Pertanika Journal of

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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.

The journal is available world-wide.

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

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Pertanika was founded in 1978. A decision was made in 1992 to streamline *Pertanika* into 3 journals as Pertanika Journal of Tropical Agricultural Science, Pertanika Journal of Science & Technology, and Pertanika Journal of Social Sciences & Humanities to meet the need for specialised journals in areas of study aligned with the interdisciplinary strengths of the university.

Currently, as an interdisciplinary journal of agriculture, the revamped journal, a leading agricultural journal in Malaysia now focuses on tropical agricultural research and its related fields.

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To publish journals of international repute.

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Pertanika is now over 42 years old; this accumulated knowledge has resulted in Pertanika Journal of Tropical Agricultural Science being abstracted and indexed in SCOPUS (Elsevier), Clarivate Web of Science (ESCI), EBSCO, DOAJ, Agricola, ASEAN CITATION INDEX, ISC, Microsoft Academic, Google Scholar, National Agricultural Science (NAL), and MyCite.

Citing journal articles

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

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

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Authorship

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Manuscript preparation

Most scientific papers are prepared according to a format called IMRAD. The term represents the first letters of the words ***I****ntroduction*, ***M****aterials and Methods*, ***R****esults*, ***A****nd* ***D****iscussion*. IMRAD is simply a more 'defined' version of the "IBC" (*Introduction*, *Body*, *Conclusion*) format used for all academic writing. IMRAD indicates a pattern or format rather than a complete list of headings or components of research papers; the missing parts of a paper are: *Title*, *Authors*, *Keywords*, *Abstract*, *Conclusions*, *References*, and *Acknowledgement*. Additionally, some papers include *Appendices*.

The *Introduction* explains the scope and objective of the study in the light of current knowledge on the subject; the *Materials and Methods* describes how the study was conducted; the *Results* section reports what was found in the study; and the *Discussion* section explains meaning and significance of the results and provides suggestions for future directions of research. The manuscript must be prepared according to the journal's **Instruction to Authors** (http://www.pertanika.upm.edu.my/Resources/regular_issues/Regular_Issues_Instructions_to_ Authors.pdf).

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Notification of the editorial decision is usually provided within 90 days from the receipt of manuscript. Publication of solicited manuscripts is not guaranteed. In most cases, manuscripts are accepted conditionally, pending an author's revision of the material.

As articles are double-blind reviewed, material that may identify authorship of the paper should be placed only on page 2 as described in the first-4-page format in *Pertanika*'s **Instruction to Authors** (http://www.pertanika.upm.edu.my/Resources/regular_issues/Regular_Issues_Instructions_to_Authors.pdf).

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

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

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

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

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

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

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

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

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ABSTRACTING AND INDEXING OF PERTANIKA JOURNALS

The journal is indexed in SCOPUS (Elsevier), Clarivate-Emerging Sources Citation Index (ESCI), BIOSIS, National Agricultural Science (NAL), Google Scholar, MyCite, ISC. In addition, Pertanika JSSH is recipient of "CREAM" Award conferred by Ministry of Higher Education (MoHE), Malaysia.

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Contents

Foreword

Welcome to the 4th issue of 2021 for the Pertanika Journal of Tropical Agricultural Science (PJTAS)!

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

This issue contains 10 articles; three review articles and the rest are regular articles. The authors of these articles come from different countries namely Algeria, Indonesia, and Malaysia, Nigeria and Korea.

Azimah Abd Rahman and her teammates from Universiti Sains Malaysia combined the data on the species diversity and firefly distribution in Southeast Asian countries published in 2015-2021. Based on the investigation, Malaysian and Thailand researchers are among the forerunners in the study related to fireflies in the Southeast Asian region. A total of 145 different species of fireflies were successfully identified. In addition, at least 34 tree species and one unidentified species (Poaceae family) of display trees or habitat by fireflies in Malaysia and Thailand were managed to be found as well. Further details of this study are found on page 713.

A regular article entitled "Effect of Various Immersion Time and Water Temperature on Seed Germination of *Clitoria ternatea* and *Momordica charantia*" revealed that the seed germination of *Clitoria ternatea* and *Momordica charantia* is improved through peeling the coat and soaking the seeds in water for various temperatures and periods. Seven pre-sowing treatments were practiced. From this study, it proves that pre-sowing treatments of seeds would prove its potential in the practical fields. Full information of this study is presented on page 745.

A selected article entitled "Phytochemical Analysis and Antibacterial Activities of Sidr Leaf Extract (*Ziziphus spina-christi*) against Pathogenic Bacteria in Aquaculture" studied the phytochemical components and analyze the effect of Sidr leaf extract on the growth of aquaculture-based pathogenic bacteria. The Sidr leaf extract contains phytochemicals, namely, flavonoids, alkaloids, saponins, tannins, and steroids, with antibacterial properties. Besides that, it also demonstrated moderate-to-strong inhibition to aquaculture pathogenic bacteria, except for *Vibrio vulnificus*. These results indicate that the Sidr leaf extract can be used as a natural herb to control bacterial pathogens in fish cultivation. The detailed information of this article is available on page 845.

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.

In the last 12 months, of all the manuscripts processed, 32% were accepted. This seems to be the trend in PJTAS.

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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Comparative Genomics of *Copia* and *Gypsy* Retroelements in Three Banana Genomes: A, B, and S Genomes

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ABSTRACT

In plants, the proportion of transposable elements (TEs) is generally dominated by long terminal repeat (LTR) retroelements. Therefore, it significantly impacts on genome expansion and genetic and phenotypic variation, namely *Copia* and *Gypsy*. Despite such contribution, TEs characterisation in an important crop such as banana [*Musa balbisiana* (B genome), *Musa acuminata* (A genome), and *Musa schizocarpa* (S genome)] remains poorly understood. This study aimed to compare B, A, and S genomes based on repetitive element proportions and copy numbers and determine the evolutionary relationship of LTR using phylogenetic analysis of the reverse transcriptase (RT) domain. Genome assemblies were acquired from the Banana Genome Hub (banana-genome-hub.southgreen.fr). Repetitive elements were masked by RepeatMasker 4.0.9 before Perl parsing. Phylograms were constructed according to domain analysis using DANTE (Domain-based ANnotation of Transposable Elements), alignments were made using MAFFT 7 (multiple alignments using fast Fourier transform), and trees were inferred using FastTree 2. The trees were inspected using SeaView 4 and visualised with FigTree 1.4.4. We reported that B, A, and S genomes are composed of repetitive elements with 19.38%, 20.78%, and 25.96%, respectively. The elements were identified with dominant proportions in the genome are LTR, in which *Copia* is more abundant than *Gypsy*. Based on RT phylogenetic analysis, LTR elements are

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clustered into 13 ancient lineages in which *Sire* (*Copia*) and *Reina* (*Gypsy*) are shown to be the most abundant LTR lineages in bananas.

Keywords: Banana, B, A, and S genomes, reverse transcriptase, transposable elements

INTRODUCTION

Banana (*Musa* spp.) is one of the most consumed fruits and staple food in many countries across Asia and Africa (Food and Agriculture Organization [FAO], 2019). Its diversity is represented by the number of cultivars and genome diversity (A, B, S, and T genome) (D'Hont et al., 2000). A, B, and S genomes are publicly available in Banana Genome Hub and GenBank. Hence it is possible to characterise their genome organisation by observing the repetitive elements and transposable elements (TEs). Defined as stretches of DNA that are competent to integrate into new positions in the genome, TEs are competent to increase their copy number over time, and that rely on one or more enzymatic function provided by an autonomous element (Lisch, 2013).

To date, comprehensive genome analysis remains limited to two genome assemblies (*M. acuminata* and *M. balbisiana*) (D'Hont et al., 2012; Davey et al., 2013) despite two recent whole-genome sequencings (WGSs) of *Musa itinerans* (Wu et al., 2016) and *M. schizocarpa* (Belser et al., 2018) have been accomplished. However, these genome data can be conducted into a comparative study of *M. acuminata* (A genome) and *M. balbisiana* (B genome), a promising study to learn the structure and character of a gene or a gene family. For example, Nugrahapraja et al. (2021) successfully identified and characterised the pectin methylesterase (PME) gene family among A and B genome bananas from the comparative study of the genome. On the other hand, the characterisation of repetitive elements can also be studied by comparing these genome data. The characterisation of repetitive elements within the bananas' chromosomal genome is relatively easier than other plant species owing to the size of the paradoxically small genome (1 C ~ 600 Mbp) (Doležel et al., 1994). Plants repetitive sequences make up a genome proportion of 20% in *Arabidopsis* and more than 80% in *Zea mays* (Kaul et al., 2000; Vitte et al., 2014), which is highly dominated by long terminal repeat (LTR).

LTR elements are an extensive group, and their immense diversity is further divided into an enormous number of families. In eukaryote, the families are grouped into two superfamilies: *Copia/Ty1* and *Gypsy/Ty3*, characterised by their terminal repeat on both ends, flanking the sequence (Wicker et al., 2007). Once thought of as 'junk DNA', TEs have been known to create a variety of alterations of genes expression and function. It leads to numerous studies to inquire how TEs have played a crucial part in plant genome dynamics. LTR elements (*Copia* and *Gypsy*) have a significant impact in contributing to flowering plants diversity, evolutionary adaptation, and genome expansion (Ragupathy et al., 2013). Through a process called exaptation

(Hoen & Bureau, 2015), TEs could be evolutionarily adapted as functional genes, such as *Fhy33/Far* (light-responsive genes), *Sleeper* (transcriptional regulator in plant development), and *Mustang* (transcriptional regulator) families (Joly-Lopez et al., 2016; Knip et al., 2013; Lin et al., 2007).

Considering banana's potential development and challenges as important crop species, notably the characterisation of chromosomal genome organisation, *in silico* analysis was performed to characterise the structure of repetitive elements. This study also aimed to dissect the LTR (*Copia* and *Gypsy*) phylogeny of three banana genomes: A, B, and S genomes. In the future, such research can be used in genome mapping, evolutionary studies, omics studies, and further depict the dynamic of transposable elements.

MATERIALS AND METHODS

Data Retrieval

Whole genome sequence (WGS) of A (*Musa acuminata* 'DH-Pahang'), B (*Musa balbisiana* 'Pisang Klutuk Wulung'), and S genomes (*Musa schizocarpa*) (Belser et al., 2018; D'Hont et al., 2012; Davey et al., 2013; Martin et al., 2016) were used in the study. Complete WGS of three genomes were downloaded directly from Banana Genome Hub through the download menu of genome_sequences (https://banana-genome-hub.southgreen.fr/species-list) (Droc et al., 2013).

Repeat Masking and Parsing

Fasta format of WGS then masked using RepeatMasker 4.0.9 (Smit et al., 2015) implemented in Perl 5.8.0. The data were aligned using RMBlast 2.9.0 while tandem repeats were analysed using TRF 4.9.0 (Benson, 1999). Dfam 3.0 protein database (Hubley et al., 2016) and RepBase v20181026 (Jurka et al., 2005) were used as a library to identify the repeats. As for RepBase library, one should contact the Genetic Information Research Institute (GIRI) to attain the non-commercial license. The dictionary of parsing was built using build_dictionary.pl against RM.out and genome. The results of RepeatMasker were parsed with one_code_to_find_them_all.pl using fuzzy matching (Bailly-Bechet et al., 2014). CSV (comma-separated values) files, which comprised LTR, transposons, elem_sorted, and copy number created from parsing, were visualised using Office 365.

Phylogeny Analysis

Phylogram was constructed by harnessing the reverse transcriptase (RT) conserved domain of transposable elements class I using DANTE (Novák et al., 2010). The domain opted for its conserved bases. Thus, it could be easily used to dissect the diversity of superfamilies or lineages. The RT detection used the algorithm of robust alignment from the LAST program. The alignment was performed against REXdb Viridiplantae 3.0, a database for plant repetitive elements (Neumann et al., 2019). Extracted domains were aligned by MAFFT

7 (fft-NS-i), a progressive fast Fourier transform alignment program (Katoh & Standley, 2013). Aligned operational taxonomical units (OTUs) were transformed into a tree using FastTree 2 (Price et al., 2010). The tree was constructed with GTR+CAT substitution model, tree search normal+NNI+SPR, JC joins evaluation, and Shimodaira-Hasegawa (SH) test (1000) as a bootstrap alternative (Shimodaira, 2002). The tree produced was manually inspected using SeaView 4 with a bootstrap threshold of 0.5 (Gouy et al., 2010). Finally, the edited tree was visualised and annotated using FigTree 1.4.4 (Rambaut, 2018).

RESULTS AND DISCUSSION

Results

Repeats Genome Proportion and Copy Number. The structure of the banana genome represents its total repeats, repetitive elements proportion, and copy number (Figure 1). Overall, repeats covered from three bananas were observed proportional to their genome size. Repetitive elements composed 19.38%, 20.78%, and 25.96% of B, A, and S genomes, respectively. The proportions of *Copia* and *Gypsy* account for 8.79% and 7.51% in B genome, 9.32% and 8.12% in A genome, and 12.66% and 9.70% in S genome, a significant fraction of the repetitive elements. On the other hand, the proportion of elements such as non-LTR retroelements [short interspersed retrotransposable element (SINE), long interspersed retrotransposable element (LINE)], DNA elements were relatively low with less than 2% of the genome. Copy number visualisation shows that tandem repeats are abundant, followed by LTRs (*Copia* and *Gypsy*), while other elements are less abundant.

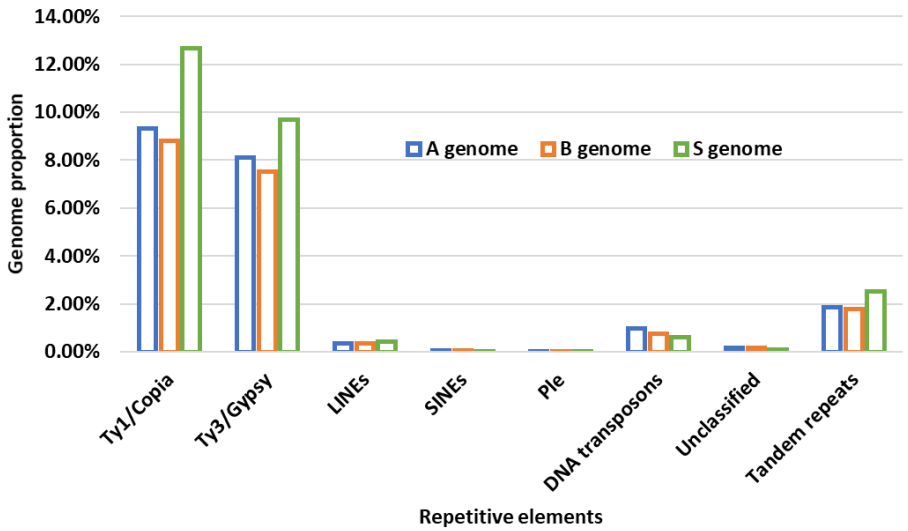


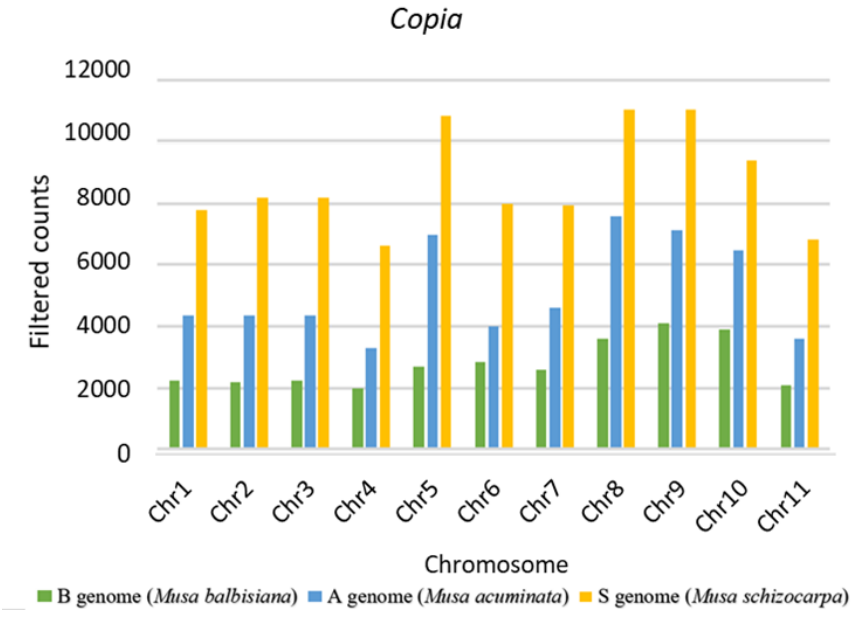
Figure 1. Proportion of repetitive elements in A, B, and S genome

Parsed Transposable Elements. As shown in Table 1, masked sequences analysis, was then parsed to produce a more explicit non-bias depiction of LTRs (*Copia* and *Gypsy*) copy number. Figure 2 shows a virtually similar trend in B, A, and S genomes that *Copia* is far more abundant than *Gypsy* in genomic and chromosomal levels as well. A glimpse at the copy number of *Copia* and *Gypsy* in the S genome illustrates an incredible abundance compared to two other genomes.

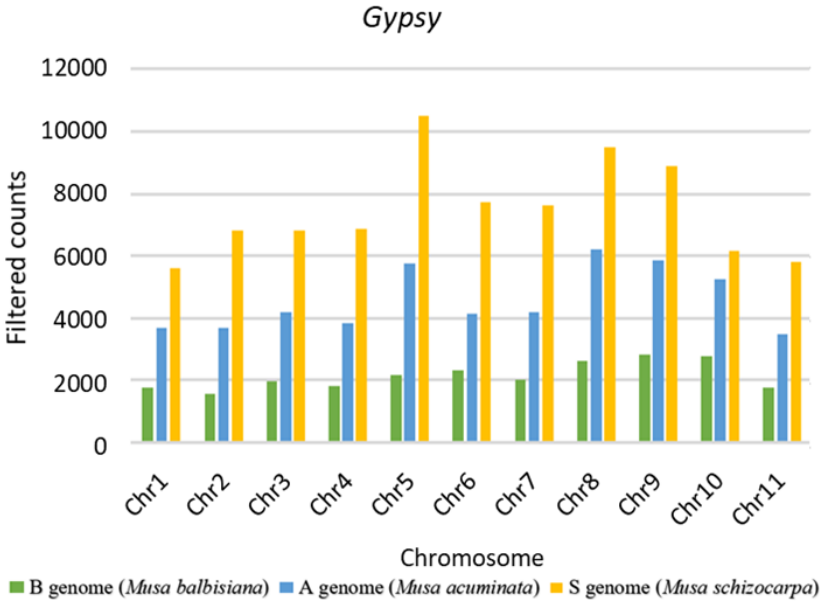
Table 1

*Copy number of LTRs (*Copia *and* Gypsy*)*

Species	LTRs copy number	
	Copia	*Gypsy*
A genome (*Musa acuminata*)	68857	59495
B genome (*Musa balbisiana*)	61070	46075
S genome (*Musa schizocarpa*)	95849	82317



(a)



(b)

Figure 2. Copy number of (a) *Copia* and (b) *Gypsy* in A, B, and S genomes described in total copies and chromosomal level

Ratio and divergence of parsed *Copia* and *Gypsy* elements were plotted as shown in Figure 3. Copies of the element are represented by blue dots, providing a general illustration of potentially full-length and active elements and those which have degraded over time. Provided that the blue dots are abundant, the ratio of elements is close to 1, possessing a low divergence. Thus, the ubiquity of *Copia* and *Gypsy* was inferred due to potentially active elements in which their bases were relatively less degraded. Figure 3 also shows an accordance trend as depicted in Figure 2 as more active and abundant *Copia* elements.

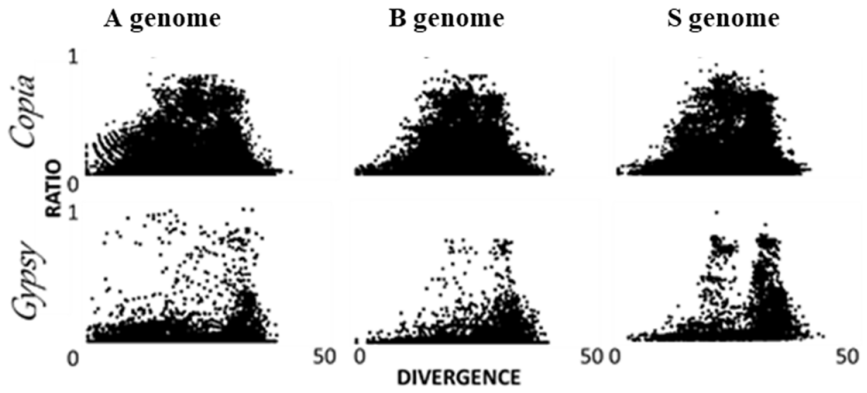


Figure 3. Parsed retroelements: divergence and ratio values of *Copia* and *Gypsy* in B, A, and S genomes

Figure 4 illustrates the ratio vs divergence of parsed elements compared from Chr1 of *M. balbisiana* to gain further perspective on how abundant and less abundant elements differ from each other regarding elements' activity. As seen from Figure 4, LINE and SINE are less abundant than LTR, such as *Copia*. On the other hand, DNA elements (*MuDR*, *Helitron*, and *hAT*) are relatively scarce. Therefore, they tend to be degraded (a ratio close to zero).

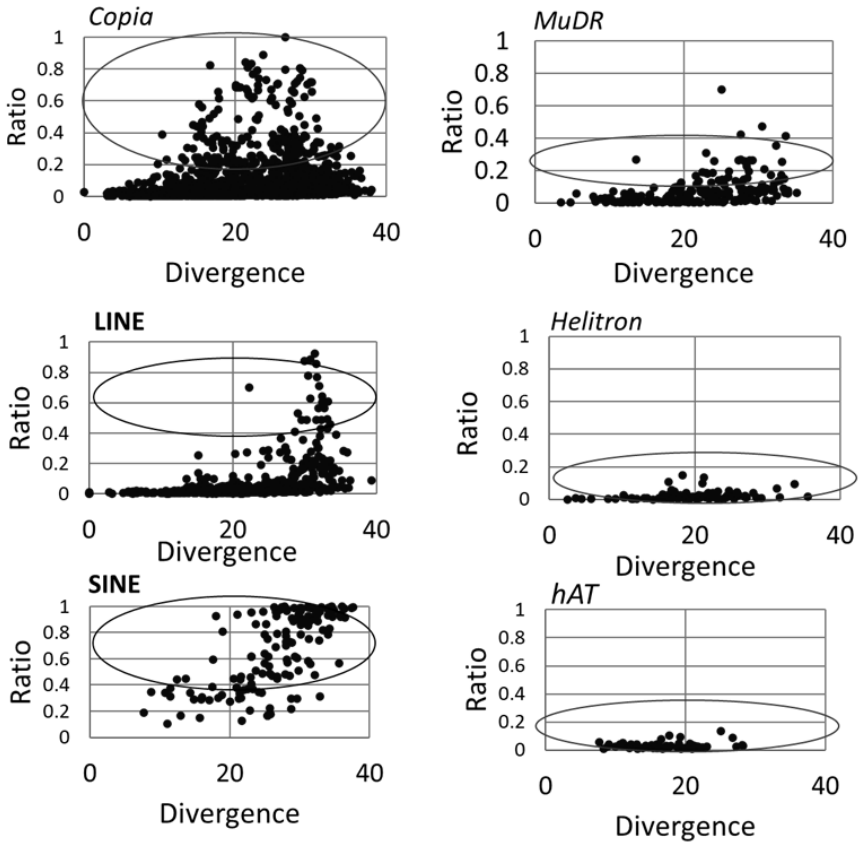


Figure 4. Ratio vs divergence comparison of *Copia*, class II elements (*MuDR*, *Helitron*, *hAT*), and non-LTRs (class LINE and SINE)

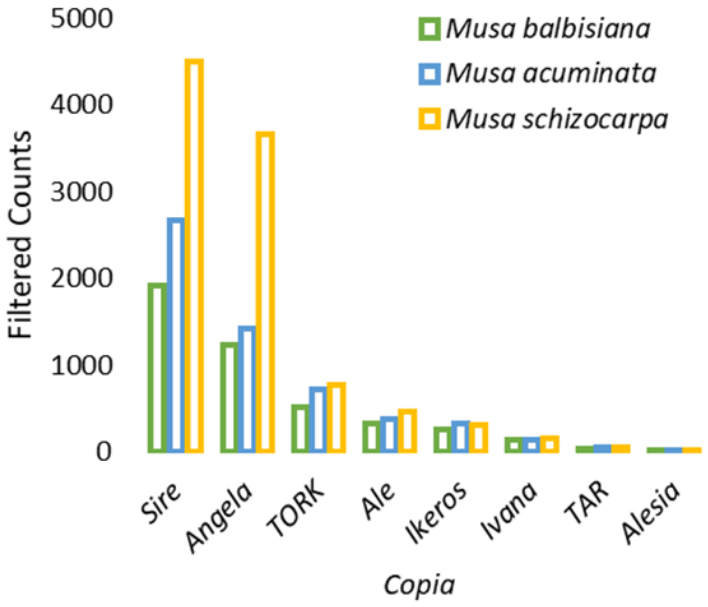
Reverse Transcriptase (RT) Domain Detection and Phylogenetic Analysis. *Copia* and *Gypsy* elements detected based on the RT domain are shown in Table 2. *Copia* lineages detected are *Ale*, *Alesia*, *Angela*, *Ikeros*, *Ivana*, *Sire*, *TAR*, and *Tork*. At the same time, *Gypsy* comprises *Galadriel*, *Tekay*, *Reina*, *CRM*, and *Retand*. The abundance of *Copia* and *Gypsy* lineages were further visualised as shown in Figure 5; the former is dominated by *Sire* lineages while the latter is shown mainly composed by *Reina* lineages.

Table 2

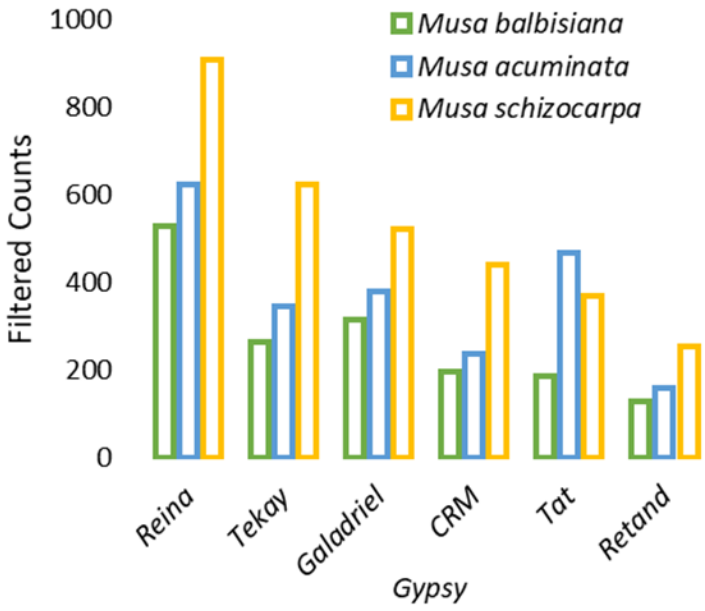
Hierarchical classification of Copia *and* Gypsy *in plants according to Neumann et al. (2019)*

Copia		Gypsy
*Ale**	Chromovirus	*Chlamyvir*
Alesia		*Tcn1*
*Angela**		*Galadriel**
Bianca		*Tekay**
Bryco		*Reina**
Lyco		*CRM**
Gymco-I, II, III, IV		*Chromo-unclass*
*Ikeros**	Non-chromovirus	*Phygy*
*Ivana**		*Selgy*
Osser		*OTA/Athila*
*Sire**		*OTA/TAT/Tat-I, II, III*
*TAR**		*Ogre*
*Tork**		*Retand**

Note. Asterisk (*): Identified elements based on RT domain: 13 elements in total comprising 8 *Copia* lineages and 5 *Gypsy* lineages



(a)



(b)

Figure 5. Filtered counts of (a) *Copia* and (b) *Gypsy* lineages in B genome (*Musa balbisiana*, green), A genome (*Musa acuminata*, blue), and S genome (*Musa schizocarpa*, yellow) showing numbers of non-bias

RT domains of *Copia* and *Gypsy* were constructed into inferred trees, as shown in Figure 6. Regardless of the type of genomes, both elements could be clustered into evolutionary lineages including two significant *Copia* and *Gypsy* superfamily clusters. *Copia* cluster encompassed two major lineages, designated *Sirevirus* clade and *Tork* clade. At the same time, *Gypsy* was divided into chromovirus and non-chromovirus. Major clades/lineages of *Copia* and *Gypsy* could be subdivided into several lineages as mentioned in filtered count results. Topologies acquired from individual genomes could be consistently inferred through a joint phylogram, as represented in Figure 6.

DISCUSSION

Stood at around 20%, the numbers of bananas' repeats proportions, including transposable elements (TEs) are less than the sister group in a subclass of Commelinids (the core of monocots) such as corn (*Zea mays*), wheat (*Triticum* sp.), and barley (*Hordeum vulgare*) with a proportion of more than 80% (Vitte & Panaud, 2005). Based on the size of the genome, with the S genome being the largest and the B genome being the smallest of all three, the proportions of TEs correlated with the size of the genome. The results are supported by the collinearity between regression analysis of various plants genomes against the proportion of TEs (Kidwell, 2002). This trend is similar to previous repeats calculations, although the results were

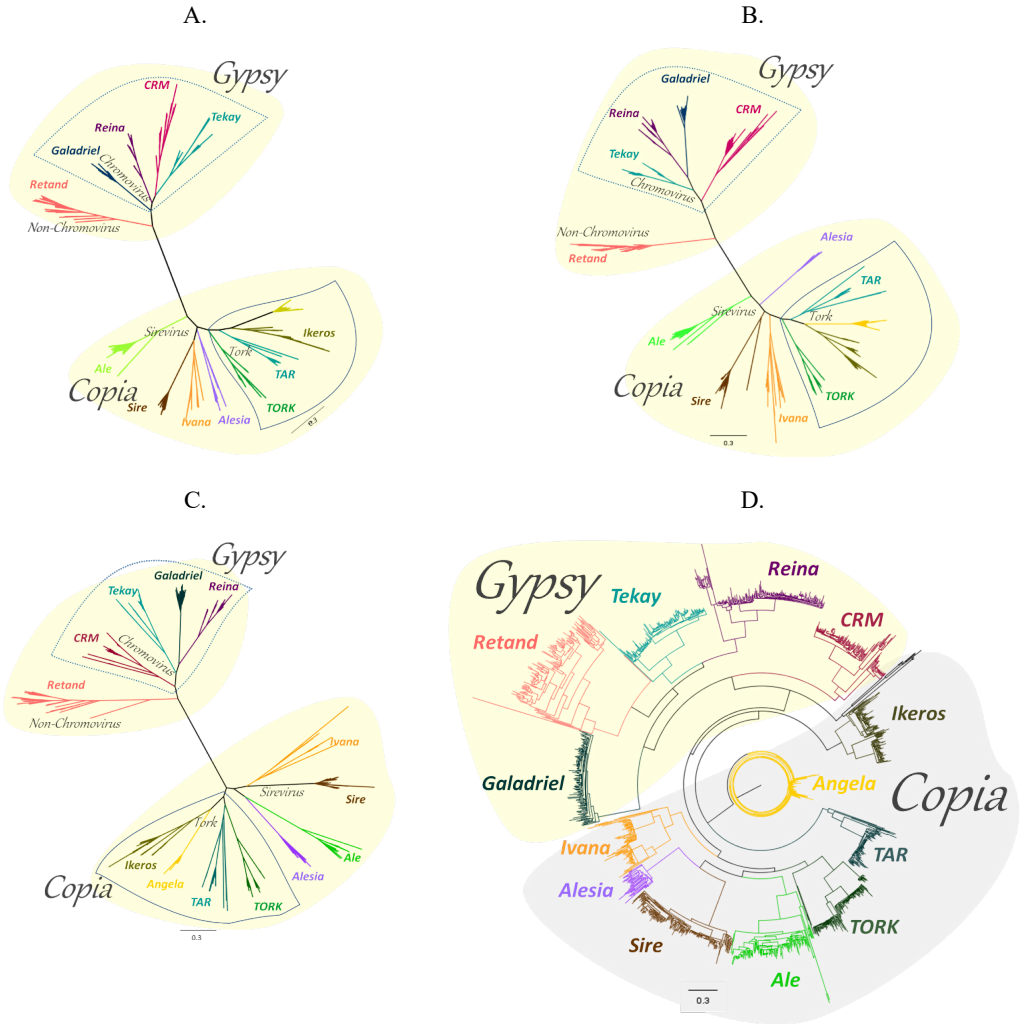


Figure 6. RT domain of *Copia* and *Gypsy* in banana phylogenetics with (A) *Musa acuminata* (AA), (B) *Musa balbisiana* (BB), (C) *Musa schizocarpa* (SS), and (D) joint phylogram of the three bananas

higher, making up about 30% of the genome (Hřibová et al., 2010). That said, TEs, particularly LTR retroelements (*Copia* and *Gypsy*), could transpose and contribute to genome expansion. At the same time, LTRs' underlying mechanism and contributions in affecting epigenetic and phenotype in bananas need further inquiry.

The dominance and prominent copy number of *Copia* and *Gypsy* in plants are not excluded in bananas. As elaborated before, the ubiquity of *Copia* and *Gypsy* resulted from the copy and paste transposition action, actively multiplying through intermediary RNA (Wicker et al., 2007). In other types of plants, *Gypsy* could outnumber *Copia*

in terms of proportion or copy number, for example, in Poales, such as *Oryza* spp. (Zhang & Gao, 2017). In the case of bananas, the abundance of *Gypsy* that somewhat lower compared to *Copia* could be understood due to its element association with spatial distributions: in the banana's genome, *Gypsy* is scattered broadly in heterochromatin, thus hampering the transcription cues from accessing the sequences in transposition (Domingues et al., 2012).

The activity of retroelements transposition could be described by their ratios close to 1. At the same time, divergences are close to zero (Bailly-Bechet et al., 2014). By contrast, DNA elements' cut and paste mechanism were shown less abundant and prone to degrade. In the meantime, non-LTR retroelements were less active to multiply. Although regarded as non-autonomous retroelements, SINEs maintain the transposition through which LINE transposition machinery enzymes are involved. The mechanism of LINE-dependent SINE transposition is also facilitated by SINE ability in recruiting RNA Pol III while LINE depends on RNA Pol II; the ratios and divergences of LINE were encountered similar to SINE patterns (Dewannieux et al., 2003).

Protein coding structures identified from domain searching were classified to chromodomain (CHD), endonuclease (ENDO), GAG, integrase (INT), protease (PROT), ribonuclease H (RH), reverse transcriptase (RT), transposase (TPase), and archeal ribonuclease H (aRH) according to REXdb (Neumann et al., 2019). Not only did we found the RT domain belonged to *Copia* and *Gypsy*, but *Parsaretrovirus/ Caulimovirus* RT within the genome described as endogenous banana streak virus (eBSV) was identified (Chabannes et al., 2013). In terms of *Copia* and *Gypsy* lineages abundance, *Sire* and *Reina* were consistently found the most ubiquitous LTR element in three genomes of the B, A, and S genomes, while other plants might differ. For instance, *Angela/Tork* and *TAT/Athila* are found prominent in paraphyletic monocots group of rice (*Oryza* spp.), *Sorghum* spp., foxtail millet (*Setaria italica*), and sugarcane (*Saccharum* spp.) (Du et al., 2010) while particular lineage such as *CRM* is absent in *Saccharum officinarum* (Domingues et al., 2012). As an addition to REXdb, a variety of lineages were grouped based on some classifications comprised GyDB (*Gypsy* Database) (Llorens et al., 2009) and unified classification (Wicker & Keller, 2007).

CONCLUSION

This study unravelled the bananas genome's overall composition, focusing on repetitive elements proportion among three genomes, *Copia* and *Gypsy* potential characteristics and their phylogeny. *Copia* and *Gypsy* were inferred as potentially active and full-length elements. Their phylogeny was illustrated in several lineages in which *Sire* and *Reina* account for a significant percentage of LTR ubiquity.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Review Article

Fireflies in South East Asia: Species Diversity, Distribution, and Habitat (2015-2021)

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ABSTRACT

Fireflies are one of the most famous luminous insects that emit bioluminescence. The most famous fireflies in Southeast Asia are *Pteroptyx*, of the order Coleoptera and the Lampyridae family. This review paper combined the data on the species diversity and firefly distribution in Southeast Asian countries such as Malaysia, the Philippines, Indonesia, Cambodia, Myanmar, Singapore, Sri Lanka, Papua New Guinea, Laos, Thailand, and Vietnam published in 2015-2021. Some countries have limited data and no studies to identify firefly species and their habitat from 2015 to 2021; the data before 2015 was used. Furthermore, the lack of studies by Southeast Asian researchers regarding the richness of firefly species has been reviewed. Malaysian and Thailand researchers are among the forerunners in the study related to fireflies in the Southeast Asian region compared to other Southeast Asian countries. Lastly, not much is known about the display trees or habitat of fireflies in many areas such as the Philippines, Indonesia, Cambodia, Myanmar, Singapore, Sri Lanka, Papua New Guinea, Laos, Thailand, and Vietnam. More studies are warranted to be conducted in the future on firefly species and their habitat.

Keywords: Bioluminescence, Coleoptera, fireflies, insects, Lampyridae, *Pteroptyx*, Southeast Asia

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INTRODUCTION

Insects include about 5.5 million species that have been documented based on the projected richness of species on a global scale (Stork, 2018). Beetles belong to the Coleoptera insect order, in which there are approximately 350,000 identified species (Slipinski et al., 2011). Beetles first started to evolve around about 297 million years old (Zhang et al., 2018) and account for insects makeup 38% of all recognised species presently (Stork, 2018). Coleoptera is also the most significant insect order, and fireflies belong to this order (Mckenna & Farrell, 2009). The beetles/insects in this Coleoptera order are also the most numerous and most prosperous insects on this earth (Slipinski et al., 2011). Although the number of beetles identified is almost 25% of all animal species living on earth, many more species are unidentified (Grove & Stork, 2000). McDonald (2003) estimates that 12 million of the 30 million Arthropod species found on earth are beetle species. However, according to Nielsen and Mound (1999), the number of beetle species described is unclear, but the species may be between 300,000 and 450,000.

Fireflies are not 'flies,' but rather beetles belonging to the Lampyridae family. Flies have one pair of wings, whereas all other winged insects have two or four pairs of wings (Dawood & Saikim, 2016). Coleoptera fireflies, which belong to the Lampyridae family, are widely known for their bioluminescent courtship sign used by the adults of several species (Lloyd, 2008) believed that synchronisation enhances the female's ability to distinguish the male's flash patterns in particular (Buck & Buck, 1968). Bioluminescence lampyrid in fireflies originates from the larval stage (Martin et al., 2017), where it acts as an aposematic signal (De Cock & Matthysen, 2003; Marek et al., 2011) that tells predators that they are unpalatable (Vencl et al., 2016). It could also be a possible tactic to attract prey (Bechara & Stevani, 2018). Attested per Moiseff and Copeland (2010), synchronous flashing is a behavioural technique to reduce visual clutter. In contrast, the the current study indicates that bioluminescence created by flying adult fireflies is often used to deter bat predators (Leavell et al., 2018). Bioluminescence is the result of the conversion of chemical energy into photons (Figure 1).

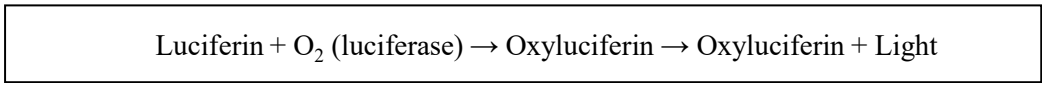

Figure 1. Bioluminescence process (Bechara & Stevani, 2018)

Note. Luciferin: Oxidation of a substrate; O_2 (Luciferase): Catalysed molecular oxygen; Oxyluciferin: Singlet excited-state product

Thus, using identical chemical routes involving the luciferin compound, Elateridae, Rhagophthalmidae, Phengodidae, and Lampyridae emit light, as do various structurally alike to luciferases (Day et al., 2009). Therefore, fireflies of the Coleoptera order in the Lampyridae family are among the night's most attractive and majestic insects. These fireflies look enticing because they have a special bioluminescent (Lewis & Cratsley, 2008; Ohba, 2004) display that emits flickering light, which makes them a potentially significant insect conservation species (Jusoh et al., 2018; Lewis et al., 2020) and sustainable ecotourism attraction (Jusoh et al., 2018). Bioluminescence is created when an enzyme called luciferase catalyses the reaction, which manifests as a flashing light emitted by fireflies (Nur Khairunnisa et al., 2016). *Pteroptyx* fireflies can produce sharp flashes with an average flashing duration of 320 milliseconds per flash, including several species of fireflies that possess 'perfect synchronisation' (Abdul Razak & Sulaiman, 2016). Foo and Dawood (2016) reported that the *Pteroptyx bearni* is the dominant firefly species found in the mangrove forest of Kawang in Sabah, Malaysia, which can be a firefly tourism spot.

Unfortunately, there was a reduction in the incidence and abundance of numerous species of fireflies in the last few decades (Lauff, 2017; Lloyd, 2018). The vast areas of mangroves on the river banks in South East Asia have been cleared for shrimp farming, flood mitigation, or oil palm plantations. These affected areas have become ill-suited for the growth and reproduction of *Pteroptyx* firefly larvae and its snail prey (Jusoh et al., 2010b; Jusoh & Hashim, 2012; Thancharoen, 2012; Wong & Yeap, 2012). Plants play an essential role in an insect's life-cycle because it is used as a stage for insect mating and food or egg-laying (Kaiser et al., 2017). However, there are also other factors, such as habitat change (Sánchez-Bayo & Wyckhuys, 2019), use of pesticides (Disque et al., 2019) as well as light pollution (Firebaugh & Haynes, 2016), that cause a decrease in the variety and quantity of fireflies. Since 2007, the firefly population has declined by 42 per cent over ten years due to land clearing along the river (Nadirah et al., 2020).

Prasertkul (2018) stated that Malaysian researchers have begun to perform various large-scale surveys, and *Pteroptyx* (especially *Pteroptyx tener*) studies have been conducted along some Malaysian rivers including, those in Selangor, Rembau, Linggi, Kerteh, and Kuala Sepetang. These studies provide valuable information on firefly abundance, distribution, seasonal variation, and the connection between plant species and fireflies. However, according to Prasertkul (2018), researchers in Thailand are less interested in studying *Pteroptyx* congregations since most recent studies have only focused on biodiversity surveys, conservation approaches, life cycle studies, and detailed habitat descriptions. Although, as Jusoh et al. (2020) stated, synchronous flashing fireflies of the genus *Pteroptyx* are found all across Southeast Asia, although little is known about

their biodiversity. According to them, recent investigations in Malaysia have shown the well-known population-level phylogeographic structure of the *P. tener* and *P. bearni*, implying the existence of crypto species. Second, morphological and genetic similarities between *Pteroptyx balingiana* and *Pteroptyx malaccae* have prompted debate over the former's validity as a different species. Consequently, they recommended that research be conducted to expand the geographical, taxonomic, and genetic sampling of Southeast Asian fireflies to understand the species biodiversity better. Similarly, according to Chen et al. (2019), basic information regarding biodiversity and its evolutionary history is still insufficient.

This review has gathered and analysed data/list, species diversity, and firefly distribution in Southeast Asia countries, such as Malaysia, Philippines, Indonesia, Cambodia, Myanmar, Singapore, Sri Lanka, Papua New Guinea, Laos, Thailand, and Vietnam papers published from 2015 to 2021. Meanwhile, this paper also collected data on display trees and firefly habitat areas and factors that influence selecting certain trees as their habitat and display trees.

FIREFLY LIST AND SPECIES DIVERSITY IN SOUTHEAST ASIAN COUNTRIES

Lampyridae is a cosmopolitan family made up of seven sub-families, 67 genera, and more than 2,000 species of fireflies that have been described worldwide (Da Silveira & Mermudes, 2014; Hu & Fu, 2018; Mu et al., 2016), most of which are found in tropical regions around the world (Hu & Fu, 2018). More than 400 species have been identified in Southeast Asia and the Indo-Pacific regions, most of which belong to the Luciolinae family (Ballantyne et al., 2015). There is only one species of *Pteroptyx* Olivier (*Maipo* sp. nov.) firefly identified in Hong Kong (Ballantyne et al., 2011), but Southeast Asia is the home to this species (Ballantyne et al., 2019; Jusoh et al., 2018). The firefly species primarily found in Southeast Asian countries, such as Malaysia, Thailand, Indonesia, Vietnam, and the Philippines, are *P. malaccae*, *Luciola pupilla*, and *P. tener* (Abdul Razak & Sulaiman, 2016). In addition, *Pteroptyx* is an East Asian and Southeast Asian descent genus with 18 species found from Hong Kong (Ballantyne et al., 2015, 2019; Jusoh et al., 2018), south to Southeast Asia (Ballantyne, 2001), and west to the Madras region of India (Ballantyne et al., 2011; Ballantyne & McLean, 1970).

The *P. tener* population was first discovered in Thailand mangrove forest in 2015 (Sriboonlert et al., 2015). They also mentioned that this was an uncommon occurrence for this species compared to the other main species in the area (*Pteroptyx malaccae* and *Pteroptyx valida*). Therefore, they suggested that extensive research utilising molecular data, morphology and behaviour, such as lightning analysis, is needed to learn more about *P. tener*'s distribution and boundaries over the Thai-Malay peninsula. It is because morphological differences in *P. tener* may come to light of variances in geographical location. Whereas in southern Thailand, there are nine species of *Pteroptyx*, once recorded in Peninsular Malaysia (Jusoh et

al., 2018) (Figure 2 for *Pteroptyx* species distribution in Thailand), of which *P. tener* was one of the most widely recorded species (Foo & Dawood, 2017). Jaikla et al. (2020) found *P. tener* only found at one site in Surat Thani province in Thailand. The identification of this species in Surat Thani validated the findings by Sartsanga et al. (2018) and Sriboonlert et al. (2015). The *P. tener* is found to be prevalent in Peninsular Malaysia, such as in Sungai Sepetang, Perak (Hazmi & Sagaff, 2018; Sulaiman et al., 2017), Sungai Bernam, Selangor (Shahara et al., 2017), Sungai Johor, Johor (Sulaiman et al., 2016), and Sungai Chukai, Kemaman (Mahmod et al., 2018).

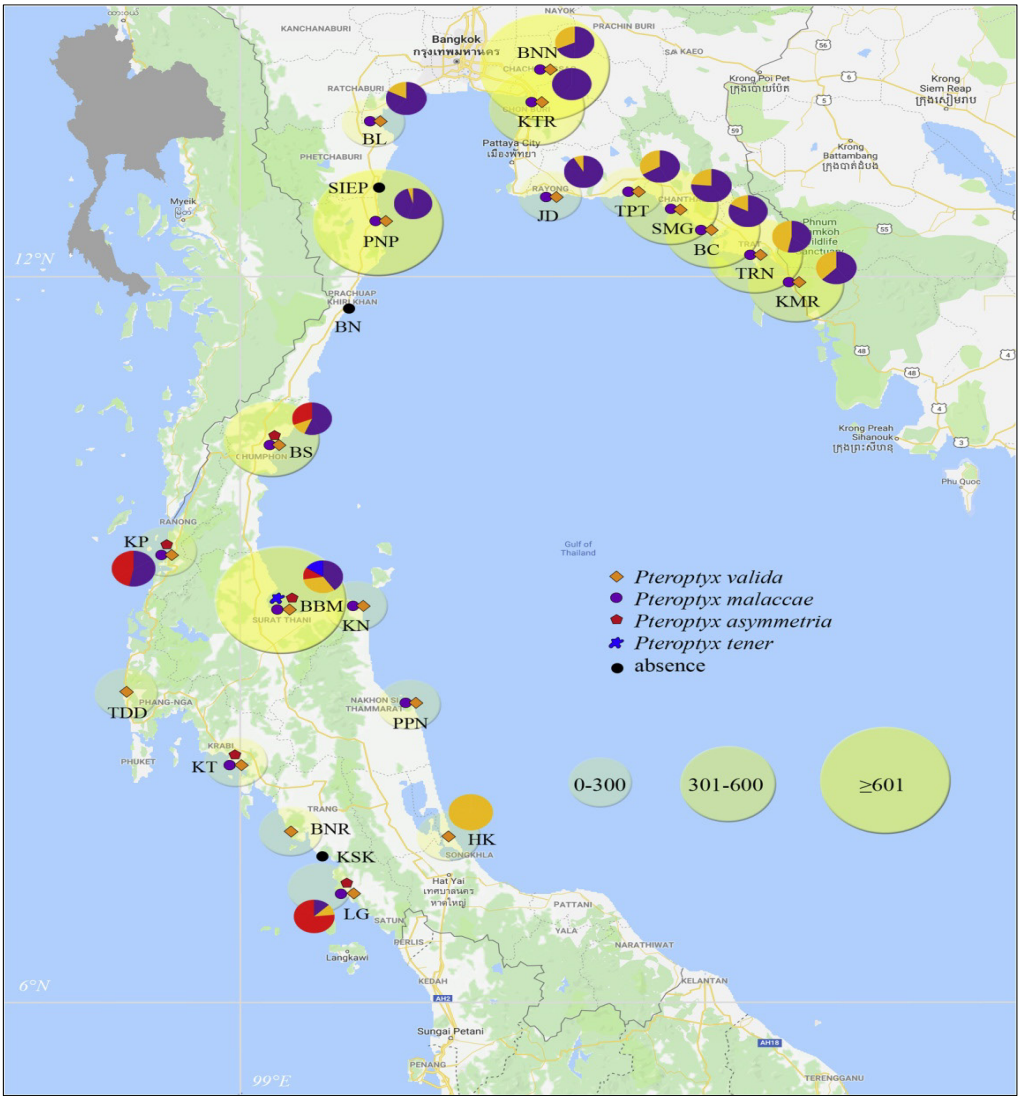

Figure 2. *Pteroptyx* species distribution in Thailand (Jaikla et al., 2020)

Sriboonlert et al. (2015) have also examined *Pteroptyx* museum specimens from two major insect museums in Thailand. From the examination, they discovered 319 *P. malaccae* and 53 *P. valida* specimens collected from 47 locations in ten provinces between 1993 and 2005, including Chanthaburi, Nakhon Pathom, and Nakhon Si Thammarat, Pathum Thani, Phang Nga, Phetchaburi, Samut Sakhon, Samut Songkhram, Sukho Thai, and Trat. The examination again discovered that 117 specimens were *P. malaccae* and 15 were *P. valida*, taken from 11 locations in six provinces between 1999 and 2008, including Chanthaburi, Nakhon Si Thammarat, Pattani, Phetchaburi, Samut Songkhram, and Trat. Using a range of species boundary analyses, Jusoh et al. (2020) discovered that *P. tener* populations along the west coast of Peninsular Malaysia differ from those on the east coast and in Borneo, although no physical differences. However, the investigation was unable to differentiate *P. bearni* from Borneo and eastern Peninsular Malaysia or distinguish *P. balingiana* and *P. malaccae* as distinct species, suggesting that these populations may be specific or indicative of a new species.

