Pertanika Journal of

TROPICAL AGRICULTURAL SCIENCE

JTAS

VOL. 42 (4) NOV. 2019

PERTANIKA JOURNALS

A scientific journal published by Universiti Putra Malaysia Press

Journal of Tropical Agricultural Science

About the Journal

Overview

Pertanika Journal of Tropical Agricultural Science (JTAS) is the official journal of Universiti Putra Malaysia published by UPM Press. It is an open-access online scientific journal which is free of charge. It publishes the scientific outputs. It neither accepts nor commissions third party content.

Recognized 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.

JTAS 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 to authors around the world regardless of the nationality.

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 three journals as Journal of Tropical Agricultural Science, Journal of Science & Technology, and Journal of Social Sciences & Humanities to meet the need for specialised journals in areas of study aligned with the interdisciplinary strengths of the university.

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Notification of the editorial decision is usually provided within ten to fourteen weeks 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.

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In the peer-review process, three referees independently evaluate the scientific quality of the submitted manuscripts.

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

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

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2. The CEE sends the article-identifying information having been removed, to three reviewers who are specialists in the subject matter represented by the article. The CEE requests them to complete the review in three 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 field.

3. The CEE, in consultation with the Editor-in-Chief (EiC), examines the reviews and decides whether to reject the manuscript, invites the author(s) to revise and resubmit the manuscript. The CEE may seek additional reviews. Final acceptance or rejection rests with the CEE and EiC, who reserve the right to refuse any material for publication. In rare instances, the manuscript is accepted with almost no revision. Almost without exception, reviewers' comments (to the author) are forwarded to the author. If a revision is indicated, the editor provides guidelines 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 author(s) may also submit a rebuttal if there is a need especially when the author disagrees with certain comments provided by reviewer(s).

5. The CEE sends the revised paper out for re-review. Typically, at least one of the original reviewers will be asked to examine the article.

6. When the reviewers have completed their work, the CEE in consultation with the EiC and EBMs examine their comments and decide whether the paper is ready to be published, needs another round of revisions, or should be rejected.

7. If the decision is to accept, an acceptance letter is sent to all the author(s), the paper is sent to the Press. The article should appear online in approximately three months.

   The Publisher ensures that the paper 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 Publisher. 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 article appears in the pages of the Journal and is posted online.

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Vol. 42 (4) Nov. 2019

PERTANIKA JOURNALS

A scientific journal published by Universiti Putra Malaysia Press

JTAS

Journal of Tropical Agricultural Science

AN INTERNATIONAL PEER-REVIEWED JOURNAL

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ABSTRACTING AND INDEXING OF PERTANIKA JOURNALS

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Foreword

Welcome to the Fourth Issue of 2019 for the Journal of Tropical Agricultural Science (JTAS)!

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

This issue contains 15 articles; 2 are short communications and the rest are regular articles. The authors of these articles come from different countries namely China, Ecuador, Indonesia, Malaysia and Nigeria.

Articles submitted in this issue cover various scopes of Tropical Agricultural Science including animal production, aquaculture, biotechnology, fisheries sciences, food and nutrition development, forestry sciences, genetics and molecular biology, horticulture, soil and water sciences and zoology.

Hui Yin Fan and her team mates from Universiti Malaysia Sabah explored the potential of blends of fish gelatin and sodium alginate as gelling agents and a delivery system for virgin coconut oil (VCO). They found out that the viscoelasticity and firmness of the puddings were increased with the blending of high fish gelatin content with sodium alginate. Details of this study is available on page 1209.

A regular article entitled “Effect of Monocalcium Phosphate Supplementation on the Growth Performance, Carcass Characteristic, Gut Histomorphology, Meat and Bone Quality of Broiler Chickens” discussed on the effect of different concentration of monocalcium phosphate supplementation on the growth performance, carcass characteristics, gut morphology, meat quality, and bone quality of broiler chickens. It concluded that this supplementation brought the positive effect on the growth performance, gut histomorphology and bone quality of broiler chickens without affecting the carcass characteristics and meat quality. The detailed information of this article is presented on page 1237.

Bun Poh Keong and his colleagues from Universiti Pendidikan Sultan Idris discussed the optimal preservation and storage regimes of total RNAs from different fish tissues. The results showed that satisfactory amount of RNA from dorsal fin and gonad was produced by preserving fish tissues in RNA*later* with subsequent storage at -80°C, but not from muscle tissues. Further details of the study are found on page 1289.

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, 34% were accepted. This seems to be the trend in Pertanika Journals for JTAS.

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

JTAS 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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Pertanika J. Trop. Agric. Sc. 42 (4): 1173 - 1184 (2019)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Effects of Cinnamon Bark Essential Oil (*Cinnamomum burmannii*) on Characteristics of Edible Film and Quality of Fresh Beef

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ABSTRACT

The effects of cinnamon bark essential oil on the characteristics of edible film and the quality of fresh beef were investigated to determine its ability to extend fresh beef shelf life. Films were prepared by incorporating cinnamon bark essential oil (0, 0.5, 1.0, 1.5 and 2%) into a film mixture of tapioca starch and glycerol. The selected film and control solution were applied to evaluate fresh beef quality. The concentration of cinnamon bark essential oil did not affect the thickness but significantly affected the water vapor transmission rate, tensile strength, elongation and antibacterial activity of edible film. The water vapor transmission rate was found to be lowered with the increasing cinnamon bark oil concentration. An increase of tensile strength was observed with increasing oil concentration up to 1.5%. The elongation of film was significantly reduced to 58.56% while an improved antibacterial activity of edible film resulted by oil addition. Overall, results indicate that 2% cinnamon bark oil enriched film preserves freshness of beef. The addition of cinnamon bark oil to an edible coating is effective in reducing microbial growth and lipid oxidation while the edible film enriched with 2% cinnamon

ARTICLE INFO

Article history:
Received: 4 April 2016
Accepted: 14 August 2019
Published: 13 November 2019

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ISSN: 1511-3701
e-ISSN: 2231-8542

bark oil would maintain the freshness of beef as long as 15 days during storage.

Keywords: Beef, cinnamon, edible film, essential oil, preservation

INTRODUCTION

Beef is one of the meat types most commonly consumed as a protein source globally. However, beef has a fairly short shelf life due to its high moisture content and high fat content (68% and 11%) (Gaman & Sherrington, 1994) which can facilitate the proliferation of microbial spoilage and rancidity due to oxidation reactions.

Edible films can increase the shelf life and maintain the quality of food through selective permeability to moisture and oxygen transfer, and by inhibiting the oxidation of fat and additives into the carrier material (Kester & Fennema, 1986). Additives which are usually added to the edible film are antimicrobial substances derived from spices. Spices that can be used as antimicrobial substances in the edible film are ginger essential oil (Miksusanti & Masril, 2013), nutmeg essential oil (Matan, 2012) and cinnamon essential oil, which have been applied to edible film made from whey protein (Bahram et al., 2013), apple puree (Du et al., 2009), chitosan (Hosseini et al., 2009) or gelatin-chitosan (Gómez-Estaca et al., 2010). Some other antimicrobial substances that have been used as preservatives in meat are β-CD-citral which has been added to a chitosan edible coating (Chen et al., 2014), oregano oil in whey protein isolate films (Zinoviadou et al., 2009), and clove-oregano essential oil in a film made from milk protein (Oussalah et al., 2004).

Cinnamomum burmannii (cinnamon) is a subtropical plant from the *Lauraceae* family and reported as an important export commodity of Indonesia. Cinnamon contains active substances in the form of trans-cinnamaldehyde (60.72%), eugenol (17.62%), and coumarin (13.39%) (Wang et al., 2009). The processing of cinnamon bark into essential oils by a steam distillation resulted in active substances such as cinnamaldehyde (92.84%), cinnamyl acetate (2.34%), α-copaene (1.56%), coumarin (1.01%), delta-cadinene (0.70%), 1.8-cineol (0.66%), isopropyl acetate (0.57%), and α-terpineol (0.32%) (Khasanah et al., 2013).

The objective of this study was to investigate the effects of cinnamon bark essential oil's addition on the characteristics of edible film and the quality of fresh beef.

MATERIALS AND METHODS

Cinnamon Bark Essential Oil

Cinnamon barks were obtained from Bubakan village, Girimarto, Wonogiri (Indonesia). Essential oil was distilled from cinnamon bark using a steam distillation method. The cinnamon bark was ground in advance using a grinding machine into particle sizes of 7-15 mesh. The ground cinnamon bark was distilled for four hours (Khasanah et al., 2013).

Preparation of Edible Film

Edible films were prepared by dissolving tapioca starch (5 g) in distilled water (100 mL) at 70°C for 30 min. The solution was cooled down to 60°C and then glycerol (2 mL) was added. The solution was then heated at 60°C and stirred on a hotplate (Heidolph MR 3001 K) with a magnetic stirrer for 30 min. The solution was cooled to 30°C and cinnamon bark essential oil (0; 0.5; 1; 1.5 and 2%) and Tween 80 (1:1) were added. The solution was cast onto a 20 x 20 x 2 cm^3 plastic plate followed by cabinet drying at 75°C for 5 h. The dried films were cooled for 10 minutes in ambient conditions and peeled from the casting surface. The films were stored in a plastic container containing silica gel for further analysis. Each film was prepared in duplicate.

Analyses of Edible Film Characteristics

The characteristics of edible film were analyzed based on its thickness (McHugh et al., 1994), tensile strength (Gontard et al., 1993), water vapor transmission rate (Gontard et al., 1993), elongation (Gontard et al., 1993), and antibacterial activity (Manab et al, 2011). A micrometer (Krisbow 0.001 mm) was used to determine the thickness of films at five different spots of films. Tensile strength and elongation of the films were determined with Zwicki I Z 0.5 Universal Testing Machine. For water vapor transmission rate analysis, films were sealed in test cells with silica gel inside. The test cells were then stored in jars (70% RH by 27% w/v NaCl) at 28±2°C and weighed at 1 h interval during 8 hours. The water vapor transmission rate was calculated from the slope of the weight gain vs. time plot. Agar diffusion method was used to determine the antibacterial activity of films against *Pseudomonas flourescens* FNCC 0071 as meat spoilage indicator bacterium. Films (5 mm diameter) were placed on nutrient agar plate containing 10^6 CFU/mL bacteria, and incubated at 37°C for 24 h. The inhibition zones were measured in mm.

Application of Edible Film on Fresh Beef

The selected concentration (2%) of cinnamon bark oil edible films that performed the best characteristics was applied to beef. An edible film solution without oil addition was used to coat the control beef sample. The tenderloin part of beef was obtained from Jagalan Slaughterhouse (Surakarta, Indonesia) and cut into 30-45 g pieces (4 x 2.5 x 2.5 cm). The beef samples were dipped into edible film solutions and allowed to drip off and were then dried at 70°C for 1 hour. The coated samples were stored in styrofoam and wrapped with plastic in cold storage at 4 ± 1°C. Analyses were carried out periodically at 0, 5, 10, 15 and 20 days of storage. Each beef sample was prepared in duplicate.

Quality Analyses of Fresh Beef

The quality of fresh beef was assessed by considering total plate count (Utami et al., 2014), total volatile bases (Min et al., 2007) and thiobarbituric acid (Tokur et al., 2006).

Data Analysis

The data on edible film characteristics were subjected to one-way analysis of variance (ANOVA) at 0.05 significance level and differences in the mean values were determined by Duncan's test ($p<0.05$) by SPSS Statistics 16 program. The paired-sample T-test was conducted to determine the significant difference between control and treatment beef samples.

RESULTS AND DISCUSSION

Thickness

The addition of cinnamon bark essential oil did not affect the thickness of the films (Table 1). Edible film thicknesses ranged from 0.114 to 0.176 mm. Similar thicknesses were shown by the previous research on edible films made from apple puree incorporated with cinnamon oil, ranging from 0.127 to 0.137 mm (Du et al., 2009). However, Friedman et al. (2000) reported that increasing the concentration of cinnamon oil affected the film thickness, causing the total solids in the films to increase.

Water Vapor Transmission Rate

Water vapor transmission rate indicates the water vapor barrier properties of the edible films (Bahram et al., 2013). The results of water vapor transmission rate analysis are shown in Table 1. The water vapor transmission rate of the edible film without the addition of essential oil was 23.52 g/h.m^2. At a concentration of 2%, cinnamon bark oil was able to reduce the water vapor transmission rate to 21.38 g/h.m^2. Lower value of water vapor transmission rate indicates better protection from moisture of food. The addition of essential oils can enhance the film's hydrophobic compounds and thus reducing the water vapor transmission rate (Sánchez-González et al., 2011). This is because water vapor absorption occurs only in the hydrophilic portion of the molecule.

Table 1

Characteristics of edible film incorporated with various concentrations of cinnamon bark essential oil

Concentration of essential oil (%)	Mean thickness (mm)^{ns}	Mean water vapor transmission rate (g/h.m^2)*	Mean tensile strength (MPa)*	Mean elongation percent (%)*	Mean inhibition zone (mm)
0	0.114±0.024	23.52^{b}±0.45	0.69^{a}±0.06	159.91^{c}±2.44	13.75^{a}±3.18
0.5	0.130±0.028	22.24^{ab}±0.32	1.65^{b}±0.08	96.99^{b}±1.66	16.81^{a}±1.86
1	0.170±0.001	22.11^{ab}±0.65	1.57^{b}±0.24	89.25^{ab}±1.44	18.62^{ab}±1.59
1.5	0.175±0.046	22.20^{ab}±0.65	1.46^{b}±0.07	83.29^{ab}±30.93	22.65^{b}±0.49
2	0.176±0.001	21.38^{ab}±1.29	1.23^{c}±0.09	58.56^{a}±1.23	28.6^{c}±0.85

Note.* Significant; ^{ns} Not Significant; $^{a-c}$ Means in the same column with different letters are significantly different at $p < 0.05$; ± SD values

Tween 80 also affects the water vapor transmission rate of the films. The addition of Tween 80 can increase the permeability of the film (Carneiro-da-Cunha et al., 2009). However, the results of this study are not in accordance with the opinion of Carneiro-da-Cunha et al. (2009). The water vapor transmission rate of edible film with the addition of cinnamon bark oil decreased with an increasing concentration of essential oil. Another factor that affected water vapor transmission rate is the film thickness. According to McHugh et al. (1994), in hydrophilic films there is a positive relationship between the film thickness and the water vapor transmission rate. The addition of cinnamon bark essential oil also reduced the water vapor transmission rate of edible film made of cheese whey from 22.20 g.mm/kpa.day.m^2 to 17.56 g.mm/kpa.day.m^2 and further improved the film barrier properties against water vapor (Bahram et al., 2013).

Tensile Strength

Table 1 shows the tensile strength values of the films. Tensile strength values were increased by up to 1% after the addition of essential oils but then showed a decrease with 2% additional treatment. The negative control had 0.69 MPa of tensile strength value, whereas edible films with the addition of cinnamon bark essential oils had tensile strength values between 1.23 MPa and 1.65 MPa. The improved tensile strength of the film after the addition of cinnamon oil might be caused by changes in the water content of the film. Hosseini et al. (2009) reported that water content reduction of film made of chitosan with incorporated cinnamon oil led to a decreased tension and an increased tensile strength. The higher the concentration of essential oils in the edible film, the lower the tensile strength of the resultant film. A higher concentration of fat and *Zataria multiflora* essential oil added to film made from sodium-casein caused a decrease in the tensile strength of the film (Broumand et al., 2011). Weakening film tensile strength can be attributed to essential oil being added to the film solution, which induced the development of a heterogeneous film structure and influenced the tensile strength of the film (Zinoviadou et al., 2009). Carneiro-da-Cunha et al. (2009) reported that the addition of Tween 80 could reduce the tensile strength of film. In addition, Du et al. (2009) found out that the addition of essential oil of cinnamon could lower the tensile strength of film significantly.

Elongation

Edible film elongation percentages are shown in Table 1. The addition of cinnamon bark essential oil lowered the value of the elongation of the film significantly, from the original 159.91% to 58.56%. The addition of essential oil is able to create a compact film structure, thereby improving continuity in a network of polysaccharides, which leads to a decrease in the elongation. Cross-linker effect was produced by a strong interaction of polymer and cinnamon oil which reduced the free volume and the molecular mobility

of the polymer (Hosseini et al., 2009). However, the addition of Tween 80 affected the elongation percentage of the film negatively. Increasing the concentration of Tween 80 could cause a decrease in the elongation percentage of the film (Carneiro-da-Cunha et al., 2009). Rojas-Grau et al. (2006) reported that edible film made from apple puree with the addition of cinnamon bark essential oil had a decreased elongation percentage. Furthermore, Hosseini et al. (2009) also reported that the elongation percentage of edible film made of chitosan also decreased with the addition of cinnamon bark essential oil.

Antibacterial Activity

Antibacterial activity analysis was performed using the agar diffusion method. In an experiment by Manab et al. (2011), the analysis was performed using *Pseudomonas fluorescence* as spoilage indicator bacterium. The inhibition zones of the edible films are shown in Table 1. The inhibition zone of the edible film containing 2% of essential oils was 28.6 mm, which was higher than the negative control, with only 13.75 mm. Comparing with the control film, the addition of cinnamon bark essential oil in the edible film increased the inhibition zone between 3.06 and 14.85 mm. The higher the concentration of cinnamon bark essential oil in the edible film, the larger the inhibition zones. A higher concentration of essential oil can significantly increase the inhibition zones (Hosseini et al., 2009). Essential oil is well known for its antimicrobial compounds and its ability to control food spoilage and the growth of pathogenic bacteria (Du et al., 2009). Cinnamon oil contains about 85% of the active antimicrobial compound cinnamaldehyde (Friedman et al., 2004). According to El-Baroty et al. (2010), cinnamaldehyde can penetrate the membrane of microorganisms and react with enzymes and proteins as well as the membrane phospholipid bilayer, which causes disruption of microbial and enzyme systems or interferes with the function of the genetic materials. Bahram et al. (2013) reported that edible films made from whey protein when added with cinnamon bark essential oil and using *Pseudomonas putida* as spoilage indicator bacterium, had increased inhibition zones from 0 mm to 22.18 mm.

Based on the analysis of the characteristics of edible films, the best essential oil concentration is 2% because the treated film exhibited higher tensile strength and antibacterial activity compared with the films applied with the other essential oil concentrations.

Total Plate Count

The TPC of beef ranged from 4.17 to 4.23 log CFU/g (Figure 1) on the first day of storage and then increased significantly over 20 days of storage. The TPC of the negative control sample rose to 7.13 log CFU/g on the 20th day of storage and beef coated with edible coating enriched with cinnamon bark oil rose to 6.03 log CFU/g on the 20th day. The TPC of beef coated with edible coating enriched with cinnamon bark oil was significantly lower compared with the

control. Chen et al. (2014) reported that in the control sample and beef coated with a chitosan solution enriched with β-CD-citral, the TPC increased during storage, but the treatment samples showed a lower TPC than the control sample.

After 20 days of storage, the TPC of all beef samples (Figure 1) exceeded the limit of microbiological quality standard of fresh beef established by the National Standardization Agency of Indonesia (Standard Nasional Indonesia [SNI] 3932:2008, 2008), which is equal to 6 log CFU/g. The control beef exceeded the limit of microbiological quality standard on day 10, with a TPC of 6.26 log CFU/g, while beef coated with edible coating enriched with essential oil exceeded the limit of microbiological quality standard on day 20, with a TPC of 6.03 log CFU/g.

Total Volatile Bases (TVB) Content

The TVB values of the meat samples are shown in Figure 2. The TVB value of the control was 12.68 mg N/100 g and then rose to 46.69 mg N/100 g on the 20th day of storage. The TVB value of the beef coated with edible coating enriched with cinnamon bark essential oil was 12.63 mg N/100 g on the first day of storage and then rose to 30.53 mg N/100 g on the 20th day of storage. The TVB value of the control was significantly higher than the TVB value of the beef coated with edible film enriched with cinnamon bark essential oil. Chen et al. (2014) reported that the TVB value of the control beef and beef with chitosan coating and β-CD-citral increased during storage, but beef with chitosan coating treatment and β-CD-citral showed lower TVB values compared with the controls. Thus, citral addition may decrease the TVB value of beef.

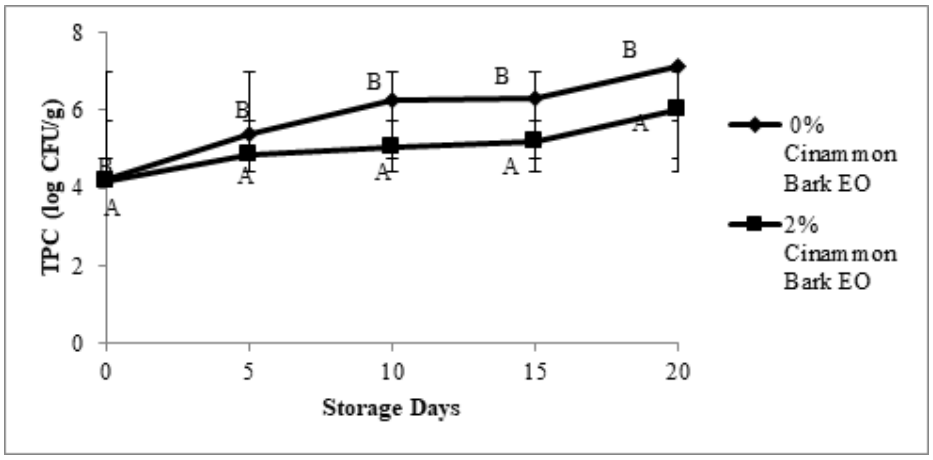

Figure 1. Total Plate Count (TPC) values of the control beef and beef coated with edible film enriched with 2% cinnamon bark essential oil during storage at low temperature (4 ± 1^{0}C) (Means with different letters are significantly different at $p < 0.05$; ± SD values)

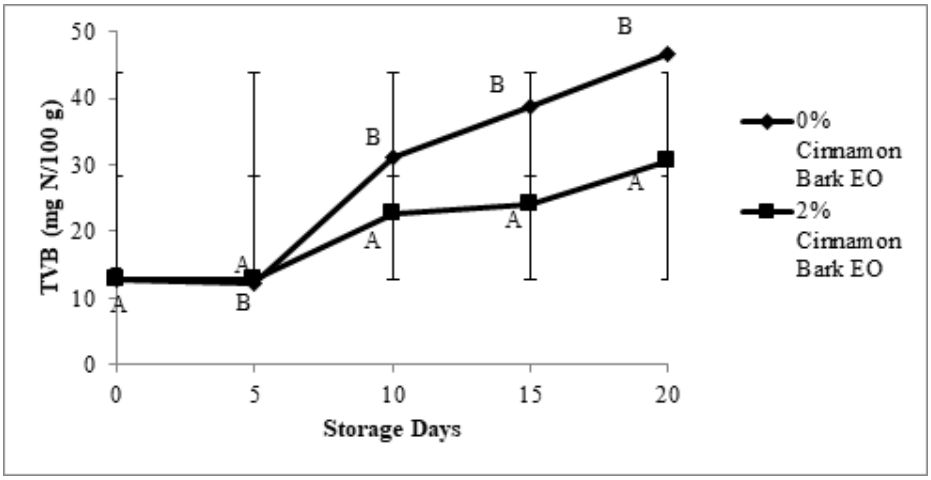

Figure 2. Total Volatile Bases (TVB) values of the control beef and beef coated with edible film enriched with 2% cinnamon bark essential oil during storage at low temperature (4 ± 1^{0}C) (Means with different letters are significantly different at $p < 0.05$; ± SD values)

An indication of livestock meat quality deterioration is an increase of TVB content. Xiao et al. (2014) indicated that the maximum TVB value for food products was 30 mg N/100 g. The TVB value of the beef control had exceeded the pre-defined maximum limit, which was equal to 31.10 mg N/100 g on day 10, and then to 46.69 mg N/100 g on day 20. Beef coated with edible film enriched with cinnamon bark essential oil passed the limit on day 20, with a TVB value of 30.53 mg N/100 g.

Thiobarbituric Acid

Measuring the thiobarbituric acid (TBA) value is one of the methods used to detect fat oxidation. The test is related to the level of aldehydes present in the oil. The TBA values of the control and beef coated with edible film enriched with cinnamon bark essential oil are shown in Figure 3. The TBA value of the control sample was 0.05 mg malonaldehyde/kg on day 1 and then rose to 0.68 mg malonaldehyde/kg on the 20th day of storage. The TBA value of the beef coated with edible film enriched with essential oil was originally 0.04 mg malonaldehyde/kg and then rose to 0.47 mg malonaldehyde/kg on day 20. The TBA value of the control was significantly higher compared with the TBA value of beef coated with edible film enriched with cinnamon bark essential oil. Chidanandaiah et al. (2009) also reported that the TBA values of the control and beef with a sodium alginate coating increased during storage, but beef with sodium alginate coating treatment had a lower TBA value compared with the controls.

The maximum TBA value that is still acceptable is 2.0 mg malonaldehyde per kg of meat. If the value exceeds this level, then the meat is considered rancid and unsafe for human consumption (Campo et al., 2005). Meanwhile, according to Kuo and Chu (2003), meat products with a TBA value

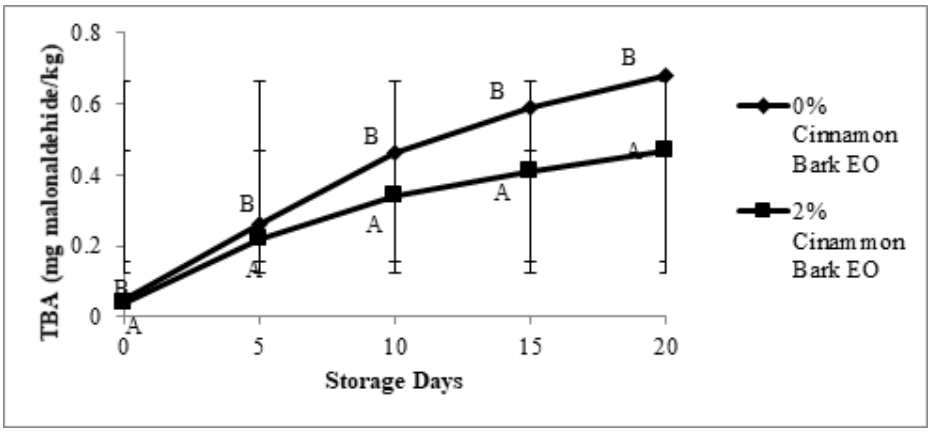

Figure 3. Thiobarbituric Acid (TBA) values of the control beef and beef coated with edible film enriched with 2% cinnamon bark essential oil during storage at low temperature (4 ± 1^{0}C) (Means with different letters are significantly different at $p < 0.05$; ± SD values)

between 0.5 and 2.0 malonaldehyde/kg have a rancid flavor. In the current experiment, the TBA value of the control exceeded the predetermined limit, which was equal to 0.59 mg malonaldehyde/kg on day 15 and 0.68 mg malonaldehyde/kg on day 20. These results indicate that the control beef is not suitable for consumption on day 15 of storage and onwards. However, based on TBA value, the beef coated with edible film enriched with 2% cinnamon bark essential oil is still suitable for human consumption because the TBA value has not exceeded the prescribed limit, which is equal to 0.47 mg malonaldehyde/kg.

Based on TPC and TVB analyses, the edible film enriched with 2% cinnamon bark essential oil is not suitable to preserve fresh beef at refrigerated temperature (4± 1^{o}C) for 20 days. However, the quality of beef coated with edible film enriched with cinnamon bark essential oil is still maintained after 15 days storage at refrigerated temperature. Edible film probably is more suitable to be used for processed meat rather than raw meat which could be stored at refrigerated temperature. The shelf life of cooked pork sausages coated with edible coating enriched with clove oil under refrigeration storage is 20 days (Lekjing, 2016).

CONCLUSION

Addition of cinnamon bark essential oil on the edible film does not affect the thickness of the film but it lowers water vapor transmission rate and results to elongation of the film. Also, increasing cinnamon bark essential oil improves tensile strength and antibacterial activity of the film. Overall, based on the film characteristics, a 2% cinnamon bark essential oil enriched film formula will improve preservation of fresh beef. The addition of 2% cinnamon bark oil to an edible coating is effective in reducing microbial growth and lipid oxidation as reflected in total plate count, total volatile

bases and thiobarbituric acid values of the beef samples. Thus, the application of edible film enriched with 2% cinnamon bark oil would maintain the freshness of beef as long as 15 days during storage.

ACKNOWLEDGEMENT

This work was partially financially supported by a research project of PNBP UNS No: 624/UN27.11/PL/2015 from Universitas Sebelas Maret, Indonesia.

REFERENCES

Bahram, S., Rezaei, M., Soltani, M., Kamali, A., Ojagh, S. M., & Abdollahi, M. (2013). Whey protein concentrate edible film activated with cinnamon essential oil. *Journal of Food Processing and Preservation*, *38*(3), 1251-1258. doi: 10.1111/jfpp.12086

Broumand, A., Emam-Djomeh, Z., Hamedi, M., & Razavi, S. H. (2011). Antimicrobial, water vapor permeability, mechanical and thermal properties of casein based *Zataria multiflora* Boiss. extract containing film. *LWT Food Science and Technology*, *44*(10), 2316–2323. doi:10.1016/j.lwt.2011.07.002

Campo, M. M., Nute, G. R., Hughes, S. I., Enser, M., Wood, J. D., & Richardson, R. I. (2005). Flavour perception of oxidation in beef. *Journal of Meat Science*, *72*(2), 303-311. doi: 10.1016/j.meatsci.2005.07.015

Carneiro-da-Cunha, M. G., Cerqueira, M. A., Souza, B. W. S., Souza, M. P., Teixeira, J. A., & Vicente, A. A. (2009). Physical properties of edible coatings and films made with a polysaccharide from *Anacardium occidentale* L. *Journal of Food Engineering*, *95*(3), 379–385. doi:10.1016/j.jfoodeng.2009.05.020

Chen, H., Shu, J., Li, P., Chen, B., Li, N., & Li, L. (2014). Application of coating chitosan film-forming solution combined β-CD-citral inclusion complex on beef fillet. *Journal of Food and Nutrition Research*, *2*(10), 692-697. doi: 0.12691/jfnr-2-10-7

Chidanandaiah, Keshri, R. C., & Sanyal, M. K. (2009). Effect of sodium alginate coating with preservatives on the quality of meat patties during refrigerated (4±1°C) storage. *Journal of Muscle Foods*, *20*(3), 275–292. doi: 10.1111/j.1745-4573.2009.00147.x

Du, W. X., Olsen, C. W., Avena-Bustillos, R. J., McHugh, T. H., Levin C. E., & Friedman, M. (2009). Effects of allspice, cinnamon, and clove bud essential oils in edible apple films on physical properties and antimicrobial activities. *Journal of Food Science*, *74*(7), M372-M378. doi: 10.1111/j.1750-3841.2009.01282.x

El-Baroty, G. S., Abd El-Baky, H. H., Farag, R. S., & Saleh, M. A. (2010). Characterization of antioxidant and antimicrobial compounds of cinnamon and ginger essential oils. *African Journal of Biochemistry Research*, *4*(6), 167-174.

Friedman, M., Henika, P. R., Levin, C. E., & Mandrell, R. E. (2004). Antibacterial activities of plant essential oils and their components against *Escherichia coli* O157:H7 and *Salmonella enterica* in apple juice. *Journal of Agricultural and Food Chemistry*, *52*(19), 6042–6048. doi: 10.1021/jf0495340

Friedman, M., Kozukue, N., & Harden, L. A. (2000). Cinnamaldehyde content in foods determined by gas chromatography–mass spectrometry. *Journal of Agricultural and Food Chemistry*, *48*(11), 5702–5709. doi: 10.1021/jf000585g

Gaman, P. M., & Sherrington, K. B. (1994). *Ilmu pangan: Pengantar ilmu pangan, nutrisi dan mikrobiologi* [Food science: Introduction to food science, nutrition and microbiology]. Yogjakarta, Indonesia: Universitas Gadjah Mada Press.

Gómez-Estaca, J., Lacey, A. L., López-Caballero, M. E., Gómez-Giillen, M. C., & Montero, P. (2010). Biodegradable gelatin-chitosan films incorporated with essential oil as antimicrobial agents for fish preservation. *Food Microbiology*, *27*(7), 889-896. doi: 10.1016/j.fm.2010.05.012

Gontard, N., Guilbert, S., & Cuq, J. L. (1993). Water and glycerol as plasticizer affect mechanical and water barrier properties of an edible wheat gluten film. *Journal of Food Science*, *58*(1), 206-211. doi: 10.1111/j.1365-2621.1993.tb03246.x

Hosseini, M. H., Razavi, S. H., & Mousavi, M. A. (2009). Antimicrobial, physical and mechanical properties of chitosan-based films incorporated with thyme, clove and cinnamon essential oils. *Journal of Food Processing and Preservation*, *33*(6), 727-743. doi: 10.1111/j.1745-4549.2008.00307.x

Kester, J. J., & Fennema, O. R. (1986). Edible films and coatings: A review. *Food Technology*, *40*(12), 47–59.

Khasanah, L. U., Anandito, R. B. K., Utami, R., Muhammad D. R. A., & Lukman, M. (2013). Pemisahan komponen minyak atsiri (*Cinnamomum burmanii*) dengan destilasi fraksinasi pada berbagai tingkat suhu destilasi [Separation of essential oil components (*Cinnamomum burmanii*) by fractionation distillation at various distillation temperature levels]. In S. I. Santoso, B. T. Eddy, A. Hermawan, J. Triastono, E. Kushartanti, F. D. Ariyanti, … K. Budirahardjo (Eds.), *Proceedings of Agribusiness National Conference 2013* (pp. 353-359). Semarang, Indonesia: Badan Penerbit Universitas Diponegoro Semarang.

Kuo, C. C., & Chu, C. Y. (2003). Quality characteristic of Chinese sausages made from PSE pork. *Journal of Meat Science*, *64*(4), 441-449. doi: 10.1016/S0309-1740(02)00213-9

Lekjing, S. (2016). A chitosan-based coating with or without clove oil extends the shelf life of cooked pork sausages in refrigerated storage. *Meat Science*, *111*, 192-197. doi:10.1016/j.meatsci.2015.10.003

Manab, A., Sawitri, M. E., Al Awwaly, K. U., & Purnomo, H. (2011). Antimicrobial activity of whey protein based edible film incorporated with organic acids. *African Journal of Food Science*, *5*(1), 6–11.

Matan, N. (2012). Antimicrobial activity of edible film incorporated with essential oils to preserve dried fish (*Decapterus maruadsi*). *International Food Research Journal*, *19*(4), 1733-1738.

McHugh, T. H., Aujard, J. F. & Krochta J. M. (1994). Plasticized whey protein edible films: Water vapor permeability properties. *Journal of Food Science*, *59*(2), 416-419. doi: 10.1111/j.1365-2621.1994.tb06980.x

Miksusanti, H, & Masril K. I. (2013). Antibacterial and antioxidant of uwi (*Dioscorea alata* L.) starch edible film incorporated with ginger essential oil. *International Journal of Bioscience, Biochemistry and Bioinformatics*, *3*(4), 354-356. doi: 10.7763/IJBBB.2013.V3.230

Min, J. S., Lee, S. O., Jang, A., Jo, C., Park, C. S., & Lee, M. (2007). Relationship between the concentration of biogenic amines and volatile basic nitrogen in fresh beef, pork, and chicken meat. *Asian-Australasian Journal of Animal Sciences*, *20*(8), 1278-1284.

Oussalah, M., Caillet, S., Salmieäri, S., Saucier, L., & Lacroix, M. (2004). Antimicrobial and antioxidant effects of milk protein-based film containing essential oils for the preservation of whole beef muscle. *Journal of Agricultural and Food Chemistry*, *52*(18), 5598–5605. doi: 10.1021/jf049389q

Rojas-Grau, M. A., Avena-Bustillos, R. J., Friedman, M., Henika, P. R., Martiän-Belloso, O., & Mchugh, T. H. (2006). Mechanical, barrier, and antimicrobial properties of apple puree edible films containing plant essential oils. *Journal of Agricultural and Food Chemistry*, *54*(24), 9262-9267. doi: 10.1021/jf061717u

Sánchez-González, L., Vargas, M., González-Martínez, C., Chiralt, A., & Cháfer, M. (2011). Use of essential oils in bioactive edible coatings. *Food Engineering Reviews*, *3*(1), 1–16. doi: 10.1007/s12393-010-9031-3

Standard Nasional Indonesia. (2008). *Mutu karkas dan daging sapi SNI 3932:2008* [Quality of beef carcass and meat SNI 3932:2008]. Jakarta, Indonesia: Indonesian National Standards.

Tokur, B., Korkmaz, K., & Ayas, D. (2006). Comparison of two thiobarbituric acid (TBA) methods for monitoring lipid oxidation in fish. *Journal of Fisheries and Aquatic Sciences*, *23*(3-4), 331–334.

Utami, R., Kawiji, Nurhartadi, E., Putra, A. Y. T., & Setiawan, A. (2014). The effect of cassava starch-based edible coating enriched with *Kaempferia rotunda* and *Curcuma xanthorrhiza* essential oil on refrigerated patin fillets quality. *International Food Research Journal*, *21*(1), 413–419.

Wang, R., Wang, R., & Yang, B. (2009). Extraction of essential oils from five cinnamon leaves and identification of their volatile compound compositions. *Innovative Food Science and Emerging Technologies*, *10*(2), 289–292. doi:10.1016/j.ifset.2008.12.002

Xiao, K., Gao, G., & Shou, L. (2014). An improved method of detecting pork freshness based on computer vision in on-line system. *Sensor and Transducers*, *169*(4), 42-48.

Zinoviadou, K. G., Kroutsoumanis, K. P., & Biliaderis, C. G. (2009). Physico-chemical properties of whey protein isolate films containing oregano oil and their antimicrobial action against flora of fresh beef. *Meat Science*, *82*(3), 338–345. doi:10.1016/j.meatsci.2009.02.004

Pertanika J. Trop. Agric. Sc. 42 (4): 1185 - 1208 (2019)

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Journal homepage: http://www.pertanika.upm.edu.my/

Antibacterial Properties of Kelulut, Tualang and Acacia Honey against Wound-Infecting Bacteria

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ABSTRACT

Bacterial infection is the most common cause of contamination that affects wound healing. This study aims to investigate the bacteriostatic and bactericidal effects of three varieties of Malaysian honey represented by two polyfloral honey varieties - Kelulut and Tualang, as well as one monofloral honey – Acacia, against eight common bacteria that infect wounds. The factors contributing to the antibacterial properties of honey such as acidity, peroxide compounds, and non-peroxide compounds, were determined using the agar well diffusion assay method and compared with medical-grade Manuka honey used in wound care (UMF 18 +). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using honey concentrations of 1.3% to 90% (w/v). The MICs for Kelulut, Tualang, and Acacia ranged from 5% to 12.5% (w/v), 12.5% to 30% (w/v), and 25% to 50% (w/v) respectively. Meanwhile, the MBCs were found to range from 5% to 12.5% (w/v), 12.5% to 90% (w/v), and 25% to 90% (w/v) respectively. Kelulut showed the highest inhibition activity. The antibacterial properties of Malaysian honey were generally comparable to Manuka. However, Kelulut bore the closest resemblance and was highly dependent on an acidic environment as the major antibacterial factor. This effect was further

ARTICLE INFO

Article history:
Received: 16 May 2019
Accepted: 20 August 2019
Published: 13 November 2019

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ISSN: 1511-3701
e-ISSN: 2231-8542

supported by the presence of peroxide and non-peroxide compounds.

Keywords: Antibacterial properties, honey, non-peroxide activity

INTRODUCTION

Honey consists of mostly glucose, fructose and trace elements of minerals, vitamins, proteins and amino acids. It can be classified into monofloral and polyfloral honey (Bradbear, 2009) based on botanical resources. Monofloral honey is produced by bees that have been foraging predominantly on one type of plant only, while polyfloral honey originates from several types of plants (Matzen et al., 2018; Ranneh et al., 2018; Samat et al., 2014). Honey possesses a number of pharmacological benefits such antibacterial (Kateel et al., 2018), anti-inflammatory (Liu et al., 2013; Nooh & Nour-eldien, 2016), antioxidant (Liu et al., 2013; Ranneh et al., 2018), and wound healing or tissue repair characteristics (Adewumi & Ogunjinmi, 2011; Rashidi et al., 2016). Recently, the usage of honey as an antibacterial agent in the treatment of ulcers (Kateel et al., 2018), wounds (El-malek et al., 2017), and other surface infections (Mcloone et al., 2016) is gaining traction in the medical industry.

Microbial infections are caused by the presence of microbes such as bacteria, fungi, and viruses; the most common being bacterial infections that often affect wound healing in humans (Yang et al., 2017). Sufficient numbers of bacteria can cause repair mechanisms, such as graft and flap formation, to fail (Sussmann & Bates-Jensen, 2012). Wound infections can be caused by Gram-positive and Gram-negative bacteria. *Staphylococcus* sp., *Streptococcus* sp., *Enterococcus* sp., *Escherichia coli*, *Klebsiella* sp., *Proteus* sp. and *Pseudomonas aeruginosa* are among the common causes of bacterial infections in foot ulcers (Kateel et al., 2018), skin ulcers (Yang et al., 2017), post-surgical wounds (Kasithevar et al., 2017) and chronic wounds (El-malek et al., 2017; Nasir et al., 2016; Sienkiewicz et al., 2016). In Malaysia, *Staphylococcus aureus* (Mustafa et al., 2015; Nasir et al., 2010, 2016), *P. aeruginosa* (Nasir et al., 2010, 2016), and *Klebsiella pneumoniae* (Low et al., 2017; Nasir et al., 2010) are commonly associated with wound infections. These bacteria are capable of developing multidrug resistance towards antibiotics, giving rise to superbugs such as methicillin-resistant *S. aureus* (MRSA) (Bereket et al., 2012), multidrug-resistant *P. aeruginosa* (Bereket et al., 2012), and carbapenem resistant *K. pneumoniae* (Low et al., 2017). There has been a call to reduce the usage of antibiotics in order to prevent the emergence of such resistant bacteria. Hence, it is important to explore antibacterial agents that do not involve the usage of antibiotics.

The antibacterial properties of honey are attributed to its osmotic effect (Mandal & Mandal, 2011; Molan, 1992), acidity (Bogdanov, 1997; Molan, 1992), and presence of peroxide and non-peroxide compounds (Kwakman et al., 2010; Zainol et al., 2013). The osmotic effect

of honey is due to its low water content, which is produced by strong interactions between sugar and water molecules, thus reducing the amount of water available for microorganisms (Mandal & Mandal, 2011). During the ripening of nectar, enzymatic action produces gluconic acid, which in turn increases the acidity of the honey (Molan, 1992). Since the optimum pH range for bacteria is from 7.2 – 7.4 (Molan, 1992), the pH of honey, which is between 3.4 and 5.4, inhibits bacterial growth (Bogdanov, 1997). Aside from osmotic and acidic characteristics, peroxide and non-peroxide compounds were identified as dominant bio-active components responsible for the antibacterial properties of most types of honey (Irish et al., 2011; Kwakman & Zaat, 2012; Mandal & Mandal, 2011). Peroxide compounds, usually represented by hydrogen peroxide (H_2O_2), cause an increase in oxidative stress, which is beneficial when it comes to controlling bacterial colonization in wound areas (Brudzynski et al., 2011; Zainol et al., 2013). The presence of non-peroxide compounds, such as phenolic compounds (Kwakman & Zaat, 2012), antimicrobial peptides (AMP) (Kwakman et al., 2011; Kwakman & Zaat, 2012), flavonoids, leptosperin (Roberts et al., 2015), and methylglyoxal (MGO) (Kwakman et al., 2011; Kwakman & Zaat, 2012) are considered unique since the compounds were not presented in all honey for inhibiting bacterial growth. In Malaysia, various types of honey, such as Tualang, Kelulut, Acacia, and Gelam, have been reported to possess antibacterial properties due to the presence of the aforementioned factors (Zainol et al., 2013).

This study aims to explore the antibacterial properties of Malaysian honey against common infectious bacteria. Tualang, Kelulut, and Acacia honey were selected to be evaluated against eight bacterial strains: *S. aureus*, *Streptococcus pyogenes*, *Enterococcus faecalis*, *E. coli*, *P. aeruginosa*, *Salmonella typhimurium*, *Proteus mirabilis* and *K. pneumoniae*. These bacteria are widely known to cause wound infections (El-malek et al., 2017; Kasithevar et al., 2017; Kateel et al., 2018; Nasir et al., 2016; Sienkiewicz et al., 2016; Yang et al., 2017) and have the potential to develop drug resistance (Bereket et al., 2012; Low et al., 2017). We believe that this is the first study to consider a large number of bacteria strains associated with wound contamination in local patients (Low et al., 2017; Mustafa et al., 2015; Nasir et al., 2010, 2016) that have not been evaluated with Malaysian Kelulut and Acacia. The antibacterial properties were evaluated based on the bacteriostatic and bactericidal effects through the determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The naturally acidic environment, and peroxide and non-peroxide compounds were identified as factors responsible for the antibacterial properties of honey. The antibacterial properties of Malaysian honey were evaluated and compared with the medical-grade Manuka honey used for wound care (UMF 18 +).

MATERIALS AND METHODS

Honey Samples

Honey samples were obtained from a local apiarist (Bee Park Pahang Sdn. Bhd., Bayu Gagah Marketing Sdn. Bhd., and Federal Agricultural Marketing Authority (FAMA) Corporation Sdn. Bhd., Malaysia) in sterile glass bottles. Prior to obtaining the honey samples, the quality and authenticity of all honey samples were approved by Malaysian Agriculture Research and Development Institute (MARDI), and Food Quality and Safety Research and Development (UNIPEQ). The three types of Malaysian honey considered in this study were Tualang, Kelulut, and Acacia. The commercially available medical-grade Manuka honey (*Comvita*® Wound care UMF 18+, New Zealand) was used as a basis of comparison in order to validate the reliability of this study.

Bacteria

Eight wound-associated bacteria commonly known to infect wounds were used in this study. These bacteria were kindly supplied by the Department of Pathology and Laboratory Medicine, International Islamic University Malaysia Medical Centre (IIUMMC), and Central Laboratory Universiti Malaysia Pahang (UMP), all labelled as standard strains from the American Type Culture Collection (ATCC, US). Three of the eight bacteria were Gram-positive bacteria – *S. aureus* ATCC 6538, *S. pyogenes* ATCC 19615, and *E. faecalis* ATCC 29212, while the other five bacteria were Gram-negative – *E. coli* ATCC 8739, *P. aeruginosa* ATCC 9027, *S. typhimurium* ATCC 14028, *P. mirabilis* ATCC 12453 and *K. pneumoniae* ATCC BAA 1144. The bacteria were cultured on nutrient or soy agar and incubated at 37ºC for 24 h, during which they were known as primary cultures. Working bacterial cultures were prepared by inoculating a loop of primary culture into the sterile screw-capped test tubes containing 10 mL of nutrient or soy broth. These cultures were incubated in an incubator shaker (Infors AG CH-4103 Bottmingen) for 24 h at 37ºC and rotational speed of 150 rpm.

Preparation of Honey Samples

The honey samples were diluted to a range of concentrations in preparation for future assays. For the evaluation of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), Kelulut, Tualang, Acacia, and Manuka samples were first diluted to a range of 30% and 90% (w/v) in water, then further diluted in broth to a final concentration of 1.25% to 30% through a stepwise two-fold dilution. For both evaluations, sugar-based (SB) samples containing the major sugar compounds which are commonly present in honey were used as artificial honey. These sugar compounds are fructose, glucose, maltose, and sucrose (Ahmed & Othman, 2013; Jalil et al., 2017). High concentrations of SB solutions were prepared by mixing 40% (w/v) fructose (Sigma, US), with 30% (w/v) glucose (Sigma, US), 8% (w/v) maltose (Sigma, US), and 2% (w/v) sucrose (Sigma, US).

In addition to MIC and MBC evaluations, efforts were also made to verify the presence of peroxide compounds and the factors contributing to antibacterial properties possessed by each type of honey by studying the effects of acidity, peroxide compounds, and osmotic pressure. In this case, each honey sample was diluted to a final concentration ranging between 30% and 90% (w/v).

Minimum Inhibitory Concentration (MIC)

The MIC of each bacterium was determined by using the method previously described in Tan et al. (2009) and Zainol et al. (2013) with slight modifications. This assay was performed in a sterile 96-well flat-bottomed polystyrene microtitre plates (Nunclon™, Thermo Fisher Scientific). A working bacteria culture was prepared accordingly and adjusted to a final concentration of 1 x 10^8 colony forming units (CFU/mL), which is equal to 0.5 McFarland standard. The adjusted concentration of bacteria at 0.5 McFarland was prepared based on optical density by diluting the working bacteria culture in fresh sterile broth until the absorbance ranged from 0.08 and 0.13 (Franklin et al., 2012). The absorbance of the prepared cultures was measured using UV-viscometer (Shimadzu, Japan) at a reference wavelength of 600 nm. The honey samples, prepared as described in the previous section, were dispensed into the test and control wells. From each concentration of honey sample, 190 µL was aseptically transferred into the prepared 96-well plate containing 10 µL of adjusted cultures. Cultures without honey samples served as the positive control while wells containing only nutrient broth and honey samples served as negative controls.

The samples were incubated in the incubator shaker (Infors AG CH-4103 Bottmingen) at 37ºC, 120 rpm for 24 h. The absorbance of the samples was measured at a time prior to incubation (known as $t = 0$), and at an elapsed time after 24 h of incubation (known as $t = 24$) at a wavelength of 590 nm. The percentage of growth inhibition, also known as MIC was calculated for each sample using Equation (1).

$$MIC = [1 - \frac{Absorbance\ of\ the\ test\ well}{Absorbance\ of\ corresponding\ control\ well}] \times 100\%$$

Eq. 1

The MIC values were expected to fall between 0% and 100%, where 0% indicated no effect on bacterial growth, and 100% indicated detrimental effects on bacterial growth. The MIC value refers to the lowest concentration of a test material which results in up to 95% growth inhibition in the test organism. All MIC values from Kelulut, Tualang, and Acacia were compared to those obtained from Manuka, and SB solutions. In addition, the growth of the bacteria was plotted to study the inhibition profiles of each honey sample. The growth inhibition responses for all tested bacteria were plotted with respect to honey concentrations ranging from 5% to 90% (w/v).

Minimum Bactericidal Concentration (MBC)

The MBC evaluation is a continuation of the MIC assessment. The MIC does not identify whether or not the bacteria is killed. Therefore, an evaluation of MBC was required. To evaluate MBC, the wells which did not show visible growth after the MIC evaluation were taken into consideration. One loopful of suspension from clear wells were subcultured on freshly prepared Trypticase Soy Agar (TSA) using the spread plate method (Zainol et al., 2013). The cultures were spread evenly on the agar surface before being incubated at 37ºC for 24 h. These samples were then examined for colony formation, which were taken to be a sign of bacterial growth. Three biological replicates were performed for each test. The concentration honey was considered as bacteriostatic if growth occurred after being cultured on the TSA, and bactericidal when inhibition of growth persisted (Zainol et al., 2013). The lowest concentration showing no growth of test organisms was considered to be the MBC. All MBC values from Kelulut, Tualang, and Acacia were compared to those obtained from Manuka, and SB solutions.

Preparation of Untreated (UT), Peroxide Non-peroxide (PNP), and Non-peroxide (NP) Samples

In this section, an attempt was made to identify the role of acidic pH, and peroxide and non-peroxide compounds that contribute to antibacterial properties of honey. Each honey sample was diluted in deionised water to a final concentration from 30% to 90% (w/v). For this assay, seven honey samples that were not subjected to any catalase treatment were prepared at a volume of 2 mL each and were denoted as untreated honey (UT). In UT samples, the acidic pH, peroxide compounds, and non-peroxide compounds were preserved because no elimination agents were added. This preparation was repeated to evaluate peroxide non-peroxide (PNP), and non-peroxide (NP) activity to verify the role of peroxide compounds in contributing to antibacterial properties. A non-acidic honey sample can be achieved through titration using of 5% (w/v) NaOH until the pH of the honey reached 7.0. A non-acidic, peroxide-free sample requires both titration using 5% (w/v) NaOH, and addition of catalase solution to the honey samples to catalyze the decomposition of hydrogen peroxide to water (Kwakman et al., 2010, 2011). The pH for the prepared solutions were measured. The UT, PNP and NP solutions are described in Table 1.

As for the catalase solution, a concentration of 4000 unit/mL was used (Adams et al., 2008). It was prepared by adding 10 mg of catalase (Sigma, US) to ultra-pure water to make a final volume of 5 mL. It has been suggested that the efficacy of catalase in removing peroxide compounds in water should be tested (Brudzynski et al., 2011; Zainol et al., 2013). To do so, six different sets of experiments were conducted: 1) 50% (w/v) Kelulut, Tualang, Acacia, and Manuka in pure water; 2) 50% (w/v) of honey in pure water with an addition of catalase solution; 3) 50% (w/v) of honey in pure water with an addition of

1% (w/v) hydrogen peroxide (Bendosen, Malaysia); 4) 50% (w/v) of honey in pure water with an addition of 1% (w/v) hydrogen peroxide and catalase solution; 5) 1% of hydrogen peroxide in pure water; and 6) 1% of hydrogen peroxide with an addition of catalase solution. The solutions were tested in the same way as agar well diffusion assay. Each test was carried out in triplicate and the average values were calculated. The catalase solution was considered effective in removing hydrogen peroxide compounds if there was no increase in the inhibition zone of sample after the addition of 1% hydrogen peroxide.

