammond et al., 1997). The cDNA sequence encoded for forming a Ca^{2+} binding site is highly conserved among C2-PLDs (J. Li et al., 2020). The Ca^{2+}-mediated activation is essential for the activity of C2-PLDs but not for PX/PH-PLDs (J. Li et al., 2020). It indicates that the PLD isoforms have different specific functions and are regulated by different plant molecules or hormones. Therefore, specific isoforms of PLD play an important role in the catabolism of phospholipids during ripening and senescence. The elevated levels of Ca^{2+} and pH in the cytoplasm stimulate the activity of PLD due to ripening.

Membrane Lipid Catabolism

The cell membrane, composed of fatty acid-based lipids and proteins, is an important structure of all cells. Phospholipid bilayer and proteins are the basic structures of the plasma membrane. Phospholipids and their catalytic products are important for various cellular mechanisms as signalling molecules, including fruit ripening. There are five types of phospholipids in the membrane, including phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and phosphoinositides (PIPs). The structure of the plasma membrane can be seen in Figure 3. The structure and composition of plasma membrane undergo modification catalysed by several major enzymes, namely PLA,

PLB, PLC, and PLD, as the fruit ripens, thus leading to membrane deterioration and softening of the fruit. These enzymes cleave their substrate at different positions of phospholipid molecules.

Ethylene binds to the ethylene receptor at the onset of ripening to produce a signal for the expression of ripening-related genes, including PLD. In addition, an increase in Ca^{2+} and a decrease in pH in the cytoplasm trigger the PLD autocatalytic activity. PLD cleaves the substrate at the phosphodiester bond to release the head group. PLD catalyses the conversion of PC as a main substrate to PA and choline. PA is then converted to diacylglycerols by phosphatidate phosphatase. Then, diacylglycerols are further catabolised to free fatty acids such as linoleic and linolenic acids by lipolytic acyl hydrolase (LAH). Lipoxygenase (LOX) catalyses the conversion of linoleic and linolenic acids to hydroperoxides. Hydroperoxide lyase (HPL) will convert hydroperoxide products from linoleic and linolenic acids to aldehydes such as hexanal, *trans*-2-hexenal, or *cis*-3-hexenal. These aldehydes are converted to 1-hexanol, *trans*-2-hexen-1-ol, and *cis*-3-hexen-1-ol by alcohol dehydrogenase (ADH) (Genovese et al., 2021). The intermediate compounds or products generated along the pathway, such as alkanes, fatty alcohols, and fatty acids, cause membrane destabilisation by forming lipid microvesicles, gel-phase lipids, and non-bilayer lipid structures. Therefore, the biophysical property of the membrane is altered, thus contributing to the softening of the fruit.

Inhibition of Ripening by Hexanal Treatment

The activity of PLD leads to membrane deterioration during ripening. Rapid ripening shortens fruits' shelf-life. The binding of ethylene to the ethylene binding receptor results in the expression of PLD and other ripening-related genes (Tiwari & Paliyath, 2011). PLD activity is also triggered by the cytoplasmic Ca^{2+} and hydrogen ion (H^{+}) elevation through stresses, membrane disruption, and ion leakage (Paliyath et al., 2008). Therefore, inhibiting PLD activity is important to prolong fruits' shelf-life. Many studies have been done to develop pre- or post-harvest technologies by inhibiting PLD activity. Alcohols such as *n*-butanol and 2-butanol can also inhibit PLD activity (L. Li et al., 2022). In addition, PLD activity can be inhibited by hexanal (El Kayal et al., 2017). It has been reported that PLD inhibition by hexanal treatment resulted in significant shelf-life enhancement in bananas (*Musa acuminata*) (L. Li et al., 2022).

The application of hexanal treatment on raspberry increased the expression of transcription activator for calcium-binding protein called calmodulin (El Kayal et al., 2017). Calcium signalling is crucial in a wide array of cellular functions, which involve calcium ions as a messenger in response to stresses. Calmodulin is a calcium sensor which binds to calcium to produce a signal that triggers the expression of genes related to cell wall metabolism. Cell wall degradation is one of the important events that causes fruit softening and decay.