According to Jaikla et al. (2020), *P. valida* was the first to be detected in 11 provinces in Thailand, namely in Chachoengsao, Chon Buri, Chumphon, Songkhla, Krabi, Phang Nga, Phetchaburi, Prachuap Khiri Khan, Ranong, Rayong, and Trang. The study also discovered the first species of *Pteroptyx asymmetria* in Chumphon and *P. malaccae* in Krabi province. In Malaysia, *P. malaccae* has only been found once in Sarawak, in Limbang (Jusoh et al., 2018). However, Abdullah et al. (2020) recently found this species along the Niah River in Miri, Sarawak. This species has been identified in Sabah (Foo & Dawood, 2015) and in Muar (Johor), Chukai (Terengganu), and Pahang Tua River (Pahang) (Jusoh et al., 2018). *Pteroptyx malaccae* was found in Malaysia in smaller populations, generally in sympathy with *P. tener*, found in much larger groups (Jusoh et al., 2011, 2018). In contrast to what happened in Thailand, *P. malaccae* developed enormous assemblages in trees along river banks in sympathy with *P. valida* Olivier (Prasertkul, 2018).

In the meantime, *P. asymmetria* has only been found in Malaysia's western peninsula (Jusoh et al., 2018). Thus, Jaikla et al. (2020) found that *P. asymmetria* is restricted to locations on the southern coast of Thailand, which has a climate similar to Malaysia. The research on firefly abundance, distribution, seasonal variation, and the relationship between plant species and fireflies is very significant since, based on the findings by Dawood and Saikim (2016), the occurrence of *P. bearni* has been reviewed in Sabah, as well as an alarming decrease in the population of some fireflies in Likas, triggered by the loss of mangroves. One study found that *P. bearni* no longer exists in Likas (Dawood & Saikim, 2016). Apart from *P. bearni*, *Pteroptyx gelasina* is also no longer found in Likas (Dawood & Saikim, 2016). However, most fireflies remain a mystery; even relevant information is practically similar to the distribution of

the species (Mobilim & Dawood, 2020). Geographically and genetically, *P. bearni* Olivier was divided into two groups: one in the eastern Malaysian Peninsula and another one in Borneo, showing colour differences between the two groups (Jusoh et al., 2014). Figure 3 shows the geographical distribution of the *Pteroptyx* samples used by Jusoh et al. (2020) in their study covering Peninsular Malaysia (East and West), Borneo, and Thailand. While, Table 1 below lists the species of fireflies found in several countries in Southeast Asia, including Malaysia, the Philippines, Indonesia, Cambodia, Myanmar, Vietnam, Laos, Brunei, Papua New Guinea, Singapore, and Thailand, from 2015-2021.

Not much is known about the fauna of fireflies in many parts of Southeast Asia, such as Laos, Cambodia, Philippines, Indonesia, Vietnam, Timor-Leste, Myanmar, Singapore, and Brunei, where many species are less known due to lack of studies on the abundance and identification of firefly species-flashes compared to Thailand and Malaysia. The majority of the firefly genera found in Indonesia are endemic fireflies found in Sumatra, Kalimantan, and Papua, although some, such as *Diaphanes javanus*, may be found on Java island (Puspitaningrum et al., 2017). In Vietnam, an unusual genus of lampyrids has been found by Jeng et al. (2007), namely *Oculogryphus fulvus* Jeng. Later, Jeng et al. (2011) have discovered a second species of the enigmatic lampyrid genus *Oculogryphus* known as *Oculogryphus bicolor* sp. in Huong Son, Ha Tinh Province, Vietnam. The Philippines is the first country outside Malaysia (Sabah and Sarawak) to record a new rare Southeast Asian firefly *Pygoluciola Satoi* (Ballantyne, 2008). Ballantyne and Lambkin (2009, 2013) and Ballantyne et al. (2015) include essential references that are partly linked to the Luciolinae fireflies present in the Philippines. Ohba and Meyer-Rochow (2012) have recorded the existence of a Guinea firefly known as *Pteroptyx effulgens* occur on the same tree (unknown species) at Open Bay (New Britain) and Kaw (New Ireland) in Papua New Guinea. Similarly, the study conducted by Iamba et al. (2021) in the Balsa plantations area of East New Britain Province, Papua New Guinea, in 2020 have found the same species, namely *P. effulgens*.

Electromethes gen. n. (Omethidae) and *Electotreta* gen. n. (Lampyridae), as well as two species, (*Electromethes alleni* sp. n. and *Electotreta rasnitsyni* sp. n.), were discovered in Baltic amber in Myanmar (Kazantsev, 2012a). Both taxa appear to be linked to the East Asian omethid (*Electromethes*) and ototretin (*Electotreta*). Again fossils of the genus of *Eoluciola* gen. n., and species *Eoluciola varang* sp. n., are described from the same place (Baltic amber), Myanmar. The taxa are placed in between Luciolini and Pristolycini, i.e. Luciolinae (Kazantsev, 2012b). The fossil genus of firefly, *Protoluciola* gen. n., and a new species, *Protoluciola albertalleni* Kazantsev sp. n., have been discovered Cretaceous Burmese amber, Myanmar (Kazantsev, 2015). While Jeng et al. (2003) reported the distribution of *Luciola substriata* in Myanmar.

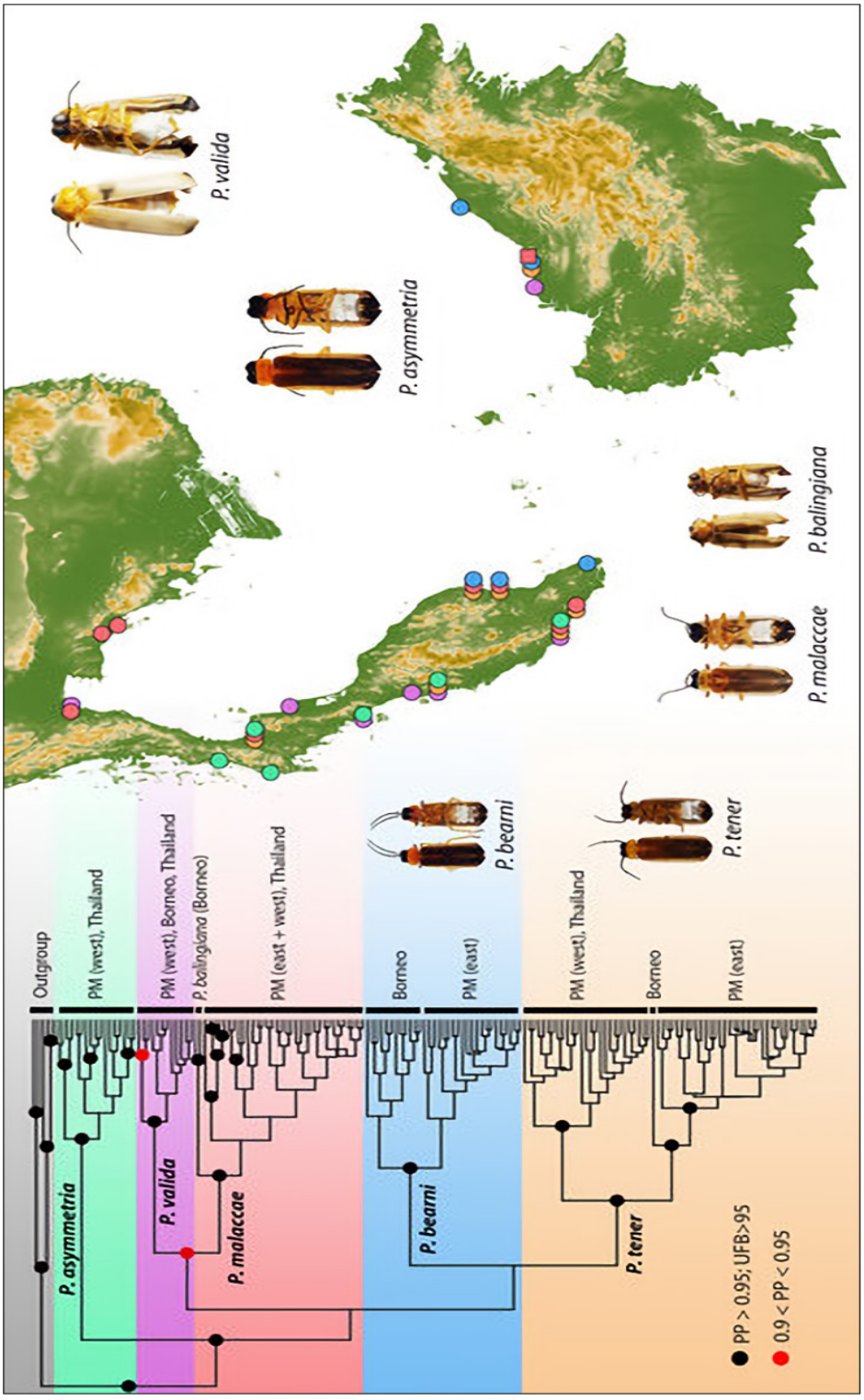

Figure 3. The geographical distribution of *Pteroptyx* samples used by Jusoh et al. (2020) in their study

Tan (2018) documented five genera of fireflies on Pulau Ubin, Singapore, including *P. valida*, *Diaphanes* sp., *Colophotia* c.f. *praeusta*, *Curtos* sp., and presumably *Stenocladius* sp. and *Diplocladon* sp. in a study performed from 2012 to 2016. While, *Curtos* sp., which he discovered in his research, might be the first to be found in Singapore. Figure 4 shows the distribution of many species related to habitat type in the western and eastern parts of Pulau Ubin, Singapore, with *Curtos* sp. only found in secondary forests at one location in Bukit Tinggi, and *Diaphanes* sp. occurring in secondary forests in the eastern and western parts of Pulau Ubin (Tan, 2018). From February 2009 to April 2010, Chan et al. (2012) conducted a nationwide survey in Singapore to determine the species richness, distribution, and abundance of fireflies at 14 sites across five habitats. The survey discovered three genera in the Luciolinae family, two in the Lampyrinae family, one in the Ototretinae family, one in the Rhagophthalmidae family, and 11 species (some of them are unknown), including *Luciola* sp. 2. *Luciola* sp. 2 is noteworthy because specimens were collected from freshwater swamp forests in Singapore's central catchment region and do not align with recognised *Luciola* species' characterisation.

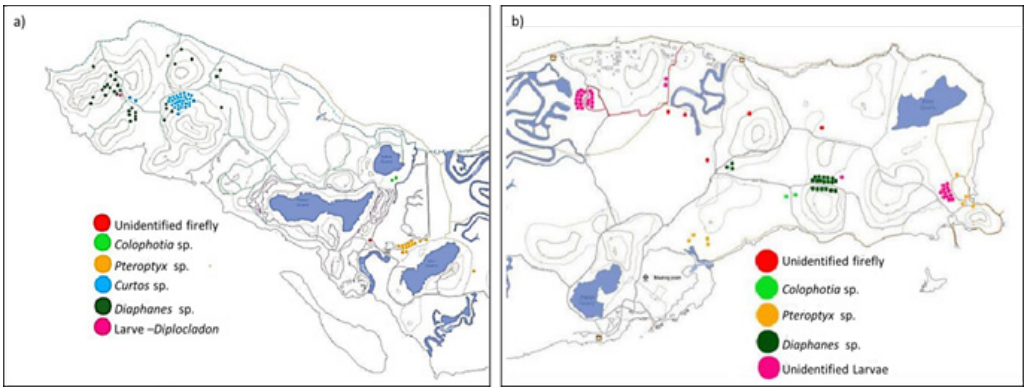

Figure 4. Records of fireflies in Pulau Ubin, Singapore; a) Western; b) Eastern (Tan, 2018)

Pteroptyx valida Olivier, *Stenocladius* sp., and *Diplocladon* sp. were all verified in the same research; however, *Pteroptyx bearni* Olivier and *Lucidina wallacei* Pic were not. While, throughout a survey performed by Jusoh et al. (2021) in the Nee Soon Swamp Forest (NSSF) on October 9, 2018, October 11, 2018, January 11, 2019, and January 18, 2019, targeting specimens that fit the description of *Luciola* sp. 2 sensu have documented four specimens (three males and one female) were collected (all discovered on January 2019). Figure 5 displays a map of Singapore that illustrates the location of *Luciola singapura* Jusoh & *Ballantyne* sp. nov. in the Nee Soon Swamp

Forest (NSSF), as well as other *Luciola* species in Asia and the Pacific islands that were examined and evaluated in a study conducted by Jusoh et al. (2021).

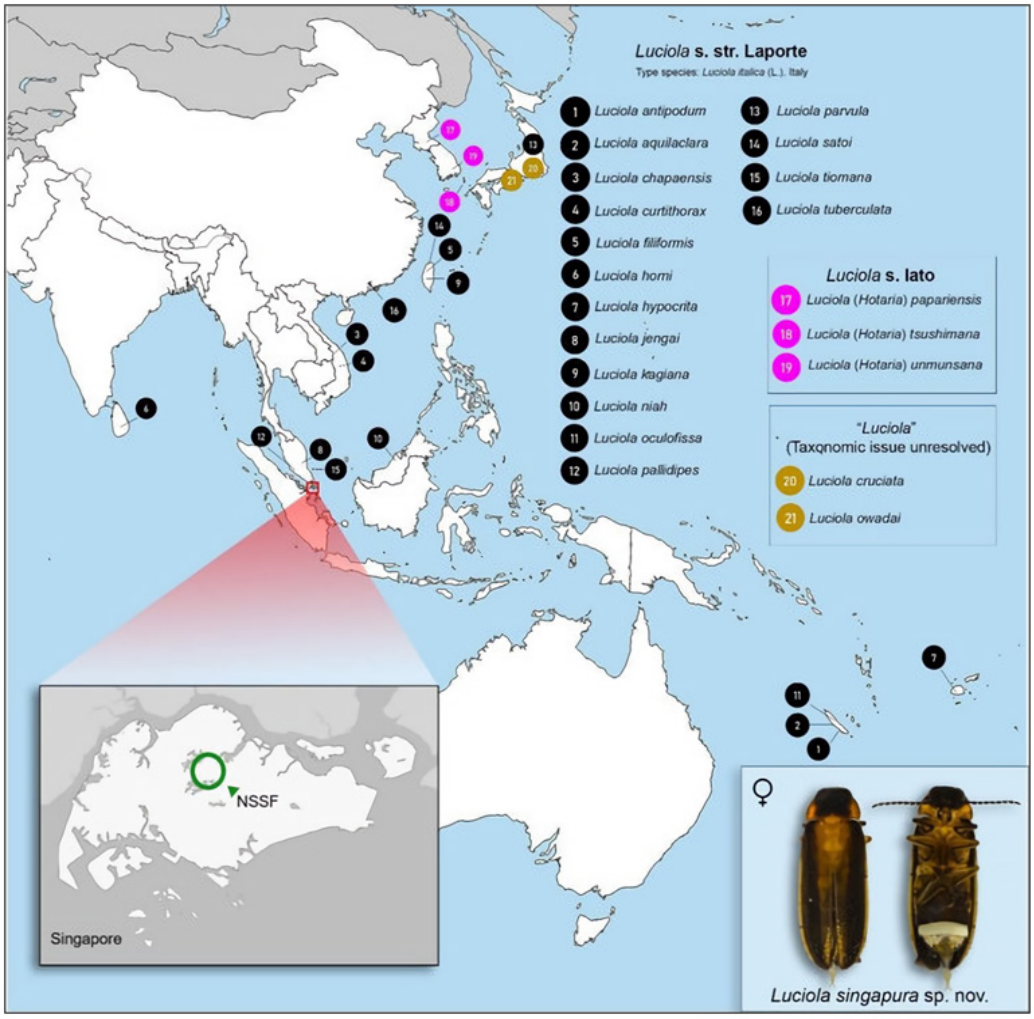

Figure 5. Luciola Singapura Jusoh & *Ballantyne* sp. nov. in the Nee Soon Swamp Forest (NSSF) as well as other localities of *Luciola* species in Asia and the Pacific Islands (Jusoh et al., 2021)

In Sri Lanka, the study of the taxonomy of fireflies for the first time began in the early 18th century (Wijesekara & Wijesinghe, 2003). Wijekoon et al. (2012) conducted a study on the regional diversity of fireflies of the subfamily Luciolinae in 2010 in Sri Lanka, covering grassland areas in Uva and Sabaragamuwa and Central, North-Central, West, South, East, North, and North regions-West. The findings of this study revealed that there are nine different species of firefly, all of which are *Luciola* species

(*Luciola antennalis*, *Luciola candezei*, *Luciola chinensis*, *Luciola horni*, *Luciola humeralis*, *Luciola intricata*, *Luciola melaspis*, *Luciola nicollieri*, and *Luciola vespertina*) (Figure 6 and Table 1). The survey was conducted by Wijekoon (2013) from 2010 to 2012 in nine provinces in Sri Lanka [same as the study area in Wijekoon et al. (2012)] showed 13 species of fireflies belonging to six genera (*Asymmetricata*, *Curtos*, *Diaphenes*, *Lamprigera*, *Luciola*, and *Stenocladius*) were recorded. The species recorded were *Asymmetricata humeralis*, *Curtos costipennis*, *Diaphenes lutescens*, *Diaphenes vitrifera*, *Luciola cerata*, *Luciola cingulata*, *Luciola dubia*, *Luciola extricans*, *Luciola horni*, *Luciola melaspis*, *Luciola praeusta*, *Lamprigera tenebrosa*, and *Stenocladius* sp. Wijekoon et al. (2016) classified specimens in the National Sri Lankan collection in Colombo as *Abscondita chinensis*, *Abscondita perplexa*, and *Abscondita promelaena*, as well as documenting *Luciola doriae* without commenting on its probable position. They also validated the presence of *Luciola humeralis* and *Luciola impressa* in Sri Lanka, classifying both as *Asymmetricata* genus (Figure 6).

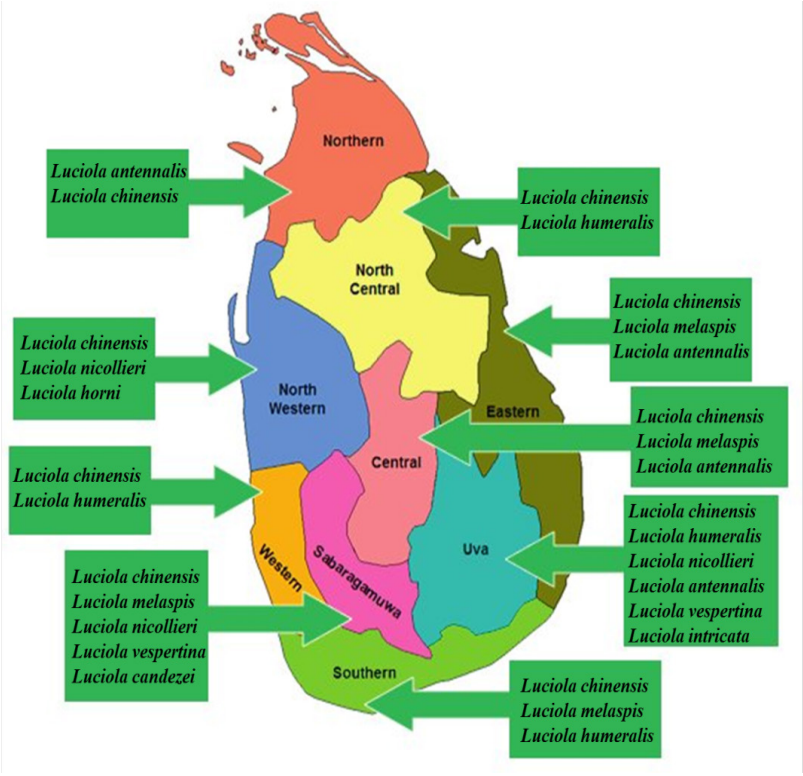

Figure 6. Firefly species discovered in nine provinces of Sri Lanka in 2010 (Wijekoon et al., 2012)

Table 1

Firefly species found in Southeast Asian countries from 2015-2021

No.	Species	Country	References
1	*Abscondita anceyi*	Thailand	Wattanachaiyingcharoen et al. (2016)
2	*Abscondita anceyi* (Olivier 1883)	Myanmar	Ballantyne et al. (2016); Olivier (1883)
3	*Abscondita berembun* Nada sp. nov.	Malaysia	Ballantyne et al. (2019)
4	*Abscondita chinensis*	Sri Lanka, Thailand	Wattanachaiyingcharoen et al. (2016); Wijekoon et al. (2016)
5	*Abscondita chinensis* (L. 1767)	Cambodia, Myanmar, Thailand, Vietnam	Ballantyne et al. (2016); Linnaeus (1767)
6	*Abscondita jerangau* Nada sp. nov.	Malaysia	Ballantyne et al. (2019)
7	*Abscondita pallescens*	Indonesia, Malaysia	Ballantyne et al. (2019)
8	*Abscondita perplexa*	Sri Lanka	Wijekoon et al. (2016)
9	*Abscondita perplexa* (Walker 1858)	Cambodia	Ballantyne et al. (2016); Walker (1858)
10	*Abscondita promelaena*	Sri Lanka	Wijekoon et al. (2016)
11	*Abscondita promelaena* (Walker 1858)	Myanmar	Ballantyne et al. (2016)
12	*Abscondita promelaena* (Walker) comb. nov.	Myanmar	Ballantyne et al. (2013)
13	*Asymmetricata circumdata*	Thailand	Pronak et al. (2018); Wattanachaiyingcharoen et al. (2016)
14	*Asymmetricata ovalis*	Thailand	Pronak et al. (2018); Wattanachaiyingcharoen et al. (2016)
15	*Atripennis* Pic 1934	Malaysia	Ballantyne et al. (2019); Pic (1934)
16	*Atyphella testaceolineata* Pic, 1939	Indonesia	Ballantyne and Lambkin (2009); Pic (1939)
17	*Australoluciola* sp.	Thailand	Sartsanga et al. (2017)
18	*Australoluciola baduria* sp. nov.	Indonesia	Ballantyne and Lambkin (2013)

Table 1 *(Continued)*

No.	Species	Country	References
19	*Australoluciola japenensis* sp. nov.	Indonesia	Ballantyne and Lambkin (2013)
20	*Baolacus lajoyei* Pic, 1915	Laos, Malaysia, Vietnam	Janisova and Bocakova (2013); Pic (1915)
21	*Ceylanidrilus bipartitus* Pic, 1911	Sri Lanka	Janisova and Bocakova (2013); Pic (1911)
22	*Colophotia brevis*	Malaysia	Jusoh et al. (2018)
23	*Colophotia* c.f. *praeusta*	Singapore	Tan (2018)
24	*Colophotia praeusta*	Malaysia	Jusoh et al. (2018)
25	*Curtos cerea*	Indonesia, Thailand	Annisa (2016); Wattanachaiyingcharoen et al. (2016)
26	*Curtos* sp.	Singapore	Tan (2018)
27	*Curtos* sp. 1	Indonesia	Annisa (2016)
28	*Curtos* sp. 2	Indonesia	Annisa (2016)
29	*Diaphanes javanus*	Indonesia	Annisa (2016); Puspitaningrum et al. (2017)
30	*Diaphanes* sp.	Indonesia, Singapore	Puspitaningrum et al. (2018); Tan (2018)
31	*Diaphanes* sp.2	Thailand	Wattanachaiyingcharoen et al. (2016)
32	*Diaphanes* sp.3	Thailand	Wattanachaiyingcharoen et al. (2016)
33	*Diaphanes* sp.4	Thailand	Wattanachaiyingcharoen et al. (2016)
34	*Diplocladon* sp.	Singapore	Jusoh et al. (2021); Tan (2018)
35	*Drilaster* (*Apodrilus*) *agcoensis* n. sp.	Philippines	Janisova and Bocakova (2013)
36	*Drilaster axillaris* Kiesenwetter, 1879	Borneo, Indonesia, Malaysia, Philippines, Sri Lanka, Thailand, Vietnam	Janisova and Bocakova (2013)
37	*Drilaster* (*Kerincius*) *medioniger* n. sp.	Indonesia	Janisova and Bocakova (2013)
38	*Electromethes alleni* sp. n.	Myanmar	Kazantsev (2012a)

Table 1 *(Continued)*

No.	Species	Country	References
39	*Electotreta rasnitsyni* sp. n.	Myanmar	Kazantsev (2012a)
40	*Emasia* gen. nov.	Borneo	Bocakova and Janisova (2010)
41	*Emasia dentata* sp. n.	Borneo	Bocakova and Janisova (2010)
42	*Eoluciola varang* sp. n.	Myanmar	Kazantsev (2012b)
43	*Eugeusis nigripennis* Pascoe, 1887	Myanmar, Thailand, Vietnam	Janisova and Bocakova (2013); Pascoe (1887)
44	*Eugeusis palpator* Westwood, 1853	Sri Lanka	Janisova and Bocakova (2013); Westwood (1853)
45	*Falsophaeopterus fruhstorferi* Pic, 1911	Indonesia	Janisova and Bocakova (2013); Pic (1911)
46	*Hyperstoma marginata* Wittmer, 1979	Sri Lanka	Janisova and Bocakova (2013); Wittmer (1979)
47	*Inflata indica*	Thailand	Sriboonlert and Wonnapinij (2019)
48	*Inflata indica* (Motschulsky 1854) comb. nov.	Thailand	Ballantyne et al. (2015); Motschulsky (1854)
49	*Lamellipalpodes annandalei* Maulik, 1921	Myanmar, Thailand	Janisova and Bocakova (2013); Maulik (1921)
50	*Lamprigera tenebrosa*	Thailand	Pronak et al. (2018)
51	*Lamprigera yunnana*	Thailand	Wattanachaiyingcharoen et al. (2016)
52	*Lampyris noctiluca*	Indonesia	Ratnawulan et al. (2020)
53	*Lloydiella japenensis* sp. n.	Indonesia	Ballantyne and Lambkin (2009)
54	*Luciolinae angusticollis* Olivier 1886	Philippines	Ballantyne et al. (2016); Olivier (1886)
55	*Luciola antennalis*	Sri Lanka	Wijekoon et al. (2012)
56	*Luciolinae apicalis* (Eschscholtz 1822)	Philippines	Ballantyne et al. (2016); Eschscholtz (1822)
57	*Luciola aquatilis* Thancharoen	Thailand	Sumruayphol and Chaiphongpachara (2019)
58	*Luciola candezei*	Sri Lanka	Wijekoon et al. (2012)
59	*Luciola chinensis*	Sri Lanka	Wijekoon et al. (2012)
60	*Luciola curtithorax*	Thailand	Wattanachaiyingcharoen et al. (2016)
61	*Luciola doriae*	Sri Lanka	Wijekoon et al. (2016)

Table 1 *(Continued)*

No.	Species	Country	References
62	*Luciola horni*	Sri Lanka	Wijekoon et al. (2012)
63	*Luciola humeralis*	Sri Lanka	Wijekoon et al. (2012, 2016)
64	*Luciola impressa*	Sri Lanka	Wijekoon et al. (2016)
65	*Luciola indica*	Thailand	Wattanachaiyingcharoen et al. (2016)
66	*Luciola intricata*	Sri Lanka	Wijekoon et al. (2012)
67	*Luciola melaspis*	Sri Lanka	Wijekoon et al. (2012)
68	*Luciola nicollieri*	Sri Lanka	Wijekoon et al. (2012)
69	*Luciola picea*	Indonesia	Annisa (2016)
70	*Luciola singapura* Jusoh & Ballantyne sp. nov.	Singapore	Jusoh et al. (2021)
71	*Luciola* sp.	Malaysia	Mobilim and Dawood (2020)
72	*Luciola* sp.1	Indonesia	Annisa (2016)
73	*Luciola* sp.2	Indonesia	Annisa (2016)
74	*Luciola* sp.3	Thailand	Wattanachaiyingcharoen et al. (2016)
75	*Luciola trilucida*	Thailand	Wattanachaiyingcharoen et al. (2016)
76	*Luciola vespertina* (*Luciola praeusta* complex)	Sri Lanka	Wijekoon et al. (2012)
77	*Luciola* WFA	Malaysia	Jusoh et al. (2018)
78	*Luciolinae bicoloriceps* Pic 1924	Philippines	Ballantyne et al. (2016); Pic (1924)
79	*Luciolinae bicoloripes* Pic 1927	Vietnam	Ballantyne et al. (2016); Pic (1927)
80	*Luciolinae brahmina* Bourgeois 1890	Cambodia	Ballantyne et al. (2016); Bourgeois (1890)
81	*Luciolinae delauneyi* Bourgeois 1890	Vietnam	Ballantyne et al. (2016); Bourgeois (1890)
82	*Luciolinae deplanata* Pic 1929	Vietnam	Ballantyne et al. (2016); Pic (1929)
83	*Luciolinae infuscata* (Erichson 1834)	Philippines	Ballantyne et al. (2016); Erichson (1834)
84	*Luciolinae maculipennis* Olivier	Malaysia	Ballantyne et al. (2016)
85	*Luciolinae recticollis* Olivier 1900	Indonesia	Ballantyne et al. (2016); Olivier (1900)

Table 1 *(Continued)*

No.	Species	Country	References
86	*Luciolinae sordida* Olivier 1909	Indonesia	Ballantyne et al. (2016); Olivier (1909)
87	*Luciolinae substriata* Gorham 1880	Indonesia, Myanmar	Ballantyne et al. (2016); Gorham (1880)
88	*Luciolinae succincta* Bourgeois 1890	Cambodia	Ballantyne et al. (2016); Bourgeois (1890)
89	*Luciolinae varia* Olivier 1908	Indonesia	Ballantyne et al. (2016); Olivier (1908)
90	*Medeopteryx amilae* (Satô) comb. nov.	Philippines	Ballantyne and Lambkin (2013)
91	*Medeopteryx flagrans* (Ballantyne) comb. nov.	Indonesia	Ballantyne and Lambkin (2013)
92	*Medeopteryx fulminea* (Ballantyne) comb. nov.	Indonesia	Ballantyne and Lambkin (2013)
93	*Medeopteryx* sp.	Thailand	Pronak et al. (2018)
94	*Mimophaeopterus jacobsoni* Pic, 1930	Indonesia	Ballantyne and Lambkin (2013); Pic (1930)
95	*Ototreta drescheri* Pic, 1937	Indonesia	Janisova and Bocakova (2013); Pic (1937)
96	*Oculogryphus fulvus* Jeng	Vietnam	Jeng et al. (2007)
97	*Ototreta subvittata* Pic, 1943	Indonesia, Malaysia	Janisova and Bocakova (2013); Pic (1943)
98	*Ototreta weyersi* E. Olivier, 1900	Borneo, Indonesia, Malaysia, Philippines, Sri Lanka, Thailand, Vietnam,	Janisova and Bocakova (2013); Olivier (1900)
99	*Poluninius selangoriensis* (*Pteroptyx testacea*)	Malaysia	Jusoh et al. (2018)
100	*Protoluciola albertalleni* sp. n.	Myanmar	Kazantsev (2015)
101	*Pteroptyx asymmetria*	Thailand, Malaysia	Abdullah et al. (2019); Asri et al. (2020); Jaikla et al. (2020); Jusoh et al. (2018); Sartsanga et al. (2018)
102	*Pteroptyx balingiana*	Malaysia	Jusoh et al. (2018)
103	*Pteroptyx bearni* Ballantyne	Malaysia	Foo and Dawood (2017)

Table 1 *(Continued)*

No.	Species	Country	References
104	*Pteroptyx bearni* or *Pteroptyx similis*	Malaysia	Abdullah et al. (2020); Chey (2008, 2010, 2011); Dawood and Saikim (2016); Foo and Dawood (2015); Foo et al. (2017); Jusoh et al. (2018)
105	*Pteroptyx effulgens*	Papua New Guinea	Iamba et al. (2021); Ohba and Meyer-Rochow (2012)
106	*Pteroptyx galbina*	Malaysia	Ballantyne et al. (2015); Jusoh et al. (2018)
107	*Pteroptyx gelasina*	Malaysia	Chey (2008, 2011); Dawood et al. (2018)
108	*Pteroptyx gombakia* sp. nov.	Malaysia	Ballantyne et al. (2015)
109	*Pteroptyx malaccae*	Malaysia, Thailand	Abdullah et al. (2019, 2020); Chey (2010); Dawood and Saikim (2016); Foo and Dawood (2015); Jaikla et al. (2020); Sartsanga et al. (2018)
110	*Pteroptyx malaccae* Gorham	Malaysia, Thailand	Asri et al. (2020); Foo and Dawood (2017); Sumruayphol and Chaiphongpachara (2019)
111	*Pteroptyx malaccae* Group 2	Malaysia	Jusoh et al. (2018)
112	*Pteroptyx malaccae* Group 3	Malaysia	Jusoh et al. (2018)
113	*Pteroptyx malaccae* Group 4	Malaysia	Jusoh et al. (2018)
114	*Pteroptyx sayangia* sp. nov.	Malaysia	Ballantyne et al. (2015)
115	*Pteroptyx surabayia* sp. nov.	Indonesia	Ballantyne et al. (2015)
116	*Pteroptyx tener*	Indonesia, Malaysia, Thailand	Abdullah et al. (2019); Chey (2010); Dawood and Saikim (2016); Foo and Dawood (2015); Hazmi and Sagaff (2018); Jaikla et al. (2020); Jusoh et al. (2018); Othman et al. (2018); Sari et al. (2014); Sartsanga et al. (2018); Shahara et al. (2017); Sriboonlert et al. (2015)
117	*Pteroptyx tener* Olivier	Indonesia, Malaysia	Asri et al. (2020); Ballantyne and Lambkin (2013); Foo and Dawood (2017); Salleh et al. (2019)

Table 1 *(Continued)*

No.	Species	Country	References
118	*Pteroptyx valida*	Malaysia, Singapore, Thailand	Abdullah et al. (2019); Dawood and Saikim (2016); Foo and Dawood (2015); Jaikla et al. (2020); Sartsanga et al. (2018); Tan (2018)
119	*Pteroptyx valida* Group 2	Malaysia	Jusoh et al. (2018)
120	*Pteroptyx valida* Olivier	Malaysia, Singapore, Thailand	Foo and Dawood (2017); Jusoh et al. (2021); Sumruayphol and Chaiphongpachara (2019)
121	*Pygatyphella huonensis* (Ballantyne, 1968)	Indonesia	Ballantyne (1968); Ballantyne and Lambkin (2009)
122	*Pygatyphella japenensis* sp. n.	Indonesia	Ballantyne and Lambkin (2009)
123	*Pygatyphella nabiria* sp. n.	Indonesia	Ballantyne and Lambkin (2009)
124	*Pygoluciola satoi*	Philippines	Ballantyne (2008)
125	*Pygoluciola dunguna*	Malaysia	Nada and Ballantyne (2018)
126	*Pygoluciola* sp.1	Thailand	Wattanachaiyingcharoen et al. (2016)
127	*Pyrocoelia analis*	Indonesia, Malaysia, Thailand	Annisa (2016); Jusoh et al. (2018); Wattanachaiyingcharoen et al. (2016)
128	*Pyrocoelia praetexta* Olivier	Thailand	Sumruayphol and Chaiphongpachara (2019)
129	*Pyrocoelia* sp.	Malaysia	Roslan and Sulaiman (2015)
130	*Pyrocoelia* sp.2	Thailand	Wattanachaiyingcharoen et al. (2016)
131	*Pyrophanes appendiculata* Olivier	Indonesia, Philippines	Ballantyne et al. (2015)
132	*Pyrophanes beccarii* Olivier	Indonesia	Ballantyne et al. (2015)
133	*Pyrophanes elongata* Ballantyne sp. nov.	Philippines	Ballantyne et al. (2015)

Table 1 *(Continued)*

No.	Species	Country	References
134	*Pyrophanes quadrimaculata* Olivier	Philippines	Ballantyne et al. (2015)
135	*Pyrophanes semilimbata* (Olivier)	Indonesia, Malaysia, Philippines	Ballantyne et al. (2015)
136	*Pyrophanes similis* Olivier	Indonesia, Philippines	Ballantyne et al. (2015)
137	*Pyrophanes similisimma* sp. nov.	Indonesia	Ballantyne et al. (2015)
138	*Pygoluciola wittmeri*	Malaysia	Mobilim and Dawood (2020)
139	*Sclerotia aquatilis*	Thailand	Pronak et al. (2018)
140	*Stenocladius* sp.	Singapore	Jusoh et al. (2021); Tan (2018)
141	*Trisinuata* sp.	Thailand	Pronak et al. (2018)
142	*Trisinuata* sp. 2	Thailand	Wattanachaiyingcharoen et al. (2016)
143	*Trisinuata microthorax* (Olivier) comb. nov.	Indonesia	Ballantyne and Lambkin (2013)
144	*Trisinuata similispapuae* (Ballantyne) comb. nov.	Indonesia	Ballantyne and Lambkin (2013)
145	*Vesta menetriesi*	Indonesia	Annisa (2016)

DISPLAY TREES/HABITAT OF FIREFLIES ACROSS SOUTHEAST ASIAN COUNTRIES

According to Buck and Buck (1966), tree species used by fireflies as a gathering/convergence area are known by several terms, such as 'mangrove fireflies' or 'firefly trees'. Meanwhile, Chey (2004) and Jusoh et al. (2010a, 2010b) called these trees 'display trees'. Fireflies often randomly pick their spot to converge (Buck & Buck, 1966). However, they often like display trees for a number of reasons, such as proximity to larval prey food, arrangement of leaves conducive for mating, and nectar-like or rubber-like food-providing trees for adults to feed (Jusoh et al., 2010a, 2010b). Sriboonlert et al. (2015) previously associated the volatile distribution of *P. tener* in southern Thailand with the cleavage of mangrove forests. However, Cheng et al. (2020) argued that there was a lack of efforts to consider or clarify how the species had achieved its current distribution in selected river systems in Southeast Asia.

Fireflies have a wide range of habitats, including mangroves, rivers, and inland highlands (Ballantyne et al., 2011). *Pteroptyx* species that inhabit mangroves are mostly in *Sonneratia caseolaris* (L.)

Engler (Sonneratiaceae), *Nypa fruticans* Wurmb. (Arecaceae), *Acanthus ilicifolius* L. (Acanthaceae), *Rhizophora apiculata* Blume (Rhizophoraceae), *Rhizophora mucronata* Lamarck, and *Bruguiera gymnorrhiza* (L.) Lamarck (Rhizophoraceae) (Jusoh et al., 2010a, 2010b; Ohba & Wong, 2004). A study conducted by Prasertkul (2018) on the occurrence of fireflies (*P. malaccae* and *P. valida*) at a park surrounded by an urban area in Samut Prakan Province found that the fireflies were in several tree species including *Sonneratia caseolaris*, *Hibiscus tiliaceus*, *Terminalia catappa*, *Ficus* sp. 1 (Banyan tree), *Ficus* sp. 2 (Fig tree), *Cerbera odollam*, *Albizia procera*, *Bambusa* sp., *Tamarindus indica*, *Pterocarpus indicus*, and *Erythrina variegata*. Of all the plant species, fireflies often use *Sonneratia caseolaris* as a pilgrimage platform, while *Terminalia catappa* (an exotic ornamental plants) are also often used mainly during the rainy season.

According to Jusoh et al. (2018), *P. bearni*, which is found in mangrove areas in Peninsula Malaysia, is restricted to the East Coast in areas bathed in saltwater, while in Miri, Sarawak, this species has been carried far into the river system, which is about 16 km from Mat Shah Jetty, to Maloi, 1 km past the Taniku jetty. The collection of this species in Likau and Niah is located near the estuary. Jusoh (2015) found that *P. galbina* had gathered around the river area but was not limited to mangrove forests. This species was spotted about 30 kilometres from the sea, flying from Niah alone along the forest trail near the river (Jusoh et al., 2018). Meanwhile, *Pteroptyx* Olivier, from the subfamily Luciolinae, is found in many mangrove swamps shrub trees (Jusoh et al., 2018). See Table 2 for a list of firefly species identified in Southeast Asian countries.

Of all 23 study locations located in 16 provinces along the Thai coast, it was found that there were three dominant (90%) mangrove species [*Sonneratia caseolaris* (L.) Engl., *Avicennia* sp. and *Rhizophora* sp.] selected by fireflies as display trees. Recent findings indicate that there are six mangrove species from three families, namely *Acanthaceae*, *Lythraceae*, and *Rhizophoraceae*, which constitute 92.5 per cent of all display trees, while 7.5% are associated with mangrove trees (Jaikla et al., 2020). *Pteroptyx* fireflies like trees with a higher proportion of openings or open spaces in the canopy, whereas trees surrounded by open areas of 0-25% are seldom as display trees. *Pteroptyx* fireflies never occupy a tree covered by a thick canopy (Jaikla et al., 2020). In Peninsular Malaysia, fireflies were discovered congregating on several tree species along the downstream riparian zone, especially the *Sonneratia* spp. (Jusoh et al., 2018; Shahara et al., 2017; Sulaiman et al., 2016, 2017). They are found associated with some riparian flora, such as *Sonneratia caseolaris*, *Hibiscus tiliaceus*, *Nypa frutican*, *Acrotichum aureum*, *Areca cathechu*, *Ficus* spp., and *Oncosperma tigillarium* (Juliana et al., 2012; Khoo et al., 2012), which are also found in Thailand (Prasertkul, 2018).

Table 2

Firefly display trees in Malaysia and Thailand

No.	Species	Country	References
1	*Acacia auriculiformis* A. Cunn.	Thailand	Jaikla et al. (2020)
2	*Albizia procera*	Thailand	Prasertkul (2018)
3	*Aegiceras floridum*	Malaysia	Foo and Dawood (2016)
4	*Avicennia* sp.	Thailand; Malaysia	Abdullah et al. (2020); Foo and Dawood (2016, 2017); Jaikla et al. (2020)
5	*Bambusa* sp.	Thailand	Prasertkul (2018)
6	*Barringtonia* sp.	Malaysia	Mahmod et al. (2018)
7	*Bruguiera* sp.	Thailand; Malaysia	Abdullah et al. (2020); Jaikla et al. (2020)
8	*Bruguiera parviflora*	Malaysia	Abdullah et al. (2020)
9	*Ceriops* sp.	Thailand	Jaikla et al. (2020)
10	*Cerbera odollam*	Thailand	Prasertkul (2018)
11	*Cocos nucifera* L.	Thailand	Jaikla et al. (2020)
12	*Cyperus involucratus* Rottb.	Thailand	Jaikla et al. (2020)
13	*Derris* sp.	Malaysia	Abdullah et al. (2020)
14	*Excoecaria agallocha*	Malaysia	Foo and Dawood (2017)
15	*Excoecaria indica* L.	Malaysia	Foo and Dawood (2017)
16	*Ficus benjamina* L.	Thailand	Jaikla et al. (2020)
17	*Ficus* sp. 1	Thailand	Prasertkul (2018)
18	*Ficus* sp. 2	Thailand	Prasertkul (2018)
19	*Guilandina bonduc* L.	Malaysia	Mahmod et al. (2018)
20	*Hibiscus* sp.	Thailand; Malaysia	Abdullah et al. (2020); Foo and Dawood (2016, 2017); Jaikla et al. (2020); Prasertkul (2018)
21	*Lumnitzera* sp.	Thailand	Jaikla et al. (2020)
22	*Lumnitzera littorea*	Malaysia	Foo and Dawood (2016)
23	*Nypa fruticans*	Thailand; Malaysia	Foo and Dawood (2017); Prasertkul (2018)
24	*Pandanus* sp.	Malaysia	Abdullah et al. (2020)
25	*Rhizophora* sp.	Thailand; Malaysia	Abdullah et al. (2020); Foo and Dawood (2016, 2017); Jaikla et al. (2020)
26	*Rhizophora apiculata*	Malaysia	Foo and Dawood (2017)

Table 2 *(Continued)*

No.	Species	Country	References
27	*Sonneratia caseolaris* (L.) Engl.	Thailand; Malaysia	Cheng et al. (2017); Hazmi and Sagaff (2018); Jaikla et al. (2020); Mahmod et al. (2018); Prasertkul (2018)
28	*Sonneratia ovata* Backer	Thailand	Jaikla et al. (2020)
29	*Tamarindus indica*	Thailand	Prasertkul (2018)
30	*Terminalia catappa*	Thailand	Prasertkul (2018)
31	*Thespesia populnea* (L) Sol.	Thailand	Jaikla et al. (2020)
32	*Thespesia populnea*	Malaysia	Abdullah et al. (2020)
33	Unidentified species from the family Poaceae	Thailand	Jaikla et al. (2020)
34	*Xylocarpus granatum*	Malaysia	Abdullah et al. (2020)
35	*Xylocarpus* sp.	Thailand	Jaikla et al. (2020)

CONCLUSION

The study successfully identified a total of 145 different species of fireflies throughout Southeast Asian countries (such as Malaysia, Philippines, Indonesia, Cambodia, Myanmar, Singapore, Sri Lanka, Papua New Guinea, Laos, Thailand, and Vietnam. While the authors also failed to find studies in Brunei) based on studies conducted by previous researchers. Data on the species of fireflies found in countries such as the Philippines, Indonesia, Cambodia, Myanmar, Singapore, Sri Lanka, Borneo, Papua New Guinea, Laos, and Vietnam are incredibly outdated due to the lacking of research undertaken in the country.

This review has managed to find at least 34 tree species and one unidentified species (Poaceae family) of display trees or habitat by fireflies in Malaysia and Thailand. For other countries such as Indonesia, Cambodia, Myanmar, Singapore, Sri Lanka, Borneo, Papua New Guinea, Laos, Thailand, and Vietnam, there are no studies were found to identify tree species inhabited by fireflies. The studies conducted in those countries only focused on firefly species and did not study the habitat areas and display trees chosen by fireflies. More research regarding fireflies, including species richness, abundance, distribution, seasonal variation, and habitat, should be conducted in Southeast Asia.

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Effect of Various Immersion Time and Water Temperature on Seed Germination of *Clitoria ternatea* and *Momordica charantia*

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ABSTRACT

The seeds of *Clitoria ternatea* and *Momordica charantia* were subjected to seven pre-sowing treatments, *i.e*., control (T_0), peeled coat and soaked in 5 °C for 24 hours (T_1), peeled coat and soaked in 37 °C for 24 hours (T_2), peeled coat and soaked in 5 °C for 48 hours (T_3), peeled coat and soaked in 37 °C for 48 hours (T_4), peeled coat and soaked in 5 °C for 48 hours (T_5), peeled coat and soaked in 37 °C for 72 hours (T_6). The study revealed that peeling the coat and soaking seeds in water for various temperatures and periods improved seed germination. The highest germination and germination energy percentage of *C. ternatea* were observed in T_2, namely 94.95% and 23.69%, respectively, while the lowest germination (0%) and germination energy (0%) was found in T_6. The highest germination and germination energy percentage of *M. charantia*, namely 64.38%, and 16.10%, respectively, were found in T_3, while the lowest germination (10.67%) and germination energy (2.17%) were observed in T_0. The germination may vary for both seeds used in the study as *C. ternatea* and *M. charantia* are different in type. The pre-sowing treatments of seeds would prove its potential in the practical fields.

Keywords: *Clitoria ternatea*, germination, herbal plants, *Momordica charantia*, seed germination

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INTRODUCTION

Herbal medicinal plants have been used as a significant source for the cure of human ailments since time immemorial, and as such, they had a significant impact on global health (Nimesh, 2018; Patwekar et al., 2016).

Medicinal herbs have become popular worldwide in the latter half of the 20th century due to the widespread recognition of the value of conventional medicines and the integration of natural source derivatives in pharmaceuticals (Alsarhan et al., 2014). In addition, Enioutina et al. (2017) stated that in many developed countries, herbal remedies had been accepted as an essential and alternative remedy under strict regulations and oversight (Alsarhan et al., 2014) because they are more effective, healthy, and have fewer side effects than modern medicine.

Clitoria ternatea L., also known as butterfly pea, is a plant that belongs to the Fabaceae family and has been used traditionally for various medicinal purposes (G. K. Kumar & Thomas, 2012). Furthermore, *C. ternatea* is a very well-known herb used in Ayurvedic medicine for different ailments. Ayurvedic 'Medya Rasayana' is a rejuvenating recipe mainly containing extracts from *C. ternatea* and is specially designed to treat patients' neurological disorders. It is also used to enhance intellect (G. K. Kumar & Thomas, 2012). Nguyen et al. (2011) added that cliotides could be found in all the parts of this plant. Cliotides are the peptides that have potent anti-microbial properties against *Escherichia coli*.

Momordica charantia is a flowering vine known as bitter gourd or bitter melon, belongs to the Cucurbitaceae family (D. S. Kumar et al., 2010). The bitter gourd fruits are used as a tonic, stomachic, stimulant, emetic, antibilious, laxative, and alterative in Ayuverdic medicine. *Momordica charantia* has been used in numerous Asian traditional medicine systems for a long time. Bitter gourd, like other bitter-tasting foods, stimulates digestion. According to clinical experience and traditional reports, *M. charantia* rarely has harmful effects because it is a demulcent and mild inflammation modulator.