Evaluation of Non-peroxide and Peroxide Activities in UT, PNP, and NP Samples

After the sample preparation, peroxide and non-peroxide activity were determined using the method previously described in Moussa et al. (2012) with slight modifications. These evaluations were performed on one type of Gram-negative bacteria (*S. aureus* ATCC 6538) and one type of Gram-positive bacteria (*E. coli* ATCC 8739). These bacteria were selected for their dominance in wound infection, clear and measurable inhibition zone on agar, and their potential of developing antibiotic-resistance. Nutrient agar was prepared according to the manufacturer's instructions. It was allowed to cool after being autoclaved at 100 kPa, 121ºC for 20 min. After a uniform swirling, the agar was poured into petri dishes and stored at 4ºC for 30 min to solidify. The bacteria cultures were prepared and adjusted to 0.5 McFarland standard, which is equivalent to 1.5 x 10^8 CFU/mL. All agar plates were inoculated using the

Table 1

Detailed description of UT, PNP and NP solutions

Solution	Honey	pH	Activity
UT	Kelulut	2.37 ±0.13	Honey was diluted with deionized water. Solutions with concentrations of 30% to 90% (w/v) of honey samples were prepared.
	Tualang	3.88 ±0.04	
	Acacia	4.25 ±0.09	
	Manuka	3.80 ±0.03	
PNP	Kelulut	7.37 ±0.22	Honey was diluted with deionized water and followed by titration with 5% (w/v) NaOH to neutralize the pH. Solutions with concentrations of 30% to 90% (w/v) of honey samples were prepared.
	Tualang	7.35 ±0.22	
	Acacia	7.27 ±0.11	
	Manuka	7.23 ±0.29	
NP	Kelulut	7.40 ±0.15	Honey was diluted with catalase solution at concentration of 4000 unit/ mL and followed by titration with 5% (w/v) NaOH to neutralize the pH. Solutions with concentrations of 30% to 90% (w/v) of honey samples were prepared.
	Tualang	7.29 ±0.27	
	Acacia	7.30 ±0.22	
	Manuka	7.27 ±0.24	

Note. The symbol ± represents the standard deviation, which was calculated between the three biological replicates

spread plate technique by spreading 100 µL of the adjusted 0.5 McFarland culture on the surface of the agar. After inoculation, wells of 6 mm in diameter were cut into the agar surface and filled with 80 µL of the test solutions. Manuka honey (UMF 18+) was taken to be the positive control, while sterile ultra-pure water and 4000 units/mL catalase solution were used as the negative control wells. Plates were incubated at 37ºC for 24 h. The diameters of the clear inhibition zones were measured in millimeter (mm), inclusive of the diameter of the well. Three biological replicates were performed for each assay.

RESULTS

Results of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Manuka honey has been very well studied in terms of its antibacterial properties against a number of bacteria (Adams et al., 2008; Henriques et al., 2011; Roberts et al., 2015). However, studies on the antibacterial properties of Malaysian honey varieties such as Kelulut and Tualang are limited (Ismail, 2016). Acacia, a monofloral honey available in Malaysia, is produced using the nectar of the *Acacia mangium* tree. It is known for its antioxidant properties and is associated with therapeutic medicinal effects such as inhibition of cancer cell growth (Salleh et al., 2017). Recently, Acacia has been studied for its antibacterial properties against several infectious bacteria and was found to possess both bacteriostatic and bactericidal effects (Zainol et al., 2013). Hence, this study includes Acacia in order to further understand its activities against infectious bacteria.

In this study, the MIC and MBC of Kelulut, Tualang, and Acacia were determined by using Manuka and SB used as the basis of comparison. The minimum bacteriostatic and bactericidal concentrations for all the bacterial strains used in this study are tabulated in Table 2. From the eight listed bacteria, Kelulut was observed to inhibit the growth of *P. aeruginosa*, *E. coli* and *P. mirabilis* growth at low concentrations of 3.75%, 7.5%, and 7.5% (w/v) respectively when compared with Tualang and Acacia. This value was 4.0, 1.3, and 1.7- fold stronger than Manuka. These findings are consistent with results shown by previous studies that examined the antibacterial activity of stingless bee honey from Borneo against bacteria associated with animals (Tuksitha et al., 2018). The MIC of Kelulut was approximately 10% (w/v) for *S. pyogenes*, similar to Manuka. However, other bacteria such as *K. pneumoniae*, *S. typhimurium*, *S. aureus* and *E. faecalis* were 2.0, 1.2, 2.0, and 1.3 times were more susceptible to Manuka respectively when compared to Kelulut. Acacia was the least strong bacteriostatic honey with MIC ranging between 30% and 50% while Tualang had MIC ranging between 20% and 40%. However, the results showed a different pattern for the MBC evaluation. Manuka honey was observed to be a stronger bactericidal agent for all bacteria except *P. aeruginosa* as compared to Kelulut, Tualang and Acacia

Table 2

Minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Malaysian honey

	Kelulut		Tualang		Acacia		Manuka		SB	
	MIC†	MBC†	MIC†	MBC†	MIC†	MBC†	MIC†	MBC†	MIC†	MBC†
E. coli ATCC 8739	7.5%	40%	25%	>90%*	40%	>90%*	10%	12.5%	40%	>90%*
P. aeruginosa ATCC 9027	3.75%	12.5%	20%	40%	30%	50%	15%	20%	40%	>90%*
K. pneumoniae ATCC BAA 1144	12.5%	30%	30%	80%	40%	>90%*	6.25%	10%	40%	>90%*
S. typhimurium. ATCC 14028	7.5%	25%	20%	60%	40%	>90%*	6.25%	10%	40%	>90%*
P. mirabilis ATCC 12453	7.5%	25%	25%	90%	40%	>90%*	12.5%	15%	50%	>90%*
S. aureus ATCC 6538	10%	30%	20%	50%	30%	>90%*	5%	5%	50%	>90%*
S. pyogenes ATCC 19615	10%	20%	30%	90%	40%	>90%*	10%	20%	40%	>90%*
E. faecalis ATCC 29212	20%	50%	40%	>90%*	50%	>90%*	15%	25%	50%	>90%*

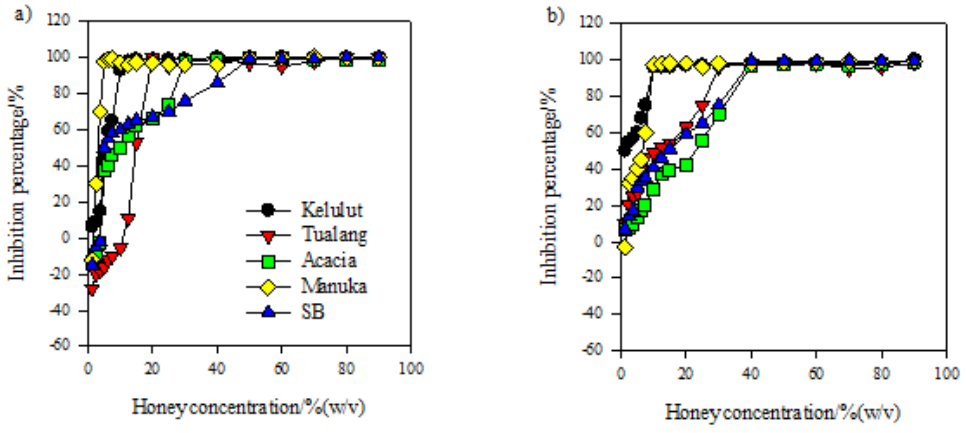
Note. The symbol * represents the highest percentage of honey concentration tested. † denotes a statistically significant difference between the mean of the sampled population and the hypothesised population mean. P-values were calculated based on the Student t-test at confidence interval of 95%. The test was performed on five biological replicates

honey. Kelulut was recorded to be the most potent antibacterial agent to *P. aeruginosa*, indicated by the lowest MIC and MBC values in both evaluations. These results are in good agreement with the previous work (Tuksitha et al., 2018).

Results of Growth Inhibition Profile of Gram-positive and Gram-negative Bacteria

Our second attempt began by studying bacterial growth response when Kelulut, Tualang, and Acacia samples were added to Gram-positive and Gram-negative bacteria. The experiments were performed with Manuka and SB as a basis of comparison. The results obtained were in the form of growth inhibition percentages at concentrations ranging from 1.25% to 90% (w/v). These results are shown in Figure 1 (for Gram-positive bacteria) and Figure 2 (for Gram-negative bacteria). In both figures, negative inhibition percentage may indicate that the concentration of glucose in honey was not enough to inhibit bacterial growth through osmotic pressure, but instead sufficient to support bacterial growth. Similar negative growth inhibition percentages were reported by previous research on bacteria such as *Bacillus cereus*, *Enterobacter clocae*, *P. mirabilis* and *Streptococcus agalactiae* (Tan et al., 2009; Zainol et al., 2013).

Klebsiella pneumoniae. According to Figure 2, Kelulut inhibited most of the Gram-negative bacteria at concentrations as low as 1.25% (w/v) except for *K. pneumoniae*. *Klebsiella pneumoniae* was observed to be more susceptible to Manuka and Kelulut – concentrations of 7.5% (w/v) already began to affect bacteria growth. On the contrary, the growth of this strain was less affected by Tualang and Acacia at concentrations less than 15% (w/v).



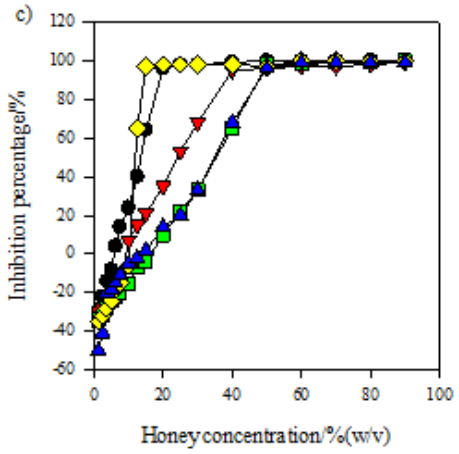


Figure 1. Growth inhibition percentage for Gram-positive bacteria of: a) *Staphylococcus aureus* ATCC 6538, b) *Streptococcus pyogenes* ATCC 19615, and c) *Enterococcus faecalis* ATCC 29212. Kelulut (circle, black), Tualang (triangle downward, red), Acacia (square, green), Manuka (diamond, yellow), and SB (triangle upward, blue)

Escherichia coli and Salmonella typhimurium. Our findings showed that less effort was needed to inhibit the growth of *E. coli* – the lowest concentration of all honey samples and SB solutions produced growth inhibition percentages ranging from 30% (Tualang), to 70% (Kelulut). However, the percentage of inhibition increased gradually when this strain was exposed to Acacia and Tualang. It took a concentration of more than 50% (w/v) for this strain to be 100% inhibited. *S. typhimurium* has similar response trends to *E. coli* when exposed to Tualang and Acacia.

Pseudomonas aeruginosa. As for *P. aeruginosa*, 100% growth inhibition was observed at a 3.75% (w/v) concentration of Kelulut, thus demonstrating its susceptibility to this variety of honey. However, it showed a strong resistance towards Manuka and Tualang. There was no growth inhibition for concentrations less than 12.5% (w/v) of Manuka, but the percentage abruptly jumped to 97% when the concentration was increased to 15% (w/v). A similar pattern was observed in Tualang. A mild resistance was demonstrated towards Acacia at concentrations of less than 5% (w/v).

Proteus mirabilis. Unlike other Gram-negative bacteria, *P. mirabilis* showed a strong resistance to Acacia honey. The addition of this honey only began to take effect at a concentration of 15% (w/v). The strongest growth inhibition for this strain occurred at the lowest concentration of Kelulut, which inhibited growth by 76%.

Staphylococcus aureus. This strain has different responses to different types of honey. The growth of *S. aureus* was more susceptible to Kelulut, starting at the lowest concentration of 1.25% (w/v). However, it was less susceptible to Tualang, requiring concentrations above 12.5% (w/v) for growth inhibition to begin. There was an abrupt increase in growth inhibition from 10% to 100% when the concentration of Tualang was increased from 12.5% to 20% (w/v). Interestingly, rather than reaching a plateau at absolute inhibition, the percentage dropped by ±5% at 95% and then slowly increased to 100% inhibition. This suggests that this strain may have developed resistance towards Tualang in the 25 - 70% (w/v) concentration range.

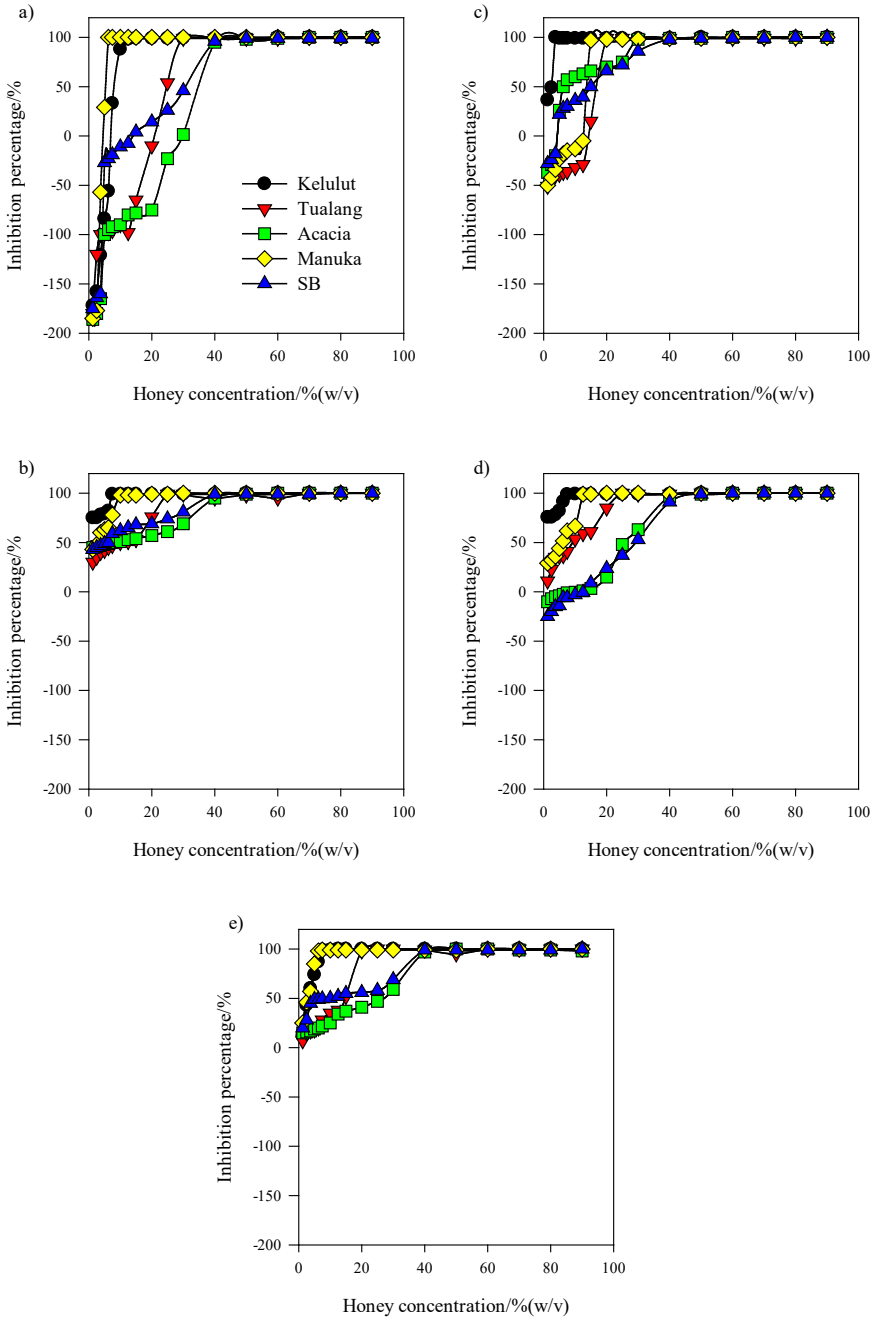


Figure 2. Growth inhibition percentage for Gram-negative bacteria of: a) *Klebsiella pneumoniae* ATCC BAA 1144, b) *Escherichia coli* ATCC 8739 c) *Pseudomonas aeruginosa* ATCC 9027, d) *Proteus mirabilis* ATCC 12453, and e) *Salmonella typhimurium* ATCC 14028. Kelulut (circle, black), Tualang (triangle downward, red), Acacia (square, green), Manuka (diamond, yellow) and SB (triangle upward, blue)

***Streptococcus pyogenes*.** The growth inhibition of *S. pyogenes* was promising when cultured with Kelulut and Manuka. There was a gradual increase in growth inhibition as the concentration increased. Besides that, this strain has shown similar trends of growth inhibition when exposed to Tualang and Acacia, with slightly lower percentage for Acacia.

***Enterococcus faecalis*.** This strain appeared to be the strongest of all the Gram-positive bacteria tested in this study when cultured with the different varieties of honey. Growth inhibition of *E. faecalis* occurred at 6.25%, 12.5%, 10%, and 20% (w/v) for Kelulut, Manuka, Tualang, and Acacia respectively, all requiring concentrations of more than 6.25%.

When exposed to SB solutions, two out of five Gram-negative bacteria showed strong resistance towards SB solutions. These bacteria were *P. mirabilis* and *K. pneumoniae*. Their growth inhibitions only started at 15% (w/v). Meanwhile, growth inhibition was only shown at concentrations of 5% (w/v) in *P. aeruginosa*, indicating a possible mild resistance. Both *E. coli* and *S. typhimurium* showed similar growth inhibition trends with a slightly lower growth inhibition percentage for *S. typhimurium*. As for Gram-positive bacteria, SB solutions have a are able to begin inhibiting the growth of *S. pyogenes* starting with the lowest SB concentration. The patterns of growth inhibition by SB solutions are similar to that of Acacia honey for both Gram-positive and Gram-negative bacteria, suggesting that the antibacterial properties of Acacia are more likely caused by the presence of high sugar content. The factors contributing to antibacterial properties of Kelulut and Tualang were then investigated.

Results of Role of Peroxide and Non-peroxide Compounds in Antibacterial Properties of Honey

The previous section has shown that the antibacterial properties possessed by Acacia could be due to its high sugar content. Here, an attempt was made to study how peroxide and non-peroxide compounds in Kelulut, Tualang, and Acacia affected the growth of *E. coli* and *S. aureus* as compared to the effects of Manuka. The inhibition zones of UT, PNP, and NP are indicated in Figure 3 for *S. aureus* (Figure 3a to 3c) and *E. coli* (Figure 3d to 3f).

UT solution preserved all factors when no elimination agents were added. As for the prepared PNP solution, it preserved peroxide and non-peroxide compounds while neutralizing the natural acidic characteristic of honey. Lastly, the prepared NP solution only preserved non-peroxide compounds and eliminated acidic and peroxide compounds.

In general, the UT solutions of Kelulut, Tualang, and Manuka showed similar patterns of inhibition in which the growth inhibition started at a concentration of 30% (w/v), and inhibition activity increased as the concentration increased. The growth inhibition of Acacia UT solution, however,

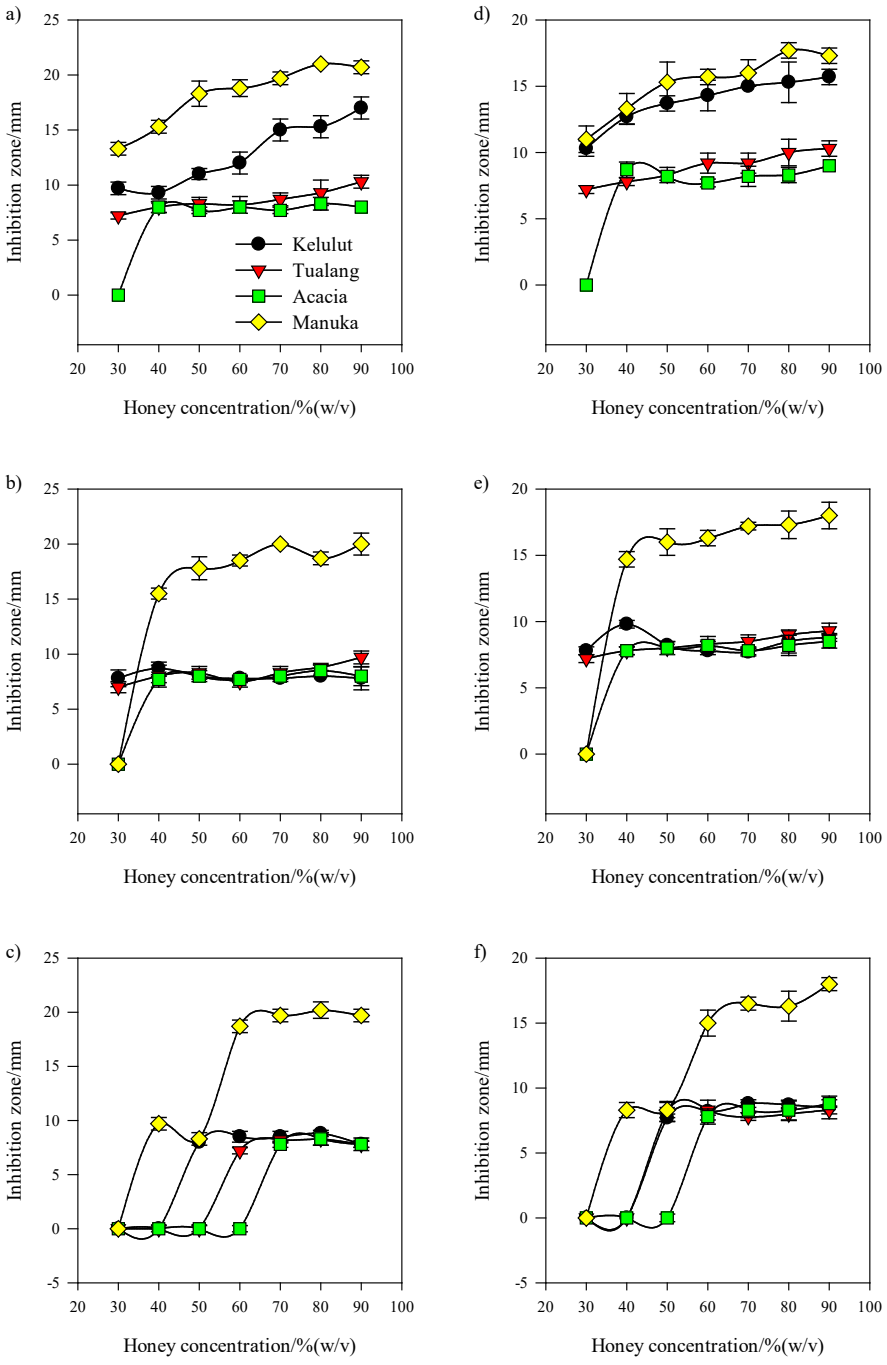


Figure 3. Inhibition activity of UT, PNP, and NP solution against *Staphylococcus aureus* ATCC6538 [(a) to (c)], and *Escherichia coli* ATCC8739 [(d) to (f)]. Kelulut (circle, black), Tualang (triangle, red), Acacia (square, green) and Manuka (diamond, yellow)

only began to appear at a concentration of 40% (w/v), and an increase in inhibition continued at higher concentrations. Among the Malaysian honey samples, both *S. aureus* and *E. coli* were most susceptible to the Kelulut UT solution, in which the inhibition activity was noticed to be as high as 1.7-fold compared with Tualang and 2.1-fold compared with Acacia.

The PNP solution demonstrated inhibition activity similar to the UT solution for all tested honey samples with no significant difference ($p>0.05$), except for Kelulut. The UT solution of Kelulut was dominated the growth inhibition assay when compared with other Malaysian honey varieties. However, the Kelulut PNP solution showed a pattern similar to that of to Tualang and Acacia instead of being comparable to Manuka PNP solution. Even so, large inhibition zones of 8.7 ±0.58 mm and 9.8 ±0.58 mm were recorded against *S. aureus* and *E. coli* by Kelulut PNP solution at a low concentration of 40% (w/v). These actions were noticed to be as high as 1.1-fold higher than the inhibition activity against *E. coli* when compared with *S. aureus*.

NP solutions for Kelulut, Tualang, and Acacia showed inhibition activities at different concentrations. The NP solution for Kelulut was the first to demonstrate inhibition activity against *E. coli* at a concentration of 50% (w/v). It was then followed by Tualang at 60% (w/v) and Acacia at 70% (w/v). For *S. aureus*, both NP solutions for Kelulut and Tualang began to inhibit growth at 50% (w/v), followed by Acacia at 60% (w/v). However, despite the inhibition of bacterial growth, these Malaysian honey samples showed no increment in the diameter of inhibition zone when higher concentrations of solutions were used for both *S. aureus* and *E. coli*. In contrast, the NP solution for Manuka began to show inhibition activity at a concentration of 40% (w/v) which continued to rise at higher concentrations against both *S. aureus* and *E. coli*.

DISCUSSION

Manuka honey is a monoflora honey produced from the nectar of *Leptospermum scoparium*, also known as the Manuka tree. This honey is widely known for its antibacterial properties due to the presence of an active non-peroxide compound called methylglyoxal (MGO) (Adams et al., 2009). MGO is a major bactericidal factor in Manuka (Kwakman et al., 2011). Besides MGO, osmotic pressure, acidic pH, leptosin, and hydrogen peroxide are also factors responsible for the antibacterial properties in Manuka (Carter et al., 2016; Roberts et al., 2015). Manuka has been found to have both bacteriostatic and bactericidal effects on numerous Gram-positive and Gram-negative pathogenic bacteria (Kwakman et al., 2011). Other than being antibacterial, Manuka has been reported to possess various pharmacological effects such as healing properties (Carter et al., 2016), antiviral properties (Watanabe et al., 2014) and antiulcer properties (Almasaudi et al., 2016). To date, Manuka is a widely accepted honey variety that has been accepted for usage during the medical care of wounds

due to its potent antibacterial properties. The term Unique Manuka Factor (UMF) is a global standard used to classify bioactive compounds that exist in Manuka honey. Manuka honey with a UMF value of more than 10 has been strongly recommended for wound care instead of honey of low or unknown potency (Tan et al., 2009). As a result, Manuka has been frequently used as a basis of comparison when it comes to determining the potency of antibacterial properties in honey.

In Malaysia, the distribution of Kelulut honey is lower compared to that of the common honeybee. Limited knowledge about this type of honey has resulted in less popularity in industrial production, and lack of research regarding its medicinal properties (Jalil et al., 2017). Kelulut honey has been found to possess beneficial effects such as antibacterial, antioxidant and anti-inflammatory properties (Jalil et al., 2017), and was proven to have bacteriostatic and bactericidal effects on various Gram-positive and Gram-negative bacteria (Tuksitha et al., 2018; Zainol et al., 2013). This study attempted to evaluate the antibacterial properties of Kelulut against bacteria commonly associated with wound infection. Several earlier studies have investigated the antibacterial properties of Kelulut against other pathogenic bacteria. When the results of this study were compared with the results of previous studies, the MIC of Kelulut in this study was found to be within the same range, studies – 3% to 20% (Tuksitha et al., 2018; Zainol et al., 2013). The MBC was also within the same range as previous studies – 1% to 32% (Tuksitha et al., 2018; Zainol et al., 2013). The MIC values obtained in this study were between 5% and 20%, while the MBC values were found to be between 12.5% and 50%.

Among the infectious bacteria tested, *P. aeruginosa* and *Acinetobacter baumannii* were found to be most susceptible to Kelulut. The MIC and MBC of *P. aeruginosa* were the lowest when compared with other honey samples including Manuka. Previous studies have reported that Kelulut is capable of stopping the growth of *P. aeruginosa* at a range between 5% and 10%, and kills the bacteria after 10% (Tuksitha et al., 2018). Meanwhile, other studies have reported that Kelulut can simultaneously stop and kill the growth of *P. aeruginosa* at a concentration of 20% (Zainol et al., 2013). In this study, the MIC and MBC values were recorded at 3.75% and 12.5% respectively. *Pseudomonas aeruginosa* is a ubiquitous opportunist pathogen that is distributed throughout the environment, particularly in moist habitats. It is the cause of many illnesses such as endocarditis, folliculitis, keratitis, meningitis, pneumonia, urinary tract infections, and wound infections. Wound infections due to *P. aeruginosa* have given significant rise to persistent infections in burn patients and patients with chronic venous leg ulcers because it is a multidrug-resistant organism. The bacteriostatic and bactericidal abilities of Kelulut should be investigated further as a possible antibacterial agent against drug-resistant, wound-infecting bacteria such as *P. aeruginosa*. Kelulut was the only local

honey in this study that possessed both bacteriostatic and bactericidal effects against all the strains of bacteria tested. Similar effects were absent for Tualang and Acacia. This was consistent with findings reported by previous antibacterial studies (Tuksitha et al., 2018; Zainol et al., 2013).

Tualang is a popular polyflora Malaysian honey produced by *Apis dorsata* (Boukraâ, 2014). It has been reported to possess various pharmacological benefits such as antibacterial, antioxidant, and anti-inflammatory properties (Ahmed & Othman, 2013). Tualang was found to possess both bacteriostatic and bactericidal effects against numerous pathogenic Gram-positive and Gram-negative bacteria (Tan et al., 2009; Zainol et al., 2013). In this study, the antibacterial properties of Tualang were evaluated against bacteria commonly known to infect wounds. The MIC of Tualang was found to be within the previously reported range when tested against general pathogenic bacteria – 6.25% to 25% (Tan et al., 2009; Zainol et al., 2013). In previous studies, Tualang produced higher or unidentified MBC values between 12.5% and 50% when tested against the pathogenic bacteria (Tan et al., 2009; Zainol et al., 2013). After analyzing both MIC and MBC values, the ranges obtained for Tualang in this study were found to be slightly higher when compared with the reported range for general pathogenic bacteria. These differences may be due to the difference in origin of nectar, batch of honey collected, and technical variation while performing the experiment such as the amount of bacterial suspension used, the type of agar or broth, and the diluent used. Among the tested bacteria, *P. aeruginosa* recorded the lowest MIC and MBC, thus being the most susceptible to Tualang. The comparison between Gram-positive and Gram-negative bacteria showed that they were equally susceptible to Tualang. This finding was concurrent with some of the previous reports (Tan et al., 2009).

Acacia honey demonstrated a similar inhibition pattern to sugar based solutions. Acacia honey is a monofloral honey produced by *Apis mellifera* or *Apis cerana* using nectar from the plant *Acacia mangium* (Bradbear, 2009; Samat et al., 2014). Acacia is one of the most widely commercially available types of honey in Malaysia reported to contain various pharmacological benefits such as antibacterial properties. Previous antibacterial studies against pathogenic bacteria reported that Acacia honey possessed both bacteriostatic and bactericidal effects with a MIC value between 15% and 25%, and MBC value between 25% and 50% (Zainol et al., 2013). In this study, we found that Acacia possessed the least potent antibacterial properties against the bacteria tested. This was based on the high MIC values, and the high or unidentified MBC values observed. This study found that the MIC and MBC for Acacia ranged from 30% to 50%, and 50% to 90% respectively. Similar to Tualang, it was apparent that the ranges of MIC and MBC were higher in this study when compared with previous antibacterial studies.

Honey antibacterial properties can be attributed to acidic pH, and presence of peroxide and non-peroxide compounds. In this study, we considered these properties to determine the factors contributing to antibacterial properties in honey. Among the Malaysian honey samples, Kelulut was found to have the most potent antibacterial properties against all tested Gram-positive and Gram-negative bacteria associated with wound infection. By evaluating the contributing factors, the potent antibacterial properties of Kelulut were attributed mostly to its naturally strong acidic environment (pH 2.37 ±0.13). A previous study showed that the acidic pH found in Kelulut ranged from 3.29 to 3.71 (Chan et al., 2017), about 1.6-fold higher than in this study. When we compared the pH of Kelulut to Tualang and Acacia samples, the Kelulut had the lowest pH, about 1.8 to 1.6-fold lower than the other samples. The strong acidic environment of Kelulut may provide a partial explanation for its potent antibacterial properties. The strong acidity of Kelulut was found to equally affect both Gram-positive and Gram-negative bacteria which were represented by *S. aureus* and *E. coli* in this study.

In addition to pH, other factors such as peroxide and non-peroxide compounds could be major contributors towards the antibacterial properties of honey. Peroxide compounds are usually represented by hydrogen peroxide (H_2O_2) (Irish et al., 2011; Kwakman & Zaat, 2012; Mandal & Mandal, 2011). In order to evaluate the presence of peroxide compounds, the honey was diluted to a concentration of 30% to 50% (w/v) (Kwakman & Zaat, 2012; Molan, 1992). By doing so, the enzyme glucose oxidase was activated, thus oxidizing glucose into gluconic acid and H_2O_2 (White et al., 1963). Out of all the Malaysian honey samples tested, Kelulut demonstrated a higher inhibition activity at a concentration between 30% and 50% (w/v). The active peroxide activity in Kelulut noticeably affected the Gram-negative *E. coli*, more than the Gram-positive *S. aureus*. The inhibition activity demonstrated was 1.1-fold higher against *E. coli* compared to *S. aureus*. The results shown in this study support the active contribution of H_2O_2 towards the antibacterial properties of honey at concentrations of 30% to 50% and simultaneously demonstrated that peroxide compounds do contribute to the antibacterial properties of Malaysian honey against wound-infecting bacteria. However, a larger number of infectious bacteria should be investigated further to better understand the effect of peroxide activities on wound-associated bacteria.

As for non-peroxide factors, honey reportedly possesses compounds such as MGO, bee-defensin-1, leptosperin, phenolic acids, flavonoids, and jelleins (Ahmed & Othman, 2013; Jalil et al., 2017; Roberts et al., 2015; Salleh et al., 2017). Compounds such as MGO were extensively studied and were revealed to cause various antibacterial mechanisms including cell wall disruption and lysis (Henriques et al., 2011; Nishio et al., 2016), disruption in gene expression patterns (Blair et al., 2009) and DNA degradation (Brudzynski et al., 2011). It

is important to identify the availability of non-peroxide compounds and their actions as they may contribute to the production of potent antibacterial properties in honey (Kwakman et al., 2010, 2011). In this study, we removed acidic and peroxide compounds to determine the effect of non-peroxide activity in the honey samples. This technique of neutralizing the known factors was suggested by previous studies that determined the action of the compound that contributed to antibacterial properties of honey (Kwakman et al., 2010, 2011). By doing so, the presence of non-peroxide compounds and their role in producing antibacterial properties in Malaysian honey against wound-infecting bacteria can be observed. This study proved the presence of active non-peroxide compounds in Kelulut, Tualang, and Acacia. Our conclusion is that the non-peroxide activity may be due to the presence of flavonoid and phenolic compounds previously identified in these honey varieties (Ahmed & Othman, 2013; Jalil et al., 2017; Salleh et al., 2017). We found that non-peroxide activity equally affected Gram-positive and Gram-negative bacteria. However, it may be unfair to simply conclude the effect of non-peroxide activity in Malaysian honey in this study since only a single representative species for each Gram-positive and Gram-negative bacteria were considered. Nevertheless, we recommend further investigation on non-peroxide compounds in Malaysian honey against more species of wound-infecting bacteria in order to thoroughly understand the effects of these compounds. With all due consideration, this study has successfully demonstrated the presence of active non-peroxide compounds that contribute to antibacterial properties in Malaysian honey against two common infectious bacteria, *S. aureus* and *E. coli*.

As with any study, there are a few limitations that have to be considered during the course of this study, thus making further investigation crucial for building a complete understanding of the properties of Malaysian honey varieties. In the present study, drug resistant bacteria strains were not considered during the evaluation of honey's antibacterial properties. Further studies should evaluate the antibacterial properties of honey against these drug-resistant strains. The efficacy of honey as an antibacterial agent can be compared to the drugs commonly used to treat drug-resistant strains of bacteria such as Methicillin Resistant *Staphylococcus aureus* (MRSA). The outcome will confirm whether Malaysian honey is a suitable alternative for antibiotics, and whether the bacteria can develop resistance against honey. Should Malaysian honey proves to be an efficient and effective solution, it can slow the emergence of drug-resistant bacterial strains due to overuse of antibiotics. It can then be used to treat drug-resistant bacterial infections such as those caused by MRSA (Bereket et al., 2012), multidrug resistant *P. aeruginosa* (Bereket et al., 2012), carbapenem resistant *K. pneumonia* (Low et al., 2017), and B-lactam resistant *E. coli* (Jacoby & Sutton, 1985).

The antibacterial properties of honey are attributed to the presence of active compounds in the honey, partially due to the pollen and nectar collected by the corresponding bees. The present study considered a single sample of Malaysian Kelulut, Tualang and Acacia for antibacterial evaluation. Honey samples collected from different regions of Malaysia (e.g. Borneo and Peninsular Malaysia) may have variations in available compounds due to the variation in pollen and nectar sources for each region. Further studies should consider evaluating the antibacterial properties of honey samples from different regions of Malaysia to identify the available compounds and their effect on the antibacterial properties of honey.

CONCLUSION

Malaysian honey, especially Kelulut, proved to be dependent on acidic environment as a major antibacterial factor. This is further supported by the presence of peroxide and non-peroxide compounds. The antibacterial properties of Malaysian honey varieties were generally comparable to Manuka. The closest resemblance was demonstrated by Kelulut. In some cases, Kelulut showed equivalent or better antibacterial activity than Manuka, especially against *P. aeruginosa*. The antibacterial potency of Malaysian honey against microorganisms associated with wounds suggests the potential of honey as an alternative therapeutic agent, particularly for wound infection. Hence, this study proposes the usage of Kelulut at a concentration of 50% (w/v) or more to simultaneously stop and kill bacteria that are commonly found to infect wounds.

ACKNOWLEDGEMENT

The authors would like to thank the Ministry of Education Malaysia in providing the fund for this project under the Fundamental Research Grant Scheme (FRGS; Grant Number: FRGS/1/2017/STG05/UMP/02/5), Universiti Malaysia Pahang (UMP) for the research facilities and IIUMC for providing the clinical bacterial strains.

REFERENCES

Adams, C. J., Boult, C. H., Deadman, B. J., Farr, J. M., Grainger, M. N. C., Manley-Harris, M., & Snow, M. J. (2008). Isolation by HPLC and characterisation of the bioactive fraction of New Zealand Manuka (*Leptospermum scoparium*) honey. *Carbohydrate Research*, *343*(4), 651–659. doi: 10.1016/j.carres.2007.12.011

Adams, C. J., Manley-harris, M., & Molan, P. C. (2009). The origin of methylglyoxal in New Zealand Manuka (*Leptospermum scoparium*) honey. *Carbohydrate Research*, *344*(8), 1050–1053. doi: 10.1016/j.carres.2009.03.020

Adewumi, A., & Ogunjinmi, A. (2011). The healing potential of honey and propolis lotion on septic wounds. *Asian Pacific Journal of Tropical Biomedicine*, *1*(1), 55–57. doi: 10.1016/S2221-1691(11)60123-8

Ahmed, S., & Othman, N. H. (2013). Review of the medicinal effects of Tualang honey and a comparison with Manuka honey. *Malaysian Journal of Medicine Sciences*, *20*(3), 6–13.

Almasaudi, S. B., El-Shitany, N. A., Abbas, A. T., Abdel-dayem, U. A., Ali, S. S., Al Jaouni, S. K., & Harakeh, S. (2016). *Antioxidant, anti-*

inflammatory, and antiulcer potential of Manuka honey against gastric ulcer in rats. Retrieved May 15, 2019, from http://downloads.hindawi.com/journals/omcl/2016/3643824.pdf

Bereket, W., Hemalatha, K., Getenet, B., Wondwossen, T., Solomon, A., Zeynudin, A., & Kannan, S. (2012). Update on bacterial nosocomial infections. *European Review for Medical and Pharmacological Sciences*, *16*(8), 1039–1044.

Blair, S. E., Cokcetin, N. N., Harry, E. J., & Carter, D. A. (2009). The unusual antibacterial activity of medical-grade *Leptospermum* honey: Antibacterial spectrum, resistance and transcriptome analysis. *European Journal of Clinical Microbiology and Infectious Diseases*, *28*(10), 1199–1208. doi: 10.1007/s10096-009-0763-z

Bogdanov, S. (1997). Nature and origin of the antibacterial substances in honey. *LWT - Food Science and Technology*, *30*(7), 748–753. doi: 10.1006/fstl.1997.0259

Boukraâ, L. (Ed.). (2014). *Honey in traditional and modern medicine*. New York, NY: Taylor and Francis Group.

Bradbear, N. (2009). *Bees and their role in forest livelihoods*. Retrieved May 15, 2019, from http://www.fao.org/3/a-i0842e.pdf

Brudzynski, K., Abubaker, K., St-martin, L., & Castle, A. (2011). Re-examining the role of hydrogen peroxide in bacteriostatic and bactericidal activities of honey. *Frontiers in Microbiology*, *2*(213), 1–9. doi: 10.3389/fmicb.2011.00213

Carter, D. A., Blair, S. E., Cokcetin, N. N., Bouzo, D., Brooks, P., Schothauer, R., & Harry, E. J. (2016). Therapeutic Manuka honey: No longer so alternative. *Frontiers in Microbiology*, *7*(569), 1–11. doi: 10.3389/fmicb.2016.00569

Chan, B. K., Haron, H., Talib, R. A., & Subramaniam, P. (2017). Physical properties, antioxidant content and anti-oxidative activities of Malaysian stingless kelulut (*Trigona* spp.) honey. *Journal of Agriculture Science*, *9*(13), 32–40. doi: 10.5539/jas.v9n13p32

El-malek, F. F. A., Yousef, A. S., & El-assar, S. A. (2017). Hydrogel film loaded with new formula from Manuka honey for treatment of chronic wound infections. *Journal of Global Antimicrobial Resistance*, *11*, 171–176. doi: 10.1016/j.jgar.2017.08.007

Franklin, R. C., Matthew, A. W., Jeff, A., Michael, N. D., George, M. E., Mary, J. F., … Barbara, L. Z. (2012). *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: Approved standard — Ninth Edition*. Retrieved May 15, 2019, from https://mafiadoc.com/download/methods-for-dilution-antimicrobial-susceptibility-tests-_5a19a8801723dd4f2ac359bc.html

Henriques, A. F., Jenkins, R. E., & Burton, N. F. (2011). The effect of Manuka honey on the structure of *Pseudomonas aeruginosa*. *European Journal of Clinical Microbiology and Infectious Diseases*, *30*(2), 167–171. doi: 10.1007/s10096-010-1065-1

Irish, J., Blair, S., & Carter, D. A. (2011). The antibacterial activity of honey derived from Australian flora. *PLoS ONE*, *6*(3), e18229. doi:10.1371/journal.pone.0018229

Ismail, W. I. W. (2016). A review on beekeeping in malaysia history, importance and future direction. *Journal of Sustainability Science and Management*, *11*(2), 70–80.

Jacoby, G. A., & Sutton, L. (1985). β-Lactamases and β-Lactam resistance in *Escherichia coli*. *Antimicrobial Agents and Chemotherapy*, *28*(5), 703–705.

Jalil, M. A. A., Kasmuri, A. R., & Hadi, H. (2017). Stingless bee honey, the natural wound healer: A review. *Skin Pharmocology and Physiology*, *30*(2), 66–75. doi: 10.1159/000458416

Kasithevar, M., Periakaruppan, P., & Muthupandian, S. (2017). Antibacterial efficacy of silver nanoparticles against multi-drug resistant clinical isolates from post-surgical wound infections. *Microbial Pathogenesis*, *107*, 327–334. doi: 10.1016/j.micpath.2017.04.013

Kateel, R., Bhat, G., Baliga, S., Augustine, A. J., Ullal, S., & Adhikari, P. (2018). Antibacterial action of tropical honey on various bacteria obtained from diabetic foot ulcer. *Complementary Therapies in Clinical Practice*, *30*(1), 29–32.

Kwakman, P. H. S., te Velde, A. A., de Boer, L., Vandenbroucke-Grauls, C. M. J. E., & Zaat, S. A. J. (2010). How honey kills bacteria. *Federation of American Societies for Experimental Biology*, *24*(7), 2576–2582. doi: 10.1096/fj.09-150789

Kwakman, P. H. S., te Velde, A. A., de Boer, L., Vandenbroucke-Grauls, C. M. J. E., & Zaat, S. A. J. (2011). Two major medicinal honeys have different mechanisms of bactericidal activity. *PLoS ONE*, *6*(3), 3–9. doi: 10.1371/journal.pone.0017709

Kwakman, P. H. S., & Zaat, S. A. J. (2012). Antibacterial components of honey. *International Union of Biochemistry and Molecular Biology*, *64*(1), 48–55. doi: 10.1002/iub.578

Liu, J., Ye, Y., Lin, T., Wang, Y., & Peng, C. (2013). Effect of floral sources on the antioxidant, antimicrobial, and anti-inflammatory activities of honeys in Taiwan. *Food Chemistry*, *139*(1–4), 938–943. doi: 10.1016/j.foodchem.2013.02.015

Low, Y., Yap, P. S., Jabar, K. A., Ponnampalavanar, S., Karunakaran, R., Velayuthan, R., … Teh, C. S. (2017). The emergence of carbapenem resistant *Klebsiella pneumoniae* in Malaysia: Correlation between microbiological trends with host characteristics and clinical factors. *Antimicrobial Resistance and Infection Control*, *6*(5), 1–13. doi: 10.1186/s13756-016-0164-x

Mandal, M. D., & Mandal, S. (2011). Honey: Its medicinal property and antibacterial activity. *Asian Pacific Journal of Tropical Biomedicine*, *1*(2), 154–160. doi: 10.1016/S2221-1691(11)60016-6

Matzen, R. D., Leth-espensen, J. Z., Jansson, T., Nielsen, D. S., Lund, M. N., & Matzen, S. (2018). *The antibacterial effect* in vitro *of honey derived from various Danish flora*. Retrieved May 15, 2019, from http://downloads.hindawi.com/journals/drp/2018/7021713.pdf

Mcloone, P., Warnock, M., & Fyfe, L. (2016). Honey: A realistic antimicrobial for disorders of the skin. *Journal of Microbiology, Immunology and Infection*, *49*(2), 161–167.

Molan, P. (1992). The antibacterial activity of honey. 1. The nature of the antibacterial activity. *Bee World*, *73*(1), 5–28. doi: 10.1080/0005772X.1992.11099109

Moussa, A., Noureddine, D., Mohamed, H. S., & Abdelmelek, M. (2012). Antibacterial activity of various honey types of Algeria against *Staphylococcus aureus* and *Streptococcus pyogenes*. *Asian Pacific Journal of Tropical Medicine*, *5*(10), 773–776. doi: 10.1016/S1995-7645(12)60141-2

Mustafa, M., Hamed, S., Iiizam, E. M., Sharifa, A. M., & Win, T. (2015). Surgical site infections: Predisposing factors and prevention. *Journal of Dental and Medical Sciences*, *14*(11), 101–106.

Nasir, N. M., Halim, A. S., Singh, K. B., Dorai, A. A., & Haneef, N. M. (2010). Antibacterial properties of Tualang honey and its effect in burn wound management: A comparative study. *BMC*

Complementary and Alternative Medicine, *10*(1), 1–7. doi: 10.1186/1472-6882-10-31

Nasir, N. M., Lee, B. K., Yap, S. S., Thong, K. L., & Yap, S. L. (2016). Cold plasma inactivation of chronic wound bacteria. *Archives of Biochemistry and Biophysics*, *605*, 76–85. doi: 10.1016/j.abb.2016.03.033

Nishio, E. K., Ribeiro, J. M., Oliveira, A. G., Andrade, C. G. T. J., Proni, E. A., Kobayashi, R. K. T., & Nakazato, G. (2016). Antibacterial synergic effect of honey from two stingless bees: *Scaptotrigona bipunctata* Lepeletier, 1836, and *S. postica* Latreille, 1807. *Scientific Report*, *6*, 1–8. doi: 10.1038/srep21641

Nooh, H. Z., & Nour-eldien, N. M. (2016). The dual anti-inflammatory and antioxidant activities of natural honey promote cell proliferation and neural regeneration in a rat model of colitis. *Acta Histochemica*, *118*(6), 588–595. doi: 10.1016/j.acthis.2016.06.006

Ranneh, Y., Ali, F., Zarei, M., Akim, A., & Abd, H. (2018). Malaysian stingless bee and Tualang honeys: A comparative characterization of total antioxidant capacity and phenolic profile using liquid chromatography-mass spectrometry. *LWT - Food Science and Technology*, *89*, 1–9. doi: 10.1016/j.lwt.2017.10.020

Rashidi, M. K., Mirazi, N., & Hosseini, A. (2016). Effect of topical mixture of honey, royal jelly and olive oil-propolis extract on skin wound healing in diabetic rats. *Wound Medicine*, *12*, 6-9.

Roberts, A. E. L., Brown, H. L., & Jenkins, R. (2015). On the antibacterial effects of manuka honey: Mechanistic insights. *Research and Reports in Biology*, *6*, 215–224. doi: 10.2147/RRB.S75754

Salleh, M. A. M., Eshak, Z., & Ismail, W. I. W. (2017). Acacia honey induces apoptosis in human breast adenocarcinoma cell lines (MCF-7). *Jurnal Teknologi*, *79*(4), 9–16. doi: 10.11113/jt.v79.9882

Samat, S., Nor, N. A., Hussein, F. N., & Wan Ismail, W. I. (2014). Effects of Gelam and Acacia honey acute administration on some biochemical parameters of Sprague Dawley rats. *BMC Complementary and Alternative Medicine*, *14*(1), 146. doi: 10.1186/1472-6882-14-146

Sienkiewicz, M., Monika, Ł., & Kowalczyk, E. (2016). The ability of selected plant essential oils to enhance the action of recommended antibiotics against pathogenic wound bacteria. *Burns*, *3*(43), 310–317. doi: 10.1016/j.burns.2016.08.032

Sussmann, C., & Bates-Jensen, B. M. (Eds.). (2012). *Wound care: A collaborative practice manual for health professionals* (4th ed.). Philadelphia, USA: Lippincott Williams and Wilkins.

Tan, H. T., Rahman, R. A., Gan, S. H., Halim, A. S., Hassan, S. A., Sulaiman, S. A., & Kirnpal-Kaur, B. (2009). *The antibacterial properties of Malaysian Tualang honey against wound and enteric microorganisms in comparison to Manuka honey*. Retrieved May 15, 2019, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2753561/pdf/1472-6882-9-34.pdf

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

Watanabe, K., Rahmasari, R., Matsunaga, A., Haruyama, T., & Kobayashi, N. (2014). Anti-influenza viral effects of honey *in-vitro*: Potent high activity of Manuka honey. *Archives of Medical Research*, *45*(5), 359–365. doi: 10.1016/j.arcmed.2014.05.006

White, J. W., Subers, M. H., & Schepartz, A. I. (1963). The identification of inhibine, the antibacterial factor in honey, as hydrogen peroxide and its origin in a honey glucose-oxidase system. *Biochimica et Biophysica Acta*, *73*(1), 57–70. doi: 10.1016/0926-6569(63)90108-1

Yang, H., Wang, W., Tan, Y., Zhang, D., Wu, J., & Lei, X. (2017). Investigation and analysis of the characteristics and drug sensitivity of bacteria in skin ulcer infections. *Chinese Journal of Traumatology*, *20*(4), 194–197. doi: 10.1016/j.cjtee.2016.09.005

Zainol, M. I., Yusoff, K. M., Yasim, M., & Yusof, M. (2013). *Antibacterial activity of selected Malaysian honey*. Retrieved May 15, 2019, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3716576/pdf/1472-6882-13-129.pdf

Pertanika J. Trop. Agric. Sc. 42 (4): 1209 - 1217 (2019)

PERTANIKA JOURNALS

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Evaluation of Fish Gelatin and Sodium Alginate Blend as Gelling Agents for Pudding Containing Virgin Coconut Oil

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ABSTRACT

Virgin coconut oil (VCO) is known for its functional properties but it is yet to be widely developed into food products. Lipid and gelling agents are crucial ingredients for the rheological characteristics of milk puddings. This study aimed to explore the potential of blends of fish gelatin and sodium alginate as gelling agents and a delivery system for VCO. A total of 15 pudding formulations were generated from a mixture design approach to determine the optimized proportions of VCO (6-13%), fish gelatin (2-6%), sodium alginate (0.15-0.75%), and water (65-75%). All 15 pudding formulations exhibited strong elastic characteristic with their *G*' (storage modulus) values higher than the *G*" (loss modulus). Formulations with high contents of gelatin (6%) and sodium alginate (0.28-0.75%) exhibited strong gel characteristics (tan $\delta < 0.1$). The optimized formulation consisted of 10.68% VCO, 3.41% fish gelatin, 0.59% sodium alginate, and 68.33% water, with desirability of 0.874 against the viscoelastic properties and firmness of a commercial pudding. A significant increase was observed in firmness and free fatty acid (FFA) value of the optimized pudding from 3 weeks onwards, over the weekly evaluation of 4 weeks storage at 4 ± 1°C. The sensory assessment showed that rancidity of pudding was not significantly detected by panels throughout the 4 weeks of storage period.