Ca^{2+} inhibits leaf abscission and tissue senescence by crosslinking pectic acid residues to rigidify the cell wall (Hepler, 2005). Hexanal treatment affects the cell wall and lipid membrane metabolism, thus leading to shelf-life improvement. Nevertheless, the interaction mechanism between hexanal and Ca^{2+} signalling in inhibiting PLD expression remains unclear. Furthermore, hexanal treatment greatly reduced annexin expression and calcium accumulation during PLD expression in raspberries (El Kayal et al., 2017). It is suggested that annexin forms Ca^{2+} channels to transport Ca^{2+} across the plasma membrane, leading to the increase of cytoplasmic Ca^{2+} concentration, which triggers the expression of lipid membrane metabolism-related genes, including PLD (El Kayal et al., 2017). It shows the role of calcium signalling in the hexanal-induced membrane stabilisation mechanism by regulating the expression of genes related to cell walls and lipid membrane metabolisms.

BIOSAFETY OF HEXANAL

Numerous studies have ensured that hexanal usage in agricultural and food industries does not harm the environment and humans. *Trichogramma* spp. are biological control agents that protect some fruits against parasites (Mohan et al., 2017a). The growth of *Trichogramma* spp. (*Trichogramma chilonis* and *Trichogramma pretiosum*) was not affected by 0.02%–0.06% hexanal formulation treatment (Mohan et al., 2017a). A biosafety study of hexanal has been done on honey bees (*Apis cerana indica*, *Apis mellifera*, and *Apis florea*) (Mohan et al., 2017b). It is reported that 0.02%–0.06% hexanal formulation treatment was safe for all tested honey bee species (Mohan et al., 2017b). Green lacewing (*Chrysoperla zasrowi sillemi*) is an important biological control agent used to control the growth of a wide range of parasites such as aphids (*Myzus persicae*), coccids (*Coccoidea*), mealy bugs (*Pseudococcidae*), and mites (*Tetranychus urticae*) (I. J. Nair et al., 2020). The exposure of hexanal formulation (0.02%–0.06%) to green lacewing was harmless, with less than 30% mortality (Mohan et al., 2020). Similarly, hexanal treatment using the direct spray method on *Chrysoperla* grubs and 0.02% hexanal formulation on *Trichogramma japonicum* revealed that hexanal was not toxic to insects (Karthika et al., 2015).

Earthworms are known as a "farmer's friend" due to their activities in soil, which benefit both soil and crops (Gunasekaran et al., 2015). No ill effect was found on earthworm (*Eudrilus eugeniae*) exposed to hexanal formulation with concentration ranging from 0.02-0.06% (Gunasekaran et al., 2015). The toxicological studies of hexanal exposure to animals have also been reported (Cho et al., 2021). The exposure of hexanal vapour at 600, 1,000, and 1,500 ppm concentrations to the Fischer 344 rat strain showed potential pulmonary toxicity to the animals (Cho et al., 2021). The genes involved in the intracellular signalling cascade associated with pulmonary toxicity were identified by analysing the expressed mRNA (Cho et al., 2021). It suggested that

hexanal exposure could be toxic to humans at those concentrations. On the other hand, the study of hexanal toxicity in the human cell lines (HeLa, A549 and HepG2) has also been reported (Gunasekaran et al., 2015). Hexanal was found to be toxic to all tested cell lines (HeLa, A549, and HepG2) at a concentration of more than 2,000 ppm based on lactate hydrogenase (LDH) and tetrazolium (MTT) assay (Gunasekaran et al., 2015). The recommended spray concentration for pre- and post-harvest applications was below 0.04% (400 ppm), which is safe for human cell lines (Gunasekaran et al., 2015). Therefore, using hexanal to improve the shelf-life and quality of fruits is safe for the environment and human health.

HEXANAL APPLICATION IN ENHANCEMENT OF SHELF-LIFE OF FRUIT

Hexanal has been found to have antimicrobial properties and the ability to enhance the shelf-life of fruits (Khan & Ali, 2018; Song et al., 1996). Pre-harvest treatment of 1% hexanal followed by post-harvest treatment of 1 ppm 1-methylcyclopropene (1-MCP) on cherry (*Prunus avium*) enhanced its shelf-life and sensory attributes (Sharma et al., 2010). Guava (*Psidium guajava*) treated with 0.015% (v/v) of hexanal showed enhanced quality and shelf-life of up to four weeks of storage (Gill et al., 2016)—meanwhile, the treatment of 0.02% hexanal dip on mango var. Neelum (*Mangifera indica*) extended its shelf life (Jincy et al., 2017). The PLD activity and ethylene evolution rate were also significantly reduced (Jincy et al., 2017). The ethylene evolution rate increases as the fruit ripens. The reduction of PLD activity and ethylene evolution rate leads to delayed ripening, extending the mango var Neelum (*M. indica*) shelf-life (Jincy et al., 2017).