Orthodox and recalcitrant seeds resulted from pollen grains' ovule double fertilization (Rajjou et al., 2012). Seed germination begins with the mature seed absorbing water and ends with the emergence of the embryo *via* the surrounding structures (Nonogaki et al., 2010). One of the vital agricultural goals is to achieve rapid and uniform germination and seedling emergence when seeds are sown. The seed industry uses priming treatments to improve the efficiency of commercial seed lots. These treatments include regulated seed ingestion followed by dehydration to their original water content to enable storage. These treatments are thought to enhance the pre-germinative metabolic process, implying that the primed seeds germinate faster than untreated seeds. The seeds are mostly more or less ellipsoid and often pitted or rugulose in a pattern of drying. The seed coat with woody exotesta of several layers is derived from the outer integument and papery endotesta (Corner,

1976). The coat of the individual seed is often watertight. Each seed contains a large embryo and little endosperm. The seeds are often germinated quickly once the seed coat is punctured (The Seed Site, 2013). However, literature examining the effect of seed priming treatments of *C. ternatea* and *M. charantiais* is scarce. Therefore, the study was conducted to investigate the effect of pre-sowing treatments on the germination of *C. ternatea* and *M. charantia* to add knowledge in this field.

MATERIALS AND METHODS

Seed Collection and Preparation

Seeds of *C. ternatea* and *M. charantia* were collected from the residents of Pagoh and Simpang Renggam, respectively, in Johor. Uniform seed sizes from each species were selected for the treatments to reduce non-treatments variation because seed size is a crucial plant trait that affects seed germination and further seedling establishment (Mog et al., 2017). The present study was conducted in batches under controlled conditions for three months, from July to September 2018. The parameters of the study were water temperature and time immersion. The pre-sowing treatments used in the study were based on Hossain et al. (2005) with a slight modification as stated in Table 1.

Table 1
Pre-sowing treatments of seeds

No.	Pre-sowing treatments
T_0	Control (intact seeds without peeling coat and soaking)
T_1	Peeled coat and soaked seeds in 5 °C for 24 hours
T_2	Peeled coat and soaked seeds in 37 °C for 24 hours
T_3	Peeled coat and soaked seeds in 5 °C for 48 hours
T_4	Peeled coat and soaked seeds in 37 °C for 48 hours
T_5	Peeled coat and soaked seeds in 5 °°C for 72 hours
T_6	Peeled coat and soaked seeds in 37 °C for 72 hours

Three replicates of 50 seeds each were used for testing the germination. The seeds were placed on the top of a filter paper soaked with distilled water in Petri dishes (5 seeds per Petri dish). Water was replenished as needed. All the Petri dishes were left at room temperature. The effects of pre-sowing treatments were assessed periodically by counting germinated seeds. The first and final counts were taken on days 2 and 14, respectively. The mean from all batches was recorded as a result.

Germination (%), Germination Energy (%), and Germination Period

The germination period was recorded. The germination, defined as the development of a plant from seed after a period of dormancy, was calculated. The germination energy was also determined, defined as the germination percentages at the peak of daily germination. In addition, germination energy is also used as a measure of germination pace. It was thought to be an indicator of the vigor of the seedling. The germination (%) (1), germination energy (%) (2), and germination period (3) were calculated using the formula cited by Czabator's index (1962):

$$\text{Germination} = \frac{\text{Total number of seeds germinated}}{\text{Total number of seeds in all replicates}} \times 100 \quad [1]$$

$$\text{Germination energy} = \frac{\text{Number of seed germinated daily}}{\text{Total number of seeds in all replicates}} \times 100 \quad [2]$$

$$\text{Germination period} = \text{Days to maximum seeds germination} \quad [3]$$

Statistical Analysis

Data were statistically analyzed by using Microsoft Excel to evaluate the potential treatment differences. The analysis of variance (ANOVA) at $\alpha = 0.05$ and the means separation were also included for the analysis in this study.

RESULTS

The seed germination of *C. ternatea* started on day two after the sowing and continued up to the next 14 days. Rapid germination was found in most treatments except for T_5 (uncoated and soaked seeds in 5 °C for 72 hours) and T_6 (uncoated and soaked seeds in 37 °C for 72 hours), as stated in Table 2. The seeds in T_5 germinated on day four after sowing, while seeds in T_6 were not germinated.

For *C. ternatea*, germination and germination energy differed significantly for seeds given different pre-sowing treatments (one-way ANOVA: $F_{(6,42)} = 872.31$, $P < 0.0001$ for germination, $F_{(6,42)} = 881.57$, $P < 0.0001$ for germination energy). The highest germination (%) (94.95 ± 3.81) and germination energy (%) (23.69 ± 0.91) of *C. ternatea* were recorded in T_2 (uncoated and soaked seeds in 37 °C for 24 hours) followed by T_4 (uncoated and soaked seeds in 37 °C for 48 hours), T_1 (uncoated and soaked seeds in 5 °C for 24 hours), T_0 (untreated), T_3 (uncoated and soaked seeds in 5 °C for 48 hours), and T_5 (uncoated and soaked seeds in 5 °C for 72 hours) (Tables 2 and 3).

Table 2

Mean germination (%) of Clitoria ternatea *at different treatments*

Days	Treatments						
	T_0	T_1	T_2	T_3	T_4	T_5	T_6
2	2.67	19.33	96.67	0	45.33	0	0
4	4.00	21.33	98.00	0.67	46.67	0	0
6	4.67	22.67	98.67	0.67	55.33	0	0
8	4.67	20.00	98.00	0.67	64.67	0.67	0
10	5.33	18.67	93.33	0.67	62.00	0.67	0
12	6.00	18.67	90.00	1.33	60.00	0.67	0
14	6.00	17.33	90.00	1.33	59.33	0.67	0
Mean	4.76 ± 1.18*	19.71 ± 1.80*	94.95 ± 3.81*	0.76 ± 0.46	56.19 ± 7.52*	0.38 ± 0.36	0 ± 0

Note. * Significant different at $\alpha = 0.05$

Table 3

Mean germination energy (%) of Clitoria ternatea *at different treatments*

Days	Treatments						
	T_0	T_1	T_2	T_3	T_4	T_5	T_6
2	0.67	4.83	24.17	0	11.33	0	0
4	1.00	5.33	24.50	0.17	11.67	0	0
6	1.17	5.67	24.67	0.17	13.83	0	0
8	1.17	5.00	24.17	0.17	16.17	0.17	0
10	1.33	4.67	23.33	0.17	15.50	0.17	0
12	1.50	4.67	22.50	0.33	15.00	0.17	0
14	1.50	4.33	22.50	0.33	14.83	0.17	0
Mean	1.19 ± 0.29	4.93 ± 0.45*	23.69 ± 0.92*	0.19 ± 0.11	14.05 ± 1.88*	0.10 ± 0.09	0 ± 0

Note. * Significant different at $\alpha = 0.05$

For *M. charantia*, germination and germination energy differed significantly for seeds given different pre-sowing treatments (one-way ANOVA: $F_{(6,42)} = 60.39$, $P < 0.0001$ for germination, $F_{(6,42)} = 60.38$, $P < 0.0001$ for germination energy).The highest germination (%) and germination energy (%) of *M. charantia* are 64.38 ± 5.21 and 16.10 ± 1.30 respectively found in T_3 (uncoated and soaked seeds in 5 °C for 48

hours), followed by T_5 (uncoated and soaked seeds in 5 °C for 72 hours), T_1 (uncoated and soaked seeds in 5 °C for 24 hours), T_6 (uncoated and soaked seeds in 37 °C for 72 hours), T_2 (uncoated and soaked seeds in 37 °C for 24 hours), and T_4 (uncoated and soaked seeds in 37 °C for 48 hours), and the lowest germination (%) (10.67 ± 8.08) and germination energy (%) (2.67 ± 2.02) was recorded in T_0 (untreated seeds) (Tables 4 and 5).

Table 4

Mean germination (%) of Momordica charantia *at different treatments*

Days	Treatments						
	T_0	T_1	T_2	T_3	T_4	T_5	T_6
2	0	30.00	3.33	55.33	4.00	24.67	10.00
4	1.33	34.67	6.00	60.00	6.67	36.00	18.00
6	7.33	38.67	13.33	62.67	14.67	48.00	26.00
8	11.33	38.67	15.33	68.67	14.67	48.00	31.33
10	16.00	40.00	16.67	68.67	15.33	46.00	31.33
12	18.67	42.00	18.67	68.00	15.33	44.00	30.00
14	20.00	44.00	20.67	67.33	16.67	42.00	29.33
Mean	10.67 ± 8.08*	38.29 ± 4.68*	13.43 ± 6.47*	64.38 ± 5.21*	12.48 ± 4.98*	41.24 ± 8.41*	25.14 ± 8.15*

Note. * Significant different at α = 0.05

Table 5

Mean germination energy (%) of Momordica charantia *at different treatments*

Days	Treatments						
	T_0	T_1	T_2	T_3	T_4	T_5	T_6
2	0	7.50	0.83	13.83	1.00	6.17	2.50
4	0.33	8.67	1.50	15.00	1.67	9.00	4.50
6	1.83	9.67	3.33	15.67	3.67	12.00	6.50
8	2.83	9.67	3.83	17.17	3.67	12.00	7.83
10	4.00	10.00	4.17	17.17	3.83	11.5	7.83
12	4.67	10.50	4.67	17.00	3.83	11.00	7.50
14	5.00	11.00	5.17	16.83	4.17	10.50	7.33
Mean	2.67 ± 2.02*	9.57 ± 1.17*	3.36 ± 1.62*	16.10 ± 1.30*	3.12 ± 1.25*	10.31 ± 2.10*	6.28 ± 2.04*

Note. * Significant different at α = 0.05

DISCUSSION

Based on the results, it seems that water temperature and time immersions were critical factors in *C. ternatea* and *M. charantia* seeds as reported by D. S. Kumar et al. (2010) and Hossain et al. (2005) in *Terminalia chebula* and *Andrographis paniculata*. Many research have been carried out to develop effective seed treatments methods to break the dormancy and accelerate the germination rate (Hossain et al., 2005). The seeds must be subjected to physical and chemical treatments to artificially achieve rapid and synchronous germination (Azad et al., 2010). These include uncoating seeds at both ends by piercing, nicking, filling, or clipping (Catalan & Macchiaveloli, 1991) and soaking the seeds in different water temperatures, different time immersion, and different concentrations of acid treatments (Kobmoo & Hellum, 1984).

Generally, temperature influenced germination in three ways: moisture, hormone development, and enzyme activity. The seeds must imbibe water to germinate, so enough moisture is available (Dove, 2010). Seeds with hard coats have improved germination when treated before sowing (Hossain et al., 2005). Hossain et al. (2005) added that the drupes germinate slowly and irregularly from untreated seeds. The study's findings show that uncoated seeds of *C. ternatea* soaked in warm water (37 ^{o}C) increased germination and germination energy. Around 95% of *C. ternatea* seeds germinate after being clipped at the end so that the embryo is not harmed and soaked in warm water for 24 hours. G. K. Kumar and Thomas (2012) have shown that seeds of *A. paniculata*, pre-treated by soaking in 35 ^{o}C water, showed up to 80% germination. The study results for *C. ternatea* also supported G. K. Kumar and Thomas (2012). On the other hand, the study results for *M. charantia* supported the finding of Hossain et al. (2005). Hossain et al. (2005) have shown that the seeds of *T. chebula*, pre-treated by soaking in cold water for 48 hours, provides the highest germination value (4.41) and germination energy (58.9%).

CONCLUSION

From the study, it can be concluded that pre-sowing treatments of seeds would prove its potential in the practical fields. Among the treatments applied in the experiment for *Clitoria ternatea* are that seeds that were depulped at two ends and soaked in 37 ^{o}C for 24 hours (T_2) were found more effective to rate germination percentage and energy. In contrast, the seeds of *Momordica charantia* were effectively germinated in T_3 (uncoated and soaked seeds at 5 ^{o}C for 48 hours). In addition, it can be concluded that germination may vary for both seeds used in the study as the seeds of *C. ternatea* and *M. charantia* are different in type.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Host-Parasitic Relationships between *Tetrastigma rafflesiae* and *Rafflesia azlanii* and *Rafflesia cantleyi* in Belum-Temenggor Forest Complex, Perak, Malaysia

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ABSTRACT

Rafflesia is a holoparasitic plant that depends solely on its host for its nutrients, given that during the early stage of its life, this parasite lives inside the host vine. The lack of host specificity and preference information for *Rafflesia* can largely be attributed to the absence of a comprehensive taxonomic study in *Tetrastigma*. Without the host, the *Rafflesia* will not be able to survive. Therefore, this research was conducted to study the host-parasitic relationships between the two species using anatomical dissection and micrographic images using a light microscope (LM) and scanning electron microscope (SEM). The anatomical study consisted of three stages of *Rafflesia* buds; the emergence of cupule stage, cupule-bract transition stage, and bract stage attached with the host. All samples underwent sliding techniques and were observed using LM and SEM. Based on the results, the anatomical characteristics of the host-parasite for the cupule stage evidenced penetration of the parasite-affected tissues inside the vascular bundles with the visibility of the flower bud. However, during other stages, the penetration of parasite-affected tissues to the vascular bundles was disrupted and cannot be seen using this sliding technique. The endoparasite of *Rafflesia* invades the

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host only towards the phloem region in the early stage. In contrast, in late buds for both species, the *Rafflesia* tissue invaded both the host xylem (proximal region) and phloem. The parasite intrusion movement for both *Rafflesia* species showed a pointed tissue towards the host as this was believed to minimise the damage of the host plant. A new method using the paraffin wax technique might improve the sectioning and provide a more precise relationship dissection. The information from this study is expected to provide baseline information and an understanding of the host-parasitic relationship between the species. In addition, further anatomical studies with the different stages of buds will offer a better understanding of their relationship with the host.

Keywords: Holoparasite, host-parasite, *Rafflesia azlanii*, *Rafflesia cantleyi*, *Tetrastigma rafflesiae*

INTRODUCTION

Approximately 4,530 of the 369,000 flowering plant species (1.2%) are parasitic and had evolved at least 12 times across the angiosperms (Bell et al., 2011; Těšitel, 2016; Twyford, 2018). Parasitic plants can be divided based on photosynthetically active hemiparasites, or holoparasitic due to a lack of photosynthetic activity. They rely entirely on a host for carbon, whether they are facultative or obligate parasites and whether they attach to the host's roots or stem (Twyford, 2018). *Rafflesia* is a holoparasitic plant with no chlorophyll and depends solely on its host for its water and nutrients (Wicaksono, 2015). Parasitic plants have long inspired interest from botanists, horticulturalists, and evolutionary biologists because they directly connect with a suitable host plant, allowing them to absorb nutrients and water from the host (Twyford, 2017). A modification of host metabolism and morphology was accomplished through specific parasites' structures called haustoria (Cocoletzi et al., 2016). Cameron et al. (2007) reported that the ability of haustorium to access the host's vascular tissue and withdraw resources is a crucial adaption that needs to be understood more. Twyford (2018) reported that holoparasites develop terminal haustoria at the meristematic tip of the primary root, then penetrate the host epidermis and cortex, and attach to the host vasculature followed by further plant growth, flowering, and senescence. Holoparasites are predominantly phloem feeders that typically retain a xylem connection and obtain all mineral nutrients, amino acids, soluble carbon, and water from the host. Hemiparasites are predominantly xylem feeders that obtain reduced carbon and nitrogen from the host. Hibberd and Jeschke (2001) reported that some 3,000 species of parasitic angiosperm among 17 families had been documented. Unfortunately, only a small number of these parasitic plants have been studied.

Mursidawati and Sunaryo (2012) studied the general observation of the *Rafflesia patma* Blume endophytes within its host plant. Their study observed three phases of *R. patma* growth: penetration,

invasion, and establishment. The penetration phase occurred during the early germination stage. In this invasion phase, the flower bud starts to grow and affect the host tissue, the establishment phase where the flower establishes as a mature flower bud prior to anthesis. Later updated in Mursidawati and Irawati (2017), the fourth phase, named the conductive stage, involved flower establishment and saw nutrients were obtained from the host.

In another study, Nikolov et al. (2014a) conducted a study on *Rafflesia cantleyi* Solms-Laubach, *Rafflesia tuan-mudae* Becc., *Rhizanthes lowii* (Becc.) Harms and *Sapria himalayana* Griffith on the flower development and the endophytic movement within its host plant. The study revealed that the endophyte was probably developed directly from proembryo instead of an embryo proper and concluded that Rafflesiaceae produced modified vegetative bodies that differed from other holoparasitic angiosperms once grouped with Rafflesiaceae.

Mursidawati et al. (2019) studied *patma* and *Tetrastigma rafflesiae* (Miq.) Planch in which the former grew from a protocorm inside the cambium tissue of the latter. *Rafflesia patma* spread within *T. rafflesiae* vascular cambium tissue linearly, but not as a continuous strand. It was suspected that the parasitic endophyte spread inside the host vascular cambium and was pushed farther away from its origin point to another part of the host as the host vine cambium fusiform initial cells divided and enlarged over months. It resulted in the endophyte not forming a long continuous strand, analogous to a fungal mycorrhizal hyphal network, within its host plant, but instead forms small meristematic cell clusters that spread as the vascular cambium expands, allowing it to be squeezed out between initial fusiform cells and spread through the host body. A recent study on tissue differentiation of early and late bud flowers of *R. patma* was conducted by Mursidawati and Wicaksono (2020). They revealed the three types of flower tissues: proximal region and tissue with non-elongated cells in the middle and distal regions. There has been limited understanding of the host-parasite association and variation in collecting and attracting host solutes. More studies are needed especially related to the host-parasitic relationship, to compare the species and different stages of buds. This study aimed to gain a better understanding of the host-parasitic relationship between *Rafflesia* and *Tetrastigma* species. The study of host-parasitic relationships between *Rafflesia* and *Tetrastigma* may provide the opportunity to understand the pathways and cells involved in the solute transfer and the physiological impact of changes in the cell structure caused by the presence of the parasite itself.

METHODS

Study Site and Field Data Collection

This study was conducted in Belum-Temenggor Forest Complex (BTFC) with the coordinate of 5° 20’ 0” North, 101° 22’ 0” East. The area is the biggest continuous forest complex in Peninsular Malaysia

in Perak (Razak et al., 2015). Belum-Temenggor has a tropical climate with an annual rainfall reaching 3,000 mm per year with an average temperature throughout the year ranges from 24 to 29.9℃ (Aiman Hanis et al., 2014). The humidity ranges from 70% to 98%, with high rainfall in April and October and low rainfall in February and July (Aiman Hanis et al., 2014). BTFC consisted of Royal Belum State Park, Gerik Forest Reserve, Banding Forest Reserve, Amanjaya Forest Reserve, and Temenggor Forest Reserve (Malaysian Nature Society [MNS], 2013). This study consisted only of Gerik Forest Reserve and Banding Forest Reserve (Figure 1).

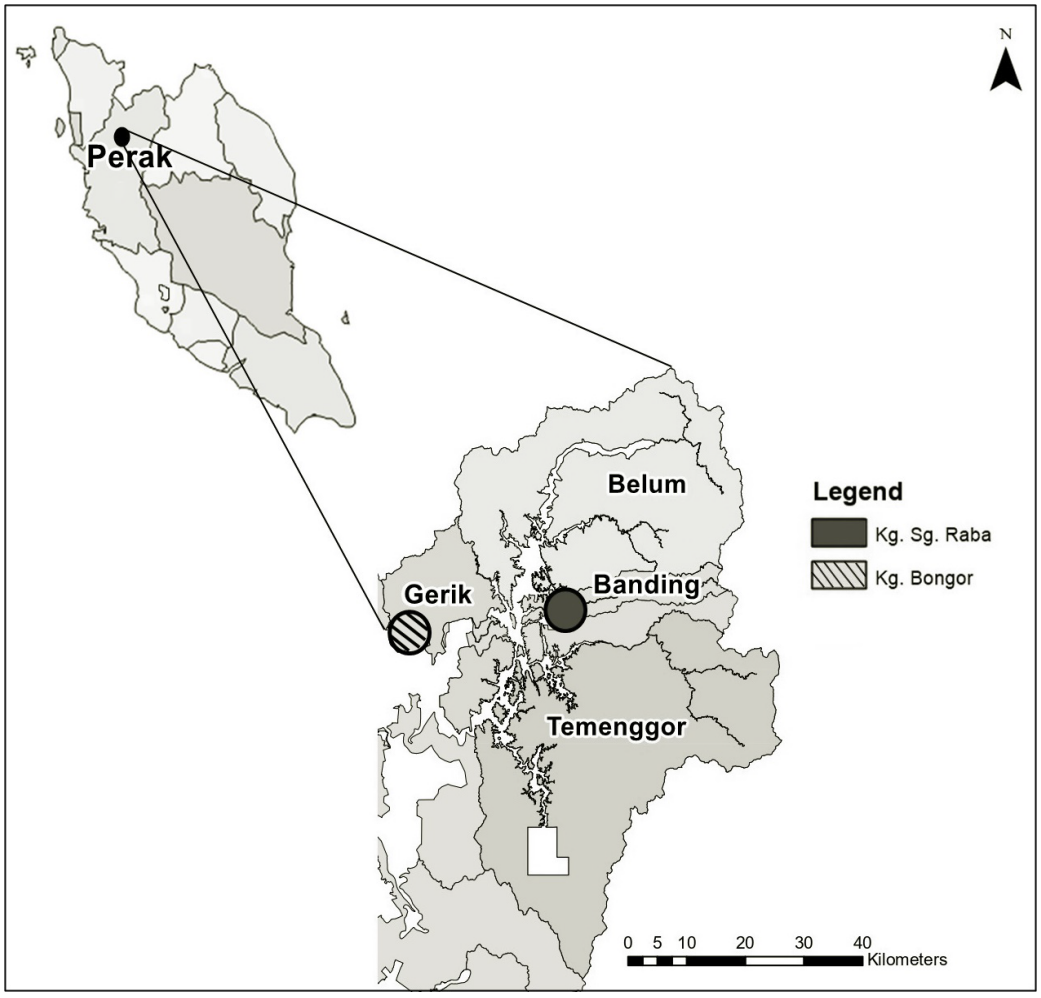

Figure 1. Location of study sites represented in circles

The samples collected consisted of three stages of *Rafflesia* buds referred to by Susatya (2020) attached with the *Tetrastigma* vine (Figure 2). These are (1) cupule stage, where the emergence of cupule stage is marked as cp in the figure, (2) cupule-bract transition stage (CBT), where the bracts are present when the cupule parts (host tissue) are primarily seen and gradually replaced by bracts marked as 'br', (3) bract stage which referred as a visible bud that fully covered by bracts where the host tissues are no longer can be seen on top of the bud. From the field observation, the number of bud samples between the *R. azlanii* Latiff & M. Wong and *R. cantleyi* species is not equal. For *R. azlanii*, only the cupule stage samples were collected and whereas for *R. cantleyi*, only the CBT stage and bract stage. In addition, Young *T. rafflesiae* stems and roots were collected to study the anatomical features of the host plant. One of the authors, a taxonomist from Universiti Kebangsaan Malaysia (UKM) involved in the identification process.

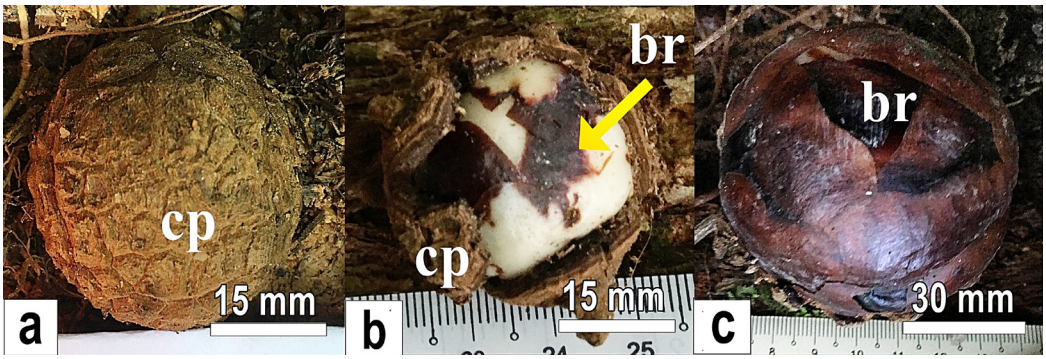

Figure 2. The bud development stage of *Rafflesia*: (a) cupule stage of *Rafflesia azlanii*, (b) cupule-bract transition stage of *Rafflesia cantleyi*, (c) bract stage of *Rafflesia cantleyi*

Note. cp = Cupule; br = Bract

Samples obtained from the field underwent the process of fixation, which involved a preparation process in a bottle containing concentrated acetic acid (AA): 70% alcohol with a ratio of 1:3 with a minimum of 48 hours. Voucher specimens of slides (UKMB40462, UKMB40463, and UKMB40464) were deposited at the Herbarium Unit, UKM.

Anatomical Method

The collected samples (Figure 3) were preserved in the AA solution and transferred to 70% ethanol for the fixation process and long-term storage. Then, they were sectioned by freehand using a sliding microtome (Leica SM2000R, Leica Camera, Germany) at a thickness of 10-15 µm. According to Tolivia and Tolivia (1987), the

samples were then stained using a few drops of safranin, Alcian blue with distilled water, dehydrated with 50%, 70%, 95%, and 100% alcohol mounted using Eupharal on the slides. Anatomical features were observed and captured using a light microscope (Olympus VS120, Olympus Corporation, Germany) with an attached digital camera.

For the micrographology study using SEM, the samples were cut into 1 cm² × 1 cm² and oven-dried for a week before the samples were completely dried up to the critical point. The specimens were placed in a drying device for 30 minutes and then affixed to the stab using a double face or colloidal silver cellophane sticker. The samples were routed to the top for scanning electron microscopy. The gold plating was conducted using a plating machine (Bal-Tec SCD 050, BalTec Corporation, USA). The observation process was conducted using the electron scanning microscope (Philips XL-30, Philips, the Netherlands) using a series of enlargements of 150×, 300×, 700×, 1,000×, 5,000×, to 10,000×. From the slides, anatomical features were observed, and the features were described and characterised.

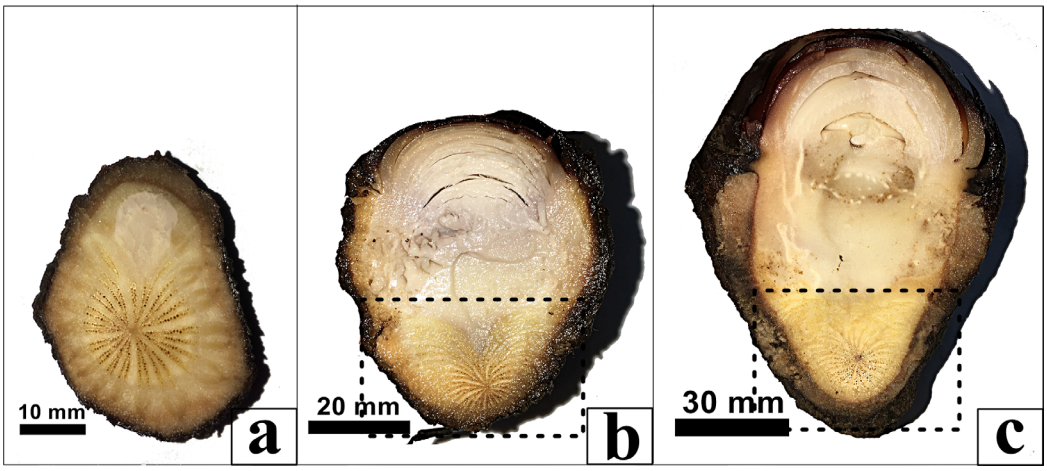

Figure 3. The transverse section of buds with the host: (a) cupule stage of *Rafflesia azlanii*, (b) cupule-bract transition stage of *Rafflesia cantleyi*, (c) bract stage of *Rafflesia cantleyi*. The dotted area referred to the anatomical part used in the study

Note. cp = Cupule; br = Bract

RESULTS AND DISCUSSION

Anatomy under Micrographology LM

Non-parasite of *Tetrastigma rafflesiae*. Figures 4(a) and 4(b) show *T. rafflesiae* root and stem images under the transverse section. Figure 4(a) is the root portion, and Figure 4(b) is the young stem of the host. Observation on the root image indicated a layer of periderm cell marked as 'PER' located at the root's outer surface, with a bit

of pith located in the centre of the root stem near the vascular bundle. The small cortex (C) in Figure 4 can be seen between the periderm and vascular bundles, consisting of a few layers of parenchyma tissues. The vascular bundles are radially arranged alternately with eight branches of phloems (PH) and xylems (XY) (Figure 4). The vascular cambium contains meristematic tissues that lie between the phloem and xylem tissues. For the young stem shown in Figure 4(b), there is also one layer of the epidermis but a large pith located near the vascular bundles in the centre of the root stem. A small cortex between the epidermis and vascular bundles consists of 12 to 15 layers of parenchyma cells and vascular bundles. They are enclosed with a single ring that contains 50 branches of phloems and xylems. The vascular bundles of non-parasitised hosts clearly show the presence of xylems and phloems without any disruption from the parasite tissues. The primary phloems are associated with a sizeable pericyclic fibre strand (Pace et al., 2018) on its outermost part, as shown in Figure 4(a) and Figure 4(b). The descriptions of anatomical characteristics given below are based on the summary of specimens examined. Results from detailed measurements are presented in Table 1.

The early stages of wood lianas show a self-supporting phase. They are adapted to grow across gaps and reach host supports, whereby older stages can absorb and reduce potentially catastrophic mechanical stresses resulting from the movement of the host plant (Lopes et al., 2008). Therefore, there is a structural difference between young stem or old and root or stem part. The root of *T. rafflesiae* shows a thick layer of parenchyma under the periderm, while the young stem only has a single layer of parenchyma. This thick periderm was to allow the root to penetrate inside the ground easier. Syamsurina (2018) mentioned that

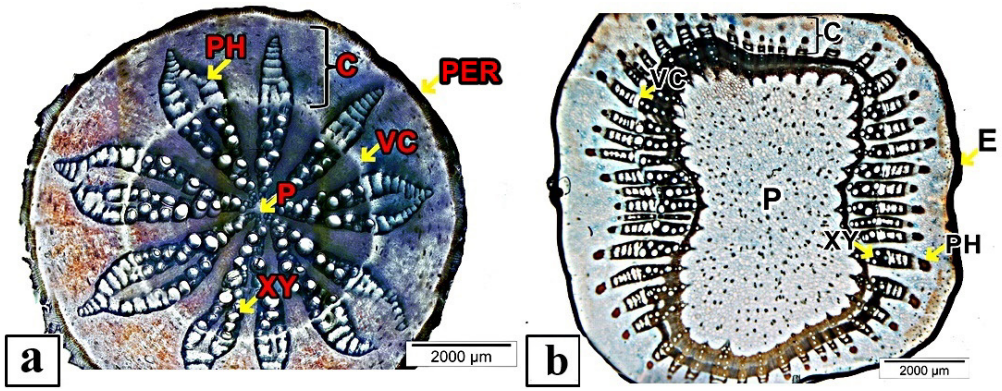

Figure 4. Transverse section of (a) *Tetrastigma rafflesiae* root, (b) young *Tetrastigma rafflesiae* stem (Scale: a, b = 2,000 µm)

Note. P = Pith; E = Epidermis; PER = Periderm; C = Cortex; PH = Phloem; VC = Vascular cambium; XY = Xylem

the *T. rafflesiae* root contains more layers of the epidermis to protect the root as it will go deep inside the ground with compact soil. The difference between the roots and stems of the host is the pith structure of the stem and is 23 bigger in length size than the root pith (Table 1). The vascular bundles of the stem have six times the branches compared to the root. However, the length of vascular bundles in the root is three times longer than in the stem.

Table 1

Anatomical characteristics measurements (mean) of Rafflesia azlanii*,* Rafflesia cantleyi*, and* Tetrastigma rafflesiae

Characteristics	*Tetrastigma rafflesiae*		*Tetrastigma rafflesiae* with *Rafflesia azlanii*	*Tetrastigma rafflesiae* with *Rafflesia cantleyi*	
	Root	Stem	Cupule stage	CBT stage	Bract stage
Thickness of epidermis/ periderm (µm)	252	201	442	430	357
Length of pith (µm)	224	5,318	191	486	662
Distance from epidermis/periderm to pith (µm)	6,503	2,490	6,536	10,752	15,885
Cortex length (µm)	1,657	1,570	1,836	2,611	5,408
Vascular bundle shape	Alternate	Enclosed with single ring	Alternate	Alternate	Alternate
No. of vascular bundle branch	8	50	7	8-9	12-14
Distance from vascular cambium to epidermis/ periderm (µm)	1,770	1,140	2,604	2,786	9,434
Length of vascular bundle (µm)	5,522	1,659	4,332	8,073	Not clear
Length of parasite-affected tissue (longest) (µm)	Not applicable	Not applicable	5,422	Not clear	Not clear
Distance from parasite-affected tissue to epidermis (µm)	Not applicable	Not applicable	6,366	Not clear	Not clear
Flower bud presence	Not applicable	Not applicable	Yes	Not clear	Not clear

Note. CBT = Cupule-bract transition stage

Rafflesia azlanii* and *Rafflesia cantleyi* buds with *Tetrastigma rafflesiae. The transverse section of *T. rafflesiae* infected by *R. azlanii* for cupule stage are shown in Figures 5(a) and (b), the *R. cantleyi* buds with the cupule-bract transition (CBT) stage is shown in Figure 5(c) and bract stage in Figure 5(d). *Rafflesia azlanii* buds show the periderm cells marked as 'PER' located at the root's outer surface with a bit of pith located in the centre of the root stem near the vascular bundle. The parasite-affected tissues marked as 'PAT' penetrate the xylem tissues in Figures 5(a) and 5(b). Parasite cells normally have a larger nucleus with usually two nucleoli (Rutherford, 1970). According to Pérez-de-Luque (2013), epidermal cells at the haustorium apex were enlarged to form the intrusive cells where the cortex cells divided for the penetration process. Nikolov et al. (2014b) reported that typical angiosperm holoparasites developed a haustorium that absorbed host nutrients and water. However, in Rafflesiaceae, the endophyte was not called haustorium since it does not connect an external shoot to the host. Thus, the parasite-affected tissue (PAT) resembles the host tissue stretched by the parasite growth.

The flower bud shape marked as 'FB' in Figures 5(a) and 5(b) shows a teardrop-shaped body only on the cupule stage. It agrees with Nikolov et al. (2014b), who reported that the *Rhizanthes lowii*, a parasite under Rafflesiaceae, also clearly showed a teardrop-shaped protocorm with a smooth texture. In this study, the flower bud of the *R. azlanii* was located between the cortex and parasite-affected tissues. The vascular bundles of the host are arranged in an alternate manner that contains seven branches of phloems and xylems. Figure 5(b) shows vascular cambium, which contains meristematic tissue between the phloem and xylem tissues. The periderm cell is seen in the figure for the CBT stage of *R. cantleyi* with *T. rafflesiae*. The image shows a bit of pith, 'P' in Figure 5(c), located in the centre of the root stem close to the vascular bundles. The parasite-affected tissues penetrated the host xylem tissues and ruptured Figures 5(c) and 5(d). Vascular bundles are arranged in an alternate manner that contains eight to nine phloem and xylem tissues Figure 5(c). A total of 12 to 14 branches of ruptured phloems and xylems [Figure 5(d)] next to the parasite-affected tissue can be seen in Figure 5(d). Based on the observation in Figure 5, the parasite penetrated and stayed in the host xylem. Nikolov et al. (2014b) supported this and claimed that *Rafflesia* endophytes live in the xylem area and later rises to the host epidermal layer.

From the observation, as the development stage progresses, the number of vascular bundles branches increases as the size increases. Parasite-affected tissue penetration was unclear in two stages of bud development (i.e., CBT stage and bract stage). According to Mursidawati et al. (2019), regarding the development of endoparasite on *R. patma*, they found that the parasite grows without any visible vascular tissues within the cambium as cell clusters. It can be seen within the parasite-

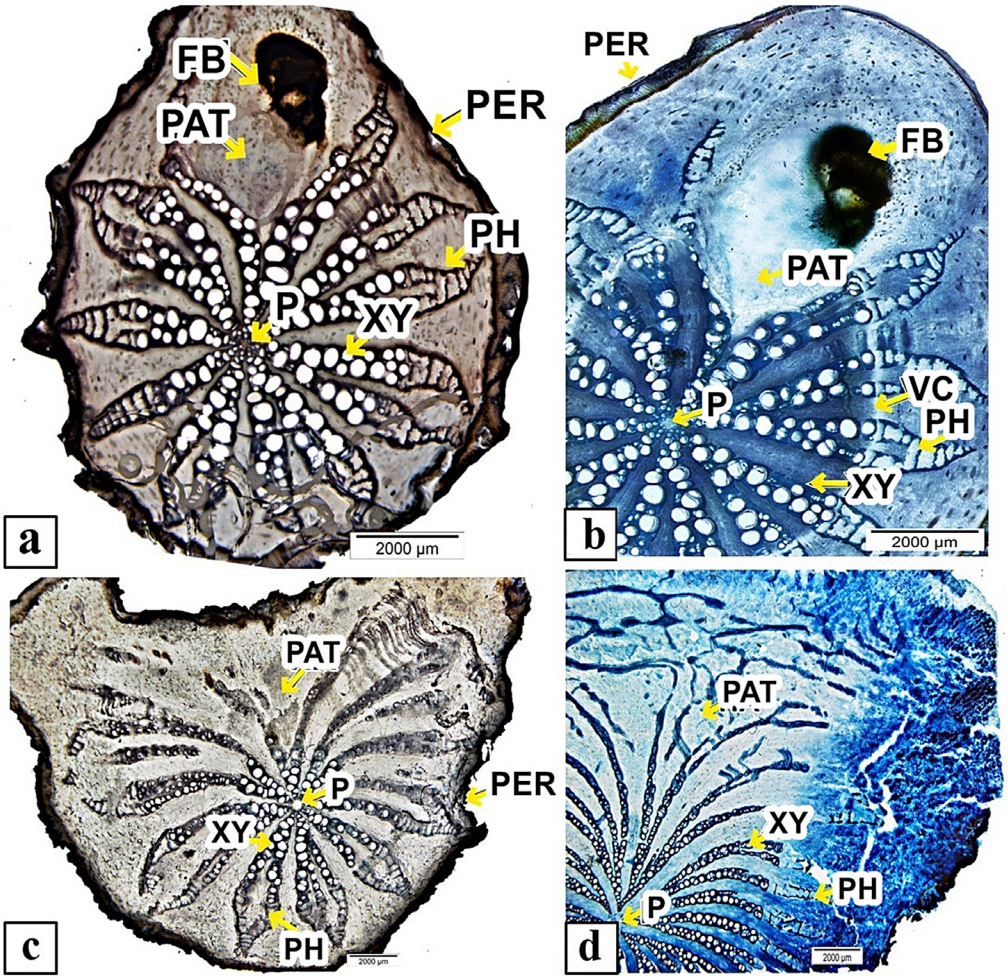

Figure 5. Transverse section of *Rafflesia azlanii* buds that attached with host (a) and (b), transverse section of *Rafflesia cantleyi* buds that attached to the host (c) and (d) (Scale: a, b, c, d = 2,000 µm)

Note. PER = Periderm; FB = Flower bud; PAT = Parasite-affected tissue; VC = Vascular cambium; PH = Phloem; XY = Xylem; P = Pith

affected tissues in Figure 4. Mursidawati et al. (2020) claimed that the flower tissue comprises three types of proximal region, tissue with non-elongated cells in the middle and distal regions. However, these tissues cannot be seen using this method because of redundant tissues due to the thick size of the specimen cutting.

As for host-parasitic interaction, it was found that at the early stages of the bud for both species, the parasitic intrusion of *Rafflesia* invading the *T. rafflesiae* only goes across the phloem region while the xylem host remains untouched. However, in late buds for both species, the *Rafflesia* tissue invaded both the xylem and phloem

of the host. A. Wicaksono (personal communication, December 18, 2020) mentioned that for *Rhizanthes infanticida*, which is also under the Rafflesiacea family, the parasite penetrated deeply into the host's xylem until reaching its core. Unlike *R. azlanii* and *R. cantleyi*, the penetration of the parasite extended only to the proximal area of the host xylem for the last bud. The movement of PAT in Figures 5(a) and 5(b) clearly shows a pointed tissue towards the host xylem region. This type of movement differed from other endoparasites observed in *Cytinus* species in De Vega et al. (as cited in Mursidawati et al., 2020, p. 112). The study shows that in *Cytinus* species, the parasite occupies the entire region of the xylem as sinker cells, whereas in Figure 5(a) and Figure 5(b), the parasite only penetrates towards one or two vascular bundles, as seen in *R. patma* (Mursidawati et al., 2020), while the remaining vascular bundles continued to grow normally. As shown in Figure 5, the number of host vascular bundles infected by *Rafflesia* has increased by stages. One vascular bundle infected for cupule stage, two vascular bundles for CBT stage and three host vascular bundles ruptured by *Rafflesia* for bract stage for *R. azlanii* and *R. cantleyi*. This movement was believed to minimise the host vascular damage to allow the host to live as the *Rafflesia* flower is huge and takes longer time to develop in the host tissue. It could answer how the host can tolerate numerous *Rafflesia* buds on the vine and still survive and manage to supply nutrients to the world's biggest flower. More buds growing in multiple angles will cause more damage to the host compared to more buds growing in the same growth direction (Mursidawati et al., 2020).

Anatomy under Micrographology SEM

Tetrastigma rafflesiae. Figures 6(a) and 6(b) show the transverse sections of *T. rafflesiae* root and young stem captured from micrographology SEM. There is no penetration of any parasite-affected cells since it was a non-infected host. For the root section, only one layer of epidermis cell is located at the root's outer surface, with a bit of pith located at the pith of the root stem. A small cortex area is observed between the epidermis and vascular bundle consisting of a few layers of parenchyma tissues. Vascular bundles are arranged alternately with 10-12 branches of phloems and xylems. There was no ruptured cell by the parasite. There is also one layer of epidermis cell at the outer surface for the young stem in Figure 6(b). A large-sized pith can be seen in the centre of the root stem near the vascular bundles, with a small cortex located between the epidermis and vascular bundles. It is in accordance with the previous report stating that the vascular bundles are arranged in an enclosed manner with a single ring containing the phloem and xylem (Crang et al., 2018). According to Marcati et al. (2014), the root xylem of *Citharexylum myrianthum* has wider vessels than the stem xylem. It can be seen in Figure 5 where the root xylem vessels were wider and larger than the stem xylem. The root wood is more vulnerable to embolism than a stem wood, and this could be better to have wider vessels under water stress (Marcati et al., 2014).

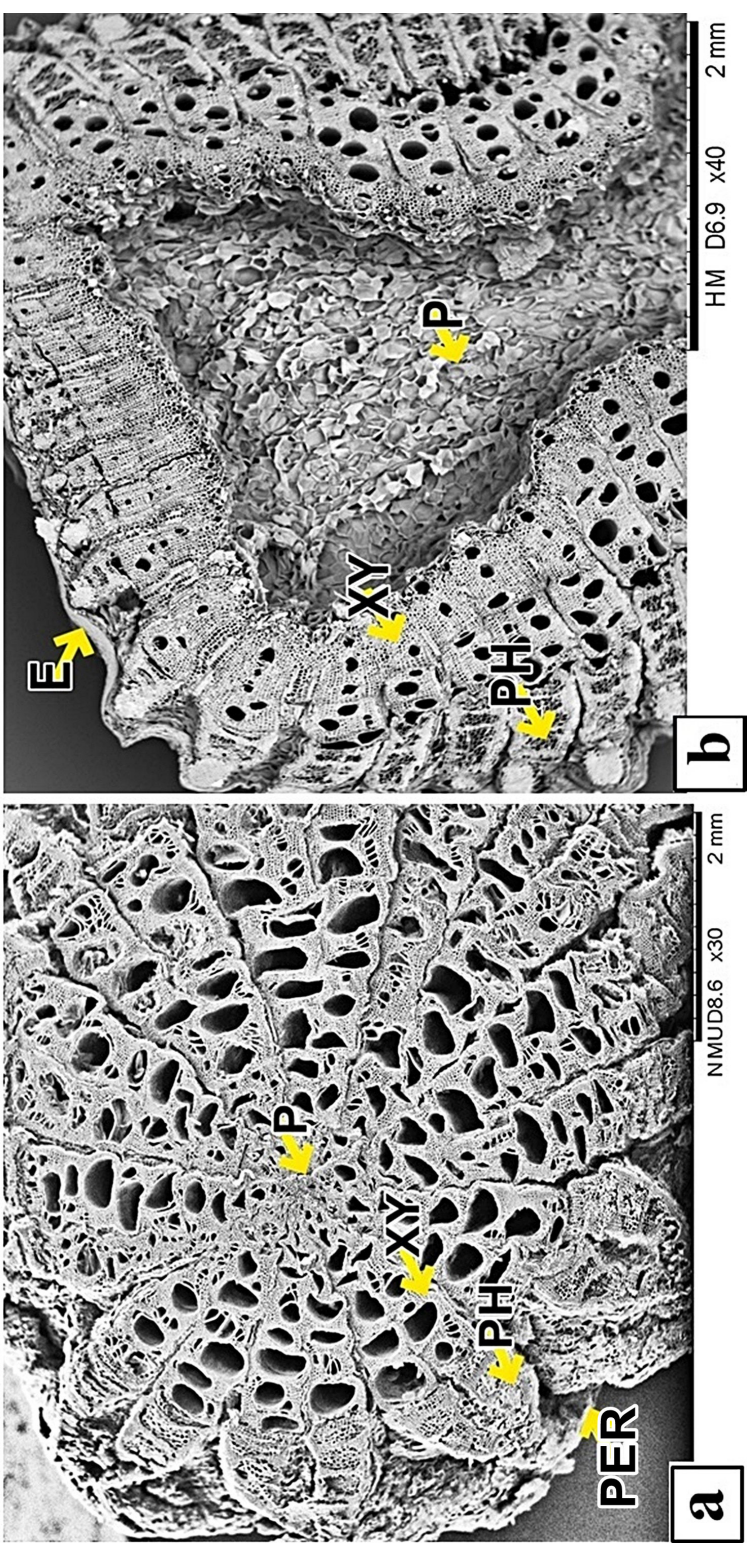

Figure 6. Transverse section SEM micrographs of (a) *Tetrastigma rafflesiae* root, (b) transverse section of *Tetrastigma rafflesiae* young stem (Scale: a, b = 2 mm)

Note. E = Epidermis; PER = Periderm; PH = Phloem; XY = Xylem; P = Pith

Rafflesia azlanii* and *Rafflesia cantleyi* buds with *Tetrastigma rafflesiae. Figure 7(a) and Figure 7(b) show that *T. rafflesiae* are infected by *R. cantleyi* in the cupule stage (a) and the bract stage (b). In contrast, Figure 7(c) shows that *T. rafflesiae* is infected by *R. azlanii* during the cupule stage. The *R. azlanii* bud attached to *T. rafflesiae* shows the periderm cell located at the root outer surface with a bit of pith located in the centre of the root stem. From the figure, the penetration of parasitic *Rafflesia* inside the infected host tissues into the xylem can be seen clearly. Vascular bundles are arranged alternately and contain eight to twelve branches of phloems and xylems, as shown in Figure 7(c). For the same stage for *R. cantleyi*, the periderm cell is located at the outer surface of the root, with a bit of pith located in the centre of the root stem. Parasite-affected tissue penetrating the host xylem tissues and rupturing the tissues. Vascular bundles of *T. rafflesiae* are arranged in an alternate manner containing seven to eight branches of phloems and xylems and are ruptured by parasitic *Rafflesia* tissue. For the brown bracts stage, the *R. cantleyi* bud shows the PAT located across the vascular bundles of infected *T. rafflesiae* in Figure 7(b). A study on *R. patma* conducted by Mursidawati et al. (2020a) found that the vascular bundle was found in the middle-late of the perigone lobe where oddly shown only xylem vascular element in the middle. It was concluded that the absence of phloem might signify the nature of holoparasite as it does not produce its foods. However, in this study, only *Rafflesia* buds were selected without the flowers. Therefore, no xylem was found. The *R. patma* involved flower only, with xylem, no phloem and one type of vascular parenchyma. Furthermore, it was believed that the vascular parenchyma might be involved in the distribution of water and nutrients (Mursidawati et al., 2020). It agrees with de Vega et al.'s study (as cited in Mursidawati et al., 2020, p. 112), using *Cytinus* (Cytinaceae), a holoparasitic plant parenchyma tissue that mediates water transport between host-parasite xylem.

Figure 7(b) shows a shoot apex of *R. cantleyi* that formed. Nikolov et al. (2014b) mentioned that the shoot meristem in Rafflesiaceae grows through dense and hard host vine tissue before it emerges. It is to protect the developing flora meristem as it erupts through the host. The pith is narrow and located in the centre of the root stem near the vascular bundles. Parasite-affected tissues penetrated the xylem tissues and ruptured the tissues. As a result, vascular bundles of *T. rafflesiae* are arranged in an alternate manner that contains eight to twelve branches of phloems and xylems. According to Cocoletzi et al. (2016), a holoparasite is a parasite that establishes both the xylem and phloem connections from the host. It can be seen clearly in Figures 7(a) and 7(c), where the parasite-affected tissues penetrate both host xylems and phloems.