Keywords: Fish gelatin, mixture design, pudding, sodium alginate, virgin coconut oil

ARTICLE INFO

Article history:
Received: 25 June 2019
Accepted: 17 September 2019
Published: 13 November 2019

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INTRODUCTION

Virgin coconut oil (VCO) is gaining increasing popularity in both the scientific field and the public as a functional food supplement. VCO is mechanically obtained from the kernel of the coconut under mild temperature or without heat, without undergoing chemical refining, bleaching

ISSN: 1511-3701
e-ISSN: 2231-8542

or deodorizing (Marina et al., 2009). VCO is considered to have numerous beneficial health effects attributed to its mode of processing that prevents the loss of its beneficial components such as vitamin E and polyphenols (Nevin & Rajamohan, 2008). Studies also showed that VCO possesses antibacterial, antiviral, antinociceptive, anti-inflammatory and lipid lowering effect in blood (Nevin & Rajamohan, 2008; Zakaria et al., 2011). VCO can be consumed directly, but its strong coconut scent has limited its palatability, which makes the incorporation of VCO into a food system an attractive approach. To date, the availability of food products for delivering VCO remains limited in the market.

Milk puddings that are widely consumed by children and elderly people can be effective in delivering food components with health benefits. The consumer acceptability on puddings depends on their gel characteristics, particularly rheological and textural properties that are defined by the type and the content of the fat and gelling agents as well as their interactions (Saha & Bhattacharya, 2010; Toker et al., 2013). The use of gelling agent blends in food products is able to bring synergetic effect to achieve a desirable texture (Li & Nie, 2016; Toker et al., 2013). Among gelling agents, gelatin finds maximum application in food products, such as yoghurt products, low-fat spread and sugar confectionery, owing to its low melting temperature and slow-setting gelation behaviour (Saha & Bhattacharya, 2010). Lately, fish gelatin is gaining prominence to replace the commercial mammalian gelatin and to increase the utilization of fish by-products (skins and bones) discarded from the fish processing industry as the gelatin source (Karim & Bhat, 2009). Meanwhile, alginate derived from brown seaweeds is a preferred gelling agent for restructured foods, puddings and desserts due to its fast-setting gelation (McHugh, 2003; Saha & Bhattacharya, 2010). It is therefore possible that the blends of gelatin and alginate would be potential in modulating the gelation strength of a food product. So far, there is no study conducted on food product development with the use of gelatin-alginate blends.

Therefore, the objective of this study was to develop a pudding formulation by optimizing the content of VCO, fish gelatin, sodium alginate and water using mixture design methodology, for achieving the targeted rheological and textural properties of a commercial pudding. The optimized pudding formulation was further evaluated for its texture, hydrolytic rancidity and sensory stability during storage for 4 weeks at refrigeration temperature.

MATERIALS AND METHODS

Materials

The VCO was purchased from a local VCO producer in Kudat, Malaysia. Fish gelatin and sodium alginate derived from seaweed was purchased from a food ingredient supplier (Klang, Malaysia). All other ingredients, inclusive of sugar, skimmed milk powder and soy lecithin were purchased from a local bakery ingredients supplier. Commercial pudding product was purchased from a local market.

Preparation of Puddings

Gelatin was added with water and stirred for 5 min at 50 ± 2°C. Lecithin (0.15%, w/w) was dissolved completely in VCO and then added gradually to the gelatin solution under stirring for 10 min at 50 ± 2°C to form emulsion. In a separate preparation, skimmed milk powder (7%, w/w), sugar (9.85%, w/w) and sodium alginate were mixed and dissolved in water, stirred continuously for 10 min at 85 ± 2°C, and then cooled to 50 ± 2°C. Then, the emulsion was added gently into the mixture and stirred for another 20 min at 50 ± 2°C. The puddings were poured into plastic containers, cooled to room temperature (23 ± 2°C) and stored in a refrigerator (4 ± 1°C) for 1 h prior to the analyses.

Experimental Design and Statistical Analysis

D-optimal mixture design of Design Expert software v. 7.0.0 (State-Ease Inc., Minneapolis, MN, USA) was employed in formulation study. The ranges of ingredients obtained from the preliminary study were used, which were the VCO (6-13%, w/w) (*A*), fish gelatin (2-6%, w/w) (*B*), and sodium alginate (0.15-0.75%, w/w) (*C*), and water (65-75%, w/w) (*D*) to make up to a constant amount (83%). A total of 15 pudding formulations was generated, consisting 12 formulations of different contents with 3 formulations were replicates (Table 1). The rheological behavior of puddings was evaluated by measuring the viscoelastic properties and firmness, and the values (*Y*) were fitted to three equation models of the linear, quadratic and cubic models (Equations (1)-(3)). The statistical significance of each equation was analyzed through analysis of variance (ANOVA) at $p<0.05$ using the same software. The optimized content of these ingredients in formulating pudding was generated using the numerical optimization technique of the same software by setting the viscoelastic properties and firmness measured on a commercial pudding as targeted values. Validation of the optimized pudding formulation was performed by comparing the experimental values to the predicted values, where comparisons were carried out by LSD t-test ($p<0.05$).

$$Y = b_1A + b_2B + b_3C + b_4D \quad (1)$$

$$Y = b_1A + b_2B + b_3C + b_4D + b_{12}AB + b_{13}AC + b_{14}AD + b_{23}BC + b_{24}BD + b_{34}CD \quad (2)$$

$$Y = b_1A + b_2B + b_3C + b_4D + b_{12}AB + b_{13}AC + b_{14}AD + b_{23}BC + b_{24}BD + b_{34}CD + b_{123}ABC + b_{124}ABD + b_{134}ACD + b_{234}BCD \quad (3)$$

where *Y* is the estimated responses (viscoelastic properties and firmness); *b* is the constant coefficients for linear and non-linear (interaction) term.

Viscoelastic Properties and Firmness Measurement of Puddings

Viscoelastic properties of pudding samples were determined using an AR 1500EX rheometer (TA Instruments, Delaware, USA) with a serrated parallel plate geometry of 20 mm and a gap of 1000 μm. The oscillation

Table 1
Viscoelastic properties and firmness of pudding formulations in a mixture design

Formulation	VCO (%)	Fish Gelatin (%)	Sodium alginate (%)	Water (%)	*G'* (Pa)	*G"* (Pa)	*G** (Pa)	tan δ	Firmness (N)
1	6.00	3.83	0.15	73.02	747.9182 ± 56.1185	48.0755 ± 5.8239	749.4773 ± 56.3693	0.0642 ± 0.0030	15.4300 ± 1.4142
2	12.86	4.99	0.15	65.00	4941.7273 ± 81.6387	605.8409 ± 56.3049	4979.4091 ± 88.1312	0.1236 ± 0.0097	21.6750 ± 1.6900
3	7.00	2.29	0.75	72.95	1352.7728 ± 193.9401	156.4955 ± 12.9529	1361.9546 ± 194.0686	0.1168 ± 0.0074	4.0050 ± 0.0212
4	9.47	4.56	0.45	68.52	4117.2273 ± 356.3175	443.7228 ± 43.1271	4141.6364 ± 349.5679	0.1090 ± 0.0199	13.0000 ± 0.2263
5	10.50	6.00	0.28	66.22	4191.5909 ± 235.5951	399.3637 ± 11.3522	4210.6818 ± 235.3380	0.0960 ± 0.0028	24.0700 ± 1.0607
6	12.87	4.39	0.74	65.00	40036.8182 ± 1931.6872	24510.4546 ± 3671.1698	47132.2727 ± 148.4924	0.6345 ± 0.0839	15.4700 ± 0.2121
7	7.06	6.00	0.75	69.19	9589.8728 ± 251.2672	973.6727 ± 18.8219	9701.0000 ± 164.8202	0.1011 ± 0.0003	19.5350 ± 0.0071
8	11.81	2.00	0.28	68.90	275.9227 ± 25.6680	24.4114 ± 1.5421	277.0409 ± 25.7066	0.0878 ± 0.0022	8.2100 ± 0.6081
9	12.87	4.39	0.74	65.00	56305.4896 ± 6023.2147	29331.9805 ± 2235.5136	63594.7437 ± 6232.5446	0.5437 ± 0.0433	16.2600 ± 0.8910
10	13.00	3.41	0.35	66.23	4266.5909 ± 433.8421	477.6228 ± 60.3934	4264.4091 ± 478.5827	0.1119 ± 0.0029	13.0450 ± 0.0778
11	6.94	6.00	0.17	69.89	3391.1364 ± 532.1942	226.7818 ± 64.6553	3398.6818 ± 535.4084	0.0662 ± 0.0086	21.4000 ± 0.3394
12	9.59	2.00	0.15	71.26	259.1091 ± 5.5797	44.1005 ± 0.7643	262.9591 ± 5.3804	0.1647 ± 0.0071	5.2450 ± 0.5869
13	6.00	2.00	0.36	74.64	266.6864 ± 50.0825	45.1627 ± 8.3567	270.4818 ± 50.7703	0.1713 ± 0.0016	1.8250 ± 0.1202
14	7.06	6.00	0.75	69.19	7820.5455 ± 1359.8288	690.9091 ± 143.2727	7851.0000 ± 1403.1570	0.0883 ± 0.0028	21.2350 ± 0.7990
15	7.00	2.29	0.75	72.95	1502.7273 ± 213.9320	192.4500 ± 25.3337	1514.9546 ± 215.4104	0.1287 ± 0.0014	3.7100 ± 0.0141

frequency sweeps were performed over the range of 0.1-10 Hz at 0.2 Pa. Measurements were carried out at 4°C and a constant strain of 1%. The storage modulus (G'), loss modulus (G''), complex modulus (G^*), and loss tangent ($\tan \delta = G''/G'$) were measured as a function of frequency and calculated using the TA rheometer Data Analysis software (Version V. 4.20, TA Instruments Inc.) (Alamprese & Mariotti, 2011; Toker et al., 2013). Firmness of pudding samples was measured using a TA.XTplus texture analyzer (Stable Micro Systems, Godalming, Surrey, UK) with a diameter plate of 35 mm connected to a load cell of 490.33 N. Penetration tests were performed to 30% of the initial height of the sample and the load (N) recorded was used as result (Alamprese & Mariotti, 2011).

Storage Stability of Optimized Pudding Formulation

Storage stability of pudding was assessed weekly over a period of 4 weeks at 4 ± 1°C. Texture stability of pudding in terms of firmness was measured using a TA.XTplus texture analyzer (Stable Micro Systems, Godalming, Surrey, UK) according to the method by Alamprese and Mariotti (2011). Hydrolytic rancidity was evaluated through determination of FFA values (%, as lauric acid) according to AOAC 940.28 (Association of Official Analytical Chemists [AOAC], 1999). For to FFA analysis, pudding was melted completely at 40°C under continuous stirring and centrifuged at 689 x *g* for 15 min. The upper oil layer was collected for FFA determination. Sensory stability in terms of rancidity was assessed by 30 semi-trained panelists from the Faculty of Food Science and Nutrition, Universiti Malaysia Sabah, Malaysia. Panelists were trained and standardized with a rancid oil sample (Villarino et al., 2007). Paired comparison test was used to detect rancidity in the stored puddings with freshly produced pudding used as control. At least 20 judgements out of the 30 panels were required for the pudding sample to be rancid significantly at 5% level of significance (Larmond, 1977). All tests for storage stability were carried out in triplicate. Experimental results were reported as mean value with standard deviation and significant differences at the probability level of $p<0.05$ was determined using one-way ANOVA with Tukey test (SPSS v. 21.0, SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Model Fitting

Table 1 shows the results of viscoelastic properties and firmness measured for each pudding formulation from mixture design study. Results of ANOVA suggested the quadratic and linear model were the best for the viscoelastic properties and firmness, respectively (data not shown). The VCO-fish gelatin, VCO-sodium alginate, VCO-water, fish gelatin-sodium alginate, fish gelatin-water, sodium alginate-water quadratic model terms had antagonistic effect on the viscoelastic properties of the pudding formulations. For the firmness of the puddings, the VCO and fish gelatin had the positive effect, but the sodium alginate

and water had the negative effect. Fish gelatin had the highest positive effect, while sodium alginate had the highest negative effect on the firmness of the pudding, which is in agreement with the antagonistic effect of alginate reported by Toker et al. (2013).

Table 2 shows the quadratic and linear models for the data of viscoelastic properties and firmness, respectively. Their *R*-squared values of higher than 0.74, and *p*-values of lack of fit of higher than 0.05 (data not shown) indicated the adequacy of the models in predicting the variation of the results (Henika, 1982; Sarteshnizi et al., 2015).

Viscoelastic Properties and Firmness of Puddings

As shown in Table 1, all puddings exhibited strong elastic characteristic as their *G'* values were higher than the *G"* values (Saha & Bhattacharya, 2010). The *G** values were close to the *G'* values and the tan δ values were less than 1, indicated that all pudding formulations were highly elastic (Lim & Narsimhan, 2006; Toker et al., 2013). A few pudding formulations exhibited strong gel characteristic as reflected by tan δ values less than 0.1 (Saha & Bhattacharya, 2010). The blends of high contents of fish gelatin (6%) and sodium alginate (0.28-0.75%) produced puddings with strong gel characteristics (formulations 5, 7, 14). Strong gel strength was also exhibited for puddings (formulations 1, 11) consisted of low content of sodium alginate (0.15-0.17%) when added with a lower amount of VCO. An addition of lipid can increase the *G'*, *G"* and tan δ values, attributed to the formation of protein-lipid complexes (Tolstoguzov, 2003). Thus, the low tan δ values were probably attributed to the lower VCO content. Further test found that puddings with strong gel characteristic were firmer (formulations 5, 7, 11, 14).

Table 2
Predicted models for the viscoelastic properties and firmness of pudding

Parameter	Predicted models	R^2
G'	844226.7076 *A* + 6499591.4760 *B* + 565244793.8239 *C* + 91884.8132 *D* - 4167580.2181 *AB* - 490130017.7576 *AC* - 1564627.5020 *AD* - 559354960.7603 *BC* - 7726608.7507 *BD* - 580510174.5598 *CD*	0.9560
G"	276854.5715 *A* + 4249945.2456 *B* + 352915852.8623 *C* + 57278.6239 *D* - 2514666.8587 *AB* - 303496662.5973 *AC* - 680844.8931 *AD* - 356302456.5589 *BC* - 5070906.9855 *BD* - 362593041.5131 *CD*	0.9819
*G**	907448.6305 *A* + 7693781.1451 *B* + 661989380.4897 *C* + 107537.8709 *D* - 4884583.4044 *AB* - 573277851.3198 *AC* - 1735571.9504 *AD* - 657297063.5143 *BC* - 9148713.7094 *BD* - 679910507.7974 *CD*	0.9640
tan *δ*	-0.0811 *A* + 102.0512 *B* + 6321.2461 *C* + 1.4474 *D* - 31.5686 *AB* - 5441.8332 *AC* - 7.6245 *AD* - 6213.7732 *BC* - 126.5370 *BD* - 6504.8091 *CD*	0.9720
Firmness	34.9614 *A* + 360.2353 *B* - 387.5188 *C* - 6.7183 *D*	0.9635

A: VCO, *B*: fish gelatin, *C*: sodium alginate, *D*: water

Optimization of Pudding Formulation

Optimization of pudding formulation was performed by setting the tan δ value of 0.2 and firmness value of 11 N (obtained for a commercial pudding) as targeted values. Pudding formulation comprised 10.68% VCO, 3.41% fish gelatin, 0.59% sodium alginate and 68.33% water were selected as optimized formulation based on the highest desirability of 0.874, with predicted tan δ value of 0.2 and firmness value of 11 N. The optimized formulation was validated through experiments and was found to have no significant difference (p>0.05) between the predicted and experimental values of tan δ (0.1954 ± 0.0048) and firmness (11.93 ± 2.72 N).

Storage Stability of Puddings

Figure 1 shows the results obtained from storage stability study on the optimized pudding formulation over 4 weeks at 4 ± 1°C. Firmness of pudding increased significantly (p<0.05) from 3 weeks onwards (Figure 1 (a)). According to Alexa et al., (2010), a more solid structure of spreads developed over storage period was likely attributed to the formation of fat crystal network during slow post-crystallization processes. This could possibly explain that the increase in firmness of pudding in this study was ascribed to the presence of VCO. Likewise, FFA of pudding increased significantly (p<0.05) from 3 and 4 weeks onwards (Figure 1 (b)). The increase in FFA values suggested the hydrolytic degradation of

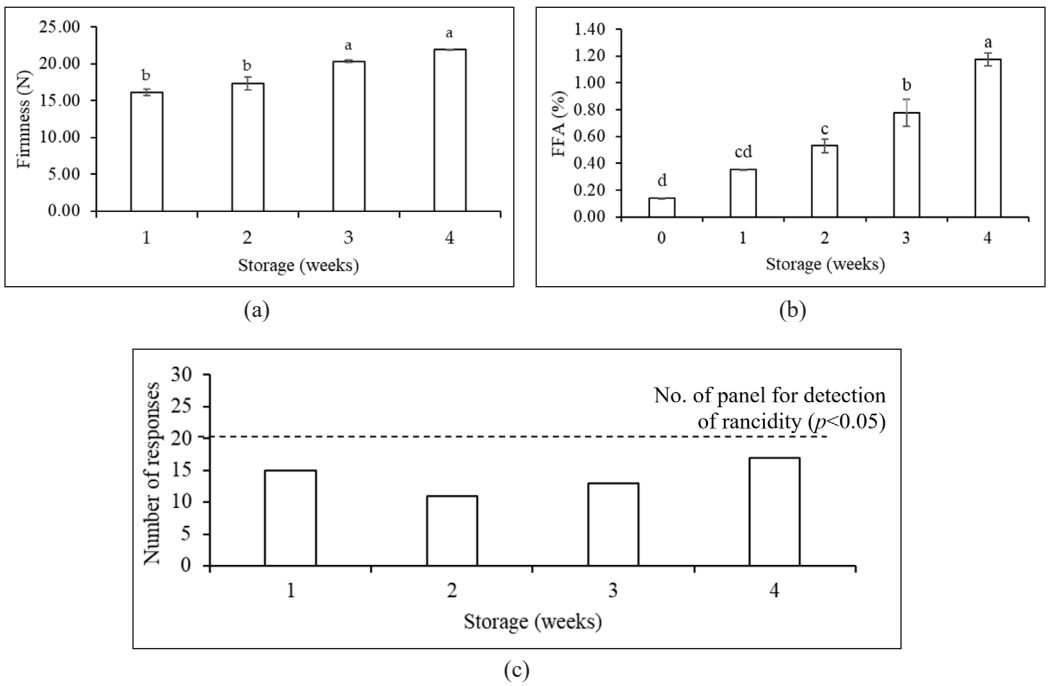

Figure 1. Results of storage stability of pudding over 4 weeks at 4 ± 1°C: firmness (a), FFA value (b), and count of panel who detected rancidity of pudding (c). Different letters indicate significant difference (p<0.05)

VCO in pudding. According to Eke-Ejiofor and Beleya (2015), the presence of the lipase activity or other hydrolytic action on lipid is responsible for the hydrolytic degradation that leads to an increase in FFA values of salad creams over storage period. The production of FFA can lead to rancidity that results in off-flavours of products (Talbot, 2016). Sensory assessment performed on pudding in this study found that rancid taste was not significant (p>0.05) when evaluated weekly (Figure 1 (c)), although significant increase in FFA values was observed from 3 weeks onwards. Results generally revealed that this pudding was stable during storage.

CONCLUSION

Optimized pudding formulation containing 10.68% VCO, 3.41% fish gelatin, 0.59% sodium alginate and 68.33% water was successfully obtained using mixture design and experimentally validated. Blending of high fish gelatin content with sodium alginate produced puddings with increased in the viscoelasticity and firmness. The firmness and FFA values of the puddings increased significantly from 3 weeks onwards, and the rancid taste was not statistically significant over 4 weeks of storage period at refrigeration temperature.

ACKNOWLEDGEMENT

Faculty of Food Science and Nutrition, Universiti Malaysia Sabah, Malaysia is gratefully acknowledged for providing the facilities and chemicals for this work.

REFERENCES

Alamprese, C., & Mariotti, M. (2011). Effect of different milk substitutes on pasting, rheological and textural properties of puddings. *LWT – Food Science and Technology*, *44*(10), 2019–2025.

Alexa, R. I., Mounsey, J. S., O'Kennedy, B. T., & Jacquier, J. C. (2010). Effect of κ-carrageenan on rheological properties, microstructure, texture and oxidative stability of water-in-oil spreads. *LWT-Food Science and Technology*, *43*(6), 843-848.

Association of Official Analytical Chemists. (1999). *Official method of analysis international* (17th ed.). Gaithersburg, USA: Association of Official Analytical Chemists.

Eke-Ejiofor, J., & Beleya, E. A. (2015). Effect of packaging materials on the storage conditions of salad cream from cassava, sweet potato and three leaf yam starches. *International Journal of Biotechnology and Food Science*, *3*(5), 57-62.

Henika, R. G. (1982). Use of response surface methodology in sensory evaluation. *Food Technology*, *36*(11), 96-101.

Karim, A. A., & Bhat, R. (2009). Fish gelatin: Properties, challenges, and prospects as an alternative to mammalian gelatins. *Food Hydrocolloids*, *23*(3), 563-576.

Larmond, E. (1977). *Laboratory methods for sensory evaluation of food*. Ottawa, Canada: Canada Department of Agriculture.

Li, J. M., & Nie, S. P. (2016). The functional and nutritional aspects of hydrocolloids in foods. *Food Hydrocolloids*, *53*, 46–61.

Lim, H. S., & Narsimhan, G. (2006). Pasting and rheological behaviour of soy protein-based pudding. *LWT- Food Science and Technology*, *39*(4), 343–349.

Marina, A. M., Man, Y. C., & Amin, I. (2009). Virgin coconut oil: Emerging functional food oil.

Trends in Food Science and Technology, *20*(10), 481-487.

McHugh, D. J. (2003). *A guide to the seaweed industry*. Rome, Italy: Food and Agriculture Organization of the United Nations.

Nevin, K. G., & Rajamohan, T. (2008). Influence of virgin coconut oil on blood coagulation factors, lipid levels and LDL oxidation in cholesterol fed Sprague–Dawley rats. *e-SPEN, the European e-Journal of Clinical Nutrition and Metabolism*, *3*(1), e1-e8.

Saha, D., & Bhattacharya, S. (2010). Hydrocolloids as thickening and gelling agents in food: A critical review. *Journal of Food Science and Technology*, *47*(6), 587–597.

Sarteshnizi, R. A., Hosseini, H., Bondarianzadeh, D., & Colmenero, F. J. (2015). Optimization of prebiotic sausage formulation: Effect of using β-glucan and resistant starch by D-optimal mixture design approach. *LWT-Food Science and Technology*, *62*(1), 704-710.

Talbot, G. (2016). The stability and shelf life of fats and oils. In P. Subramanuam (Ed.), *The stability and shelf life of food* (2nd ed., pp. 461-503). Cambridge, England: Woodhead Publishing.

Toker, O. S., Dogan, M., Caniyilmaz, E., Ersoz, N. B., & Kaya, Y. (2013). The effect of different gums and their interactions on the rheological properties of a dairy dessert: A mixture design approach. *Food Bioprocess Technology*, *6*(4), 896–908.

Tolstoguzov, V. (2003). Some thermodynamic considerations in food formulation. *Food Hydrocolloids*, *17*(1), 1-23.

Villarino, B. J., Dy, L. M., & Lizada, M. C. C. (2007). Descriptive sensory evaluation of virgin coconut oil and refined, bleached and deodorized coconut oil. *LWT-Food Science and Technology*, *40*(2), 193-199.

Zakaria, Z. A., Somchit, M. N., Jais, A. M., Teh, L. K., Salleh, M. Z., & Long, K. (2011). *In vivo* antinociceptive and anti-inflammatory activities of dried and fermented processed virgin coconut oil. *Medical Principles and Practice*, *20*(3), 231-236.

Pertanika J. Trop. Agric. Sc. 42 (4): 1219 - 1235 (2019)

PERTANIKA JOURNALS

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Journal homepage: http://www.pertanika.upm.edu.my/

The Chemical Composition, Anti-nutritional and Microbial Properties of Ensiled Cassava Root-Leaf Blends as Potential Feed in Swine Diet

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ABSTRACT

A study was conducted to determine the effects of ensiling on the proximate and mineral composition, anti-nutritional factors and microbial properties of ensiled cassava root-leaf blends at 50:50, 60:40, 70:30 and 80:20 ratios in an air tight sealed bottles and to also determine the blend that could replace maize in swine diet. The data was subjected to one-way analysis of variance in a completely randomized design. Results of the study indicated that blend 50:50 had highest value of crude protein 12.96%, while blend 60:40 had highest gross energy value of 4617.17 kcal/kg. Blend 70:30 had gross energy of 4180.95 kcal/kg and crude protein of 10.12%. Results of the mineral composition revealed that blend 50:50 had highest values of calcium 5.96 g/kg and phosphorus 1.98 g/kg. Anti-nutritional factors of the blends were drastically reduced after ensiling. Microbial load of ensiled blends revealed that only blend 70:30 recorded the presence of *Lactobacillus* spp., while *Salmonella* spp. was not detected in all the blends. It was concluded that ensiling was effective for removal of anti-nutritional factor, improving chemical composition and hygienic quality of ensiled product. Blend 70:30 was recommended as a replacement for maize in swine nutrition.

Keywords: Cassava root-leaf blends, ensiled, microbial properties, mineral contents, proximate composition, swine nutrition

ARTICLE INFO

Article history:
Received: 10 April 2019
Accepted: 23 July 2019
Published: 13 November 2019

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INTRODUCTION

The major source of energy feedstuff in Nigeria is maize where it constitutes about 30-40% of formulated swine diet. In recent times, the cost of maize has increased considerably due to increased production

ISSN: 1511-3701
e-ISSN: 2231-8542

of biofuel and droughts in some parts of Africa (United States Department of Agriculture [USDA], 2015). Consequently, the availability or supply does not meet its demand and livestock producers appear most hit in terms of scarcity and high cost of feedstuff (Hamzat et al., 2003). This has accelerated the demand to find alternative feed resources that can replace maize in swine diets and at a lower cost of production. Cassava, a nutritionally rich energy feedstuff which production is sustainable with a metabolizable energy of 3265 kcal/kg (Kanto & Juttupornpong, 2002) offers a veritable cheap alternative. Cassava can completely or partially replace maize of metabolizable energy 3300kcal/kg (Lesson & Summer, 2001) as an energy source for all classes of pigs requiring 2600-3200kcal/kg (National Research Council [NRC], 2012) in their diets (Nnadi et al., 2010).

The majority (70%) of the world's cassava is produced in Nigeria, Brazil, Indonesia, Democratic Republic of Congo and Thailand (Food and Agriculture Organization [FAO], 2015). In 2016, Nigeria produced about 46 million metric tonnes of cassava making the country the world's largest producer (FAO, 2016). Cassava root is composed almost exclusively of carbohydrate, as well as approximately 1-3% crude protein (Stupak et al., 2006). Cassava can be grown in areas with poor fertility as it is resistant to adverse environments and tolerates a range of rainfalls (Montagnac et al., 2009), thus, making its cultivation not seasonal. The crop is cultivated across the country. The high energy content and all year-round availability of cassava compared to maize makes it a potential replacement for maize in swine diet and can be relied upon to provide the anticipated relief against the increasing cost of livestock feeds. However, potential utilization of cassava roots as feedstuff is limited by some factors which include; low crude protein content (1-3%), anti-nutritional factors majorly cyanogenic glycosides and rapid perishability (Eruvbetine et al., 2002).

One of the remedies to these limitations is the supplementation of cassava root with cassava leaf which is richer in crude protein content (21%; Kanto & Juttupornpong, 2002). Cassava leaves, regarded as farm waste during harvesting of cassava roots, have been shown to be rich in protein, minerals (calcium, phosphorus) vitamins (A, B and C) and essential amino acids (leucine and lysine) (Adewusi & Bradbury, 1993). The leaves and tender stems are underutilized as they are often left to rot away on farmsteads in cassava producing areas (Aderemi et al., 2006). Nevertheless, it also contains anti-nutritional factors like the roots (Wobeto et al., 2007) and requires proper processing to reduce or eliminate these anti-nutritional factors.

Sun drying process is probably the cheapest and most common method used in the tropics for effective removal of anti-nutritional factors such as cyanide, oxalate and phytate in cassava (Cardoso et al., 2005; Wanapat, 2009). However, it is season dependent, laborious and time consuming. Ensiling is another method which is as

nearly as good as sun drying for preservation and reduction of anti-nutrients (Phuc et al., 2001). Ensiling process has been found to be an efficient means of reducing cyanide and other anti-nutritional factors to its barest negligible concentration and increasing nutritional value of cassava (Borin et al., 2005; Tetchi et al., 2012). In addition, the process has been reported to improve the nutritional composition of cassava peels (Adeleke et al., 2017), cassava mash (Oduah et al., 2015; Oluwafemi & Udeh, 2016). Furthermore, the process is cheap, simple and not season bound. According to Limon (1992), one of the advantages of cassava silage is that the preservation is guaranteed for several months, thus eliminating another limitation of cassava; rapid perishability.

However, the preservation of silage depends on the production of sufficient acid to inhibit activity of undesirable microorganisms under anaerobic conditions. Lactic acid bacteria (LAB) naturally present on forage crops are responsible for silage fermentation and also influence silage quality (Lin et al., 1992). During silage fermentation, LAB converts sugar into lactic acid and as a result, the pH is reduced and the forage is well preserved (Cai et al., 1999).

Ensiling is therefore postulated to be one of ways of tackling the problems of cassava root utilization in animal nutrition since it tends to proffer solution to most of its limitations, especially when combined with cassava leaf. Thus, this study was aimed at determining the proximate composition, mineral contents, anti-nutritional factors and microbial properties of different ratios of ensiled cassava root-leaf blends and to determine which of the blends will be nutritionally sufficient to replace maize in swine diet.

MATERIALS AND METHODS

The experiment was carried out at the Animal Nutrition Laboratory, College of Animal Science and Livestock Production located within latitude 7° 13' N and longitude 3° 26' E (Google Map, n. d.), Federal University of Agriculture, Abeokuta, Ogun State, Nigeria.

Collection and Processing of Cassava Root and Leaf

The cassava roots (TMS 30572) were harvested fresh from the demonstrating farm of the Federal university of Agriculture, Abeokuta, Ogun State. They were rinsed in water to remove the adhering dirt and sand and then grated in a locally fabricated cassava grinding machine (diesel engine of 8hp, Lagos, Nigeria). The ground (sieve size 2mm) unpeeled cassava roots were then packed in Hessian bags and placed under locally fabricated screwed pressed hydraulic presser (cassava dewatering machine) for 24 hours for the purpose of removing effluent as described by Kuye and Sanni (2002). The leaf biomass (consisting of leaves, petioles and stalk) remaining after harvesting of the roots were collected and wilted overnight to reduce 20% of the moisture content of the leaf biomass and later chopped (into 4cm long). The dewatered cassava pulp and wilted cassava leaf biomass were thereafter mixed together at different ratios.

Ensiling of Cassava Root and Leaf

Ensilage procedures were conducted for 21 days according to the methods employed by Hang (1998) in air tight cylindrical glass bottles sealed bottles with a capacity of (1400cm^3) using varying mixtures of grated, dewatered cassava pulp and dried cassava leaves at different ratios of 50:50, 60:40, 70:30 and 80:20 as described by Eruvbetine et al. (2002).The bottles were filled with the premixed materials as quickly as possible and compacted properly to eliminate remnant air so as to minimize the loss of nutrients by oxidation. A polythene sheet was used to cover the ensiled material to create anaerobic conditions for fermentation. Silages were made in quadruplicate to have a total of sixteen samples in all. The bottles were stored at room temperature and placed on the laboratory shelves. At the end of the ensiling procedure with a resultant blend of pH value range 4-5, representative sample of each of the blends were chemically analyzed.

Determination of pH. Ten (10) g of each sample was taken and crushed. Twenty (20) ml of distilled water was then added and the mixture homogenized properly. The pH was measured using a pH meter (INOLAB 730) with glass electrode (Association of Official Analytical Chemists [AOAC], 1995).

Chemical Composition of Ensiled Cassava Root-Leaf Blends

Determination of Proximate Composition. Proximate composition (dry matter, crude protein, crude fibre, ether extract and nitrogen free extract) of dewatered cassava root pulp, wilted cassava leaf biomass and samples of ECRLB were determined using standard analytical methods as described by Association of Analytical Chemists (AOAC) (1995); Dry matter was determined by oven drying at 100^o C for 24 hours, the nitrogen (N) content of the feed was determined by Kjedhal method and the crude protein was estimated as N x 6.25, ether extract was determined by Soxhlet fat analysis as; % Fat = weight of fat/ weight of sample x 100 crude fibre was determined by Weende method and calculated as; % crude fibre = $(W_1 – W_2)/W$ x 100. Where W_1 = weight before ashing, W_2=weight after ashing, W= weight of sample. Total ash was done using the furnace incineration gravimetric method, while the nitrogen free extract was calculated as: 100%- (%CP +% CF+ %EE+ % Ash). The gross energy values of the blends were determined according to standard procedures using the Adiatic Bomb Calorimeter (Model 1261; Parr Instrument Company, Moline, IL, USA).

Mineral Composition. Mineral content (calcium, magnesium, potassium, sodium, iron, copper, manganese, zinc and phosphorus) of the samples were determined according to the standard protocols described by Sodamade et al. (2013) as follows: one (1) g of each of the samples were weighed and subjected to dry ashing in a well cleaned porcelain crucible at 50^{o}C in a muffle furnace. The resultant ash was dissolved in 5.0 ml of HNO_3/HCl/ H_2O (1:2:3) and heated gently on a heating

mantle until brown fumes disappeared. Five (5.0) ml of distilled water was added to each of the sample in the crucible and heated until colourless solution was obtained. The mineral solution was filtered into a 100 ml volumetric flask through filter paper and the volume was made to the mark with distilled water. The solution was analyzed for its elemental composition using parking Elmer 403 model of atomic absorption spectrophotometer.

Anti-nutritional Composition. The anti-nutritional composition of the blends was determined before and after ensiling. Hydrogen cyanide compositions of the blends were determined through the alkaline titration procedure as described by Anhwange et al. (2011). Ten (10) grams of each of the samples were ground and soaked in the mixture of 200 cm^3 of distilled water and 10 cm^3 of orthophosphoric acid. The mixture was kept for 12 hours to release all the bounded cyanide. The mixture was then distilled until 150 cm^3 of the distillate were collected. Twenty (20) cm^3 of the distillate were poured into a conical flask containing 40 cm^3 of distilled water. Eight (8 cm^3) of ammonia solution (6 mol/dm^3) and 2 cm^3 of potassium iodide (5%) solution were added. The mixture was then titrated with silver nitrate (0.02 mol/dm^3) to faint but permanent turbidity, 1cm^3 (0.02 mol/dm^3 $AgNO_3$) is equivalent to (1.08 mg HCN). The percentage hydrocyanide was calculated with the formula:

$$Hydrocyanide\ (\%) = \frac{Titre \times 10 \times 0.27 \times 100}{1000 \times weight\ of\ sample}$$

Determination of Phytic Acid. The phytic acid was determined using the procedure described by Haritha and Jayadev (2017). About 2.0 g of each of the samples were weighed into 250 ml conical flask. One hundred (100) ml of 2% concentrated HCl acid was used to soak each sample in a conical flask for 3 hours and then filtered through a double layer of hardened filter papers. Fifty (50) ml of each filtrate was placed in 250 ml beaker and 100 ml of distilled water was added to each to give proper acidity. Ten (10) ml of 0.3% ammonium thiocyanate solution was added into each solution as indicator. Each solution was titrated with standard iron chloride solution, which contained 0.00195 g iron per ml. the end point colour was brownish-yellow which persisted for 5 minutes. The percentage phytic acid was calculated.

Determination of Tannin. For the determination of tannin, protocols described by Makkar et al. (1993) was adopted. About 400 mg of each of the samples were placed into two conical flasks and 40 ml diethyl ether containing 1% acetic acid (v/v) was added, then the mixtures were properly mixed to remove the pigment materials. Each supernatant was carefully discarded after 5 minutes and 20 ml of 70% aqueous acetone was added and the flasks was sealed with cotton plug covered with aluminum foil and then kept in electrical shaker for 2 hours for extraction. Each content in the flask was filtered through Whatman filter paper and

samples (filtrates) were for analyzed. Fifty (50 ml) of tannin extract from each sample was taken into test tubes and volume of each was made up to 1.0 ml with distilled water, 0.5 ml Folin-Ciocalteu reagent was added to each and mixed properly. Then 2.5 ml of 20% sodium carbonate solution was added and mixed. The mixtures were kept for 40 minutes at room temperature, after which absorbance was taken using spectrophotometer and concentration was estimated from the tannic acid standard curve.

Determination of Oxalate. Oxalate was determined by using the method described by Haritha and Jayadev (2017). One (1) gram of each sample was placed in a 250 ml volumetric flask, 190 ml of distilled water and 10 ml of 6 m HCl was added. Each mixture was warmed on a water bath at 90°C for 4 hours and the digested samples were centrifuged at a speed of 2,000 rpm (10,000 × *g*) for 5 minutes after which the supernatant was then diluted to 250 ml. Three (3) 50 ml aliquots of each supernatant were evaporated to 25 ml, and then the brown precipitate was filtered off and washed. The solutions were titrated with concentrated ammonia solution in drops until Salmon pink colour of methyl orange changed to faint yellow. The solutions were heated on a water bath to 90°C and the oxalate was precipitated with 10 ml of 5% calcium dichloride ($CaCl_2$) solution. The solutions were allowed to stand overnight and then centrifuged. Each precipitate was washed into a beaker with hot 25% tetraoxosulphate (vi) acid (H_2SO4), diluted to 125 ml with distilled water and after warming to 90°C it was titrated against 0.5 ml potassium tetraoxomanganate (vii) ($KMnO_4$).

Microbial Counts. The Microbial composition of the blends was analysed by using plate count method as described by Cai et al. (1998): Ten (10) g of each of the samples were blended with 90 ml of sterilized distilled water, then a further tenfold serial dilutions ranging from 10^{-1} to 10^{-5} were prepared and incubated anaerobically at 30°C for 48h on lactobacilli de Man, Rogosa and Sharpe (MRS) agar (Difco lab.Inc., Detroit, MI, USA) after which the numbers of *Lactobacillus* spp. were measured by the plate count method. Coliform bacteria were cultivated on blue light broth agar incubated at 30°C for 48h. *Pseudomonas* spp. were investigated on a sterile nutrient agar (oxoid) by pour plate method. Incubation of the plates was carried out at 37°C for 24hours. After incubation, the organisms were enumerated and purified by successive streaking on fresh agar plates. Pure cultures of the organisms on slants were stored at 4°C prior to identification. *Salmonella* spp. were sought on Salmonella-Shigella (SS) agar after a pre-enrichment on Rapport-Vassiliates soja (RVS) agar according to standard method. Total bacteria count was investigated on potato dextrose agar by spread plate method. The media used were prepared and incubated according to the labelled manufacturer's instructions. The colonies were enumerated and expressed as colony forming unit (CFU) per gram of fresh matter.

Statistical Analysis

Determined chemical compositions of the blends were subjected to one-way analysis of variance in a completely randomized design using Statistical Analysis System (SAS) (2000). The means of treatments showing significant differences were compared using Tukey's test. Statistical significance was accepted at P≤0.05.

RESULTS

Results of the proximate composition of ensiled cassava root-leaf blends are as shown in Table 1.The crude protein (CP), crude fibre (CF) and ether extracts (EE) values of the blends ranged from (7.21-12.96%), (18-22%) and (10-13.50%) respectively with blend 50:50 recording the highest (P<0.05) values while blend 80:20 recorded the least (P<0.05) values. Gross energy values across the blends ranged from (4180.95- 4671.17 kcal/kg) with blend 60:40 recording the highest value (4671.17 kcal/kg) while 70:30 recorded the least value (4180.95 kcal/kg) as compared to other blends.

The mineral composition of different proportions of ensiled cassava root- leaf blends is presented in Table 2. Highest (P<0.05) concentrations of calcium (Ca), magnesium (Mg), pottassium (K), manganesse (Mn), iron (Fe) and phosphorus (P) were recorded for blend 50:50, while blend 70:30 recorded highest (P<0.05) concentrations of sodium (Na) and copper (Cu) and blend 80:20 recorded highest zinc (Zn) concentration as compared to other blends.

The anti-nutritional factors of cassava root-leaf blends before and after ensiling were as shown in Table 3. There were significant (P<0.05) differences in the ECRLB anti-nutrients (tannin, phytate, HCN and oxalate) determined in this study both before and after ensiling.

The microbial load of ensiled cassava root-leaf blends is presented in Table 4. Total bacterial count and coliform count differed significantly (P<0.05) across the blends. Salmonella species were not detected, while Pseudomonas count and Lactobacillus counts did not differ significantly (P>0.05) across the blends.

Table 1
Proximate composition of ensiled cassava root-leaf blends

Composition	Cassava root- leaf ratio				SEM	P. value	DCRP	WCLB
	50:50	60:40	70:30	80:20				
CP (%)	12.96^{a}	10.54^{b}	10.12^{b}	7.21^{c}	0.533	0.021	1.54	24.46
CF (%)	22.00^{a}	20.00^{b}	19.00^{c}	18.00^{d}	0.382	0.034	20.42	28.65
EE (%)	13.50^{a}	12.00^{b}	11.00^{c}	10.00^{d}	0.333	0.031	0.98	25.00
Ash (%)	3.50^{a}	2.80^{b}	2.80^{b}	2.50^{c}	0.103	0.028	2.38	2.98
NFE (%)	35.54^{d}	48.51^{c}	49.78^{b}	52.29^{a}	1.676	0.016	45.00	38.76
DM (%)	87.50^{c}	94.50^{a}	90.00^{b}	91.50^{b}	1.584	0.018	92.35	81.75
Energy(kcal/kg)	4508.00^{b}	4671.17^{a}	4180.95^{d}	4331.63^{c}	38.54	0.046	4227	3957

Note. $^{a, b, c, d}$: means in the same row with different superscripts are significantly different (P<0.05). CP - crude protein; CF - crude fibre; EE - ether extract; NFE - nitrogen-free extract; SEM - Standard error of means; P. value - Probability value. DCRP - dewatered cassava root pulp, WCLB - wilted cassava leaf biomass

Table 2
Mineral composition of ensiled cassava root-leaf blends

Minerals	Cassava root- leaf ratio				SEM	P. value
	50:50	60:40	70:30	80:20		
Calcium (g/kg)	5.96[a]	5.78[b]	4.24[d]	4.33[c]	1.053	0.035
Phosphorus (g/kg)	1.98[a]	1.56[b]	0.43[d]	0.58[c]	0.167	0.024
Magnesium (g/kg)	3.45[a]	3.24[b]	2.65[c]	2.51[d]	0.097	0.028
Potassium (g/kg)	7.72[a]	7.56[b]	6.10[c]	5.46[d]	2.475	0.016
Sodium (g/kg)	1.92[c]	2.01[b]	2.20[a]	2.11[ab]	0.272	0.013
Manganese (mg/kg)	1.73[a]	1.44[b]	0.85[c]	0.66[d]	0.117	0.022
Iron (mg/kg)	49.65[a]	47.56[b]	22.24[c]	19.15[d]	3.549	0.036
Copper (mg/kg)	4.60[a]	4.20[b]	3.38[c]	3.12[d]	0.069	0.041
Zinc (mg/kg)	17.75[a]	16.50[b]	12.01[c]	10.57[d]	0.740	0.044

Note. [a, b, c, d]: means in the same row with different superscripts are significantly different (P<0.05). SEM - Standard error of means; P. value - Probability value

Table 3
Anti-nutritional factors of cassava root-leaf blends before and after ensiling

Parameters	Cassava root-leaf ratio				SEM	P. value
	50:50	60:40	70:30	80:20		
Before ensiling						
Tannin (%)	0.026[a]	0.017[b]	0.010[c]	0.006[d]	0.002	0.010
Phytate (%)	0.031[d]	0.108[a]	0.036[c]	0.048[b]	0.009	0.013
Oxalate (%)	0.029[a]	0.019[b]	0.017[c]	0.013[d]	0.002	0.021
HCN (mg/kg)	0.510[a]	0.475[a]	0.427[b]	0.330[c]	0.021	0.011
After Ensiling						
Tannin (%)	0.012[b]	0.018[a]	0.005[c]	0.003[c]	0.002	0.012
Phytate (%)	0.014[c]	0.052[a]	0.018[c]	0.023[b]	0.006	0.032
Oxalate (%)	0.013[a]	0.011[b]	0.007[c]	0.006[c]	0.004	0.031
HCN (mg/kg)	0.022[a]	0.017[b]	0.014[c]	0.017[b]	0.004	0.010

Note. [a, b, c, d]: means in the same row with different superscripts are significantly different (P<0.05). HCN - Hydrocyanide; SEM - Standard error of means; P. value - Probability value

Table 4
Microbial load of ensiled cassava root- leaf blends after ensiling

Microbial counts (x10^{3}CFU/g)	Cassava root-leaf ratio				SEM	P. value
	50:50	60:40	70:30	80:20		
Total bacteria count	1.210[b]	0.811[c]	0.810[c]	1.610[a]	0.100	0.000
Coliform	0.200[b]	0.300[a]	0.167[b]	0.200[b]	0.017	0.004
Pseudomonas	0.210	0.100	0.137	0.210	0.022	0.224
Lactobacillus	0.000	0.000	0.033	0.000	0.008	0.441
Salmonella	ND	ND	ND	ND	--	--

Note. [a, b, c, d]: means in the same row with different superscripts are significantly different (P<0.05). ND - Not detected; SEM - Standard error of means; P. value - Probability value

DISCUSSION

The feed ingredient that will replace maize successfully in swine diet must have chemical composition similar to those of maize. Proximate composition of the blends reveals that blend 70:30 recorded a crude protein (CP) value of 10.12% which is close to the CP of maize 8.9- 10.0 % as reported by (Osei et al., 1999; Sproule et al., 1988). This implies that it could be used to replace maize in swine diet. The crude protein values for blends 50:50, 60:40 and 80:20 (12.96, 10.54 and 7.21%) reported in this study were higher than values of (12.14, 9.48 and 7.0%) reported by Eruvbetine et al. (2002) for similar proportion of cassava roots and leaf mixture. The difference might be attributed to the processing methods employed, cassava variation and the environmental conditions. Motarjemi (2002), had earlier reported higher nitrogen content incorporated into ensiled cassava roots was found to improve protein quality as well as enhancing nutrient bio availability. The nutritional requirement of pigs (NRC, 2012) reveals that pigs require moderate quantities of protein for growth and development thus making the blends good sources of protein for all classes of swine. As the level of cassava leaves inclusion in the blends increased, there was a corresponding increase in the CP, CF and EE values thus confirming the fact that cassava leaves are good sources of protein, fibre and fat (Akinfala et al., 2002). The crude fibre range (18-22%) obtained in this study was higher as compared to the ones reported in previous literatures Anja et al. (2016; 10.7%) and Ngiki et al. (2014; 12.6%) in cassava root and leaf meal mixture. This higher crude fibre contents may be attributed to the peels, leaves and petioles (leaf biomass) contained in the blends and could be suitable for sows as higher dietary fibre has been reported to be beneficial to sows as it affects sows' colostrum composition (Loisel et al., 2013). However, some form of physical treatment may be needed for efficient utilization by other classes of pigs (Sauer et al., 1991) if ECRLB is to replace maize in their diet. An average crude fibre of 1.93% has been reported for maize by previous researchers (Osei et al., 1999; Sproule et al., 1988). Crude fibre helps in the maintenance of normal peristaltic movement of the intestinal tract and stimulate gut health, hence; diets containing lower fibre could cause constipation and eventually lead to colon disease (Okon, 1983). The ash contents (2.50-3.50%) reported in this study were higher than the recommended ash range of (1.5-2.5%) for nuts, seeds and roots in order to be suitable for animal feeds (Pomeranz & Clifton, 1981). The improved ash contents of the blends were due to the outer peels and leaf biomass contained in the blends, indicating higher mineral profile of the blends as compared to the value of 1.6% reported for maize (Zhai, 2002) and thus, better sources of mineral element in the swine diet. As the level of cassava inclusion in the blends increased, there was a corresponding increase in the level of Nitrogen free extract (NFE) values. Cassava roots have been reported to have higher level of starch and soluble sugars when compared

to maize (Eruvbetine & Adejobi, 2000) and this could be responsible for the higher gross energy values recorded. The gross energy values of the blends were higher than the values reported by Oso et al. (2014; 3919 kcal/kg) and Akapo et al. (2014; 3374.68 kcal/kg) for unpeeled cassava root meal used for feeding broiler chickens and values of (4058 kcal/kg) and (3832 kcal/kg) for cassava root silages registered by (Arajuo et al., 2016; Silva et al., 2008) respectively. In comparison, the gross energy values reported in this study were higher than the average value of maize (4003 kcal/kg) reported in literatures (Osei et al., 1999; Sproule et al*.,* 1988; Zhai, 2002). This further confirms that the blends are potential sources of energy in animal diet and that blend 70:30 which has a gross energy close to maize could conveniently replace maize in swine diet.

The role of minerals in swine metabolism is well documented (Close, 1999). Among other functions, they are important for carbohydrate, fat and protein metabolism and are involved in nutrient transfer across cell membranes (Close, 1999). Cassava leaves have been reported to be rich sources of minerals and vitamins (Buitrago et al., 2002), which could be responsible for the higher concentration of minerals in blend 50:50 that had higher ratio of leaves. Magnesium has been reported to be involved in maintaining electrical potential in leaves and activation of some enzyme systems (Ferrao et al., 1987). Also, calcium in conjunction with phosphorus, magnesium and Manganese is responsible for bone formation. The mineral profile of the blends reported in this study are higher than those reported by Akapo et al. (2014) and Omosuli (2014) for unpeeled cassava roots and boiled cassava roots respectively. The reason for improved mineral content reported in this study could be attributed to the processing differences as fermentation has been said (Motarjemi, 2002) to enhance micronutrient availability. The mineral profile reported in this study proofs sufficient for daily physiological needs of various classes of pigs as established by (Kinh, 2002).

It is an established fact that cassava contains anti-nutritional compounds that affect digestibility and absorption of nutrients (Graf et al., 1987), thus, determination of anti-nutrients is of great necessity. The synergistic efforts of ensiling employed in this study are believed to be responsible for the reduced ANFs values observed after the ensiling procedure. The reduction level of tannin concentration across the blends; 50:50 (54%), 60:40 (53%), 70:30 (50%) and 80:20 (50%) is similar to the reports of (Nakagawa et al., 2002) that stated that about 40-50% of tannin is lost after processing. Anti-nutritional characteristics of tannin include: antioxidant, inhibiting starch and protein digestibility and also hinder iron and thiamin absorption (Bravo, 1998; Silva & Silva, 1999). Phytate is another anti-nutritional compound found in abundance in cassava roots (Marfo et al., 1990). The phytate range (0.14-0.052g/100g**)** reported after processing in this study was lower than the values reported by Oboh (2006; 7.05mg/g) during the fermentation of cassava peels

and Omosuli (2014; 0.79mg/100g) in boiled cassava roots. Phytate reduction has been attributed to the activity of the endogenous phytate enzyme from the raw ingredient and inherent microorganisms which are capable of hydrolyzing the phytic acid in the fermented food preparations into inositol and orthophosphate (Sandberg & Andlid, 2002). Phytates have been known to form insoluble salts with metals thus, making them unavailable for absorption in the body (Igbabul et al., 2014), so reduced level of phytates in feeds improves the availability and absorption of required metal ions in the body. Oxalates are anti-nutrients that negatively affect Ca and Mg bioavailability, form complexes with protein and inhibit peptic digestion (Massey, 2007; Oboh, 1986). Oxalate contents (0.006-0.013g/100g) reported in this study falls within the recommended level of (<0.05%) ingestion by non-ruminants (Rahman et al., 2012). The greater percentage reduction of HCN concentration across the blends (50:50; 95.7%), (60:40; 96.42%), (70:30; 96.72%) and (80:20; 94.85%) after processing establishes the fact that fermentation is a very effective process for eradication of endogenous cyanic compounds in cassava roots as reported by previous researchers (Essers et al., 1996; Igbabul et al., 2014; Kirmayo et al., 2000; Tetchi et al., 2012). A 50% decrease in HCN during the addition of cellulolytic bacteria to improve the quality of cassava flour was reported by Meryandini et al. (2011), while (70-75%) and 85% decrease was observed by Achinewu et al. (1998) and Kobawila et al. (2005) after 72 hours fermentation respectively. The HCN level reported in this study is safe for inclusion by pigs since it is below the lethal dose of 1-3 mg HCN/kg diet reported by Constable et al. (2017) for monogastric animals.

Indigenous natural fermentation has been reported to involve mixed colony of microorganisms (Kobawila et al., 2005). The growth and succession pattern of these organisms were reported to be dependent on factors such as water activity, pH, and substrate (Blandino et al., 2003), thus the microbial as well as resulting physiochemical interactions eventually regulate the number and types of microorganisms that survives to the end of the fermentation process (Brauman et al., 1996; Padonu et al., 2009). The lowest coliform count observed with blend 70:30 may be attributed to the presence of Lactobacillus species in this particular blend which were not present in other blends. Species of lactic acid bacteria have been shown to inhibit coliforms or Enterobacteriacea (Lo et al., 2010). Presence of coliforms in foods may indicate contamination/inadequate conditions of hygiene but which does not confer a significant risk to human health (Alves & Setter, 2000) and consequently animal health. The main source of contamination includes humans, sewage, utensils, processing equipment and environment, handling and storage conditions and rodents (Eze et al., 2008). Presence of coliform counts in the silages produced in this study agrees with the findings of (Napasirth et al., 2015) in silage of cassava residues (cassava

leaves, peel and pulp). Lactobacillus species were only found in blend 70:30 and was present in abundance (3.3 x 10^5CFU/g). No particular reason could be attributed to this. When lactic acid bacteria especially lactobacilli reach at least 10^5 CFU/g of fresh matter, silage can be well preserved (Napasirth et al., 2015). Epiphytic lactic acid bacteria are naturally present in forage crops and are responsible for silage fermentation and also influence silage quality (Lin et al., 1992) by reducing the pH and inhibiting the growth of other harmful bacteria as a result, forage is preserved (Cai et al., 1999). The absence of *Lactobacillus* spp. in other blends could be due to the presence of other fermenting bacteria which are embedded in the total bacteria counts but are not revealed in this study as it is observed that these blends (60:50, 60:40, and 80:20) had a higher concentration of TBC than blend 70:30. The salmonella count recorded zero count for all the blends. Salmonella are pathogenic organisms, the fact that these organisms were not found in the blends is highly positive and confirms the safety of the silage because its presence can pose a risk to animal health in the form of infections, intoxication and feed poisoning (Cai et al., 1999).