In addition, the activity of enzymes related to ripening and fruit softening, such as pectinmethlyesterase, catalase, peroxidase, and polygalacturonases in mango, was reduced after being treated with 0.02% hexanal using the direct-spray method (Preethi et al., 2021). Pre-harvest spray of 0.02% (v/v) hexanal was able to preserve the firmness of nectarines for at least 45 storage days at 2°C (Kumar et al., 2018). The onset of internal browning and mealiness was delayed for about eight days (Kumar et al., 2018). In addition, the expression of three PLD genes was significantly down-regulated (Kumar et al., 2018). The shelf-life of bananas was extended by spraying them with 2%–3% hexanal on days 15 and 30 pre-harvest and dipping them in hexanal post-harvest (Yumbya et al., 2018). However, pre-harvest 2%–3% hexanal spray showed better fruit preservation (Yumbya et al., 2018).

Antibacterial and Antifungal Properties of Hexanal

The ability of hexanal to prolong fruit shelf-life correlates with its antibacterial and antifungal properties, which are listed in Table 1. *Salmonella* Typhimurium is one of the major causes of foodborne illness (Hanning et al., 2009; He et al., 2021). It

is resistant to many antibiotics, such as ampicillin, chloramphenicol, streptomycin, tetracycline, and sulphonamides (D. V. T. Nair et al., 2018). The treatment of hexanal on fruit-based foods may be beneficial to prevent the infection due to its antibacterial activity while enhancing shelf-life. The exposure of 50 µl hexanal on *Salmonella* Typhimurium did not show any inhibitory effect for 30 min of exposure time. The inhibitory effect started to show from 45–75th min. No growth was observed for more than 90 min of hexanal exposure (Lamba, 2007). Moreover, hexanal inhibited pathogenic bacteria usually found in raw food, such as *Escherichia coli*, *Salmonella* Enteritidis, and *Listeria monocytogenes* (Lanciotti et al., 2004). It is reported that 150 ppm of hexanal caused significant inhibition of *Listeria monocytogenes* growth (Lanciotti et al., 2004). Hexanal (150 ppm) also inhibited the growth of *E. coli* and *Salmonella* Enteritidis at the lag phase inoculated at levels of 10^4–10^5 CFU/g (Lanciotti et al., 2004). Since hexanal is a hydrophobic molecule, it easily interacts with hydrophobic membranes and causes damage to the bacterial cell (Helander et al., 1997).

Table 1
Antibacterial and antifungal activities of different methods of hexanal treatment

Treatment method	Concentration	Microorganism	Reference
Hexanal vapour	50 µl/L	*Monilinia fructicola* and *Monilinia laxa*	Baggio et al. (2014)
Hexanal vapour	450 µl/L	*Botrytis cinerea*, *Monilinia fructicola*, *Sclerotinia sclerotiorum*, *Alternaria alternata*, and *Colletotrichum gloeosporioides*	Song et al. (2007)
Hexanal vapour	40 µmol/L	*Penicillium expansum*	Fan et al. (2006)
Hexanal dipping	0.02% (v/v)	*Lasiodiplodia theobromae*	Seethapathy et al. (2016)
Hexanal vapour	800 ppm	*Colletotrichum gloeosporioides* and *Lasiodiplodia theobromae*	Dhakshinamoorthy et al. (2020)
Hexanal vapour	5.02 µl/L	*Colletotrichum gloeosporioides* and *Lasiodiplodia theobromae*	Dhakshinamoorthy et al. (2020)
Haxanal vapour	150 ppm	*Listeria monocytogenes*, *Escherichia coli*, and *Salmonella* Enteritidis	Lanciotti et al. (2004)
Hexanal containing broth	400 µl/L	*Aspergillus flavus*	Li et al. (2021)
Hexanal added to a trypticase soy agar plate	50 µl	*Salmonella* Typhimurium	Lamba (2007)
In vitro (disc-diffusion method) Hexanal dissolved in dimethyl sulfoxide	0.015–500 ppm	*Moraxella catarrhalis*, *Escherichia coli*, *Streptococcus pyogenes*, *Staphylococcus aureus* and *Salmonella* Enteritidis (no significant antibacterial activities against these bacteria)	Bisignano et al. (2001)