The micrographs of the SEM image cannot be used for measurement due to the shrinkage process during the drying process that changed the size of the samples. The longest length for each characteristic was

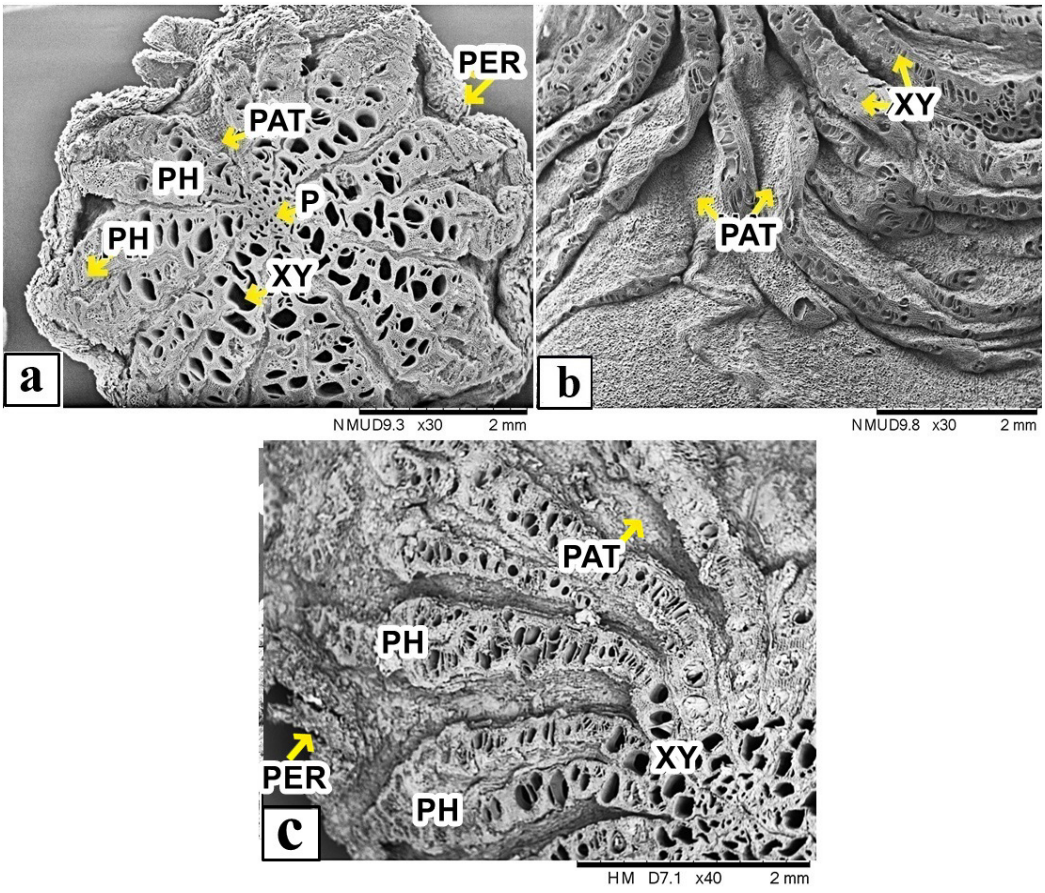

Figure 7. SEM micrographs of transverse section of (a-b) *Rafflesia cantleyi* attached to the host and (c) cross section of *Rafflesia azlanii* bud attached with the host (Scale: a, b, c = 2 mm)

Note. PER = Host periderm; PH = Host phloem; XY = Host xylem; PAT = Parasite-affected tissue of the host; P = Host pith

when the buds reached the bract stage as the size was bigger than other stages. Only a young stem of *T. rafflesiae* has a different vascular bundle shape, enclosed with a single ring. Only the parenchyma cells layer can be seen clearly for the young stem due to the softness of the wood and caused a less thick layer on the slide. The length of the vascular bundle and the parasite-affected tissues cannot be seen clearly for the CBT and bracts stages. It is due to the penetration process by the parasite tissue in the host (woody part) and caused the slow growth of the buds. The microscopic analyses of host-parasitic relationships between both *Rafflesia* species and the hosts revealed the presence of pointed intrusions in the point of attachment. The pointed intrusions which penetrated inside between 1-3 of vascular bundles of *T. rafflesiae* were believed to minimise the damage. In this study, the penetration of *Rafflesia* inside the host has

shown a similar trend where the number of host vascular bundles infected increased by stages for both species in which one vascular bundle infected for cupule stage, two vascular bundles for CBT stage and three host vascular bundles ruptured by *Rafflesia* for bract stage. It can be speculated that fewer ruptured host vascular bundles may lead to the longevity of *Rafflesia* growth in the host.

CONCLUSION

This anatomical study used a sliding technique to demonstrate the early stage of *Rafflesia* buds (i.e., cupule stage). The anatomical characteristics in the later stages cannot be observed clearly. The parasitic intrusion of *Rafflesia* invading the host only goes across the phloem region for the early stage. In contrast, in the late bud for both species, the *Rafflesia* tissue invaded both the host's xylem (proximal region) and phloem. The movement of parasite intrusion of *Rafflesia* for both species has shown a pointed tissue towards the host as this was believed to minimise the damage of the host plant. A light microscope with a digital camera is sufficient to observe the penetration of the parasite-affected tissues inside the vascular bundle. In contrast, the SEM is more suitable for observing details. The sliding technique used has damaged the samples of soft bud tissues. Thus, an alternative method should be applied to get a better view of the anatomical structures of both species. Further work using different methods such as the rotating technique using the paraffin wax method may improve the study as shown in other *Rafflesia* studies. In addition, similar future studies may be conducted to study the structure of vascular bundles between species with greater detail. Furthermore, the wood anatomy of the host can be studied in detail in terms of differences and similarities among different species. It will enhance our understanding of the interaction between *Rafflesia* species with their host.

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Growth of *Acacia mangium* at Different Stand Ages and Soil Physicochemical Properties in Sarawak, Malaysia

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ABSTRACT

The information on soil physicochemical properties is vital for the optimum wood biomass production in forest plantation management. The objective of this study was to determine the topsoil physicochemical properties under different *Acacia mangium* stand ages and their effect on the growth parameters. Five plots were established randomly within each five different stand ages. In all sample plots, the diameter at breast height (DBH) and the total height of standing trees were measured. Soil samples were collected at a depth of 0 to 20 cm at three random points in each plot, then mixed to get a composite before determining physical and chemical properties. DBH mean and the total height of *A. mangium* increased as stand age increased. The mean annual volume increment maximised at the 8.5 years old stand with 27.9 $m^3 ha^{-1} yr^{-1}$. Survival rate and stem density decreased as stand age increased. Principal component analysis (PCA) results showed that the most important soil physical properties were soil organic matter, silt and sand contents, bulk density, and moisture content. For soil chemical parameters, exchangeable magnesium (Mg), cation exchange capacity (CEC), total carbon (TC), total nitrogen (TN), and carbon-to-nitrogen (C/N) ratio were the influential soil variables. Soil pH, available phosphorus (P), and clay content were negatively correlated with the growth development of *A. mangium* trees. Observations suggest that multiple soil variables are essential for the success of the *A. mangium* plantation.

Keywords: *Acacia mangium* plantation, growth, principal component analysis, soil properties, stand age

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INTRODUCTION

Acacia mangium is one of the major species used in the timber plantation industry in Malaysia. This species is a unique timber tree in the tropical forest. It is fast-growing, tolerant to poor soil, and can undergo nitrogen-fixing by itself (Lee et al., 2015). Due to better site adaptability and growth performance, more than 85% of the Compensatory Forest Plantation Project (CFPP) area had been planted with *A. mangium* (Hashim et al., 2015). The plantation in Sarawak is established mainly in the central lowland region, which generally comprises elevated wave-cut platforms developed from sedimentary rocks. The central region of the state area is primarily dominated by the red-yellow Podzolic soils (Lee et al., 2015). In forest plantations, this soil type is usually low in the water holding capacity. It has poor infertility that is likely to limit the tree to grow efficiently (Lee et al., 2015). The soil status in the plantation area commonly dictates the type of vegetation planted on the land (Binkley & Giardina, 1998; Bonifacio et al., 2008). Thus, only a few high tolerance tree species can be grown in the plantation areas.

The capability of the soil to store nutrients is vital for the tree to survive. The physicochemical properties of soil support trees stand to grow efficiently and productively in every condition, such as disease susceptibility and harsh weather. The growth and wood quality are also highly influenced by the physicochemical properties of the soil (Rigatto et al., 2004). According to Blake and Hartge (1986), generally, most of the total volume of surface soil consists of 50% solids (45% of soil particles with less than 5% of organic matter) and about 50% of pore space (occupied with water and air).

In forest plantation management, soil quality can be defined as biomass production due to soils capacity (Jamil et al., 2016). Soil provides roots to anchor, grow, contain pore spaces for air, and preventing the plants from becoming waterlogged. It supports the tree stands planted in a high degree of slope and during the harsh weather from collapsing. The physical properties of soil are responsible for the rooting activities, holding the water availability, and easing water absorption by plants. They hold the soil nutrients in exchangeable ions that are available for plant roots to absorb. Soil can also hold a certain amount of oxygen in pore spaces and other gasses (Binkley & Fisher, 2019). Soil chemical properties are also crucial for a plant to survive. The deficiency of certain nutrients in the soil makes them inaccessible and unavailable to the tree. The problem can increase the susceptibility of diseases and insects, leading to infections of the tree. The *Eucalyptus* forest in Mexico had experienced stunted growth due to phosphorus (P) and nitrogen (N) deficiencies in the soil resulting in low biomass production of eucalyptus (Fenn et al., 2006). The addition of phosphorus might contribute to the tree growth, carbon, and nitrogen storage to the litter and woody biomass. It was reported that the application of phosphorus fertilisers to the *A. mangium*

stands resulted in an increase in stem diameter and volume at stands between 4 and 5 years old (Hardiyanto & Wicaksono, 2008). Phosphorus also is an essential nutrient which when lacking, will limit tree growth, especially in tropical areas (Zás & Serrada, 2003).

An increasing amount of nitrogen fertiliser in an *A. mangium* plantation in Sumatra, Indonesia, did not affect the growth. This is because there was the nitrogen-fixing ability for this tree species (Hardiyanto & Wicaksono, 2008). Furthermore, additional potassium (K) and calcium (Ca) did not affect diameter growth. They also recorded that applying lime (increasing the soil pH level) and potassium fertilisers to tree stands did not positively affect growth. The leguminous crop plantations, such as *A. mangium*, needs a good P and N in the soil (Bini et al., 2013).

PCA can provide insight to find the main factors affecting the classification of vegetation and soil (Li et al., 2018). This analysis is widely used in many studies to explain the expected effect of soil and vegetation on geography, ecology, forestry, and soil science (Eni et al., 2012). Several researchers had documented the interrelationship of soil and vegetation in rainforest and forest plantation using this analysis approach (Eni et al., 2012; Li et al., 2018; Matali & Metali, 2015; Salehi & Maleki, 2012).

The most effective soil factor on the separation of two Poplar plantations in the north of Iran was also determined using PCA (Salehi & Maleki, 2012). They found the accumulation of clay content, soil organic matter, nitrogen, phosphorus, and potassium in the first axis of PCA, also known as principal component 1 (PC1). The clay content had a positive correlation to PC1. In contrast, the other four soil parameters had a negative correlation to PC1. Eni et al. (2012) studied the mangrove swamp in Tinapa, Nigeria, and analysed PCA soil parameters. They recorded that the soil parameters with the most variation in PC1 were exchangeable sodium (Na) and exchangeable magnesium (Mg). For principal component 2 (PC2) at their study site, they found that soil organic matter (SOM) and total nitrogen (TN) were heavily loaded on the axis to represent the second group, which was the most influenced variation of soil parameters.

In an *A. mangium* plantation in Andalau Forest Reserve, Sungai Liang, Brunei, it was reported that the total N and P have positively associated with PC1. In contrast, soil organic layer depth and exchangeable Ca were negatively associated with the axis (Matali & Metali, 2015). On the other hand, the total Mg, exchangeable K, available P, and gravimetric water content were positively correlated to PC2. Matali and Metali (2015) used PCA biplot to find the association of vegetation parameters to the soil nutrients concentration. They found that pH was the most influential component of the *A. mangium* plantation in Andalau Forest Reserve Brunei.

Previous studies on the effects of soil physicochemical properties on the growth parameters of *A. mangium* stands in natural

and planted forest areas have been done in Sarawak by Hardiyanto and Wicaksono (2008), Perumal et al. (2015), and Tanaka et al. (2015). However, information on soil physicochemical properties within a large scale of a commercially planted forest of different stand ages, particularly on *A. mangium*, is still unavailable. This warrant a study to provide information regarding the soil status on *A. mangium* and its relation to growth performance, particularly in Sarawak. Thus, our study aimed to determine the topsoil physicochemical properties at different stand ages of *A. mangium* and their relationship with growth parameters.

MATERIALS AND METHODS

Study Site

The study was conducted at a forest plantation owned by Daiken Sarawak Sdn. Bhd., the Malaysian and Japanese joint venture companies, incorporated on February 15, 1994. The plantation area is located approximately 60 km from the town of Bintulu in Sarawak, Malaysia at 03˚21.347' N and 113°27.129' E and within 30 to 160 m asl (height above mean sea level). The site receives a total annual rainfall of 2749 mm, and the average temperature ranges from 23 °C to 32 °C (Soil Survey Report, 1996). The plantation was originally a secondary forest and was planted with *A. mangium*. In general, the soil type is a well-drained Bekenu series, subgroup Typic Hapludult, Paleudult, Kanhapludult, and Kandiudult characterised by fine, loamy, siliceous, isohyperthermic, and red-yellow to yellow. The main factor restricting the use of this soil is its low fertility status (Paramananthan, 2000). However, the initial soil conditions were considered to be similar for all stands. The terrain was undulating with a slope of less than 6˚ and covered by the same vegetation type. Planting spacing of 3 x 3 m is used, which resulted in 1,111 trees per hectare.

Field Sampling

A chronosequence approach was employed in this study. Five different stand ages, namely, 4.3, 5.8, 8.5, 10.8, and 12.7 years old, were chosen for this study. These stands were selected to represent growth performance spaced about two years apart. A total of five 30 m × 30 m (4,500 m^2) sample plots were established randomly within each of five different stand ages. Thus, a total of 25 plots are assigned to the plantation for this study. At the planting spacing of 3 x 3 m, there were 100 trees initially within a 30 m × 30 m plot size. Thus, the real trees are 500 for each stand age at the time of planting. The DBH of standing trees was measured by using diameter tape (Yamayo, Japan). The top or total height of the standing trees was measured using the trigonometry principle (Philip, 1994). The Suunto clinometer (Forestry Suppliers, Inc., Canada) and rangefinder (Bushnell®, USA) were used to measure a tree's slope and horizontal distance, respectively.

Determination of *Acacia mangium* Tree Growth

Growth is the increasing dimension of each tree in a forest stand for a given time. Growth can be measured by the mean annual increment (MAI) of the tree parameter. MAI is the dimension of a tree over the age of the tree. The basal area of the tree is the cross-sectional area of a tree stem measured at breast height which is 1.3 m from the ground. The geometrical shape of the *A. mangium* tree is considered a cone shape along the tree's stem. The form factor for a conical shape tree is 0.33 (Philip, 1994). Using basal area, total tree height, and form factor of 0.33, total tree volume was calculated as 0.33 multiply by basal area multiply by total tree height. MAI of growth parameters, including DBH, height, basal area, and volume of each tree, were calculated by dividing these parameters by the tree age. Stand density or stocking is calculated by counting the number of trees per ha. The survival rate is calculated by counting the number of trees survived divided by the number of seedlings planted initially and expressed as a percentage.

Determination of Soil Physical Properties

The soil properties of each stand age were determined by collecting soil samples. Each stand age was represented by one plot. Three random points within each selected plot were identified for soil sampling. Soil samples were collected at a 0 to 20 cm depth (topsoil) using a hand auger (Eijkelkamp, Netherland). The field sampling method for soil sampling in all the study plots was adapted from Wasli et al. (2009). The soil samples from each point were mixed to get a composite sample for each soil layer. Particle size distribution was determined using the pipette method (Gee & Bauder, 1986). In this method, the inorganic soil particle was separate into the sand, silt, and clay fractions (Soil Survey Staff, 1999). Soil bulk density was on the 100 cc core sampler (Eijkelkamp, Netherland) with the dry mass ratio to the bulk volume of the soil core. This experiment was carried out by drying the soil samples at 105 °C overnight (Blake & Hartge, 1986). The soil organic matter (%) was determined using the loss on ignition method (Schulte et al., 1991). Soil moisture was determined by the American Society for Testing and Materials (ASTM) D2216-19 (2019) laboratory standard test method. Soil moisture content (%) of each sample was calculated as the ratio of soil moisture to the mass of the oven-dry soil.

Determination of Soil Chemical Properties

Soil pH was measured in distilled water (H_2O) in the soil to a solution ratio of 1:5 using the glass electrode method (denoted as 'pHw' and 'pHk'). Total carbon (TC) content was determined using the loss on ignition method. Total nitrogen (TN) content was determined by Kjeldahl acid digestion using Digesdahl® (USA) and tested with Hach DR/890 Portable Colorimeter (Hash, USA) (Bremner, 1996). The CEC and the contents

exchangeable bases (Ca, Mg, K, and Na) were measured after successive extraction (three times) using 1 M ammonium acetate (NH_4-OAc) adjusted to pH 7.0 and 10% sodium chloride (NaCl), respectively (Gee & Bauder, 1986). The concentrations of Ca, Mg, K, and Na were determined with the atomic absorption spectrophotometer (AAS) (Thermo Scientific, Ice Series 3500, USA). Available phosphorus content was measured using the Bray II method (Bray & Kurtz, 1945) with a V-630 ultraviolet-visible (UV) spectrophotometer at a wavelength of 710 nm (JASCO, USA).

Data Analysis

The data of soil physicochemical properties were statistically analysed using one-way analysis of variances (ANOVA) to determine any significant differences between stand ages. Tukey's HSD (honestly significant difference) tests were used to analyse the differences among means. PCA with varimax rotation was performed to identify the variables that accounted for the most variations in the datasets of soil physicochemical properties. PCA was also performed to establish the correlation between soil properties and stand growth parameters. This analysis is used to find the linear combinations of the soil physicochemical properties to make the interpretation of the results easier to be observed. The PCA identifies the principal components (PCs) that are the dominant factors to determine the correlative effect among soil physicochemical properties and between physicochemical properties and growth parameters. PC with eigenvalues of 1.0 or more will be selected for the dominant factors. The PCA is also used to determine the most influential factors of soil physicochemical properties contributing to the growth of different analysis parameters. Finally, the correlation analysis was concluded to determine which variables among the soils physicochemical properties and growth parameters correlate and measure the strength of their associations.

All analyses were conducted using IBM SPSS Statistics 24.0.

RESULTS

Growth Characteristics of *Acacia mangium*

The survival rate and stem density decreased as stand age increased. The mean survival rate and stem density of 3.7 years stand was 60.8% and 676 stem ha^{-1}, respectively, while in the 12.7 years old stand, only 27.4% survived or 304 stem ha^{-1} left standing (Table 1). Mean DBH and the total height of *A. mangium* increased as stand age increased. MAI of volume initially increased with stand age and reached the maximum at 27.9 $m^3 ha^{-1} yr^{-1}$, then it decreased from stand age 8.5 years onwards (Figure 1).

Table 1

Mean of DBH, height, stem density, and survival rate of different Acacia mangium *stand ages*

Stand age (year)	DBH (cm)	Ht (m)	Mean density (stems ha^{-1})	Survival rate (%)
3.7	15.5^{a}*	17.6^{a}	676^{b}	61
5.8	19.4^{b}	20.7^{b}	473^{ab}	43
8.5	26.5^{c}	26.3^{c}	367^{a}	33
10.8	26.9^{c}	26.6^{d}	324^{a}	29
12.7	28.2^{d}	27.6^{d}	304^{a}	27

Note. *Means within the column with the same letter are not significantly different; DBH = Diameter at breast height; Ht = Height

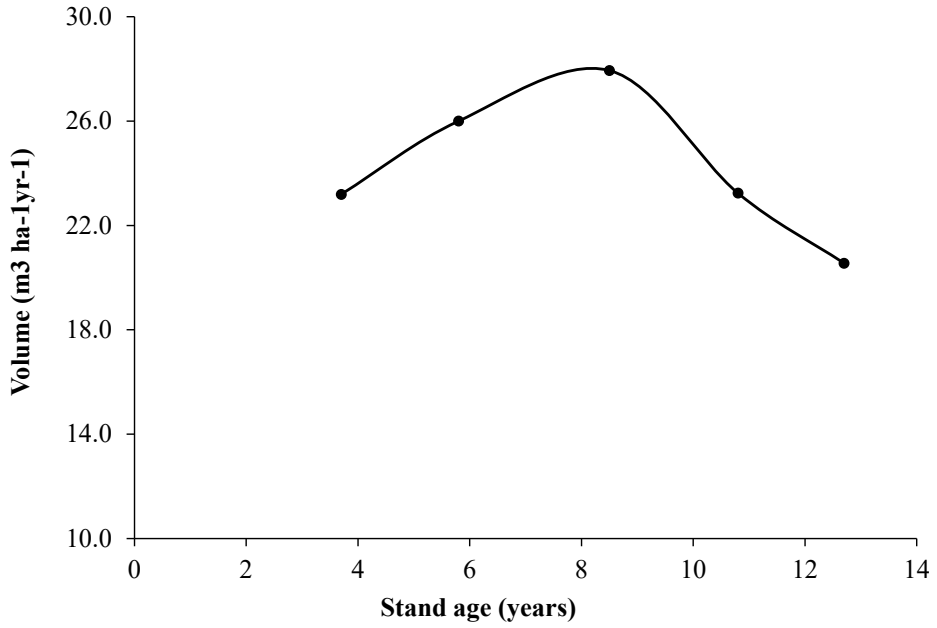

Figure 1. Mean annual wood volume increment of *Acacia mangium* according to stand ages

Soil Physicochemical Properties in Different Stand Ages

Topsoil (0 cm to 20 cm) is the most important layer for plant growth. It consists of O and A horizons containing accumulated humus or organic matter and is available for plant nutrients. Soil physical properties of different stand ages of *A. mangium* plantation are shown in Table 2. Although not significant, there was an increasing trend of SOM with the stand age. Our results indicated that the organic content in the

soil increased gradually with age from 5.8 to 12.7 years old stand. The increased level of organic contents in the older stand soil might be influenced by the development of the root mat at the surface horizon (Ishizuka et al*.,* 1998). The soil moisture content ranged from 13.99% to 16.92% and did not differ among the stand ages. The clay, silt, and sand contents range from 24.70% to 28.49%, 13.53% to 19.41%, and 58.55% to 61.95%, respectively, with no significant variation throughout the stand ages.

The soil chemical properties within the topsoil depth in various ages of *A. mangium* plantation are shown in Table 3. We observed that pH values dropped (more acidic) as stand age increased from 4.67 to 3.19. Bulk density in all stand ages was similar in all stand ages except for 8.5 years old stand. The topsoil of 8.5 years old stand recorded significantly low bulk density than the other stand ages.

The CEC values in all study areas were relatively low, ranging from 6.84 to 8.53 cmolc kg^{-1}. The oldest stand of 12.7 years old was observed to have a significantly low CEC value compared to other stand ages except for 3.7 years old stand age. The value of TC and TN content in the planted area appeared to be similar for all stand ages. The TC and TN contents in all stand ages ranged from13.67 g kg^{-1} to 24.16 g kg^{-1} and 0.41 g kg^{-1} to 1.02 g kg^{-1}, respectively. In general, TC and TN values decrease with the increment of stand ages but increase back at 12.7 years old stand age. The C/N ratio was within 30.12 to 41.79, where the values did not differ between stand ages.

Table 2

Soil physical properties within different stand ages of Acacia mangium *plantation*

Stand age (year)	SOM (%)	Moisture content (%)	Clay (%)	Silt (%)	Sand (%)	Bulk density (g mL^{-1})
3.7	3.86±0.61^{ab}	15.11±3.03^{a}	28.49±0.91^{a}	15.92±0.54^{a}	58.55±2.58^{a}	1.55±0.28^{b}
5.8	2.82±0.44^{a}	13.99±3.06^{a}	25.39±2.01^{a}	14.34±1.70^{a}	60.27±3.65^{a}	1.54±0.22^{b}
8.5	2.97±0.32^{a}	16.34±3.03^{a}	24.96±12.20^{a}	13.53±6.32^{a}	61.51±16.37^{a}	0.31±0.12^{a}
10.8	2.35±0.33^{a}	16.25±1.16^{a}	24.70±2.30^{a}	15.65±2.74^{a}	59.65±0.50^{a}	0.73±0.84^{b}
12.7	4.16±1.12^{a}	16.92±1.06^{a}	25.65±4.15^{a}	17.54±1.06^{a}	61.95±5.11^{a}	0.69±0.30^{b}

Note. Means ± standard deviation = Values in the same column followed by different letters indicate significant differences between stand ages at $P < 0.05$ using Tukey's HSD test; SOM = Soil organic matter

Table 3

Soil chemical properties within different stand ages of Acacia mangium *plantation*

Stand age (year)	pH (H_2O)	CEC (cmolc kg^{-1})	TC (g kg^{-1})	TN (g kg^{-1})	C/N ratio	Exch. Ca (cmolc kg^{-1})	Exch. Mg cmolc kg^{-1})	Exch.K (cmolc kg^{-1})	Exch. Na (cmolc kg^{-1})	Available P (mg kg^{-1})
3.7	4.67±0.17^{b}	7.13±0.81^{ab}	22.43±3.52^{a}	0.67±0.25^{a}	37.39±16.04^{a}	1.04±0.65^{a}	0.23±0.99^{b}	0.10±0.35^{a}	0.06±0.01^{a}	12.81±1.96^{b}
5.8	3.66±0.02^{a}	8.53±0.67^{b}	18.38±2.59^{a}	0.60±0.22^{a}	30.63±16.11^{a}	0.28±0.03^{a}	0.17±0.36^{a}	0.10±0.15^{a}	0.06±0.01^{a}	9.00±0.96^{b}
8.5	3.54±0.25^{a}	8.48±0.50^{b}	17.26±1.86^{a}	0.41±0.32^{a}	31.51±9.555^{a}	0.66±0.17^{a}	0.45±0.17^{b}	0.18±0.15^{b}	0.06±0.00^{a}	6.05±0.38^{a}
10.8	3.25±0.11^{a}	7.92±0.82^{b}	13.67±1.88^{a}	0.48±0.45^{a}	28.98±4.83^{a}	0.54±0.41^{a}	0.33±0.59^{b}	0.11±0.21^{a}	0.07±0.01^{a}	8.36±1.11^{b}
12.7	3.19±0.32^{a}	6.84±0.73^{a}	19.15±1.05^{a}	0.80±0.61^{a}	31.39±16.56^{a}	0.42±3.38^{a}	0.22±0.21^{b}	0.11±0.23^{a}	0.05±0.01^{a}	10.17±4.69^{b}

Note. Means ± standard deviation = Values in the same row followed by different letters indicate significant differences between stand ages at $P < 0.05$ using Tukey's HSD test; CEC = Cation exchange capacity; TC = Total carbon; TN = Total nitrogen; C/N ratio = Carbon-to-nitrogen; ratio; Exch. Ca = Exchangable calcium; Exch. Mg = Exchangable magnesium; Exch. K = Exchangable potassium; Exch. Na = Excahangable sodium; Available P = Available phosphorus

The exchangeable Ca and Na mean values among all stand ages were statistically similar. *Acacia mangium* planted areas were observed to have similar exchangeable Mg mean values in all stand ages except for 5.8 years old stand. For exchangeable K, the 8.5 years old stand age topsoil was significantly higher than the other stand ages. The available P mean values in 8.5 years old stand age area was found to be significantly lower than the other stand ages. The available P increased back after 8.5 years old, probably due to the accumulation of P when the *Acacia mangium* stands reached their maturity at 8.5 years after planting. The demand for phosphorus is high during the initial years of plant growth. It decreases in later years due to internal recycling (Fernandez et al., 2000).

Correlation among the Soil Physicochemical Properties

The principal component analysis generated the three most significant principal component scores (PC1, PC2, and PC3), which explains 70.03% of the total variability. Table 4 shows the loading values of the first three principal components. These loadings explain the contribution of each variable in a principal component. Variables with coefficients ≥ ±0.70 were selected and considered significant (Eni et al., 2012). The underline number refers to the variable loads on that component that are significant (coefficients ≥ ±0.70). For example, the first component score, PC1, has five soil physicochemical characteristics that exhibited high positive factor loadings. The parameters included SOM (0.855), TC (0.855), exchangeable Mg (0.805), CEC (0.796), and available P (0.774) that was accounted for 28.14% of the total variance in all parameters.

The second component score, PC2, was loaded heavily with four soil parameters that accounted for 25.27% of the total variance. Two of them exhibit negative factor loadings, which were C/N ratio (-0.899) and sand content (-0.843). In comparison, other two-factor loadings were silt content (0.865) and TN (0.726) that were positive. It is indicated that the C/N ratio and sand content had an inverse effect on silt content and TN in the study area. The third component score, PC3, has three variables that accounted for 16.63% of the total variance parameters. One of them exhibited negative factor loading, which was moisture content (-0.949). At the same time, the rests were bulk density (0.860) and pH (0.704) that were positive. This suggests that soil moisture content is negatively correlated with bulk density and pH level. Overall, PCA results illustrated the basic soil physical properties that significantly influence SOM, silt, sand contents, bulk density, and moisture content. While, for soil chemical parameters, exchangeable Mg, CEC, TC, TN, and C/N ratio were sets of influential soil variables.

The loading plot of PC1 and PC2 were selected to show the degree of intercorrelation and association of the soil physicochemical properties of the study area. Loadings of PC1 and PC2 show that all the components were partitioned inordinate

space (Figure 2). In the PC1 vector, soil organic matter and TC were shown to have the farthest point indicating that they had the most significant influence on the soil variables. Exchangeable Mg and CEC also showed that cation retention capacity also significantly affect soil properties.

Table 4

Rotated component of soil physicochemical properties in all Acacia mangium *stand ages*

Soil properties	Principal components		
	1	2	3
SOM	.855*		.400
TC	.855		.400
Exch. Mg	.805		-.332
CEC	.796		
Available P	.774		.381
Exch. Na	.633		-.364
Clay content	.533	.426	
C/N ratio		-.899	
Silt content		.865	
Sand content	.329	-.843	
TN		.726	
Bulk density			.860
Moisture content			-.949
pH			.704
Exch. K	.463	-.318	-.647
Exch. Ca			
Eigenvalues	**4.502**	**4.043**	**2.660**
% Variance	**28.136**	**25.267**	**16.627**
Cumulative explanation	**28.136**	**53.403**	**70.030**

Note. *Variables underlined with eigenvectors (coefficients) ≥ ±0.70 are considered significant; SOM = Soil organic matter; TC = Total carbon; Exch. Mg = Exchangable magnesium; CEC = Cation exchange capacity; Available P = Available phosphorus; Exch. Na = Exchangable sodium; C/N ratio = Carbon-to-nitrogen; ratio; TN = Total nitrogen; Exch. K = Exchangable potassium; Exch. Ca = Exchangable calcium

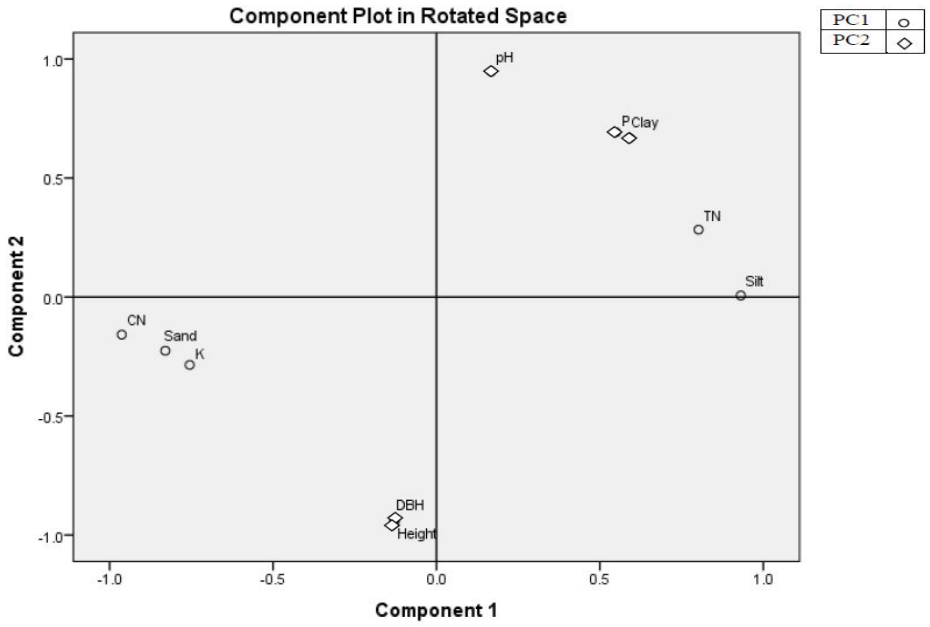

Figure 2. Distribution of soil variables of all stand ages concerning PC1 and PC2

Correlation between Soil Physicochemical Properties and Growth Parameters

The PCA of the physicochemical properties in topsoil (0 cm to 20 cm depth) incorporating stand growth parameters in five different stand ages was performed to determine the association between physicochemical properties and growth parameters (DBH and height). The analysis generated the three most significant principal component scores (PC1, PC2, and PC3), explaining 73.137% of the total variability (Table 5). The first component, PC1, was loaded heavily with five soil physicochemical parameters that accounted for 42.60% of the variation. It exhibited three positive factor loadings, which were C/N ratio (0.961), sand content (0.843), and exchangeable K (0.750). There were two negative factor loading exhibits in PC1: silt content (-0.935) and TN (-0.795).

The second component score, PC2, was loaded heavily with three-factor loadings, consisting of two growth parameters and one soil chemical property that accounted for 17.07% of the variation. In addition, they were stand DBH (0.951), height (0.948), and soil pH level (-0.861). Finally, the third principal component, PC3, was loaded with two soil physicochemical properties that accounted for 13.47% of the variance include TC (0.969) and SOM (0.969).

The soil physicochemical and stand growth parameters in PC1 and PC2 were selected to construct the loading plot in Figure 3. The available phosphorus and clay content in PC2 was also selected as they showed a significant correlation to

the growth parameters (Table 5). There was a negative effect on available P and clay content toward DBH and height. This loading plot helped show the degree of intercorrelation and association of those soil physicochemical properties to the growth parameters in the study area. The distance between variables in the loading plot depicted the strength of correlation among variables. The farther the distance represents, the stronger correlation.

Table 5

Rotated component of soil physicochemical properties and growth parameters in all Acacia mangium *stand ages*

Soil and growth parameters	Principal component				
	1	2	3	4	5
C/N ratio	.961*	.117			-.147
Silt content	-.935				-.120
Sand content	.843	.240	.165	-.148	-.146
TN	-.795	-.141	.406	-.132	
Exch. K	.750	.253	.198	.415	-.273
CEC	.657	.130	-.446	.266	.207
Clay content	-.570	-.481	.407	.136	.462
Available P	-.552	-.511	.508		.238
DBH	.165	.951		.150	-.146
Height	.166	.948		.149	-.151
pH (DIW)	-.200	-.861	.354		.189
TC			.969		.171
SOM			.969		.171
Exch. Mg	.196	.285		.877	-.260
Exch. Na		.221	-.325	.863	.125
Exch. Ca		-.479	.308	.730	
Moisture content	-.116	.349	-.111	.169	-.874
Bulk density	-.347	-.116	.415		.811
Eigenvalues	**7.667**	**3.073**	**2.425**	**2.053**	**1.302**
% Variance	**42.593**	**17.074**	**13.470**	**11.407**	**7.236**
Cumulative explanation %	**42.593**	**59.666**	**73.137**	**84.543**	**91.779**

Note. *Variables underlined with eigenvectors (coefficients) ≥ ±0.70 are considered significant; C/N ratio = Carbon-to-nitrogen ratio; TN = Total nitrogen; Exch. K = Exchangable potassium; CEC = Cation exchange capacity; Available P = Available phosphorus; DBH = Diameter at breast height; DIW = Deionised water; TC = Total carbon; SOM = Soil organic matter; Exch. Mg = Exchangable magnesium; Exch. Na = Exchangable sodium; Exch. Ca = Exchangable calcium

In the loading plot, it shows that all the components were partitioned inordinate space. It was observed that the soil pH level, available P, and clay content were correlated. The vast opposite distance between pH, available P, clay content, DBH, and height in the loading plot (Figure 3) revealed that these variables are significantly negatively correlated to the growth parameters. The pH level is observed to have the farthest point from the growth parameters compared to the other soil properties. Hence, the soil pH level has the most significant influence on the DBH and height in the study (Table 5; Figure 3).

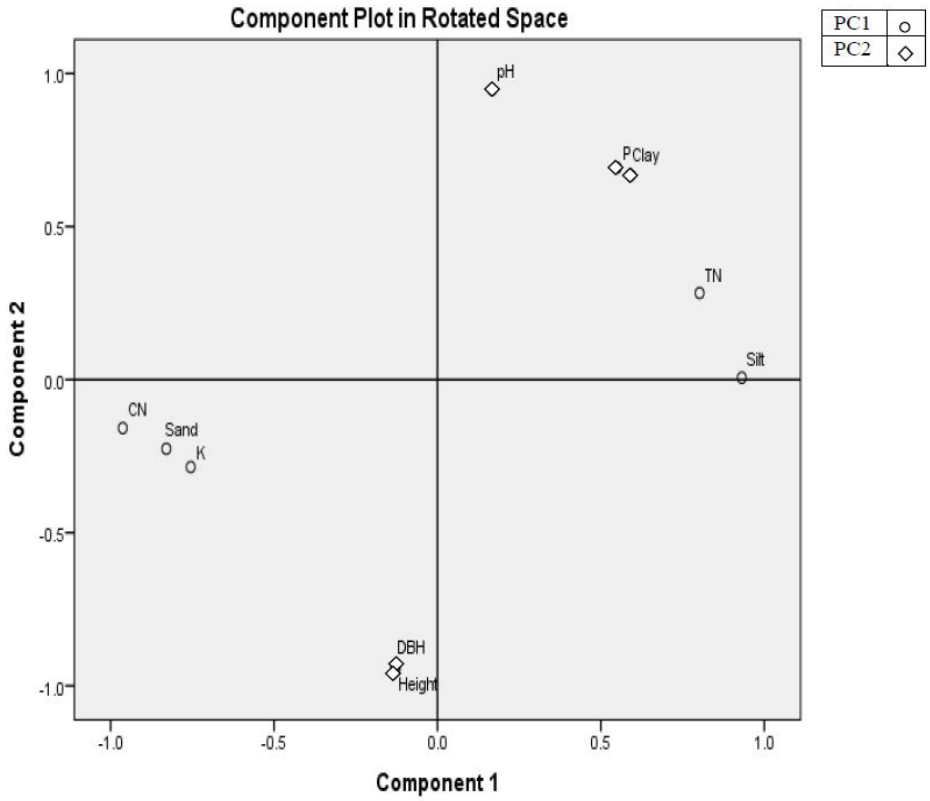

Figure 3. Distribution of soil variables and growth parameters of all stand ages concerning PC1 and PC2

Pearson correlation coefficient was used to determine the correlation among physicochemical properties and growth parameters and measure the strength of the association (Table 6). The result showed the soil pH was strongly ($P < 0.01$) negatively correlated to DBH ($r = -0.852$) and height ($r = -0.848$). Soil available P was also observed to show significant negative correlation ($P < 0.05$) to stand DBH ($r = -0.609$) and height

(r = -0.607). Similarly, clay content was negatively correlated to DBH (r = -0.595) and height (r = -0.597). It suggests that an excessive amount of available P and clay content in the soil might affect DBH and height.

Table 6

Pearson correlation coefficient of soil physicochemical and stand growth parameters

Parameters	DBH (cm)	Height (m)
DBH (cm)	1.000	
Height (m)	1.000	1.000
pH	-0.852**	-0.849**
SOM (%)	-0.100	-0.100
TC (g kg-1)	-0.100	-0.100
TN (g kg-1)	-0.280	-0.282
C/N ratio	0.307	0.308
CEC (cmolc kg-1)	0.189	0.182
Exch. Ca (cmolc kg-1)	-0.305	-0.300
Exch. Mg (cmolc kg-1)	0.479	0.479
Exch. K (cmolc kg-1)	0.431	0.430
Exch. Na (cmolc kg-1)	0.303	0.300
Available P (mg kg-1)	-0.609*	-0.607*
Clay (%)	-0.595*	-0.597*
Silt (%)	-0.172	-0.173
Sand (%)	0.372	0.375
Bulk density (g mL-1)	-0.291	-0.293
Moisture content (%)	0.439	0.441

Note. **Correlation significant at $P < 0.01$. *Correlation significant at $P < 0.05$; DBH = Diameter at breast height; SOM = Soil organic matter; TC = Total carbon; TN = Total nitrogen; C/N ratio = Carbon-to-nitrogen ratio; Exch. Ca = Exchangable calcium; Exch. Mg = Exchangable magnesium; Exch. K = Exchangable potassium; Exch. Na = Exchangable sodium; Available P = Available phosphorus

DISCUSSION

The DBH and height of *A. mangium* increased with age, suggesting the robustness of *A. mangium* growing rapidly and continuously in a plantation but up to a certain age as the growth decreased after 8.5 years old. It is normal to see the high growth rate of *Acacia* trees at an early age then diminish at a later age. Growth rates of *Acacia* tree are very rapid at a young age and begin to slow down after the fifth or after 8 years (Krisnawati et al., 2011). The same goes for stand basal

area and volume per ha of *A. mangium* as the tree increased with stand age; however, after 8.5 years, it started to decrease (Heriansyah et al., 2007). Lee et al. (2015) stated that the growth rate of *A. mangium* would decline rapidly at 7 or 8 years after planting. Our results also showed that 8.5 years old stand recorded the maximum growth rate. The growth of *A. mangium* levels off after 8.5 years of planting.

MAI reported in this study is relatively low compared to other studies due to the form factor used to calculate tree volume, which was 0.33. It was reported that the total standing volume of *A. mangium* in Sarawak was 177.40 m^3 ha^{-1} with MAI of 26 m^3 ha^{-1} yr^{-1} at a stand age of 7 years. The estimated productivity of 7 years old *A. mangium* plantation in Planted Forest Zone in Bintulu was 185 - 229 m^3 ha^{-1}. It averaged at 205 m^3 ha^{-1} to 8 cm top diameter with MAI at 29 m^3 ha^{-1} yr^{-1} (Gardner, 2009). The regular growth rate of *A. mangium* in Sumatra, Indonesia, ranged between 22 m^3 ha^{-1} yr^{-1} and 35 m^3 ha^{-1} yr^{-1}, depending on stand age (Harwood & Nambiar, 2014).

The organic matter within the soil is essential for soil and its overall health as it is accumulated with a large number of nutrients, moisture, and assistive bio-organisms (Bot & Benites, 2005). It helps the soil properties be in good condition to store the nutrients and water available for the tree to grow efficiently. The soil chemical variables, such as TC, exchangeable Mg, CEC, and available P, are also essential components in soil properties of the different stand ages. The soil TC is essential for *A. mangium* plantation as it is the main component of stand wood biomass (Nykvist & Sim, 2009). The CEC and exchangeable bases in the soil are directly proportional to the soil organic matter and clay content (Berry et al., 2007). The electrical charges in the soil influence the soil ability to hold the nutrients and provide buffering against soil acidification. The soil nutrients primarily exist as cations, which may increase soil fertility (McKenzie et al., 2004). Available P is essential for the growth of the stand as the lack of P element within the soil properties will result in an adverse effect on the growth and productivity, especially for leguminous plant type in tropical rainforest (Bini et al., 2013; Majid & Paudyal, 1999; Zás & Serrada, 2003).

Sand content was observed to significantly negatively affect the silt content and TN, which indicated that the soil's high amount of sand content would reduce silt content and nitrogen concentration in the study area. Soil properties dominated by high sand content lack nutrients, particularly nitrogen (Binkley & Fisher, 2019). Nath (2014) stated that the sand content is also negatively correlated to the water holding capacity in growing soil in Sivasagar, India. The negative association of moisture content to pH level suggested that high water content might increase the soil's acidity. It is inconsistent with Zhang and Wienhold (2002), which recorded that the high moisture content level will significantly alter the pH level to be higher in corn post-cropped soil.

Several soil characters may affect stand growth parameters (stand DBH and height). Results from correlation analysis and PCA were consistent regarding the negative effect of pH, available P, and clay content toward DBH and height. Soil pH showed it is the essential variable correlated to DBH and height. It indicates that the more acidic the soil, the greater the DBH and height (Figure 3). The pH values in *A. mangium* plantation in Andulau Forest Reserve, Sungai Liang, Brunei Darussalam ranged between 3.8 to 4.1 and is the most influential variable (Matali & Metali, 2015). The high density of litter and wood biomass within the older stands area might contribute to the high rate of nitrification leading to acidification. Soil pH reduction in the older stands could be related to vegetative coverage, which then caused extensive secretion of organic acids associated with accelerated organic matter decomposition (Lee et al., 2015).

Soil pH below 5.0 is ordinarily toxic to plant growth. Nutrient toxicity can occur in acid soils when the pH is 4.8 and lower due to aluminium (Al) and manganese (Mn) become more available in the soil solution and are harmful to plant roots (Slattery et al., 1999). However, *A. mangium* can survive well in acid soils, and the optimum soil pH range is 4 to 6 (Arentz et al., 1995). This tree species adapted well to acidic soils and grows satisfactorily in soils even with pH less than 4.0 (Franco & de Faria, 1997; Midgley & Turnbull, 2003).

Available P and clay content were shown to negatively correlate with DBH and height, suggesting that a high level of available P and clay can adversely affect the DBH and height growth rates. These results corroborate with Nurudin et al. (2013), stating that increased P adsorption negatively affects *Acacia* growth. An increase in P concentration resulted in a decrease in soil respiration under *A. mangium* plantation (Cao et al., 2011), indicating little or no aerobic microbial activity in the soil, resulting in poor growth. Clay has been shown to inversely correlate with Poplar plantation height growth (Salehi & Maleki, 2012). High clay content had been shown to cause limited root penetration and absence of *Acacia* fine roots (Kadir et al., 1998), thus limit tree growth. It was also reported that higher clay content correlates with low TC content (Nurudin et al., 2013), which negatively impacted wood productivity.

The results of this study can assist the plantation manager in the decision-making process in managing commercial *A. mangium* plantations. This study demonstrated that soil physical and chemical properties are vital in understanding the health condition of a plantation. Determining soil properties will ultimately help managers manage planted forests effectively because the soil physicochemical properties available for tree growth are affected by management practices that include site preparation plantation establishment, silvicultural treatment, and harvesting systems.

CONCLUSION

The growth rate of the *Acacia mangium* tree is very rapid at the early stage but slowed down as it gets old. Tree DBH, total height,

basal area, and volume increased as stand age increased. However, the survival rate and mean annual increment decreased with age. The stand growth and yield culminate at the age of 8.5 years old. Information of the soil physicochemical properties status in forest plantation areas of various age stands is vital to understand the growth performance of the *A. mangium* plantation. The PCA analyses showed that the physical soil properties that are most influential were SOM, silt and sand contents, bulk density, and moisture content, while for chemical parameters, exchangeable Mg, CEC, TC, TN, and C/N ratio. In general, soil pH, available P, and clay content showed the most variables. They are significantly negatively correlated to DBH and height of *A. mangium* stand. The PCA helped to reveal some relationships between some soil properties and stand parameters. These results help broaden the understanding of the interrelationship and association of soil properties on the growth parameters of *A. mangium* plantation.

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Chemical Profiles of *Terminalia catappa* LINN Nut and *Terminalia subspathulata* KING Fruit

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ABSTRACT

Terminalia catappa and *Terminalia subspathulata* are two species of the Combretaceae family of medium to large forest trees. The fruits of *T. catappa* are known for the edible nuts commonly known as tropical almonds due to their similarity in taste with almonds of commerce. Therefore, the chemical profiles of the fruits of the two *Terminalia* species were examined to ascertain their potential value for food or health uses. Gas chromatography-mass spectrometry (GCMS) and ultrahigh-pressure liquid chromatography-electrospray ionisation tandem mass spectrometry (UHPLC-ESI-MS/MS) techniques were employed to profile the extracts to ensure good coverage of the classes of metabolites of the fruit extracts. The GCMS results revealed that *T. catappa* nuts were rich in palmitic acid (33.2%), linoleoyl chloride (29.1%), and oxacyclohexadecan-2-one commonly known as pentadecanolide (16.2%). In comparison, the major constituents of *T. subspathulata* fruits were palmitic acid (18.1%) and its methyl ester, methyl palmitate (9.3%). Furthermore, a total of 38 compounds were putatively identified in the 70% aqueous methanolic extracts of both species via UHPLC-ESI-MS/MS analysis, comprising three organic acids, sixteen hydrolysable tannins, ten phenolic acids, eight flavonoids, and a diarylheptanoid. The GCMS- and liquid chromatography-mass spectrometry-(LCMS-) LCMS-based metabolite profiles obtained in the present study have revealed the diversity of chemical constituents in the *T. catappa* nuts and *T. subspathulata* fruits, potentially valorised as functional foods

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nutraceutical ingredients for plant-based health products.

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INTRODUCTION

Plants have served as a source of food and health remedies for man and animals from time immemorial (Tugume & Nyakoojo, 2019). Today, plants still play an important role in traditional and complementary medicine, featuring in many countries' primary health care systems (Shewamene et al., 2020). It is because plants contain a diverse array of metabolites with useful pharmacological properties. However, only a small percentage of the plant kingdom has been explored for their nutritive and/or therapeutic potentials (Noorhosseini et al., 2020). One possible reason for this phenomenon is the absence or lack of scientific evidence supporting their importance and potential value (Liebelt et al., 2019). Plants are valued based on their chemical composition, the relative concentrations of their chemical constituents, and their biological potentials. Therefore, there is a need to fill this gap in information so that their potential value as food, nutraceutical, and future medicines can be fully realised and valorised.