CONCLUSION

The study concluded that ensiling improved nutritional qualities of the blends with respect to chemical composition, hygienic quality as well as anti-nutrient reduction and the blends were well preserved. Ensiled cassava root-leaf mixture of 70:30 had chemical composition (energy and crude protein) similar to the widely standardized and reported composition of maize in literatures and could therefore be suggested to replace maize in swine diets. Further study is recommended on the evaluation of this blend in swine diet.

ACKNOWLEDGEMENT

The authors wish to acknowledge the Animal Nutrition Laboratory Technologists (Mrs. Ebun Adejumoke Oyedele and Mr. Olusegun Samuel Balogun) of the College of Animal Science and Livestock Production, Federal University of Agriculture Abeokuta for their support during the Laboratory analysis of samples from the study.

REFERENCES

Achinewhu, S. C., Barber, L. I., & Ijeoma, I. O. (1998). Physicochemical properties and garification (*gari* yield) of selected cassava cultivars in Rivers State, Nigeria. *Plant Foods and Human Nutrition*, *52*(2), 133-140.

Adeleke, B. S, Akinyele, B. J, Olaniyi, O. O., & Jeff-Agboola, T. A. (2017). Effect of fermentation on chemical composition of cassava peels. *Asian Journal of Plant Science and Research*, *7*(1), 31-38.

Aderemi, F. A., Alabi, O. M., & Lawal, T. E. (2006). Utilization of whole cassava meal by egg type chicken. In A. M., Raji, J. A., Oluokun, & S. O. Odukoya (Eds.), *Proceedings of the 11th Annual conference of Animal Science Association of Nigeria* (pp. 73-75). Ibadan, Nigeria: Animal Science Association of Nigeria - Nigerian Institute of Animal Scientists (ASAN-NIAS).

Adewusi, S. R. A., & Bradbury, J. H. (1993). Carotenoids in cassava: Comparison of open-column and HPLC methods of analysis. *Journal of the Science of Food and Agriculture*, *62*(4), 375–383.

Akapo, A. O., Oso, A. O., Bamgbose, A. M., Sanwo, K. A., Jegede, A. V., Sobayo, R. A., … Olorunsola, R. A. (2014). Effects of feeding cassava (*Manihot esculenta* Crantz) root meal on growth performance, hydrocyanide intake and haematological parameters of broiler chicks. *Journal of Tropical Animal Health Production*, *46*(7), 1167–1172.

Akinfala, E. O., Aderibigbe, A. O., & Matanmi, O. (2002). Evaluation of the nutritive value of whole cassava plant as replacement for maize in the starter diets for broiler chicken. *Livestock Research for Rural Development*, *14*(6), 23-30.

Alves, A. A. C., & Setter, T. L. (2000). Response of cassava to water deficit: Leaf area growth and Abscisic acid. *Crop Science*, *40*(1), 131-137.

Anhwange, B. A., Asemave, K., Ikyenge, B. A., & Oklo, D. A. (2011). Hydrogen cyanide content of *Manihot utilissima*, *Colocasia esculenta*, *Dioscorea bulbifera* and *Dioscorea dumetorum* tubers found in Benue State. *International Journal of Chemistry*, *3*(4), 69-71.

Anja, A., Amaha, N., & Urge, M. (2016). Effects of inclusion rate of cassava (*Manihot esculenta* Crantz) leaf and root mixture on feed intake and growth rate of grower chicks. *Journal of Biology, Agriculture and Healthcare*, *6*(5), 52-59.

Araujo, D. D., Amorin, A. B., Saleh, M. A. D., Curcelli, F., Perdigon, P. L., Bicudo, S. J., & Berto, D. A. (2016). Nutritional evaluation of integral cassava root silages for growing pigs. *Animal Nutrition*, *2*(3), 149-153.

Association of Official Analytical Chemists. (1995). *Official methods of analysis* (15th ed.). Gaithersburg, USA: AOAC International.

Blandino, A., Al-Aseeri, M. E., Pandiella, S. S., Cantero, D., & Webb, C. (2003). Cereal-based fermented foods and beverages. *Food Research International*, *36*(6), 527-543.

Borin, K., Lindberg, J. E., & Ogle, R. B. (2005). Effect of variety and preservation method of cassava leaves on diet digestibility by indigenous and improved pigs. *Animal Science*, *80*(3), 319-324.

Brauman, A., Kéléké, S., Malonga, M., Miambi, E., & Ampe, F. (1996). Microbiological and biochemical characterization of cassava retting, a traditional lactic acid fermentation for foo-foo (cassava flour) production. *Applied and Environmental Microbiology*, *62*(8), 2854-2855.

Bravo, L. *(*1998). Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition Review*, *56(11),* 317*–3*33.

Buitrago, J., Ospina, B., Gil, J. L., & Aparico, H. (2002). *Cassava roots and leaf meal as the main ingredient in poultry feeding: Some experiences in Colombia*. Retrieved April 9, 2019, from https://pdfs.semanticscholar.org/17be/9576e64e27fec37748ce6b21a90b6b33ac43.pdf?_ga=2.162281276.1499235407.1563780248-563722479.1563179160

Cai, Y., Benno, Y., Ogawa, M., & Kumai, S. (1999). Effect of applying lactic acid bacteria isolated from forage crops on fermentation characteristics and aerobic deterioration of silage. *Journal of Dairy Science*, *82*(3), 520–526.

Cai, Y., Benno, Y., Ogawa, M., Ohmomo, S., Kumai, S., & Nakase, K. (1998). Influence of *Lactobacillus* spp. from an innoculant and of *Weissella* and *Leuconostoc* spp. from forage crop on silage. *Applied and Environmental Microbiology*, *64*(8), 2982-2987.

Cardoso, A. P., Mirione, E., Ernesto, M., Massaza, F., Cliff, J., Haque, R. M., & Bradbury, J. H. (2005). Processing of cassava roots to remove cyanogens. *Journal of Food Composition and Analysis*, *18*(5), 451 – 460.

Close, W. H. (1999). Organic minerals for pigs. In T. P. Lyons & K. A. Jacques (Eds.), *Proceedings of Biotechnology in the Feed Industry*: *15th Alltech's Annual Symposium* (pp. 51-60). Loughborough, England: Nottingham University Press.

Constable, P. D., Hinchcliff, K. W., Done, S. H., & Gruenberg, W. (2017). *11-diseases of the hemolymphatic and immune systems*: *Veterinary medicine* (11th ed.). London, United Kingdom: Elsevier Health Sciences.

Eruvbetine, D., & Adejobi, P. K. (2000). Preparation of cass-soya concentrate for inclusion in poultry diet (in vitro studies). Nigerian Journal of Animal Production, 27(1), 50-54.

Eruvbetine, D., Tajudeen, I. D., Adeosun, A. T., & Olojede, A. A. (2002). Cassava (*Manihot esculenta*) leaf and tuber concentrate in diets for broiler chickens. *Bioresource Technology*, *86*(3), 277-281.

Essers, A. J. A., Van der Grift, R. M., & Voragen, A. G. J. (1996). Cyanogen removal from cassava roots during sun-drying. *Journal of Food Chemistry*, *55*(4), 319-325.

Eze, V. C., Okoye, J. I., Agwung, F. D., & Nnabueke, C. (2008). Chemical and microbiological evaluation of soybeans flours bought from local markets in Onitsha, Anambra State, Nigeria. *Continental Journal of Applied Science*, *3*, 39-45.

Ferrao, J. E. M., Ferro, A. M. B. C., & Antures, A. M. G. (1987). Bambara groundnut (*Vigna subterranean*): Aspect of its nutritive value. *Gracia deorta seriede Estudos Agronomics*, *14*, 35-39.

Food and Agriculture Organization. (2015). *Food outlook biannual report on global food markets.* Retrieved December 20, 2015, from http://www.fao.org.

Food and Agriculture Organization. (2016). *Pigs and animal production and health.* Italy, Rome: FAO.

Google Map. (n. d). *Google maps directions for locations coordinates*. Retrieved October 2, 2016, from http://www.earthgoogle.com

Graf, E., Empson E., & Eaton, J. W. (1987). Phytic acid. A natural antioxidant. *Journal of Biological Chemistry*, *262*(24), 11647-11650.

Hamzat, R. A., Tiamiyu, A. K., & Raji, A. M. (2003). Effect of dietary inclusion of Kola Pod Husk (KPH) on growth performance of West African Dwarf (WAD) goats. In *Proceedings of 28th Annual Conference of Nigeria Society of Animal Production* (pp.21-273). Ibadan, Nigeria: Nigerian Society of Animal Production.

Hang, D. T. (1998). *Digestibility and nitrogen retention in fattening pigs fed different levels of ensiled cassava leaves as a protein source and ensiled cassava root as energy source*. Retrieved January 09, 2018, from http://www.1rrd.org/1rrd10/3/hang1.htm

Haritha, M. C., & Jayadev, A. (2017). Determination of tannin, phytate and total oxalate content in selected varieties of cassava (*Manihot esculenta* Crantz) tubers. *International Journal for Research in Applied Science and Engineering Technology*, *5*(11), 141-145.

Igbabul, B. D., Amove, J., & Twadue, I. (2014). Effect of fermentation on the proximate composition, anti-nutritional factors and functional properties of cocoyam (*Colocasia esculenta*) flour. *African Journal of Food Science and Technology*, *5*(3), 67-74.

Kanto, U., & Juttupornpong, S. (2002). *Clean cassava chips for animal feeding in Thailand*. Retrieved October 23, 2015, from https://pdfs.semanticscholar.org/fa64/7eb9dee0b139757486f539b6cd5871b62671.pdf

Kimaryo, V. M., Massawe, G. A., Olasupo, N. A., & Holzapfel, W. H. (2000). The use of a starter culture on the fermentation of cassava for the

production of "kivumde" a traditional Tanzanian food product. *International Journal of Food Microbiology*, *56*(2-3), 179 – 190.

Kinh, L. V. (2002). Determination of protein and amino acid for pregnant sow and lactating sow. *Science and Technology Journal of Agricultural Rural Development*, *10*, 25-28.

Kobawila, S. C., Loumbe, D., Keleke, S., Hounhouigan, J., & Gamba, C. (2005). Reduction of the cyanide content during fermentation of cassava root and leaf to produce bikedi and ntoba mbodi, two food products from Congo. *African Journal of Biotechnology*, *4*(7), 689-696.

Kuye, A., & Sanni, L. O. (2002). Analysis of the equilibrium moisture sorption data for lafun and soyaflour. *Journal of Modeling, Design and Management of Engineering System*, *1*(1), 63-70.

Lesson, S., & Summer, J. D. (2001). *Scott's nutrition of chicken* (4th ed.). Guelph, Canada: University Book.

Limon, R. L. (1992). *Ensilage of cassava products and their use as animal feed*. Retrieved April 1, 2019, from ftp://ftp3.us.freebsd.org/pub/misc/cd3wd/1004/_ag_animal_feed_roots_tubers_bananas_unfao_en_lp_103300_.pdf

Lin, C., Bolsen, K. K., Brent, B. E., & Fung, D. Y. C. (1992). Epiphytic lactic acid bacteria succession during the pre-ensiling and ensiling periods of alfalfa and maize. *Journal of Applied Bacteriology*, *73*(5), 375-387.

Lo, W. U., Ho, P. L., & Chow, K. H. (2010). Fecal carriage of CTX-M type extended-spectrum beta-lactamase-producing organisms by children and their household contacts. *Journal of Infection*, *60*(4), 286-292.

Loisel, F., Farmer, C., Ramaekers, P., & Quesnel, H. (2013). Effects of high fiber intake during late pregnancy on sow physiology, colostrum production and piglet performance. *Journal of Animal Science*, *91*(11), 5269-5279.

Makkar, H. P. S., Blummer, M., Browy, N. K., & Becker, K. (1993). Gravimetric of tannins and their correlation with chemical and protein precipitation methods. *Journal of the Science of Food and Agriculture*, *61*(2), 161-165.

Marfo, E. K., Simpson, B. K., Idowu, J. S., & Oke, O. L. (1990). Effect of local food processing on phytate levels in cassava, cocoyam, yam, maize, sorghum, rice, cowpea, and soybean. *Journal of Agricultural Food Chemistry*, *38(7),* 1580–*1585.*

Massey, L. K. (2007). Food oxalate: Factors affecting measurement, biological variation and bio availability. Journal of the American Dietetic Association, 107(7), 1191-1194.

Meryandini, V., Melani, A., & Sunarti, T. C. (2011). Addition of cellulolytic bacteria to improve the quality of fermented cassava flour. *African Journal of Food Science and Technology*, *2*(2), 30-35.

Montagnac, J. A., Davis, C. R., & Tanumihardjo, S. A. (2009). Nutritional value of cassava for use as a staple food and recent advances for improvement. *Comprehensive Review of Food Science*, *8*(3), 181-194.

Motarjemi, Y. (2002). Impact of small scale fermentation technology on food safety in developing countries. *International Journal of Food Microbiology*, *75*(3), 213-229.

Nakagawa, T., Yokozawa, T., Terasawa, K., Shu, S., & Juneja, L. R. (2002). Protective activity of green tea against free radical and glucose-mediated protein damage. *Journal of Agricultural Food Chemistry*, *50(8),* 2418–*2422.*

Napasirth, V., Napasirth, P., Sulinthone, T., Phommachanh, K., & Cai, Y. (2015). Microbial population, chemical composition and silage

fermentation of cassava residues. *Journal of Animal Science*, *86*(9), 842–848.

National Research Council. (2012). *Nutrient requirements of swine* (11th ed.). Washington, USA: The National Academy Press.

Ngiki, Y. U., Igwebuike, J. U., & Moruppa, S. M. (2014). Effects of replacing varying dietary levels of maize with cassava root-leaf meal mixture on haematology and carcass characteristics of broiler chickens. *International Journal of Science and Technology*, *3*(6), 363-373.

Nnadi, P. A., Omeke, B. C., & Okpe, G. C. (2010). Growth and reproductive performances of weaner pigs fed maize replaced cassava diet. *Animal Research International*, *7*(3), 1257-1263.

Oboh, G. (2006). Nutrient enrichment of cassava peels using a mixed culture of *Saccharomyces cerevisae* and *Lactobacillus* spp. solid media fermentation techniques. *Electronic Journal of Biotechnology*, *9*(1), 64-79.

Oboh, S. O. *(*1986). *Biochemical composition and utilization of sweet potato* (*Ipomoea batatas*) *in ruminant rations* (Unpublished Doctoral thesis)*, University of Ibadan, Nigeria.*

Oduah, N. O., Adepoju, P. A., Longe, O., Elemo, G. N., & Oke, O. V. (2015). Effects of fermentation on the quality and composition of cassava mash (*gari*). *International Journal of Food Nutrition and Safety*, *6*(1), 30-41.

Okon, B. D. (1983). *Studies on the chemical composition and nutritive values of the fruits of African star apple* (Unpublished Master's thesis), University of Calabar, Nigeria.

Oluwafemi, G. I., & Udeh, C. C. (2016). Effect of fermentation periods on the physiochemical and sensory properties of *gari*. *Journal of Environmental Science, Toxicology and Food Technology*, *10*(1), 37-42.

Omosuli, S. V. (2014). Effects of processing on the chemical and anti-nutritional properties of cassava roots. *Journal of Botanical Sciences*, *3*(1), 27-31.

Osei, S. A., Dei, H. K., & Tuah, A. K. (1999). Evaluation of quality protein maize as a feed ingredient for layer pullet. *Journal of Animal and Feed Sciences*, *8*(2), 181 – 189.

Oso, A. O., Akapo, O., Sanwo, K. A., & Bamgbose, A. M. (2014). Utilization of unpeeled cassava (*Manihot esculenta* Crantz) root meal supplemented with or without charcoal by broiler chickens. *Journal of Animal Physiology and Animal Nutrition*, *98*(3), 431-438.

Padonou, S. W., Hounhouigan, J. D., & Nago, M. C. (2009). Physical, chemical and microbiological characteristics of *lafun* produced in Benin. *African Journal of Biotechnology*, *8*(14), 3320-3325.

Phuc, B. H. N., Lindberg, J. E, Ogle, B., & Thomke, S. (2001). Determination of the nutritive value of tropical biomass products for monogastrics using Rats: Effects of drying temperature, ensiling and level of inclusion of cassava leaves and sweet potato vines. *Asian-Australian Journal of Animal Science*, *14*(7), 986-993.

Pomeranz, V., & Clifton, D. (1981). Properties of defatted soybean, peanut field pea and pecan flours. *Journal of Food Science*, *42*, 1440-1450.

Rahman, M. M., Abdullahi, R. B., & Wankhadijah, W. E. (2012). A review of oxalate poisoning in domestic animals: Tolerance and performance aspects. *Journal of Animal Physiology and Animal Nutrition*, *97*(4), 606-614.

Sandberg, A. S., & Andlid, T. (2002). Phytogenic and microbial phytases in human nutrition. *International Journal of Food Science Technology*, *37*(7), 823-833.

Sauer, W. C., Mosenthin, R., Ahrens, F., & Denhartog, L. A. (1991). The effect of source of fibre on ileal amino acid digestibility and bacterial nitrogen excretion in growing pigs. *Journal of Animal Science*, *69*(10), 4070 -4077.

Silva, M. A. A., Furlan, A. C., Moreira, I., Parano, D., & Martins, E. N. (2008). Nutritional evaluation and performance of cassava root silage with or without whole soybean in nursery piglets. *Revista Brasileira de Zootecnia*, *37*(8), 1441-1449.

Silva, M. R., & Silva, M. A. *(*1999). Nutritional aspects of phytates and tannins. *Revista de Nutrição*, *12*(1), 21-32.

Sodamade, A., Bolaji, O. S., & Adeboye, O. O. (2013). Proximate analysis, mineral contents and functional properties of *Moringa oleifera* leaf protein concentrate. *Journal of Applied Chemistry*, *4*(6), 47-51.

Sproule, A. M., Serna-Saldivar, S. O., Bockholt, A. J., Rodney, L. W., & Knabe, D. A. (1988). Nutritional evaluation of tortillas and tortilla chips from quality protein maize. *Cereal Food World*, *33*(2), 233-236.

Statistical Analysis System. (2000). *SAS/STAT® user's guide*. Cary, USA: SAS Institute Incorporation.

Stupak, M., Vandeschuren, H., Gruissem, W., & Zhang, P. (2006). Biotechnological approaches to cassava protein improvement. *Trends in Food Science Technology*, *17*(12), 634- 641.

Tetchi, F. A., Solomon, O. W., Celah, K. A., & Georges, A. N. (2012). Effects of cassava variety and fermentation time on biochemical and microbiological characteristics of raw artisanal starter for attiéké production. *Innovative Romanian Food Biotechnology*, *10*(3), 40-47.

United States Department of Agriculture. (2015). *World agricultural supply and demand estimates*. Retrieved October 23, 2015, from http://www.usda.gov/oce/commodity/wasde/latest.pdf

Wanapat, M. (2009). Potential uses of local feed resources for ruminant. *Tropical Animal Health Production*, *41*(7), 1035-1039.

Wobeto, C., Correa, A. D., De Abreu, C. M. P., Dos Santos, C. D., & Pereira, H. V. (2007). Antinutrients in the cassava (*Manihot esculenta* Crantz) leaf powder at three ages of the plant. *Food Science and Technology*, *27*(1), 108-112.

Zhai, S. W. (2002). *Nutritional evaluation and application of quality protein maize Zhong Dan 9409 in laying hen feed* (Unpublished Master's thesis), Northwestern Agricultural and Forestry University, China.

Pertanika J. Trop. Agric. Sc. 42 (4): 1237 - 1250 (2019)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Effect of Monocalcium Phosphate Supplementation on the Growth Performance, Carcass Characteristic, Gut Histomorphology, Meat and Bone Quality of Broiler Chickens

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ABSTRACT

The aim of this study was to investigate the effect of different concentration of monocalcium phosphate supplementation on the growth performance, carcass characteristics, gut morphology, meat quality, and bone quality of broiler chickens. A total of 108 day-old male broiler chicks (Cobb 500) were randomly divided into 3 treatment groups with 6 replicates and each replicate consist of 6 birds. Treatment 1 (control) was fed available commercial starter and finisher diets containing dicalcium phosphate. Treatment 2 and Treatment 3 were fed the same commercial diet but with added 0.5% and 1.0% of monocalcium phosphate respectively. Weekly body weight, feed intake and feed conversion ratio were calculated to determine their growth performance. A total of 12 chickens per each treatment group were selected randomly and slaughtered for gut histomorphology, carcass characteristics, meat, and bone quality assessment at the end of the 42 days study period. In the present study, birds supplemented with 0.5% MCP showed significant increase ($p > 0.05$) in growth performance (body weight, body weight gain, and feed intake), gut histomorphology (villi height), and bone quality (bone weight, diaphysis diameter, medullary canal diameter, lateral wall thickness, medial wall

ARTICLE INFO

Article history:
Received: 14 June 2019
Accepted: 23 July 2019
Published: 13 November 2019

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ISSN: 1511-3701
e-ISSN: 2231-8542

thickness, and bone breaking strength). There were no significant differences ($p > 0.05$) in the carcass characteristics and meat quality between treatment groups. In summary, monocalcium phosphate supplementation could be the key to reduce culling of lame broiler birds based on the positive effect on the growth performance, gut histomorphology and bone quality of broiler chickens without affecting the carcass characteristics and meat quality.

Keywords: Bone quality, broilers, carcass characteristic, growth performance, gut histomorphology, meat quality, monocalcium phosphate

INTRODUCTION

Prosperous as it is on the surface, the broiler industry faces numerous challenges in accordance with the rapidly growing population. To keep up with the demands, extensive researches have been by the combination of genetics enhancement and feed enrichment to boost the feed conversion ratio (FCR) and growth rate in broiler chickens (Gonzalez-Ceron et al., 2015). This knowledge has greatly contributed to the poultry industry, giving birth to fast-growing strains such as Cobb 500 and Ross 308. These breeds are considerably larger than their predecessors and have more amount of muscles especially in the breast area due to very efficient FCR.

In spite of its remarkably rapid growth rate, the undeniable issue regarding leg problems in broiler chickens continues to cause economic losses in the broiler industry. This is due to the late development of bones compared to the fast development of muscles. Other than that, the pressure of the muscle weight on the bones also contributes to leg problems. Examples of common leg problems in broiler chicken are Valgus (VVD), crooked toes, tibial dyschondroplasia (TD), vertebral deformities, twisted legs, osteoporosis of the proximal femur, femoral head necrosis, rupture of the gastrocnemius tendon and rickets (Dinev, 2012). According to Knowles et al. (2008), over 27.6% of birds at the mean age of 40 days showed poor locomotion and 3.3% were almost unable to walk. This is unsettling for the broiler industry, as the skeletal deformities have been observed to cause pain and movement problems, resulting in lameness of the bird, limited movement which leads to loss of appetite and low feed consumption, thus resulting poor growth rate, increased culling rate, increased fatality, and increased carcass condemnation and degrading at slaughter.

In general, broiler chicken receives an inadequate amount of calcium and phosphorus. This is because the calcium (Ca) and phosphorus (P) from vegetable-based diet only fulfil 30% of the amount required by the body, and only half of it will be absorbed by the intestines (Eeckhout & De Paepe, 1994). Supplementing inorganic source of calcium and phosphorus such as monocalcium phosphate (MCP) into the commercial diet are potentially favourable in providing the extra Ca and P needed by the body (Liu et al., 2013). Theoretically, properties of MCP include improvement of digestion and the efficiency of carbohydrate, protein, fat, mineral, and energy metabolism in the body. Thus, the objective of this

study was to investigate the effect of supplementing different concentrations of MCP on the growth performance, gut histomorphology, carcass characteristics, meat quality, and bone quality in commercial broilers.

MATERIALS AND METHODS

Birds, Husbandry and Diets

All experimental procedures were conducted in accordance with the Institutional Animal Care and Use Committee of Research Policy at Universiti Putra Malaysia (UPM). A total of 108 day-old male broiler chicks (Cobb 500) was obtained from a local hatchery. Immediately after arrival, the chicks were weighed and randomly allocated into 3 dietary treatments placed in battery cages with wired flooring in an open-sided house. Each treatment consisted of 6 replications, with 6 broilers per replication. The birds were vaccinated intraocularly with live Newcastle disease (ND) and infectious bronchitis (IB) vaccine on day 7 followed by infectious bursal disease (IBD) vaccine on day 14. Throughout the experimental period, water and feed were provided ad libitum to the birds. The broilers were fed commercial starter and finisher diets from 0 to 21 and 22 to 42 days respectively. The birds were provided with three types of diets. The dietary treatments were Treatment 1 (T1) commercial diet without any supplementation (control); Treatment 2 (T2) commercial diet supplemented with 0.5% MCP and Treatment 3 (T3) commercial diet supplemented with 1.0% MCP. The nutritive value of the starter and finisher diets are presented in Table 1.

Table 1
Nutrient content of starter and finisher diets

	Treatments					
	Starter (0-21 days)			Finisher (22-42 days)		
	T1	T2	T3	T1	T2	T3
Nutrient content						
ME (MJ/kg)	12.90	12.90	12.90	13.30	13.30	13.30
Crude protein (%)	22.00	22.00	22.00	20.00	20.00	20.00
Crude fibre (%)	5.00	5.00	5.00	8.00	8.00	8.00
Crude fat (%)	4.50	4.50	4.50	4.00	4.00	4.00
Moisture (%)	13.00	13.00	13.00	13.00	13.00	13.00
Ash (%)	8.00	8.00	8.00	8.00	8.00	8.00
Calcium (%)	0.90	1.06 (+18%)	1.19 (+32%)	0.85	0.98 (+15%)	1.05 (+23%)
Phosphorus	0.45	0.49 (+9%)	0.53 (+17%)	0.42	0.45 (+7%)	0.47 (+11%)
Cost						
Diets (RM)	132.00	132.00	132.00	130.000	130.000	130.000
MCP (RM)	0.00	2.00	4.00	0.00	2.00	4.00
Total (RM)	132.00	134.00	136.00	130.000	132.00	134.00

Note. T1: 0% MCP (control); T2: 0.5% MCP; T3: 1.0% MCP

Sample and Data Collection

For the growth performance, weekly body weight (BW) and feed intake (FI) were recorded per replicate for the calculation of body weight gain (BWG) and feed conversion ratio (FCR). At day 42, 12 broilers were randomly selected from each treatment group and slaughtered for gut histomorphology, carcass characteristics, meat, and bone quality analysis. The birds were slaughtered at the Department of Animal Science abattoir, Faculty of Agriculture, UPM according to the Halal slaughter procedure (Abdulla et al., 2015).

Gut Histomorphology

Intestinal samples were collected to study the intestinal histomorphology. Five (5) cm of duodenum, jejunum, and ileum were harvested flushed with 10% neutral buffered formalin solution and set overnight. The intestinal samples were then excised, dehydrated in a tissue processing machine and embedded in paraffin wax. Each sample was cut about 4 mm on a slide. Then the samples were stained with haematoxylin and eosin, mounted and viewed under a Nikon DS-U2/L2 light microscope. The villi height and crypts depth were examined, captured, and measured with NIS-Elements D software. The height of the villi were measured from tip to crypt transition while crypts depth were measured at the invagination between two villus. This procedure was conducted at the Histopathology Lab, Faculty of Veterinary Medicine, UPM.

Carcass Characteristics

The carcasses were dissected manually, and the following parameters were recorded: final live weight, kill-out weight, de-feathered weight, dressing percentage, breast muscle weight, drumsticks weight, wings weight, neck weight, head weight, shank weight, full gizzard weight, empty gizzard weight, gastrointestinal tract weight, heart weight, and liver weight.

Meat Quality Analysis

Determination of pH. The right pectoralis major (breast muscle) was collected, snap-frozen in liquid nitrogen (-195°C) and stored at −80°C to preserve the pH properties of the meat. After 24 hours, the samples were crushed using mortar and pestle until it became fine particles. The samples were then homogenized by using a homogenizer. The pH was then taken by a portable pH meter (Mettler Toledo, AG 8603, Switzerland).

Determination of Colour. Thirty (30) g of breast meat from samples of each treatment groups were analysed using a colour flex spectrophotometer (Hunter Lab Reston, VA, USA). The meat samples were bloomed at room temperature (27°C) for 30 minutes prior to analysis. The colour flex spectrophotometer was then standardized and properly set up as required. Once the meat samples were analysed, the colour flex spectrophotometer produced L*, a*, and b* results according to the gross appearance of meat samples' colour.

Drip Loss Measurement. Forty (40) g of breast meat from the samples of each treatment groups were weighed and recorded as initial weight (W1). The meat samples were kept in vacuum-packed plastic bags and kept in the freezer at 4°C. After 48 hours, the samples were removed from the bags, gently blotted with a tissue to dry and final weight was taken (W2). This procedure was done to determine the amount of water loss during storage. The percentage of drip loss will be calculated as (W1-W2)/ W1 x 100.

Cooking Loss Measurement. Thirty (30) g of breast meat from the samples of each treatment groups were weighed and recorded as initial weight (W1). The muscle samples were placed in vacuum-packed plastic bags and fully immersed in the water bath at 80°C for 20 minutes. After cooking, the samples were removed from the water bath and the bags were allowed to cool down to room temperature for 15 minutes. The cooked samples were blotted with tissue paper and weighed, the weight was recorded as final weight (W2). The cooking loss was determined by the amount of water loss from cooking and the percentage was calculated using the formula (W1-W2)/ W1 x 100.

Bone Quality Analysis

Tibiotarsus Length and Weight. The right drumstick from the samples of each treatment groups was taken flesh intact. The samples were kept frozen in the freezer (-20°C) for 36 hours. Then, the sample was thawed and boiled in boiling water (95°C) for 10 minutes. After that, the samples were cooled down to room temperature. The flesh, bone cap, and patella were then removed manually by hand revealing the tibiotarsus. The bones were air-dried for 24 hours at room temperature. Then, the tibiotarsus length was measured using Vernier dial calliper (0-100mm/0.02mm) and weighed. Each bone was put a mark on the midpoint.

Bone-breaking Strength. The bone-breaking strength of tibiotarsus samples was measured using a three-point bending test. The tibiotarsus samples from each treatment group were fixed one by one on a universal testing machine (Model 1000R, with 5000N load cell) and the amount of force required to break the bone when applied at a constant speed of 10 mm/min and distance between supports of 50 mm was recorded.

Tibiotarsal Index. Thickness of the medial and lateral wall of three tibiotarsus samples from each treatment groups was measured using a Vernier dial calliper at the point of breakage (midpoint). Medullary canal diameter was calculated by subtracting the thickness of medial and lateral walls from the diameter at diaphysis. This procedure was done following the bone-breaking strength procedure. Tibiotarsal index was measured as (diaphysis diameter – medullary canal diameter)/ diaphysis diameter x 100.

Ash, Calcium and Phosphorus Content. The tibiotarsus samples from each treatment group were dried in an oven for 24 hours at 105°C. After that, the samples were weighed

and recorded as (W1). Then, the samples were placed in a pre-weighed crucible and put in a muffle furnace at 600°C for 6 hours and were re-weighed and recorded as (W2). Ash content of the tibiotarsus samples was calculated as (W1-W2)/W2 X 100. The ashes samples were then diluted and carried out in a fume cupboard. Dilution process was done by diluting 2 grams of three ashes sample from each treatment group into 100 mL of fuming hydrochloric acid (37%). The mixture was then mixed well. A volume of 0.6 mL of the mixture was pipetted into a test tube containing 14.4 mL of water. The samples were then sealed and sent to for calcium and phosphorus analysis by using atomic absorption spectrometry (Perkin Elmer Analyst 400) and autoanalyser (Lachat Instruments QuikChem 8000Series FIA + System) respectively. This procedure was done following the tibiotarsal index procedure. Upon getting the results, the percentage of calcium and phosphorus in the ashes samples were calculated as calcium level (mg/L) x 100/W1 X 0.0025 and phosphorus level (mg/L) x 100/W1 X 0.0025 respectively.

Statistical Analysis

JMP® Version 11. NC: SAS Institute Inc. software was used to analyse all the data collected. ANOVA with control, Dunnett's test were used to compare means between treatment groups. The data were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

Growth Performance

The impact of supplementing MCP on the growth performance of broilers are presented in Table 2. There were significant differences on the BW, BWG, and FI among treatment groups. Broilers supplemented with 0.5% MCP (T2) had the highest BW, BWG, and FI throughout the 42 days study period. In the case of rapid growth, an adequate nutritional supply of both Ca and P become crucial for the fast-growing broiler. Thus, additional of Ca and P are needed for those birds. According to the Applegate and Angel (2014), chicks require about 1.0% Ca with 0.45% of available P. However, there were reports that chicks needed about 0.65 to 0.7% of total P because the corn-soybean meal diet contained only about 0.2 to 0.25% P due to the indigestibility of phytate (Mitchell & Edwards, 1996). Therefore, supplementing Ca and P were found to increase the growth performance in young broilers (Hamdi et al., 2015). In normal practices, dicalcium phosphate (DCP) is usually added in commercial broiler feed. Even though research has found that the amount of Ca and P availability in MCP are higher than di- or tri sources, farmers are still reluctant to practiced MCP supplementation due to the higher feed cost (Ogino, 1979). In different studies, the addition of 0.6% and 2% MCP were found to increase the growth performance of hybrid African catfish and mirror carp fishes respectively (Kim et al., 1998; Mgbenka & Ugwu, 2005). A feed grade MCP used in this study is a high quality Ca and P source that is used as a feed additive or premix in

the animal feed industry. Nonetheless, there was limited study conducted on the effect of MCP supplementation in commercial broilers.

No significant difference was observed in the FCR for all treatment groups. However, numerically lower parameters were observed in broilers supplemented with 1.0% MCP (T3). These findings were concurrent with Abdulla et al. (2017), who found that broilers fed with diets containing 1.5% of Ca had lower BW and BWG. Agreeing to Mutucumarana et al. (2014), excessive Ca interact with inorganic P causing the Ca to be indigestible. As a result, it will alter the intestinal pH to be more acidic or at pH 5.0. The acidic environment in the small intestine will then decrease the digestibility and absorption of P. Huge Ca and P imbalance will occur if they continue to consume high Ca diet leading to lower FI and higher FCR which was exhibited in this current study (Adeola & Walk, 2013).

Gut Histomorphology

The effect of MCP supplementation on the villus height and crypt depth of broilers are shown in Table 3. There was a significant difference in the villus height among treatment groups. Supplementing 0.5% MCP (T2) was found to increase the villus height in the jejunum and ileum. This finding was supported by Pohl et al. (2012), who reported that inclusion of 1.5% of

Table 2
Effect of MCP supplementation on the growth performance of broilers

Parameters	Treatments			SEM
	T1	T2	T3	
1-21 days				
Initial body weight (g)	54.06	51.61	52.69	0.21
Body weight (g)	971.02^{b}	982.52^{ab}	940.82^{a}	10.01
Body weight gain (g)	916.96^{b}	930.91^{ab}	888.13^{a}	9.17
Feed intake (g)	1205.37	1211.91	1176.92	11.81
FCR	1.24	1.23	1.25	0.01
22-42 days				
Body weight (g)	2396.89^{b}	2488.74^{a}	2365.34^{b}	25.12
Body weight gain (g)	2342.83^{b}	2437.13^{a}	2312.65^{b}	20.92
Feed intake (g)	4462.71^{b}	4604.51^{a}	4410.21^{b}	21.93
FCR	1.90	1.89	1.91	0.01
Feeding Cost				
Starter/bird (RM)	3.17	3.25	3.20	
Finisher/bird (RM)	8.47	8.96	8.67	
Total/bird (RM)	11.64	12.21	11.87	

Note. $^{a, b, c}$ values with superscript within column are significantly different at $P< 0.05$.
T1: 0% MCP (control); T2: 0.5% MCP; T3: 1.0% MCP

calcium formate (CaFo) increased the duodenal villus height compared to the lower and higher concentration of CaFo in broiler chickens. Increased villus height of the intestine corresponds to increase digestive and absorptive functions of the intestine due to the enhanced absorptive surface area leading to feeding efficiency (Zhang & Adeola, 2017). In addition to growth promoting activity, minerals such as Ca and P play a major role in the immune response where mineral inclusion above requirements is required to boost immune responses (Dozier et al., 2003). It will affect the gut pH, gastrin production, acid secretion, epithelial cell proliferation, and nutrient absorption leading to bacteriostatic effect which could have a possible effect on gut histomorphology and villus height (Pohl et al., 2012). When the size and height of intestinal villi increase, nutrient absorption in gastrointestinal tract will be enhanced and result in better feed efficiency of the chicken which was observed in the present study (Ruttanavut & Yamauchi, 2010).

Besides, there was also a significant difference in the crypt depth between treatment groups. Broilers supplemented with 0.5% MCP (T2) had the shortest crypt depth in the duodenum, jejunum, and ileum compared to the other treatment birds. The crypts base or also known as the villus factory are constantly dividing so that the villi structure will be maintained hence more villi will be developed (Chwen et al., 2013). Crypt depth measurements are affected by the enterocytes differentiating activity. Shorter crypt depth results in a slower turnover of the intestinal mucosa resulting in lower maintenance requirement (Gao et al., 2008). As a result, more energy is focused on the production and growth rate where heavier BW and BWG were exhibited by T2 birds. In contrast, a deeper crypt depth observed in T3 birds indicates increased production rate of the enterocyte and migration up the villi. When the turnover is faster, higher maintenance is required for villi regeneration. A deeper crypt is also resulted due to inflammation from

Table 3
Effect of MCP supplementation on the intestinal histomorphology of broilers

Parameters	Treatments			SEM
	T1	T2	T3	
Villus height				
Duodenum (µm)	1038.39	1107.82	1063.99	42.22
Jejunum (µm)	610.91^{b}	765.11^{a}	585.17^{b}	21.50
Ileum (µm)	394.87^{ab}	430.61^{a}	321.98^{b}	7.91
Crypt depth				
Duodenum (µm)	127.85^{b}	122.23^{b}	176.12^{a}	2.29
Jejunum (µm)	162.65^{a}	103.27^{c}	130.35^{b}	3.54
Ileum (µm)	95.32^{ab}	89.72^{b}	104.42^{a}	2.19

Note. $^{a, b, c}$ values with superscript within column are significantly different at $P< 0.05$.
T1: 0% MCP (control); T2: 0.5% MCP; T3: 1.0% MCP

pathogens and toxin which may fasten the tissue turnover (Gao et al., 2008).

Carcass Characteristics

The impact of supplementing MCP on the carcass characteristics of broilers are presented in Table 4. No significant differences were observed in all the parameters among treatment birds. A similar finding was reported by Hamdi et al. (2017), where different sources of Ca and P had no effect on the productive performance of broiler chickens for both 21 and 35 days old. However, higher parameters were observed in broilers supplemented with 0.5% MCP (T2). The quality and quantity of carcass are determined by environmental and genetic factors. According to Abdulla et al. (2017), one of the major contributors to the environmental aspect is the nutrient received by the animal. For example, T2 broilers had a higher dressing percentage, heavier breast muscle, drumsticks, neck, head, and shank probably due to the higher BWG. In addition, heavier gizzard and gastrointestinal tract may be due to the high FI and enhanced gut histomorphology of T2 broilers.

In a report by Al Daraji et al. (2011), birds fed with a too low or too high level of dietary Ca had lower carcass weight due to lower BW and BWG which was demonstrated in T3 birds. This was in accord with findings by Han et al. (2016), which

Table 4
Effect of MCP supplementation on the carcass characteristics of broilers

Parameters	Treatments			SEM
	T1	T2	T3	
Final live weight (kg)	2.34[b]	2.44[a]	2.31[b]	20.92
Kill out weight (%)	97.64	97.72	97.98	0.37
De-feathered weight (%)	93.42	93.41	93.95	0.22
Dressing percentage (%)	73.81	73.86	72.62	0.70
Breast muscle (%)	18.48	22.01	21.44	0.97
Drumsticks (%)	11.38	11.44	11.31	0.86
Wings (%)	9.57	9.41	10.55	0.73
Neck (%)	2.13	2.31	2.22	0.45
Head (%)	2.90	3.12	2.91	0.15
Shank (%)	4.01	4.21	4.05	0.71
Full gizzard (%)	1.57	1.84	1.32	0.17
Empty gizzard (%)	1.25	1.38	1.22	0.07
Gastrointestinal tract (%)	15.29	15.44	14.40	1.13
Heart (%)	0.49	0.49	0.50	0.01
Liver (%)	2.54	2.54	2.07	0.44

Note. [a, b, c] values with superscript within column are significantly different at $P< 0.05$.
T1: 0% MCP (control); T2: 0.5% MCP; T3: 1.0% MCP

suggested that Ca and P imbalance in dietary feed compressed muscle growth and meat production of broiler chickens.

Meat Quality

The effect of MCP supplementation on the meat quality of broilers is shown in Table 5. All experimental broiler birds slaughtered on day 42 showed no significant differences in the meat quality parameters. The result was consistent to Li et al. (2016), who measured the pH values and the cooking loss and significant differences were not observed in broiler groups fed different levels of P. Nevertheless, the meat colour of the breast meat showed significant differences between groups. High P diets resulted in increased lightness (L*) and redness (a*) values (Li et al., 2016). Although broilers supplemented with 1.0% MCP (T3) demonstrated the highest values for all the meat quality parameters in the current work, no significant change in the meat quality was observed. This could be attributable to the P levels in the diet which might not be deficient or excessive enough to affect the meat quality (Rath et al., 2000).

Higher dietary P in broiler feed results in higher P content in the breast meat. It was found that deficient or excessive incorporation of P levels in feed caused detrimental effects on meat quality by decreasing the intramuscular fat content (IMF) and fatty acids of the breast meat (Li et al., 2016). Driver et al. (2006) found that broken clavicles were associated with bloody breast meat affecting the redness of the muscle, but in most cases, the bleeding occurred without any detectable fracture of the bone. This may occur due to Ca and P imbalances in broilers where bleeding occurs from hairline fractures of the bone as a result of poor mineralization. Calcium is also required in activities of blood-clotting proteins that may explain the phenomenon. It has been demonstrated that removing DCP from broiler finisher diet resulted in an increase of blood-splashed breast meat (Chen & Moran, 1994).

Table 5
Effect of MCP supplementation on the meat quality of broilers

Parameters	Treatments			SEM
	T1	T2	T3	
Drip loss (%)	1.78	2.04	2.71	0.50
Cooking loss (%)	19.95	23.60	24.28	3.52
pH	6.26	6.16	6.41	0.29
Colour:				
Lightness (L*)	49.05	47.80	52.04	3.03
Redness (a*)	5.547	5.34	6.43	1.15
Yellowness (b*)	18.88	18.56	20.28	1.60

Note. [a, b, c] values with superscript within column are significantly different at P< 0.05.
T1: 0% MCP (control); T2: 0.5% MCP; T3: 1.0% MCP

Bone Quality

The impact of supplementing MCP on the bone quality of broilers is presented in Table 6. Experimental broilers supplemented with varying concentration of MCP in the diet resulted in some significant differences in the bone quality parameters. Broilers supplemented with 0.5% MCP (T2) had the highest significant values for bone weight, diaphysis diameter, medullary canal diameter, lateral wall thickness, medial wall thickness, and bone breaking strength. Bone quality is a composite of properties that makes bone resist fractures. The present experiment showed that T2 birds had better bone mineralization compared to the other treatments. Supplemental amount of Ca in diets resulted in better tibia mineralization, in particular, higher levels of breaking strength, length, weight, ash weight, and ash Ca contents (Han et al., 2016).

On the other hand, chickens supplemented with 1.0% MCP (T3) demonstrated a higher Ca and P percentages in the bone ash but the weakest bone breaking strength. This was supported by the findings of Hurwitz et al. (1995), where insufficient or excess supply of one or both minerals (Ca and P) interfered with homeostasis of the second one. This homeostasis imbalance resulted in reduced bone mineralization. This was in agreement with an earlier study that observed increased tibia ash and mineral contents were due to high dietary Ca and P content (Nelson et al., 1990). However, according to Venalainen et al. (2006), increase in bone ash content was not proportionate to bone breaking strength. The resistance to breaking of long bones such as tibia and femur appeared to be affected by the Ca and P content in starter diet, while events of broken clavicles were only influenced by the type of diet fed during

Table 6
Effect of MCP supplementation on the bone quality of broilers

Parameters	Treatments			SEM
	T1	T2	T3	
Bone length (mm)	93.67	95.33	91.67	3.76
Bone weight (g)	7.87^{b}	8.45^{a}	6.65^{c}	1.45
Diaphysis diameter (mm)	7.81^{b}	8.95^{a}	7.89^{b}	1.21
Medullary canal diameter (mm)	4.99^{a}	5.67^{a}	4.16^{b}	0.31
Lateral wall thickness (mm)	1.65^{b}	2.31^{a}	1.73^{b}	0.54
Medial wall thickness (mm)	1.16^{b}	2.01^{a}	0.97^{b}	0.20
Tibiotarsal index	35.81	46.05	36.20	0.51
Bone breaking strength (N)	300.06^{ab}	336.59^{a}	256.20^{b}	8.66
Ash (%)	56.37	56.44	56.56	1.71
Ca (%)	2.40^{b}	3.24^{a}	3.69^{a}	0.40
P (%)	1.21^{b}	2.07^{a}	2.28^{a}	0.29

Note. $^{a, b, c}$ values with superscript within column are significantly different at P< 0.05.
T1: 0% MCP (control); T2: 0.5% MCP; T3: 1.0% MCP

grower-finisher phase (Driver et al., 2006). Williams et al. (2003), provided evidence that rapid growth rates, rather than genotype, had a greater effect on bone mineralization. A fast-growing commercial line of broilers showed decreased tibial mineralization and increased bone porosity as compared with a slow growing line of broilers.

CONCLUSION

Dietary inclusion of 0.5% MCP was found to enhance the growth performance, gut histomorphology, and bone quality of broiler chickens. Higher values were also obtained in the carcass characteristics without affecting the meat quality. Therefore, MCP could be used effectively as a Ca and P supplement in the commercial broiler industry to reduce culling of birds with musculoskeletal problems in particularly lameness and skeletal deformities. MCP supplementation may have a greater effect in layers or broiler breeders because of their longer rearing periods and increased calcium requirements.

ACKNOWLEDGEMENT

The authors would like to thank the staff of the Department of Animal Science, Faculty of Agriculture, UPM for their technical assistant. The project was funded by the Putra Grant - Putra Young Initiative (GP-IPM), Universiti Putra Malaysia (Grant no: 9638400).

REFERENCES

Abdulla, N. R., Loh, T. C., Akit, H., Sazali, A. Q., Foo, H. L., Kareem, K. Y., … Rahim, R. A. (2017). Effects of dietary oil sources, calcium and phosphorus levels on growth performance, carcass characteristics and bone quality of broiler chickens. *Journal of Applied Animal Research*, *45*(1), 423-429.

Abdulla, N. R., Zamri, A. N. M., Sabow, A. B., Kareem, K.Y., Nurhazirah, S., Ling, F. H., … Loh, T. C. (2015). Physico-chemical properties of breast muscle in broiler chickens fed probiotics, antibiotics or antibiotic-probiotic mix. *Journal of Applied Animal Research*, *45*(1), 64-70.

Adeola, O., & Walk, C. L. (2013). Linking ileal digestible phosphorus and bone mineralization in broiler chickens fed diets supplemented with phytase and highly soluble calcium. *Poultry Science*, *92*(8), 2109-2117.

Al Daraji H. J., Al Mashadani, H., Mirza, H., Al Hayani, W., & Al Hassani, A. (2011). Effects of feeds containing different fats on certain carcass parameter of Japanese quail. *ARPN Journal of Agriculture and Biological Science*, *6*(1), 6-11.

Applegate, T. J., & Angel, R. (2014). Nutrient requirements of poultry publication: History and need for update. *The Journal of Applied Poultry Research*, *23*(3), 567-575.

Chen, X., & Moran, E. T. (1994). Response of broilers to omitting dicalcium phosphate from the withdrawal of feed: Live performance, carcass downgrading and further processing yields. *The Journal of Applied Poultry Research*, *4*(1), 69-82.

Chwen, L. T., Foo, H. L., Thanh, N. T., & Choe, D. W. (2013). Growth performance, plasma fatty acids, villous height and crypt depth of preweaning piglets fed with medium chain triacylglycerol. *Asian-Australasian Journal of Animal Sciences*, *26*(5), 700-704.

Dinev, I. (2012). Leg weakness pathology in broiler chickens. *Japan Poultry Science Association*, *49*(2), 63-67.

Dozier, W. A., Davis, A. J., Freeman, M. E., & Ward, T. L. (2003). Early growth and environmental implications of dietary zinc and copper concentrations and sources of broiler chicks. *British Poultry Science*, *44*(5), 726-731.

Driver, J. P., Pesti, G. M., Bakalli, R. I., & Edwards, H. M. (2006). The effect of feeding calcium- and phosphorus-deficient diets to broiler chickens during the starting and growing-finishing phases on carcass quality. *Poultry Science*, *85*(11), 1939–1946.

Eeckhout, W., & De Paepe, M. (1994). Total phosphorus, phytate-phosphorus and phytase activity in plant feedstuffs. *Animal Feed Science and Technology*, *47*(1-2), 19-29.

Gao, J., Zhang, H. J., Yu, S. H., Wu, S. G., Yoon, I., Quigley, J., … Qi, G. H. (2008). Effects of yeast culture in broiler diets on performance and immunomodulatory functions. *Poultry Science*, *87*(7), 1377-1384.

Gonzalez-Ceron, F., Rekaya, R., Anthony, N. B., & Aggrey, S. E. (2015). Genetic analysis of leg problems and growth in a random mating broiler population. *Poultry Science*, *94*(2), 162-168.

Hamdi, M., López-Vergé, S., Manzanilla, E. G., Barroeta, A. C., & Pérez, J. F. (2015). Effect of different levels of calcium and phosphorus and their interaction on the performance of young broilers. *Poultry Science*, *94*(9), 2144-2151.

Hamdi, M., Solà Oriol, D., Franco Rosselló, R., Aligué i Alemany, R. M., & Pérez, J. F. (2017). Comparison of how different feed phosphates affect performance, bone mineralization and phosphorus retention in broilers. *Spanish Journal of Agricultural Research*, *15*(3), e0605. doi: 10.5424/sjar/2017153-11149

Han, J., Wang, J., Chen, G., Qu, H., Zhang, J., Shi, C., … Cheng, Y. (2016). Effects of calcium to non-phytate phosphorus ratio and different sources of vitamin D on growth performance and bone mineralization in broiler chickens. *Revista Brasileira de Zootecnia*, *45*(1), 1-7.

Hurwitz, S., Plavnik, I., Shapiro, A., Wax, E., Talpaz, H., & Bar, A. (1995). Calcium metabolism and requirements of chickens are affected by growth. *The Journal of Nutrition*, *125*(10), 2679-2686.

Kim, J. D., Kim, K. S., Song, J. S., Lee, J. Y., & Jeong, K. S. (1998). Optimum level of dietary monocalcium phosphate based on growth and phosphorus excretion of mirror carp, *Cyprinus carpio*. *Aquaculture*, *161*(1-4), 337–344.

Knowles, T. G., Kestin, S. C., Haslam, S. M., Brown, S. N., Green, L. E., Butterworth, A., … Nicol, C. J. (2008). Leg disorder in broiler chickens: Prevalence, risk factors and prevention. *PLOS One*, *3*(2), e1545.

Li, X., Wang, J., Wang, C, Zhang, C, Li, X., Tang, C., & Wei, X. (2016). Effect of dietary phosphorus levels on meat quality and lipid metabolism in broiler chicken. *Food Chemistry*, *205*, 289-296.

Liu, J. B., Chen, D. W., & Adeola, O. (2013). Phosphorus digestibility response of broiler chickens to dietary calcium-to-phosphorus ratios. *Poultry Science*, *92*(6), 1572–1578.

Mgbenka, B. O., & Ugwu, L. L. C. (2005). Aspects of mineral composition and growth rate of the hybrid African catfish fry fed inorganic phosphorus-supplemented diets. *Aquaculture Research*, *36*(5), 479-485.

Mitchell, R. D., & Edwards Jr, H. M. (1996). Effects of phytase and 1, 25-dihydroxycholecalciferol on phytate utilization and the quantitative requirement for calcium and phosphorus in young broiler chickens. *Poultry Science*, *75*(1), 95-110.

Mutucumarana, R. K., Ravindran, V., Ravindran, G., & Cowieson, A. J. (2014). Influence of dietary calcium concentration on the digestion of nutrients along the intestinal tract of broiler chickens. *Journal of Poultry Science*, *51*(4), 392–401.