Meanwhile, a previous study showed that *in vitro* (disc-diffusion method) antibacterial activity of hexanal (0.015–500 ppm) dissolved in dimethyl sulfoxide (DMSO) was not significant against all tested bacterial strains such as *Moraxella catarrhalis*, *E. coli*, *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Salmonella* Enteritidis (Bisignano et al., 2001). It indicated that the effectiveness of hexanal as an antibacterial volatile compound depends on the treatment methods and the types of fungal or bacterial strains. Therefore, the study for optimisation of hexanal treatment is important to ensure its effectiveness in enhancing the shelf-life of fruit by killing the bacteria and fungus that cause fruit spoilage.

Monilinia fructicola and *Monilinia laxa* are plant pathogenic fungi that cause brown rot disease in fruits (Martini & Mari, 2014). It is reported that hexanal vapour treatment at a concentration of 50 µl/L reduced the development of brown rot disease on peaches (*Prunus persica*) (Baggio et al., 2014). The growth of *M. fructicola* and *M. laxa* in the peaches was also inhibited (Baggio et al., 2014). Similarly, the treatment of 450 µl/L of hexanal vapour on major post-harvest fungal pathogens such as *Botrytis cinerea*, *M. fructicola*, *Sclerotinia sclerotiorum*, *Alternaria alternata*, and *Colletotrichum gloeosporioides* for 24 hr inhibited the growth of all tested fungal pathogens (Song et al., 2007). The treatment of 900 µl/L hexanal vapour reduced the decay of raspberry (*Rubus idaeus*) and lesion development on peaches (*Prunus persica*) for at least two days at 20 and 7°C (Song et al., 2007).

The viability of *Penicillium expansum* in apples was reduced following 24 hr of 40 µmol/L hexanal vapour treatment (Fan et al., 2006). Similarly, the treatment of 0.02% hexanal dipping inhibited the growth of stem-end rot disease *Lasiodiplodia theobromae* in mango (Seethapathy et al., 2016). In growth, *C. gloeosporioides* and *L. theobromae in vitro* were inhibited after exposure to 800 ppm hexanal vapour (Dhakshinamoorthy et al., 2020). In addition, the exposure for 3 hr of 5.02 µl/L hexanal vapour treatment on bananas significantly reduced the infection of *C. gloeosporioides* and *L. theobromae* (Dhakshinamoorthy et al., 2020).

Hexanal also exhibited antifungal activity against *Aspergillus flavus* at a minimal inhibitory concentration of 0.4 µl/ml (S.-F. Li et al., 2021). It was found that several metabolites such as phosphatidylcholine, riboflavin, D-sorbitol, D-ribose, D-mannitol, L-malic acid, and deoxyinosine in *A. flavus* were significantly reduced (S.-F. Li et al., 2021). From the metabolite analyses, it was suggested that the inhibition of *A. flavus* growth by hexanal involved the membrane synthesis, adenosine triphosphate binding cassette (ABC) transport system, and tricarboxylic acid (TCA) cycle (S.-F. Li et al., 2021). It evidenced the potential of hexanal as an effective antifungal agent to prevent fruit spoilage. The study of the effect of vapour pressure on the antifungal activity of volatile compounds revealed that the antifungal activity was enhanced as the

vapour pressure increased using hexanal as a model molecule (Gardini et al., 1997). The increase in temperature led to the increase in vapour pressure, thus enhancing the antifungal activity of hexanal vapour against *Aspergillus niger* (Gardini et al., 1997).

CONCLUSION

Hexanal is a naturally biosynthesised compound from plants and is safe for the environment and humans. Hexanal has promising potential to be used in pre- or post-harvest technology to enhance the shelf-life and quality of fruits. Moreover, hexanal exhibits antibacterial and antifungal activities that could help prolong the shelf-life of fruits. However, the efficiency of hexanal treatment depends on several factors, such as formulation, method of treatment, and type of fruit. Hexanal is a potent PLD inhibitor, the key enzyme in lipid membrane deterioration. However, the inhibition mechanism of PLD by hexanal at the molecular level is still unclear. The expression analysis of PLD and other ripening-related genes using quantitative real-time PCR (qRT-PCR) could be used to understand the mechanism of PLD inhibition by hexanal. In addition, determining PLD crystal structure by X-ray crystallography could reveal the possible binding site of hexanal to PLD.

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