The genus *Terminalia* belongs to the Combretaceae family of plants, which comprises flowering plants of about 530 species of trees, shrubs, and lianas (Christenhusz & Byng, 2016). Several *Terminalia* species are reported to be medicinal and commonly used in Indian traditional medicine or Ayurveda. Despite its widespread use and reports on the efficacy of several of its members, many remain unexplored for their potential uses (Cock, 2015). Studies on several *Terminalia* species in the past, including *Terminalia arjuna*, *Terminalia bellirica*, *Terminalia chebula*, and *Terminalia catappa*, have shown them to be rich sources of phenolic compounds, flavonoids, triterpenoids, and saponins (Cock, 2015; Zhang et al., 2019). These species were also reported to exhibit important pharmacological properties such an anti-inflammatory, hepatoprotective, antimalarial, antidiabetic, and antimicrobial activities (Cock, 2015; Das et al., 2020; Zhang et al., 2019). However, many of these reports were mainly focused on the properties of the leaf, root, stem, and bark or stem bark parts of the plants. Other parts or organs' chemical and biological properties notably the fruits and nuts plentiful from some of these species, are lesser-known and remain unexplored.

Terminalia catappa Linn. is regarded as a wonder plant among the *Terminalia* species and also seemed to be the most prescribed medicinally (Cock, 2015). It is a large tropical tree that grows predominantly in the tropical regions of Asia, Africa, and Australia (Anand et al., 2015). The leaves, bark, and roots have been used in Ayurvedic medicine to treat several ailments such as hypertension, dysentery, and diarrhoea (Cock, 2015; Oyeleye et al., 2018). The ripe fruits of *T. catappa* are the source of

an edible nut known as 'tropical almonds' or 'Indian almonds', which can be eaten raw or roasted, and reported to be a rich source of fatty acids with high nutritive properties (Siew et al., 2015), and anti-cardiovascular potential (Kalita et al., 2018). A phytochemical study on an antifungal fraction of *T. catappa* leaf revealed the presence of punicalin, punicalagin, gallic acid, and isovitexin derivative (Terças et al., 2017). In addition, chemical characterisation of the phenolic-rich extracts of *T. catappa* revealed eleven (gallic acid, catechin, chlorogenic acid, caffeic acid, ellagic acid, epicatechin, rutin, quercitrin, isoquercitrin, quercetin, and kaempferol), and eight (gallic acid, catechin, caffeic acid, ellagic acid, resveratrol, rutin, quercetin, and kaempferol) phenolic compounds in the leaf and stem bark, respectively (Oyeleye et al., 2018). Furthermore, the flavonoid profiles of the bark, fruit, and wood have also been reported (Venkatalakshmi et al., 2016).

Meanwhile, *T. subspathulata* is native to Malaysia and Singapore (National Board Parks [NParks], 2015). Aside from being highly valued for the use of its hardwood, especially in making canoes, very little is known about its medicinal use by the local population of Malaysia, as well as its chemical profile. However, the recent listing of this wild species in the list of endangered species prompted its inclusion in this research study (NParks, 2015).

Gas chromatograph (GC) and liquid chromatography (LC) are two chromatographic separation techniques extensively used to study phytochemical constituents. Over the past few decades, the development of mass spectroscopy (MS) has contributed substantially to the scope of applications of both GC and LC. Hyphenation of GC or LC with MS technique has made the separation and identification of compounds in complex mixtures more effective and efficient. Gas chromatography-mass spectrometry (GC-MS) is primarily used to analyse compounds that are adequately volatile and stable under the high temperature of GC conditions. Some polar compounds, such as those with a number of hydroxyl groups, can be derivatised and subsequently subjected to GC-MS analysis (Patel et al., 2010). In contrast, compounds with low volatility, whose volatility cannot be increased even on derivatisation, can be analysed using liquid chromatography-mass spectrometry (LC-MS). Generally, LC-MS allows the analysis of compounds with a broader range of polarity and minimal sample preparation (Perez et al., 2016). Given the different scope of application of GC-MS and LC-MS, using both platforms can offer a more comprehensive view of the metabolite profiles of plant extracts, which are usually complex mixtures of compounds with diverse polarity.

While there is a number of studies regarding the phytochemical contents of the different parts of *T. catappa* (Oyeleye et al., 2018; Terças et al., 2017; Venkatalakshmi et al., 2016), there is still a lack of comprehensive phytochemical profile of its fruits. Only flavonoids from fruits of *T. catappa* have been reported

previously by Venkatalakshmi et al. (2016). Besides, the chemical composition of *T. subspathulata* has not yet been reported. Therefore, in the present study, the chemical constituents of *T. catappa* nuts and *T. subspathulata* fruits were profiled using GC-MS and ultrahigh-pressure liquid chromatography-electrospray ionisation tandem mass spectrometry (UPLC-ESI-MS/MS) techniques. Application of both GC-MS and LC-MS will allow the establishment of a more comprehensive phytochemical profile of the fruits of the two *Terminalia* species.

MATERIALS AND METHODS

Collection and Identification of Plant Materials

The fresh, unripe fruits of *T. catappa* and *T. subspathulata* were collected from the campus grounds of Universiti Putra Malaysia (UPM) and the Sultan Idris Shah Forest Education Center (SISFEC) in May 2017. Voucher specimens of each species, SK3336/18 and SK3337/18, respectively, were deposited in the mini-herbarium of the Biodiversity Unit, Institute of Bioscience (IBS), UPM.

Solvents and Chemicals

Analytical grade methanol, n-hexane, and LC-MS grade water were purchased from Merck (Germany). LC-MS grade methanol and formic acid were supplied by Fisher Scientific (Belgium). Deionised water was obtained from the Milli-Q purification system (Millipore, USA).

Sample Processing and Extraction

The fresh fruits of *T. catappa* and *T. subspathulata* were cleaned under running water immediately after collection, drained, and pat-dried off any excess water. The edible kernel (nut) of *T. catappa* was removed from the fibrous husk for chemical analysis. For *T. subspathulata*, however, the whole fruit was used for the chemical analysis due to its smaller size. The processed nuts of the former and fruits of the latter were air-dried at ambient temperature. The dried samples were pulverised and extracted with 70% aqueous methanol using a solid to liquid ratio of 1:30 (w/v). The extraction was assisted with 30 min ultrasonication (SK8210HP Shanghai KUDOS Ultrasonic Instrument Co. Ltd., China) with the sonication frequency set to 53 kHz. The sonicator bath temperature was maintained between 25–30 °C. The solvent extract was drained, collected, and set aside, while the plant residue was re-extracted with fresh solvent. The extraction procedure was repeated four times to ensure that the extraction was adequately exhaustive, and the solvent extract was collected for each batch. The collected extracts were pooled, filtered, and concentrated under *vacuo*, using a rotary evaporator (Heidolph GmbH and Co. K.G., Germany), freeze-dried, and kept at -20 °C prior to analysis.

GCMS Analysis

One gram of the crude methanolic extracts of the *T. catappa* nuts and *T. subspathulata* fruits were separately reconstituted in methanol and then solvent-partitioned into n-hexane to separate the fatty acids

and other non-polar metabolites from other metabolites of higher polarity. The hexane fractions were freeze-dried and subjected to GCMS analysis using the QP2010 Ultra GCMS system (Shimadzu, Japan). The system was equipped with Rxi-5ms fused silica capillary column (30 m length, 0.25 mm ID, 0.25 µm film thicknesses, composed of 5% diphenyl and 95% dimethyl polysiloxane). The analysis was carried out by gradient temperature program, starting with an initial temperature of 50 °C for 3 min, increased to 300 °C at 3 °C/min, and then 300 °C constantly for another 10 min. The sample was eluted at 1 µL, with the mass conditions: ionization voltage of 70 eV, helium flowing at 11.8 mL/min, ion source temperature of 250 °C, and scan range m/z 40-700 amu. The compound identification was carried out by comparing the mass data of the hexane fractions to NIST 11 (National Institute of Standard Technologies, Mass Spectra) and FFNSC 1.3 (Flavor and Fragrance Natural and Synthetic Compounds) libraries.

UHPLC-ESI-MS/MS Analysis

The UHPLC-ESI-MS/MS analysis of the 70% aqueous methanolic extracts of *T. catappa* nuts and *T. subspathulata* fruits was carried out using Q Exactive™ Focus Hybrid Quadrupole Orbitrap mass spectrometer (Thermo Scientific, USA). The system was equipped with Dionex Ultimate 3000 UHPLC. Separation was performed by Acquity UHPLC BEH C18 column (100 x 2.1 mm, 1.7 µm) at a flow rate of 0.4 mL/min using a gradient elution of LC-MS grade water (A): acetonitrile (solvent B) with an additional 0.1% formic acid. The solvent was eluted gradient as follows: 5% B (0-2 min), 5-70% B (2–32 min), 70-100% B (32–37 min), 100% B (37-40 min), and 100-5% B (40-48 min). The sample injection volume for the analysis was 2 µL and observed under UV at 210, 254, 270, and 360 nm wavelengths. Mass data acquisition was performed in negative and positive ion modes, using electrospray ionization (ESI) technique at capillary voltage 3.5 kV, sheath gas 80 arb, and non-transfer tube temperature 320 °C, where the total ion chromatogram (TIC) was recorded from 150 to 1,500 amu. Data processing was performed using Thermo Xcalibur™ 2.2 software (Thermo Scientific, USA).

RESULTS AND DISCUSSION

GCMS Metabolite Profile of Hexane Fractions

The GCMS spectral data of the hexane fractions of *T. catappa* nuts and *T. subspathulata* fruits are presented in Figures 1 and 2, respectively, together with the total ion chromatograms of the individual samples. Several of the detected metabolites were common to both *T. catappa* nuts and *T. subspathulata* fruits. These were the saturated fatty acids, palmitic acid and stearic acid, fatty acid esters, methyl palmitate, methyl linoleate, and decanedioic acid bis(2-ethylhexyl) ester, as well as a fatty acid chloride, linoleoyl chloride. Although both *T. catappa* nuts and *T. subspathulata* fruits were rich in palmitic acid, the content in *T. catappa* nuts was almost twice (33.2%) of that found in *T.*

subspathulata fruits (18.1%). *Terminalia catappa* nuts were also rich in fatty acid chloride, linoleoyl chloride (29.1%), and the cyclic lactone, oxacyclohexadecan-2-one, commonly known as pentadecanolide (16.2%). Linoleoyl chloride, however, was a minor constituent (2.1%) in *T. subspathulata* fruits, whereas pentadecanolide was not detected in *T. subspathulata* fruits. The major constituents of *T. subspathulata* were palmitic acid and its methyl ester, methyl palmitate (9.3%). Meanwhile, moderate amounts of stearic acid (4.8-5.7%) were present in both species.

Saturated and polyunsaturated fatty acids are important ingredients for cosmetics and other botanical-based health products. These fatty constituents have been linked to antioxidants (Bouazzi et al., 2020) and antimicrobial activities (Desbois & Smith, 2010; Pinto et al., 2017; Oyeleye et al., 2018). Palmitic acid and stearic acid are very common saturated fatty acids found in both plants and animals and have exhibited good antibacterial and antifungal properties (Karimi et al., 2015). Meanwhile, linoleoyl chloride identified from the essential oils of leaf *Kaempferia galanga* Linn. showed promising anti-nociceptive and anti-inflammatory activities, as well as larvicidal and repellent effects (Bhuiyan et al., 2008). The compound is a useful reagent for synthesising fatty acid esters of hydroxy fatty acids (FAHFAs), which are known to have beneficial biological effects such as antidiabetes and anti-inflammation (Gowda et al., 2020). Meanwhile, pentadecanolide is one of the major components of *Angelica archangelica* L. species. It has been reported to be a potent and selective inhibitor of rat liver cyclic AMP-dependent protein kinase (Wang & Polya, 1996). Pentadecanolide has been synthesised and has important applications as a fragrance ingredient (Belsito et al., 2011). Decanedioic acid bis-(2-ethylhexyl) ester was previously isolated from endophytic bacteria, which exhibited antimicrobial activity (Mohamad et al., 2018; Tambekar et al., 2017).

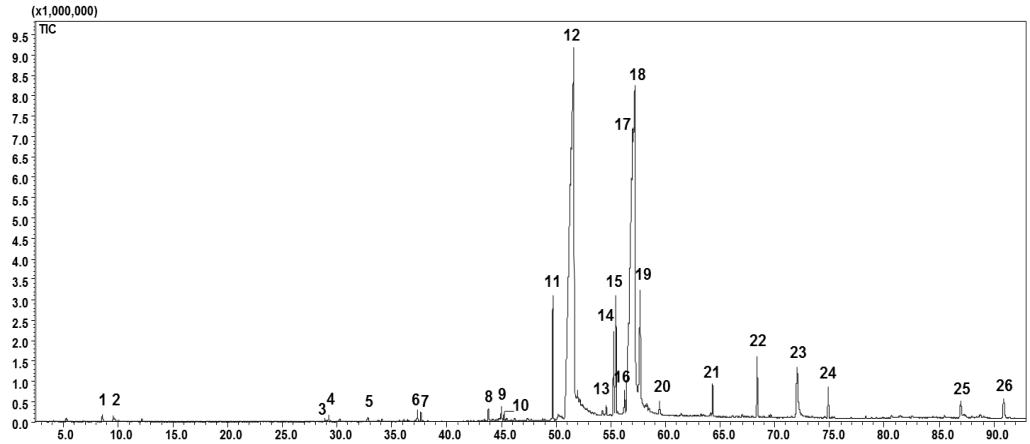
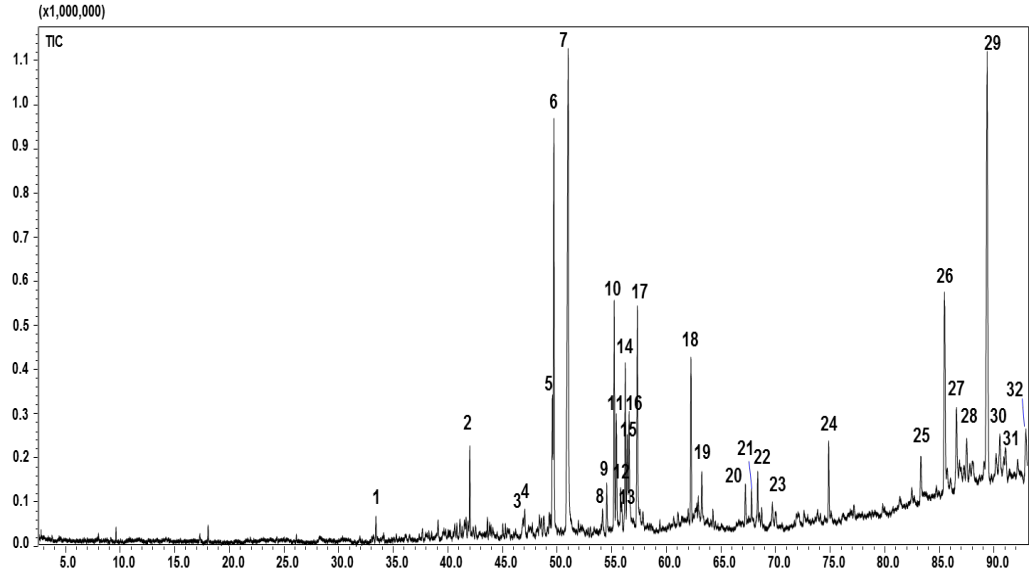


Figure 1. GCMS spectral data of metabolites identified in hexane fraction of *Terminalia catappa* nut

Peak	RT (mins)	Compound ID	Molecular weight	Molecular formula	RI	RI*	SI %	Area %
1	8.58	5,6-Dihydro-2*H*-pyran-2-one	98	$C_5H_6O_2$	939	927	83	0.13
3	28.91	Tetradecene	196	$C_{14}H_{28}$	1391	1403	94	0.06
4	29.27	Tetradecane	198	$C_{14}H_{30}$	1399	1400	95	0.10
5	32.86	Propyl 4-hydroxybenzoate	180	$C_{10}H_{12}O_3$	1482	1480	87	0.12
6	37.38	1-Hexadecene	224	$C_{16}H_{32}$	1592	1602	94	0.19
7	37.68	Hexadecane	226	$C_{16}H_{34}$	1599	1600	96	0.17
8	43.85	Myristic acid	228	$C_{14}H_{28}O_2$	1760	1769	95	0.24
9	45.02	1-Octadecene	252	$C_{18}H_{36}$	1792	1801	95	0.24
10	45.27	Octadecane	254	$C_{18}H_{38}$	1799	1800	95	0.16
11	49.73	Methyl palmitate	270	$C_{17}H_{34}O_2$	1926	1925	95	2.78
12	51.55	Palmitic acid	256	$C_{16}H_{32}O_2$	1980	1977	80	33.15
14	55.28	Methyl linoleate	294	$C_{19}H_{34}O_2$	2095	2093	93	1.92
15	55.48	1,16-Hexadecanediol	258	$C_{16}H_{34}O_2$	2101	2097	78	2.78
16	56.27	Stearic acid methyl ester	298	$C_{19}H_{38}O_2$	2127	2127	90	0.49
17	57.02	Linoleoyl chloride	298	$C_{18}H_{31}ClO$	2151	2139	77	29.10
18	57.21	Oxacyclohexadecan-2-one	240	$C_{15}H_{28}O_2$	2157	2144	83	16.22
19	57.70	Stearic acid	284	$C_{18}H_{36}O_2$	2173	2167	91	4.79
21	64.31	Bis(2-ethylhexyl) adipate	370	$C_{22}H_{42}O_4$	2399	2414	96	0.77
22	68.40	Phthalic acid, 2,4-dimethylpent-3-yl octyl ester	376	$C_{23}H_{36}O_4$	2551	2540	81	1.42
23	72.06	Glyceryl monooleate	356	$C_{21}H_{40}O_4$	2693	2689	86	2.48
24	74.88	Decanedioic acid bis-(2-ethylhexyl) ester	426	$C_{26}H_{50}O_4$	2808	2812	94	0.71

Note. RT: Retention time; RI: Retention index of identified compound; RI*: Retention index of compound identified in NIST library; SI: Similarity index; A%: Percentage composition; nd: Not determined

Figure 1. (Continued)



Peak	RT (min)	Compound ID	Molecular weight	Molecular Formula	RI	RI*	SI %	Area %
1	33.42	Pentadecane	212	$C_{15}H_{32}$	1497	1500	88	0.45
2	41.98	Heptadecane	240	$C_{17}H_{36}$	1712	1700	90	1.55
3	46.87	Phytone	268	$C_{18}H_{36}O$	1846	1841	80	0.18
4	47.03	2,6,10,15-Tetramethylheptadecane	296	$C_{21}H_{44}$	1851	1852	88	0.62
6	49.69	Methyl palmitate	270	$C_{17}H_{34}O_2$	1927	1925	95	9.29
7	51.01	Palmitic acid	256	$C_{16}H_{32}O_2$	1967	1977	94	18.09
8	54.16	2,6,10,14-Tetramethylnonadecane	324	$C_{23}H_{48}$	2063	2051	80	0.34
10	55.24	Methyl linoleate	294	$C_{19}H_{34}O_2$	2096	2093	91	4.92
11	55.44	Methyl linolenate	292	$C_{19}H_{32}O_2$	2103	2098	84	2.83
12	55.83	Phytol	296	$C_{20}H_{40}O$	2115	2106	85	0.82
13	55.92	Oxiraneundecanoic acid, 3-pentyl-, methyl ester, cis-	312	$C_{19}H_{36}O_3$	2118	2129	74	0.55
14	56.25	Methyl stearate	298	$C_{19}H_{38}O_2$	2129	2127	88	3.20
15	56.43	Linoleoyl chloride	298	$C_{18}H_{31}ClO$	2135	2139	86	2.14
16	56.60	Eicosyl heptafluorobutyrate	494	$C_{24}H_{41}F_7O_2$	2140	2131	78	3.25
17	57.35	Stearic acid	284	$C_{18}H_{36}O_2$	2165	2167	91	5.74
18	62.26	Methyl eicosanoate	326	$C_{21}H_{42}O_2$	2331	2328	94	3.75
19	63.25	Arachidic acid	312	$C_{20}H_{40}O_2$	2366	2366	86	1.01
20	67.23	2-Monopalmitin	330	$C_{19}H_{38}O_4$	2510	2498	86	0.98
21	67.80	Methyl docosanoate	354	$C_{23}H_{46}O_2$	2532	2530	91	0.51
22	68.36	Phthalic acid, 2,4-dimethylpent-3-yl octyl ester	376	$C_{23}H_{36}O_4$	2553	2540	76	0.92
24	74.86	Decanedioic acid bis(2-ethylhexyl) ester	426	$C_{26}H_{50}O_4$	2812	2812	92	1.73

Note. RT: Retention time; RI: Retention index of identified compound; RI*: Retention index of compound identified in NIST library; SI: Similarity index; A%: Percentage composition; nd: Not determined

Figure 2. GCMS spectral data of metabolites identified in hexane fraction of *Terminalia subspathulata* fruit

UHPLC-ESI-MS/MS Metabolite Profile of 70% Aqueous Methanolic Extracts

UHPLC-ESI-MS/MS gives accurate molecular ion mass and fragmentation patterns of analytes. It gives an exact identity of metabolite present without ambiguity (Xiao et al., 2012). In the present study, the UHPLC-ESI-MS/MS spectral data of the 70% aqueous methanolic extracts of *T. catappa* nuts and *T. subspathulata* fruits were acquired in both negative and positive ionisation modes. However, the metabolites in both extracts were poorly ionized in the positive ionisation mode. Thus, metabolite identification and annotation were carried out only on the negative ionisation mode. Metabolite identification was carried out by comparing the mass spectrometry (MS) data (accurate mass, negative, and positive ion modes) of the compounds analysed using Thermo Xcalibur 2.0 (Thermo Fisher Scientific Inc., USA) with MS data obtained from the literature and open-source databases such as Metabolomics Workbench, Human Metabolome Database (HMDB), PubChem, MassBank, and Metlin. For gallic acid and isovitexin, the identification was based on comparison with the pure standards. The total ion chromatogram (TIC) of the *T. catappa* nuts and *T. subspathulata* fruits extracts are provided as supplementary data SD1(A) and SD1(B), respectively. The metabolites identified in the extracts comprise organic acids, hydrolysable tannins, phenolic acids, flavonoids, and diarylheptanoids. These identified compounds were tabulated in Table 1, consisting of the retention times (RT), molecular formula, molecular ion mass, and tandem mass.

Organic Acids. Organic acids greatly impact organoleptic properties, especially related to flavour, colour, and scent (Famiani et al., 2015; Flores et al., 2012; Sandín-España et al., 2016). In addition, they are major components of ripe fruits (Walker et al., 2018) and have high antioxidant activity, which makes them an excellent remedy against many ailments (Liu et al., 2019).

The citric acid (**1**) was identified in both *T. catappa* nuts and *T. subspathulata* fruits. It showed a pseudomolecular ion at m/z 191 and tandem mass with characteristic sequential losses of carbon dioxide (CO_2) and water moieties, for example, at m/z 129 for $[M\text{-}H\text{-}CO_2\text{-}H_2O]^-$, 111 for $[M\text{-}H\text{-}CO_2\text{-}2H_2O]^-$, 85 for $[M\text{-}H\text{-}2CO_2]^-$, and m/z 67 for $[M\text{-}H\text{-}2CO_2\text{-}2H_2O]^-$. Homocitric acid (**6**), which has an additional methylene (CH_2) unit in its structure, showed a pseudomolecular ion at m/z 205 and a similar fragmentation pattern as citric acid. Comparison with literature supported the identification of the two organic acids (Al Kadhi et al., 2017; Mena et al., 2012). Compound **1** is found in many fruits, especially citrus fruits and vegetables (Abdel-Salam et al., 2014; Penniston et al., 2008). Studies have shown **1** to decrease brain lipid peroxidation and inflammation and liver damage, and DNA fragmentation (Abdel-Salam et al., 2014). Meanwhile, chebulic acid (**33**) was identified based on its pseudo molecular ion at m/z 355 and compared with the literature (Yang et al., 2012). The compound was

Table 1

Identified compounds from Terminalia catappa *nut and* Terminalia subspathulata *fruit extracts using UHPLC-ESI-MS/MS*

No.	RT (min)	Compound ID	Molecular formula	$[M-H]^-$ (m/z)*	Mass error (ppm)	MS/MS fragments (m/z)	TCN	TSF
	Organic acids							
1	0.73	Citric acid	$C_6H_8O_7$	191.0192	0.00	191, 173, 155, 147, 129, 111, 87, 85, 67	+	+
6	1.45	Homocitric acid	$C_7H_{10}O_7$	205.0347	-0.49	191, 173, 155, 143, 125, 111, 87, 67	+	+
33	13.24	Chebulic acid	$C_{14}H_{12}O_{11}$	355.0304	0.84	355, 325, 337 310, 307, 175	+	-
	Hydrolysable tannins							
2	0.87	HHDP glucose	$C_{20}H_{18}O_{14}$	481.0615	-0.62	481, 421, 301, 275,	+	+
4	1.07	Punicalin (α/β isomer)	$C_{34}H_{22}O_{22}$	781.0522	-0.38	781, 601, 600, 575, 448, 392, 301, 298	+	+
5	1.38	Punicalagin	$C_{48}H_{28}O_{30}$	1083.0590	0.28	1083, 781, 601, 600, 301	+	+
7	1.63	bis-HHDP glucose	$C_{34}H_{24}O_{22}$	783.0682	0.13	783, 481, 451, 301, 275, 229	+	+
8	1.72	2-*O*-Galloypunicalin	$C_{41}H_{26}O_{26}$	933.0644	1.07	781, 721, 600, 575, 450, 425, 301	+	+
10	2:42	Punicalagin isomer	$C_{48}H_{28}O_{30}$	1083.0590	0.28	1083, 781, 601, 600, 301	+	+
12	3.36	bis-HHDP glucose isomer	$C_{34}H_{24}O_{22}$	783.0682	0.13	783, 481, 301, 275, 221	+	+
13	4.25	Corilagin	$C_{27}H_{22}O_{18}$	633.0733	0.79	633, 463, 301, 174	-	+
17	5.22	Galloy-bis-HHDP glucose	$C_{41}H_{28}O_{26}$	935.0809	1.92	935, 633, 481, 301, 299,	+	+
18	5.73	DigalloylHHDP glucose	$C_{34}H_{26}O_{22}$	785.0844	0.76	785, 633, 483, 419, 169	+	-

Table 1 *(Continued)*

No.	RT (min)	Compound ID	Molecular formula	$[M-H]^-$ (m/z)*	Mass error (ppm)	MS/MS fragments (m/z)	TCN	TSF
	Hydrolysable tannins							
19	5.87	Flavogallonic acid	$C_{21}H_{11}O_{13}$	469.0046	0.64	469, 425, 301, 300	+	+
20	6.15	Corilagin isomer	$C_{27}H_{22}O_{18}$	633.0733	0.79	633, 463, 301, 174	+	+
24	7.57	Ellagic acid pentoside	$C_{19}H_{14}O_{12}$	433.0412	1.15	433, 301, 229	+	+
29	9.41	Ellagic acid deoxyhexoside	$C_{20}H_{16}O_{12}$	447.0571	1.57	447, 315, 301, 299, 270, 151	+	-
32	11.61	2,3-Di-*O*-methylellagic acid	$C_{16}H_{10}O_8$	329.0303	1.82	329, 314, 299, 298	+	+
38	15.71	Tri-*O*-methylellagic acid	$C_{17}H_{12}O_8$	343.0463	2.62	328, 313, 298, 270, 269	+	-
	Phenolic acids							
3	0.98	Glucogallin	$C_{13}H_{16}O_{10}$	331.0669	1.21	331, 271, 211, 169, 125	+	+
9	1.98	Protocatechuic acid	$C_7H_6O_4$	153.0183	-3.27	153, 109, 108	+	+
11	2.93	Gallic acid	$C_7H_6O_5$	169.0132	-2.96	169, 125, 107	+	+
14	4.51	Digalloyglucose	$C_{20}H_{20}O_{14}$	483.0783	1.65	483, 331, 313, 169	+	+
15	4.94	Caffeic acid	$C_9H_8O_4$	179.0344	0.00	179, 135, 117, 107	+	+
16	4.97	Brevifolin carboxylic acid	$C_{13}H_8O_8$	291.0151	3.44	291, 247, 219, 191, 175	+	-
21	6.28	Trigalloylglucoside	$C_{27}H_{24}O_{18}$	635.0884	0.00	635, 483, 465, 313, 301, 169	+	-
22	6.53	*p*-Coumaric acid	$C_9H_8O_3$	163.0391	-2.45	163, 119, 93	+	-

Table 1 *(Continued)*

No.	RT (min)	Compound ID	Molecular formula	$[M-H]^-$ (m/z)*	Mass error (ppm)	MS/MS fragments (m/z)	TCN	TSF
	Phenolic acids							
28	9.31	Methyl gallate	$C_8H_8O_5$	183.0293	0.00	183, 169, 168, 139	+	+
35	13.32	Fertaric acid	$C_{14}H_{14}O_9$	325.0565	1.54	325, 310, 193, 149	+	+
	Flavonoids							
23	7.25	Luteolin-*C*-hexoside	$C_{21}H_{20}O_{11}$	447.0933	1.34	447, 429, 357, 327, 285	-	+
25	8.28	Isovitexin	$C_{21}H_{20}O_{10}$	431.0985	1.62	431, 341, 311, 283, 281, 269	-	+
26	8.30	Quercetin-3-*O*-deoxy-hexosylhexoside	$C_{27}H_{30}O_{16}$	609.1475	3.12	609, 301, 300, 271, 151	+	+
27	8.52	Quercetin-3-*O*-hexoside	$C_{21}H_{20}O_{12}$	463.0891	3.02	463, 445, 301, 300, 271, 151	+	+
30	11.31	Apigenin	$C_{15}H_{10}O_5$	269.0455	1.86	269, 241, 225, 183, 157, 151, 117	-	+
31	11.49	Isookanin	$C_{15}H_{12}O_6$	287.0562	2.09	287, 269, 177, 151, 135, 125, 109, 107	-	+
36	13.40	Naringenin	$C_{15}H_{12}O_5$	271.0613	2.21	271, 187, 177, 151, 119	-	+
37	14.26	Isorhamnetin	$C_{16}H_{12}O_7$	315.0508	0.95	315, 301, 300, 283, 271, 151	-	+
	Other compounds							
34	13.27	Demethoxycurcumin	$C_{20}H_{18}O_5$	337.1079	0.89	337, 321, 306, 291, 191, 177, 161	+	-

Note. RT: Retention time; CPD ID: Compound identity; TCN: *Terminalia catappa* nuts; TSF: *Terminalia subspathulata* fruits; +: Detected; -: Not detected; HHDP: Galloyl-hexahydroxydiphenoyl

* Values based on total ion chromatogram (TIC) of *Terminalia catappa* for compounds common to both extracts

detected only in the nuts of *T. catappa* but not in the fruits of *T. subspathulata*. It has been previously reported in the fruits of *T. chebula* (Avula et al., 2017).

Hydrolysable Tannins. The two extracts were found to be rich in hydrolysable tannins, comprising of twelve ellagitannins (**2**, **4**, **5**, **7**, **8**, **10**, **12**, **13**, **17**, **18**-**20**) and four ellagic acid derivatives (**24**, **29**, **32**, and **38**), as shown in Table 3. Ellagitannins are polymeric structures consisting of galloyl and hexahydroxydiphenoyl (HHDP) units esterified with a polyol, usually glucose. A characteristic reaction of ellagitannins is releasing the bislactone and forming an HHDP group, which eventually lactonizes to produce ellagic acid (Zhu et al., 2015). It leads to the observation of a characteristic base peak at m/z 301 corresponding to that of a deprotonated ellagic acid in the mass spectra of ellagitannins, in addition to the characteristic neutral losses of galloyl (152 amu), gallic acid (170 amu), HHDP (302 amu), galloylglucose (332 amu), HHDP glucose (482 amu), and galloyl-HHDP-glucose (634 amu) residues (Regueiro et al., 2014). Compound **2** was identified as HHDP glucose based on $[M-H]^-$ at m/z 481 and tandem mass at m/z 301 ($[M-H\text{-glucose}]^-$) corresponding to the HHDP residue, in agreement with the report of Singh et al. (2016). Compounds **7** and **12** were identified as bis-HHDP glucose isomers based on pseudomolecular ions at m/z 783 ($[M-H]^-$) and tandem mass at m/z 481 ($[M-H-HHDP]^-$). Meanwhile, **17**, with $[M-H]^-$ at m/z 935, tandem mass at m/z 633 ([M-H-HHDP] and m/z 481 for a further loss of galloyl moiety, was identified as galloyl-bis-HHDP glucose. This tandem fragmentation pattern was also observed for **18** with $[M-H]^-$ at m/z 785. Compound **18** was thus identified as digalloylHHDP glucose, present only in *T. catappa*. This class of ellagitannins have been reported previously in the bark of *Eucalyptus globulus* Labill. (Santos et al., 2011) and the unripe fruits of *T. arjuna* (Singh et al., 2016). Based on comparison with literature data, **4**, with $[M-H]^-$ at m/z 781, was identified as punicalin. Both compounds **5** and **10** showed the same $[M-H]^-$ (m/z 1083) and tandem mass, including losing an HHDP moiety at m/z 781. Thus **5** and **10** were identified as isomeric forms of punicalagin, differing from **4** by an additional moiety of HHDP (Mena et al., 2012; Mininel et al., 2014). The two compounds have been previously reported in the fraction *T. catappa* leaf, which exhibited antifungal activity against *Candida* species (Venkatalakshmi et al., 2016). Meanwhile, **8**, which exhibited $[M-H]^-$ at m/z 933, 152 amu higher than **4**, was identified as 2-*O*-galloylpunicalin. Isomeric forms of corilagin were also identified in the extracts, as **13** and **20**, with $[M-H]^-$ at m/z 633 and tandem mass at m/z 463 $[M-H-170]^-$ and 301 $[M-H-169-162]^-$, resulting from inductive cleavage of galloyl acid and galloylglucose (Nuengchamnong & Ingkaninan, 2017; Pfundstein et al., 2010). The fragmentation pathways of the ellagitannins are provided in the supplementary data SD2 and SD3. Corilagin has been reported in the leaves of *T. catappa* and is known for its strong

antioxidant property (Kinoshita et al., 2007), in addition to anti-tumour, hepatoprotective, and anti-inflammatory activities (Li et al., 2018).

Flavogallonic acid (**19**) was identified based on its $[M-H]^-$ at m/z 469, and tandem mass at m/z 425 for $[M-H-CO_2]^-$, and 300 for $[M-H-169]^-$ indicating the loss of gallic acid (Pfundstein et al., 2010). Flavogallonic acid has been previously reported in the fruits of *T. chebula* (Sarabhai et al., 2013). Several ellagic acid derivatives (**24**, **29**, **32**, and **38**) were also detected in the extracts. Ellagic acid has been reported previously as a constituent in the leaf and stem bark of *T. catappa* (Oyeleye et al., 2018), thus the detection of these ellagic derivatives in the present work is not surprising. Compounds **24** and **29**, with $[M-H]^-$ at m/z 433 and 447, were identified as ellagic acid pentoxide and ellagic acid deoxyhexoside, respectively. Both compounds exhibited characteristic tandem mass for ellagic acid at m/z 301, produced following the losses of pentosyl (132 amu) and deoxyhexosyl (146 amu) moieties from the respective pseudomolecular ions (Pinheiro et al., 2018). Compounds **32** and **38**, with $[M-H]^-$ at m/z 329 and 343, respectively, were identified as methoxylated derivatives of ellagic acid, based on tandem mass resulting from successive losses of methyl group (CH_3) groups (Kumar et al., 2015). The fragmentation pathways of the ellagic acid derivatives are provided in the supplementary data SD4 and SD5. Ellagic acid has been reported to have strong antioxidant, antiproliferative, chemopreventive, and antiatherogenic properties (Larrosa et al., 2010). The antioxidant activity of ellagic acid has been attributed to the stability of its free radical (Regueiro et al., 2014). Ellagic acid pentoside has been reported to be a constituent of the vitamin C rich berries of camu-camu (*Myrciaria dubia*) (Fracassetti et al., 2013). It is also present in walnuts, together with ellagic acid-2-rhamnoside (Bulló et al., 2011).

Phenolic Acids. Phenolic acids have been reported as one of the major contributors to sensory quality, colour, nutritional, and antioxidant properties of edible foods from the plant kingdom (Cheynier, 2012; Kumar & Goel, 2019). Compounds **3**, **11**, **14**, **21**, and **28** were identified as gallic acid and its derivatives. Gallic acid (**11**), with $[M-H]^-$ at m/z 169 and tandem mass at m/z 125 ($[M-H-CO_2]^-$), was identified by comparison with the standard compound. Oyeleye et al. (2018) has previously reported the presence of gallic acid in the leaf and stembark of *T. catappa*. Compound **3**, with $[M-H]^-$ at m/z 331, was identified as glucogallin, a monogalloylglucose, based on tandem mass at m/z 211 and 169 for sequential loss of the glucose moiety, 162 amu (Avula et al., 2017; Kumar et al., 2015). Compounds **14** and **21**, with $[M-H]^-$ at m/z 483 and m/z 635, were identified as digalloylglucose and trigalloylglucose, respectively, based on the tandem mass arising from losses of the corresponding number of galloyl moieties (Singh et al., 2016). Compound **28**, with $[M-H]^-$ at m/z 183, identified as methyl gallate, showed characteristic tandem mass at m/z 168 and 125 for sequential methyl and CO_2 moieties losses.

Other phenolic acids present in the extracts were the naturally common acids, protocatechuic (**9**) and caffeic acids (**15**), identified based on comparison with literature values (Buiarelli et al., 2010; Maity et al., 2013; Wang et al., 2012). Compound **9** is found in many edible and medicinal plants and protects against cardiovascular diseases and neoplasms (Oniszczuk et al., 2019; Szumiło, 2005). Compound **15** has been reported to be one of the metabolites in hazelnut and most edible fruits and has been reported to have anticancer potential (Ghirardello et al., 2010).

Compound **35**, with $[M-H]^-$ at m/z 325, was identified as fertaric acid, an ester formed from ferulic acid bound to tartaric acid. The compound showed characteristic losses of the corresponding fragments of ferulic acid and tartaric acid moieties (Pati et al., 2014). Fertaric acid is mostly found in grapes (Gris et al., 2013; Mozetič et al., 2006). The nut extract of *T. catappa* showed the presence of *p*-coumaric acid (**22**) and its isocoumarin derivative, brevifolin carboxylic acid (**16**). The former acid, with $[M-H]^-$ at m/z 163, was identified from the tandem mass at m/z 119 for $[M-H-CO_2]^-$ and 93 for $[M-H-CO_2-C_2H_2]^-$ (Kumar et al., 2015), while the latter acid, with $[M-H]^-$ at m/z 291, was identified based on the tandem mass at m/z 247 for $[M-H-CO_2]^-$, 219 for $[M-H-CO_2-CO]^-$, and m/z 191 for $[M-H-CO_2-2CO]^-$ (Zhu et al., 2015). Hydroxycinnamic acids such as **15**, **22**, and **35** have been reported to provide many health benefits, including antioxidant, anti-inflammatory, anti-collagenase, antimicrobial, and anti-tyrosinase (Adisakwattana, 2017; Alam et al., 2016; Taofiq et al., 2017).

Flavonoids. Flavonoids have been reported for numerous positive effects on human health and are present in many plants (Ganeshpurkar & Saluja, 2017; Howes, 2018). In the present study, eight flavonoids were identified in the fruit extract of *T. subspathulata* (**23**, **26**-**27**, **30**, **31**, **36**, and **37**). Compound **23**, with $[M-H]^-$ at m/z 447 and tandem mass of m/z 357 for $[M-H-90]^-$ and m/z 327 for $[M-H-120]^-$, was identified as luteolin-*C*-hexoside (Chen et al., 2016; Otłowska et al., 2018). Compounds **26** and **27**, with $[M-H]^-$ at m/z 609 and 463, respectively, were identified as quercetin derivatives based on the characteristic base peak ions at m/z 301 and tandem mass m/z 271 and 151 (Kumar et al., 2017; Yang et al., 2012). Losses of the deoxyhexosylhexose (m/z 308) and hexose (m/z 162) moieties allowed the identification of **26** as quercetin-3-*O*-deoxyhexosylhexoside and **27** as quercetin-3-*O*-hexoside (Kumar et al., 2015). Glycosylation at the C-3 position of these compounds was determined by the higher relative abundance of their radical aglycone $[Y_0 - H]^-$ ion (m/z 300) than the Y_0^- ion (m/z 301) (Buzgaia et al., 2020). Compound **27** has been reported in the fruit, leaf, and stem bark of *T. catappa*, while quercetin-3-*O*-glucoside was present in the leaf (Oyeleye et al., 2018). Compound **25**, with $[M-H]^-$ at m/z 431, was identified as isovitexin by comparison with a commercial standard. The tandem mass for the apigenin aglycone was observed at m/z 269 ($[M-H-gluc]^-$)

while apigenin itself could be identified as compound **30**, which exhibited [M-H]$^-$ at m/z 269. It showed characteristic fragments at m/z 151 and 117, arising from $^{1,3}A^-$ and $^{1,3}B^-$ retro-Diels Alder (rDA) cleavages of the flavonoid skeleton (Deseo et al., 2020; Otłowska et al., 2018). While the tandem mass m/z 151 is common for flavonoids, m/z 117 is characteristic apigenin, a flavone with a hydroxy group (OH) at the B ring. Compound **31**, with [M-H]$^-$ at m/z 287, and tandem mass m/z 269 for water loss was identified as isookanin based on the proposed fragmentation pathway shown in Figure 3. The respective losses of $C_6H_5O_2$ and $C_9H_7O_4$ moieties from the molecular ion to give the fragment ions at m/z 177 and 109 were also in agreement with Yang et al. (2016). Compound **36** with [M-H]$^-$ at m/z 271, and compound **37** with [M-H]$^-$ at m/z 315, were identified as naringenin and isorhamnetin, respectively, in agreement with literature values (Fathoni et al., 2017; Kumar et al., 2015). Compound **37** has been detected previously in the bark, fruit, and wood of *T. catappa* (Venkatalakshmi et al., 2016).

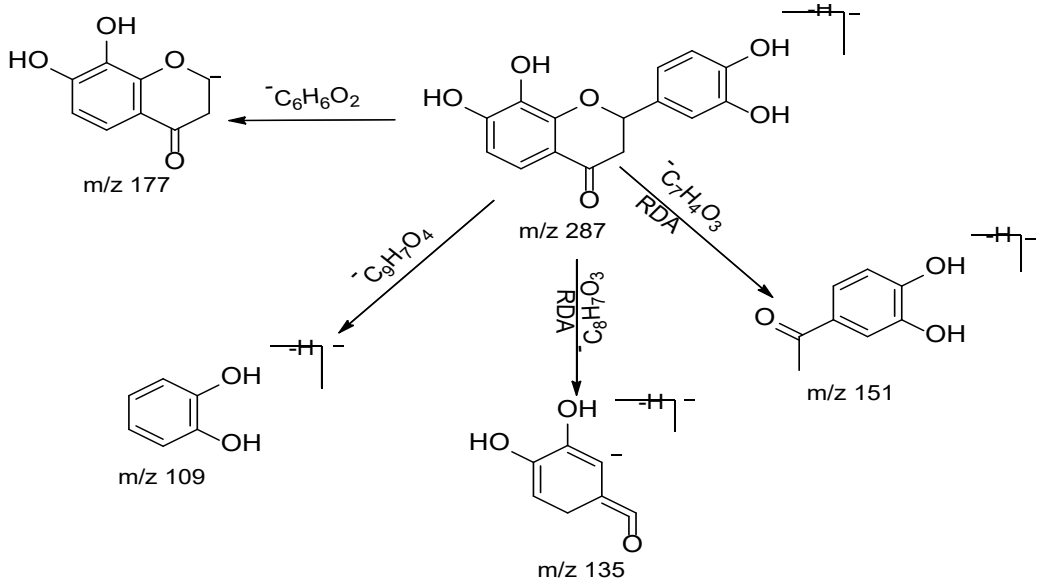


Figure 3. Proposed fragmentation pathways for isookanin **31**

Other Constituents. Compound **34**, with [M-H]$^-$ at m/z 337, was identified as demethoxycurcumin, based on a comparison of tandem mass proposed fragmentation pathway depicted in Figure 4. The compound loses a methoxy group (CH_3O) to give tandem mass m/z 306 [M-H-31]$^-$, while hetero cleavages of the -CO-CH_2-CO- linkage gave rise to tandem masses m/z 161 and m/z 177. Diarylheptanoids are characteristic constituents of the rhizomes of *Curcuma* species (Bresciani et al.,

2020; Jiang et al., 2006), which makes its detection highly surprising due to the difference in the plant family. Further isolation and purification studies will need to be carried out to validate its identification. As a class of compounds, curcuminoids have demonstrated strong antioxidant and anti-inflammatory activities, among others (Opara & Chohan, 2014).

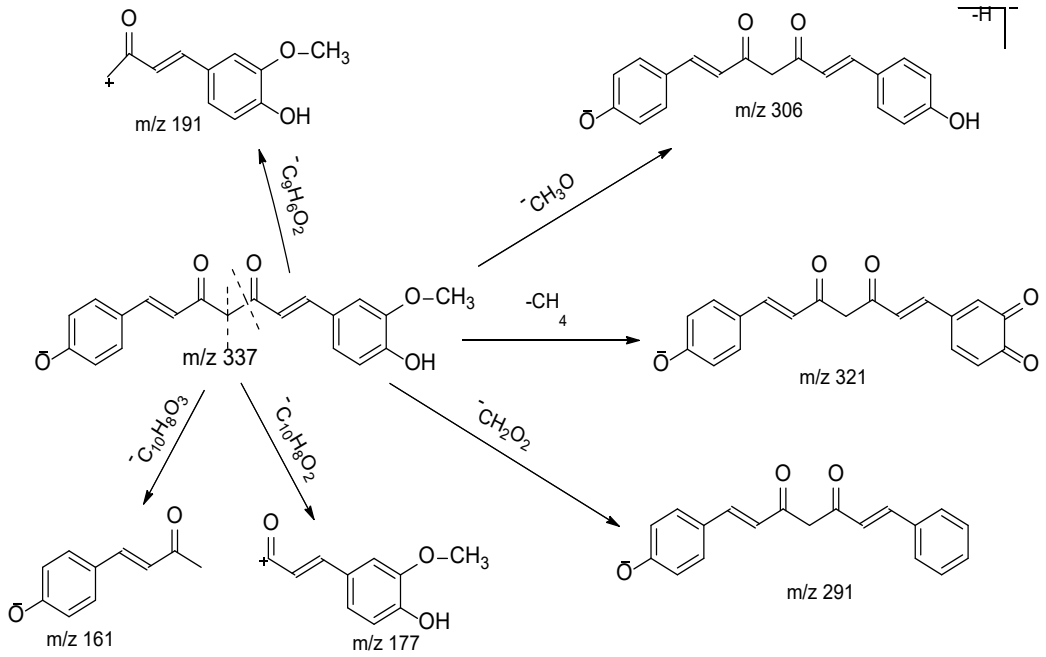


Figure 4. Proposed fragmentation pathways for demethoxycurcumin 34

CONCLUSION

The mass spectrometric analysis revealed the phytochemical diversity of *T. catappa* nuts and *T. subspathulata* fruits. The GCMS profiling of the n-hexane fractions of the crude extracts revealed the presence of metabolites from the classes of saturated and polyunsaturated fatty acid, fatty acid ester, fatty acid chloride, fatty alcohol, macrocyclic lactone, alkane, alkenes, and unsaturated carboxylic acid. On the other hand, via UHPLC-ESI-MS/MS analysis, a total of 38 compounds, comprising organic acids, hydrolysable tannins, phenolic acids, flavonoids, and diarylheptanoids were identified in the methanolic extracts of both species. The outcome of this study provides insight into the chemical profile of *T. catappa* nuts and *T. subspathulata* fruits. The phytochemical diversity of these underutilised fruits and nuts reveals their potential for further exploitation and development as functional foods and nutraceutical ingredients for plant-

based health products. However, a detailed quantitative and toxicity analysis of the extracts or their bioactive components is necessary to reach these goals.