Nelson, T. S., Harris, G. C., Kirby, L. K., & Johnson, Z. B. (1990). Effect of calcium and phosphorus on the incidence of leg abnormalities in growing broilers. *Poultry Science*, *69*(9), 1496-1502.

Ogino, C. (1979). Availability of dietary phosphorus in carp and rainbow trout. *Nippon Suisan Gakkaishi*, *45*, 1527-1532.

Pohl, S. A., Caldwell, D. J., Lee, J. T., Coppedge, J. R., Dunn-Horrocks, S. L., Stringfellow, K. D., … Farnell, M. B. (2012). Effects of dietary calcium formate inclusion on broiler performance, skeletal development, and gut maturation. *The Journal of Applied Poultry Research*, *21*(2), 311–317.

Rath, N. C., Huff, G. R., Huff, W. E., & Balog, J. M. (2000). Factors regulating bone maturity and strength in poultry. *Poultry Science*, *79*(7), 1024-1032.

Ruttanavut, J., & Yamauchi, K. (2010). Growth performance and histological alterations of intestinal villi in broilers fed dietary mixed minerals. *Asian Journal of Animal Sciences*, *4*(3), 96-106.

Venalainen, E., Valaja, J., & Jalava, T. (2006). Effects of daily metabolisable energy, calcium, and phosphorus on bone mineralization, leg weakness and performance on broiler chickens. *British Poultry Science*, *47*(1), 301-310.

Williams, B., Waddington, D., Murray, D. H., & Farquharson, C. (2003). Bone strength during growth: Influence of growth rate on cortical porosity and mineralization. *Calcified Tissue International*, *74*(3), 236-245.

Zhang, F., & Adeola, O. (2017). Techniques for evaluating digestibility of energy, amino acids, phosphorus, and calcium in feed ingredients for pigs. *Animal Nutrition*, *3*(4), 344–352.

Pertanika J. Trop. Agric. Sc. 42 (4): 1251 - 1262 (2019)

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TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Evaluation of *Enterobacter* sp. Strain G87 as Potential Probiont against *Vibrio harveyi* Infection in *Artemia* Nauplii and Asian Seabass (*Lates calcarifer*) Larvae

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ABSTRACT

Probiotic has gained many interests as an alternative method in preventing and treating diseases in aquaculture. The benefits include improving feed value, inhibition of pathogenic microorganisms, anti-mutagenic and anti-carcinogenic activity, growth promoting factors, and increase host immune response. This research was carried out in order to evaluate the potential of probiont *Enterobacter* sp. G87 in conferring protection to *Artemia* and seabass larvae against *Vibrio harveyi* infection. In preliminary *in vivo* test, *Artemia* nauplii was treated with *Enterobacter* sp. G87 at three different concentrations 10^4, 10^6 and 10^8 CFU mL^{-1} and challenged with *V. harveyi* at 10^5 CFU mL^{-1}. After challenged, significant increased survival was found in *Artemia* (78±2%) treated with 10^6 CFU mL^{-1} of *Enterobacter* sp. G87 compared with challenged group with no probiont added (48±2%). From the results, two concentrations of probiont (10^6 and 10^8 CFU mL^{-1}) were selected to be used in seabass larvae *in vivo* challenge assay. After challenged with *V. harveyi* at 10^5 CFU mL^{-1} highest survival was found in seabass larvae treated with 10^6 CFU mL^{-1} of *Enterobacter* sp. G87 (95±3%). Additionally, *Enterobacter* sp. G87 was also able to reduce *Vibrio* counts both in *Artemia* and seabass larvae culture. This study showed that probiont *Enterobacter* sp. G87 was able to protect *Artemia* nauplii and seabass larvae from *Vibrio harveyi* infection and has a potential to be further studied in a larger scale.

Keywords: *Artemia*, *Enterobacter*, larvae, probiotics, seabass, *Vibrio harveyi*

ARTICLE INFO

Article history:
Received: 23 July 2019
Accepted: 09 October 2019
Published: 13 November 2019

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ISSN: 1511-3701
e-ISSN: 2231-8542

INTRODUCTION

World is predicted to undergo food crisis in 2050 if the alternative to captured fisheries could not be found (Béné et al., 2015). Fish landing have declined drastically for the past 10 years due to overfishing and environmental issue (Food and Agriculture Organization [FAO], 2015). Aquaculture is the best option to fulfill the protein requirement of the country which projected to grow more than 100% production within five years ahead (FAO, 2016).

However, major disease outbreaks have been reported within the aquaculture industry around the world due to the increased in stocking density, over-crowding and poor husbandry management along with the rapid growth of aquaculture (Tan et al., 2016). The annual economic losses associated with diseases worldwide are estimated to be in excess of US$9 billion per year (Ruwandeepika Hettipala Arachchige, 2010). One of the common disease outbreaks is bacterial infection known as vibriosis which is commonly caused by *Vibrio harveyi* (Talpur, 2014). *Vibrio harveyi* is one of the *Vibrio* sp. which is an important aquaculture pathogen that can infect large number of marine animals (Li et al., 2011).

In Malaysia, Asian Seabass is one of the top demand species from the locals; probably due to its unique taste and reasonable price. In 2017, production seabass in Malaysia was recorded nearly at 30,000 metric tons (Department of Fisheries [DOF], 2017). The production of seabass increased through the years due to high demand from consumers which make seabass culture to be a profitable industry. The annual production of worldwide for this type of fish increased from 93,422 metric tons in 2012 to 101,231 metric tons in 2013 (FAO, 2016). Production of seabass is greatly affected by the occurrence of vibriosis, which causes heavy mortality of more than 50% (Ransangan & Mustafa, 2009).

The use of antibiotic as preventive measures are limited in most country including Malaysia because of its negative effect to environment, human health and causing antimicrobial ressistance. Alternatively, the use of probiotic is one of the best option to control diseases in aquatic environment (Harikrishnan et al., 2011). Among the common microorganism used as probiotics are *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bacillus* sp., *Lactococcus* sp. and also yeast *Saccharomyces cerevisiae* (Salamoura et al., 2014).

As for now, probiotic usage in Malaysia has been a popular option however, there are still lack of local probiotic products. This mainly because of limitation of knowledges, awareness, resources, research and development on local probiotics strain. Among the earliest study done on application of probiotics in aquaculture in Malaysia was by Al-Dohail et al. (2009) who reported the beneficial effects of *L. acidophilus* on growth performance and immune response of African catfish *Clarias gariepinus*. *Lactobacillus plantarum* was also proven to be able to reduce *Vibrio* loads in culture water of *Portunus pelagicus* larvae as well as improving the survival rate of the larvae (Talpur et al., 2013)

Live feeds are crucial at early stages of larvae. *Artemia* is one of the live feeds that are commomly used for marine larvae. One of the pathway to intorduce probiotics to the cultured hosts are by using *Artemia* as the medium or transporter. Enrichment of *Artemia* using probiotics able to increase the nutrients contents as well as provide protection towards any pathogens since feed could be a possible carrier for diseases as well (Hai, 2015).

Potential probiont *Enterobacter* sp. G87 was isolated from gut of healthy adult Asian seabass. In earlier studies, our lab had confirmed the antagonistic properties of this strain against *V. harveyi* strain NBRC 15634 in *in vitro* assay. Thus, this study was undertaken to determine the ability of potential probiont *Enterobacter* sp. G87 in protecting *Artemia* which is one of an important live feed in larviculture as well as the most preferable model for a preliminary *in vivo* test prior testing to the real host (Frans et al., 2013). The study also includes on the effect of *Enterobacter* sp. G87 towards seabass larvae after being challenged with *V. harveyi*.

MATERIALS AND METHODS

Artemia Nauplii and Seabass Larvae

The *Artemia* (Bio-Marine, USA) cyst was obtained from the laboratory of Fish Health Laboratory, Faculty of Agriculture, UPM. Meanwhile, larvae of seabass (*Lates calcarifer*) at size average of 1 inch were obtained from fish farm in Banting, Selangor. The fish were acclimatized for 24 hr in separate tanks prior use for experiments. Any presence of pathogenic vibrios were tested by taking few samples of fish and streak on Thiosulphate Citrate-Bile Salt (TCBS, Difco Company, USA) agar.

Bacterial Cultures and Growth Condition

The potential probiont *Enterobacter* sp. G87 and pathogenic *V. harveyi* strain NBRC 15634 were obtained from Fish Health Laboratory, Department of Aquaculture, UPM. *Enterobacter* sp. G87 was previously isolated from the gut of healthy adult seabass *Lates calcarifer*. Tryptic soy agar (Difco Company, USA) + 1.5% NaCl was used to culture *Enterobacter* sp. G87 and TCBS media was used for *V. harveyi*. Both isolates were incubated at 30ºC for 24 hr prior to use. Meanwhile for broth cultures, the isolates were inoculated in TSB (Difco Company, USA) + 1.5% NaCl and incubated 24 hr in the innova®42 incubator shaker (Eppendorf, Germany) series at 120 rpm, 30ºC prior used in challenged assay. Concentrations were adjusted accordingly using spectrophotometer and McFarland Standard.

Preliminary Challenged Assay using *Artemia*

Artemia cyst (Bio-Marine brand) was cultured for 24 hr using sterile seawater (SSW) at 28-30°C with continuous aeration and light intensity. After 24 hr of incubation, 20 hatched *Artemia* nauplii were divided into falcon tube containing 30 ml SSW. All treatments were run in triplicate. *Artemia* nauplii were immersed for 24 hr with *Enterobacter* sp. G87 at concentration of 10^8, 10^6 and 10^4 CFU mL^{-1} which were

selected based on *in vitro* results in previous studies. *Vibrio harveyi* at concentration of 10^5 CFU mL^{-1} was added into the respective falcon tubes after 24 hr. Control was run with no bacteria added. The tubes were placed on the orbital shaker, 50rpm at room temperature. *Artemia* was fed with yeast once daily. Experiment was stopped when the group that was challenged with *V. harveyi* only reached 50% mortality. The mortality and water quality parameters (salinity, pH and dissolved oxygen) were recorded everyday. Experiment on each of the group was run in triplicates and water quality was checked daily.

Challenge Assay on Seabass Larvae

The larvae were acclimatized for 24 hr prior to use. Next, 20 larvae were divided into 5L aquarium contained 2L SSW with continuous aeration. In this assay, two concentrations of probiont *Enterobacter* sp. G87 were used (10^8 and 10^6 CFU mL^{-1}) based on the findings from preliminary *in vivo* assay using *Artemia* and challenged with 10^8 CFU mL^{-1} of *V. harveyi*. Larvae were pre-incubated with probiont *Enterobacter* sp. G87 on the first day and challenged with *V. harveyi* on the next day (after 24 hr). No bacteria either probiont or pathogen were added in the control. Mortality and water quality were checked and recorded every day. Experiment was stopped until group challenged with *V. harveyi* with no probiont reached 50% mortality. Each of the treatment group was run in triplicate according to Table 1 and Table 2 and water quality was checked daily.

Table 1
Treatments for preliminary in vivo *challenged using* Artemia *nauplii*

Label	Treatment
C	Positive Control (with no addition of bacteria)
T1	*Vibrio harveyi* 10^5 CFU mL^{-1} (Negative Control)
T2	*Enterobacter* G87 10^8 CFU mL^{-1}
T3	*Enterobacter* G87 10^6 CFU mL^{-1}
T4	*Enterobacter* G87 10^4 CFU mL^{-1}
T5	*Enterobacter* G87 10^8 CFU mL^{-1} + *Vibrio harveyi* 10^5 CFU mL^{-1}
T6	*Enterobacter* G87 10^6 CFU mL^{-1} + *Vibrio harveyi* 10^5 CFU mL^{-1}
T7	*Enterobacter* G87 10^4 CFU mL^{-1} + *Vibrio harveyi* 10^5 CFU mL^{-1}

Table 2
Treatments for seabass larvae in vivo *challenge assay*

Label	Treatment
C	Positive Control (with no addition of bacteria)
VH	*Vibrio harveyi* 10^8 CFU mL^{-1} (Negative Control)
CT1	*Enterobacter* G87 10^6 CFU mL^{-1}
CT2	*Enterobacter* G87 10^8 CFU mL^{-1}
T3	*Enterobacter* G87 10^6 CFU mL^{-1} + *Vibrio harveyi* 10^8 CFU mL^{-1}
T4	*Enterobacter* G87 10^8 CFU mL^{-1} + *Vibrio harveyi* 10^8 CFU mL^{-1}.

Vibrio Counts in *Artemia* and Seabass Larvae

Five *Artemia* nauplii from each tank were separated from culture water using sieve. Next, *Artemia* was suspended in 1 mL SSW and meshed using sterile mortar and pestle. Serial dilutions were made up to 10^8 CFU mL^{-}1. In order to determine *Vibrio* loads in larvae, 10 µl of each diluted sample was plated on TCBS agar. The plates were incubated at room temperature for 24 hr. The colonies of *Vibrio* formed were counted using ROCKER galaxy 230 colony counters and calculated as CFU mL^{-1} using this formula:

Concentration of bacteria =
(Number of CFU)/(Volume plated × Total dilution)

In order to determine the *Vibrio* counts in seabass larvae the same method as *Artemia* was applied.

Statistical Analysis

All the data collected were analyzed using One-way Analysis of Variance (ANOVA). Multiple comparison tests (Tukey test) were used (IBM SPSS Statistic 20 software) in order to determine the significance among groups. Results were expressed as the mean ± standard error and the differences were considered significant at $p<0.05$.

RESULTS

Preliminary Challenged Assay using *Artemia*

After four days of observation, the survival rate of *Artemia* treated with *Enterobacter* sp. G87 (T2, T3 and T4) were between 77-82% for all concentrations. The results demonstrated that *Enterobacter* sp. G87 was not harmful to the *Artemia* (Figure 1). The highest survival was shown at concentration of 10^8 CFU mL^{-1} (T2, 82±2%). In challenged group, after *V. harveyi* was added to the

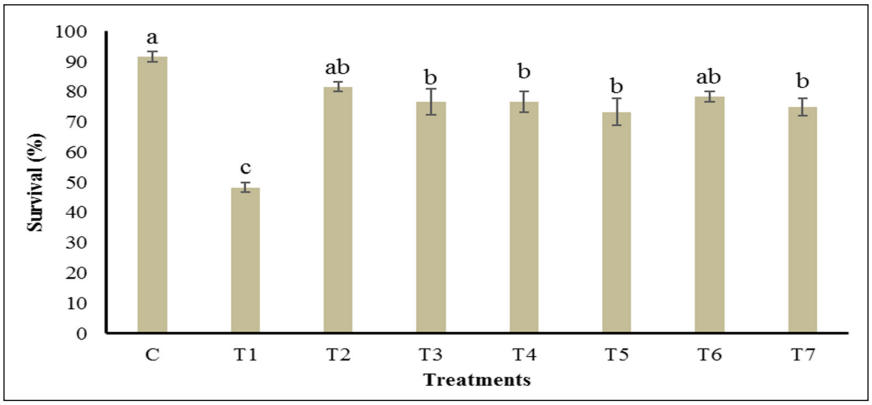

Figure 1. The survival rate of *Artemia* nauplii after pre-incubated with different concentrations of *Enterobacter* sp. G87 (10^8, 10^6, 10^4 CFU mL^{-1}) and challenged with 10^5 CFU mL^{-1} of *Vibrio harveyi*. Error bars indicate standard error. Mean with different alphabet letters indicate significant difference ($p<0.05$). *Note*. C: Control; T1: *Vibrio harveyi* 10^5 CFU mL^{-1}; T2: *Enterobacter* sp. G87 10^8 CFU mL^{-1}; T3: *Enterobacter* sp. G87 10^6 CFU mL^{-1}; T4: *Enterobacter* sp. G87 10^4 CFU mL^{-1}; T5: *Enterobacter* sp. G87 10^8 CFU mL^{-1} + *Vibrio harveyi* 10^5 CFU mL^{-1}; T6: *Enterobacter* sp. G87 10^6 CFU mL^{-1} + *Vibrio harveyi* 10^5 CFU mL^{-1}; T7: *Enterobacter* sp. G87 10^4 CFU mL^{-1} + *Vibrio harveyi* 10^5 CFU mL^{-1}

respective treatments, the survival rate for group T5, T6, T7 was between 73-78% which was significantly higher compared to group with *V. harveyi* only (T1, 48±2 %). *Artemia* treated with 10^6 CFU mL^{-1} (T6) showed the highest survival (78±2%) after being challenged with *V. harveyi*. The results showed that *Enterobacter* sp. G87 was capable to confer protection to *Artemia* against *V. harveyi* infection (Figure 1).

Challenged Assay on Seabass Larvae

After four days of experiment, results demonstrated the survival of seabass larvae treated with potential probiont *Enterobacter* sp. G87 at concentration of 10^6 CFU mL^{-1} (CT1, 93±2 %) showed no significant different with the control (98±2%) indicated this concentration was not harmful to the larvae. Moreover, after challenged with *V. harveyi*, this concentration provided full protection (T3, 95±3%) to the larvae with significant difference compared to the control group with *V. harveyi* only (VH, 45±3%). However, pre-incubation of seabass larvae at concentration of 10^8 CFU mL^{-1} of *Enterobacter* sp. G87 (CT2) reduced the survival to 55±5% which was significantly different compared to control group and no protection was observed after challenged. Results suggest this concentration was too high and not suitable

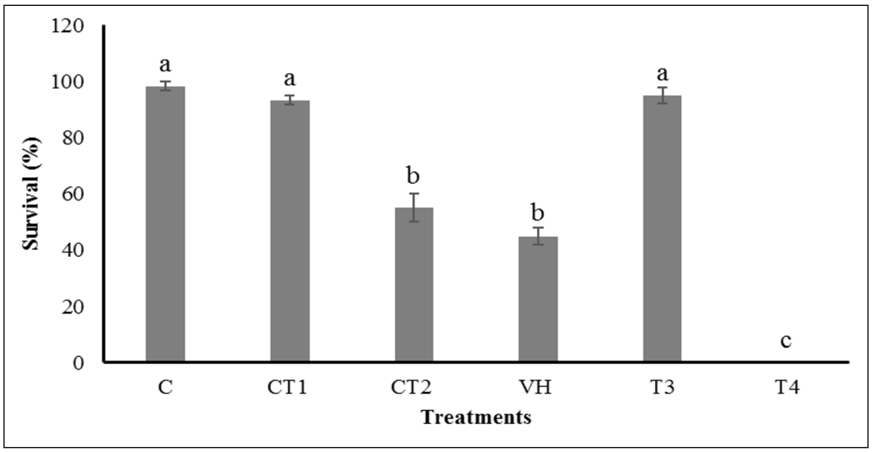

Figure 2. The survival rate of seabass larvae after pre-incubated with different concentrations of *Enterobacter* sp. G87 (10^8 and 10^6 CFU mL^{-1}) and challenged with 10^5 CFU mL^{-1} of *Vibrio harveyi.* Error bars indicate standard error. Mean with different alphabet letters indicate significant difference (p<0.05). *Note*. C: Control; CT1: *Enterobacter* sp. G87 10^6 CFU mL^{-1}; CT2: *Enterobacter* sp. G87 10^8 CFU mL^{-1}; VH: *Vibrio harveyi* 10^8 CFU mL^{-1}; T3: *Enterobacter* sp. G87 10^6 CFU mL^{-1} + *Vibrio harveyi* 10^8 CFU mL^{-1}; T4: *Enterobacter* sp. G87 10^8 CFU mL^{-1} + *Vibrio harveyi* 10^8 CFU mL^{-1}

for the larvae (Figure 2).

***Vibrio* Counts**

All concentrations of probiont *Enterobacter* sp. G87 (T5, T6, T7) were able to reduce the numbers of *Vibrios* in *Artemia* significantly at the end of the assay compared with control group of *V. harveyi* only (T1) (Table 3).

Meanwhile in seabass challenge assay, probiont *Enterobacter* sp. G87 at

concentration of 10^6 CFU mL^{-1} (T3) was able to reduce the numbers of *Vibrios* significantly compared to T1. However, at concentration of 10^8 CFU mL^{-1} (T4) no reduction in numbers of *Vibrios* was observed (Table 4).

Table 3
Vibrio *count in* Artemia *after pre-incubated with* Enterobacter *sp. G87 and challenged with* 10^5 *CFU* mL^{-1} *of* Vibrio harveyi

Treatments	Description	Log10(CFU mL^{1})
T1	*Vibrio harveyi* 10^5 CFU mL^{-1}	9.4 ± 0.0^a
T5	*Enterobacter* sp. G87 10^8 CFU mL^{-1} + *Vibrio harveyi* 10^5 CFU mL^{-1}	5.3 ± 0.1^b
T6	*Enterobacter* sp. G87 10^6 CFU mL^{-1} + *Vibrio harveyi* 10^5 CFU mL^{-1}	6.4 ± 0.2^b
T7	*Enterobacter* sp. G87 10^4 CFU mL^{-1} + *Vibrio harveyi* 10^5 CFU mL^{-1}	7.2 ± 0.1^b

Note. Mean with different alphabet letters indicate significant difference ($p<0.05$)

Table 4
Vibrio *counts in seabass larvae after pre-incubated at different concentrations of* Enterobacter *sp. G87 and challenged with* 10^8 *CFU* mL^{-1} *of* Vibrio harveyi.

Treatments	Descriptions	Log10 (CFU mL^{-1})
T1	*Vibrio harveyi* 10^8 CFU mL^{-1}	5.2 ± 0.0^a
T3	*Enterobacter* sp. G87 10^6 CFU mL^{-1} + *Vibrio harveyi* 10^8 CFU mL^{-1}	0.0 ± 0.0^b
T4	*Enterobacter* sp. G87 10^8 CFU mL^{-1} + *Vibrio harveyi* 10^8 CFU mL^{-1}	5.1 ± 0.1^a

Note. Mean with different alphabet letters indicate significant difference ($p<0.05$)

DISCUSSION

In this study, probiotic *Enterobacter* sp. G87 was tested to discover its potential to confer protection in *Artemia* and seabass larvae culture against *V. harveyi* infection. The results demonstrated the ability of *Enterobacter* sp. G87 to increase the survival of seabass larvae and *Artemia* nauplii when challenged with *V. harveyi*. The probiont also able to reduce the numbers of *Vibrio* in host after challenged.

Enterobacter is a Gram negative, rod shaped and facultative bacteria which is widely distributed in soil, water, intestinal tract of animals as well as sewage (Rogers, 2017). There are few reports on *Enterobacter* sp. which highlighted its potential as probiotics in *in vitro* assay. Wendy et al. (2014) reported on *Enterobacter ludwigii* inhibitory activity against two pathogenic strains; *Vibrio parahaemolyticus* and *Aeromonas hydrophila* in *in vitro* assay. *Enterobacter hormaechei* which was isolated from grey mullet (*Mugil cephalus*) was also reported to have antagonistic properties against *Vibrio cholera* in well diffusion assay (Ghosh et al., 2011). However, to our knowledge, there is less information on the application of *Enterobacter* sp. in *in vivo* study.

In this study, *Artemia* was used as host in the preliminary *in vivo* assay. *Artemia* is an important live feed for a variety of

finfish and shellfish and are given to over 85% of aquaculture species around the world. It is therefore necessary to control the bacterial population of *Artemia* to minimize the danger of bacterial infection before their use in culture systems (Lamari et al., 2014). With the presence of probiotic, it could provide protection for *Artemia* in terms of immunity and disease (Balcázar et al., 2007). Furthermore, it could act as a carrier to introduce probiotics to the targeted host (Seenivasan et al., 2012). Enrichment and bioencapsulation of *Artemia* have been widely applied in marine fish and crustacean culture around the world as it can enhance the nutritional value of *Artemia* (Immanuel, 2016). For an example, study done by Jamali et al. (2014) reported rainbow trout larvae fed with *Artemia* enriched with *Bacillus* sp. had a higher growth and survival rate.

The survival of *Artemia* was high after enrichment with probiont *Enterobacter* sp. G87. All concentrations tested were able to provide protection to the *Artemia* after challenged with *V. harveyi*. The optimum concentration was at 10^6 CFU mL^{-1}, where full protection in *Artemia* was observed. Similar finding was observed in *Artemia* when challenged with *Vibrio parahemolyticus* and *V. cholera* after being enriched with two probiotics, *L. acidophilus* and *Lactobacillus sporogenes* which showed higher survival (72%) compared to the normal *Artemia* (Immanuel, 2016). In the previous study, concentration of 10^6 CFU mL^{-1} had been identified as optimal concentration of *L. sporogenes* to attain good survival and growth in *Artemia* (Jacobsen et al., 1999; Seenivasan et al., 2012). *Bacillus* spp. JAQ04 and *Micrococcus* spp. JAQ07 at concentration 10^6 CFU mL^{-1} also resulted in better survival of *Artemia* (70%) when challenged with *Vibrio alginolyticus* compared to control group (20%) (Shazwani et al., 2015).

After being challenged, the numbers of *Vibrio* in *Artemia* was determined to observe the vibrios reduction if any. Results demonstrated the ability of *Enterobacter* sp. G87 in reducing the numbers of *Vibrio* in *Artemia*. This suggests the protection maybe due to antibacterial activity or the colonization factor provided by *Enterobacter* sp. G87. Mohan et al. (2014) proved this theory when two probiotics *Alteromonas* sp. and *Actobacterium* sp. were able to colonize better, grow faster resulting in high counts compared to pathogens (*V. harveyi* and *Aeromonas* sp.) when introduced together to *Artemia*.

Seabass larvae was chosen as species of interest because of its high economic importance for larvae culture and aquaculture (Frans et al., 2013). The role of beneficial probiotic to limit and to control environmental pathogens which become particularly important in the future of aquaculture, especially with regard to increasing number of antibiotic resistant strains of bacteria (Haq et al., 2012).

In seabass larvae challenge assay, *Enterobacter* sp. G87 at concentration of 10^6 CFU mL^{-1} showed higher survival after challenged with *V. harveyi* compared to 10^8 CFU mL^{-1}. The results suggest that higher concentration of probionts may not be

suitable in conferring protection for seabass larvae due to high mortality observed after challenged.

High amount of probiont sometimes may not be suitable in protecting the host against pathogenic infection. It might harm the host instead which leads to bad effect on the host's health (Martínez Cruz et al., 2012; Tuan et al., 2013). High concentration of probiotics may deplete the oxygen content in the water due to a very fast rate of bacterial colonization which can disturbed the oxygen level in water (Yaminudin, 2017). Suzer et al. (2008) also reported that high concentration of probiotics was not good for husbandry parameter of the culture water. Hence, it is crucial to use probiotics in a correct concentration in order to exert optimum beneficial effects on the growth and survival of hosts (Bagheri et al., 2008).

The ability of *Enterobacter* sp. G87 in conferring protection of *Artemia* and seabass larvae against *V. harveyi* was in line with study done by Capkin and Altinok (2009) that reported the survival of rainbow trout *Oncorhynchus mykiss* challenged with bacteria *Yersinia ruckeri* which caused Yersioniosis diseases was increased when fed with feed supplemented with *Enterobacter cloacoe* for 60 days. LaPatra et al. (2014) also demonstrated the use of *Enterobacter* strain C6-6 in rainbow trout which showed higher survival after challenged with *Flavobacterium psychrophilum* in both *in vitro* and *in vivo* assay.

At the end of challenge assay, the pathogen count was done to investigate the reduction numbers of *Vibrio* in *Artemia* and seabass larvae. Results revealed the potential of *Enterobacter* sp. G87 in reducing the numbers of *Vibrio* after being challenged. *Enterobacter* sp. G87 at 10^6 CFU mL^{-1} was able to reduce the numbers of *Vibrio* count completely in seabass larvae. This suggest the protection provided by *Enterobacter* sp. G87 may be due to its ability to produce antimicrobial compound or compete for the colonization sites with the pathogens. Water quality parameters were within optimal range during the experiment indicated it did not contribute to the mortality of *Artemia* and seabass larvae.

The potential probiotic *Enterobacter* sp. G87 showed capability to enhance the survival of *Artemia* and seabass larvae and able to provide protection against *V. harveyi* infection at concentration of 10^6 CFU mL^{-1}. The results were relevant with the definition of probiotic which an adequate amount of live microbial which has a beneficial effect on the host by modifying the host associated or ambient microbial community, ensuring improvement by use of the feed or enhancing its nutritional value, enhancing the host response towards disease, or by improving the quality of its environment (Verschuere et al., 2000). Other than that, when the culture system was provided with potential probiont, it will be ingested naturally by the host (Mahdhi et al., 2011).

CONCLUSION

This research was done to observe the ability of potential probiont *Enterobacter* sp. G87 to protect *Artemia* nauplii and seabass larvae against *Vibrio harveyi*. The results showed

that *Enterobacter* sp. G87 at concentration of 10^6 CFU mL^{-1} was the most effective to protect *Artemia* and seabass larvae against *V. harveyi* infection and reduced the numbers of *Vibrio*. Thus, *Enterobacter* sp. G87 was proven to have potential as a good probiotic for seabass larval culture and as enrichment of *Artemia*. The used of potential probiotic can be advantageous for the aquaculture production.

ACKNOWLEDGEMENTS

This research was supported by Ministry of Higher Education Malaysia through Fundamental Research Grant Scheme (FRGS/2013/5524396) and Higher Institution Centres of Excellence (HiCoE), Ministry of Science, Technology and Innovation (MOSTI) Malaysia (Vote no.: 6369100). Special thanks to Putra Group Initiative Grant (IPB-UPM, Vote no.: 9553100).

REFERENCES

Al-Dohail, M. A., Hashim, R., & Aliyu-Paiko, M. (2009). Effects of the probiotic, *Lactobacillus acidophilus*, on the growth performance, haematology parameters and immunoglobulin concentration in African catfish (*Clarias gariepinus*, Burchell 1822) fingerling. *Aquaculture Research*, *40*(14), 1642-1652.

Bagheri, T., Hedayati, S. A., Yavari, V., Alizade, M., & Farzanfar, A. (2008). Growth, survival and gut microbial load of rainbow trout (*Onchorhynchus mykiss*) fry given diet supplemented with probiotic during the two months of first feeding. *Turkish Journal of Fisheries and Aquatic Sciences*, *8*(1), 43-48.

Balcázar, J. L., Rojas-Luna, T., & Cunningham, D. P. (2007). Effect of the addition of four potential probiotic strains on the survival of pacific white shrimp (*Litopenaeus vannamei*) following immersion challenge with *Vibrio parahaemolyticus*. *Journal of Invertebrate Pathology*, *96*(2), 147-150.

Béné, C., Barange, M., Subasinghe, R., Pinstrup-Andersen, P., Merino, G., Hemre, G. I., & Williams, M. (2015). Feeding 9 billion by 2050–Putting fish back on the menu. *Food Security*, *7*(2), 261-274.

Capkin, E., & Altinok, I. (2009). Effects of dietary probiotic supplementations on prevention/ treatment of yersiniosis disease. *Journal of Applied Microbiology*, *106*(4), 1147-1153.

Department of Fisheries. (2017). *Perangkaan perikanan tahunan 2017* [Annual fishery statistics 2017]. Putrajaya, Malaysia: DOF.

Food and Agriculture Organization. (2015). *The state of food insecurity in the world*. Rome, Italy: FAO.

Food and Agriculture Organization. (2016). *The state of world fisheries and aquaculture: Contributing to food security and nutrition for all*. Rome, Italy: FAO.

Frans, I., Dierckens, K., Crauwels, S., Van Assche, A., Leisner, J., Larsen, M. H., & Rediers, H. (2013). Does virulence assessment of *Vibrio anguillarum* using sea bass (*Dicentrarchus labrax*) larvae correspond with genotypic and phenotypic characterization?. *PLOS One*, *8*(8), e70477.

Ghosh, S., Ringø, E., Deborah, G. S. A., Rahiman, K. M., & Hatha, A. A. M. (2011). *Enterobacter hormaechei* bac 1010 from the gut of flathead grey mullet as probable aquaculture probiont. *Journal of Nature Science and Sustainable Technology*, *5*(3), 189.

Hai, N. V. (2015). The use of probiotics in aquaculture. *Journal of Applied Microbiology*, *119*(4), 917-935.

Haq, M. B., Vijayasanthi, P., Vignesh, R., Shalini, R., Chakraborty, S., & Rajaram, R. (2012). Effect of probiotics against marine pathogenic bacteria on *Artemia franciscana. Journal of Applied Pharmaceutical Science*, *2*(4), 38.

Harikrishnan, R., Balasundaram, C., & Heo, M. S. (2011). Fish health aspects in grouper aquaculture. *Aquaculture*, *320*(1-2), 1-21.

Immanuel, G. (2016). Bioencapsulation of brine shrimp *Artemia* nauplii with probionts and their resistance against *Vibrio* pathogens. *Journal of Fisheries and Aquatic Science*, *11*, 323-330.

Jacobsen, C. N., Nielsen, V. R., Hayford, A. E., Møller, P. L., Michaelsen, K. F., Paerregaard, A., & Jakobsen, M. (1999). Screening of probiotic activities of forty-seven strains of *Lactobacillus* spp. by *in vitro* techniques and evaluation of the colonization ability of five selected strains in humans. *Applied and Environmental Microbiology*, *65*(11), 4949-4956.

Jamali, H., Tafi, A. A., Jafaryan, H., & Patimar, R. (2014). *Effect of enriched* Artemia parthenogenetica *with probiotic (*Bacillus *spp.) on growth, survival, fecal production and nitrogenous excretion in rainbow trout (*Oncorhynchus mykiss*) larvae*. Retrieved July 22, 2019, from https://www.omicsonline.org/open-access/effect-of-enriched-artemia-parthenogenetica-with-probiotic-bacillus-spp-on-growth-survival-fecal-production-and-nitrogenous-excretion-in-rainbow-trout-oncorhynchus-mykiss-larvae-2332-2608-2-111.pdf

Lamari, F., Sadok, K., Bakhrouf, A., & Gatesoupe, F. J. (2014). Selection of lactic acid bacteria as candidate probiotics and *in vivo* test on *Artemia* nauplii. *Aquaculture International*, *22*(2), 699-709.

LaPatra, S. E., Fehringer, T. R., & Cain, K. D. (2014). A probiotic *Enterobacter* sp. provides significant protection against *Flavobacterium psychrophilum* in rainbow trout (*Oncorhynchus mykiss*) after injection by two different routes. *Aquaculture*, *433*, 361-366.

Li, M. F., Wang, C. L., & Sun, L. (2011). A pathogenic *Vibrio harveyi* lineage causes recurrent disease outbreaks in cultured Japanese flounder (*Paralichthys olivaceus*) and induces apoptosis in host cells. *Aquaculture*, *319*(1-2), 30-36.

Mahdhi, A., Hmila, Z., Chaieb, K., Kamoun, F., & Bakhrouf, A. (2011). Probiotic properties of halophilic *Bacillus* strains enhance protection of *Artemia* culture against pathogenic *Vibrio*. *Aquatic biology*, *13*(3), 225-231.

Martínez Cruz, P., Ibáñez, A. L., Monroy Hermosillo, O. A., & Ramírez Saad, H. C. (2012). *Use of probiotics in aquaculture*. Retrieved July 22, 2019, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3671701/pdf/ISRN.MICROBIOLOGY2012-916845.pdf

Mohan, S. S., Kumar, J. B., Ajitha, M. L., & Babu, M. M. (2014). Probiotics from *Artemia* and its application on controlling the bacterial pathogens in aquaculture system. *International Journal of Pharma and Bio Sciences*, *5*(4), 1168 - 1172.

Ransangan, J., & Mustafa, S. (2009). Identification of *Vibrio harveyi* isolated from diseased Asian seabass *Lates calcarifer* by use of 16S ribosomal DNA sequencing. *Journal of Aquatic Animal Health*, *21*(3), 150-155.

Rogers, K. (2017). Enterobacter *bacteria genus*. Retrieved July 22, 2019, from Encyclopedia Britannica website: https://www.britannica.com/science/Enterobacter

Ruwandeepika Hettipala Arachchige, D. (2010). Expression of virulence genes of Vibrios belonging to the harveyi clade in the brine shrimp *Artemia* (Doctoral dissertation), Ghent University, Belgium.

Salamoura, C., Kontogianni, A., Katsipi, D., Kandylis, P., & Varzakas, T. (2014). Probiotic fermented milks made of cow's milk, goat's milk and their mixture. *Journal of Biotechnology*, 185, S37 - S125.

Seenivasan, C., Radhakrishnan, S., Muralisankar, T., & Bhavan, P. S. (2012). Influence of combined probiotics *Lactobacillus sporogenes* and *Bacillus subtilis* on survival, growth, biochemical changes and energy utilization performance of *Macrobrachium rosenbergii* (De Man, 1879) post larvae. *Journal of Ecobiotechnology*, *4*(1), 29-34.

Shazwani, N., Pipudin, M., Jasmin, M. Y., Ina-Salwany, M. Y., Harmin, S. A., & Karim, M. (2015). Evaluation of antagonism activity of potential Malaysian probiont strains, *Bacillus* spp. JAQ04 and *Micrococcus* spp. JAQ07 in *in vitro* condition and on *Artemia franciscana* against *Vibrio alginolyticus*. *Journal of Fisheries and Aquatic Science*, *10*(4), 300.

Suzer, C., Çoban, D., Kamaci, H. O., Saka, Ş., Firat, K., Otgucuoğlu, Ö., & Küçüksari, H. (2008). *Lactobacillus* spp. bacteria as probiotics in gilthead sea bream (*Sparus aurata,* L.) larvae: Effects on growth performance and digestive enzyme activities. *Aquaculture*, *280*(1), 140-145.

Talpur, A. D., Ikhwanuddin, M., Abdullah, M. D. D., & Bolong, A. M. A. (2013). Indigenous *Lactobacillus plantarum* as probiotic for larviculture of blue swimming crab, *Portunus pelagicus* (Linnaeus, 1758): Effects on survival, digestive enzyme activities and water quality. *Aquaculture*, *416*, 173-178.

Talpur, D. (2014). *Mentha piperita* (Peppermint) as feed additive enhanced growth performance, survival, immune response and disease resistance of Asian seabass, *Lates calcarifer* (Bloch) against *Vibrio harveyi* infection. *Aquaculture*, *420*, 71-78.

Tan, L. T. H., Chan, K. G., Lee, L. H., & Goh, B. H. (2016). *Streptomyces* bacteria as potential probiotics in aquaculture. *Frontiers in Microbiology*, *7*, 79.

Tuan, T. N., Duc, P. M., & Hatai, K. (2013). Overview of the use of probiotics in aquaculture. *International Journal of Research in Fisheries and Aquaculture*, *3*(3), 89-97.

Verschuere, L., Rombaut, G., Sorgeloos, P., & Verstraete, W. (2000). Probiotic bacteria as biological control agents in aquaculture. *Microbiology and Molecular Biology Reviews*, *64*(4), 655-671.

Wendy, F. T., Hena, M. A., Wong, S. K., Idris, M. H., Sharifuzzaman, S. M., & Ina-Salwany, M. Y. (2014). *Enterobacter ludwigii,* a candidate probiont from the intestine of Asian seabass. *Journal of Science and Technology in the Tropics*, *10*(1), 5–14.

Yaminudin, N. J. (2017). *Evaluation of potential probionts from marine shellfish for black tiger shrimp* Penaeus monodon *(Fabricius, 1978) larviculture against vibriosis* (Master dissertation), Universiti Putra Malaysia, Malaysia.

Pertanika J. Trop. Agric. Sc. 42 (4): 1263 - 1271 (2019)

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Short Communication

Antibiotic Sensitivity and Pathogenicity of *Aeromonas veronii* Isolated from Diseased Red Hybrid Tilapia in Malaysia

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ABSTRACT

This article reports the antibiotics sensitivity and pathogenicity of *Aeromonas veronii* 5L isolated from diseased red hybrid tilapia. The antibiotic sensitivity of *A. veronii* 5L was determined towards 13 antimicrobial agents. Then, the bacterial inoculums ranging between 0 and 10^6 CFU/mL were used for intraperitoneal challenge in red hybrid tilapia juveniles. *Aeromonas veronii* 5L showed resistance to ampicillin, streptomycin and sulfamethoxazole/trimethoprim. Following intraperitoneal exposure, mortality was observed as early as 24 h post infection leading to a total of 56.7% cumulative mortality at 10^2 CFU/mL, and 66.7% from 10^3 to 10^6 CFU/mL. Clinical signs and gross lesions including abdominal distension, detachment of

ARTICLE INFO

Article history:
Received: 4 July 2019
Accepted: 15 August 2019
Published: 13 November 2019

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ISSN: 1511-3701
e-ISSN: 2231-8542

scales and ulceration on the body surface, inflammation around the operculum and based of fins, hemorrhage of internal organs, and accumulation of fluid in abdominal cavity. Histopathological examinations revealed generalized congestion of the brain, necrotic hepatocytes, generalized tubular necrosis of kidney and multifocal necrosis of spleen with splenic infarction. The $LD_{50\text{-}336h}$ of *A. veronii* 5L was determined at $1.9 \times 10^{3.73}$ CFU/mL. This study revealed the capability of *A. veronii* as another important pathogen in tilapia culture in Malaysia.

Keywords: *Aeromonas veronii*, antibiotic sensitivity, fish disease, pathogenicity, tilapia

INTRODUCTION

Aeromonas veronii infection has been reported in several fish species around the world including carp (*Cyprinus carpio*), oscar (*Astronotus ocellatus*), Chinese longsnout catfish (*Leiocassis longirostris*), Nile tilapia (*Oreochromis niloticus*) and pond loach (*Misgurnus anguillicaudatus*) (Cai et al., 2012; Dong et al., 2015; Sreedharan et al., 2011; Yu et al., 2010; Zhu et al., 2016). Affected fish exhibit either abdominal distension, abnormal swimming behavior, loss of appetite, reddish foci on the skin, dark bodies, hemorrhages all over the body surface and ulceration with muscular necrosis, severe haemorrhage of internal organs, liver congestion, enlarged spleen and kidney, enteritis and gut displayed a significant amount of yellowish liquid (Eissa et al., 2015; Sreedharan et al., 2011; Yu et al., 2010; Zhu et al., 2016).

Tilapia (*Oreochromis* spp.) culture in Malaysia is commonly affected by *Streptococcus agalactiae*, *S. iniae* and *Aeromonas hydrophila* infections (Amal et al., 2010; Ismail et al., 2016; Nur-Hidayahanum et al., 2016; Rahmatullah et al., 2017). Recently, the isolation of *A. veronii* from diseased cultured red hybrid tilapia (Nile tilapia × Mozambique tilapia (*O. mossambicus*)) that concurrently infected with Tilapia Lake Virus (TiLV) has been reported in Malaysia (Amal et al., 2018). However, the pathogenicity of the isolated *A. veronii* was not conducted. Thus, we report the antibiotics sensitivity and pathogenicity of *A. veronii* isolated from diseased red hybrid tilapia in Malaysia. In this study, we revealed the virulent and capability of *A. veronii* as another important pathogen in tilapia culture in Malaysia in the future.

MATERIALS AND METHODS

Aeromonas veronii 5L was used in this study. It was isolated from diseased red hybrid tilapia juvenile that was concurrently infected with TiLV (Amal et al., 2018). The isolate was identified as *A. veronii* based on Gram staining, oxidase and catalase tests, API®20NE system (bioMérieux, Marcy l'Etoile, France), PCR and sequencing analyses. Stock of *A. veronii* 5L was cultured in *Aeromonas* medium base agar (AMBA) (Oxoid, Hampshire, United Kingdom) and incubated for 24 h at 30°C. Then, 10

colonies of the isolate were inoculated into 100 mL of tryptic soy broth (TSB) (Merck, Darmstadt, Germany), and incubated in an orbital incubator at 0.75 × *g* for 24 h at 30°C. The bacterial concentrations were calculated based on standard ten-fold serial dilutions and spread plating methods onto AMBA, prior to fish challenge experiment.

The antibiotic sensitivity of *A. veronii* 5L was evaluated using the Kirby-Bauer disc diffusion method (Clinical and Laboratory Standards Institute [CLSI], 2016). Direct colony suspension of *A. veronii* with adjusted turbidity equivalent to 0.5 McFarland standard was used. A total of 13 representative antimicrobial agents (Oxoid, London, UK), including ampicillin (10 µg), cefotaxime (30 µg), cefepime (30 µg), cephalothin (30 µg), gentamycin (10 µg), kanamycin (30 µg), streptomycin (10 µg), nalidixic acid (30 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), chloramphenicol (30 µg), nitrofurantoin (300 µg), ciprofloxacin (5 µg) and tetracycline (30 µg) were tested in duplicate. The resistance profiles (resistant, intermediate or susceptible) were interpreted using recommended criteria (CLSI, 2013, 2016). The multiple antibiotic resistance (MAR) index was determined according to Krumperman (1985). A MAR index higher than 0.2 indicated high-risk exposure to these antibiotics.

A total of 180 red hybrid tilapia juveniles weighing 20.5 ± 0.8 g were used for challenge experiment. The fish were divided into six groups and each group was in triplicate with 10 fish/replicate, including the control group. At the start of the experiment, each group was exposed intraperitoneal (IP) with different bacterial concentration that ranged between 10^2 and 10^6 CFU/mL of live *A. veronii* 5L, while the non-infected control group was similarly exposed to sterile phosphate-buffered saline. Each fish was IP injected with final volume of 100 µL of the inoculum.

Following infection, continuous aeration was provided to all groups, while feed was given twice daily. The experiment was conducted for 336 h, during which the clinical signs, gross lesions and mortality pattern were recorded. Freshly dead fish were collected for bacterial isolation and identification. The LD_{50} of *A. veronii* 5L infection in the fish was calculated at 336 h post infection (hpi), according to formula described by Ramakrishnan (2016). The mean ± SD of water quality parameters during the study period were as follows: dissolved oxygen at 5.60 ± 0.50 mg/L, pH at 7.2 ± 0.5, water temperature at 27.10 ± 0.50°C, ammonia at 0.02 ± 0.01 mg/L, and nitrite at 0.10 ± 0.50 mg/L.

Quantitatively, the brain, kidney, liver and spleen of dead fish were collected for histopathological changes observation (Amal et al., 2018). Following fixation in 10% buffered formalin for 24 - 48 h, the samples were processed in tissue processor, embedded in paraffin, sectioned at 4 µm thick and stained routinely with Harris haematoxylin and eosin (HE) for histological study. The sections were

examined and photographed using Nikon Eclipse 50i Japan microscope and The Nikon NIS-Element D 3.2 Image Analyser (Nikon Instruments Inc., USA).

RESULTS

Aeromonas veronii 5L displayed strong resistance to ampicillin (range: 0 mm), streptomycin (0 mm) and sulfamethoxazole/trimethoprim (0 mm), while intermediately sensitive to kanamycin (16 - 17 mm). The isolate was sensitive to cefotaxime (31 - 33 mm), cefepime (28 – 29 mm), cephalothin (23 - 24 mm), gentamycin (18 mm), tetracycline (28 mm), ciprofloxacin (33 mm), nalidixic acid (30 - 31 mm), nitrofurantoin (24 mm) and chloramphenicol (30 mm). The MAR index was 0.23.

Fish mortality was observed as early as 24 hpi in fish infected with 10^2, and 10^4 to 10^6 CFU/mL of *A. veronii* 5L (Figure 1). In general, the fish mortality showed increasing pattern until the end of the experimental period. Mortality among fish infected with 10^4 and 10^6 CFU/mL reached peak at 66.7% as early as 264 hpi, followed by those infected with 10^5 CFU/mL at 312 hpi, with 10^3 CFU/mL at 336 hpi. However, infection with 10^2 CFU/mL peaked with 56.7% cumulative mortality at 336 hpi. *Aeromonas veronii* 5L was successfully re-isolated from all dead fish. No fish mortality was observed in negative control group. The $LD_{50\text{-}336h}$ of *A. veronii* 5L in red hybrid tilapia juveniles was determined at $1.9 \times 10^{3.73}$ CFU/mL.

Infected fish tended to isolate from the schooling group, appeared lethargy, less responsive to stimuli, loss of appetite,

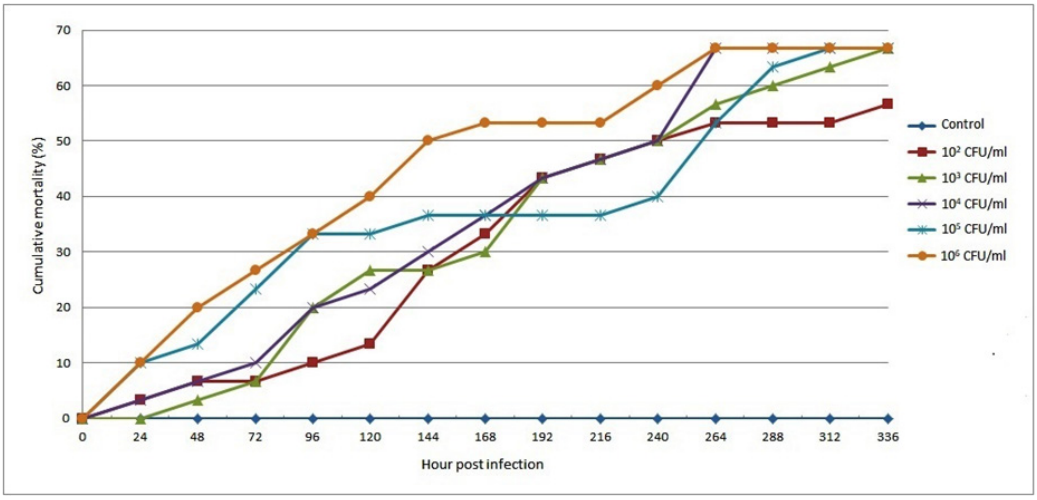

Figure 1. Cumulative mortality patterns of red hybrid tilapia juveniles following intraperitoneal challenge with various concentration of *Aeromonas veronii* 5L up to 336 hours

showed distended abdomen, occasional detachment of scales and ulceration on the body surface, pale, inflammation around the operculum and based on fins, skin hemorrhages and ulceration of fins (Figure 2). Post-mortem examination revealed hemorrhagic liver, enlarged gall bladder, accumulation of fluid in abdominal cavity, inflammation and hemorrhage of the brain.

The histopathological assessment showed generalized congestion of the brain (Figure 3). Kidneys showed generalized tubular necrosis with mild haemorrhage. The histopathological lesions occasionally observed in the liver include necrosis of hepatopancreatic cells and generalized necrosis of the hepatocytes. Generalized multifocal necrosis and multifocal melano macrophage centre were observed in the spleen. Besides, hyperplasia of red pulp and splenic infarctions were also observed.