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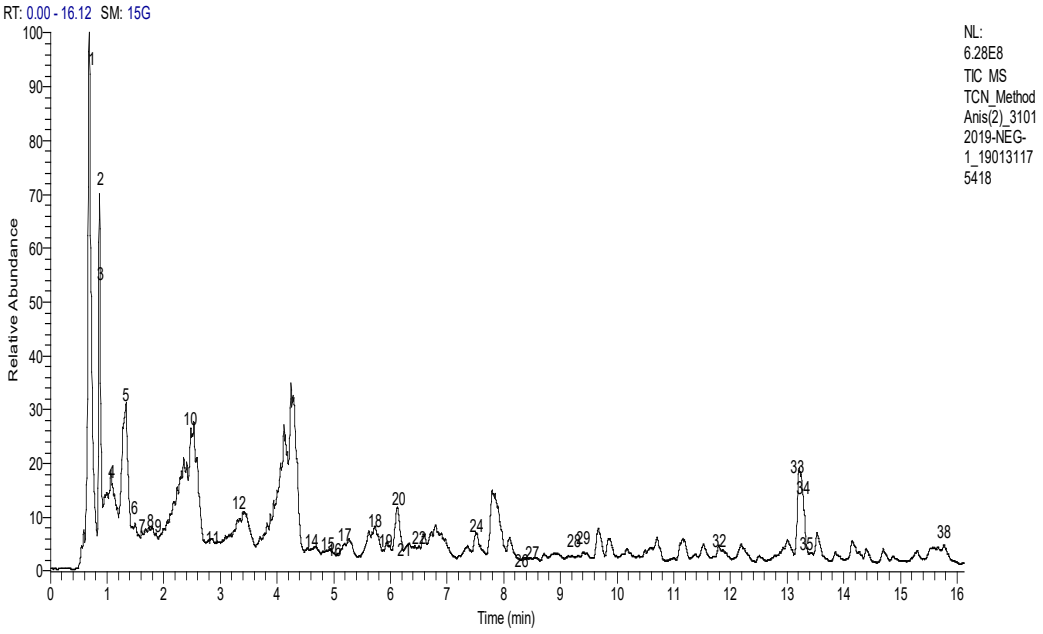
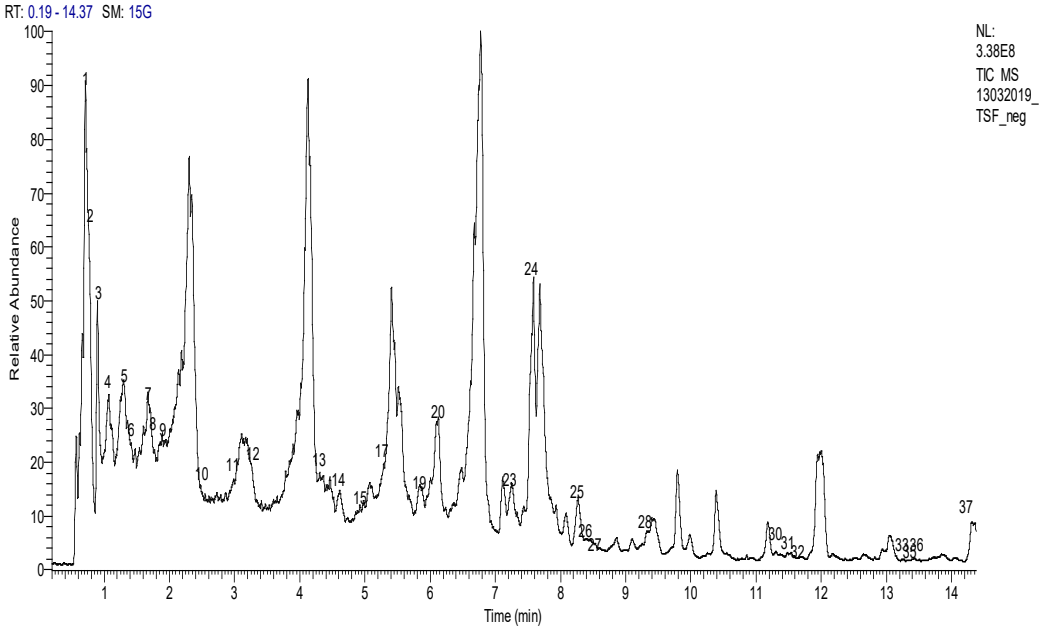
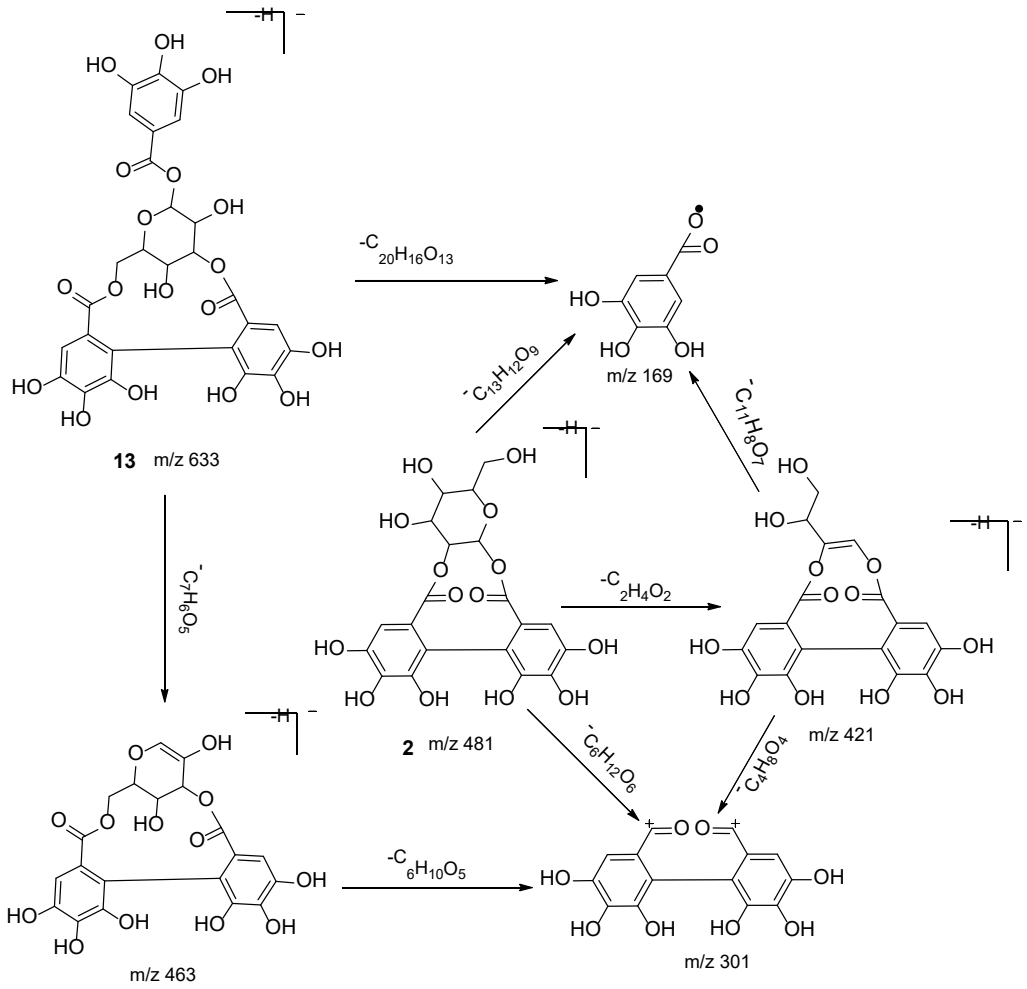
SUPPLEMENTARY DATA



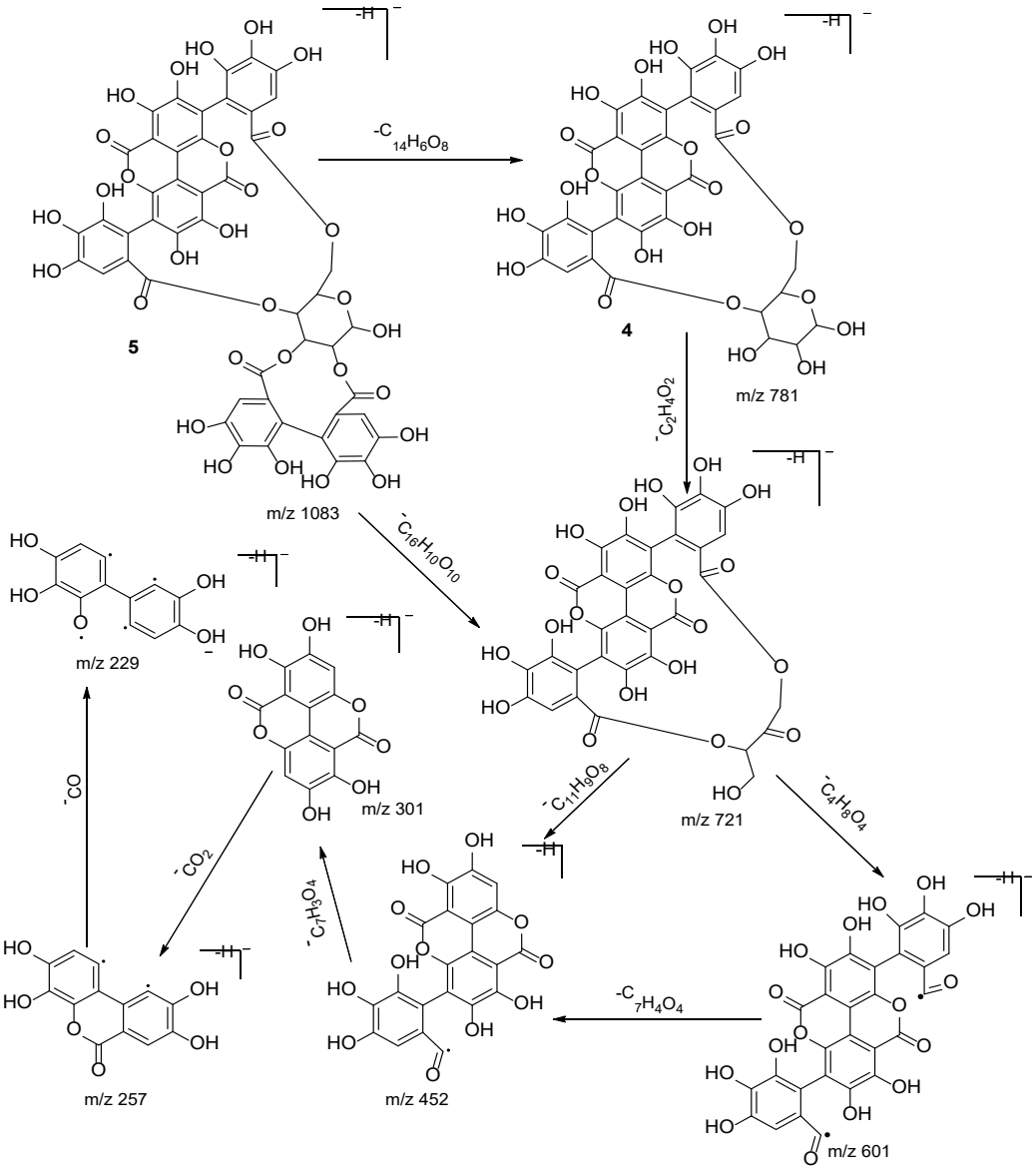
SD1(A). LCMS total ion chromatogram of *Terminalia catappa* nuts in negative mode



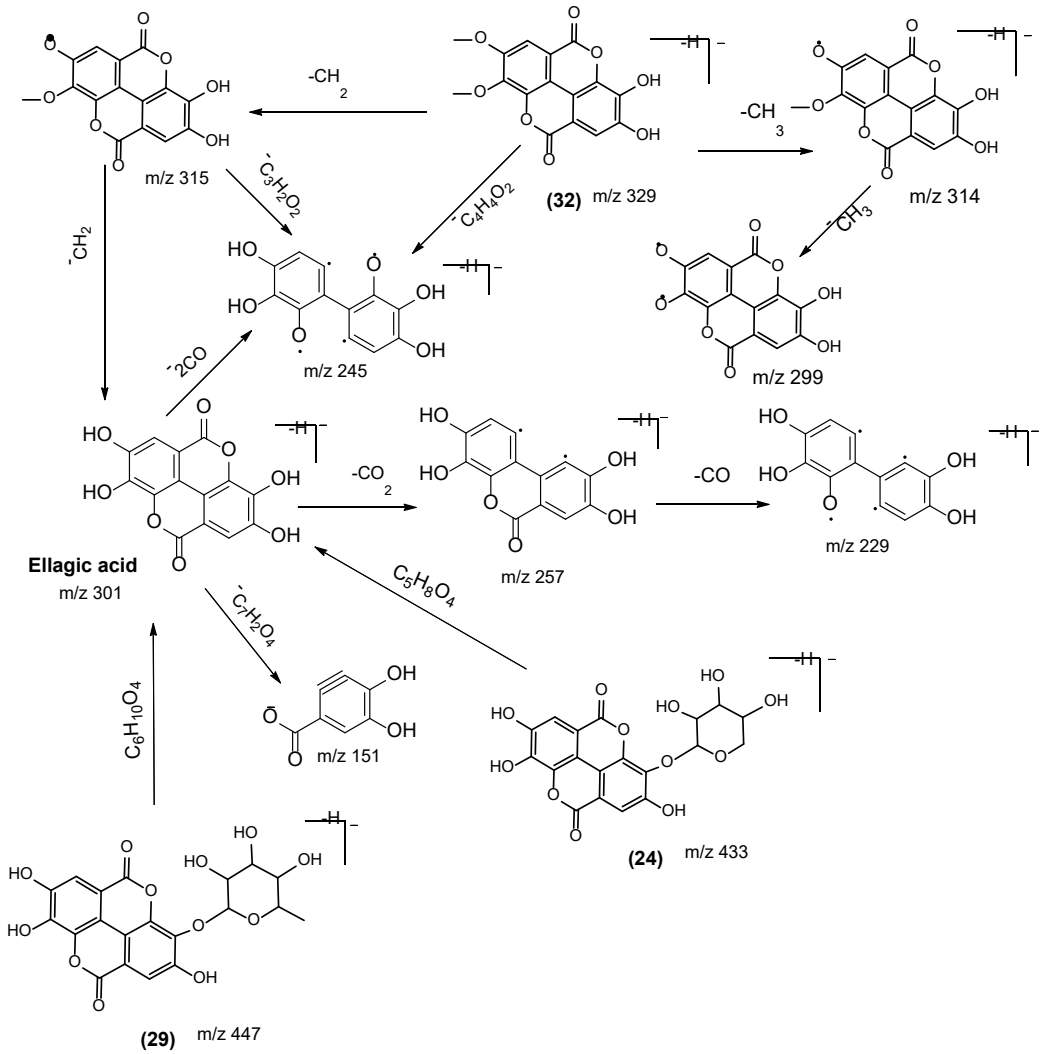
SD1(B). LCMS total ion chromatogram of *Terminalia subspathulata* fruits in negative mode



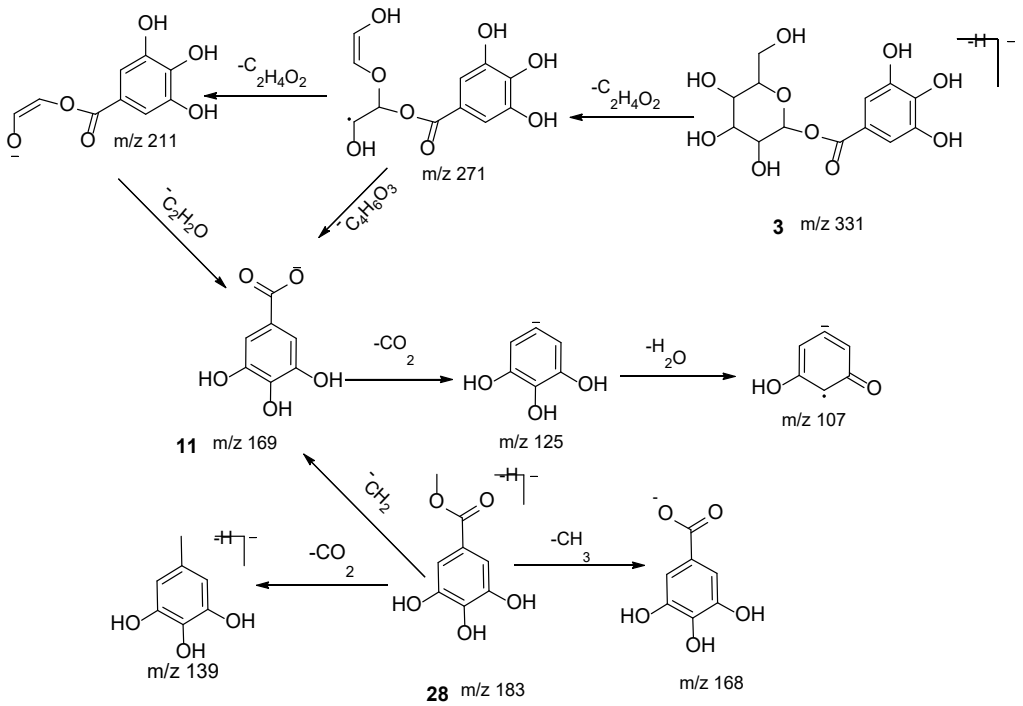
SD2. Proposed fragmentation pattern of HHDP glucose **2** and corilagin **13**



SD3. Proposed fragmentation pattern of punicalagin **5** and punicalin α/β **4**



SD4. Proposed fragmentation pattern of ellagic acid pentoside **24**, ellagic acid deoxyhexoside **29** and 2,3-di-*O*-methylellagic acid **32**



SD5. Proposed fragmentation pattern of gallic acid **11**, glucogallin **3**, and methyl gallate **28**

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Review Article

Bio-Compost Behaviour as Soil Additive by Food Waste Pretreatment on the Growth of *Abelmoschus esculentus* L.: A Systematic Review

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ABSTRACT

Food waste (FW) has always been a significant issue faced by almost all countries worldwide. The rise in FW does not only influence one's food supply, yet the greenhouse gas (GHG) emission such as methane (CH_4) and carbon dioxide (CO_2) gas leads to global warming and health issues. This paper reviews the primary FW treatments available in all countries. Most advanced countries have accomplished that the least cost and most efficient FW treatment is composting. Among all the composting methods available, vermicomposting (VC) that uses redworms (*Eisenia fetida*) produces nutrients rich bio-compost, as proven in the existing literature. Furthermore, bio-compost produced by the VC method nourishes plant growth. In this study, the primary research data sources are 78 scientific articles over the last few years. This research is the consensus on VC as the FW treatment. Besides, briefly discuss the FW pretreatment methods, the effect of bio-compost on soil properties, and their corresponding effects on the growth of *Abelmoschus esculentus* L.

Keywords: *Abelmoschus esculentus* L., food waste treatment, pretreatment methods, soil additive, vermicomposting

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INTRODUCTION

Sustainable Development Goals (SDGs), implemented in 2015, are meant to be accomplished by 2030. Among the 17 goals listed, precisely in the 12th target, the concept of food loss and waste management were considered. It stipulates that by 2030, food waste (FW) will be halved globally at both the consumer and trade levels. Besides, the number of food losses throughout the supply chain should be reduced, including losses in post-harvest areas (Gao et al., 2017).

It is due to the FW that significantly contributes to greenhouse gas (GHG) emissions. It accounts for around twenty percent of overall GHG emissions in developing countries (Vinoth Kumar & Kasturi Bai, 2008). Therefore, many treatments have been proposed and conducted to reduce the environmental effects of FW products (Gao et al., 2017). For instance, landfill, composting, incineration, and anaerobic digestion are the common treatment practiced.

Composting is the natural method of decomposition of organic matter that produces bio-compost, increasing soil fertility. Among the other FW treatments, composting can be done in various ways. Aerobic digestion, compost of bin mechanical aerated pile, vermicomposting (VC), vessel composting, and windrow is some of the proven methods (Sundberg, 2005).

VC is a method that can enhance the beneficial use of FW into rich soil amendments and nutrients (Arancon et al., 2003). VC is a composting method that uses red worms for the degradation of foodstuffs into bio-compost. Good quality composts can be produced by VC (Sundberg, 2005). However, redworms have sensitive skin, and these delicate skins can only live under certain circumstances in the environment. Findings stated that acidic conditions might harm the redworms (Pierre et al., 2020).

Food consumption by redworms plays a vital role in producing high-quality bio-compost. Different pH of FW treated by the VC method using redworms produces a different standard of bio-compost. The VC method can also give significant results for bio-compost with different pretreatment methods on FW. Researchers agreed that high-quality bio-compost obtained from VC could improve soil nutrients (Shen et al., 2015).

Hence, this review paper consists of few parts; where the first part of the review is the properties of FW in terms of the pH value followed by the FW pretreatment methods. The next part comprises the FW treatments available. Bio-compost behavior on soil properties is discussed further in the next part of the review. The last part of the review consists of the nutrient required to grow the lady's finger (*Abelmoschus esculentus* L.) by bio-compost as the organic fertilizer.

PROPERTIES OF FOOD WASTE

One of the largest waste generated in Malaysia, which is almost half of the entire volume of waste generated daily, is known as the FW (Arvanitoyannis et al., 2008), according to a study published by

the National Solid Waste Management Department in 2012. The production quality of this amount of FW is about $800 billion where it accounts for almost 7% of the global annual GHG emissions (Slorach et al., 2019).

At sites, approximately 2.29 million tons of wasted food are sent to landfills globally, resulting in around 11 million tons of CO_2 emissions (Hao et al., 2015). Globally, the cost of FW is projected to be approximately USD 2.6 trillion, including a portion of USD 1 trillion is mainly on GHG emissions (Jain et al., 2018). Although consumers are to be blamed, FW also occurs because of distributors, restaurants, and marketers (Gao et al., 2017).

Among the FW from kitchen scrap, fruit peels, or even expiry date food items, it can be signified into strong and weak acidic FW. Strong acid FW is the FW with a pH of 1 to 3, whereas weak acid FW is the FW with a pH of 4 to 6 (Sundberg et al., 2013). Due to the suitable and adequate surroundings for redworms to carry out their composting process, the pH of FW in VC plays a significant role (Boulter et al., 2000). Therefore, suitable pH can increase the quality of bio-compost produced.

Composting of traditional agricultural waste can be enhanced with additives. Additive items such as alkaline minerals, lime, and ash may also contain pH-balancer compounds, which raise compost pH levels (Sasaki et al., 2003). Compost from alkaline items can advertise micro-organism behavior due to potential inhibitory effects due to high alkalinity (Yu & Huang, 2009). When composting wastage, a high acidity stage at the beginning of the process is often possible (Sundberg, 2005).

However, extended low pH condition leads to a problem in FW composting. It has been proven in Scandinavia from past research. The slow decomposition of FW prolongs the composting period (Wong et al., 2009). These are the effects of the dominating acids contained in the FW. The pH values of bio-compost were generally neutral and are provided by the processing of CO_2 and organic acids released during microbial activity (Albanell et al., 1988).

FOOD WASTE PRETREATMENT METHODS

To make the therapy more successful, every treatment performed has its pretreatment process. Pretreatment of the organic substrate was first introduced at the beginning of the 1920s and, owing to the intensive use of energy or chemicals, was regarded as the costliest phase. Study papers relating to pretreatment approaches are known to increase tremendously compared to 1990 results (Yin et al., 2016). Apart from this, pretreatment methods affect bio-compost quality.

Specific pretreatment procedures for solid waste can be used, such as mechanical (ultrasound and lysis), thermal, chemical, and biological pretreatment (Tun & Juchelková, 2019). Thermal and chemical, accompanied by ultrasonic and microwave pretreatment, are also some of the pretreatments in use. However, the pretreatment results of FW are different and depend greatly on the process

for pretreatment and the composition of the substrates (Krishna & Kalamdhad, 2014). All the pretreatment stated falls under physical, chemical, physicochemical, and biological pretreatments.

The approach by pretreatment methods and subsequent bioprocessing approaches can therefore be preferred. Most of the chemical, mechanical, and thermal processes research are concerned (Delgenes et al., 2003). The rest may be based on a synthesis of many approaches (Salihu & Alam, 2016). Pretreatment involving alkaline solution has a higher possibility of affecting the quality of bio-compost due to the pH condition for worms.

Physical Pretreatment

Without any chemical reactions or additional microorganisms, the pretreatment process is called physical pretreatment (Zheng et al., 2014). The purpose of this pretreatment is to minimize the particle size and increases the surface area of FW. It is also known as mechanical pretreatment that reduces the FW particles without generating any products. Perhaps, one of the significant disadvantages is that the process requires energy. Advantages of this pretreatment include less operational costs, high disintegration potential, uses simple operating process, and no transformation in chemical properties involved (Bernstad & la Cour Jansen, 2012). The common operation usually depends on the robustness that acts on the FW surfaces. Some of the common physical pretreatments are discussed below.

Milling Pretreatment. In this technique, the particle size of FW is reduced. Thus, it promotes the disarrangement of the lignocellulosic material crystallinity. The method provides a reduction of the FW that is below 2.0 mm. Hence, it helps in increasing the particle surface area for a continuous hydrolysis method (Paritosh et al., 2017). By implementing this pretreatment method, the processing time has been highly reduced. Also, the amount of water consumed is reduced. However, energy consumption for this method is still high (Seidl & Goulart, 2016).

Ultrasonication Pretreatment. Ultrasonication is one of the efficient in FW pretreatment. It is a technique that uses ultrasonic waves that stimulates pressure deviation (Batista Meneses et al., 2020). This pretreatment is combined many processes that involve chemical reactions, combustion, shearing, and pyrolysis. This combination increases the efficiency of the pretreatment method. Apart from that, Seidl and Goulart (2016) have described that the productivity of this pretreatment truly depends on the properties of FW.

Extrusion Pretreatment. This method mainly focuses on producing objects. FW is forced into a frame with the preferred size and shape. When the FW exits the frame, it usually experiences expansion (Batista Meneses et al., 2020). FW has its effect when this pretreatment method is introduced. For instance, minimization in the size of the material automatically increases its

surface area, and visible transformation in FW occurs. Process efficiency is improved by one of the common ways practiced: combining extrusion methods with any acid or alkaline treatments (Duque et al., 2017).

Thermal Pretreatment. One of the fastest pretreatment methods is thermal pretreatment. Another significant gain is that solid FW is sterilized so that unwanted microbes that may impact methane (CH_4) or hydrogen rates are deactivated. Thermal pretreatment is the most general method of application in which biomass substrates are solubilized by heat. It was used at 50 to 270 ℃ at temperatures for a long time to boost organic particulate disintegration (Deepanraj et al., 2017). Results indicate that the solubility and biodegradability of organic compounds are considerably improved by heat pretreatment.

The pH control can further boost the performance and better understanding of FW thermal pretreatment. During external auxiliary energy sources, the dewatering option is called thermal drying (Ragazzi et al., 2007). Gandhi et al. (2018) investigated the effect of thermal pretreatment on kitchen waste at various temperatures (37 °C, 50 °C, and 60 °C) to study the influence on hydrolysis. Therefore, pretreatment at 60 °C was shown to be beneficial, with a total chemical oxygen demand (TCOD) removal rate of 79.2%. In addition, oven drying makes the dehydration process efficient in a short duration (Salihu & Alam, 2016).

Physicochemical Pretreatment

Physicochemical pretreatment has effects on either the physical or chemical properties of FW. Steam explosion pretreatment, liquid hot water pretreatment, supercritical CO_2 explosion pretreatment, microwave pretreatment, and plasma pretreatment are examples of physical-chemical pretreatment.

Steam Explosion Pretreatment. This pretreatment is similar to hydrothermal pretreatment. The FW is briskly heated in a reactor filled with saturated steam at high temperatures and pressure. The FW is kept in the reactor for seconds to several minutes based on the FW properties (Sun et al., 2009). The vapor formed in the reactor penetrates the FW and breaks the chemical bonds. The most significant advantage of this technique is that it is an environmentally friendly method. However, since it is still a developing technology, improvements in reactor design need to be explored. Besides, the operational mode of the reactor and the addition of catalysts enhances the FW pretreatment (Song et al., 2018).

Liquid Hot Water Pretreatment. This pretreatment retains the temperature of the liquid water at 140 °C and 240 °C. The water can easily invade the cell wall and break down the cells at high temperatures (Xu et al., 2019). The benefits are that no chemical additives are introduced in this method. Also, low toxicity compounds are not formed. Besides, a common reactor can be used instead of a corrosion-resistant reactor

due to the low formation of byproducts (Yu & Huang, 2009).

Supercritical Carbon Dioxide Explosion Pretreatment. This pretreatment uses supercritical CO_2 because it is a green solvent that is not flammable and volatile. After the pretreatment, solid-liquid extraction is conducted because the solvent can be removed easily. Typical process variables such as the flow and temperature are to be adjusted to maximize the yields. Besides, combining this method with ultrasonication and extrusion gives better results (Batista Meneses et al., 2020).

Microwave Pretreatment. Microwave pretreatment uses microwaves to irradiate lignocellulosic materials (Kamaruzzaman et al., 2018). This pretreatment method can speed up all the biological, physical, and chemical processes due to the high heat supply and higher collision rate of ions (Batista Meneses et al., 2020). However, this method is strongly dependent on the properties of FW.

Plasma Pretreatment. The addition of ozone (O_3) in this plasma pretreatment helps in the lignin degradation process in FW. In addition, it helps to enhance the following process, which is the hydrolysis process (Kamaruzzaman et al., 2018). This pretreatment is also called ozonation pretreatment. Plasma produces highly reactive compounds that can degrade cellulose into glucose. Thus, this pretreatment can be easily break down the complex structure of lignocellulosic materials (Vanneste et al., 2017).

Biological Pretreatment

Biological pretreatment can be classified as environmentally friendly and requires less capital investment. However, perhaps, this pretreatment is only effective in controlled environmental conditions and requires a longer time for the growth of microbes. Compared with the other methods, the biological methods below usually require low energy and chemical input. However, the longer pretreatment time and the release of odor are some of its main disadvantages. Examples of biological pretreatments are enzymatic pretreatment, microbial consortium pretreatment, and fungal pretreatment (Batista Meneses et al., 2020).

Microbial Consortia. Some advantages of using the microbial consortia method are that it increases functional robustness, has high productivity, and has high stability when biochemically degrading the FW (Batista Meneses et al., 2020). However, by using this method, FW degradation rates and physicochemical changes are easily afflicted. Therefore, it is known as one of the main disadvantages of the method. Those factors are the physical, enzyme activity, chemical, and biological factors (Sukumaran et al., 2005).

Fungal Species Pretreatment. Fungi are known as a type of microorganisms where the cell walls are made up of chitin. Some of

the species are brown rot, soft rot, and white rot. These fungi are characterized based on their capability to break down the structures in different types of FW. One of the major advantages of implementing this method is the low usage of chemical reagents and low energy consumption. However, the disadvantage is that the incubation period is long (Batista Meneses et al., 2020).

Enzymatic Pretreatment. With the help of microorganisms as fungi, protozoa, plants, and bacteria, cellulose of the FW can be easily transformed through cellulases, a synergistic enzyme complex. It is called the hydrolysis of cellulose. Therefore, this is known as enzymatic pretreatment. It also helps in the generation of energy (Sukumaran et al., 2005). Enzyme application has several benefits: low capital investment, energy cost-effectiveness, usage of reagent is less and causes no environmental issues. However, low profit is generated as a longer incubation period is needed for the enzymes to carry out the process.

Chemical Pretreatment

Chemical pretreatment uses chemicals to deviate the chemical and physical properties of lignocellulose in FW. Most researchers are interested in this treatment because of its higher productivity for better bioconversion performance (Nzioka et al., 2016). Some pretreatment classified as chemical pretreatment is alkali pretreatment, acid pretreatment, and ionic liquids pretreatments.

Alkali Pretreatment. The primary purpose of alkali pretreatment is to solubilize lignin in the FW. The commonly used reagents for this pretreatment are potassium, calcium, ammonium, and sodium hydroxides. Sodium hydroxide is known as the most effective reagent among others (Nzioka et al., 2016). Several studies researched the advantages of the alkali pretreatment method.

Alkaline pretreatment using that calcium hydroxide has also been found to be easy and effective. Moreover, calcium hydroxide, $Ca(OH)_2$, is cheap, and the handling method is easy. The FW-alkali ratio must be optimized depending on the solid content and alkaline concentration used for alkaline pretreatment. Ammonia-based alkali pretreatment is famous for its less corrosive, less toxic, and higher recovery rate. Although alkali pretreatment can effectively remove lignin, the major disadvantage is the recovery of the alkaline reagent added.

Acid Pretreatment. During acid pretreatment, hydronium ions can degrade hemicellulose chains into glucose monomers (Lloyd & Wyman, 2005). Organic acids and inorganic acids can be used. Sulfuric acid and hydrochloric acid are examples of inorganic acids, whereas formic acid and oxalic acid are examples of organic acids. Concentrated acids work efficiently at low temperatures, while dilute acids are effective at high temperatures. However, most of the concentrated acids are harmful and have high maintenance costs. Therefore, dilute acids are commonly used in industrial (Baruah et al., 2018).

Ionic Liquids. Ionic liquids are solvents with a melting point of less than 100 ℃, comprised of cations and anions. In this process, the cations and anions break down cellulose and lignin. As a result, the ionic liquids can undergo a recovery process to be used again. Some of the advantages are that these liquids are non-volatile, non-toxic, and the primary benefit is that the cations and anions react based on the properties of the ionic liquids used (Chen et al., 2017). Thus, ionic liquids are often known as green solvents. The ordinary used ionic liquids are the imidazolium salts (Zhang & Matsuto, 2010).

Combined Pretreatment

Combined pretreatment is a combination of more than one pretreatment method. Researches have reported that combined pretreatment has effective results (Salihu & Alam, 2016). However, some of the pretreatments are proved based on the laboratory scale. Thermal-chemical pretreatment is one of the commonly combined pretreatment practices.

Thermal-Chemical Pretreatment. The thermal-chemical pretreatment method is a method that has a combination of chemical and thermal pretreatment. Kullavanijaya and Chavalparit (2020) stated that for thermal-chemical pretreatment, an hour-soaked sample in 3% sodium hydroxide (NaOH) solution is heated in an autoclave at (121 ℃, 103.4 kPa) for half an hour has the highest soluble chemical oxygen demand (SCOD) content. From the study by Kullavanijaya and Chavalparit (2020), the cellulose content of the sample is has degraded. Studies have been found that the best organic matter solubilization was achieved using thermochemical pretreatments when NaOH/L is used as an alkaline agent (Álvarez-Gallego et al., 2015).

Hence, the total solid and volatile solid of the bio-compost sample is decreased. As a result, the water holding capacity and the nutrient content in the soil may be affected. However, ammonium ions (NH_4^+) concentration increases because of the rise in organic nitrogen-containing degradation following thermal-chemical pretreatment (Lee et al., 2019). It is an industry-scale pretreatment that was implemented successfully. The results of thermal-chemical pretreatment depend on the form of the substrate, the reaction temperature, and the time of reaction for FW pretreatment (Salihu & Alam, 2016).

Moreover, for thermal-chemical pretreatments, the rate of biodegradability is like volatile solid solubilization. Thermal-chemical pretreatments help in the breakdown of macromolecules into monomers (Zhang & Matsuto, 2010). It enhances the composting process as the worms can easily consume the FW. However, a variety of pretreatment thermal-chemical conditions has not been explored subsequently. A combination of thermal and chemical pretreatment methods can improve FW degradation, as reported by Lee et al. (2019).

FOOD WASTE TREATMENTS

Proper FW treatment selection benefits the country in the environmental and economic sectors. Anaerobic digestion, composting, incineration, landfilling, and heat-humidity reactions are common FW management methods for handling FW (Gao et al., 2017). Anaerobic digestion (AD) is a valid technology that produces biogas and digestate from the decaying process of organic matter without oxygen. Besides, a landfill is a typical approach with the downside of an enormous property area and a high degree of GHG emission known as CH_4 gas.

Incineration is used to generate heat energy which can significantly minimize waste volume (Gao et al., 2017). However, the incineration process is expensive and involves heavy energy use and technology. On the other hand, heat-moisture reaction treatment can effectively remove bacteria and odors (Gao et al., 2017). Chen et al. (2017) have analyzed the discharge of hazardous trace elements by stimulating the heat-moisture treatments on FW samples. Lastly, composting is referred to as decomposition or the natural process of 'rotting' of organic matter under controlled conditions with the help of microorganisms.

Composting as the Treatment

Composting is known as the most efficient FW treatment, among others. Various researchers have studied and supported this treatment. The number of composting installations has risen since the late 1990s for several reasons (Zhang & Matsuto, 2010). Besides, according to Lim et al. (2016), composting had the lowest cost overall compared to other treatments. Alternative approaches have been discovered in the past few years. However, composting has been the best way to fix the FW disposal affecting the environment globally. Composting is carried out to control and reduce FW in compliance with the SDGs.

Perhaps, composting produces a dark nutrient-rich material from organic matter known as "bio-compost" or "humus" (Nagavallemma et al., 2006). Bio-compost incorporating enhances the soil structure, texture, and tilth (Chauhan et al., 2008). As there are plenty of environmental problems and soil destruction caused by fertilizers and pesticides, bio-composts have developed to be of great significance (Argun et al., 2017). S. S. Kumar (2013) justified that the application of bio-compost is said to promote plant growth and productivity in several crops.

Besides, the production of bio-compost can be an optional method to usual waste disposal methods nowadays and decrease the amount of chemical fertilizer used in agricultural sectors (Ozores-Hampton et al., 2019). The factors that can differentiate for the compost production are the amount of land acquired, the type and quantity of waste to be compost, economic value, and labor force (Apagu, 2012). Some of the common systems at large-scale composting are the windrow method, in-vessel systems, and static pile systems, while small-scale composting systems are compost tumbler, bokashi bin, and VC (Sundberg, 2005).

A summary of the GHG emission rate between AD, composting, and VC is presented in Table 1. The emission rate of CO_2 and CH_4 gas in both AD and VC are higher than composting. It defines that the FW decomposition rate is higher in these two FW treatments compared to composting. Besides, nitrous oxide (N_2O) largely contributes to GHG emissions by destroying stratospheric ozone (Aronson & Allison, 2012). Hence, lower N_2O emission by VC, causing it to be the environmental-friendly treatment that is safe and hygienic (Othman et al., 2012).

Table 1

Summary of greenhouse gas (GHG) emission rate between anaerobic digestion (AD), composting, and vermicomposting (VC)

Emission rate ($mg.m^{-2}h^{-1}$)	AD	Composting	VC	Reference
CO_2	2950	882	1675	Chan et al. (2011)
CH_4	9.54	2.17	4.76	
N_2O	1.59	1.48	1.17	

Note. CO_2 = Carbon dioxide; CH_4 = Methane; N_2O = Nitrous oxide

Vermicomposting is the Effective Method. During certain conditions, a mixture of all these methods can make high-quality compost more efficient. From all these methods above, VC is known as an effective technique in composting. VC is a more environmentally sustainable natural method for FW (Othman et al., 2012). VC is not the decomposition by microorganisms, yet it is an enzymatic deterioration by the earthworm digestive system. VC uses worms to consume FW. Earthworms can consume almost all types of FW, and the amount of food is comparable with their body weight per day. Soil nutrients are rich in the excrete (castings) from the worms.

Earthworms feed on and migrate through the digestive system of biological waste and excrete it in granule shape. Good quality composts can be produced by VC that involves no physical material turning. VC is an excellent way to turn diverse solid residue types into soil additives (Cao et al., 2016). Earthworms enhance the growth of bacteria and actinomycetes through the passage of soil by them. Actinomycetes are produced at a higher rate in the presence of worms (Nagavallemma et al., 2006).

It is widely embraced around the globe, including Asia, Africa, Europe, and North and South America (Arancon et al., 2008). In the United States, potential benefits of VC, such as stabilization, have been extensively publicized (Cao et al., 2016). Besides, there are more than 1800 species of earthworms worldwide (Arancon et al., 2004). Among them, Savigny (*Eisenia fetida*) is known as the common worm

used in VC. It is also known as "manure worm" ,"compost worm", "red wiggler", and "redworm". Redworm is considered one of the toughest worms that can easily adapt to its surroundings.

Redworms are commonly found all around the world, especially on Canadian farms. It can be found easily on piles of manure that are left for a long time. Redworms have rings on their body that is made from circular muscles. The muscle expands, and contracts, making the worms move as they shorten and lengthen the body (Angima et al., 2011). This phenomenon gives them an extraordinary feature as they can move in all directions. In addition, moisture is needed for the lubrication of the worm's motion. All these features well suited them for breaking down organic matter.

Redworms are also known as multifaceted VC earthworms because they can resist high temperatures and has high reproductive rates (Munroe et al., 2007). Besides, their food ingestion rate is high as well. On the other hand, compost worms have some requirements. Firstly, they need an optimum living environment called bedding. Second, they need a constant food supply. Then, moisture content should be controlled by approximately more than half water content by weight percentage. Third, a proper air supply is needed for the worms as living things. Finally, they cannot sustain themselves in extreme temperatures.

BIO-COMPOST EFFECT ON SOIL PROPERTIES

The primary function of bio-compost is to increase soil fertility. Based on the studies and researches regarding the benefits of bio-compost as a soil additive, bio-compost has influenced the soil properties as stated below.

Soil Fertility

Loss and imbalance of organic matter in the soil affect global production in agriculture (Dastpak et al., 2020). Conversely, a rise in organic matter has a significant impact on the soil. Bio-compost from FW provides high nutrient values that minimize the usage of chemical fertilizer to increase soil fertility. The usage of bio-compost has been proved to support plant growth in various studies (Kuang et al., 2012).

Physical Properties

Lower bulk density increases the overall porosity of the soil. It is one of the advantages of bio-compost on soil properties. The increase in soil density and the overall porosity can be achieved by bio-compost which helps lower the soil density and increase the organic matter. Besides, water retention of the soil can be further improved by adding bio-compost to the soil. Bio-compost applied to the soil with increased water capacity can usually prevent the drainage of excess nutrients into nearby soil resources compared to sandy soils with higher porosity. Therefore, bio-compost

can be used as an enhancer for the soil in terms of physical properties and sometimes boost the production of the crops. Thus, soil nutrients and soil properties are improved and preserved with bio-compost (Ahmad et al., 2016).

Chemical Properties

Moisture Content. The moisture content impacts bacteria and prevents useful microorganisms that serve as composters if their moisture content is too poor. However, the anaerobic state creates an unpleasant odor when the humidity content is too high, disrupting the VC process. VC process would be successful at a moisture content between 50 and 60% (Bhat et al., 2017). However, the properties of compost, such as humidity, pH value, and carbon to nitrogen (C: N) ratio, are influenced by the type of FW. The main benefit of bio-compost on soil properties is that it can conserve more water than mineral soil, preventing the plants from wilting quickly. With optimum moisture content, the rate of mineralization and decomposition is faster. Apart from that, bio-compost can simultaneously minimize water and fertilizer usage as bio-compost has higher moisture content with various nutrients (Ozores-Hampton et al., 2019).

Soil Nutrients. Compared to Bhat et al. (2017), VC has increased in all total macronutrients and a drop in total sulfide. Phosphorus (P), nitrogen (N), and potassium (K) were shown to be crucial components of the plant growth and production processes (Iqbal et al., 2012). Magnesium (Mg) minimizes heavy metal stress by adding to plant photosynthesis (Pierre et al., 2020). Traditionally, N has been known as the primary plant nutrient. The proteins building cell and plant tissue are an integral part. Besides, N is also the leading determinant of plant growth of all the primary plant nutrients. Plants lacking N have yellowish leaves and steep growth. Plant growth and crop production typically increase with N content in soils, as it is one of the essential elements in chlorophyll.

Therefore, the increased use of the soil from VC provides a larger amount of N for growth plants (Othman et al., 2012). N continues to increase as the dry mass increase due to the CO_2 emissions, wastewater depletion by evaporation, and nitrogen-fixing operations. The C: N ratio can influence the FW decomposition rate during the composting process (Garg et al., 2012). C is used as the energy supply, whereas N creates proteins during the composting phase using microorganisms. The higher the C: N ratio, the lower the decay process, and N is immobilized throughout the composting period. However, FW has an adequate ratio of C: N that can compost the substances (Wapa et al., 2014).

Soil pH. Bio-compost influences soil pH depending on the soil's original pH and the pH of the bio-compost. Using bio-compost as neutral or slightly alkalizing pH or pH buffer can increase soil acidity, especially in soils of higher concentrations (Othman et al., 2012). In addition, the changes in pH can lead to changes in soil particles' surface

load that affects their ability to link the available nutrients. Therefore, to produce an effective bio-compost, pH is an essential consideration.

Electrical Conductivity. Plant excess salt such as sodium ion (Na^+), magnesium ion (Mg^{2+}), potassium ion (K^+), and chloride ion (Cl^-) have a substantial adverse effect on soil structure. Electrical conductivity (EC) in the soil can be improved by bio-compost. Bio-compost has a high amount of salt, which is proportional to redworms consumption. Over time, EC levels in the soil will decrease because of crop nutrient absorption and leaching. Therefore, the addition of bio-compost can increase the EC level in the soil.

BIO-COMPOST EFFECTS ON THE GROWTH OF *ABELMOSCHUS ESCULENTUS* L.

Vermicompost may have physical and chemical properties that affect plant development and overall morphology in various ways. Vermicompost can be used on a variety of crops, including field and horticultural crops. The use of vermicompost in conjunction with inorganic fertilizers increased crop yields in crops such as potato, rapeseed, groundnut, black gram, rice, mulberry, and marigold (A. Kumar et al., 2018). Positive effects from the addition of vermicompost to the soil in tomato crops have also been reported (Gutiérrez-Miceli et al., 2007). As a result, vermicompost has the potential to be a valuable ingredient in sustainable agriculture.

Abelmoschus esculentus L. is a flu-creating plant of the malevolent family. The most common name of this plant is either okra or Lady's finger (Muqtadir et al., 2019). The plant is grown around the world in tropical, subtropical, and mild temperate areas. Throughout the tropics, it is known as a summer vegetable. Besides, the *A. esculentus* L. is acknowledged as the oldest crop (Gemede, 2015). N is a vital element that helps in the synthesis of useful nutrients and cell development.

The plant growth and pod of *A. esculentus* L. will increase with the supply of N. P enhances fruiting and fruit development and is directly involved in the living mechanisms of a plant. N, P, and K rates and ratios required by the *A. esculentus* L. have not been studied extensively. Zinc (Zn) and boron (B) are some of the main micronutrients needed by the *A. esculentus* L. based on the studies (Sajid et al., 2012). The deficiency of Zn affects the formation of strip tissue on the leaves. The presence of B increases with the decreasing pH value of the soil (Waqas et al., 2018).

Sodium (Na), magnesium (Mg), and calcium (Ca) are also known to be the principal elements in the lady's finger pods. Iron (Fe), manganese (Mn), and nickel (Ni) were also reported to be found on the *A. esculentus* L. (Moyin-Jesu, 2007). Research on *A. esculentus* L. cultivated with varying proportions of municipal solid waste vermicompost (MSWVC) amendment ratios (0%, 20%, 40%, 60%, 80%, and 100%) with agricultural soil was conducted. Based on the analysis, yield and physiological

reactions of *A. esculentus* L. were the lowest at 0% and highest at 60% (Srivastava et al., 2018).

The previous study has found that the use of plant nutrients by VC is improved (Nagavallemma et al., 2006), and it makes the use of water more effective for soil reception (Ellery & Kai, 2010). Gurav and Pathade (2011) suggested that castings by worms will be high content in N, P, K, and other micronutrient based on the FW chosen. Therefore, it is justified that the addition of bio-compost increases the yield of *A. esculentus* L. (Muhammad Firdaus et al., 2018). Plant growth determines the quality of bio-compost in terms of nutrient content. A wide variety of essential nutrients in plants which is not provided by chemical fertilizers can be supplied by good quality bio-compost.

The research indicates that bio-compost may also be used to generate an organic change to soil with strong yields for *A. esculentus* L. (Srivastava et al., 2018). Overall changes in soil, carotenoids, and protein content have been increased substantially with 20%, 40%, and 60% with 65 days with germinations. In addition, VC is proven to enhance the water holding capacity, nutrient profile, and soil microbial response in the lady's finger plant (Weber et al., 2014).

To sum up, bio-compost can nurture a particular plant species. Some essential plant metabolites like N, P, and K in FW are transformed into chemical means that are more accessible for the plants during material transit through the worms of the gut. Furthermore, several valuable nutrients needed by plants have been shown to have been significantly rich in bio-compost (Cao et al., 2016). Thus, the addition of bio-compost increases the plant growth and performance of the fruit produced (Arancon et al., 2003).

CONCLUSION

This review article has accumulated the primary details that can be applied in the research of bio-compost as soil additive by food waste (FW) pretreatment on the *Abelmoschus esculentus* L. growth. Firstly, the advantages of composting FW help reduce waste and act as organic fertilizer. Based on the compost methods reviewed above, the vermicompost (VC) method is the most convenient composting method for this research. This method requires less labor and produces high-quality compost compared to others. Bio-compost produced by VC can be an alternative to chemical fertilizer which is pricier. Pretreatment methods on the FW provide effective results during VC.

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Phytochemical Analysis and Antibacterial Activities of Sidr Leaf Extract (*Ziziphus spina-christi*) against Pathogenic Bacteria in Aquaculture

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ABSTRACT

The success rate of aquaculture is highly influenced by several factors, including optimum water quality, feed management, and microorganism control. Several microorganisms interfere with the quality of media and fish culture, *i.e.*, fish growth. *Aeromonas* and *Vibrio* are the main pathogenic bacteria that disrupt fish growth and cause mortality. Sidr leaf (*Ziziphus spina-christi*) extract contains phytochemicals that have antibacterial properties. This study aimed to identify the phytochemical components and analyze the effect of Sidr leaf extract on the growth of aquaculture-based pathogenic bacteria. Sidr leaf extract was obtained using ethanol and tested via phytochemical analysis, chemical analysis, prediction of activity spectra for substances (PASS) examination, and inhibition capability against *Aeromonas hydrophila*, *Aeromonas caviae*, *Aeromonas sobria*, *Pseudomonas putida*, *Pseudomonas aeruginosa*, *Streptococcus agalactiae*, *Vibrio vulnificus*, *Vibrio harveyi*, *Vibrio parahaemolyticus*, and *Vibrio alginolyticus*. The results showed that Sidr leaf extract contained phytochemicals, namely, flavonoids, alkaloids, saponins, tannins, and steroids. Gas chromatography-mass spectrometry analyses showed that the Sidr leaf extract contained 30 compounds with antiseborrheic effects. PASS analysis demonstrated that 15 compounds (64.51% level) have potential as antibacterial, with

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a probability activity value of more than 0.300. The inhibition test showed that the Sidr leaf extract exhibited moderate-to-strong inhibition against pathogenic bacterial growth, except for *V. vulnificus*, for which it produced a weak inhibition. The results indicate that Sidr leaf extract can be used as a natural herb to control bacterial pathogens in fish cultivation.

Keywords: *Aeromonas*, antibacterial, aquaculture, Sidr leaf extract, *Vibrio*

INTRODUCTION

Aquaculture plays an essential role in the Indonesian economy, and its production increases annually. Fish production from the marine culture in 2016 reached 9,773,055 tons and increased to 9,808,494 tons in 2017, and it is projected to reach 12 million tons in 2023 (Statistics Indonesia [BPS], 2020). This target projection was launched to meet both domestic and export needs. Tran et al. (2017) stated that there is a continuous increase in international market demand. Therefore, considerable effort should be exerted to achieve production and fulfill food safety and food security.

One of the efforts to increase aquaculture fish production is to avoid antibiotics while improving fish culture and survival rate. Feeds with good nutrition can also provide well-maintained fish growth (Prabu et al., 2017). Microorganisms in fish farming grow naturally and, in several cases, are intentionally added to maintain water quality and fish survival. However, several organisms interfere with fish growth through infection and cause mortality to cultured fish (Bentzon-Tilia et al., 2016). The pathogenic bacteria that frequently disrupt fish culture are generally members of *Aeromonas* and *Vibrio*. In *Aeromonas*, fish infected include *A. sobria*, *A. caviae*, and *A. hydrophila*, whereas those from vibrios comprise *V. vulnificus* and *V. harveyi* (Atujona et al., 2018; Monteiro et al., 2018; Pan et al., 2017). Several antibiotics, including tetracycline, oxolinic acid, and florfenicol, have suppressed pathogenic bacteria. However, these antibiotics leave a residue, and certain fish are resistant to antibiotics. The improper utilization of antibiotics in aquaculture can result in multidrug-resistant bacteria in media culture (Igbinosa et al., 2017). *Aeromonas* isolated from fish farming in Denmark has shown 69% resistance to oxytetracycline, 20% resistance to oxolinic acid, and resistance to florfenicol for several isolates. Antibiotic-resistant bacteria can spread and infect humans (Monteiro et al., 2018). Therefore, minimizing the use of antibiotics is needed to prevent the development of resistant bacteria. The addition of microalgae (*Spirulina platensis* and *Chorella vulgaris*) to aquafeed was reported by Joshua and Zulperi (2020) to improve fish immunity. Other natural antibacterial sources have been used in conventional medicine, such as *Ziziphus* (Al-Mutairi et al., 2016), and may prove useful to preventing disease outbreaks and supporting sustainable aquaculture.

The Sidr plant (*Ziziphus spina-christi*) is a tropical plant that has been used as

herbal medicine to treat fever, dandruff, eye diseases, and inflammation. The studies show that Sidr leaves have antibacterial properties as demonstrated by the ability to inhibit the growth of *Escherichia coli*, *Klebsiella* spp., *Pseudomonas aeruginosa*, and *Salmonella* sp. (Al-Mutairi et al., 2016). Ethanol- and methanol-derived Sidr leaf extracts can also inhibit the growth of various Gram-positive and Gram-negative bacteria (Khaleel et al., 2019; Temerk et al., 2017). These effects are due to the phytochemical contents of Sidr leaves, such as alkaloids, flavonoids, and saponins (Asgarpanah & Haghighat, 2012). Sidr leaves also contain phenols with a lethal concentration 50 (LC_{50}) concentration of 21.40 µg/mL. These serve as a source of antioxidants for pharmaceuticals (Khaleel et al., 2019). Based on their phytochemical content, Sidr leaves can be a source of natural antibiotics in aquaculture. Such a natural source would be beneficial because it can reduce antibiotic residues in cultured products and bacteria resistance in the aquaculture environment. This study aimed to determine the phytochemical compounds and the performance of Sidr leaf extract to control various bacterial pathogens in aquaculture *in vitro*.

MATERIALS AND METHODS

Materials

Dry Sidr leaves (*Ziziphus spina-christi*) were obtained from Sukoharjo District, Central Java, Indonesia. Aquatic pathogenic bacteria (*A. hydrophila*, *A. caviae*, *A. sobria*, *P. putida*, *P. aeruginosa*, and *S. agalactiae*) were isolated from tilapia from Magelang, Central Java, Indonesia. Additionally, *V. vulnificus*, *V. harveyi*, *V. parahaemolyticus*, and *V. alginolyticus* were obtained from the Center for Brackish Water Cultivation Fisheries Jepara, Central Java, Indonesia. The bacteria were cultured on trypticase soy agar, tryptone soya broth, nutrient broth, and nutrient agar (Merck, Germany).

Preparation of Sidr Leaf Extract

Sidr leaf extract was prepared according to Al-Mutairi et al. (2016) with several modifications. A total of 200 g dried Sidr leaves were sieved using a 60-mesh sieve. The resulting Sidr leaf powder was then immersed in 96% ethanol at a ratio of 1:10 (vol/vol) and then ultrasonicated for 1 hr. The powder was then macerated for 24 h and filtered using Whatman No. 1 filter paper with a vacuum pump to speed up the process. The aqueous filtered part was evaporated in a rotary evaporator at a temperature of 45°C–60°C for 2 hr until the extract thickened. The extract was stored at ±5°C for phytochemical and antibacterial tests.

Phytochemical Analysis of Sidr Leaf Extract

Phytochemical analysis was conducted to identify the compounds in Sidr leaf extract, including flavonoids, tannins, alkaloids, saponins, and steroids. The phytochemical procedure was performed according to Temerk et al. (2017). The flavonoid test

was conducted by adding 1 mL of 10% sodium hydroxide (NaOH) to a 3 mL extract. A yellow extract color exhibited the presence of flavonoids. The tannin test was conducted using one to two drops of 1% iron (III) chloride ($FeCl_3$) to 1 mL extract. A positive reaction showed a color change to green-black or dark-blue color. The alkaloid test was conducted by adding 1 mL of 1% hydrogen chloride (HCl) to 3 mL extract in a test tube. The mixture was then heated for 20 min, chilled, and filtered. Two drops of Mayer's reagent were then added to 1 mL of extract. A thick residue indicated the presence of alkaloids. The saponin test was conducted by shaking a 2 mL aliquot in a test tube for 2 min. A foam formation indicated a positive reaction. Finally, the steroid test was conducted by adding five drops of concentrated sulfuric acid (H_2SO_4) to 1 mL extract in a test tube. The appearance of a red color indicated the presence of steroids.

Chemical Component Analysis of Sidr Leaf Extract with Gas Chromatography-Mass Spectrometry (GC-MS)

Phytochemical analysis with GC-MS was conducted according to Ads et al. (2017). A total of 1 µL sample was injected into the GC-MS equipment (GC17A MSQP 5000 Shimadzu, Japan) using a TG-SQC column 15 MX (0.25 mm × 0.25 µm) at an injector temperature of 250°C. The oven temperature was set to 50°C and increased to 150°C with an average increase of 7°C/min. Then, heating was continued until 250°C with an average rate of 5°C/min and to 290°C with an average rate of 10°C/min. The extract was injected in split mode. The results were then matched with the mass spectra peaks in the Wiley library (Stein et al., 2011).

Prediction of Activity Spectra for Substances (PASS)

PASS analysis was conducted according to Parasuraman (2011) using the PASS web tool. The PASS web tool interprets active biological spectra with a two-dimensional structure and predicts probability to be active and probability to be inactive (Pa: Pi) ratio. The research was conducted in two phases: (1) accessing the Pub Chem server (https://pubchem.ncbi.nlm.nih.gov/) to obtain the canonical simplified molecular-input line-entry system (SMILES) information and (2) predicting the biological activity via PASS analysis using the website, http://www.way2drug.com/PASSOnline/index.php, by entering the canonical SMILES structure (Riyadi et al., 2020).

Inhibition Test

The inhibition test was conducted according to Motamedi et al. (2014) using the disk method. As much as 0.1 mL cultured bacteria with 10^8 cfu/mL density were obtained and spread onto an agar plate. Sterilized 6-mm-diameter paper disks were immersed in various Sidr leaf extracts prepared using distilled water (100, 300, 500, and 800 mg/L) for 1 hr. Additionally, tetracycline (10 mg/L) was used as the positive control, and distilled water was

used as the negative control. The paper disks were then placed onto inoculated pathogenic bacterial agar and incubated for 24–48 hr. The clear zone produced around the paper disk was measured as the inhibition zone. The test was conducted in triplicate at each extract concentration.

RESULTS AND DISCUSSION

Phytochemistry of Sidr Leaf Extract and Their Properties

The phytochemical screening tests (Figure 1) showed that the Sidr leaf extract contained secondary metabolite compounds, such as flavonoids, alkaloids, saponins, tannins, and steroids.

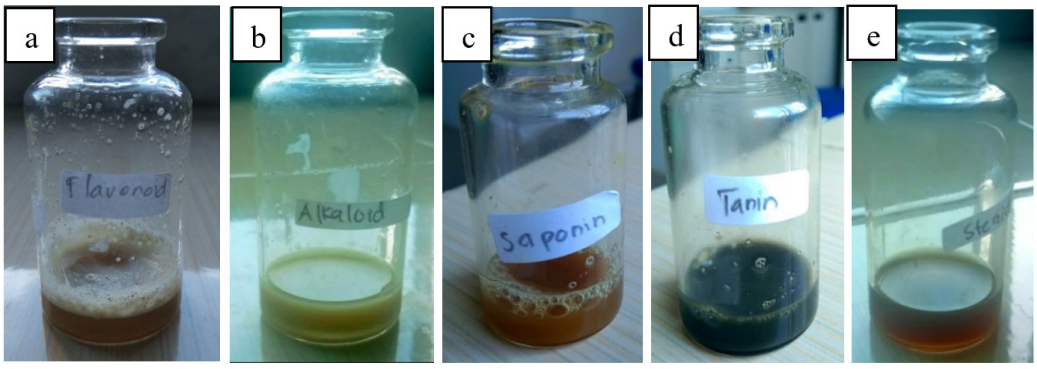

Figure 1. Phytochemicals contained in Sidr (*Ziziphus spina-christi*) leaf extract

Note. a) flavonoid, b) alkaloid, c) saponin, d) tannin, and e) steroid

Table 1

Phytochemicals identified in Sidr (Ziziphus spina-christi) *leaf extract*

No.	Compound	Color	Result analysis
1.	Flavonoid	Yellow	+ (Positive)
2.	Alkaloid	Thick sediment	+ (Positive)
3.	Saponin	Bubble	+ (Positive)
4.	Tannin	Blueish green color	+ (Positive)
5.	Steroid	Red color	+ (Positive)
6.	Triterpenoid	Brownish red	+ (Positive)

Flavonoids, phenolic compounds, triterpenic acids, and polysaccharides have been reported as the major phytoconstituents of *Zizyphus* species (Soni & Malik, 2021). However, various studies have reported different phytochemical contents in *Z. spina-christi* leaf extracts. According to Asgarpanah and Haghighat (2012), Sidr leaf phytochemical content consists of saponins, alkaloids, and flavonoids.

Different results are shown by Ibrahim et al. (2015), who found alkaloids to be the significant phytochemical of Sidr leaf from Nigeria. Ermias et al. (2011) and Alhassan et al. (2019) found that steroid, flavonoid, tannin, lipid, anthraquinone, saponin, and alkaloid are present in *Z. spina-christi* leaf extracts. By contrast, Suliman and Mohammed (2018) found that the phytochemical components of Sidr leaf from Sudan do not contain steroids. Furthermore, Taghipour et al. (2020) stated that the ethanol extract of Iranian Sidr leaf contains alkaloids, tannins, saponins, and flavonoids. This variation in phytochemical content is likely due to differences in climate and environmental conditions where the plant grows. Environmental and climatic conditions, especially temperature, soil type, and age of the plant, affect herbs' chemical content and functional properties (Gull et al., 2012; Inbathamizh & Padmini, 2013). Mbunde et al. (2018) also showed that the areas where herbs grow, such as the coast, highlands, and mountains with different soil types, cause differences in phytochemical content such as phenolics and flavonoids.

The phytochemical components of Sidr leaf extract (Table 1) can have antibacterial properties. For example, flavonoids have antibacterial, antioxidant, and anti-inflammatory properties (Adamczak et al., 2020). Alkaloids possess antibacterial and antifungal properties. Saponins function as barriers against pathogenic bacteria, improve immunity, and have antibacterial, antioxidant, anticancer, and antidiabetic activities. Tannin is a compound that binds proteins and forms water-insoluble compounds. Tannins, as an antibacterial, bind proteins in the bacterial cell wall and coagulate the materials to inhibit bacterial growth (Dangoggo et al., 2012; Ravi et al., 2016). Steroids, consisting of cholesterol and ergosterol, are components of cell membranes. Fusidic acid is a steroid compound that can prevent Gram-positive and Gram-negative bacterial infections (Doğan et al., 2017).

Sidr Leaf Compound Components and PASS Results

Figure 2 and Table 2 describe the GC-MS analysis results on Sidr leaf compounds.

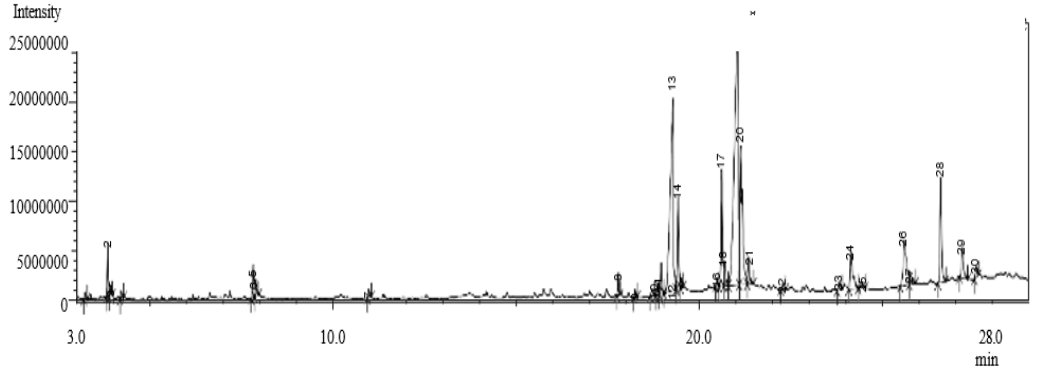

Figure 2. GC-MS chromatogram of the Sidr (*Ziziphus spina-christi*) leaf extract

Table 2

*Phytochemical compounds identified in Sidr (*Ziziphus spina-*christi) leaf extract obtained using chemical component analysis of Sidr leaves via GC-MS*

Peak#	Retention time	Area	Area (%)	Name
1	3.245	1426541	0.23	1-butanol, 3-methyl-
2	3.860	11016330	1.79	Pyrrolidine-.alpha.,.alpha.,.alpha.',.alpha.'-d4
3	3.940	3423281	0.56	(O-D) ethanol ethen-2-d-ol(E) CAS
4	4.226	2301742	0.37	N-methoxy formamide
5	7.826	9238879	1.50	1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane-6,7-endo,endo-diol
6	7.895	5171975	0.84	1,3,3-trimethyl-2-oxabicyclo[2.2.2]octan-6,7-endo,endo,diol
7	10.979	2836928	0.46	Methanamine, N-[3-methyl-2-butenylidene]
8	17.792	4892173	0.79	Neophytadiene
9	18.250	1847442	0.30	3,7,11,15-tetramethyl-2-hexadecen-1-ol
10	18.716	2585580	0.42	Hexadecanoic acid, methyl ester
11	18.869	2749748	0.45	n-hexadecanoic acid
12	18.954	8944771	1.45	n-hexadecanoic acid
13	19.276	112224191	18.23	n-hexadecanoic acid
14	19.422	24469703	3.98	Hexadecanoic acid, ethyl ester (CAS)
15	19.510	2191255	0.36	Farnesyl acetate 2
16	20.474	1807268	0.29	11-octadecenoic acid, methyl ester
17	20.611	31375266	5.10	2-hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-(CAS)
18	20.690	7944588	1.29	14-pentadecenoic acid
19	21.033	194401927	31.58	cis-9-hexadecenal
20	21.119	69774864	11.34	Ethyl oleate

Table 2 *(Continued)*

Peak#	Retention time	Area	Area (%)	Name
21	21.345	8269672	1.34	Heptadecanoic acid, ethyl ester (CAS)
22	22.287	2009621	0.33	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester (CAS)
23	23.822	2855504	0.46	Di-(9-octadecenoyl)-glycerol
24	24.133	18521526	3.01	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester (CAS)
25	24.440	1983710	0.32	1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester
26	25.586	28747798	4.67	9-tetradecenal, (Z)-
27	25.760	5323334	0.86	Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester (CAS)
28	26.583	31995999	5.20	Squalene
29	27.172	11436638	1.86	Behenic alcohol
30	27.553	3734209	0.61	Solanesol
		615502463	100.00	

Based on the GC-MS analysis, the Sidr leaf extract contained 30 chemical compounds (Table 2). These 30 chemical compounds comprised the following: 1-butanol, 3-methyl; optical density (OD) ethanol; pyrrolidine; n-methoxy formamide; two kinds of 1,3,3-trimethyl-2-oxabicyclo [2.2.2] octane-6,7-endo, endo-diol; methenamine, N-[3-methyl-2-butenylidene]; neophytadiene; 3,7,11,15-tetramethyl-2-hexadecen-1-ol; hexadecanoic acid, methyl ester; three kinds of n-hexadecanoic acid; farnesyl acetate 0.4780.4370.807; 11-octadecenoic acid, methyl ester; 2-hexadecen 3,7,11,15-tetramethyl-[R-[R*,R*-(E)]]-0.5490.4170.736; 14-pentadecenoic acid; cis-9-hexadecenal; ethyl oleate; heptadecanoic acid, ethyl ester; hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethaned iyl ester; di-(9-octadecenoyl)-glycerol; hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester; 1,2-benzene dicarboxylic acid, mono (2-ethylhexyl) ester; 9-tetradecenal, (Z)-; octadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester; squalene; behenic alcohol; and solanesol. Based on chemical analysis, two major components were contained in large quantities, *i.e*., n-hexadecanoic acid (31.58%) and cis-9 hexadecenal (18.23%). By contrast, Moustafa et al. (2016) found that the main chemical compounds of *Z. spina-christi* leaf extract are n-hexadecanoic acid, tetradecanoic acid, and cis-vaccenic acid, and Abu-Raghif et al. (2017) identified considerable amounts of n-hexadecanoic acid and the flavonoid 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-. These different results are likely due to differences in the growing environment (Inbathamizh & Padmini, 2013). However, these studies have something in common. They contain n-hexadecanoic acid in the chemical composition of Sidr leaf, and n-hexadecanoic acid has antioxidant, anticancer, and antiinflammatory properties (Aparna et al., 2012; Mazumder et al., 2020).

The chemical compounds in the Sidr leaf extract can act as antibacterial agents. The mechanism for inhibiting microbial growth involves the destruction of the bacterial cytoplasm cell membrane's integrity and triggering intracellular leakage (Bouyahya et al., 2019). This phenomenon is achieved by accumulating hydrophobic groups on phospholipids, causing cell death (Huang et al., 2019; Kinnunen et al., 2012). Phenolic components can inhibit cell DNA and RNA synthesis (Ulanowska et al., 2006). Additionally, the terpenoid group can damage cell membrane efficacy and interfere with microbial growth (Wu et al., 2016). The most significant antibacterial compound detected in the Sidr leaf extract was cis-9 hexadecenal (31.58%). Pyrrolidine, neophytadiene, farnesyl acetate, and squalene were also detected. Cis-9 hexadecenal is a family of agrochemicals used to prevent and eradicate pests (Mensah-Agyei et al., 2020). Pyrrolidine is an alkaloid class compound known to function as an antifungal and antibacterial agent (Thawabteh et al., 2019). Neophytadiene and farnesyl acetate are members of the terpenoid group. Neophytadiene is an

antimicrobial, antioxidant, antipyretic, and anti-inflammatory agent (Swamy et al., 2017). Neophytadiene, together with sitosterol components, can inhibit the growth of *Vibrio parahaemolyticus* (Raman et al., 2012).

Meanwhile, farnesyl acetate is a volatile component that can inhibit the growth of Gram-positive bacteria, namely, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Micrococcus luteus* (Boussaada et al., 2008). Squalene, a steroid class member, detected in the Sidr leaf extract, is also an antioxidant and antibacterial agent that can inhibit *Pseuduomonas aeruginosa* (Awang-Jamil et al., 2021).

The fatty acids detected in the Sidr leaf extract were saturated fatty acids (25.39%) consisting of hexadecenoic acid and octadecanoic acid, whereas the unsaturated fatty acids (11.63%) included ethyl oleic and 11-octadecanoic acids. Fatty acids can also have antimicrobial activity. Fatty acids with an atomic C length less than or equal to 6 can inhibit the growth of Gram-negative bacteria (Yoon et al., 2018). Fatty acids with C atoms between 10 and 12 can inhibit yeast growth, whereas those with a C atomic length of more than 12 can inhibit the growth of Gram-positive bacteria (Potocki et al., 2021; Yoon et al., 2018). Additionally, oleic fatty acid acts as a bactericidal against the development of mycobacteria and *S. aureus* (Orhan et al., 2011). Stearic acid and palmitic acid also have an inhibitory effect but are not as significant as oleic acid (Ababutain et al., 2019).

PASS analysis was applied to understand further the potential antimicrobial role of the compounds mentioned above in combating bacterial pathogens in aquaculture (Table 3).

Table 3

PASS analysis of compounds identified in Sidr leaf (Ziziphus spina-christi) *extract*

No.	Compound	Amount (%)	Antibacterial	
			Pa	Pi
1	1-butanol, 3-methyl-	0.23	0.298	0.061
2	Pyrrolidine	1.79	0.379	0.003
3	(OD) ethanol	0.56	ND	ND
4	N-methoxy formamide	0.37	0.424	0.025
5	1,3,3-trimethyl-2-oxabicyclo [2.2.2] octane-6,7-endo, endo-diol	1.50	0.470	0.019
6	1,3,3-trimethyl-2-oxabicyclo [2.2.2] octane-6,7-endo, endo-diol	0.84	0.470	0.019
7	Methanamine, N- [3-methyl-2-butenylidene]	0.46	0.372	0.037
8	Neophytadiene	0.79	0.363	0.040

Table 3 *(Continued)*

No.	Compound	Amount (%)	Antibacterial	
			Pa	Pi
9	3,7,11,15-tetramethyl-2-hexadecen- 1-ol	0.30	0.417	0.026
10	Hexadecanoic acid, methyl ester	0.42	0.263	0.076
11	n-hexadecanoic acid	0.45	0.300	0.060
12	n-hexadecanoic acid	1.45	0.300	0.060
13	n-hexadecanoic acid	18.23	0.300	0.060
14	Acid	3.98	0.186	0.022
15	Farnesyl acetate0.4780.4370.807	0.36	0.437	0.023
16	11-octadecenoic acid, methyl ester	0.29	0.298	0.061
17	2-hexadecen 3,7,11, 15-tetramethyl- [R- [R *, R * - (E)]] - 0.5490.4170.736	5.10	0.417	0.026
18	14-pentadecenoicacid	1.29	0.356	0.042
19	cis-9-hexadecenal	31.58	0.400	0.030
20	Ethyl oleate	11.34	0.270	0.073
21	Heptadecanoic acid, ethyl ester	1.34	0.186	0.022
22	Hexadecanoic acid, 1- (hydroxymethyl) -1.2-ethaned iyl ester	0.33	0.277	0.069
23	Di- (9-octadecenoyl)–glycerol	0.46	0.340	0.046
24	Hexadecanoic acid, 2-hydroxy-1- (hydroxymethyl) ethyl ester	3.01	0.295	0.062
25	1,2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester	0.32	0.309	0.057
26	9-tetradecenal, (Z) -	4.67	0.400	0.030
27	Octadecanoic acid, 2- hydroxy-1- (hydroxymethyl) ethyl ester	0.86	0.295	0.062
28	Squalene	5.20	0.295	0.062
29	Behenic alcohol	1.86	0.225	0.008
30	Solanesol	0.61	0.424	0.025

Note. Pa = Probability to be active ; Pi = Probability to be inactive

Fifteen compounds (64.51% levels) with a probability to be active (Pa) value of more than 0.300 were found, indicating that they possess antibacterial activity. The bioactive compounds detected were as follows: 1,3,3-trimethyl-2-oxabicyclo [2.2.2] octane-6,7-endo, endo-diol (Pa: 0.470); farnesyl acetate 0.4780.4370.807 (Pa: 0.437); N-methoxy formamide (Pa: 0.424); and solanesol (Pa: 0.424). Interestingly, cis-9-hexadecenal (palmitic acid), a main chemical compound in Sidr leaf, also has antimicrobial activity (Pa: 0.400). Previously, Kumar and Rajakumar (2016) found cis-9-hexadecenal to have anti-inflammatory, nematicide, pesticide, lubricant, antiandrogenic, flavor, hemolytic 5-alpha reductase inhibitor, antioxidant, and hypocholesterolemic properties.

Pseudo-peptide pyrrolidinedione natural products, namely moiramide B and andrimid, represent a new class of antibiotics that target bacterial fatty acid biosynthesis (Pohlmann et al., 2005). N-methyl formamide isolated from the red algae *Portieria hornemannii* (Lyngbye) P.C.Silva is effective against two plant pathogenic bacteria (Sivakumar et al., 2017). Additionally, Chen et al. (2007) showed that solanesol has significant inhibitory effects against *E. coli*, *Mycobacterium phlei*, *P. aeruginosa*, and *S. aureus* but poor inhibitory effects against *Bacillus circulans* and *Bacillus subtilis*. The PASS forecasting tool was used to estimate Pa:Pi (active, inactive ratio) at the forecast thresholds of Pa > 30%, Pa > 50%, and Pa > 70% according to Parasuraman (2011). The forecast's average accuracy was approximately 95% based on the leave-out cross-validation calculation. The PASS forecast accuracy depends on detailed knowledge of the spectrum of biological activity for each compound available in the PASS training package. Thus, the estimation of biological activity is precise (Filimonov et al., 2014).

Inhibition Test of Sidr Leaf Extract against Several Aquaculture Pathogenic Bacteria

In vitro testing showed that the Sidr leaf extract can be used to control several aquaculture-based pathogenic bacteria. Table 4 and Figure 3 show that the Sidr leaf extract at a concentration of 500 ppm was almost as effective as tetracycline, especially for *A. hydrophila*, *A. sobria*, *S. agalactiae*, and *V. parahaemolyticus*. By contrast, the extract was less effective at controlling *A. caviae*, *P. aeruginosa*, and *V. vulnificus*.

Table 4

*Average inhibition zone size caused by Sidr leaf (*Ziziphus spina-christi*) extract when exposed to pathogenic bacteria found in aquaculture settings*

Pathogenic bacteria	Average inhibition zone (mm)				
	Tetracycline (mg/L)	100 (mg/L)	300 (mg/L)	500 (mg/L)	800 (mg/L)
Aeromonas hydrophila	15.20	2.87	9.50	13.17	6.87

Table 4 *(Continued)*

Pathogenic bacteria	Average inhibition zone (mm)				
	Tetracycline (mg/L)	100 (mg/L)	300 (mg/L)	500 (mg/L)	800 (mg/L)
Aeromonas caviae	8.67	6.57	6.20	4.50	6.20
Aeromonas sobria	5.40	13.90	10.43	12.67	4.53
Pseudomonas putida	15.17	4.83	5.87	9.23	9.33
Pseudomonas aeruginosa	12.10	4.83	5.87	5.80	6.07
Streptococcus agalactiae	8.73	11.00	10.97	8.47	7.90
Vibrio vulnificus	12.70	2.87	3.80	2.50	0.00
Vibrio harveyi	18.67	12.20	9.23	0.00	3.43
Vibrio parahaemolyticus	13.00	3.40	7.23	12.37	6.17
Vibrio alginolyticus	12.03	10.67	6.93	3.83	00.00

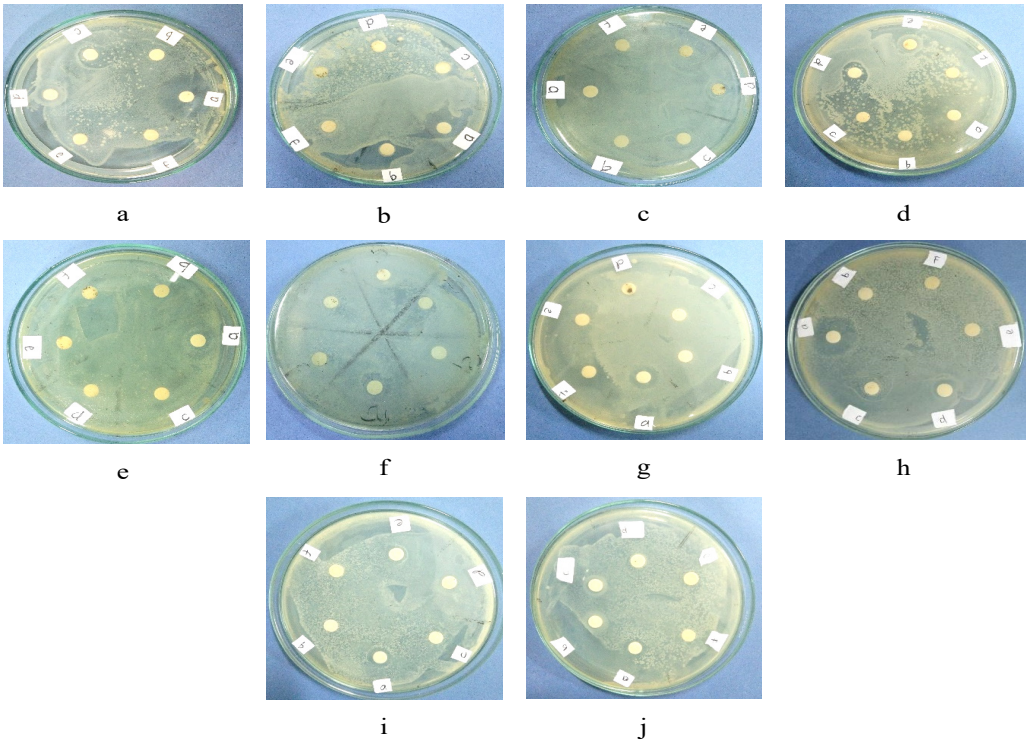

Figure 3. Visual examples of the inhibition zone caused by the Sidr leaf (*Ziziphus spina-christi*) extract when exposed to pathogenic bacteria from aquaculture settings

Note. a) *Aeromonas hydrophila*, b) *Aeromonas caviae*, c) *Aeromonas sobria*, d) *Aeromonas putida*, e) *Pseudomonas aeruginosa*, f) *Streptococcus agalactiae*, g) *Vibrio vulnificus*, h) *Vibrio harveyi*, i) *Vibrio parahaemolyticus*, and j) *Vibrio alginolyticus*

Based on the *in vitro* results, the Sidr leaf extract can inhibit the growth of 10 pathogenic bacteria, with the degree of inhibition ranging from moderate to strong, except for *V. vulnificus* (Table 4 and Figure 3). The 10 bacteria tested were pathogenic to farmed fish and shrimp. *Aeromonas* are pathogenic bacteria that cause diseases in freshwater fish, such as common carp (Baba et al., 2016), catfish (Sarjito et al., 2018a), tilapias, eel, and goldfish (Algammal et al., 2020). Meanwhile, members of *Vibrio* cause a common disease among groupers, such as marine fish (Sarjito et al., 2009), mud crabs (Sarjito et al., 2018b), shrimp (Novriadi, 2016; Sarjito et al., 2018c), and cultured shellfish (Novriadi, 2016). In addition, these pathogenic bacteria can be transmitted to humans through fresh seafood (Praja & Safnurbaiti, 2018).

For inhibition tests, the potency of inhibition is indicated by the zone diameter (strong: 10–20 mm, moderate: 5–10 mm, and weak: less than 5 mm) (Pargaputri et al., 2016). The zone diameter observed from the Sidr leaf extract strongly showed inhibition of *V. parahaemolyticus* and *Aeromonas* bacteria at a dose of 500 mg/L, except for *A. caviae*. Similarly, the Sidr leaf extract strongly inhibited vibrios (*V. harveyi* and *V. alginolyticus*), *A. sobria*, and *S. agalactiae* at 100 mg/L. The Sidr leaf extract produced weak and moderate inhibitions for *V. vulnificus* and *P. aeruginosa*, respectively. Therefore, the inhibition test results indicated that the Sidr leaf extract could be used as a natural antibiotic in fish cultivation.

The antibacterial properties of the Sidr leaf extract are consistent with a previous research report that methanolic extracts from Apocynaceae and Lamiaceae moderately inhibit *A. hydrophila* with an inhibition zone of 9.67 mm (Haniffa & Kavitha, 2012). Additionally, Sidr leaf extract is antibacterial agent that is both bacteriostatic, *i.e.*, able to inhibit the growth of bacteria, and bactericidal, *i.e.*, able to kill bacteria (Abdel-Fatah et al., 2016; Mulyani et al., 2021). For example, Motamedi et al. (2014) showed that Sidr leaf extract is effective against *S. aureus* with the same value of minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) of 8 mg/mL. Al-Mutairi et al. (2016) reported that Sidr leaf extract could inhibit the growth of *P. aeruginosa* with a MIC of 100 mg/L. Mulyani et al. (2021) demonstrated that MIC and MBC of Sidr leaf extract impede *E. coli* with a zone diameter of 11.1 mm were at 50 % and 75 %, respectively. Moreover, Brito et al. (2015) demonstrated that Sidr leaf extract could inhibit several antibiotic-resistant bacterial strains and acts against rhabdomyosarcoma cells (Abu-Raghif et al., 2017). Table 2 shows that the lowest concentration of Sidr leaf extract used in the present study (100 mg/L) could inhibit tested aquatic pathogenic bacteria. Based on these data, it seems likely that Sidr extract is potentially a natural antibiotic in aquaculture. The results in this study are in line with those of Alhassan et al. (2019), Al-Mutairi et al. (2016), Khaleel et al. (2019), and Temerk et al. (2017). The results indicate

that the Sidr leaf extract has the potential as an antibacterial agent. Thus, Sidr leaf extract should be tested as an alternative antibacterial agent to inhibit pathogenic bacterial growth in aquaculture.

CONCLUSION

The Sidr leaf extract contains phytochemicals, namely, flavonoids, alkaloids, saponins, tannins, and steroids, with antibacterial properties. GC-MS analysis showed that the Sidr leaf extract has 30 compounds that function as an antiseborrheic. These compounds include the following: 1-butanol, 3-methyl; (OD) ethanol; pyrrolidine; n-methoxy formamide; two molecules of 1,3,3-trimethyl-2-oxabicyclo [2.2.2] octane-6,7-endo, endo-diol; methenamine, n-[3-methyl-2-butenylidene]; neophytadiene; 3,7,11,15-tetramethyl-2-hexadecen-1-ol; hexadecanoic acid, methyl ester; three kinds of n-hexadecanoic acid; acid; farnesyl acetate 0.4780.4370.807; 11-octadecenoic acid, methyl ester; 2-hexadecen-3,7,11, 15-tetramethyl-[R-[R*, R*-(E)]]-0.5490.4170.736; 14-pentadecenoic acid; cis-9-hexadecenal; cis-9-hexadecenal; ethyl oleate; heptadecanoic acid, ethyl ester; hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester; di-(9-octadecenoyl)-glycerol; hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester; 1,2 benzene dicarboxylic acid, mono (2-ethylhexyl) ester; 9-tetradecenal, (Z); octadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester; squalene; behenic alcohol; and solanesol. PASS analysis demonstrated 15 compounds (64.51% level) having antibacterial activity and a Pa value of more than 0.300. The inhibition test demonstrated that the Sidr leaf extract exhibits moderate-to-strong inhibition to aquaculture pathogenic bacteria, except for *V. vulnificus*, which produced a weak inhibition. These results indicate that the Sidr leaf extract can be used as a natural herb to control bacterial pathogens in fish cultivation.

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Review Article

Efficacy of Anti-termite Extracts from Four Saharan Plants against the Harvester Termite, *Anacanthotermes ochraceus*

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ABSTRACT

This study aimed to examine the anti-termite potential of four Saharan plant extracts, namely, the apple of Sodom or rubber bush, C*alotropis procera*; pergularia, *Pergularia tomentosa*; jimsonweed, *Datura stramonium*, and Egyptian henbane, *Hyoscyamus muticus* from Bechar (southwest of Algeria) on workers of the harvester termite, *Anacanthotermes ochraceus* (Isoptera: Hodotermitidae). A direct contact application test was conducted with five fractions from aqueous extracts of each part of plant species (leaves, stems) using hexane, dichloromethane, ethyl acetate, butanol, and exhausted fraction. A repellent test was realized with aqueous extracts (10%) of plant species leaves and stems. According to the direct contact application test, all tested plants fractions showed termiticidal activities with different degrees. Butanolic fractions presented the best effects from leaves of *C. procera* and *P. tomentosa* with median lethal time (LT_{50}) = 231.03 and 244.96 min, respectively. In the second test, wood samples were exposed to termite attack for four weeks, and the weight loss percentage was determined. The weight loss ranged from 0.034 to 16.90% at concentrations of 10% of plant extracts. The best repellent effect was obtained from leaves of *C. procera* (weight loss = 0.034%) and leaves

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of *D. stramonium* (weight loss = 1.29%). It was concluded that some Saharan plants are a good source of anti-termite compounds, especially *C. procera*.

Keywords: *Anacanthotermes ochraceus*, direct contact application, repellent test, Saharan plants

INTRODUCTION

Termites are one of the most agriculturally important insects (Sahay et al., 2014). Nevertheless, despite some advantages of termites in soil reclamation, they are often called "silent destroyer" (Kamble & Thakor, 2017).

About 2,500 termite species worldwide, and about 10% are economically important as pests and consumption of cellulose-based plant materials (Ranjith et al., 2015). The termites are among the most problematic pests in agriculture and urban area. They cause significant annual crop loss and damage to buildings, especially in semi-arid tropics and sub-humid (Mokeddem et al., 2017). In addition, the harvester termite, *Anacanthotermes ochraceus* (Isoptera: Hodotermitidae), found in North Africa and Western Asia, is highly destructive on wood.

Previously, the control of termites is completely based on chemical products, particularly synthetic insecticides, such as persistent organochloride and organophosphate insecticides (Ranjith et al., 2015).

Indiscriminate use of chemical pesticides to control insect pests has contributed to a number of biological and environmental hazards. These man-created problems have further resulted in phytotoxicity, mammalian toxicity, biomagnification of pesticide residue, insect resistance, insect resurgence, and increased cost of production (Dipendra et al., 2012). Therefore, replacing synthetic insecticides with bio-rational control is universally acceptable and practical (Ranjith et al., 2015).

With the restriction of synthetic chemical use, termite control is currently focusing on various safer strategies. A wide range of toxic plants, repellent, or have some anti-feeding properties are considered as being insecticidal (Dodji et al., 2016).

There has been a growing interest in termite control by using plants that are known to have chemicals that protect them from different insect pests and microorganisms. Insecticides of plant origin have received significant attention due to their effectiveness on many economically important harmful insects and environmental compatibility. In addition, plant products are considered safer and an alternative to toxic synthetic chemicals (Dipendra et al., 2012). Therefore, various plant extracts have been studied for their toxicity, repellency, and attractancy on termites and insect species (Nazeer et al., 2016).

This study is the continuation of work carried out, done in our laboratory. It was demonstrated that the aqueous extracts of four Saharan plant species, namely, the apple of Sodom or rubber bush, *C. procera*; pergularia, *P. tomentosa*; jimsonweed, *D. stramonium*, and Egyptian henbane, *H. muticus*, were effective against *A. ochraceus*

using direct contact application (Bourmita et al., 2013). This investigation aims to evaluate the efficacy of fractions from aqueous extracts from these plants on *A. ochraceus*. In addition, a repellent test was also conducted with these aqueous extracts (10%) to evaluate their full insecticidal effect.

MATERIALS AND METHODS

Plant Materials

The plants collected from the Bechar region, Southwestern Algeria and used in this study were: *Calotropis procera* (Asclepiadaceae) CA04/02, *Pergularia tomentosa* (Asclepiadaceae) CA00/44, *Datura stramonium* (Solanaceae) CA00/50, and *Hyoscyamus muticus* (Solanaceae) CA00/43 (Cheriti, 2002). The plants were identified, and voucher specimens were deposited at the herbarium of Phytochemical and Organic Synthesis Laboratory (POSL), Bechar University, Bechar, Algeria.

Plant Extract Preparations

The leaves of each plant species were dried for seven to fifteen days in the shade at an ambient temperature of 25 to 35 °C. The dried leaves (150 g) were powdered and extracted with 1.5 L of distilled water for 3 hr, and the obtained filtrates were evaporated to remove one-third of water and extracted with four solvents. In a separating funnel, we performed an extraction was performed with solvents. After stirring, each portion of solvent (100 mL) remained in contact with the aqueous phase for 20 min. The operation was repeated four times for each solvent. Five fractions were obtained (hexane, dichloromethane, ethyl acetate, butanol, and exhausted fraction). The extracts were concentrated under reduced pressure at 45 °C, and the obtained residues were stored at 5 °C until use for testing against *A. ochraceus*.

Collection of Termite

The termite, A. ochraceus, was collected on Bechar University (UTMB), Bechar, Algeria, and acclimatized under laboratory conditions 28 to 30 °C and 70 to 80% relative humidity in total darkness. The captured termites were immediately separated from the deadwood and placed inside a room containing wooden sticks as a food source.

Biological Tests

Direct Contact Application Method. Whatman No.1 filter papers were soaked with 400 µL of each extract (dilution: 10%) and put with ten workers of *A. ochraceus* in Petri dishes (90 mm diameter). Filter papers were given as a sole source of food for insects. Filter papers soaked with 400 µL of distilled water were used as control. The mortality was calculated every 30 min for 24 hr.

Determination of Survival Duration

The average percent mortality of termite was corrected by using Schneider-Orelli's formula (Schneider-Orelli, 1947) before subjected subjecting it to regression analysis as in Probit analysis by Finney (1971) for

determination of the median lethal time, LT_{50} values, as in Bourmita et al. (2013). Schneider-Orelli's corrected mortality formula was as follows:

$$M_c = \frac{M_2 - M_1}{100 - M_1} X100$$

where,

M_C = Corrected mortality (%)
M_2 = Mortality in the treated population (%)
M_1 = Mortality in the control population (%)

Repellent Test (Weight Loss)

The test was performed to check the repellent effect of each aqueous extract from leaves and stems of four plant species. The test was carried out by using wood pieces treated with different extracts. A sufficient number of white wood pieces were cut and well dried, and weighed. All samples have constant dimensions (20 x 5 x 2 cm).

The wood samples were treated with 10% aqueous extracts dilution for 1 hr in plastic bottles. The treated wood samples were air-dried and implanted in termite-infested areas (Lahmar region, southwest of Algeria). They were exposed to termite attacks for four weeks (López-Naranjo et al., 2014), then collected, cleaned, dried, and weighed to determine the weight loss (Figures 1 and 2).

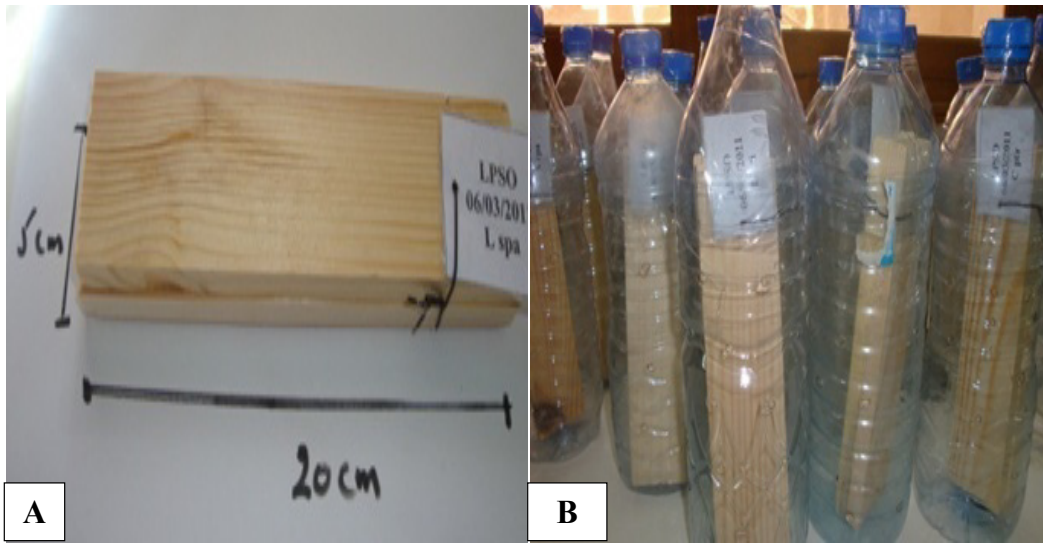

Figure 1. (A) Dimension of wood sample; (B) Samples treated with 10% aqueous extracts for 1 hr in plastic bottles

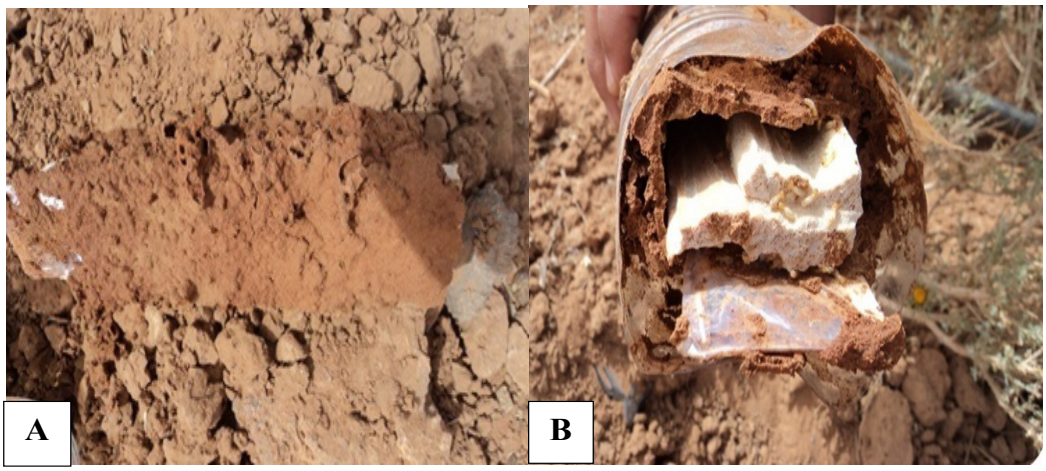

Figure 2. (A) Samples exposed to termite attack for four weeks; (B) Positive trapping of wood attacked by termite after four weeks.

Weight Loss Analysis

After four weeks of the exposure period, the wood samples were removed, cleaned, dried during the day, and weighed to determine the weight loss.

The difference between damaged and intact wood samples was measured, and the weight loss percentage was calculated as follows:

$$\% \text{ weight loss} = (W_3\text{-}W_4 / W_3) \times 100$$

where,

W_3 = weight of test block after treatment

W_4 = weight of test block after exposure to termite attack (Funke et al., 2015)

The weight loss in wood sample was recorded as percentage

RESULTS

The aqueous plant extracts from *C. procera*, *P. tomentosa*, *D. stramonium*, and *H. muticus* were evaluated for their anti-termite efficiency against *A. ochraceus* by direct contact application and repellent test. The results are illustrated in Table 1 and Figure 3 below: as for the termite mortality after 8 hr, it was 100% with 10% of the leaves extract of the plant after 24 hr of treatment compared with the control using distilled water.

The test result showed that the LT_{50} of the butanolic extracts of the leaves of *C. procera*, *P. tomentosa*, and *H. muticus* were 231.030, 244.960, and 257.520 min, respectively; and that of the ethyl acetate extracts of the leaves of *D. stramonium* was 265.089 min.

Repellent Test (Weight Loss)

The weight loss in a wood sample due to termite attack was recorded in percentage, as shown in Figure 3. The results corresponded with the extent of termite attack on the wood samples treated with aqueous

Table 1

Effect of different plant extracts on median lethal time (LT_{50}) of Anacanthotermes ochraceus *population*

Plant species	Extract	Regression equation	Coefficient of regression (R^2)	Median lethal time (LT_{50}) (in min)
Pergularia tomentosa	*n*-hexane	y = 2.728x - 2.295	R^2 = 0.645	472.193
	Dichloromethane	y = 2.794x - 2.352	R^2 = 0.646	427.910
	Ethyl acetate	y = 2.747x - 2.260	R^2 = 0.644	439.423
	n-butanol	y = 2.737x - 1.539	R^2 = 0.842	244.960
	Exhausted fraction	y = 2.781x - 2.608	R^2 =0.575	544.134
Hyoscyamus muticus	*n*-hexane	y = 2.937x - 2.538	R^2 = 0,687	368.607
	Dichloromethane	y = 2.850x - 2.493	R^2 = 0.687	425.718
	Ethyl acetate	y = 2.838x - 2.496	R^2 = 0.689	437.821
	n-butanol	y = 3.050x - 2.353	R^2 = 0.721	257.520
	Exhausted fraction	y = 2.950x - 2.893	R^2 = 0.623	473.797
Calotropis procera	*n*-hexane	y = 2.686x - 1.965	R^2 = 0.760	391.800
	Dichloromethane	y = 2.676x - 1.485	R^2 = 0.840	265,089
	Ethyl acetate	y = 2.633x - 1.541	R^2 = 0.814	304.950
	n-butanol	y = 2.835x - 1.701	R^2 = 0.805	231.030
	Exhausted fraction	y = 2.868x - 2.817	R^2 = 0.626	531.600
Datura stramonium	*n*-hexane	y = 2.889x - 2.600	R^2 = 0.684	430.026
	Dichloromethane	y = 3.061x - 2.756	R^2 = 0.666	341.830
	Ethyl acetate	y = 2.655x - 1.435	R^2 = 0.841	265.290
	n-butanol	y = 3.236x - 3.129	R^2 = 0.564	325.126
	Exhausted fraction	y = 2.847x - 2.509	R^2 = 0.689	434.023

extracts of *C. procera*, *D. stramonium*, *H. muticus*, and *P. tomentosa*.