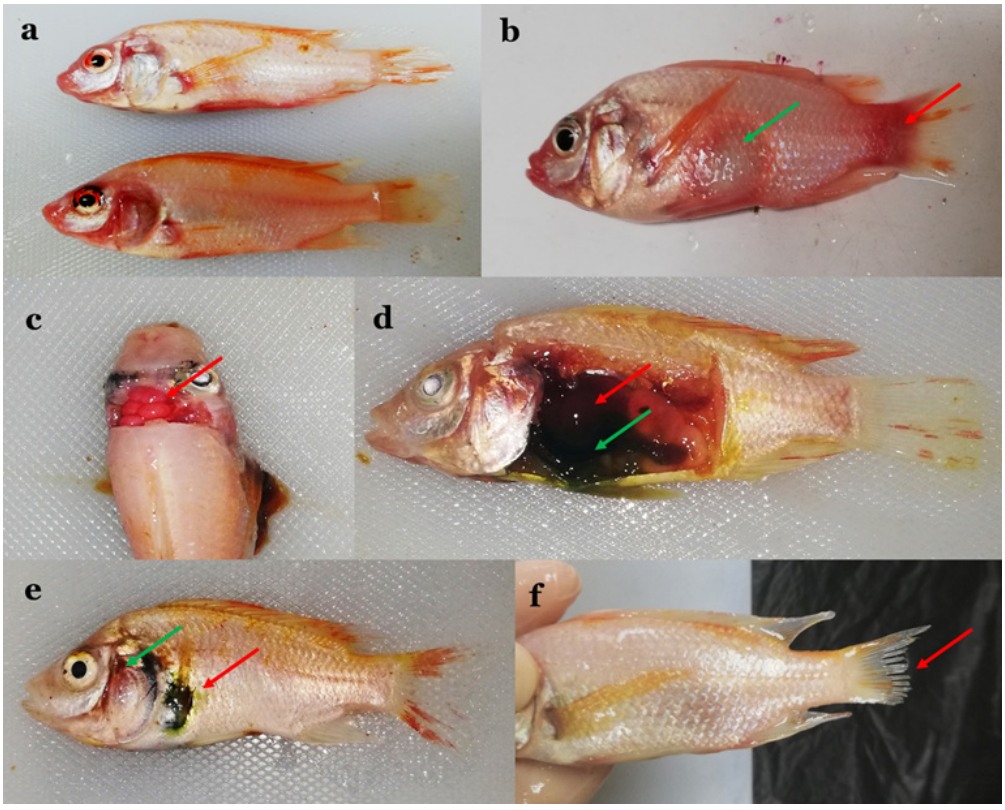

Figure 2. Gross lesions of juvenile red hybrid tilapia following intraperitoneal infection by *Aeromonas veronii* 5L. a) Inflammation around the operculum area and fins base; b) Ulceration around the abdomen (green arrow) and inflammation at the base of caudal fin (red arrow); c) Inflammation and haemorrhage of the brain (red arrow); d) Haemorrhage liver (red arrow) and enlarged gall bladder (green arrow); e) Inflammation around operculum area (green arrow) and distension of abdominal area (red arrow) due to fluid accumulation; f) Severe ulceration of caudal fin (red arrow)

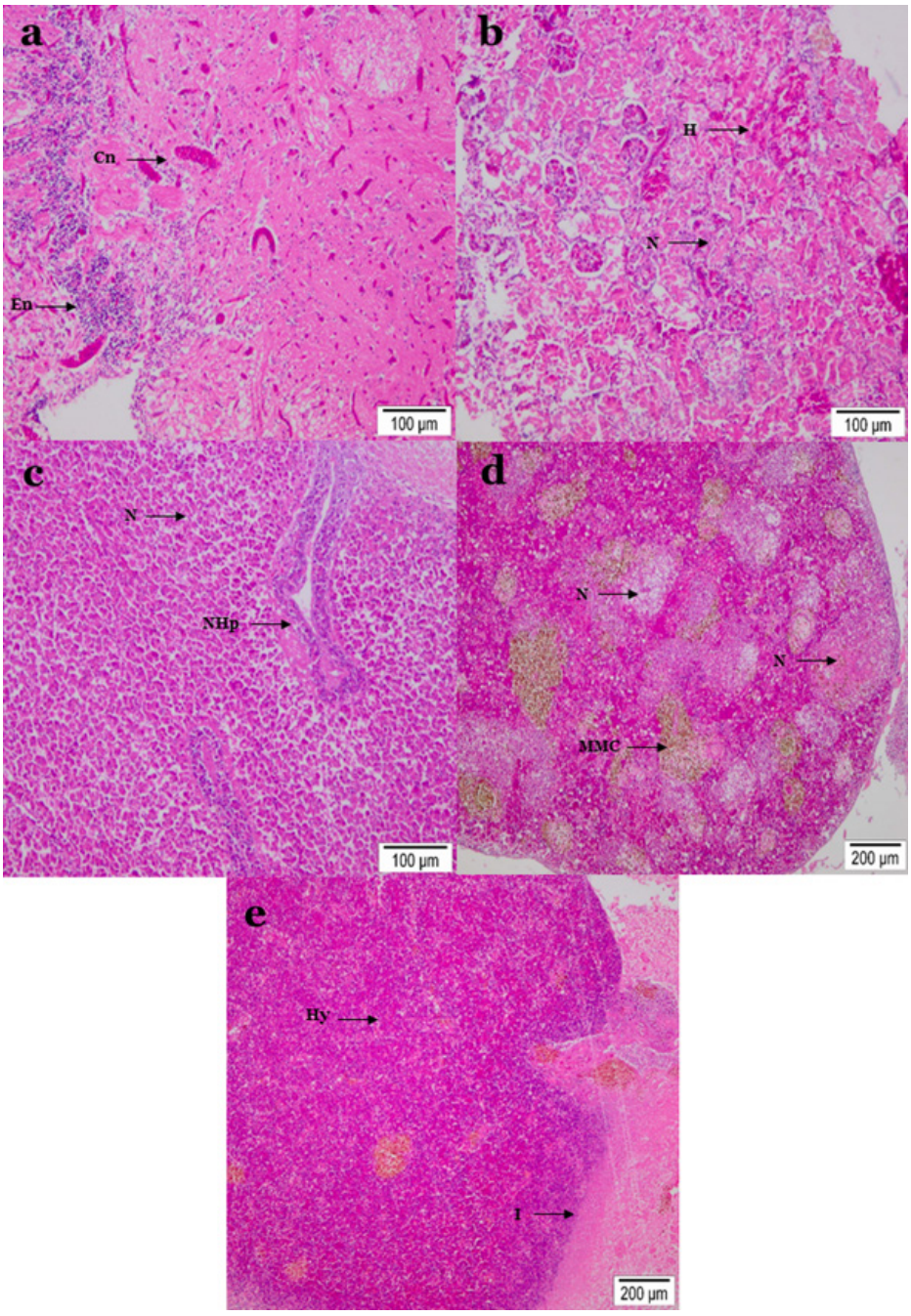

Figure 3. Histopathological lesions of juvenile red hybrid tilapia following intraperitoneal injection by *Aeromonas veronii* 5L. a) Encephilitis (En) with presence of generalized congestion (Cn), brain, HE, ×100. b) Generalized tubular necrosis (N) with mild haemorrhage (H), kidney, HE, ×100. c) Necrosis of hepatopancreatic cells (NHp) and generalized necrosis (N), liver, HE, ×100. d) Generalized multifocal necrosis (N) and increased in sizes and number of multifocal melano macrophage centre (MMC), spleen, HE, ×100. e) Hyperplasia of red pulp (Hy) and splenic infarction (I), spleen, HE, ×100

DISCUSSION

Clinical infection by *A. veronii* in fish has not been reported in Malaysia. However, disease outbreak causing daily mortality between 300 and 1800 cultured red hybrid tilapia juveniles was recorded in 2018, resulting in approximately 25% mortality (Amal et al., 2018). The outbreak was due to concurrent infection involving TiLV infection and *A. veronii*. In this study, experimental infection resulted in mortality as early as 24 hpi, with peak cumulative mortality rate of 66.7%. With low $LD_{50\text{-}336h}$ at $1.9 \times 10^{3.73}$ CFU/mL, this *A. veronii* 5L isolate was considered virulent. The virulence of *A. veronii* was also observed in Nile tilapia juvenile in Thailand, where *A. veronii* at 8.9×10^6 CFU/fish killed 100% of fish within 24 hpi (Dong et al., 2017).

In this study, infected fish displayed typical aeromonad infection (Eissa et al., 2015; Sreedharan et al., 2011; Yu et al., 2010; Zhu et al., 2016), suggesting common signs of haemorrhagic septicemic as in the virulent motile aeromonad septicaemic strain (Dias et al., 2016). In Saudi Arabia, experimentally infected Nile tilapia by *A. veronii* showed hemorrhage at the base of all fins, generalized external hemorrhage on the body skin, congestion and enlargement of internal organs, enteritis, and intestine filled with transparent fluids (Hassan et al., 2017). The results of histopathological changes in this study also indicated septicaemia, which are in agreement with previous study in Thailand (Dong et al., 2017).

Antibiotic sensitivity study indicated that *A. veronii* 5L was isolated from the fish that previously had high-risk exposure to the tested antibiotics. Nevertheless, in this study, cefotaxime, cefepime, cephalothin, gentamycin, tetracycline, ciprofloxacin, nalidixic acid, nitrofurantoin and chloramphenicol could still be used to treat infections by *A. veronii*. Similarly, Hassan et al. (2017) also indicated that the capability of chloramphenicol, nitrofurantoin and nalidixic acid to control *A. veronii* infection in tilapia.

CONCLUSION

This study revealed the virulent and capability of *A. veronii* as another important pathogen in tilapia culture in Malaysia in the future, besides *S. agalactiae*, *S. iniae* and *A. hydrophila*. Moreover, while the production of tilapia keeps increasing in this country, the results should also alarm the tilapia industry, especially to the farm operators and authorities.

ACKNOWLEDGEMENT

This work was supported by the Higher Institution Centre of Excellence, the Ministry of Education, Malaysia (Vot number: 6369100).

REFERENCES

Amal, M. N. A., Koh, C. B., Nurliyana, M., Suhaiba, M., Nor-Amalina, Z., Santha, S., … Zamri-Saad, M. (2018). A case of natural co-infection of Tilapia Lake Virus and *Aeromonas veronii* in a Malaysian red hybrid tilapia (*Oreochromis niloticus* × *O. mossambicus*) farm experiencing high mortality. *Aquaculture*, *485*(2), 12-16.

Amal, M. N. A., Zamri-Saad, M., Siti-Zahrah, A., Zulkafli, R., Misri, S., Nur-Nazifah, M., & Shahidan, H. (2010). Prevalence of *Streptococcus agalactiae* in tilapia kept in different water bodies. *Online Journal of Veterinary Research*, *11*(2), 153-162.

Cai, S. H., Wu, Z. H., Jian, J. C., Lu, Y. S., & Tang, J. F. (2012). Characterization of pathogenic *Aeromonas veronii* bv. *veronii* associated with ulcerative syndrome from Chinese longsnout catfish (*Leiocassis longirostris* Günther). *Brazilian Journal of Microbiology*, *43*(1), 382-8.

Clinical and Laboratory Standards Institute. (2013). *Performance standards for antimicrobial susceptibility testing; Twenty-third informational supplement (CLSI document M100-S23)*. Retrieved July 03, 2019, from https://www.facm.ucl.ac.be/intranet/CLSI/CLSI-M100S23-susceptibility-testing-2013-no-protection.pdf

Clinical and Laboratory Standards Institute. (2016). *Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria (3rd ed., CLSI document M45)*. Retrieved July 03, 2019, from https://clsi.org/media/1450/m45ed3_sample.pdf

Dias, M. K. R., Sampaio, L. S., Proietti-Junior, A. A., Yoshioka, E. T. O., Rodrigues, D. P., Rodriguez, A. F. F., … Tavares-Dias, M. (2016). Lethal dose and clinical signs of *Aeromonas hydrophila* in *Arapaima gigas* (Arapaimidae), the giant fish from Amazon. *Veterinary Microbiology*, *188*(5), 12-15.

Dong, H. T., Nguyen, V. V., Le, H. D., Sangsuriya, P., Jitrakorn, S., Saksmerprome, V., … Rodkhum, C. (2015). Naturally concurrent infections of bacterial and viral pathogens in disease outbreaks in cultured Nile tilapia (*Oreochromis niloticus*) farms. *Aquaculture*, *448*(11), 427-435.

Dong, H. T., Techatanakitarnan, C., Jindakittikul, P., Thaiprayoon, A., Taengphu, S., Charoensapsri, W., … Senapin, S. (2017). *Aeromonas jandaei* and *Aeromonas veronii* caused disease and mortality in Nile tilapia, *Oreochromis niloticus* (L.). *Journal of Fish Diseases*, *40*(10), 1395-1403.

Eissa, I. A. M., Maather, E., Mona, S., Desuky, E., Mona, Z., & Bakry, M. (2015). *Aeromonas veronii* biovar sobria a causative agent of mass mortalities in cultured Nile tilapia in El- Sharkia governorate, Egypt. *Life Science Journal*, *12*(5), 90-97.

Hamid, N. H., Hassan, M. D., Md-Sabri, M. Y., Hasliza, A. H., Hamdan, R. H., Afifah, M. N. F., … Fuad, M. M. (2016). *Studies on pathogenicity effect of* Aeromonas hydrophila *infection in juvenile red hybrid tilapia* Oreochromis *sp.* Retrieved July 03, 2019, from https://pdfs.semanticscholar.org/2072/a1218179c8b2b0797f8ace555df4bb2ef9e6.pdf

Hassan, M. A., Noureldin, E. A., Mahmoud, M. A., & Fita, N. A. (2017). Molecular identification and epizootiology of *Aeromonas veronii* infection among farmed *Oreochromis niloticus* in Eastern Province, KSA. *Egyptian Journal of Aquatic Research*, *43*(2), 161-167.

Ismail, M. S., Siti-Zahrah, A., Syafiq, M. R. M., Amal, M. N. A., Firdaus-Nawi, M., & Zamri-Saad, M. (2016). Feed-based vaccination regime against streptococcosis in red tilapia, *Oreochromis niloticus* × *Oreochromis mossambicus*. *BMC Veterinary Research*, *12*(1), 194.

Krumperman, P. H. (1985). Multiple antibiotic indexing of *E. coli* to identify high-risk sources of fecal contamination of foods. *Applied and Environmental Microbiology*, *46*(1), 165-170.

Rahmatullah, M., Ariff, M., Kahieshesfandiari, M., Daud, H. M., Zamri-Saad, M., Sabri, M. Y., … Ina-Salwany, M. Y. (2017). Isolation and pathogenicity of *Streptococcus iniae* in cultured red hybrid tilapia (*Oreochromis* sp.) in Malaysia. *Journal of Aquatic Animal Health*, *29*(4), 208-213.

Ramakrishnan, M. A. (2016). Determination of 50% endpoint titer using a simple formula. *World Journal of Virology*, *5*(2), 85.

Sreedharan, K., Philip, R., & Singh, I. S. (2011). Isolation and characterization of virulent *Aeromonas veronii* from ascetic fluid of oscar *Astronotus ocellatus* showing signs of infectious dropsy. *Diseases of Aquatic Organisms*, *94*(1), 29-39.

Yu, J. H., Han, J. J., Kim, H. J., Kang, S. G., & Park, S. W. (2010). First report of *Aeromonas veronii* infection in farmed Israeli carp *Cyprinus carpio* in Korea. *Journal of Fish Pathology*, *23*(2), 165-176.

Zhu, M., Wang, X. R., Li, J., Li, G. Y., Liu, Z. P., & Mo, Z. L. (2016). Identification and virulence properties of *Aeromonas veronii* bv. sobria isolates causing an ulcerative syndrome of loach *Misgurnus anguillicaudatus*. *Journal of Fish Diseases*, *39*(6), 777-781.

Pertanika J. Trop. Agric. Sc. 42 (4): 1273 - 1288 (2019)

PERTANIKA JOURNALS

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Journal homepage: http://www.pertanika.upm.edu.my/

Genetic Diversity of *Capsicum* L. Accessions from South West Nigeria using Simple Sequence Repeats (SSR) Markers

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ABSTRACT

Pepper (*Capsicum* L.) is a widely consumed vegetable in South West Nigeria because of its nutritional and medicinal potentials. This study is aimed at evaluating genetic diversity among 30 pepper accessions collected from different pepper-growing areas in South West Nigeria using SSR markers. Amplification potentials and bands clarity were considered for selecting 17 among 29 SSR markers screened. Genetic diversity was evaluated using principal coordinates analysis (PCoA), cluster analysis (CA), and analysis of molecular variance (AMOVA). The sum of 208 alleles was detected with an average value of 12.24 alleles per locus for each accession. Genetic diversity was high in all loci with the mean value ranging from 0.23 to 0.77. The result of AMOVA showed that 2 % of the genetic diversity was due to interspecific variations while 98 % of the differences were due to intraspecific variations among accessions. The results of cluster analysis showed clearly high genetic similarity coefficient at > 71 %. The intraspecific and interspecific genetic relationships observed could be an integral part of the useful tools for genetic improvement of the genus *Capsicum*

ARTICLE INFO

Article history:
Received: 9 April 2019
Accepted: 19 August 2019
Published: 13 November 2019

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ISSN: 1511-3701
e-ISSN: 2231-8542

through breeding purposes especially the wild varieties.

Keywords: *Capsicum*, genetic diversity, Nigeria, South West, SSR

INTRODUCTION

Capsicum (L.) pepper fruit is a vegetable with colour range from red, purple to yellow when mature (Oni, 2011). The pungency properties in pepper fruit, resulting from their high concentration of capsaicinoid make them important ingredients in people's diet all over the world (Germplasm Resources Information of Network [GRIN], 2009). This characteristic makes Nigeria pepper to be on high demand. Pepper production is an important agribusiness worldwide and one of the revenue sources in South West, Nigeria (Showemimo & Olanrawaju, 2000). Food and Agriculture Organization [FAO] (2010) reported that Nigeria produced 695,000 metric tons of pepper from a total area of 77,000 ha accounting for 50 % total production in Africa. Today, pepper has become widely exploited in tropical and temperate regions because of its nutritional contents, antioxidants properties and high health-protecting factors (Christine et al., 2014; Mady et al., 2005). Abdullahi et al. (2003) reported the potentials of pepper in African medicine as sore throat treatment.

Genetic diversity is a possible guarantee in reviving some of the economic plant species that near extinction. Research on genetic resources and plant breeding is one innovational activity most relevant for agriculture sustainability (Almeida et al., 2005). In response to the report of Almeida et al. (2005), the significant efforts with collection, characterization, and conservation could help to conserve pepper for various genetic breeding programmes. In Nigeria, most past research effort on pepper was focused mainly on food (Ado, 1999; Falusi & Morakinyo, 2001; Gill, 1992; Mady et al., 2005), and taxonomic classification using anatomical structures (Nwachukwu et al., 2007) with little or no effort on genetic improvement using SSR markers. However, an effort made by Falusi (2006) on genetic diversity of *Capsicum* in Nigeria using morphological markers was not comprehensive because he used few morphological characters. This scientific gap could be filled through proper diversity study of collected accessions at the molecular level to examine the level of genetic relatedness. Molecular markers will be much more appropriate in evaluating genetic relationships among the collected accessions. Previous workers had reported number of chromosomes of 2n=24 (x=12) for the genus *Capsicum* (Morakinyo & Falusi, 1992; Nwakiti, 1981; Stebbins, 1971). High chromosome number suggests higher productivity and wide genetic base for the genus *Capsicum* (National Research Council [NCR], 2006; Silva et al. 2011). Information on the genetic diversity in the population of *Capsicum* varieties in South West, Nigeria is scanty. Hence, there is a need to investigate the genetic relationships

among collected pepper accessions for breeding purposes and genetic improvement of the genus *Capsicum*.

Anu and Peter (2003) as well as Odeigah et al. (1999) had reported the use of biochemical and molecular markers to characterize some *Capsicum* accessions in Nigeria, but both of them used SDS-polyacrylamide gel electrophoresis of seed proteins markers. However, review of pieces of literature revealed no reports on the use of simple sequence repeats (SSR) makers in evaluating the genetic diversity populations of pepper in Nigeria. Some studies on *Capsicum* using SSR markers were reported in China, Russia, and Japan with the highest degree of genetic diversity recorded compared to other molecular markers (Chen et al., 2006; Luo et al., 2006; Zhou et al., 2009). They all reported SSR makers to be most suitable in evaluating genetic diversity because of its hypervariable allelic variations attribute.

Therefore, this study focused on the use of SSR to examine genetic diversity among 30 *Capsicum* accessions collected from different pepper growing areas within South West Nigeria. This is with a view to provide scientific information for genetic improvement of the genus through breeding purposes especially the wild *Capsicum* varieties.

MATERIALS AND METHODS

Capsicum Accessions and Areas of Collection

Thirty (30) accessions of *Capsicum* were collected from rural farmers within the South West, Nigeria and the gene bank of National Centre for Genetic Resources and Biotechnology (NACGRAB) in Nigeria between December 2014 and December 2015. The sources, dates and other collection data of the accessions studied, and a map of collection areas are detailed in Table 1 and Figure 1 respectively.

Sample Preparation

Young fresh leaves of studied accessions were plucked and freeze-dried for three days and thereafter stored at -20°C in Dr. Wang Lihao's laboratory at the Institute of Vegetables and Flowers (IVR), Chinese Academy of Agricultural Sciences (CAAS), Beijing, China.

DNA Extraction and Quantification

A modified mini-preparation CTAB protocol was employed for DNA extraction while DNA quantification was conducted using NanoDrop Spectrophotometer at 260 nm (Fulton et al., 1995).

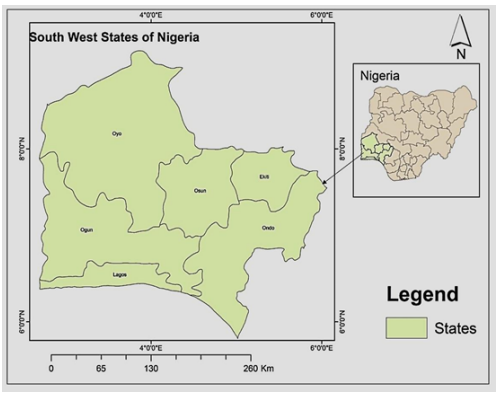

Figure 1. Map of South West Nigeria showing the study area and the vegetational zones. Scale in KM (Source: Agboola, 1979)

Table 1

Accessions number, local name, area of collection, states within South West, Nigeria and latitude and longitude where samples were collected

S/N	Accession Number	Local name	Taxonomic information	Area of collection	State	Latitude	Longitude
1	Og001	Rodo Hausa	*Capsicum chinense* Jacq.	Osiele	Ogun	7.19171	3.44524
2	Og002	Rodo Yoruba	-	Ago-Iwoye	Ogun	6.93456	3.89995
3	Og004	Rodo Hausa (yellow fruit)	*C. chinense* Jacq.	Odogbolu	Ogun	6.90826	3.66554
4	Og007	Rodo Hausa	*Capsicum annuum* L.	Ijebu-Ode	Ogun	6.82378	3.91793
5	Og010	Rodo Hausa	*C. annuum* L.+ *C. chinense* Jacq.	Iperu	Ogun	6.90826	3.66554
6	Oy018	Rodo Hausa	*C. chinense* Jacq.	Lanlate	Oyo	7.67845	3.44516
7	Ek021	Tiny rodo	*C. chinense* Jacq.	Ikole-Ekiti	Ekiti	7.78366	5.52441
8	La026	Shombo	*C. annuum* L.	Badagry	Lagos	6.43766	2.87833
9	Oy032	Rodo Yoruba	*C. chinense* Jacq.	Ijio	Oyo	7.93333	2.96670
10	On029	Green pepper tatashe	*C. annuum* L.	Igbokoda	Ondo	6.34956	4.80245
11	Oy031	Tatashe	*C. annuum* L.	Iganna	Oyo	7.97608	3.24667
12	Og006	Ijosi (original)	*C. chinense* Jacq.	Ayetoro	Ogun	7.24227	3.02362
13	Og009	Ijosi	*Capsicum frutescens* L.	Orile-Ilugun	Ogun	7.36658	3.66848
14	Oy030	Ijosi	*C. frutescens* L.	Gaa Fulani	Oyo	7.85613	3.90959
15	La011	Round shape (Ijosi)	*C. frutescens* L.	Epe	Lagos	6.58433	3.97733
16	Os016	Atawere funfun	*C. frutescens* L.	Yakoyo	Osun	7.49613	4.44109
17	On028	Atawere funfun	*C. frutescens* L.	Ondo town	Ondo	7.11111	4.85427
18	Os013	Small bawa	*C. annuum* L.	Bode-osi	Osun	7.63312	4.21323
19	On019	Big bawa	*C. annuum* L.	Owena Alade	Ondo	7.19441	5.01983

Table 1 *(Continued)*

S/N	Accession Number	Local name	Taxonomic information	Area of collection	State	Latitude	Longitude
20	Ek024	Long Bawa	*C. annuum* L.	Iye-Ekiti	Ekiti	7.98655	5.22046
21	On027	Medium size Bawa	*C. annuum* L.	Oka-Akoko	Ondo	7.46214	5.83462
22	Os033	Bawa	*C. annuum* L.	Ita osa	Osun	7.43701	4.56132
23	Oy034	Atawere	*C. chinense* Jacq.	Ipapo	Oyo	8.13008	3.50983
24	Og003	Long shombo	*C. annuum* L.	Iyana –Agbede	Ogun	Unknown	Unknown
25	NGB01010	Unknown	*C. chinense* Jacq.	NACGRAB	Unknown	Unknown	Unknown
26	NGB01066	Unknown	*C. chinense* Jacq.	NACGRAB	Unknown	Unknown	Unknown
27	NGB01240	Unknown	*C. chinense* Jacq.	NACGRAB	Unknown	Unknown	Unknown
28	NGB01017	Unknown	-	NACGRAB	Unknown	Unknown	Unknown
29	NGB01022	Unknown	*C. chinense* Jacq.	NACGRAB	Unknown	Unknown	Unknown
30	NGB01282	Unknown	-	NACGRAB	Unknown	Unknown	Unknown

Source of SSR Primers

Twenty-nine (29) SSR polymorphic microsatellite markers specific to *Capsicum* publicly available from Nicolai et al. (2013) were adopted and used for this study.

PCR Amplification Reaction

This was done using 10 µL volumes with 2 µL of 25 ng/ µL genomic DNA as a template, 5 µL of 2 × GoTaq® Green Master Mix polymerase, 0.25 µL each of both primers, and 2.5 µL of sterilized ddH_2O. The amplification reaction was performed with initial denaturation at 94 °C for 2 mins, 35 cycles of 94 °C for 3 secs, 55.0 °C for 20 secs, 72 °C for 30 secs and 72 °C for 7 mins (Sun et al., 1993).

SSR PAGE Analysis

The products were evaluated on 6 % (w/v) polyacrylamide gel electrophoresis (PAGE) for 1.5 hr in 1 X Tris/borate/EDTA buffer with 7.5 M urea at constant voltage using the manufacturer's protocol. The gels were washed in water and stained with 2 g/ mL of silver nitrate ($AgNO_3$). The size of individual DNA band was determined using DNA ladder. Bands were developed by dissolving 15 g of NaOH in 1 L of distilled H_2O and added 3 ml of formaldehyde. The gels were allowed to dry before spreading the gels on trans-illuminator and gel images were taken using a digital camera.

Statistical Analysis

After scoring of all SSR fragments, gene diversity was determined using PowerMarker software program (Liu & Muse, 2005). Shannon information index was used to determined polymorphic information content and the number of alleles (Shannon & Weaver, 1949). Popgene software version 1.31 (Yeh et al., 1999) was used to analyze genetic similarity, genetic distance, allele number, number of alleles with a frequency of greater than 5 % and less than 50 %. GenAlEx 6.501 software was used to estimate analysis of molecular variance (AMOVA), mean diversity, expected and unbiased heterozygosity (Peakall & Smouse, 2006).

Principal coordinates analysis (PCoA) was constructed using Minitab software. NTSYSpc v. 2.20 software was used to determine a genetic similarity between accessions while Jaccard's similarity coefficient of accessions was employed to construct UPGMA dendrograms (Rohlf, 2005).

RESULTS

SSR Primers Genetic Information/ Polymorphisms

Twenty-nine (29) primers were used in determining the genetic diversity between 30 *Capsicum* accessions studied. Twelve (12) SSR primers did not clearly exhibit polymorphism, thus, they were not included in the analysis. Table 2 shows the SSR primers sequences used.

Genetic Parameters Estimates of the SSR Primers

The primers generated polymorphic bands, with size ranging from 50bp to 350bp. Two hundred and eight (208) alleles were

Table 2

The sequences of the primers used for this study

Primer / Locus name	Forward Sequence (5'– 3') Reverse (5'– 3')
Epms-350	TGGGAAGAGAAATTGTGAAAGC AGGAAACATGGTTCAATGCC
Hpms1-214	AAGCTTATCCCTTTCAAATATAA ATATCTCACGTATTGCGGATTCTT
HpmsCaSIG19	TGGCCAGCTTCACACAGAGGTA TGTCACAATATTGGAGGCCAGAA
Hpms1-5	CCAAACGAACCGATGAACACTC GACAATGTTGAAAAAGGTGGAAGAC
Epms-725	CGCTCGCTACCCTTTCATTA AATTTCGGAAGGGCAAAGAT
Gpms-169	TCGAACAAATGGGTCATGTG GATGAGGGTCCTGTGCTACC
Gpms-100	TCCATACGGTTGGAGGAGAG ACTATGCTCTGCTGTGCCCT
HpmsE064	CCCTCCTTTTACCTCGTCAAAAA ATGCCAAGGAGCAATGAGAACC
Gpms-104	GCAGAGAAAATAAAATTCTCGG CAATGGAAATTTCATCGACG
HpmsE013	GCGCCAAGTGAGTTGAATTGAT CACCAATCCGCTTGCTGTTGTA
Gpms-29	CAGGCAATACGGAGCATC TGTGTTGCTTCTTGGACGAC
HpmsE008	CCCCTTAACTTTTAATTCTAGATCTGC TCGTTGTTCCTCCATCACC TCA
HpmsAT2	TGGATCCCAAAAGACTCAGAACA TATTTCCCTCAGTCGAGGTCGT
Gpms-101	CCTATCACCCTCTTTGAGCC TAAAGACCAGCCCTGGATGA
Epms-391	TTTCTTCTCTGGCCCTTTTG ACGCCTATTGCGAATTTCAG
Hpms2-24	TCGTATTGGCTTGTGATTTACCG TTGAATCGAATACCCGCAGGAG
Epms-397	GCACCCTCCCAATACAAATC GATCACGGAGAAAGCAAAGG

Source: Nicolai et al. (2013).

recorded in all 30 accessions with the mean value of 12.24 alleles per locus (Table 3).

The allele number per locus was 6 in loci H1-214 and E-725, while locus HE013 had 18. Minor allele frequencies ranged from 0.13 in locus E-350 and locus G-101 to 0.87 for locus HE008, with an average of 0.37. There was high gene diversity in all loci except HE008 (0.231). Gene diversity ranged from 0.23 in HE008 to 0.94 in G-101 with an average of 0.77. The polymorphic information content (PIC) ranged from 0.20 in HE008 to 0.93 in G-101 with a mean value of 0.75 (Table 3). The most informative markers were primers E-350, H1-214, HCaSIG19, G-169, G-100, G-104, HE013, G-29, G-101, E-391 and E-397 with PIC values of 0.91, 0.77, 0.71, 0.92, 0.91, 0.84, 0.82, 0.79, 0.93 and 0.92 respectively (Table 3).

The Allelic Pattern Across Sub-groups among Accessions

This was performed to determine allele's variability among the accessions according

Table 3

Summary of genetic parameters estimates of the SSR markers

Locus	MAF	NA	NPB	GD	PIC
Epms-350	0.13	14	7	0.92	0.91
Hpms1-214	0.37	6	3	0.79	0.77
HpmsCaSIG19	0.50	12	6	0.72	0.71
Hpms1-5	0.63	16	8	0.58	0.56
Epms-725	0.40	6	3	0.72	0.68
Gpms-169	0.17	16	8	0.92	0.92
Gpms-100	0.17	14	7	0.91	0.91
HpmsE064	0.53	12	6	0.65	0.62
Gpms-104	0.27	14	7	0.86	0.84
HpmsE013	0.37	18	9	0.83	0.82
Gpms-29	0.30	8	4	0.81	0.79
HpmsE008	0.87	8	4	0.23	0.20
HpmsAT2	0.57	8	4	0.65	0.63
Gpms-101	0.13	16	8	0.94	0.93
Epms-391	0.17	16	8	0.92	0.92
Hpms2-24	0.53	10	5	0.64	0.60
Epms-397	0.17	14	7	0.92	0.91
Total	6.27	208	104	13.04	12.73
Mean	0.37	12.24	6.12	0.77	0.75

Keys. MAF = Minor allele frequency; NA = Number of alleles per locus; NPB = Number of polymorphic bands; GD = Gene diversity; PIC = Polymorphic information content

to their sources. The numbers of different alleles (Na) ranged from 1.15 in Lagos collection to 1.79 in NACGRAB and Ogun subgroups (Table 3). The analysis showed that NACGRAB and Ogun accessions produced a higher number of different alleles (Na = 1.79), and effective alleles (Ne = 1.57 and 1.54) respectively (Table 4 and Figure 2).

Lagos had the lowest Shannon information index (I) value of 0.11 and the highest value of 0.48 was recorded for NACGRAB. Result showed that allelic pattern according to the source of accessions at ≤ 50 % was one (1) in NACGRAB, Ogun, and Oyo respectively. The diversity (H) was low across the board, with values ranging from 0.08 in Lagos to 0.33 in NACGRAB. The unbiased diversity (uh) values ranged from 0.15 in Lagos to 0.39 in NACGRAB. This shows that intraspecific diversity is low among the sub-groups. The percentage of polymorphic loci ranged from 15.38 % in Lagos to 80.77 % in NACGRAB (Table 4 and Figure 2).

Table 4

Alleles/Bands pattern according to the source of accessions within groups

Population	Ekiti	Lagos	NAGRAB	Ogun	Ondo	Osun	Oyo
No. of accessions per population	2	2	6	8	4	3	5
Na	1.20	1.15	1.79	1.79	1.50	1.45	1.63
No. alleles	97	90	102	104	100	96	104
No. alleles (≥ 5 %)	97	90	102	104	100	96	104
Ne	1.14	1.02	1.57	1.54	1.39	1.42	1.42
I	0.14	0.11	0.48	0.46	0.32	0.34	0.36
No. LComm alleles (≤ 50 %)	0	0	1	1	0	0	1
H	0.10	0.08	0.33	0.31	0.22	0.24	0.24
Uh	0.20	0.15	0.39	0.35	0.30	0.35	0.30
% of polymorphic loci	20.19 %	15.38 %	80.77 %	79.81 %	53.85 %	52.88 %	62.50 %

Keys. Na = Number of different alleles; No. alleles = Number of private alleles; Number alleles frequency > 5 % = Number of different alleles with a frequency > 5 %; Ne = Effective alleles; I = Shannon's information index; No. LComm alleles (< 50 %) = Number of locally common alleles (frequency > 5 %) found in 50 %; H = Diversity; Uh = Unbiased diversity

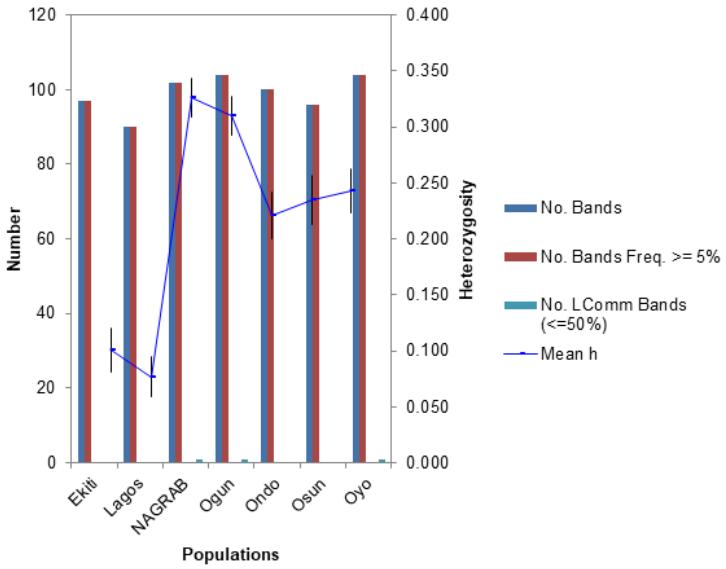

Figure 2. Allelic patterns across the seven populations

Keys. No. Bands = Number of different alleles; No. Bands Freq. > 5 % = Number of different alleles with a frequency > 5 %; No. LComm Bands (< 50 %) = Number of locally common alleles; Mean h = Mean diversity

Percentages of Molecular Variance of Accessions (AMOVA)

Table 5 shows that 2 % of the genetic diversity was due to interspecific variations while 98 % of the differences were due to intraspecific variations among accessions. This indicates higher intraspecific diversity within the accessions and less interspecific diversity among the accessions.

Principal Coordinate Analysis of Accessions of *Capsicum* based on Areas of Collection

Principal coordinate analysis of 30 *Capsicum* accessions was constructed using the SSR data matrix. Three major cluster groups were generated from the scattered plot of the PCoA from the 30 accessions. Cluster group A comprised a total of 10 accessions from (Ogun, Lagos, Osun, Ondo, Oyo, and NACGRAB) subgroups while cluster group B composed of 18 accessions from all subgroups. Cluster group C comprised 2 accessions from NACGRAB (Figure 3).

Cluster Analysis of Accessions of *Capsicum*

A dendrogram was constructed from the raw data of the seventeen (17) SSR markers. The similarity coefficient (SC) delineated the 30 accessions into two (2) main clusters A and B at SC = 0.64. However, at a similarity coefficient level of 0.88 all accessions are separated (Figure 4).

However, at similarity coefficient (SC) of 0.71, cluster group A was also segregated into six sub-cluster groups A1, A2, A3, A4, A5, and A6. The sub-cluster A1 consisted of 2 accessions (NGB01066

and NGB01012) while A2 had an isolated accession of (Og001). The sub-cluster A3 had 2 accessions (Og002 and On027) while A4 consisted of 10 accessions (Og003, Ek024, Os013, On019, Oy031, On29, Oy018, Os033, Ek021, and Og007). The sub-cluster A5 had 2 accessions (Og004 and Oy032) while A6 consisted of 3 accessions (NGB01010, NGB01017, and NGB01240). Cluster group B was further segregated into 2 sub-cluster groups B1 and B2. The sub-cluster B1 contained 7 accessions (Og006, La011, La026, NGB01282, Oy034, On028, and Os016) while sub-cluster group B2 contained 3 accessions (Og009, Oy030, and Og010) (Figure 4). In all 30 accessions investigated, eight sub-cluster groups were identified.

Table 5

AMOVA among and within accessions variations

Source	Df	SS	MS	Est. Var.	TV %	*p*-value*
Among Accns	6	114.233	19.039	0.433	2 %	< 0.001
Within Accns	23	396.867	17.255	17.255	98 %	< 0.001
Total	29	511.100		17.688	100 %	

Keys. Accns = Accessions; Df = Degree of freedom; SS = Sum of square; MS = Mean square; EV = Estimated variation; TV = Total variation; * = After 999 random permutations

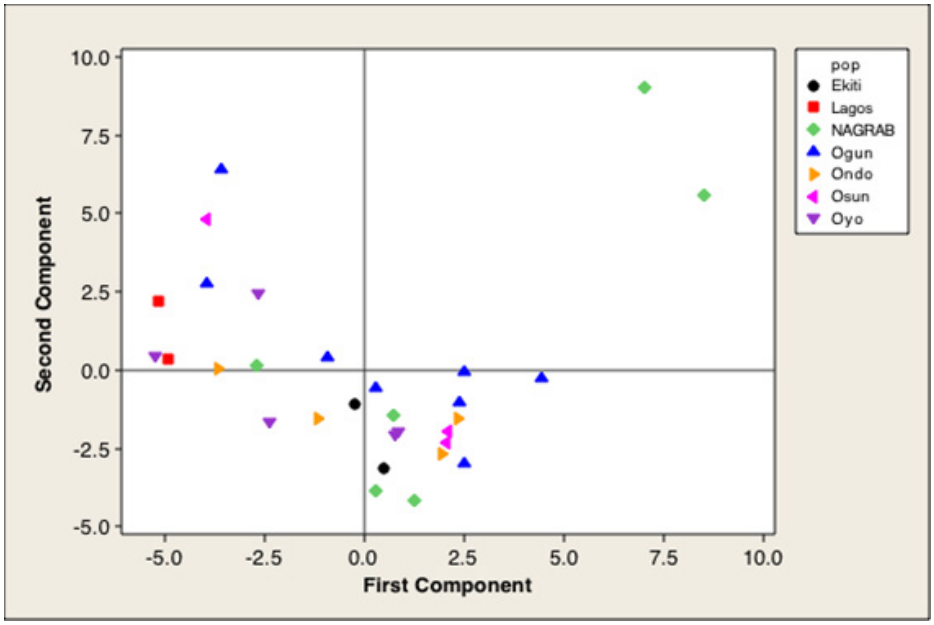

Figure 3. Scatter plot of 30 *Capsicum* accessions based on first and second components of principal coordinate analysis using SSR data on areas of collection

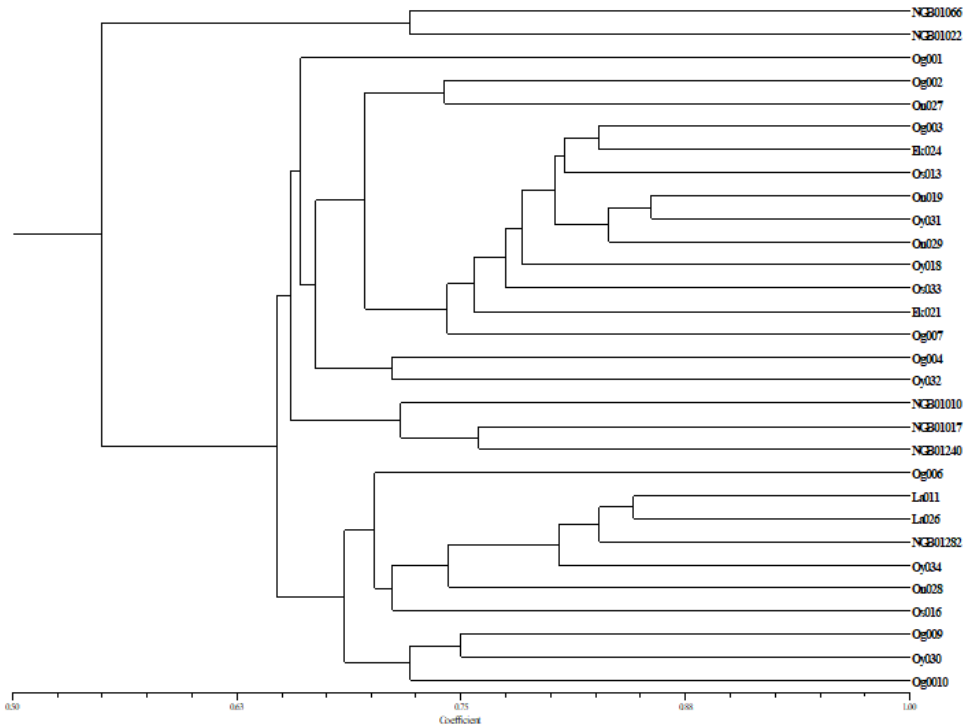

Figure 4. Dendrogram generated from SSR markers used for the 30 accessions of *Capsicum* species

Keys. A and B = Major cluster groups; A1 to A6 = Sub cluster groups of A; B1 and B2 = Sub cluster of B

DISCUSSION

Seventeen SSR markers employed in this study were very effective. This is because they segregated the accessions into varieties; based on genetic similarities. These markers effectively distinguished the accessions.

The mean observed Na per locus (12.24) detected was similar to 13.79 alleles/SSR primer reported by Zhang et al. (2016). Alleles ranges per primer (6 to 18) recorded corroborated the work of Zhang et al. (2016). They reported range of 6 to 29. However, numbers of alleles per locus recorded in three markers E725, HE013 and HE008 with (6, 8 and 10 respectively) were very close to the reported values of alleles per locus (7, 6 and 11) for exact markers by Zhang et al. (2016). This finding agreed to the reports of several authors on the varying range of allele's number per locus (Zhang et al. 2010;2016).

The mean of PIC obtained is close to the range of the previous studies by Zhang et al., (2010, 2016). Zhang et al. (2016) reported a 60 % PIC value on *Capsicum* germplasm from China, United States, Brazil, Bulgaria and Japan. The high level of polymorphisms obtained for these *Capsicum* accessions could be traced to their cultivation in pepper growing regions in South West, Nigeria. The average mean values of genetic diversity (0.77) recorded agreed to the findings of Zhang et al. (2016). These findings

suggest the effectiveness of SSR markers in the genetic intraspecific and interspecific diversity of pepper.

From this study accessions collected from the same location were grouped together, with those belonging to the same species showing closer relationships at the molecular level. This study also showed higher intraspecific diversity within the accessions and less interspecific diversity among the *Capsicum* accessions. These findings corroborate the reports of Nikolai et al. (2013). These authors worked on 46 accessions of *Capsicum* and reported high genetic diversity similar to the values observed in this study among accessions of *C. annuum*.

Furthermore, high values recorded for all the measured genetic diversity suggest allelic richness among the accessions collected from South West, Nigeria. This could be relied on in evaluating diversity for genetic improvement of pepper. However, the negligible number of private bands recorded in growing areas showed the genetic similarities of accessions and that these bands insignificantly contributed to the overall diversity of the accessions studied.

The mean allelic pattern divided all accessions into sub-groups based on the collections areas (Ekiti, Lagos, NACGRAB, Ogun, Ondo, Osun, and Oyo) with narrow or no diversity. Five out of the seven areas of collection displayed similarity in effective alleles (Ne) and private alleles (unique) while Ekiti and Lagos areas of the collection displayed lower values and unique alleles. This trend also reflected in the percentage polymorphic loci indicating high allelic diversity among accessions except for Ekiti and Lagos accessions.

The allelic pattern plot across accessions showed a gradual increase in allelic richness from Lagos to NACGRAB accessions down to Ogun, though with no sharp demarcation among Ondo, Osun and Oyo accessions. The gradual increase in allelic richness suggests strong correlation among the accessions due to similarity of alleles and exchange of a number of alleles at a particular locus. However, Ekiti accessions were isolated. This suggests weak connectivity between Ekiti accessions and other accessions and this may be due to differences in alleles and no exchange of a number of alleles. The results on genetically homogenous nature of the accessions as a result of alleles exchange agreed with the report of Balloux and Lugon-Moulin (2002) as well as NRC (2006). They reported that genetic structures reflecting the allele number exchanged between populations.

With respect to the allelic patterns again, there is a slight increase in connectivity from Lagos accessions to other areas, while there is a slight reduction from NACGRAB to other areas. However, there is a stable connection from Ondo via Osun en route Oyo accessions. This allelic connectivity finding is interesting and suggests a genetic link among accessions irrespective of an area of the collection in South West, Nigeria. This study provides additional genetic information between landraces and exotic hybridized pepper species in South West, Nigeria.

There was a very high level of diversity within accessions (98 %) and relatively low diversity among accessions (2 %). The natural interbreeding is perhaps responsible for the higher diversity within accessions compared to less genetic differences among accessions while low genetic diversity indicates low gene flow/genetic differentiation among accessions. This result corroborated the earlier findings on consistency of high genetic diversity within populations than between populations (Ganesan et al., 2014; Yang et al., 2016) on genetic diversity among *M. oleifera* and woody species respectively.

The results of CA and PCoA revealed a high degree of similarity among accessions particularly at > 71 % genetic similarity level and segregated 30 accessions into eight groups. The widely distribution of *Capsicum* accessions particularly in cluster groups A and B showed their adaptability to different areas in South West, Nigeria.

The findings in this study have enriched the understanding of the level of genetic relationships among populations of pepper in South West, Nigeria for breeding programs, thus, some wild varieties in South West, Nigeria that are underexploited but with valuable agronomic characters could be genetically improved upon for utilization as food, medicine, and also for other product development.

CONCLUSION

The intraspecific and interspecific genetic relationships observed could be an integral part of the useful tools for genetic improvement of the genus *Capsicum* through breeding purposes especially the wild varieties.

ACKNOWLEDGEMENTS

Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing, People's Republic of China is well appreciated. Nigeria Tertiary Education Trust Fund (TETFund) of the Federal Republic of Nigeria is well acknowledged. The Germplasm Unit of the National Centre for Genetic Resources and Biotechnology (NACGRAB), Ibadan, Oyo State, Nigeria is well appreciated for the supply of six accessions out of 30 pepper accessions used for this study.

REFERENCES

Abdullahi, M., Muhammad, G., & Abdulkadir, N. U. (2003). *Medicinal and economic plants of Nupe land*. Bida, Nigeria: Jube-Evans.

Ado, S. G. (1999). *Potentials of native and exotic pepper germplasm in Nigeria*: *An exploitable resource in the next millennium*. Lafia, Nigeria: Genetic Society of Nigeria Publishing.

Agboola, S. A. (1979). *An agricultural atlas of Nigeria* (1st ed.). Oxford, United Kingdom: Oxford University Press.

Almedia, C. M. C. V., Dias, L., Okabe, E. T., & Medeiros, J. R. P. (2005). Variability in genetic resources of cacao in Rondonia, Brazil. *Crop Breeding and Applied Biotechnology*, *5*(1), 318-324.

Anu, A., & Peter, K. V. (2003). Analysis of seed protein of 29 lines of *Capsicum annuum* L. by polyacrylamide gel electrophoresis. *Genetic Resources and Crop Evolution*, *50*(1), 239-243.

Balloux, F., & Lugon-Moulin, N. (2002). The estimation of population differentiation with microsatellite markers. *Molecular Ecology*, *11*(1), 155-165.

Chen, X. J., Chen, J. F., Di, H. & Lou, Q. F. (2006). RAPD diversity analysis of 5 domesticated species. *Acta Horticulturae Sinica*, *33*(1), 751–756.

Christine, E., Peters, H., & Orim, A. O. (2014). Comparative evaluation of the nutritional, phytochemical and microbiological quality of three pepper varieties. *Journal of Food and Nutrition Sciences*, *2*(3), 74-80.

Falusi, O. A. (2006). Interchromosomal connections and metaphase 1 clumping in meiosis of two *Capsicum* Linn. species in Nigeria. *African Journal of Biotechnology*, *5*(22), 2066-2068.

Falusi, O. A., & Morakinyo, J. A. (2001). Pollen and hybridization studies in some Nigerian species of peppers. *Nigerian Journal of Technology*, *1*(2), 40-43.

Food and Agriculture Organization. (2010). *Food composition table for use in Africa, FAO and US Department of Health, Education and Welfare*. Rome, Italy: FAO.

Fulton, T. M., Chunwongse, J., & Tanksley, S. D. (1995). Microprep protocol for extraction of DNA from tomato and other herbaceous plants. *Plant Molecular Biology Reporter*, *13*(1), 207–209.

Ganesan, S. K., Singh, R., Roy-Choudhury, D., Bharadwaj, J., Gupta, V., & Singode, A. (2014). Genetic diversity and population structure study of drumstick (*Moringa oleifera* Lam.) using morphological and SSR markers. *Industrial Crop and Product*, *60*(1), 316–325.

Germplasm Resources Information of Network. (2009). Capsicum *L. germplasm*. Retrieved November 18, 2018, from http://www.ars.grin.gov.cgi

Gill, L. S. (1992). *Ethno medicinal uses of plants in Nigeria*. Benin City, Nigeria: UNIBEN Publishing.

Liu, K., & Muse, S. V. (2005). Power marker: Integrated analysis environment for genetic marker data. *Bioinformatics*, *21*(1), 2128-2129.

Luo, Y. D., Li, J. G., & Li, M. F. (2006). Analysis of genetic diversity of *Capsicum* germplasm resources by using SSR markers. *Biotechnology Bulletin*, *51*(1), 337–341.

Mady, E. A., Uguru, M. I., & Ugwoke, K. I. (2005). Interrelations of growth and disease expression in pepper using principal component analysis. In *Proceeding of 30th Annual National Conference of Genetic Society of Nigeria* (pp. 1-5). Nsukka, Nigeria: Genetic Society of Nigeria Publishing.

Morakinyo, J. A., & Falusi, O. A. (1992). Chromosome behaviour in *Capsicum annuum* and *C. frutescens* and their intra and inter specific hybrids. *Nigerian Journal of Botany*, *14*(1), 135-143.

National Research Council. (2006). *Lost crops of Africa: Volume II: Vegetables, development, security and cooperation*. Washington, USA: NRC.

Nicolai, M., Cantet, M., Lefebvre, V., Sage-Palloix, A. M., & Palloix, A. (2013). Genotyping a large collection of pepper (*Capsicum* spp.) with SSR loci brings new evidence for the wild origin of cultivated *C. annuum* and the structuring of genetic diversity by human selection of cultivar types. *Genetic Resources and Crop Evolution*, *60*(1), 2375-2390.

Nwachukwu, C. U., Mbagwu, F. N., & Onyeji, A. N. (2007). Morphological and leaf epidermal features of *Capsicum annuum* and *Capsicum frutescens* Solanaceae. *Nature and Science*, *5*(3), 54-60.

Nwakiti, O. C. (1981). Sterility intraspecific hybrids of *Capsicum*. *Indian Journal of Genetics*, *41*(1), 200-204.

Odeigah, P. G., Oboh, B. O., & Aghalokpe, I. O. (1999). The characterization of Nigerian varieties of pepper, *Capsicum annuum* and *Capsicum frutescens* by SDS-polyacrylamide gel electrophoresis of seed proteins. *Genetic Resources and Crop Evolution*, *46*(1), 127-131.

Oni, M. O. (2011). Evaluation of seed and fruit powders of *Capsicum annuum* and *Capsicum frutescens* for control of *Callosobruchus maculates* (F.) *International Journal of Biology*, *3*(2), 185-188.

Peakall, R., & Smouse, P. E. (2006). GENALEX 6: Genetic analysis in excel: Population genetic software for teaching and research. *Molecular Ecology*, *6*(1) 288–295.

Rohlf, F. J. (2005). *Numerical taxonomy and multivariate analysis system*, v*ersion 2.2. Exeter software*. New York, NY: Applied Biostatistics Publishing.

Shannon, C. E., & Weaver, W. (1949). *The mathematical theory of communication*. Chicago, USA: Urbana Press.

Showemimo, F. A., & Olanrewaju, J. O. (2000). Yield performance heritability and interrelations in some quantitative traits of "Tatase" pepper (*Capsicum annum* L.). *Journal of Horticultural Science, 6(1)*, 25-30.

Silva, N., Mendes-Bonato, A. B., Sales, J. G. C., & Pagliarini, M. S. (2011). Meiotic behavior and pollen viability in *Moringa oleifera* (Moringaceae) cultivated in Southern Brazil. *Genetics and Molecular Research*, *10*(3), 1728-1732.

Stebbins, G. L. (1971). *Processes of organic evolution* (2th ed.). Upper Saddle River, USA: Pearson Prentice-Hall Press.

Sun, Y., Hegamyer, G., & Colburn, N. (1993). PCR-direct sequencing of a GC-rich region by inclusion of 10 % DMSO: Application to mouse c-jun. *Biotechniques*, *15*(3), 372-374.

Yang, H., Li, X., Liu, D., Chen, X., Li, F., Qi, X., … Wang, C. (2016). Genetic diversity and population structure of the endangered medicinal plant *Phellodendron amurense* in China revealed by SSR markers. *Biochemical Systematics and Ecology*, *66*(1), 286-292.

Yeh, F. C., Boyle, R., Yang, R. C., Ye, Z., Mao, J. X., & Yeh, D. (1999). *The user-friendly freeware for population genetic analysis version 1.31*. Edmonton, Canada: University of Alberta Press.

Zhang, B. X., Wang, L. H., Mao, S. L., & Zhang, Z. H. (2010). Research progress on pepper breeding and genetic during China's eleventh five-year plan. *China Vegetables*, *24*(1), 1-9.

Zhang. X., Zhang, Z., Gu, X., Mao, S., Li, X., Joel, C., … Zhang, B. (2016). Genetic diversity of pepper (*Capsicum* spp.) germplasm resources in China reflects selection for cultivar types and spatial distribution. *Journal of Integrative Agriculture*, *15*(9), 1991-2001.