The changes in weight loss of wood samples treated with different aqueous extracts of plants (natural resistance) compared with the wood treated with distilled water as controls are shown in Figure 3. Regarding extracts as a preservative substance, 10% of the extract concentrations strongly affect the *A. ochraceus* termite species tested. Figure 3 showed the percent weight loss of wood samples treated by aqueous extracts, initial and final weight of each sample after planting in the experimental sites for 30 days; some differences were noted. Regarding the use of aqueous extract as a preservative substance, the concentrations of 10% showed the weight loss of the leaves of *C. procera* (0.034%), the stems of *H. muticus* (1.71%), stems and

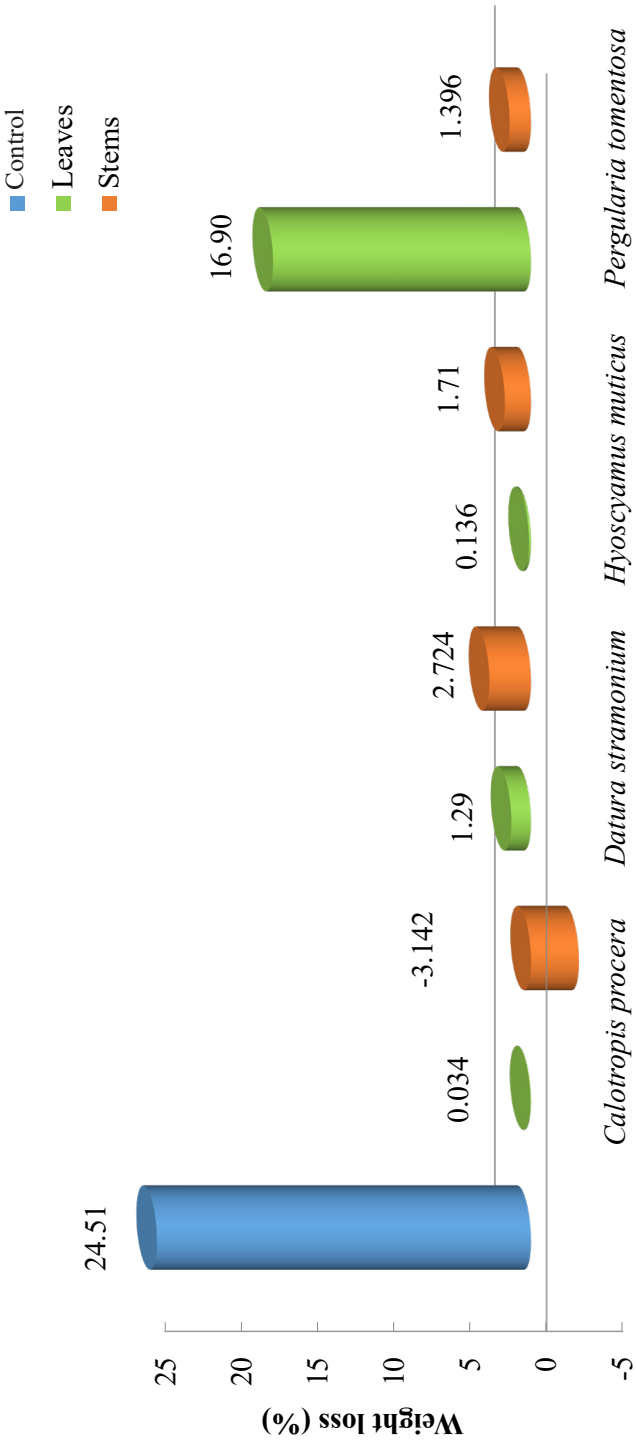

Figure 3. Weight loss of wood samples treated with 10% aqueous plant extracts after four weeks

leaves of *D. stramonium* (2.724% and 1.29%), respectively, and the leaves of *P. tomentosa* with 16.90%. Such increases indicated that the wood without preservatives (lower control) lost more than 24.51% weight compared to 10% of the plant extracts treatment. The negative weight loss of the stems of *C. procera* (-3.142%) and the stems of *P. tomentosa* (-4.396%) was due to the presence of humidity during this planting period.

DISCUSSION

The four Saharan plants, namely, *C. procera*, *P. tomentosa*, *D. stramonium*, and *H. muticus*, were selected to determine their biological activities and examined their anti-termite potential and efficacy to develop an alternative control against the termite, *A. ochraceus*. In addition, the assessment of mortality using the LT_{50} and the repellent test (weight loss) were carried out with their leaf aqueous extracts.

Vinothkumar et al. (2018) reported that among various control measures, chemical control stood first in termite management in sugarcane in Tamil Nadu, southern India. The continuous use of synthetic insecticides in termite control is known to cause aquatic and environmental pollution, lethal effects on non-target organisms and has resulted in the need to search for plant-derived compounds as an alternative (Hridayesh et al., 2016). However, many plant species can be used as botanical insecticides (Abbasipour et al., 2011a). These compounds act as fumigants, contact insecticides, repellents, and antifeedants (Zoubiri & Baaliouamer, 2014).

The result of the bioassay reported in Table 1 and Figure 3 showed the toxic action of the extracts from the leaves of *C. procera*, *P. tomentosa*, *D. stramonium*, and *H. muticus* to workers of *A. ochraceus* after 24 hr of treatment. In addition, the changes in weight loss of wood samples treated with different aqueous plant extracts compared with those treated with distilled water as control were observed (Figure 3). This change is due to their repellent effects against termite attacks. Conceivably, the plant extracts may contain bioactive substances that are toxic and deter feeding to termites. In contrast, the direct contact application method revealed that the percent mortality recorded at 10% concentration of the leaf extract of *C. procera* caused the highest mortality with LT_{50} = 231.03 min (Table 1).

The perusal of literature revealed a finding supporting results of the present work as in the study conducted in our laboratory by Bourmita et al. (2013). It demonstrated that the aqueous leaf extracts of these plants significantly affected the mortality of *A. ochraceus* workers. The study also revealed the LT_{50} of the 5% concentration of the leaf and stem extracts of *C. procera* were at LT_{50} = 54.24 min and LT_{50} = 58.54 min, respectively, and the 4% concentration of the leaf extract of *P. tomentosa* was at LT_{50} = 94.97 min.

Mueen et al. (2005) reported that C. *procera* is possessed acaricidal, schizonticidal, antimicrobial, anthelmintic, insecticidal, anti-inflammatory, antidiarrheal,

anticancerous, and larvicidal activities. In medicine, it was used to treat common ailments such as fever, rheumatism, indigestion, cough, cold, eczema, asthma, elephantiasis, nausea, vomiting, and diarrhea (Rashmi et al., 2011). Elimam et al. (2009) examined the insecticidal activity of the leaf extract of *C. procera* and reported that the aqueous leaf extract of *C. procera* showed a high level of toxicity against the larvae of mosquitoes, *Anopheles arabiensis* and *Culex quinquefasciatus*. Sharief et al. (2019) also examined the insecticidal activity of ethanolic, butane, and distilled water extracts of the leaves and flower of *C. procera* and found that they were effective against the house fly *Musca domestica*.

Butanolic extract of the leaves of *P. tomentosa* with LT_{50} = 244.960 min showed significant toxicity against *A. ochraceus*. A 100% mortality was obtained by 10% concentration of the extracts after 24 hr of treatment. *Pergularia tomentosa* has been exploited in traditional medicine as having antioxidant, antibacterial, antifungal, insecticidal, and cytotoxic activities documented by Al-Mekhlafi and Masoud (2017). The insecticidal activity of *P. tomentosa* was also reported that its crude methanol extract and its isolated cardenolides had potent antifeedant activity against the cotton leafworm, *Spodoptera littoralis* (Green et al., 2011). Furthermore, Acheuk and Doumandji-Mitiche (2013) reported that the alkaloids extract from the aerial part of *P. tomentosa* showed significant toxic and growth inhibitory effects against the fifth instar nymph of the migratory locust, *Locusta migratoria*. Miladi et al. (2018) also reported that the flowers of *P. tomentosa* exhibited insecticidal activity against *L. migratoria.* However, a comparative study of ethanolic leaf extracts from the four selected plants against the larval stages of the dengue fever mosquito vector, *Aedes aegypti*, *P. tomentosa*, showed some larvicidal properties but with less efficacy (Asiry et al., 2017).

The ethyl acetate extracts of the leaves of *D. stramonium* with LT_{50} = 265.089 min showed significant toxicity against *A. ochraceus*. Mortality of 100% was obtained by 10% concentration of the extract after 24 hr of treatment. The various parts of the plant (leaves, seeds, roots, and fruits) are used in medicine for different purposes (Tayoub et al., 2016). The plant extracts are acaricidal, insect repellent, antimicrobial, larvicidal, pesticide toxicity, antifungal, and vibriocidal (Aderonke et al., 2017). In addition, the plant comprises bioactive constituents like scopolamine, hyoscamine, tropane alkaloids, tannins, proteins, carbohydrates, sapronins, steroids, flavonoids, phenols, and glycosides (Al-snafi. 2017; Debnath & Chakraverty, 2017). Abbasipour et al. (2011b) reported *D. stramonium* aerial part extract as and insect mortality factor. The concentration at LC_{50} of acetone extract is active as a toxicant against the cowpea weevil, *Callosobrchus maculatus*, and an oviposition deterrent. Swathi et al. (2012) reported that the ethanolic leaf extract *of D. stramonium* also has significant larvicidal and repellent activities against the mosquitoes, *Aedes aegypti*, *Anopheles*

stephensi, and *Culex quinquefasciatus*. A study by Jawalkar et al. (2016) revealed that the ethanol, chloroform, and acetone extracts of *D. stramonium* seeds were effective against the rice weevil, *Sitophilus oryzae*.

The results showed that butanolic leaf extract of *H. muticus* was significantly toxic against *A. ochraceus* with LT_{50} = 257.520 min. The mortality of 100% was obtained by 10% concentration of the extracts after 24 hr of treatment. *Hyoscyamus muticus* was also reported for its tropane alkaloid content, and the main alkaloids present are scopolamine and hyoscyamine (Abed Elmaksood et al., 2016). Elsharkawy et al. (2018) reported that the methanolic extract of *H. muticus* exhibited an antifeedant effect on the fourth instar larvae of the cotton leafworm, *Spodoptera littoralis*. Abdel-Aziz et al. (2009) demonstrated that the alkaloidal chloroform fraction of the leaf and root extracts of the leaves and roots of *H. muticus* exhibited an acaricidal activity against the two-spotted spider mite, *Tetranychus urticae*.

The results obtained from our investigation showed that the plant extracts of *C. procera*, *P. tomentosa*, *D. stramonium*, and *H. muticus* used in this study have repellent and toxic effects on *A. ochraceus*. Therefore, they can be used as an effective anti-termite agent and can be suggested and used as a natural insecticide for termite control because they constitute a rich source of bioactive chemicals.

In addition to preliminary tests, developing new efficient anti-termite treatment using natural products needs bio-guided fractionation. Therefore, less efficient fractions or extracts could be neglected for more advanced tests.

CONCLUSION

The harvester termite, *Anacanthotermes ochraceus* (Isoptera: Hodotermitidae), is one of the most destructive termite pests. Therefore, using natural products of some Saharan plant species is a principal goal developed in our laboratory to discover effective and environmentally friendly termite control agents. The purpose of this study was to investigate the anti-termite effect of fractions against *A. ochraceus* from the aqueous leaf and stem extracts of four Saharan plant species, namely, the apple of Sodom or rubber bush, *Calotropis procera*, pergularia, *Pergularia tomentosa* (Asclepiadaceae), and jimsonweed, *Datura stramonium*, and Egyptian henbane, *Hyoscyamus muticus* (Solanaceae).

Of these plant species, our results have provided evidence on the efficacy of some Saharan plant species to be important sources of anti-termite treatment, especially *C. procera* and *P. tomentosa*. Therefore, our upcoming investigation will isolate and evaluate the anti-termite effect of compounds isolated from fractions and extracts from these plant species.

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Isolation and Characterization of Newcastle Disease Virus Subgenotype VII.2/VIIi from Commercial Chicken and Swan in Malaysia

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ABSTRACT

Frequent Newcastle disease (ND) outbreaks in poultry have been reported in Southeast Asia, including Malaysia. However, limited studies have been carried out on detecting the Newcastle disease virus (NDV) from non-poultry birds. In this study, the detections of NDV were carried out using tissues samples from suspected ND cases from commercial chickens and swab samples of non-poultry birds captured in bird sanctuaries. Five samples from commercial chickens and one sample from black swans were found positive for ND. They were classified as velogenic NDV based on the partial sequencing of the fusion (F) gene, which revealed the amino acid motif on the F cleavage site of ^{112}RRQKRF^{117}. In addition, phylogenetic analysis based on partial F gene showed that all NVD isolates are classified as class II genotype VII subgenotype VII.2 (VIIi) and are clustered together with NDVs isolated from chickens in 2017 in Indonesia. This finding indicates the occurrence of subgenotype VII.2 (VIIi) as the fifth panzootic of ND in Malaysia and the importance of the epidemiology of virulent NDV in various avian species.

Keywords: F gene cleavage site, genotype VII, subgenotype VII.2/VIIi

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INTRODUCTION

Newcastle disease (ND), particularly caused by the virulent Newcastle disease virus (NDV), is one of the most highly contagious diseases and causes high morbidity and mortality rates in infected chicken flocks worldwide. Hence, the disease is classified under list A poultry contagious disease by the Office International des Epizooties (World Organisation for Animal Health [OIE], 2012). Newcastle disease virus (NDV), previously known as Avian paramyxovirus type-1 (APMV-1), has recently been classified as Avian orthoavulavirus 1 (AOaV-1), under the genus of *Orthoavulavirus*, subfamily *Avulavirinae*, and family *Paramyxoviridae* (International committee on taxonomy of viruses [ICTV], 2019). Even though NDV has a single serotype, due to its genetic diversity, the virus is further classified into several genotypes and subgenotypes based on the nucleotide sequences of the F protein cleavage site and phylogenetic analysis (Dimitrov et al., 2019).

Recently, Dimitrov et al. (2019) updated the virus's classification and nomenclature by adding three new genotypes, reducing some subgenotypes, and maintaining the two existing classes and existing genotypes. The new classification has changed the genotype VII landscape, as the subgenotypes were consolidated into three subgenotypes, namely subgenotype VII.1.1, which consists of VIIb, VIId, VIIe, VIIj, and VIIl; subgenotype VII.1.2 which consists of VIIf and subgenotype VII.2, which consists of VIIh, VIIi, and VIIk.

Five global panzootic outbreaks of ND have been reported. Genotypes II, III, and IV NDVs caused the first panzootic, from the 1920s to the 1960s (Alexander, 2009). The second panzootic outbreak was caused by genotype V NDV and occurred in the late 1960s in Europe (Lomniczi et al., 1998), while subgenotype VIb caused the third in the 1980s (Kaleta et al., 1985). Genotype VII caused the fourth panzootic outbreak in Southeast Asia in 1985 and then spread through Asia, Africa, Europe, and South America (Herczeg et al., 1999). A fifth panzootic is currently caused by genotype VII and primarily by subgenotypes VII.2 (VIIh and VIIi). It has spread rapidly across Asia and the Middle East (Diel et al., 2012; Miller et al., 2015).

Since ND was first reported in Java Island, Indonesia (Kraneveld, 1926), then in Newcastle-upon-Tyne, England in 1926 (Xiao et al., 2012), outbreaks have been continuously reported in many countries. In Malaysia, the first ND outbreak was reported in Parit Buntar, Perak, in poultry flocks in 1934. Since then, ND cases associated with different virulent and avirulent NDV strains have been reported, primarily in commercial poultry birds (Aljumaili et al., 2017; Berhanu et al., 2010; Jaganathan et al., 2015; Roohani et al., 2015; Satharasinghe et al., 2016; Shohaimi et al., 2015; Tan et al., 2009, 2010). However, there is limited information on the detection of NDV in non-poultry birds and the transmission of NDV from non-poultry to commercial poultry birds in Malaysia.

Previous studies have detected virulent NDV of various genotypes, especially genotype VII in dead or alive, healthy, or clinically ill, free-living or captive non-poultry and wild birds displaying virulent properties with the presence of multiple basic amino acids at positions 112 to 116 and a phenylalanine residue at position 117 (^{112}RRQKRF^{117} and ^{112}RRRKRF^{117}). Among the bird species shown to be positive for NDVs are anseriformes (geese, wild mallards, white storks, egrets, black swans), galliformes (chickens, peacocks, pheasants, turkeys), psittaciformes (cockatoos, parrots), columbiformes (feral rock pigeon, Eurasian collared dove), falconiformes (Eurasian sparrowhawk, buzzards), and strigiformes (owls) (Miller et al., 2015; Turan et al., 2020; Vidanović et al., 2011; Wajid et al., 2017; Xie et al., 2012). Therefore, this study was conducted to determine the presence of NDV in non-poultry in bird sanctuaries and commercial chickens based on molecular characterization and thus to determine the current NDV strains circulating in the fields.

METHODOLOGY

Swab Samples from Non-Poultry Birds

Oropharyngeal and cloacal swab samples from non-poultry birds were collected in October 2017 from three different wild bird sanctuaries: Pusat Janaelektrik Sultan Salahuddin Aziz Shah, in Kapar, Selangor located in the central region of Peninsular Malaysia (3.116406, 101.324743); Pusat Konservasi Hidupan Liar Kuala Gula, in Perak, located in the northern region of Peninsular Malaysia (4.938800, 100.467408) and Putrajaya Wetland (2.962004, 101.695584). The sampling times and locations have coincided with flyways for the seasonal journey of the migratory birds from their breeding areas to Malaysia. These sampling programs were conducted together with a surveillance team from Jabatan Perlindungan Hidupan Liar dan Taman Negara Semenanjung Malaysia (PERHILITAN) Selangor and Perak under the permission certificate JPHL&TN:100-6/1/14 (25). A total of 15 bird species (n = 75), including local wild birds, resident birds, and migratory birds, were caught, swabbed, and released back to nature. A total of 150 swabs were collected (75 oropharyngeal and 75 cloacal). According to species, these swabs were then pooled in 1 mL sterile phosphate buffer saline (PBS) (1st Base, Singapore); the 15 bird species were represented by five oropharyngeal and five cloacal swabs each. The birds were handled with proper personal protective equipment (PPE), and samples were taken under sterile conditions under 2012 OIE guidelines.

Tissue Samples from Commercial Chickens

Various tissue samples from commercial chickens suspected of having ND that was submitted to the Laboratory of Vaccines and Biomolecules, Institute of Bioscience (IBS), Universiti Putra Malaysia (UPM) for NDV detection using reverse transcriptase-polymerase chain reaction (RT-PCR) from 2016 to 2017 was used in this study. A total of 20 tissue samples were received from

different poultry farms in several states around Malaysia. Each of the samples contained organs such as brains, tracheas, proventriculi, and cecal tonsils from five individual chickens.

Molecular Characterization

Virus Isolation and Propagation. Virus isolation and propagation were performed from tissue samples of commercial chickens tested positive for NDV by RT-PCR. Briefly, 30 mg of the tissue were homogenized in a 50 mL centrifuge tube (SPL Life Sciences Co. Ltd., Korea) containing 3 mL sterile PBS using tissue rupture (QIAGEN, Germany) to produce homogenates. Then, the homogenates were centrifuged at 2,500 x *g* for 5 min at 10 °C (Eppendorf 5417R, Eppendorf, Germany). Next, the supernatant was aspirated and filtered using a 0.45 µm syringe filter (Sartorius, Germany) to produce inoculum. In the case of the swab samples of non-poultry birds, the inoculums were prepared by combining the oropharyngeal and cloacal swab samples and vortexed for 30 sec. Then, the samples were centrifuged at 2,500 x *g* for 10 min at 10 °C and filtered using a 0.45 µm syringe filter to produce inoculum.

Next, each inoculum was inoculated into five 10-day-old specific-pathogenic-free embryonated chicken eggs (SPF ECE) at a 0.1 mL/eggs volume, according to OIE (2012). The eggs were incubated at 37 °C (ESCO, Singapore) for five days. The allantoic fluids from the first inoculation were then harvested and used as inoculum for the second passage. Following completion of three serial passages in 10-day-old SPF ECE, the allantoic fluids were harvested and tested for hemagglutination activity via haemagglutination (HA) spot test according to OIE (2012).

RNA Extraction. According to the manufacturer's protocol, the allantoic fluids were extracted using TRIzol® reagent (Invitrogen, USA). Briefly, 300 µL of the allantoic fluids were mixed with 750 µL of TRIzol® reagent, then vortexed and incubated for 5 min at room temperature. Then, 200 µL of chloroform (Merck, Germany) was added, vortexed, and incubated for 10 min at room temperature. Next, the samples were centrifuged at 11,200 x *g* and 10 °C for 10 min. Subsequently, 500 µL of the clear supernatant was mixed with 800 µL of isopropanol (1st Base, Singapore) then vortexed and incubated on ice for 20 min. Next, the mixture was centrifuged at 15,000 x *g*, 4 °C for 15 min, and the supernatant was discarded. The RNA was then washed with 800 µL of 70% molecular grade ethanol (Merck, Germany) then washed again with 800 µL of 100% molecular grade ethanol (Merck, Germany). Finally, the RNA pellet was air-dried for 10 min then reconstituted into 30 µL of RNAse-free water (Qiagen, the Netherlands).

Primer Design. The primer set used in this study was designed by Berhanu et al. (2010), targeting the nucleotides sequence of the F gene at region 47-581, including the F0 cleavage site. This amplification was performed using forward

primer, 5′-ATGGGC(C/T)CCAGA(C/T) CTTCTAC-3′, and reverse primer, 5′- CTGCCACTGCTAGTTGTGA TAATCC -3' , with an expected size of 535 bp.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). A standard one-step RT-PCR was performed using a One-Step RT-PCR Kit (Biotechrabbit™, Germany). Briefly, 12 µL of One-Step Mix, 2× was mixed with 1 µL of RNAse-free water, 1µL of RT-RI Blend 20×, 1 µL of forward primer (20 µM), 1 µL of reverse primer (20 µM), and finally 4 µL of RNA template. Non-template control (NTC) was used as the negative control and NDV LaSota virus as the positive control. The PCR mixture was amplified in a thermocycler (C1000 Touch™ Thermal Cycler, Bio-Rad Laboratories, USA) set for 10 min at 48 °C for reverse transcription, 3 min at 95 °C for polymerase activation, 40 cycles of 20 sec at 95 °C for denaturation, 30 sec at 58 °C for annealing, 20 sec at 72 °C for extension, and 5 min at 72 °C for a final extension.

Agarose Gel Electrophoresis. Agarose gel electrophoresis was run at 110 volts for 45 min (Analytik Jena, Malaysia). First, the PCR products were loaded into 1.5% agarose gel. Next, the gel was loaded with 4 µL of TrackIt™ 1 kb DNA Ladder (Invitrogen, USA) as marker followed by 4 µL of PCR product mixed with 1 µL of TrackIt™ Cyan/Orange Loading Buffer (Invitrogen, USA), 4 µL of negative control, and 4 µL of positive control. The gel was then visualized using the Gel-doc™ XR+ System (Bio-Rad Laboratories, USA).

Fusion Gene Sequencing and Phylogenetic Analysis. The partial F genes of the isolated NDV isolates were sent for Sanger sequencing (Repfon Glamor Sdn. Bhd., Malaysia). The raw sequences obtained were trimmed, edited, and assembled using BioEdit (Hall, 1999) to generate consensus sequences that were analyzed with the Basic Local Alignment Search Tool (BLAST) program [GenBank, National Center for Biotechnology Information (NCBI)]. The sequences of Malaysian NDV isolates were compared with reference strains representative of each genotype and subgenotype obtained from GenBank NCBI (Table 1 and Table 2) using the ClustalW method in MEGA v7.0 software. Then, phylogenetic trees were constructed using the maximum likelihood method based on the Kimura 2 parameter model (Tamura et al., 2011) with 1,000 bootstrap replicates.

Table 1

NDV reference strains representing the different classes and genotypes

Class	Genotype	Isolates/ strain	Accession number	Origin
I	I	Teal/France/100011/2010	JQ013039	France
		DE-R49/99	DQ097393	Hungary

Table 1 *(Continued)*

Class	Genotype	Isolates/ strain	Accession number	Origin
II	I	Ulster/67	AY562991	Northern Ireland
		V4 (Queensland)	M24693	Australia
	II	LaSota	DQ195265	USA
		MB061/06	GQ901891	Malaysia
	III	Mallard/CH/HLJ383/06	KY776604	China
		Miyadera	M24701	Japan
	IV	Herts/33	AY741404	UK
		Italien	EU293914	Italy
	V	Largo/71	AY562990	USA
		Anhinga/U.S (FI)/44083/93	AY562986	USA
	VI	NDV05-028	DQ439885	China
		NDV05-029	FJ766528	China
	VII	IBS002/11	KF026013	Malaysia
		IBS005/11	KR074405	Malaysia
		IBS025/13	KT355595	Malaysia
	VIII	AF2204	AF048763	Malaysia
		MB085/05	GQ901901	Malaysia
		HR09	MF285077	China
	IX	ZhJ-1/85	AF458023	China
		FJ-1/85	AF458009	China
	XI	MG/39/4/08	HQ266605	Madagascar
		MG/MEOLA/08	HQ266604	Madagascar
	XII	GD1003/2010	KC152049	China
		GD450/2011	KC152048	China
	XIII	BD-C161/2010	KY905320	Bangladesh
		KW48/2011	KU936209	Bangladesh
	XIV	NIE09-2014/2009	HF969145	Nigeria
		NIE09-2041/2009	HF969149	Nigeria

Table 1 *(Continued)*

Class	Genotype	Isolates/ strain	Accession number	Origin
II	XVI	FO/499-31/505/2008	MH392226	Nigeria
		867-2/2008	JX186997	Nigeria
	XVII	228-7/2006	KF442614	Nigeria
		903/KUDU-113/1992	KU058680	Nigeria
	XVIII	NIE10-171/2011	HF969217	Nigeria
		CIV08-042/2007	HF969218	Ivory coast

Table 2

NDV reference strains representing the different subgenotypes of NDV genotype VII

Diel et al. (2012)	Dimitrov et al. (2019)	Isolates/ strain	Accession number	Origin
VIIb	VII.1.1	Ck/SD-01-12-Ch	KJ184594.1	China
		Ck/JS-17-11-Ch	JQ013871.1	China
		Ck/SD704/12	JX840454	China
VIId	VII.1.1	Ck/MB016/07	GQ901894.1	Malaysia
		Ck/MB064/05	GQ901893.1	Malaysia
VIIe	VII.1.1	Go/GD/1/98	AF456437.1	China
		Ck/Ibaraki/SG106/1999	AB853927.2	Japan
VIIf	VII.1.2	ND/03/018	GQ338309.1	China
		Ck/ND/03/044	GQ338310.1	China
VIIh	VII.2	Ck/Makassar/003/09	HQ697256.1	Indonesia
		Ck/IBS005/11	KR074405.1	Malaysia
		Ck/IBS002/11	KR074404.1	Malaysia
VIIi	VII.2	Ck/Banjarmasin/010/10	HQ697254.1	Indonesia
		Ck/ Kulonprogo/04171317/2017	MK069429.1	Indonesia
		Ck/IBS025/13	KT355595.1	Malaysia
VIIj	VII.1.1	Dk/JLQG/2013	KJ136259.1	China
		Ck/JLJT/2012	KJ136258.1	China
VIIl	VII.1.1	Ck/IR/MAM81/2018	MH481363.1	Iran
		Ck/IR/MAM68/2017	MH481361.1	Iran
		Ck/IR/MAM55/2017	MH247187.1	Iran

Biological Characterization

Mean Death Time (MDT). MDT is the average death time for the minimum lethal dose to kill all the inoculated SPF ECE. NDV isolates were classified as velogenic, mesogenic, and lentogenic according to mean embryo death time at <60 hours, 60 to 90 hours, and >90 hours, respectively (Alexander, 1988). Briefly, a ten-fold serial dilution of allantoic fluid of NDV isolates was prepared in 1x sterile PBS (10^{-1} to 10^{-10}). Then, 0.1 mL of the inoculum was injected into the allantoic cavity of 10-day-old SPF eggs. The infected SPF eggs were incubated at 37 ^{o}C for five days with daily candling to monitor embryonic death.

Intracerebral Pathogenicity Index (ICPI). ICPI is the mean score of daily observations of each inoculated chick over eight days. NDV isolates were classified as velogenic strains with an ICPI of 1.5-2.0, mesogenic with an ICPI of 0.5-1.5, and lentogenic with an ICPI <0.5 (OIE, 2012). Briefly, the allantoic fluid with a HA titer $>2^4$ was diluted into sterile PBS (1/10). Then 0.05 mL of the inoculum was injected intracerebrally into 10 SPF chicks aged 24 to 40 hours old (Alexander & Senne, 2008; OIE, 2012).

RESULTS

Collection of Samples from Different Bird Species and Commercial Chickens

A total of 150 swab samples collected from 15 different species of migratory birds, local birds, and resident birds in bird sanctuaries and along with 20 samples from suspected cases of ND in commercial chickens, were screened for NDV detection. All the non-poultry birds were clinically healthy and not showing any overt clinical signs, while the commercial poultry birds were clinically ill and suspected of having ND (Table 3 and Table 4).

Virus Isolation and RT-PCR

Based on the virus isolation performed, a total of five cases from commercial chickens and one case from non-poultry birds (black swans) with IDs of UPM/NDV/IBS303/2016, UPM/NDV/IBS380/2017, UPM/NDV/IBS362/2016, UPM/NDV/IBS501/2017, UPM/NDVIBS599/2017, and UPM/NDV/IBS932/2017 were found to be positive for NDV. These isolates showed consistent embryonic mortality with an increased number of dead embryos following three serial passages, positives on the HA spot test and positives on the RT-PCR (Table 3). One sample from non-poultry species showed embryonic mortality and was positive on the HA spot test. However, the sample gave a negative result on RT-PCR. Meanwhile, all the other samples from non-poultry species were negative for NDV, with inconsistent or no embryonic mortality and negatives on the HA spot test and RT-PCR. The examination of the dead embryos showed lesions, small embryos with cranial hemorrhages, and cloudy allantoic fluid with the presence of petechial hemorrhages on the yolk sac (data not shown).

Table 3

NDV isolates isolated in this study

Virus ID	Location	Types of birds	Clinical signs	Samples	Embryonic mortality at different passage			F cleavage site	MDT	ICPI
					P1	P2	P3			
UPM/NDV/IBS303/2016	Pulau Pinang	Broiler, 28 days old	Dypsnea Rales Diarrhea	Trachea Cecal tonsil	0/5	1/5	3/5	^{112}RRQKRF^{117}	58.2	1.7
UPM/NDV/IBS362/2016	Sabah	Broiler, 35 days old	Dypsnea Rales Diarrhea	Proventriculus Cecal tonsil	0/5	2/5	3/5	^{112}RRQKRF^{117}	58.4	1.7
UPM/NDV/IBS380/2017	Perak	Broiler, 25 days old	Dypsnea Rales Diarrhea	Cecal tonsil	5/5	5/5	5/5	^{112}RRQKRF^{117}	56.2	1.7
UPM/NDV/IBS501/2017	Kedah	Broiler, 30 days old	Torticollis Rales Diarrhea	Brain Cecal tonsil	1/5	3/5	4/5	^{112}RRQKRF^{117}	57.6	1.7
UPM/NDV/IBS559/2017	Pulau Pinang	Broiler, 36 days old	Dypsnea Rales Diarrhea	Trachea	1/5	4/5	5/5	^{112}RRQKRF^{117}	57.0	1.7
UPM/NDV/IBS932/2017	Putrajaya	*Cygnus atratus*	Healthy	Oropharyngeal Cloacal swab	1/5	1/5	2/5	^{112}RRQKRF^{117}	58.0	1.7

Note. MDT = Mean death time; ICPI = Intracerebral pathogenicity index

Table 4

Detection of HA activities and RT-PCR assay to confirm NDV in non-poultry bird species

Species	Origin	Embryonic mortality at different passage			HA spot test	RT-PCR
		P1	P2	P3		
Actitis hypoleucos	Selangor	0/5	0/5	0/5	-ve	-ve
Xenus cinereus	Selangor	0/5	0/5	0/5	-ve	-ve
Tringa totanus	Selangor	0/5	0/5	0/5	-ve	-ve
Charadrius leschenaultii	Selangor	0/5	0/5	0/5	-ve	-ve
Vanellus indicus	Selangor	0/5	0/5	0/5	-ve	-ve
Charadrius mongolus	Selangor	0/5	0/5	0/5	-ve	-ve
Vanellus indicus	Selangor	0/5	0/5	0/5	-ve	-ve
Charadrius mongolus	Selangor	0/5	0/5	0/5	-ve	-ve
Caprimulgus affinis	Selangor	0/5	0/5	0/5	-ve	-ve
Calidris minuta	Selangor	0/5	1/5	1/5	+ve	-ve
Numenius phaeopus	Perak	0/5	0/5	0/5	-ve	-ve
Butorides striata	Perak	0/5	0/5	0/5	-ve	-ve
Egretta garzetta	Perak	0/5	0/5	0/5	-ve	-ve
Halcyon pileata	Perak	0/5	0/5	0/5	-ve	-ve
Cygnus atratus	Putrajaya	1/5	1/5	2/5	+ve	+ve

Note. HA = Hemagglutination; RT-PCR = Reverse transcriptase-polymerase chain reaction

RT-PCR Amplification of Fusion Gene

The partial F genes of the NDV isolates were amplified with the expected amplicon size of 535 bp (Figure 1). All six samples were then sent for Sanger sequencing (MATRIOUX, Malaysia) of the partial F gene.

Phylogenetic Analysis of Fusion Gene

The Malaysian NDV isolates are closely related to the previously characterized genotype VII NDV with >96.0% identity based on the partial F gene sequence alignment. The phylogenetic tree was generated based on 535 bp of the F gene segment corresponding to nucleotide position 47 to position 535, including the F cleavage site compared to NDV genotype I-XVIII as the reference strains. The phylogenetic tree analysis showed that all the identified NDV isolates were clustered into a single genotype VII (Figure 2) and subgenotype VII.2 /VIIi (Figure 3).

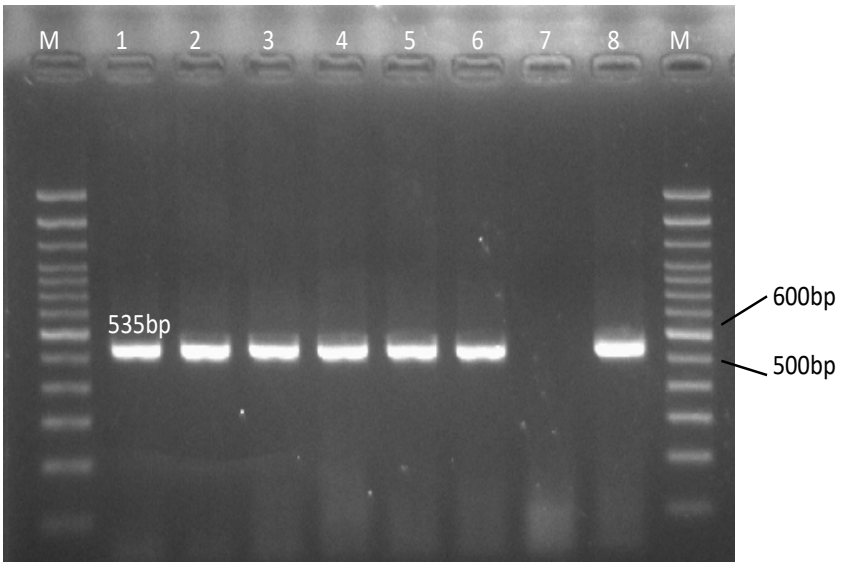

Figure 1. Agarose gel electrophoresis of NDV isolates: UPM/NDV/IBS380/2017 (lane 1), UPM/NDV/IBS599/2017 (lane 2), UPM/NDV/IBS362/2016 (lane 3), UPM/NDV/IBS501/2017 (lane 4), UPM/NDV/IBS303/2016 (lane 5), UPM/NDV/IBS932/2017 (lane 6), non-template control NTC (lane 7), and positive control LaSota (lane 8). Lane M is a molecular weight ladder

The pathotypes of the NDV isolates were then analyzed based on the amino acid sequences of the F cleavage site using MEGA v7.0. The analysis showed that all the identified NDV isolates were velogenic with the presence of multiple basic amino acid residues at position ^{112}RRQKRF^{117}, MDT of <60 hours, and an ICPI of 1.7.

Pairwise Evolutionary Distances

The pairwise evolutionary distance was constructed using the maximum composite likelihood model to estimate mean distances between newly isolated subgenotype VIIi isolates and subgenotypes VIIb, VIId, VIIe, VIIf, VIIh, VIIi, VIIl, and VIIj. Evolutionary distance estimation of nucleotides proved that Malaysia VIIi isolates are distinct from the previously reported subgenotype VIIi at 0.030 to 0.075 and other subgenotypes at 0.076 to 0.140 (Table 5). In addition, the amino acids are distinct from VIIi at 0.076 to 0.130 and other subgenotypes at 0.169 to 0.305 (Table 5), suggesting the newly isolated subgenotypes VIIi from this study have evolved from the same ancestor of the previously isolated subgenotypes VIIi. In addition, of all six NDV isolates, three isolates UPM/NDV/IBS 362/2016, UPM/NDV/IBS501/2017, and UPM/NDV/IBS599/2017, shared the highest nucleotide and amino acid identity with reference subgenotype VII.2 (VIIi) at 97% and 92.4%, respectively. Meanwhile,

Figure 2. Phylogenetic analysis of the NDV isolates identified in this study (marked with ▲), and 36 previously characterized isolates representing Class I and Class II NDV genotypes. The tree was inferred using the maximum likelihood method based on the Kimura-2 parameter model (1,000 bootstrap replicates) using MEGA v7.0 software.

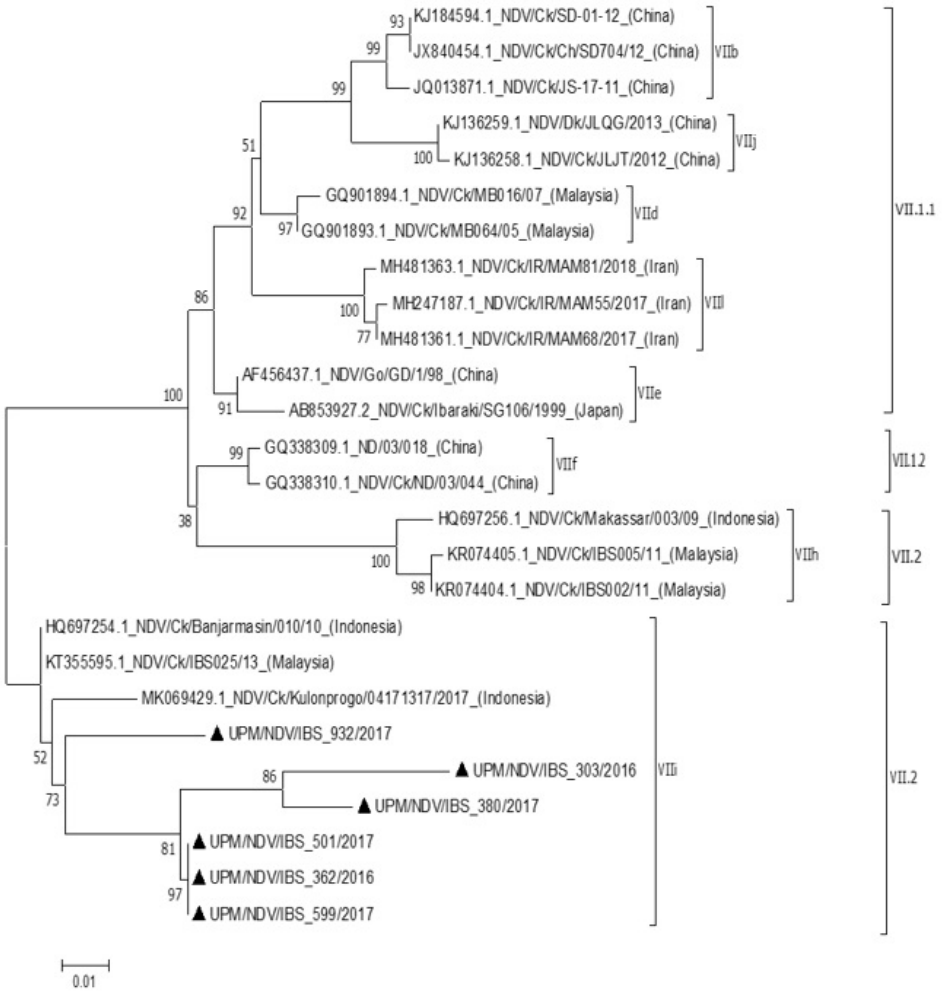

Figure 3. Phylogenetic analysis of the NDV subgenotype VII according to Diel et al.'s (2012) classification system (VIIb-VIIi) and Dimitrov et al.'s (2019) classification system (VII.1 -VII.2). NDV isolates identified in this study (marked with ▲) and 20 previously characterized isolates representing NDV subgenotypes VII.

isolate UPM/NDV/IBS303/2016 showed 93.6% and 89%, respectively; isolate UPM/NDV/IBS380/2017 showed 94.1% and 89%, respectively, and isolate UPM/NDV/IBS 932/2017 showed 92.5% and 87%, respectively.

Table 5

Evolutionary distances of nucleotide sequences (lower diagonal) and amino acid sequences (upper diagonal) estimated between the mean distances of six isolates with subgenotypes VII of the fusion (F) gene

	Isolates	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	UPM/NDV/IBS 303/2016		0.076	0.076	0.076	0.076	0.119	0.271	0.246	0.229	0.212	0.237	0.110	0.263	0.305
2	UPM/NDV/IBS 362/2016	0.052		0.034	0.000	0.000	0.110	0.229	0.203	0.186	0.178	0.203	0.076	0.229	0.297
3	UPM/NDV/IBS 380/2017	0.044	0.034		0.034	0.034	0.136	0.263	0.237	0.220	0.212	0.237	0.110	0.254	0.331
4	UPM/NDV/IBS 501/2017	0.052	0.000	0.034		0.000	0.110	0.229	0.203	0.186	0.178	0.203	0.076	0.229	0.297
5	UPM/NDV/IBS 599/2017	0.052	0.000	0.034	0.000		0.110	0.212	0.203	0.186	0.178	0.203	0.076	0.229	0.297
6	UPM/NDV/IBS 932/2017	0.067	0.047	0.069	0.047	0.047			0.195	0.178	0.169	0.212	0.130	0.229	0.280
7	Subgenotype VIIb	0.131	0.103	0.112	0.103	0.103	0.111		0.085	0.127	0.136	0.186	0.178	0.085	0.237
8	Subgenotype VIId	0.118	0.081	0.106	0.081	0.081	0.089	0.037		0.076	0.085	0.144	0.153	0.119	0.212
9	Subgenotype VIIe	0.116	0.079	0.103	0.079	0.079	0.086	0.049	0.032		0.076	0.144	0.136	0.161	0.220
10	Subgenotype VIIf	0.111	0.076	0.103	0.076	0.076	0.084	0.059	0.037	0.034		0.102	0.127	0.169	0.178
11	Subgenotype VIIh	0.128	0.099	0.116	0.099	0.099	0.108	0.081	0.069	0.062	0.057		0.161	0.203	0.246
12	Subgenotype VIIi	0.064	0.030	0.059	0.030	0.030	0.075	0.081	0.064	0.057	0.054	0.079		0.195	0.237
13	Subgenotype VIIj	0.128	0.099	0.118	0.099	0.099	0.106	0.030	0.042	0.054	0.064	0.081	0.081		0.297
14	Subgenotype VIIl	0.138	0.116	0.140	0.116	0.116	0.121	0.103	0.089	0.096	0.081	0.116	0.096	0.121	

DISCUSSION

ND has been endemic in Malaysia for decades since its first detection in 1934. Implementing a good flock health program, including a mass vaccination regime on poultry farms, can decrease ND outbreaks; however, the disease is a major threat to the industry. The result of continuing surveillance among commercial poultry farms indicates that genotype VII is the predominant strain that has been causing ND outbreaks around Malaysia since the 2000s, although viruses from genotype I, III, VI, and VIII have also been reported (Shohaimi et al., 2015).

According to OIE reports, Malaysia has experienced several major ND outbreaks in commercial poultry flocks. Since 1999, ND has been caused by avirulent and virulent genotypes. NDV genotypes I, II, III, VI, VII, and VIII have been reported in commercial poultry (Aljumaili et al., 2017; Berhanu et al., 2010; Roohani et al., 2015; Satharasinghe et al., 2016; Shohaimi et al., 2015; Tan et al., 2009, 2010). The subgenotype VII.2 (VIIh and VIIi) NDVs, which have caused the fifth ND panzootic outbreak, has been endemic in Southeast Asia, including Malaysia (Berhanu et al., 2010), Indonesia (Doan et al., 2020; Xiao et al., 2012), Vietnam (Choi et al., 2014; Le et al., 2018), Cambodia (Choi et al., 2013), and then spread to other countries (Liu et al., 2015).

There is limited information available regarding the detection of NDV in non-poultry birds, including wild birds in Malaysia. Nevertheless, previous studies have reported the detection of genotype III in peacocks, genotype VI in pigeons, genotype VII in peacocks, owls, and egrets (Shohaimi et al., 2015) as well as genotype II in parrots (Berhanu et al., 2010) in Malaysia. However, not much is known about the role of wild bird reservoirs in the exchange of virulent ND among wild birds and poultry species in Malaysia. In this study, six isolates of subgenotype VII.2/VIIi were isolated from commercial chicken farms (broiler) and wetland (black swan). According to amino acid residues at the F gene cleavage site, the six isolates were identified as virulent strains with the motif of ^{112}RRQKRF^{117}, an identification supported by results from MDT and ICPI.

Several studies have reported increasing NDV genotype VII detection among waterfowl, especially in China and Taiwan (Ke et al., 2010; Zhang et al., 2010). However, the severity of infection varies depending on the virus, host, age, co-infection, host's immune status, and environmental condition, as Alexander (2009) explained. Kaleta and Kummerfeld (2012) reported that healthy white storks could harbor virulent NDV genotype VII and thus potentially serve as a reservoir in spreading virulent NDV to susceptible bird species. Xie et al. (2012) reported a similar situation, in which NDV subgenotype VIIa was isolated from healthy wild egrets in China. Meanwhile, Vidanović et al. (2011) isolated NDV subgenotype VIId in dead mallard, feral rock pigeon, Eurasian sparrowhawk, and Eurasian collared dove during an ND outbreak in

Serbia, while Wajid et al. (2017) isolated NDV subgenotype VIIi from clinically ill wild pigeon and black swan in Pakistan. However, in another study, Wajid et al. (2018) isolated NDV subgenotype VIIi from clinically healthy wild duck, geese, and black swans in Pakistan. In contrast, Miller et al. (2015) isolated NDV subgenotype VIIi from pheasants, peacocks, parakeets, parrots, and pigeons in Pakistan, showing tremors and paralysis with 60% mortality. In this study, the velogenic NDV isolated from a black swan in the wetland area in Putrajaya does not show any obvious clinical signs. Further research to sequence the complete genome of the viruses is required to get more information regarding the epidemiology of the virus, as this study only addressed partial F gene sequencing.

Molecular characterization of F genes, especially the F cleavage site, is a reliable test for determining the pathotypes of the NDV strains (Toyoda et al., 1987) and genotype classification (Diel et al., 2012; Dimitrov et al., 2019). Based on the constructed phylogenetic tree (Figure 2), the studied NDV isolates were clustered into class II genotype VII as subgenotype VII.2/VIIi (Figure 3). This result is in line with previous studies that indicated NDV genotype VIIi or VII.2 is responsible for the fifth ND panzootic outbreak (Courtney et al., 2013; Lu et al., 2014; Miller et al., 2015; Zhang et al., 2010). The studies also indicated that subgenotype VIIi is circulating among commercial chickens and wild birds in Asia (Miller et al., 2015; Putri et al., 2017; Umali et al., 2017; Xiao et al., 2012). In addition, the detection of multiple basic amino acids at positions 112 to 116 and a phenylalanine residue at position 117 (^{112}RRQKRF^{117}) in the new isolates indicates the viruses are virulent.

According to OIE (2012), virus isolation is the standard gold method for NDV isolation and identification via inoculation into SPF ECE. This step allows NDVs to adapt in the embryonated eggs and grow to a higher virus titer. In this study, five samples from commercial chickens (UPM/NDV/IBS303/2016, UPM/NDV/IBS380/2017, UPM/NDV/IBS362/2016, UPM/NDV/IBS501/2017, and UPM/NDV/IBS599/2017) and one sample from non-poultry, black swans (UPM/NDV/IBS932/2017) showed constant embryonic mortality following three viral passages. Meanwhile, the remaining samples from wild birds showed inconsistent or no embryonic mortality patterns. As expected, only samples consistently showing embryonic mortality were found to be positive by a HA spot test and confirmed by RT-PCR using NDV specific F gene primers.

One sample from wild birds, *Calidris minuta*, showed mild on the HA test but negative for RT-PCR (Table 4), indicating the presence of other avian viruses. The HA spot test is a direct and visible macroscopic test that detects agglutination of chicken red blood cells (RBCs) but does not identify the etiological agent present in the allantoic sample tested. The HA spot test is commonly practiced in the laboratory to test for selected viruses that have hemagglutinin protein such as NDV, avian influenza virus (AIV), and

egg drop syndrome (EDS) (OIE, 2018). However, further studies failed to detect avian influenza virus (AIV), infectious bronchitis, and fowl adenovirus (data not shown). A HA spot test gives positive results when haemagglutinin on the surface of NDV binds to chicken RBCs, producing clumping or hemagglutination. However, there is a possibility of getting a false-positive result when a nonspecific reaction occurs in tested samples. In addition, the HA spot test does not distinguish between infectious viral particles and degraded viral particles since both particles can cause hemagglutination (OIE, 2012).

CONCLUSION

This study isolated NDV subgenotype VII.2 (VIIi) from commercial chicken and non-poultry birds, indicating that the current ND infection in Malaysia is caused by subgenotype VII.2 (VIIi). The same trend has been observed in neighboring countries such as Indonesia, the Philippines, Vietnam, and Cambodia, along with fifth ND panzootic outbreaks across Asia. However, the epidemiological link to the detection of the viruses in different avian species is not clear. Therefore, further studies are required to gain insight into the importance of non-poultry birds, including wild birds, as the source of virulent NDV in poultry in Malaysia.

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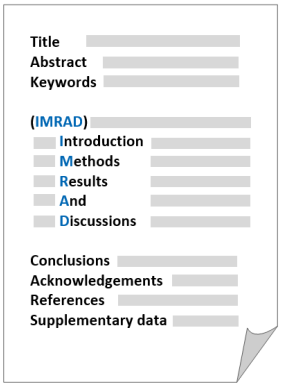

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