Zhou, J., Shen, H. L., Yang, W. C., Tan, F., Wang, Y. L., & Guo. S. (2009). Analysis of genetic diversity of *capsicum* germplasm by using SSR markers. *Acta Agriculturae Boreali-Sinica*, *24*(1), 62-67.

Pertanika J. Trop. Agric. Sc. 42 (4): 1289 - 1294 (2019)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Short communication

Optimal Preservation and Storage Regimes of Total RNAs from Different Fish Tissues

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ABSTRACT

Standardized and optimized methods in preservation and storage of RNA samples on three different tissues were elucidated on fish tissues. The treatments used included excision of fresh tissues, preservation of tissues by immersing completely in RNA*later* followed by immediate freezing at -80°C prior to RNA assays. Current data suggests that preserving fish tissues in RNA*later* with subsequent storage at -80°C produced satisfactory amount of RNA from dorsal fin and gonad, but not from muscle tissues. The optimized method will provide an alternative storage method option and enables greater usage of RNA*later* for preserving RNA from animal cells.

Keywords: Fish tissues, preservation, RNA, *RNAlater*, storage

INTRODUCTION

The advent of molecular techniques has increased the number of researches using DNA and RNA in bulk. The RNA, which is closely related to protein synthesis, is widely used in studies related to genomics and proteomics. RNA can be assayed from different parts of any organism either through conventional method or by using readily available kits (Deng et al., 2005; Junttila et al., 2009). The RNA extraction kits, for instance are usually optimized to enhance the quality and amount of RNA being assayed. High quality of RNA is essential and is a prerequisite for many downstream applications such as microarray analysis (Copois et al., 2007), gene expression (Shabihkhani et al., 2014) and other nucleic acid-based technologies.

ARTICLE INFO

Article history:
Received: 06 June 2019
Accepted: 01 August 2019
Published: 13 November 2019

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ISSN: 1511-3701
e-ISSN: 2231-8542

It is well known that RNases, which is ubiquitous, are very aggressive and can rapidly digest significant amounts of RNA (Escobar & Hunt, 2017). Therefore, sample preparation, preservation and storage free from RNases are critical for determining the quality of RNA. Traditional method to deactivate the activity of RNases is usually through deep freezing of samples with liquid nitrogen. However, fast snap-freezing facilities are often not available in certain sampling locations which are remote (Gorokhova, 2005). Moreover, freezing sampling materials with liquid nitrogen are very often do not deactivate RNases activity efficiently, resulting RNA fragmentation (Shabihkhani et al., 2014). An alternative method will be the usage of RNA*later* during RNA assay.

RNA*later* is a storage reagent that stabilizes and protects RNA by rapidly permeating into the tissues and stabilizing cellular RNA. Manufacturer's recommendation on the usage on animal tissues preservation with RNA*later* in general are not plausible. According to manufacturer's instruction, RNA*later* is not suitable for stabilization of RNA in animal tissues, whole blood, plasma, serum and tissues with high abundance of fat (QIAGEN, 2006). Despite this, the effectiveness of stabilizing effect in animal tissues should be tested to gain the optimum preservation and storage regimes for RNA, especially for samples which are difficult to obtained or rare in nature. Previous work demonstrated that preserving fresh microcrustaceans (*Artemia*) samples with RNA*later* with subsequent storage at 5°C and room temperature (19 to 22°C) were effective to maintain a huge amount of RNA in crustacean for at least eight months and one month after preservation respectively. Moreover, this study also showed no significant RNA degradations were detected when samples were deep freezed immediately into a -80°C freezer at any time of the experiment (Gorokhova, 2005). Besides that, study had shown that high RNA quality could be achieved by optimizing procurement and storage methods specifically for each cell type (Shabihkhani et al., 2014).

Thus, the objective of this study was to evaluate the applicability of optimal regime for preserving RNA from different animal tissues (using an ornamental fish) by applying optimal preservation (soak in RNA*later*) and storage condition (freeze at -80°C) .

MATERIALS AND METHODS

Sample Preparation and Preservation

Fish samples from subgenus *Poecilia* were collected from local breeding aquaria. The fish samples collected comprised of six sexually matured males and six sexually matured females (2.5 ± 1.23 cm, standard length). The fishes were euthanized in crushed ice for 10 minutes with subsequent tissues excision from dorsal fin, muscle and gonad from each sample. Approximately 10 mg of each tissue were dissected, sliced thinly and immediately soaked completely in 100 µl of RNA*later*. The RNA*later* volume used was in accordance to the

ratio suggested by the manufacturer (10 µl reagent per 1 mg tissues) (QIAGEN, 2006). A total of 36 samples were prepared for RNA assay (12 replicates per tissue type).

Storage

Preserved tissues samples were stored immediately into a -80°C freezer. The storage regimes applied were in accordance to the optimized storage condition as described by Gorokhova (2005), which exhibited high amount of RNA yield even after eight months of storage.

Extraction Procedure

Prior to RNA assay, samples were left to thaw at room temperature and subsequent transfer to a new 1.5 ml appendorf tube. Excess RNA*later* solution was dried from the samples by using paper tissues. Next, approximately 350 µl of lysis buffer were immediately added in each tube. Subsequent extraction procedures were in accordance with RNeasy Mini kit (cat. #74104, QIAGEN, Germany) handbook with minor modifications on lysate homogenization. In detail, homogenization of tissues in lysis buffer (RLT buffer) was carried out by passing the lysate through a blunt 20-gauge needle (0.9 mm diameter) for at least 20 times, until a murky solution was formed. Murky solution is an indication of well mixed lysate. Purified RNA was quantified immediately prior to freeze storage at -80°C.

Nucleic Acid (RNA) Quantification

Quantification of purified RNA was performed by using Qiagen's QIAexpert. Spectrophotometric absorbance measurements performed were: 230, 260 and 280 nm. Each purified RNA sample was loaded into 16 wells QIAexpert slide and scanned for measurements.

Statistics

Data were presented as comparisons between RNA amount in ng (mean ± SD) and ratio of $A_{260}/A_{280,}$ and between RNA amount in ng (mean ± SD) and ratio of A_{260}/A_{230} among different tissues. One-Way Independent ANOVA was performed using IBM SPSS Statistics 24 analysis software to investigate significant differences of obtained data and significance was accepted when $P < 0.05$.

RESULTS AND DISCUSSION

Protein contamination was generally low as indicated by A_{260}/A_{280} ratio, which is greater than 2.0 in all 36 purified RNA samples (Table 1). The A_{260}/A_{280} ratio of 2.0 and above indicates high purity of RNA in the eluent (Escobar & Hunt, 2017; QIAGEN, 2011). However, the A_{260}/A_{230} ratios, which were less than 1.7, indicates carry over of salts and other contaminants during purifications (Escobar & Hunt, 2017; QIAGEN, 2011) (Table 1). Possible examples of contaminants could be urea, EDTA, carbohydrates and phenolate ions, which were the ingredients commonly used in extraction buffers. According to the manufacturer's handbook, presence of contaminants could be due to mishandling of spin column, which accidentally contacted the flow through after centrifugation (QIAGEN, 2012).

Table 1
Amount of RNA in ng (mean ± SD), ratios of A_{260}/A_{280} and A_{260}/A_{230} absorbance among different tissues

Tissues	*n*	mean ± SD		
		Amount of RNA/ng	A_{260} / A_{280}	A_{260} / A_{230}
Dorsal fin	12	119.65^{a} ± 101.35	2.00 ± 0.09	1.17 ± 0.71
Muscle	12	19.30^{c} ± 16.57	2.51 ± 0.48	0.03 ± 0.04
Gonad	12	72.80^{b} ± 97.50	2.03 ± 0.16	0.74 ± 0.64

Different superscript alphabet indicates significant difference at $P < 0.05$

Total amount of RNA yield extracted from dorsal fin was the highest (119.65 ± 101.35 ng), followed by gonad (72.80 ± 97.50 ng) and RNA yield extracted from muscle produced the least amount (19.30 ± 16.57 ng) comparatively (Table 1). Samples exhibited negligible degradation of RNA (Figure 1). From our empirical results, it is reasonable to assume that the proposed preservation and storage regime is more efficient for preserving RNA from fin rays and gonad compared to muscle tissue. Perhaps due to different cells types which are having different composition and structural integrity have varied penetration affinity towards RNA*later*. With respect of tissue type, fins in fishes composed of stiff rays covered by skin, which comprised stratified squamous epithelium of various thickness (Bone et al., 1995). On the other hand, gonadal tissues consist of synchronous germ cells from oogenesis or spermatogenesis (Huang et al., 2002). In addition, muscle tissue comprised mainly of muscular cells only. To demonstrate further, in microcrustacean such as *Artemia*, *Daphnia* and copepods, the presence of an exoskeleton could be a barrier for the diffusion of aqueous sulfate salt solutions such as RNA*later* (Gorokhova, 2005). In addition, exoskeleton permeability may vary according to different species, ontogenetic stages and molting cycle of the crustacean. These biological conditions could impedes the penetration of preservative into tissues, causing different level of nucleic acids degradation prior to extraction. Further to this, low amount of RNA in muscle cells discovered in this study could be also attributed by low tolerance of this cell type towards freeze-thaw stress. For instance, degradation of 28S rRNA was prominent from samples extracted from snap-frozen autopsy tissues (Auer et al., 2014; Ross et

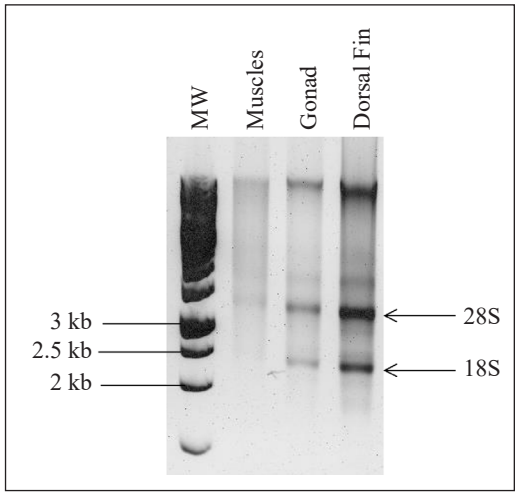

Figure 1. Analysis of RNA isolated from muscle, gonad and dorsal fin tissues using Qiagen's RNeasy Mini kit. MW: Molecular weight (1 kb)

al., 1992) and soil microbial (Pesaro et al., 2003). In the context of qPCR, samples with high amount of RNA are preferred to ease normalization of RNA, as low volume is required, dilution is carried out during generation of standard curve.

CONCLUCION

Current data suggests that preserving fish tissues in RNA*later* with subsequent storage at -80°C produced satisfactory amount of RNA from dorsal fin and gonad, but not from muscle tissues. Further studies are required to evaluate the effectiveness of RNA*later* and storage condition for stabilizing RNA in animal cells from other tissues type as well as from other animal species.

ACKNOWLEDGEMENTS

This work is supported by Ministry of Education (MOE) under FRGS 2014-0115-101-02 at Universiti Pendidikan Sultan Idris (UPSI). We are grateful for all the contributions, support and helpful discussion provided by all parties involved in this project.

COMPLIANCE WITH ETHICAL STANDARDS

The authors declared that there is no conflict of interest involved. All applicable local institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

REFERENCES

Auer, H., Mobley, J., Ayers, L., Bowen, J., Chuaqui, R., Johnson, L., … Ramirez, N. (2014). The effects of frozen tissue storage conditions on the integrity of RNA and protein. *Biotechnic and Histochemistry : Official Publication of the Biological Stain Commission*, *89*(7), 518. doi: 10.3109/10520295.2014.904927

Bone, Q., Marshall, N. B., & Blaxter, J. H. S. (1995). *Biology of fish*. Retrieved June 05, 2019, from http://www.cichlid-forum.com/articles/biology_of_fish.php

Copois, V., Bibeau, F., Bascoul-Mollevi, C., Salvetat, N., Chalbos, P., Bareil, C., … Del Rio, M. (2007). Impact of RNA degradation on gene expression profiles: Assessment of different methods to reliably determine RNA quality. *Journal of Biotechnology*, *127*(4), 549–559. doi: 10.1016/j.jbiotec.2006.07.032

Deng, M. Y., Wang, H., Ward, G. B., Beckham, T. R., & McKenna, T. S. (2005). Comparison of six RNA extraction methods for the detection of classical swine fever virus by real-time and conventional reverse transcription–PCR. *Journal of Veterinary Diagnostic Investigation*, *17*(6), 574–578. doi: 10.1177/104063870501700609

Escobar, M. D., & Hunt, J. L. (2017). A cost-effective RNA extraction technique from animal cells and tissue using silica columns. *Journal of Biological Methods*, *4*(2), 72. doi: 10.14440/jbm.2017.184

Gorokhova, E. (2005). Effects of preservation and storage of microcrustaceans in RNA*later* on RNA and DNA degradation. *Limnology and Oceanography: Methods*, *3*(2), 143–148. doi: 10.4319/lom.2005.3.143

Huang, J. D., Lee, M. F., & Chang, C. F. (2002). The morphology of gonadal tissue and male germ cells in the protandrous black porgy, *Acanthopagrus schlegeli*. *Zoological Studies*, *41*(2), 216–226.

Junttila, S., Lim, K.-J., & Rudd, S. (2009). Optimization and comparison of different methods for RNA isolation for cDNA library construction from the reindeer lichen *Cladonia rangiferina*. *BMC Research Notes*, *2*(1), 204. doi: 10.1186/1756-0500-2-204

Pesaro, M., Widmer, F., Nicollier, G., & Zeyer, J. (2003). Effects of freeze-thaw stress during soil storage on microbial communities and methidathion degradation. *Soil Biology and Biochemistry*, *35*(8), 1049–1061. doi: 10.1016/S0038-0717(03)00147-0

QIAGEN. (2006). *RNA*later® *handbook*. Retrieved June 05, 2019, from https://www.qiagen.com/us/resources/resourcedetail?id=38566945-e96b-46a4-a287-ec026849da4b&lang=en

QIAGEN. (2011). *RT² HT first strand handbook*. Retrieved June 05, 2019, from https://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=2&cad=rja&uact=8&ved=2ahUKEwjMybCBkKXkAhVBmuYKHZ4YBHcQFjABegQIAxAC&url=https%3A%2F%2Fwww.qiagen.com%2Fmq%2Fresources%2Fdownload.aspx%3Fid%3D9082d0c7-844e-45be-96c0-d5f580ef595b%26lang%3Den&usg=AOvVaw1ZLM-JmGiT2k6TKxPKXuJ3

QIAGEN. (2012). *RNeasy® mini handbook*. Retrieved June 05, 2019, from http://www.bea.ki.se/documents/EN-RNeasy%20handbook.pdf

Ross, B. M., Knowler, J. T., & McCulloch, J. (1992). On the stability of messenger RNA and ribosomal RNA in the brains of control human subjects and patients with Alzheimer's disease. *Journal of Neurochemistry*, *58*(5), 1810–1819. doi: 10.1111/j.1471-4159.1992.tb10057.x

Shabihkhani, M., Lucey, G. M., Wei, B., Mareninov, S., Lou, J. J., Vinters, H. V., … Yong, W. H. (2014). The procurement, storage, and quality assurance of frozen blood and tissue biospecimens in pathology, biorepository, and biobank settings. *Clinical Biochemistry*, *47*(4–5), 258–266. doi: 10.1016/j.clinbiochem.2014.01.002

Pertanika J. Trop. Agric. Sc. 42 (4): 1295 - 1320 (2019)

PERTANIKA JOURNALS

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Oil Palm Pollinator Dynamics and Their Behavior on Flowers of Different Oil Palm Species *Elaeis guineensis*, *Elaeis oleifera* and the *oleifera* x *guineensis* Hybrid in Ecuador

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ABSTRACT

The entomofauna and the behavioral patterns of potential pollinators were studied on female and male flowers of the oil palms *Elaeis guineensis*, *Elaeis oleifera* and *oleifera* x *guineenis* (OxG) hybrids in the Pacific coast and Amazon basin productive regions in Ecuador. Insect population studies were performed using a stratified sampling method and the determination of insect activity by monitoring insect arrivals to female flowers in anthesis. Additionally, insect pollinator pollen-transport capacity and life cycles were determined for *Elaeidobius kamerunicus*, *Grasidius hybridus*, *Couturierius constrictirostris* and *Mystrops costaricensis*. *Elaeis guineensis* female flowers were visited only by *Elaeidobius kamerunicus*, in both locations, at the Amazon basin plantation and at the Pacific coast plantation. *Elaeidobius kamerunicus* was the most abundant species (1,960 individuals) on *E. guineensis* during the dry season in Amazonia. *Elaeis oleifera* and OxG hybrids showed high numbers of *G. hybridus* (771 and 194 individuals,

ARTICLE INFO

Article history:
Received: 6 July 2019
Accepted: 10 October2019
Published: 13 November 2019

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ISSN: 1511-3701
e-ISSN: 2231-8542

respectively). *Couturierius constrictirostris* and *M. costaricensis* visited the flowers in lesser numbers. The activity studies showed that *E. kamerunicus* had diurnal behavior, while *G. hybridus* was active in the morning in the Amazon region and at dusk on the Pacific coast. *Elaeidobius kamerunicus* was the pollinator with the highest pollen loading capacity (8,273 grains/individual). The life cycle of *C. constrictirostris* was the longest (41.7 days in the Amazon region and 30.3 days on the Pacific coast), followed by *E. kamerunicus,* with 36.7 days in Amazonia and 30.3 days on the Pacific coast.

Keywords: *Couturierius*, *Elaeidobius*, *Elaeis guineensis*, *Elaeis oleifera*, hybrids, *Mystrops*

INTRODUCTION

Oil palm crops provide high revenue, foreign exchange and working places in tropical South America that present limited employment opportunities. Ecuador reported 263,840 ha of oil palm in 2016 (Salazar et al., 2016) and 257,120.93 ha in 2017 (Asociación Nacional de Cultivadores de Palma Aceitera [ANCUPA], 2018), and approximately 87% of the national production units belong to small farmers (Fomento de Exportaciones de Aceite de Palma y sus derivados [FEDAPAL], 2017). Oil palm is one of the most efficient oleaginous crops per hectare, covering the whole internal demand for vegetal oil for human consumption in many countries such as Indonesia, Malaysia, Venezuela, Ecuador, among others (Labarca & Narváez, 2009; Pacheco et al., 2017; Salazar et al., 2016; Turner & Gillbanks, 1974).

The expansion of "bud rot", known as "PC" disease, in Ecuador during the 1970s has forced growers to use the interspecific hybrids of *Elaeis oleifera* x *Elaeis guineensis* (OxG), which is tolerant to this disease and has therefore been considered as an alternative for developing new vegetal material suitable for production (Burgos, 2013; Louise et al., 2007). Research institutes and private organizations in Brazil, Costa Rica, Colombia, Venezuela and Ecuador have assessed interspecific hybrids (OxG) to increase production levels free from "bud rot" disease with promising results (Alvarado et al., 2010; Barba & Baquero, 2012; Bravo & Bernal, 2015; Teixeira Souza Júnior, 2013). However, these interspecific hybrids present a critical agronomical disadvantage in terms of low pollination levels in comparison with *Elaeis guineensis* (Alvarado et al., 2000, 2013). Assisted pollination is a common agronomic practice used to obtain good fruit set levels in commercial crops. However, this practice increases production costs and entails logistic limitations (Hacienda La Cabaña, 2009; Rosero & Santacruz, 2014). Most pollination studies have been performed on *Elaeis guineensis*, but very few have focused on *Elaeis oleifera* or the OxG hybrids. In the last few years, there has been an important increase in OxG hybrid planting areas in Ecuador. Plantations will have to renew the vegetal materials in at least 30,000 ha/year (ANCUPA, 2018), but there is a lack of information about the

OxG hybrid agronomic behavior, pollination mechanisms and pollinator adaptation to monoculture.

Two families of Coleoptera, namely Curculionidae (Baford et al., 2011; Mondragón & Roa, 1985) and Nitidulidae (*Mystrops costaricensis*) have been reported to be pollinators of oil palms (Baford et al., 2011; Syed, 1979, 1984). Curculionidae beetles, such as *Elaeidobius kamerunicus*, *Elaeidobius subvittatus*, *Couturierius* sp., and *Grasidius* sp., have been considered to be the most efficient pollinators in commercial oil palm plantations (Tuo et al., 2011) in Côte D´Ivoire, Western Africa. Further studies conducted by Syed (1984) showed that pollination by *E. kamerunicus* had increased the fruit set in *E. guineensis*. Several studies have been conducted since 1980 that introduce *E. kamerunicus* to commercial plantations to increase fruit set (Caudwell, 2002; Meléndez & Ponce, 2016; Syed, 1982). Artificial introduction of *E. kamerunicus* populations in *E. guineensis* crops the fruit set increased from 15% to 26% and resulted in a 60% increase in crop yield (Harun & Noor, 2002; Prasetyo et al., 2014; Syed, 1979; Syed & Saleh, 1988). However, there are some reports of poor fruit set apparently caused by a combination of environmental factors and poor-quality pollen that may be affecting the performance of *E. kamerunicus* (Teo, 2015).

There is high interdependence between insect pollinators and plants; male flowers are essential for pollinators to complete their life cycle, since these flowers are used for oviposition and as a food source for larvae and adults, and the leaf bracts are used to protect the pupae (Caudwell, 2002; Labarca & Narváez, 2009; Syed, 1984). Curculionidae and Nitidulidae beetles feed on the pollen grains in male flowers and transport pollen from male to female flowers (Henderson, 1986; Labarca & Narváez, 2009). The insect affinity for oil palm flowers is essential for the survival of the species since insects are attracted to the flowers only during anthesis because they respond to specific flower chemical changes during anthesis (Baford et al., 2011). Mystropinae beetles have shown diurnal visitation patterns to flowers (Núñez et al., 2005). However, other studies showed that these insects can still be active late in the afternoon, between 18:00 and 20:00 (Genty, 1985).

Due to the expansion of hybrid material, there is a need to understand the pollination mechanisms and pollinator dynamics in *E. oleifera* and the OxG hybrid in Ecuador. The present work focused on evaluating the insect diversity associated with the inflorescences of *E. guineensis*, *E. oleifera* and hybrid oil palms, the life cycles and activity of the potential pollinators and the capacity of insects to carry pollen to establish their potential as pollinators.

MATERIALS AND METHODS

Sites Description and Plant Materials

Oil palm pollinator studies were performed in two oil palm plantations, one in the Pacific coast lowlands and one in the Amazon basin lowlands in Ecuador. The first location was the Palmar del Río plantation (0°19′ S,

77°04′ W) at 290 m.a.s.l. and with annual precipitation of 3,392 mm. This plantation has 10,000 ha of oil palm crops and is located in Francisco de Orellana province (Site 1). The studies at this location were performed during the rainy and dry seasons in 2014. The second study location was the Energy and Palma plantation (1° 07′ N, 78° 45′ 50" W) in the province of Esmeraldas, which was 13,000 ha in size and located on the Pacific coast of the country (Site 2). It is located at 500 m.a.s.l., with an annual precipitation of 1,500-1,800 mm. The studies were performed during the dry season in 2015 and the rainy season in 2016 (Table 1).

The cultivated area of the plantations was divided into strata that included *E. guineensis*, *E. oleifera* and hybrid material (OxG). The insects were collected in each stratum using a randomized sampling method (Galindo, 2008).

The pollinators of flowers (male and female) of *E. guineensis and E. oleifera* and interspecific (OxG) hybrids in anthesis were studied at both localities using the same method. The hybrids (OxG) evaluated at Palmar del Río were Taisha x Avros, Taisha x LaMé and Cuarí x LaMé. At Energy and Palma, the evaluated hybrids were Unipalma (OxG) and Cuarí x LaMé. *Elaeis guineensis* and *E. oleifera* (Taisha) were evaluated at both sites. *Elaeis guineensis* was taken as the genetic material of reference because it has been the most commonly cultivated palm.

Table 1

Number of oil palm flower samples in anthesis at the two study sites: The Amazonia basin lowlands (Site 1) and the Pacific coast (Site 2)

Palm species	Female flowers		Male flowers	
	Season			
	Dry	Rainy	Dry	Rainy
Site 1 (Amazonia)				
Elaeis guineensis (Papúa)	2	2	2	2
Elaeis oleifera (Taisha)	2	2	2	2
Hybrid Taisha x Avros	17	17	17	17
Hybrid Cuarí x LaMé	24	24	24	24
Hybrid Taisha x LaMé	3	3	3	3
Site 2 (Pacific coast)				
Elaeis guineensis (Papúa)	10	10	8	8
Elaeis oleifera (Taisha)	1	1	1	1
Hybrid Unipalma	36	36	21	21
Hybrid Cuarí x LaMé	14	14	14	14

Insects Associated with Oil Palm Inflorescences

Insects were collected from male flowers during anthesis described by Hormaza et al. (2010), and 20 spikes were shaken over a white paper to gather the specimens. Insects were identified using taxonomic keys (Borror et al., 1989; Hala et al., 2012; O'Brien et al., 2004).

Diurnal and nocturnal insect activity was observed among the female inflorescences. Observations were executed every 20 minutes from 5:00 until 0:00 (midnight). Visiting insects were collected and later identified. Observations were repeated three times for four different female inflorescences (12 samples per species). The insects visiting female flowers in anthesis were trapped using a 40 x 50 cm^2 plastic sheet smeared with BIOTAC glue (IUPAC number: polybutene polymer; exporter: Marketing ARM, International, Inc., USA) combined with odorless vegetable oil to facilitate the application of the glue. The plastic film was fixed over the female inflorescences and left in place for a period of 24 hours starting at 6:00.

Life Cycle of the Insects

Eggs and immature stages of *E. kamerunicus*, *G. hybridus*, *C. constrictirostris* and *M. costaricensis* were collected from male flowers and kept in small plastic boxes (6 cm diameter) with a wet paper towel. Developmental changes were recorded daily. The working area and materials were sanitized with 0.02% formaldehyde. Insects were kept under natural light conditions at a temperature of approximately 20°C.

Pollen Carried by Potential Insect Pollinators

Elaeidobius kamerunicus, *G. hybridus*, *C. constrictirostris* and *M. costaricensis* were selected to evaluate their capacity for carrying pollen grains. Twenty specimens were collected from male inflorescences during anthesis and placed in Eppendorf tubes. Distilled water and Tween 20 were added (0.5 ml each), along with four drops of 7% safranin staining solution (Prada et al., 1998). The pollen grains were counted using a Neubauer chamber of 0.1 mm depth and 0.0025 mm^2 area (Marienfeld, Germany; Model: Fuchs-Rosenthal bright line) according to the methods described by Chinchilla and Richardson (1991) and Prada et al. (1998).

Data Analysis

Insect population percentages were calculated, and descriptive statistical analysis were used. Simpson's diversity index (D) was calculated to determine the diversity level and predominance of species associated with each oil palm type. This index was expressed as 1-D to facilitate analysis and to interpret the lowest values as indicating the dominance of one species and a value near 1.0 as indicating high diversity.

Analysis of similarities (ANOSIM) was performed in the software PAST, version 3.26, to compare insect species composition between different oil palm species in the Amazon basin (Site 1) and Pacific coast (Site 2).

For statistical analysis in pollen transport potential, an ANOVA was conducted to compare the different capacities of the insects to carry pollen. Furthermore, a Tukey test ($p<0.05$) was performed on the obtained data.

RESULTS AND DISCUSSION

The results regarding the insect pollinators found and their percentage incidence in female flowers are shown in Table 2.

Elaeis guineensis and *E. oleifera* showed a higher specificity of insects visiting the female flowers (Table 2). *Elaeis guineensis* flowers were visited by *E. kamerunicus* (a total of 2,576 specimens at the Amazon plantation and 1,429 at the Pacific coast plantation). This insect represented 100.0% of the whole pollinator population sampled among these palms. The insect diversity on palms was evaluated using Simpson's index, and the results showed that *E. guineensis* presented index values of 0.00 and 0.001 at all sampled locations and during the two seasons. These values indicate that female flowers are associated mostly with a unique pollinator species, i.e. *E. kamerunicus*. The fact that *E. kamerunicus* was introduced to the continent to pollinate *E. guineensis* might explain the affinity of this pollinator for the palm (Chinchilla et al., 1990; Syed, 1979). *Elaeis guineensis* was not visited by other Curculionidae species in high numbers, and during the dry season, this palm received a very limited number of *M. costaricensis* visits (16 specimens).

Elaeis oleifera attracted three individuals of *E. kamerunicus* (3.7%) at the Amazon basin plantation. The most common insect associated with its female flowers was *G. hybridus* at both sampling sites. Among the palms at the Pacific coast site, 404 specimens (31.5%) were collected during the rainy season, and 771 specimens (68%) were collected from the palms in the Amazonian basin during the dry season. This American native palm showed that *G. hybridus*, *C. constrictirostris* and *M. costaricensis* were associated with its female flowers in both locations, the Amazon basin and on the Pacific coast. The presence of *C. constrictirostris* was more common among the Amazon basin palms (362 specimens; 32%) during the dry season. Another species associated with *E. oleifera* was *M. costaricensis*: higher numbers were observed among the palms on the Pacific coast during the rainy season (734; 57.2%), but lower numbers were observed during the dry season (13; 28.3%). The presence of this species increased the values of Simpson's diversity index (1-D) in the coastal region. The higher variety of insects on *E. oleifera* might be explained by the coevolution between native insects and palms from America (Mondragón & Roa, 1985). *Elaeis oleifera* in comparison with *E. guineensis* and the OxG hybrids, achieved the highest 1-D index value in the Amazonian basin during the rainy season

Table 2

Pollinator population occurrence and Simpson's index (D) calculated for female flowers during anthesis

Season/ Site/ Palm species	Insect species									
	Eleidobius kamerunicus		*Grasidius hybridus*		*Couturierius constrictirostris*			*Mystrops costaricensis*	Total	Simpson's index (1-D)
Rainy season	n	(%)	n	(%)	n	(%)	n	(%)		
Amazonia										
Elaeis guineensis	152	(99.3)	1	(0.0)	0	(0.0)	0	(0.0)	153	0.01
Elaeis oleifera	3	(3.7)	59	(72.0)	20	(24.4)	0	(0.0)	82	0.74
TxA	99	(70.2)	42	(29.8)	0	(0.0)	0	(0.0)	141	0.42
CxL	253	(87.8)	34	(11.8)	1	(0.3)	0	(0.0)	288	0.21
TxL	213	(91.0)	19	(8.1)	2	(0.9)	0	(0.0)	234	0.17
Pacific coast										
Elaeis guineensis	1429	(100.0)	0	(0.0)	0	(0.0)	0	(0.0)	1429	0.00
Elaeis oleifera	0	(0.0)	404	(31.5)	144	(11.2)	734	(57.2)	1282	0.56
Unipalma	0	(0.0)	194	(100.0)	0	(0.0)	0	(0.0)	194	0.00
CxL	1	(5.9)	16	(94.1)	0	(0.0)	0	(0.0)	17	0.12
Dry season										
Amazonia										
Elaeis guineensis	2576	(100.0)	0	(0.0)	0	(0.0)	0	(0.0)	2576	0.00
Elaeis oleifera	0	(0.0)	771	(68.0)	362	(32.0)	0	(0.0)	1133	0.44
TxA	334	(85.4)	57	(14.6)	0	(0.0)	0	(0.00)	391	0.25
CxL	721	(86.6)	112	(13.4)	0	(0.0)	0	(0.00)	833	0.23
TxL	137	(92.6)	11	(7.4)	0	(0.0)	0	(0.00)	148	0.14

Table 2 *(Continued)*

Season/ Site/ Palm species	Insect species										Simpson's index (1-D)
	Eleidobius kamerunicus		*Grasidius hybridus*		*Couturierius constrictirostris*		*Mystrops costaricensis*		Total		
Pacific coast											
Elaeis guineensis	188	(92.2)	0	(0.0)	0	(0.0)	16	(7.8)	204		0.15
Elaeis oleifera	0	(0.0)	21	(45.7)	12	(26.1)	13	(28.3)	46		0.66
Unipalma	37	(34.3)	70	(64.8)	0	(0.0)	1	(0.9)	108		0.66
CxL	46	(38.3)	44	(36.7)	0	(0.0)	30	(25.0)	120		0.66

Note. TxA: Taisha x Avros; TxL: Taisha x LaMé; and CxL: Cuarí x LaMé
n: Number of specimens

(1-D = 0.74). The coevolution of native pollinators with this American palm may explain the choice of different insect species for this palm, as stated previously.

All female flowers from oil palm OxG hybrids received fewer insect visits than *E. guineensis* and *E. oleifera* flowers during anthesis. In general, hybrids present well-developed bracts that completely cover the female flowers, possibly hindering access to insects. These morphological characteristics might reduce the attraction of pollinator species (Syed, 1984).

Elaeidobius kamerunicus was the most numerous insects associated with OxG hybrid female flowers during anthesis in the Amazonian basin. The lowest percentage of *E. kamerunicus* among hybrids was 70.2% (99 specimens) for Taisha x Avros (TxA). In the Ecuadorian coastal region, hybrids such as Unipalma and CxL had a higher presence of *G. hybridus*, accounting for up to 100% of the sample, with 194 individuals on Unipalma during the rainy season. These observations could indicate that for this hybrid, the presence of *E. kamerunicus* is more strongly affected by its cultivation in areas that experience high rainfall than *G. hybridus* (Prada et al. 1998). Populations of the native insect *G. hybridus* on hybrids were similar in both studied regions, showing similar numbers during the two seasons. Female oil palm flowers in the Amazonian basin presented high quantities of associated insects during the rainy season (898 individuals) and dry season (5,081), but

at the Pacific coastal plantation, there were more insects during the rainy season (2,922) and very few insects during the dry season (478). These results suggest that this insect could be used all year long as a pollinator of hybrids in commercial plantations. In fact, *E. kamerunicus* and *G. hybridus* seem to successfully coexist on OxG hybrids, although *E. kamerunicus* populations are higher. The female flowers of the hybrids do not attract *C. constrictirostris* or *M. costaricensis* in high numbers; consequently, these pollinator species remain less important for commercial plantations. Moreover, a study conducted by Labarca and Narváez (2009) mentioned that high numbers of *M. costaricensis* are detrimental for pollination because this species did not visit female flowers in large numbers; moreover, it fed on pollen from male flowers.

The CxL hybrid was studied in both sampling areas during the two seasons, and it hosted more insects than the other hybrids. *E. kamerunicus* showed a maximum of 721 (86.6%) specimens in the Amazon basin and *G. hybridus* showed a maximum of 112 specimens (13.4%) on the Pacific coast during the dry season. The higher number of insects during the dry season is apparently due to the presence of a higher number of male flowers during the dry season, which serve as food source (Appiah & Agyei, 2013; Teo, 2015). The highest numbers of *E. kamerunicus* on the CxL hybrid were recorded in the Amazon region (87.8 and 86.6% during the rainy and dry seasons, respectively), whereas on the coast, the percentages were 5.9 and 38.3% during the rainy and dry seasons, respectively. The second most common pollinator associated with the CxL hybrid was *G. hybridus*. Even though it was not as numerous as *E. kamerunicus*, it contributed to pollen dissemination among the female flowers. This pollinator is more abundant during the rainy season in the coastal region (94.1%), which contrasts with the pattern observed for *E. kamerunicus* (5.9%). This pattern of seasonal changes in population numbers may suggest that the pollination process could occur all year long. Simpson diversity values for the CxL hybrid were low (0.21 and 0.12) at the Amazon plantation, while on the Pacific coast, during the dry season, the diversity value reached 0.66, which was similar to that for *E. oleifera*. These numbers might indicate that anthesis and the chemical composition of the attractants of female flowers of CxL are closer to those of *E. oleifera* than *E. guineensis*. The TxL hybrid presented the lowest diversity value (1-D = 0.17 and 0.14), while Unipalma achieved a relatively high diversity value during the dry season (1-D = 0.66) but a very low value in the rainy season because only *G. hybridus* was recorded among the flowers. The OxG hybrid flowers showed high diversity values due to the presence of *E. kamerunicus* and *G. hybridus* on the female flowers during anthesis.

The ANOSIM test for insect composition on female flowers showed that most of R values were between 0 and 1 and were significant (Table 3). The analysis indicated that the insect species

Table 3

Analysis of similarities (ANOSIM) of insects on oil palm species female flowers, (Permutation N: 9999; p-*value <0.0001 and R=0.2732). Pairwise comparison according to the occurrence of* Elaeidobius kamerunicus, Grasidius hybridus, Couturierius constrictirostris *and* Mystrops costaricensis

Palm species (Groups)	TxA	CxL (Site 1)	TxL	*Elaeis guineensis* (Site 1)	*Elaeis oleifera* (Site 1)	Unipalma	CxL (Site 2)	*Elaeis guineensis* (Site 2)
CxL (Site 1)	0.042 (0.0685)							
TxL	-0.04892 (0.6202)	-0.02036 (0.5076)						
Elaeis guineensis (Site 1)	0.2543 (0.0468*)	0.4461 (0.0116*)	0.3016 (0.0696)					
Elaeis oleífera (Site 1)	0.4073 (0.0054*)	0.6857 (0.0008*)	0.8492 (0.0055*)	1 (0.0294*)				
Unipalma	0.238 (0.0001*)	0.3901 (0.0001*)	0.3266 (0.0004*)	0.3988 (0.0001*)	0.3264 (0.0033*)			
CxL (Site 2)	0.1197 (0.0033*)	0.3236 (0.0001*)	-0.002726 (0.4670)	0.1735 (0.0348*)	0.1109 (0.0848)	0.1528 (0.0005*)		
Elaeis guineensis (Site 2)	0.1364 (0.0141*)	0.162 (0.0068*)	-0.01154 (0.4691)	0.2802 (0.0492*)	0.9863 (0.0004*)	0.4547 (0.0001*)	0.3778 (0.0001*)	
Elaeis oleífera (Site 2)	0.4331 (0.0046*)	0.6993 (0.0008*)	0.8056 (0.0039*)	1 (0.0296*)	0.09375 (0.2868)	0.3311 (0.0015*)	0.1164 (0.0675)	0.9685 (0.0001*)

Note. The table shows the R value and the *p*-value (in brackets)
* Statistical significance between groups

composition in each of the female flower palm species and hybrids are more similar within each group than to insect species in the other palm species. The insect communities between *E. guineensis* and *E. oleifera* are different ($p \leq 0.05$) in both study sites. There are also differences in *E. guineensis* insect composition between the study sites, however, there were not differences in *E. oleifera* when comparing both study sites. The insect composition in the hybrid CxL in the Amazon basin (Site 1) did not show significant differences when compared with the hybrids TxA, TxL and *E. guineensis*. While the difference of CxL and TxL hybrids and *E. oleifera* is high. The difference of insect composition of *E. guineensis* and *E. oleifera* is high (R=1, p=0.0294). The insect composition in the hybrid CxL at the Pacific coast (Site 2) was not different from the hybrid TxL and *E. oleifera* (Table 3).

Male flowers of *E. guineensis* and *E. oleifera* in both sampling regions showed higher numbers of pollinator insects than the OxG hybrids. The highest numbers of total counted insects were 97,749 (100%) of *E. kamerunicus* on *E. guineensis* and 4,750 of *G. hybridus* (21.1%), 3,080 of *C. constrictirostris* (13.7%) and 14,678 of *M. costaricensis* (65.2%) on *E. oleifera* on the Pacific coast during the rainy season.

Male flowers of *E. guineensis* in the Amazon region showed a very high specificity, attracting *E. kamerunicus*, with a total of 8368 specimens during the rainy season (Table 4). The size of the population was similar during the dry season (8,517), with a diversity value of 0.00, showing that *E. kamerunicus* was the only insect that visited these flowers. In contrast, the pure *E. oleifera* material did not attract individuals of *E. kamerunicus* during the study. The massive populations of *E. kamerunicus* on *E. guineensis* could be limiting the arrivals of other pollinator species to this palm (Appiah & Agyei, 2013; Genty, 1985). *Grasidius hybridus* is present in high numbers on *E. oleifera* throughout the year in eastern Ecuador; however, this species duplicated its population during the dry season. There were 1,877 individuals in Amazonia during the rainy season and 3,519 individuals during the dry season. On the coast, the opposite pattern was observed: 2,560 insects were observed during the dry season and 4,750 were observed during the rainy season. The diversity values for this palm remained between 0.49 and 0.78, indicating that the insect populations are balanced on these oil palm species, perhaps due to species coevolution (Labarca & Narváez, 2009; Meléndez & Ponce, 2016).

The high affinity of each pollinator species for pure materials implies that in small commercial plantation fields, the numbers of male flowers should be high enough to replace manually assisted pollination with entomophilous pollination. Despite these facts, *E. kamerunicus* showed a lower affinity to male flowers of Unipalma, TxA and CxL hybrids, with maximum values of 890 (69.4%), 552 (94.4%) and 848 (31.6%) recorded insects, respectively. More studies of the morphological structures and chemical composition of male flowers

Table 4

Pollinator population occurrence on male oil palm flowers during anthesis. Simpson's index (D) of diversity calculated according to the insect population sampled

Season/ Site/ Palm species	Insect species									
	Elaeidobius kamerunicus		Grasidius hybridus		Couturierius constrictirostris		Mystrops costaricensis		Total	Simpson's index (1-D)
Rainy season	n	(%)	n	(%)	n	(%)	n	(%)		
Amazonia										
Elaeis guineensis	8368	(100.0)	1	(0.0)	0	(0.0)	0	(0.0)	8369	0.00
Elaeis oleifera	1	(0.0)	1877	(73.7)	669	(26.3)	0	(0.0)	2547	0.78
TxA	552	(94.4)	33	(7.1)	0	(0.0)	0	(0.0)	585	0.11
CxL	694	(92.9)	53	(5.6)	0	(0.0)	0	(0.0)	747	0.14
TxL	117	(94.4)	7	(5.6)	0	(0.0)	0	(0.0)	124	0.11
Pacific coast										
Elaeis guineensis	97749	(100.0)	0	(0.0)	0	(0.0)	0	(0.0)	97749	0.00
Elaeis oleifera	0	(0.0)	4750	(21.1)	3080	(13.7)	14678	(65.2)	27258	0.51
Unipalma	890	(69.4)	393	(30.6)	0	(0.0)	0	(0.0)	1283	0.43
CxL	458	(56.2)	357	(43.8)	0	(0.0)	0	(0.0)	815	0.49
Dry season										
Amazonia										
Elaeis guineensis	8517	(100.0)	2	(0.0)	0	(0.0)	0	0.00	8519	0.00
Elaeis oleifera	2	(0.0)	3519	(58.6)	2489	(41.4)	0	(0.0)	6010	0.49
TxA	94	(16.4)	369	(64.3)	111	(19.3)	0	(0.0)	574	0.52
CxL	848	(31.6)	1594	(59.3)	244	(9.1)	0	(0.0)	2686	0.55
TxL	35	(21.6)	122	(75.3)	5	(3.1)	0	(0.0)	162	0.39

Table 4 *(Continued)*

Season/ Site/ Palm species	Insect species								Total	Simpson's index (1-D)
	Elaeidobius kamerunicus		Grasidius hybridus		Couturierius constrictirostris		Mystrops costaricensis			
Pacific coast										
Elaeis guieensis	47760	(100.0)	0	(0.0)	0	(0.0)	0	(0.0)	47760	0.00
E.laeis oleifera	0	(0.0)	2560	(22.0)	1871	(16.1)	7183	(61.8)	11614	0.54
Unipalma	734	(39.2)	785	(41.9)	0	(0.0)	354	(18.9)	1873	0.64
CxL	334	(36.9)	570	(63.1)	0	(0.0)	0	(0.0)	904	0.47

Note. TxA: Taisha x Avros; TxL: Taisha x LaMé; and CxL: Cuarí x LaMé
n: Number of specimens

and pollen production in OxG hybrids are needed to explain their impact on insect populations during the rainy season.

In general, OxG hybrids showed similar quantities of *E. kamerunicus* at Site 2 (Pacific coast), CxL presented 458 individuals, and Unipalma was visited by 890 insects. These numbers suffered a reduction of 30.2% during the dry season for Unipalma and 19.3% for CxL. This pattern is very similar to that in the Amazonian region, where a reduction in *E. kamerunicus* during the dry season was observed on the CxL hybrids, from 92.9% (1-D=0.14) to 31.6% (1-D=0.55) for this pollinator. The reduction in the *E. kamerunicus* population seems to allow other species to occupy the flowers because the value 1-D= 0.55 indicates an increase in diversity on CxL.

Grasidius hybridus was assessed on OxG hybrids, and its population increased during the dry season on these plants. The population of this pollinator increased from 33 (7.1%) individuals to 369 (64.3%) on TxA, and from 53 (5.6%) to 1594 (59.3%) individuals on CxL in the Amazonian region. Likewise, on the coast, on the Unipalma hybrid, the population of this insect increased from 393 (30.6%) individuals to 785 (41.9%). These observations could be useful for the future management of pollinator populations and therefore increase production levels. It can be stated that *E. kamerunicus* proliferates during the rainy season, while *G. hybridus* proliferates during the dry season. This pattern is consistent with the findings described by Ponnamma (1999), Sánchez

et al. (2004) and Syed (1984). Therefore, this information should be considered in commercial plantations where entomophiles pollination practices are used.

Couturierius constrictirostris is a pollinator restricted to *E. oleifera* materials and OxG hybrids. It increased in terms of its presence among male flowers during the dry season. This insect always remained less numerous than *G. hybridus*. In the Amazonian region, *C. constrictirostris* was not present on OxG hybrids during winter, but during the dry season, 111 (19.3%), 244 (9.1%) and 5 (3.1%) individuals were found on TxA, CxL and TxL, respectively. On *E. guineensis,* this pollinator was not observed during the collection procedures, and its populations on pure *E. oleifera* materials remained lower than those on *G. hybridus*. Its absence during the rainy season and its low numbers on OxG hybrids indicate that this insect as not a good candidate for use in assisted pollination programs on commercial plantations. In other works (Appiah & Agyei, 2013; Prasetyo et al., 2014; Teo, 2015; Yue et al., 2015), it was shown that populations of *E. kamerunicus* change with the weather conditions, suggesting that native insects could also be affected by this natural factor. The calculated diversity was higher on OxG hybrids than on *E. guineensis*, and this information suggests that hybrid male flowers have characteristics similar to their *E. oleifera* parents.

A fourth insect species was found in male inflorescences, i.e. *M. costaricensis*. The presence of this insect is restricted solely to the coastal region. It was present in high numbers in pure *E. oleifera* materials during the two sampling periods. Its presence was not recorded among the CxL hybrids, causing a decrease in their diversity index values (0.14 and 0.47). On Unipalma, the insect was not present during the rainy season, but it appeared during the dry season, with 354 specimens, and increased the diversity index value for this hybrid (0.64).

It is remarkable that male flowers provide a refuge for greater quantities of insects than female flowers; these insects are pollen feeders, and male flowers are their food source all year long.

The ANOSIM test for insect composition on male flowers showed that most of R values were between 0 and 1 and were significant (Table 5). The analysis indicated that the insect species composition in each of the male flower palm species and hybrids are more similar within each group than to insect species in the other palm species. The insect composition in the hybrid CxL in the Amazon basin (Site 1) did not show significant differences when compared with the hybrids TxL, TxA and *E. guineensis*. The insect composition on hybrid TxL did not show a significant difference with *E. oleifera*. The insect communities of *E. guineensis* in the Amazon basin (Site 1) and the Pacific coast (Site 2) were different ($p \leq 0.05$). While the insect composition in *E. oleifera* did not show significant differences between the two study sites (Table 5). The insect composition shows that *E. guineensis* is predominantly associated with *E. kamerunicus*, while *E. oleifera* is associated

Table 5

Analysis of similarities (ANOSIM) of insects on oil palm species male flowers (Permutation N: 9999; p-*value <0.0001 and R= 0.3308). Pairwise comparison according to the occurrence of* Elaeidobius kamerunicus, Grasidius hybridus, Couturierius constrictirostris *and* Mystrops costaricensis

Palm species (Groups)	TxA	CxL (Site 1)	TxL	*Elaeis guineensis* (Site 1)	*Elaeis oleifera* (Site 1)	Unipalma	CxL (Site 2)	*Elaeis guineensis* (Site 2)
CxL (Site 1)	-0.06291 (0.4873)							
TxL	-0.004842 (0.4137)	0.02949 (0.2284)						
Elaeis guineensis (Site 1)	0.2165 (0.0001*)	0.2343 (0.0001*)	0.3651 (0.0545)					
Elaeis oleifera (Site1)	0.2225 (0.0001*)	0.2336 (0.0001*)	0.3571 (0.039*)	1 (0.0268*)				
Unipalma	0.4278 (0.0001*)	0.3614 (0.0001*)	0.6496 (0.0001*)	1 (0.0001*	1 (0.0001*)			
CxL (Site 2)	0.3304 (0.0001*)	0.2693 (0.0001*)	0.7812 (0.0001*)	1 (0.0003*)	1 (0.0002*)	0.07399 (0.0321*)		
Elaeis guineensis (Site 2)	0.3476 (0.0002*)	0.306 (0.0001*)	0.9012 (0.0002*)	0.4529 (0.0085*)	1 (0.0005*)	1 (0.0001*)	1 (0.0001*)	
Elaeis oleifera (Site 2)	0.2393 (0.001*)	0.2653 (0.0016*)	0.1667) 0.144	1 (0.0636)	1 (0.0644)	1 (0.0011*)	1 (0.0018*)	1 (0.007*)

Note. The table shows the R value and the *p*-value (in brackets)
* Statistical significance between groups

with *G. hybridus*, *C. constrictirostris* and *M. costaricensis*. These results suggest that the insect composition on the hybrids have mixed characteristics from *E. guineensis* and *E. oleifera*, since the hybrid oil palm flowers attract insects that visit exclusively any of these two species. This may be due to mixed flower chemical attractant composition in the hybrids (Gomes et al., 2011).

Diurnal and Nocturnal Insect Behavior on Female Flowers

The results of observations of pollinator arrivals to female *E. oleifera* flowers are shown in Figure 1.

The curves show that *E. kamerunicus* is practically absent during the whole day on this palm regardless of the region. The opposite phenomenon was observed in the case of *G. hybridus*, which emerged in the morning and stopped moving before 17:00 in the afternoon, showing diurnal activity in the Amazonian region. A different behavior of this insect was observed on the coast, where *G. hybridus* showed crepuscular activity beginning at 17:00 and stopping before 20:00 during twilight. During its peak visiting time (18:40), visits surpassed 350 individuals; this is in contrast to the mobility observed at the Amazonian location, where the highest peak of arrivals accounted for approximately 150 individuals.

The behavior of *C. constrictirostris* was similar to that of *G. hybridus* on these *E. oleifera* palms, with the difference being that the populations were significantly reduced in comparison to those of *G. hybridus*. The highest population peaks were 53 individuals in the Amazonian region and 89 in the coastal region.

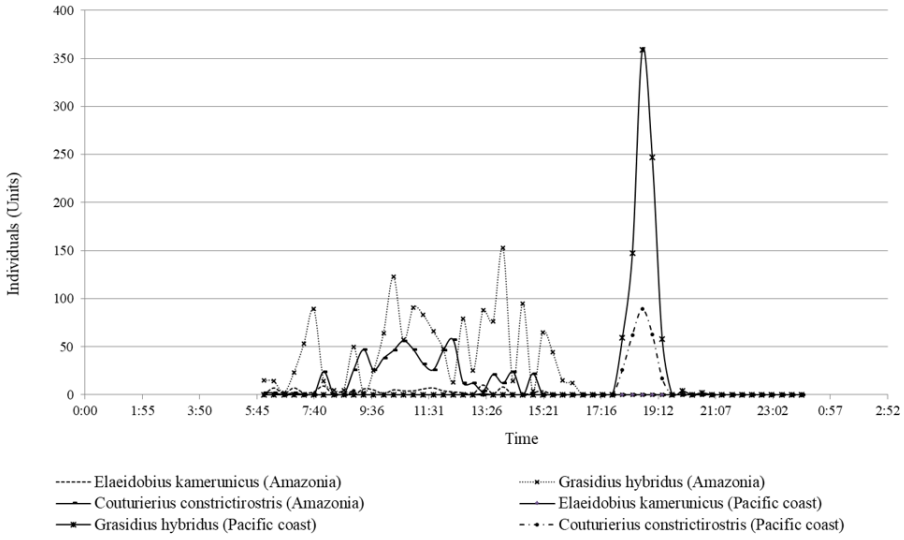

Figure 1. Diurnal and nocturnal insect activity on female *Elaeis oleifera* palm flowers

In Figure 2, the three pollinator insects show a diurnal activity pattern, and a certain temperature level (warm) is needed for their activity and mobility, since environmental factors and insect metabolism affect the mobility of pollinators that are active fliers and visit flowers at a specific time of the day ("daily activity window") (Herrera, 1990; Stone et al., 1999). In fact, Genty (1985) and Sánchez et al. (2004), in their studies showed that in moderate temperatures 22.3 and 30.1°C *E. kamerunicus* population proliferated and during the day, between 10:00 and 11:00 insects visit in high numbers the oil palm flowers. The reduced activity of *E. kamerunicus* on *E. oleifera* in comparison to *E. guineensis* could be further proof of an affinity for oil palm genetic characteristics. According to Jianjun et al. (2015), insects are linked to flowers according to anthesis phases and palm species. Figure 2 shows that *E. kamerunicus* was recorded in both geographical regions during the day.

The highest activity of *E. kamerunicus* was observed after 07:00 in the morning and before 16:00 in the afternoon. This insect showed a peak of activity, with a high number of insects (379 specimens), at 10:40 in the morning. These results are similar to those obtained by Hala et al. (2012), in studies done in Côte d´Ivoire (West Africa).

An oil palm hybrid (CxL) was observed in both the Amazonian and coastal regions. As shown in Figure 3, female flowers in anthesis are able to attract exotic (*E. kamerunicus*) and native pollinators (*G. hybridus* and *C. constrictirostris*). Nevertheless, in contrast to *E. guineensis*

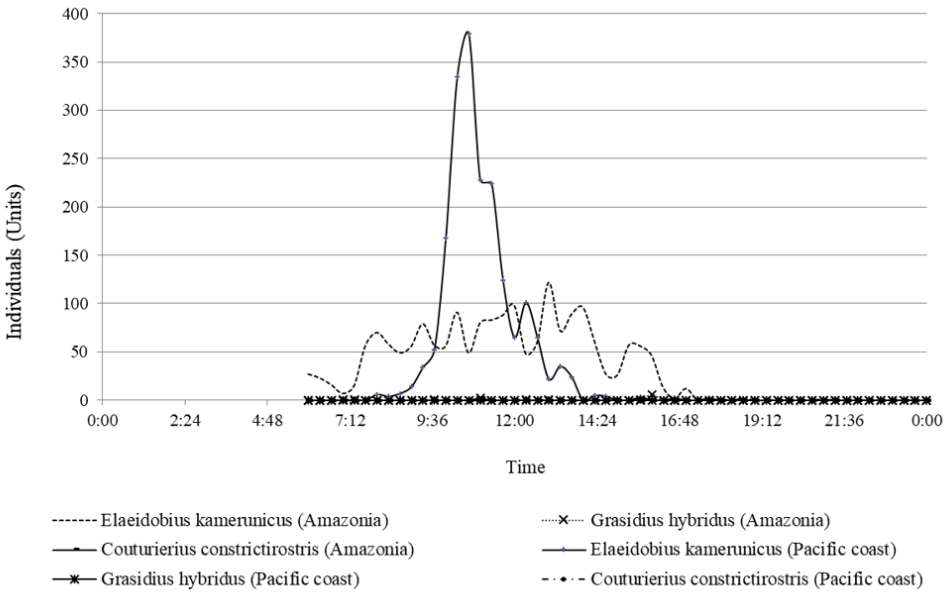

Figure 2. Diurnal and nocturnal insect activity on female *Elaeis guineensis* palm flowers

and *E. oleifera* (shown in Figures 1 and 2), the CxL hybrid flowers presented a limited number of insects. *E. kamerunicus* oscillated between 15 and 20 individuals during the highest peaks of activity (between 8:00 and 17:00). *Grasidius hybridus* was the most numerous insects during the twilight peak, reaching 86 specimens. The other native pollinator, *C. constrictirostris,* has a very similar behavior but is present in small numbers.

The presence of the three pollinator insects suggests that the genetic composition of hybrids could influence the production of certain compounds of chemicals created by the plant to attract pollinators to the flowers (Kirejtshuk & Couturier, 2010). Other hybrids were assessed: TxA and UNIPALMA. TxA was studied in the Amazonia region, and the other hybrid, UNIPALMA, was observed only on the coast. Hybrid TxA flowers attracted the three pollinator species in small numbers. On the one hand, the highest peak of *E. kamerunicus* was 16 individuals at approximately 09:30 in the morning. The maximal population of *G. hybridus* was 6 specimens presenting diurnal behavior. On the other hand, *C. constrictirostris* is even less numerous, with 4 individuals observed at 11:00 in the morning. The limited presence of pollinators during the whole day showed

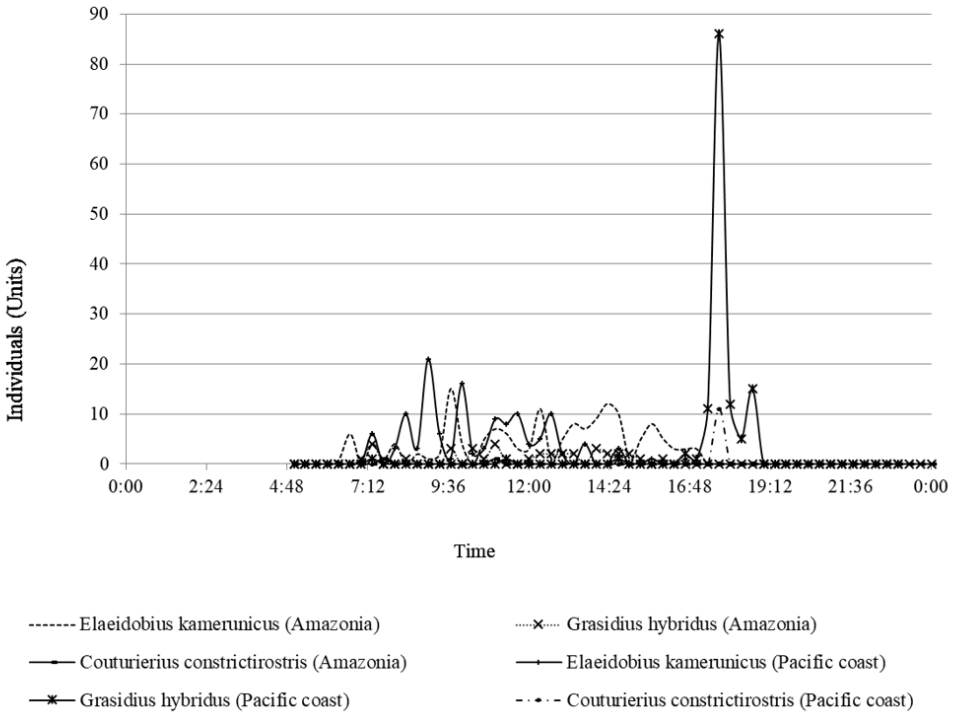

Figure 3. Diurnal and nocturnal insect activity on female flowers of CxL hybrid palms

shorter durations of insect activity on these flowers in comparison to that observed on *E. guineensis* and *E. oleifera* or CxL hybrids. The Unipalma hybrid presented a low number of insects (8 specimens) on female flowers arriving at twilight, between 18:20 and 19:20.

Pollen Transport Capacity of Pollinator Insects

The pollination capacity of each insect was assessed and is shown in Figure 4.

Elaeidobius kamerunicus was the pollinator that showed the highest pollen loading capacity per individual (8,273 pollen grains). This species is significantly different from the other three species studied in terms of the pollen loading capacity. This study presented a high standard deviation of the data, which is assumed to be a result of the fact that male and female insects were not analyzed separately. Male specimens present a high number of corporeal setae, which could increase the amount of pollen that is collected from male flowers (Syed, 1984). The second group of pollinators that showed a high capacity to transport pollen grains from male to female flowers was *G. hybridus* (3,517 grains) and *C. constrictirostris* (2,623 grains). Nevertheless, considering the population results, as *G. hybridus* numbers surpassed those of C. *constrictirostris* on female flowers, *G. hybridus* could be considered a better pollen carrier in oil palm plantations. A third statistical group appeared in this study corresponding to *M. costaricensis*, which was the insect that carried the least

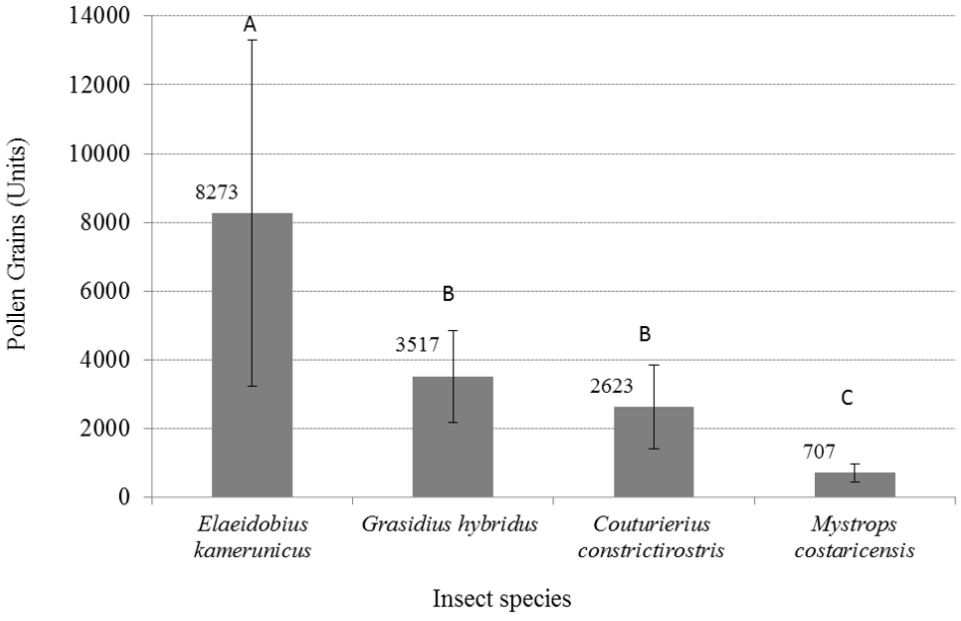

Figure 4. Pollen transport capacity per insect of four pollinator species associated with female flowers on oil palm. ANOVA comparing insects (p=0.000) and Tukey's test at a 95% confidence level

amount of pollen grains (707). The results could be influenced by several factors, including pollinator size, the presence of insect setae and population size (Ågren, 1996; Núñez et al., 2005; Prada et al. 1998; Teo, 2015). As is known, this insect is not a good pollen carrier (Labarca & Narváez , 2009) because of its size and body structure (Kirejtshuk & Couturier, 2010).

These studies were conducted in the Ecuadorian Amazonian and coastal regions, and no significant difference was found among the different insect transport capacities analyzed in each region (Figure 4). These results suggest that the insects are subjected to similar conditions among the male flowers in both regions.

Pollinator Life Cycle

The pollinator life cycles presented some variations among species. These results appear in Table 6.

Eggs and adults were collected from male flowers of oil palm species. The four pollinator species showed 3 larval stages, which were variable according to species. The longest life expectancy corresponded to *C. constrictirostris*, which presented 41.2±4.7 and 36.0±1.8 days in the Amazonia and coastal regions, respectively. *E. kamerunicus* and *G. hybridus* were similar in longevity in the different regions; the former pollinator showed 36.7±4.1 (Amazonia) and 30.3±2.8 days (Pacific coast). In the same way, *G. hybridus* showed

Table 6

Average pollinator life cycle values in the Ecuadorian Amazonia and coastal regions

	Developmental stages (days)						
Insect species	Egg	Larva 1	Larva 2	Larva 3	Pupa	Developmental cycle	Life span
Amazonia							
Elaeidobius kamerunicus	1.3±0.8	1.7±0.9	2.4±0.8	2.3±1.2	3.2±1.0	21.0±4.1	36.7±4.1
Grasidius hybridus	2.3±0.7	1.3±0.8	2.0±1.0	3.9±0.9	5.0±0.9	17.6±2.6	36.4±3.1
Couturierius constrictirostris	2.9±1.3	1.4±0.5	3.1±1.1	2.8±1.1	4.7±1.0	21.3±4.0	41.2±4.7
Pacific coast							
Elaeidobius kamerunicus	1.5±0.5	1.5±0.5	2.7±0.6	2.5±0.5	2.7±0.5	19.3±2.5	30.3±2.8
Grasidius hybridus	2.3±0.5	2.6±0.5	2.6± 0.5	3.6±0.5	3.5±0.5	17.3±1.1	31.8±0.8
Couturierius constrictirostris	2.6±0.5	2.7±0.5	2.7± 0.5	3.4±0.5	3.5±0.5	21.1±1.8	36.0±1.8
Mystrops costaricensis	2.7±0.4	1.6±0.5	2.4±0.5	3.5±0.5	2.6±0.5	13.8±1.2	26.5±1.2

a life expectancy of 36.4±3.1 and 31.8±0.8 in the two studied regions, respectively. The data obtained from the observations of *E. kamerunicus* are different from the results presented by Tuo et al. (2011), who found a life span of 59.18±8.53 days for this insect.

Mystrops costaricensis, a relatively small species of Coleoptera (Nitidulidae), presented the shortest life expectancy among oil palm pollinators (26.5±1.2 days). In the present study, the most numerous insects were *E. kamerunicus* and *G. hybridus*, which coexist and share their ecological niches in the male flowers of OxG hybrids and present good pollen grain transport capacity. The developmental cycles of both species were longer than 15 days. *E. kamerunicus* had a developmental cycle of 21.0±4.1 days in Amazonia and 19.3±2.5 on the coast; these results are within the range presented by Syed (1982) and Tuo et al. (2011). During this life cycle period, the pollinator is able to visit female flowers, pollinating them. In the same way, *G. hybridus* showed a developmental cycle of 17.6±2.6 days in Amazonia and 17.3±1.1 on the coast. According to Greenberg et al. (2005), warm temperatures can positively affect the female oviposition level or longevity in other Curculionidae species.

CONCLUSIONS

Oil palm species are associated with specific pollinator insects. *Elaeis guineensis*, material introduced from Africa, presented high affinity for hosting *E. kamerunicus* on its male flowers, and this insect was present in lesser quantities on female flowers. Only one species of pollinator was observed on flowers of *E. guineensis*, which was *E. kamerunicus*. On *E. oleifera*, the diversity of pollinators was higher, although the pollinator most associated with its flowers was *G. hybridus*, native to tropical America, suggesting coevolution between the palms and their pollinators. OxG hybrids are palms that are associated with high numbers of *E. kamerunicus* and moderate numbers of *G. hybridus*. These results should be considered in agronomic practices on commercial plantations because both insects could be useful for entomophilous pollination. The release of pollinators on plantations must consider population fluctuations during the rainy and dry seasons. In general, in Amazonia during the dry season, pollinators on female flowers are more numerous than they are during the rainy season, and the opposite was registered on the Pacific coast. An increase in the number of male flowers should permit the avoidance of agronomic practices such as manually assisted pollination, particularly in small production units, where the cost of such practices is high.

OxG hybrids offer refuge and food to three species established as their potential pollinators (*E. kamerunicus*, *G. hybridus* and *C. constrictirostris*) that seem to coexist successfully on oil palm plantations. Nevertheless, *E. kamerunicus* and *G. hybridus* are more numerous on hybrid palms, and they maintain their population numbers during the rainy and dry seasons. This pollinator diversity in hybrids could be related to their genetic origin, as they

originate from African and American palms. However, more studies regarding this hypothesis are needed.

Regarding the evaluation of the mobility patterns of pollinators among female flowers, it can be stated that regarding *E. oleifera*, more activity of insects was observed during the day in Amazonia and during twilight on the coast, i.e. *G. hybridus* and *C. constrictirostris*, respectively. For *E. guineensis*, *E. kamerunicus* showed its highest peak of activity on the coast at 10:40, and in Amazonia, the insect maintains its activity the whole morning. This pattern shows the preference of this insect for diurnal activity.

The mobility behavior of insects on CxL hybrid female flowers showed moderated arrivals of the two species (*E. kamerunicus* and *G. hybridus*) all day long. However, the high frequency of arrival of *G. hybridus* on the coast showed that this insect prefers twilight conditions for shifting plants.

The life cycle results from the present work were very similar in Amazonia and on the coast. The good pollen-transport capacity of *E. kamerunicus* and *G. hybridus* (8,273 and 3,517 pollen grains, respectively) as well as the long life expectancy of *E. kamerunicus* (36.7 days in Amazonia and 30.3 days on the coast) and *G. hybridus* (36.4 days in Amazonia and 31.8 days on the Coast) indicate that these two species have potentially useful roles as pollinators on commercial plantations. It is also important to mention that these two insect species are able to coexist on the same oil palm OxG hybrids.

ACKNOWLEDGEMENTS

The authors would like to thank Ing. Francisco Ávalos, Asociación Nacional de Cultivadores de Palma (ANCUPA) [Ecuadorian National Association of oil palm growers], PDR plantation and Energy and Palma plantation for their support during this research.

REFERENCES

Ågren, J. (1996). Population size, pollinator limitation, and seed set in the self-incompatible herb *Lythrum salicaria*. *Ecology*, *77*(6), 1779-1790.

Alvarado, A. Bulgarelli, J., & Moya, B. (2000). Germinación del polen en poblaciones derivadas de un híbrido entre *Elaeis guineensis* Jacq. y *E. oleifera* HBK, *Cortés* [Pollen germination in populations derived from a hybrid between *Elaeis guineensis* Jacq. and *E. oleifera* HBK, Cortés]. *ASD Oil Palm Papers*, *20*, 35-36.

Alvarado, A., Escobar, R., & Henry, J. (2013). *El híbrido OxG Amazon: Una alternativa para regiones afectadas por Pudrición del cogollo en palma de aceite* [The OxG Amazon hybrid: An alternative for regions affected by oil palm bud rot]. Retrieved July 05, 2019, from https://pdfs.semanticscholar.org/21a7/3430cad63889f379a181b06ed14ea410cc61.pdf?_ga=2.5480501.76810772.1571125569-567184117.1569382369

Alvarado, A., Escobar, R., & Peralta, F. (2010). El programa de mejoramiento genético de palma aceitera de ASD Costa Rica y su contribución a la industria [The oil palm genetic improvement program of ASD Costa Rica and its contribution to the industry]. *ASD Oil Palm Papers*, *35*, 13-22.

Appiah, S. O., & Agyei, D. (2013). Studies on Entomophil pollination towards sustainable production and increased profitability in the oil

palm: A review. *Elixir Agriculture*, *55*, 12878-12883.

Asociación Nacional de Cultivadores de Palma Aceitera. (2018). *Palma* [Palm]. Retrieved July 05, 2019, from http://ancupa.com/wp-content/uploads/2018/06/PALMA-Abril-ANCUPA-.pdf

Baford, A. S., Hagen, M., & Borchsenius, F. (2011). Twenty-five years of progress in understanding pollination mechanisms in palms (Arecaceae). *Annals of Botany*, *108*(8), 1503–1516.

Barba, J., & Baquero, Y. (2012). Híbridos OxG obtenidos a partir de oleíferas Taisha Palmar del Río (PDR), Ecuador. Variedad - PDR (Taisha x Avros) [Hybrids OxG obtained from Taisha *oleifera* - Palmar del Rio - Ecuador. Variety - PDR (Taisha x Avros)]. Retrieved July 05, 2019, from https://publicaciones.fedepalma.org/index.php/palmas/article/view/10690/10675

Borror, D., Triplehorn, C., & Johnson, N. (1989). *Introduction to the study of insects* (6th ed.). Springfield, USA: Saunders College Publishing.

Bravo, V., & Bernal, G. (2015). Principales problemas fitosanitarios de la palma aceitera en la región amazónica del Ecuador [Main phytosanitary problems of oil palm in the Amazon region of Ecuador]. *Revista Palma Ecuador*, *28*, 24-27.

Burgos, R. (2013). Ecuador país palmicultor [Palm grower of Ecuador country]. *Revista Palma Ecuador*, *23*, 34-45.

Caudwell, R. (2002). *Polinización con insectos en palma de aceite: ¿Es el momento para evaluar la viabilidad a largo plazo y la sostenibilidad del* Elaeidobius kamerunicus*?* [Pollination with insects in oil palm: Is it time to evaluate the long-term viability and sustainability of *Elaeidobius kamerunicus*?]. Retrieved July 05, 2019, from https://publicaciones.fedepalma.org/index.php/palmas/article/view/906/906

Chinchilla, C., & Richardson, D. (1989). Situación actual de los insectos polinizadores y la polinización en palma aceitera en Centroamérica [Current situation of pollinating insects and pollination in oil palm in Central America]. *Boletín Técnico*, *3*(2), 29-48.

Chinchilla, C., Escalante, M., & Richardson, D. (1990). Polinización en palma aceitera (*Elaeis guineensis* Jacq.) en Centroamérica II. Comportamiento de insectos [Pollination in oil palm (*Elaeis guineensis* Jacq.) in Central America II. Insect behavior]. *Turrialba*, *40*(4), 461-470.

Fomento de Exportaciones de Aceite de Palma y sus derivados. (2017). *Estadísticas* [Statistics]. Retrieved July 05, 2019, from http://www.fedapal.org/web2017/

Galindo, E. (2008). *Problemas y ejercicios de probabilidad y estadística* (3rd ed.) [Problems and exercises of probability and statistics (3rd ed.)]. Quito, Ecuador: Prociencia Editores.

Genty, P. (1985). Polinización entomófila de la palma africana en América tropical. Panel. Tema IV [Entomophilic pollination of African palm in tropical America. Panel. Topic IV]. *Revista Palmas*, *6*(3), 90-101.

Gomes, S. (2011). *Polinizadores e semioquímicos do dendezeiro híbrido (*Elaeis oleifera *(H.B.K.) CORTÉS x* Elaeis guineensis *JACQ)* [Pollinators and semiochemicals of oil palm hybrid (*Elaeis oleifera* (H.B.K.) CORTÉS x *Elaeis guineensis* JACQ)]. Viçosa, Brazil: Universidade Federal de Viçosa.

Greenberg, S., Sétamou, M., Sappington, T., Liu, T., Coleman, R., & Armstrong, J. (2005). Temperature-dependent development and reproduction of the boll weevil (Coleoptera: Curculionidae). *Insect Science*, *12*(6), 449-459.

Hacienda La Cabaña. (2009). *Polinización asistida en material* E.o *x* E.g [Assisted pollination in material of *E.o* x *E.g*]. Bogotá, Colombia: Hacienda La Cabaña S. A.

Hala, N., Tuo, Y., Akpesse, A., Koua, H., & Tano, Y. (2012). Entomofauna of oil palm tree inflorescences at La Mé Experimental Station (Côte d'Ivoire). *American Journal of Experimental Agriculture*, *2*(3), 306-319.

Harun, M. H., & Noor, M. (2002). Fruit set and oil palm bunch components. *Journal of Oil Palm Research*, *14*(2), 24-33.

Henderson, A. (1986). A review of pollination studies in the Palmae. *The Botanical Review*, *53*(3), 221-259.

Herrera, C. M. (1990). Daily patterns of pollinator activity, differential pollinating effectiveness, and floral resource availability, in a summer-flowering Mediterranean shrub. *Oikos*, *58*(3), 277–288.

Hormaza, P., Forero, D., Ruiz, R., & Romero, H. (2010). *Fenología de la palma de aceite africana (*Elaeis guineensis *Jacq.) y el híbrido interespecífico (*Elaeis oleifera *Kunt) Cortés x* Elaeis guineensis *Jacq.* [Phenology of African oil palm (*Elaeis guineensis* Jacq.) and interspecific hybrid (*Elaeis oleifera* Kunt) Cortés x *Elaeis guineensis* Jacq.]. Bogotá, Colombia: Centro de investigación de palma de aceite (Cenipalma).

Jianjun, Y., Zhen, Y., Cheng, B., Zetan, C., Weifu, L., & Fangzhen, J. (2015). Pollination activity of *Elaeidobius kamerunicus* (Coleoptera: Curculionoidea) on oil palm on Hainan Island. *Florida Entomologist*, *9*(2), 499-505.

Kirejtshuk, A., & Couturier, G. (2010). Sap beetles of the tribe Mystropini (Coleoptera: Nitidulidae) associated with South American palm inflorescences. *Annales de la Société entomologique de France (N. S.): International Journal of Entomology*, *46*(3), 367-421.

Labarca, M. V., & Narváez, Z. (2009). Identificación y fluctuación poblacional de insectos polinizadores en palma aceitera (*Elaeis guineensis* Jacquin) en el sur del lago de Maracaibo, estado Zulia, Venezuela [Population identification and fluctuation of pollinating insects in oil palm (*Elaeis guineensis* Jacquin) in the south of Lake Maracaibo, Zulia state, Venezuela]. *Revista de la Facultad de Agronomía*, *26*(3), 305-324.

Louise, C., Amblard, P., & De Franqueville, H. (2007). *Investigaciones dirigidas por el CIRAD sobre las enfermedades del complejo pudrición del cogollo de la palma aceitera en Latinoamérica* [Research conducted by CIRAD on diseases of the rotting complex of the oil palm bud in Latin America]. Retrieved July 05, 2019, from https://publicaciones.fedepalma.org/index.php/palmas/article/view/1270/1270

Meléndez, M., & Ponce, W. (2016). Pollination in the oil palms *Elaeis guineensis*, *E. oleifera* and their hybrids (OxG), in tropical America. *Pesquisa Agropecuaria Tropical*, *46*(1), 102-110.

Mondragón, V., & Roa, J. (1985). *Censo de entomofauna nativa asociada con inflorescencias masculinas y femeninas y análisis de polinización en palma africana (*Elaeis guineensis *Jacq.), palma americana (*Elaeis melanococca*) e híbrido interespecífico (*E. guineensis *x* E. melanococca*) en Colombia* [Census of native entomofauna associated with male and female inflorescences and analysis of pollination in African palm (*Elaeis guineensis* Jacq.), American palm (*Elaeis melanococca*) and interspecific hybrid (*E. guineensis* x *E. melanococca*) in Colombia]. Retrieved July 05, 2019, from https://publicaciones.fedepalma.org/index.php/palmas/article/view/82/82

Núñez, L., Bernal, R., & Knudsen, J. (2005). Diurnal palm pollination by mystropine beetles: Is it weather-related?. *Plant Systematics and Evolution*, *254*(3-4), 149–171.

O'Brien, C. W., Beserra, M., & Couturier, G. (2004). Taxonomy of *Couturierius*, new genus and *Grasidius*, genus new to South America, palm flower weevils in the Derelomini [Coleoptera, Curculionidae]. *Revue Française d'Entomologie*, *26*(4), 145-156.

Pacheco, P., Gnych, S., Dermawan, A., Komarudin, H., & Okarda, B. (2017). *The palm oil global value chain: Implications for economic growth and social and environmental sustainability*. Bogor, Indonesia: Center for International Forestry Research (CIFOR).

Ponnamma, K. N. (1999). Diurnal variation in the population of *Elaeidobius kamerunicus* on the anthesising male inflorescences of oil palm. *Planter*, *75*(881), 405-410.

Prada, M., Molina, D., Villarroel, D., Barrios, R., & Díaz, A. (1998). Efectividad de dos especies del género *Elaeidobius* (Coleoptera: Curculionidae) como polinizadores en palma aceitera [Effectiveness of two species of the genus *Elaeidobius* (Coleoptera: Curculionidae) as pollinators in oil palm]. *Bioagro*, *10*(1), 3-10.

Prasetyo, A., Purba, W., & Susanto, A. (2014). *Elaeidobius kamerunicus*: Application of hatch and carry technique for increasing oil palm fruit set. *Journal of Oil Palm Research*, *26*(3), 195-202.

Rosero, G., & Santacruz, L. (2014). *Efecto de la polinización asistida en medio líquido en la conformación del racimo en material híbrido OxG Guaicaramo S. A.* [Effect of assisted pollination in liquid medium on cluster formation of hybrid material OxG Guaicaramo S. A.]. Retrieved July 05, 2019, from https://publicaciones.fedepalma.org/index.php/palmas/article/view/11028/11013

Salazar, D., Villafuerte, D., Cuichán, M., Orbe, D., & Márquez, J. (2016). *Encuesta de superficie y producción agropecuaria continua (ESPAC) 2016* [Survey of surface and continuous agricultural production (ESPAC) 2016]. Retrieved July 05, 2019, from https://www.ecuadorencifras.gob.ec/documentos/web-inec/Estadisticas_agropecuarias/espac/espac-2016/Informe%20ejecutivo%20ESPAC_2016.pdf

Sánchez, E., Salamanca, J., Calvache, H., Ortiz, L., & Rivera, D. (2004). *Evaluación de poblaciones de polinizadores y su relación con la formación de racimos en la zona de Tumaco, Colombia* [Evaluation of pollinator populations and their relationship with cluster formation in the Tumaco area, Colombia]. Retrieved July 05, 2019, from https://publicaciones.fedepalma.org/index.php/palmas/article/view/1069/1069

Stone G. N., Gilbert, Willmer P. G., Potts S. G., Semida F., & Zalat S. (1999). Windows of opportunity and the temporal structuring of foraging activity in a desert solitary bee. *Ecological Entomology*, *24*(2), 208–221.

Syed, R. (1979). Studies on oil palm pollination by insects. *Bulletin of Entomological Research*, *69*(2), 213-224.

Syed, R. (1982). Insect pollination of oil palm: Feasibility of introducing *Elaeidobius* spp. into Malaysia. In E. Pushparajah & C. P. Soon (Eds.), *The oil palm in agriculture in the eighties: A report of the proceedings of the International Conference on Oil Palm in Agriculture in the Eighties held in Kuala Lumpur from 17-20 June, 1981, Volume 2* (pp. 263-289). Kuala Lumpur, Malaysia: Incorporated Society of Planters.

Syed, R. (1984). *Los polinizadores de la palma africana* [The pollinators of the African palm]. Retrieved July 05, 2019, from https://publicaciones.fedepalma.org/index.php/palmas/article/view/72/72

Syed, R., & Saleh, A. (1988). *Población del* E. kamerunicus *Faust en relación con la conformación del racimo* [Population of *E. kamerunicus* Faust in relation to cluster conformation]. Retrieved July 05, 2019, from https://publicaciones.fedepalma.org/index.php/palmas/article/view/191/191

Teixeira Souza Júnior, M. (2013). *Enfoque y avances del programa de mejoramiento genético de la palma de aceite en Embrapa* [Approach and

advances of the oil palm genetic improvement program in Embrapa]. Retrieved July 05, 2019, from https://publicaciones.fedepalma.org/index.php/palmas/article/view/10680/1066

Teo, T. (2015). Effectiveness of the oil palm pollinating weevil, *Elaeidobius kamerunicus*, in Malaysia. *UTAR Agriculture Science Journal*, *1*(4), 40-43.

Tuo, Y., Koua, H., & Hala, N. (2011). Biology of *Elaeidobius kamerunicus* and *Elaeidobius plagiatus* (Coleoptera: Curculionidae) main pollinators of oil palm in West Africa. *European Journal of Scientific Research*, *49*(3), 426-432.

Turner, P., & Gillbanks, R. (1974). *Oil palm cultivation and management*. Kuala Lumpur, Malaysia: Incorporated Society of Planters.

Yue, J., Yan, Z., Bai, C., Chen, Z., Lin, W., & Jiao, F. (2015). Pollination activity of *Elaeidobius kamerunicus* (Coleoptera: Curculionoidea) on oil palm on Hainan Island. *Florida Entomologist*, *98*(2), 499-506.

Pertanika J. Trop. Agric. Sc. 42 (4): 1321 - 1342 (2019)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Plant Derived Pesticides (*Citrus hystrix* DC, *Mentha* x *piperita* L., *Ocimum basilicum* L.) in Controlling Household Ants (*Tapinoma indicum* (F.), *Pheidole megacephala* (F.), *Monomorium pharaonis* (L.)) (Hymenoptera: Formicidae)

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ABSTRACT

The pest ants-related predicament is increasing in urban area and becoming a problem for most of the citizen. The most common approach for pest ants' control is the application of various insecticide spray. However, the long-term insect pest control must not be dependent on synthetic insecticides with many negative effects. Repellence and insecticidal effects from plants, *Citrus hystrix*, *Mentha piperita* and *Ocimum basilicum* have been reputed for different type of insect pests. The combination of an effective botanical insecticide and gel bait is ideal for the treatment of insect pests. In this study, the repellence and insecticidal effects of plant extracts obtained from three plant species with various concentrations (3 x 10^5, 5 x 10^5, 7 x 10^5, and 1 x 10^6 ppm) were against common urban pest ants, *Tapinoma indicum*, *Pheidole megacephala* and *Monomorium pharaonis* using repellence, insecticidal and gel bait bioassays. The three plant extracts usually repel ants with the efficacy being dependent on the ants and irrespective of plant species. Repellence and mortality of ants were negatively related to the higher percentage (100% = fully repelled; -100% = fully attracted; 0% = neither repelled nor attracted) of being repelled, the lower the death will be. Moreover, the optimal doses that make the fastest mortality of ants are

ARTICLE INFO

Article history:
Received: 25 June 2019
Accepted: 5 August 2019
Published: 13 November 2019

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ISSN: 1511-3701
e-ISSN: 2231-8542

not consistent between the insecticidal and gel bait bioassays.

Keywords: Bio-pesticide, control, household ants, plant extract

INTRODUCTION

Ants are the third most abundant household pests in urban areas, after mosquitoes and cockroaches, as they always foraging in groups have raised annoyances (Lee, 2002). Household pest ants foraging in kitchen area may result in foods contamination and the contaminated foods may not be suitable for consumption. Moreover, ants can serve as carrier of pathogens for several diseases and raised alert of health problems for some people which are more sensitive to insects' bite (Lee & Tan, 2004).

Synthetic or man-made insecticides are currently most common method used for pest ant's management as it is fast and effective. However, it is also necessary to keep in mind the side effects of the continuous use of synthetic or chemical insecticides including the growing of insect pests that are resistant to man-made insecticide, environmental pollution, possibly harmful health of the operators and causing unnecessary dwindling of species (Hebling et al., 2000; Khater, 2012). The efficacy of insecticide spray is also limited considering this method could not reach the heart of the colony which is the queen of the ant's population and only affects the foraging ants which just make up a section of the whole colony (Hanna et al., 2015). Moreover, in comparison to the laboratory, the efficacy of biological control using natural predators in the field is unstable and sometimes, ineffective, considering the constant changing environmental circumstances (Castaño-Quintana et al., 2013).

Bait integrated with toxic active ingredient is currently used to deal with problems of purging worker ants. Toxic bait is highly efficient in colony removal by indulge the pest ants with toxic attractant and utilise the common behaviour of ants, trophallaxis of which allow the ants to disperse the toxic active ingredient throughout the colony, to achieve whole colony elimination. Nevertheless, active ingredient commonly integrated in commercial bait are synthetic insecticides. Thus, botanical insecticides that can achieve the same efficiency as synthetic insecticides could be used to replace them. Botanical-based insecticides are safer, eco-friendly, species-specific, decompose quickly and have very low occurrence of insect pest resistance (Khater 2012; Pavela et al., 2010).

Plants are common in producing secondary metabolites and the toxic properties of these production have been evaluated since ancient times to use against various household insect pests (Adeyemi, 2010; George et al., 2000). Plant extracts and essential oil are becoming increasingly important to replace the synthetic active ingredient in the bait, to minimize synthetic pesticide dependency. In the present study, repellence as well as insecticidal effect of kaffir lime (*Citrux hystrix*), peppermint

(*Mentha piperita*), and basil (*Ocimum basilicum*) were evaluated against the common household pest ants including ghost ants (*Tapinoma indicum*), big-headed ants (*Pheidole megacephala*) and pharaoh ants (*Monomorium pharaonis*). The extracted crude ingredients of plants were also infused into the gel and the performance of gel bait with botanical insecticides were evaluated.

MATERIALS AND METHODS

Plants Extraction

The plant's part used for extraction of kaffir lime, peppermint, and basil were dried leaves using Soxhlet extraction (Handa, 2008). About 30-40g of dried plant leaves were placed into the thimble and a flask contained methanol (about 250-260 mL) as extracting solvent was placed below the thimble. The bottle of methanol was heated, and the vapours contacted with plant materials. The soluble active compounds from the plant material were then transferred into the vaporised methanol. The vapours then condensed, dripped back into thimble, and flowed back into the flask. The flask of methanol was slowly be replaced by methanol that contained the crude extracts of plants. The procedure of the extraction lasted approximately 5-6 hours. The solvent that contained the extract was dried for 1-2 days. About 1-3 g of plant's crude could be obtained and the extract was ready to use.

Prepared solution (concentration calculated / formula):

$$\text{Parts per million} = \frac{\text{grams of solute (g)}}{\text{grams of solution (ml)}} \text{ X } 1{,}000{,}000$$

Ants Collection

In the present study, three type of ants, ghost ants, big-headed ants, and pharaoh ants were collected with live traps from places around the School of Biological Sciences, Universiti Sains Malaysia. The ants were identified based on the descriptions by Klotz et al. (2008).

Bioassays

Repellence Bioassay. Different Concentrations (3 x 10^5, 5 x 10^5, 7 x 10^5 and 1 x 10^6 ppm) of crude extract was prepared. Whatman® No.1 filter paper (90 mm) was splatted in half with one half dipped the solution of crude extracts of various concentrations and the other half was leaving clean. Control was filter paper dipped with distilled water (Figure 1). Thirty ants were used for each treatment of each ant species. Each test was replicated three times. Number of ants at several time intervals including 1 hour after the experiment set up, 3 hours, 6 hours, 12 hours, and every 24 hours up to 3 days of both sides were determined. The behaviour or response as well as the activities of ants toward the tested plant extracts was observed during the bioassay. The repellence percentage was calculated with the following formula (Abdullah et al., 2015):

$$PR = \frac{NC - NT}{NC + NT} \text{ x } 100$$

where, NC = Number of ants on the non-treated side and NT = Number of ants on the treated side.

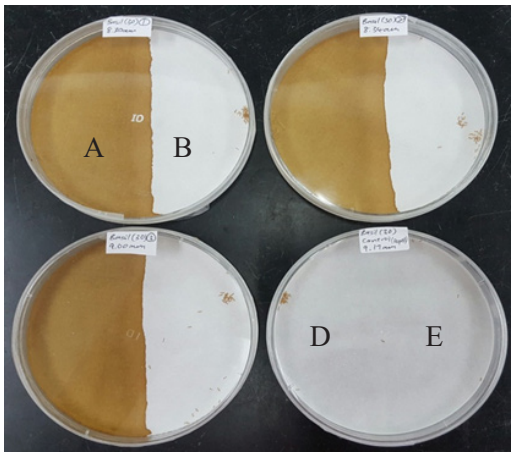

Figure 1. Repellence bioassay of 3 x 10^5 ppm of basil (treated side A and untreated side B) with replicate one, two, three (start from the left to right and bottom left) and control plates (bottom right) (side D treated with distilled water and untreated side E)

Insecticidal Bioassay. Four concentrations of crude extract (3 x 10^5, 5 x 10^5, 7 x 10^5 and 1 x 10^6 ppm) of each tested plant extract were applied on the ants' pronotum with 3 replications. Ants inoculated with distilled water used as control. Collected ant samples were rather aggressive with high mobility, thus, the whole collection tube which contained the ant samples were placed inside the refrigerator for 3-4 minutes to reduce the ants' activity or mobility. The extract solutions (0.1 µL) were then pipetted on the pronotum region. Following the bioassay, mortality of ants was assessed by counting the number of perished samples at time intervals including 1 hour after the experiment set up, 3 hours, 6 hours, 12 hours and every 24 hours and up to 3 days. The ant was considered no longer alive if the individuals remained motionless for 1-2 minutes and no respond given when the body was touched with brush. The behavioural or response as well as any activities showed by the ants toward the tested plant extracts during the bioassay were recorded.

Gel Bait Bioassay. The plant extracts were diluted with 20% sugar solution to several concentrations (3 x 10^5, 5 x 10^5, 7 x 10^5 and 1 x 10^6 ppm). Ferti-plant jelly (Fertiland Trading Co., Malaysia) was then dipped into the plant extract sugar solution, and the jelly absorb and expanded within the solution about 5 hours. The expanded jelly was then measured about 1 g and used as mimic gel bait (Figure 2). Jelly dipped with pure sugar solution was used as control. Repellence effect of the prepared gel as well as the mortality of ants due to after contact with the gel were evaluated at several time intervals including 1 hour after the experiment set up, 3 hours, 6 hours, 12 hours, and every 24 hours up to 3 days. The ant was considered no longer alive if the particular individuals remained motionless for 1-2 minutes and no respond given when the body was touched with brush. The behavioural response as well as any activities showed by the ants toward the tested plant extracts during the bioassay were recorded.

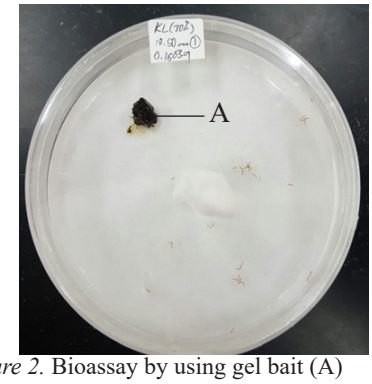

Figure 2. Bioassay by using gel bait (A)

Statistical Analysis

Results from the bioassay on repellence were recorded as percentage of which (100% = fully repelled; -100% = fully attracted; 0% = neither repelled nor attracted). For insecticide and gel bait bioassays, percentage of mortality of each tested ant species were presented. We used Pearson Correlation to demonstrate how the repellence efficacy of the crude extracts relate to ant's mortality (Hinton et al., 2014). The lethal time (LT_{50}, LT_{90}) was calculated with Probit analysis for all plant tested extracts (Akcay, 2013). Analysis of Variance (ANOVA) and significant differences among the various concentrations of the extracts were analysed by Tukey's test ($p < 0.05$) using IBM SPSS Statistic version 22.

RESULTS

Repellence Bioassay

Percentage of Repellence of Tested Plant Extracts with Various Concentrations. Based on Table 1, ghost ants had shown highly repelled from four concentrations of all tested extracts at each time interval. The percentage of repellence (PR) without negative sign had shown that ghost ants were staved off the tested plant extracts, regardless of concentrations (PR = 100% = entirely repelled, PR = 0 = neither attracted nor repelled from the tested extracts, PR = -100% = entirely attracted). Nevertheless, reaction of the tested ant species against the repellence effect showed by each plant extracts was varied. Table 2 presented that big-headed ants was not staved off by all the tested plant extracts and even constantly moving to the side that contain the crude extracts. On the other hand, pharaoh ants were repelled by various concentrations of each plant extract (Table 3) and also more likely to stay idle. Self-grooming of ghost ants could be perceived at high frequency, particularly after the ants were moving out from treated side. Big-headed ants' major workers are most likely surrounded by their minor workers, while individual's aggregation was regularly shown in pharaoh ants.

Mortality of Ants after Contact the Plant Extracts in Repellence Bioassay. There was ant's mortality in repellence bioassay. Ants could have taken the active ingredient of the plant extracts when they moved the side covered with extract solution of which lethal to the tested ants. In the treatments of peppermint (1×10^6 ppm) and basil (3×10^5 ppm), big headed ants had the highest death rate of which full mortality (100%) was observed within 48 hours (Table 5) considering the ants were only little repulsed by the tested plant extracts. The result was also suggesting that big-headed ants were more vulnerable compared to ghost ants and pharaoh ants. Ghost ants and pharaoh ants showed similar rate of mortality (Tables 4 and 6). Efficacy of repellence was negatively associated with the mortality of ants.

Table 1

Percentage of repellence of three plant extracts with various concentrations against Tapinoma indicum

Treatment	Concentration (ppm)	Percentage of repellence (%)						
		1 h	3 h	6 h	12 h	24 h	48 h	72 h
Citrus hystrix	3 x 10^5	84.44±4.443a	84.44±12.372a	88.89±11.110a	88.89±8.013a	75.56±15.555a	66.66±17.638a	57.78±18.189a
	5 x 10^5	97.78±2.223a	100.00±0.000a	100.00±0.000a	95.55±2.223a	93.33±0.000a	77.78±8.890a	57.78±15.553a
	7 x 10^5	100.00±0.000a	95.55±2.223a	97.78±2.223a	88.89±5.879a	71.11±5.879a	42.22±5.879a	48.89±12.372a
	1 x 10^6	88.89±4.443a	100.00±0.000a	93.33±3.848a	100.00±0.000a	93.33±6.667a	82.22±5.879a	60.00±10.183a
Mentha piperita	3 x 10^5	86.67±3.848a	82.22±8.890a	84.45±9.686a	80.00±10.183a	82.22±5.879a	73.33±3.848a	55.55±17.777a
	5 x 10^5	88.89±11.110a	91.11±5.879a	93.33±3.848a	93.33±0.000a	91.11±2.220a	84.44±5.880ab	48.89±14.573a
	7 x 10^5	95.55±2.223a	93.33±3.848a	97.78±2.223a	91.11±5.879a	80.00±7.696a	57.78±9.686a	46.67±3.848a
	1 x 10^6	100.00±0.000a	95.55±2.223a	95.56±4.443a	97.78±2.223a	42.22±2.223ab	42.22±2.223a	17.78±14.573a
Ocimum basilicum	3 x 10^5	80.00±13.879a	97.78±2.223a	100.00±0.000a	93.33±3.848a	100.00±0.000a	97.78±2.223ab	95.55±2.223ab
	5 x 10^5	86.67±3.848a	95.55±2.223a	82.22±2.223a	97.78±2.223a	91.11±4.443a	93.33±3.848ab	86.67±3.848ab
	7 x 10^5	91.11±4.443a	93.33±3.848a	93.33±3.848a	91.11±5.879a	86.67±7.699a	75.55±13.518a	62.22±14.573a
	1 x 10^6	91.11±2.223a	95.55±2.223a	100.00±0.000a	95.55±2.223a	97.78±2.223a	84.45±2.223ab	91.11±2.220ab

* Means followed by the same letter in each column are not significantly different (P > 0.05)

Table 2

Percentage of repellence of three plant extracts with various concentrations against Pheidole megacephala

Treatment	Concentration (ppm)	Percentage of repellence (%)						
		1 h	3 h	6 h	12 h	24 h	48 h	72 h
Citrus hystrix	3 x 10^5	73.33±0.000ab	80.00±13.879a	86.67±7.699a	75.55±13.518a	62.22±2.223a	33.33±26.667a	31.11±28.890a
	5 x 10^5	17.78±13.518a	73.33±6.667a	57.78±11.759a	44.44±19.749a	8.89±23.200a	2.22±19.371a	-4.44±14.571a
	7 x 10^5	46.67±13.876a	64.44±8.013a	73.33±3.848a	40.00±10.183a	24.44±11.113a	17.78±22.555a	17.78±22.555a
	1 x 10^6	86.67±0.000ab	86.67±6.667a	64.44±5.880a	60.00±0.000a	62.22±5.879a	53.33±3.848a	53.33±3.848a
Mentha piperita	3 x 10^5	-4.44 ±15.555a	60.00±7.696a	62.22±8.890a	53.33±13.878a	15.55±17.777a	22.22±2.223a	22.22±2.223a
	5 x 10^5	-15.56 ±24.745b	-22.22 ±13.518b	46.67±7.699a	-4.45±21.199ab	11.11±23.518a	13.33±34.210a	28.89±34.712a
	7 x 10^5	46.67±10.182ab	55.55±13.518a	75.56±8.013a	53.33±7.699a	55.56±8.013a	42.22±8.890a	42.22±9.686a
	1 x 10^6	53.33±3.848a	66.67±10.182a	73.33±6.667a	55.55±11.759a	26.67±7.7699a	26.67±7.699a	24.44±8.013a

Table 2 *(Continued)*

Treatment	Concentration (ppm)	Percentage of repellence (%)						
		1 h	3 h	6 h	12 h	24 h	48 h	72 h
Ocimum basilicum	3 x 10^5	40.00±6.667ab	51.11±8.890a	66.66±13.333a	48.89±5.879a	40.00±3.851a	40.00±3.851a	40.00±3.851a
	5 x 10^5	62.22±14.573ab	55.56±4.443a	46.67±13.333a	68.89±8.890a	4.44±17.356a	15.56±12.372a	31.11±8.890a
	7 x 10^5	55.56±11.113a	80.00±10.183a	82.22±2.223a	71.11±9.688a	64.44±8.013a	40.00±6.667a	36.67±6.938a
	1 x 10^6	62.22±9.686ab	68.90±5.878a	64.44±5.880a	57.78±9.685a	46.66±6.667a	40.00±7.696a	44.44±5.880a

* Means followed by the same letter in each column are not significantly different (P > 0.05)

Table 3

Percentage of repellence of three plant extracts with various concentrations against Monomorium pharaonis

Treatment	Concentration (ppm)	Percentage of repellence (%)						
		1 h	3 h	6 h	12 h	24 h	48 h	72 h
Citrus hystrix	3 x 10^5	88.89±4.443a	91.11±4.443a	95.56±4.443a	84.44±12.372a	31.11±27.306ab	33.33±6.667a	33.33±6.667ab
	5 x 10^5	100.00±0.000ab	100.00±0.000a	97.78±2.223a	100.00±0.000a	97.78±2.223a	88.89±5.879a	88.89±5.879a
	7 x 10^5	97.78±2.223a	95.55±2.223a	97.78±2.223a	100.00±0.000a	93.33±3.848a	64.44±17.356a	71.11±22.552a
	1 x 10^6	100.00±0.000ab	97.78±2.223a	100.00±0.000a	100.00±0.000a	100.00±0.000a	100.00±0.000ab	95.55±2.223a

Table 3 *(Continued)*

Treatment	Concentration (ppm)	Percentage of repellence (%)						
		1 h	3 h	6 h	12 h	24 h	48 h	72 h
Mentha piperita	3 x 10^5	97.78±2.223a	97.78±2.223a	97.78±2.223a	100.00±0.000a	100.00±0.000a	95.56±4.443ab	95.56±4.443a
	5 x 10^5	91.11±2.220a	95.55±2.223a	91.11±4.443a	100.00±0.000a	95.55±2.223a	75.55±9.686a	60.00±15.398a
	7 x 10^5	97.78±2.223a	100.00±0.000a	97.78±2.223a	95.55±2.223a	95.55±2.223a	88.89±4.443a	75.55±2.223a
	1 x 10^6	100.00±0.000ab	100.00±0.000a	97.78±2.223a	93.33±6.667a	86.67±7.699a	68.89±17.356a	62.22±16.024a
Ocimum basilicum	3 x 10^5	100.00±0.000ab	100.00±0.000a	95.56±4.443a	100.00±0.000a	100.00±0.000a	95.55±2.223ab	95.56±4.443a
	5 x 10^5	100.00±0.000ab	95.55±2.223a	100.00±0.000a	88.89±8.013a	73.33±13.878ab	48.89±26.201a	40.00±17.638a
	7 x 10^5	97.78±2.223a	100.00±0.000a	97.78±2.223a	100.00±0.000a	100.00±0.000a	100.00±0.000ab	95.55±2.223a
	1 x 10^6	97.78±2.223a	97.78±2.223a	100.00±0.000a	97.78±2.223a	88.89±5.879a	77.78±15.553a	0.000±0.000ab

* Means followed by the same letter in each column are not significantly different (P > 0.05)

Table 4

Mortality of Tapinoma indicum *after contacted with various concentrations of three plant extracts in repellence bioassay*

Treatment	Concentration (ppm)	Mortality (%)						
		1 h	3 h	6 h	12 h	24 h	48 h	72 h
Citrus hystrix	3×10^5	-	2.22±2.223a	4.45±2.223a	12.22±5.879a	20.00±3.333a	27.78±7.777a	37.78±4.005a
	5×10^5	-	0.00±0.000a	3.33±1.925a	6.67±1.925a	15.56±2.940a	33.33±5.774a	56.67±13.876ab
	7×10^5	-	1.11±1.110a	3.33±1.925a	8.89±4.008a	15.56±2.940a	71.11±17.248ab	90.00±10.000b
	1×10^6	-	0.00±0.000a	0.00±0.000a	5.55±2.223a	17.78±4.005a	57.78±8.011ab	98.89±1.110b
Mentha piperita	3×10^5	-	0.00±0.000a	1.11±1.110a	1.11±1.110a	3.33±1.925a	3.33±1.925a	4.45±2.223a
	5×10^5	-	1.11±1.110a	1.11±1.110a	3.33±3.333a	6.67±5.093a	13.33±8.389a	24.45±11.277a
	7×10^5	-	0.00±0.000a	2.22±2.223a	5.56±1.113a	13.33±1.925a	24.44±6.761a	47.78±10.599ab
	1×10^6	-	1.11±1.110a	1.11±1.110a	4.44±2.940a	15.56±5.557a	63.33±10.713ab	93.33±3.333b
Ocimum basilicum	3×10^5	-	0.00±0.000a	2.22±1.110a	2.22±1.110a	2.22±1.110a	3.33±1.925a	3.33±1.925a
	5×10^5	-	1.11±1.110a	3.33±3.333a	6.67±1.925a	6.67±1.925a	12.22±1.110a	20.00±3.333a
	7×10^5	-	5.55±2.223ab	11.11±1.110ab	17.78±6.187ab	22.22±9.095ab	32.22±12.223a	47.78±11.110ab
	1×10^6	-	0.00±0.000a	0.00±0.000a	1.11±1.110a	1.11±1.110a	6.66±3.333a	17.78±1.110a
Control	-	-	0.00±0.000a	0.00±0.000a	0.00±0.000a	0.00±0.000a	0.00±0.000a	0.00±0.000a

* Means followed by the same letter in each column are not significantly different ($P > 0.05$)
* '-' No test conducted due to no mortality

Table 5

Mortality of Pheidole megacephala *after contacted with various concentrations of three plant extracts in repellence bioassay*

Treatment	Concentration (ppm)	Mortality (%)						
		1 h	3 h	6 h	12 h	24 h	48 h	72 h
Citrus hystrix	3×10^5	6.67±6.667a	20.00±13.879a	30.00±20.817a	58.89±27.845ab	66.67±31.683a	75.56±22.799a	82.22±16.140a
	5×10^5	2.22±2.223a	3.33±3.333a	14.45±7.779a	42.22±20.215a	83.33±12.019a	95.56±4.443a	98.89±1.110a
	7×10^5	0.00±0.000a	12.22±5.553a	20.00±5.774a	48.89±10.599a	86.67±3.333a	98.89±1.110a	100.00±0.000a
	1×10^6	1.11±1.110a	25.56±12.372a	52.22±12.223ab	78.89±2.940ab	93.33±1.925a	95.55±2.223a	97.78±2.223a
Mentha piperita	3×10^5	0.00±0.000a	0.00±0.000a	0.00±0.000a	17.78±4.447a	50.00±6.938ab	92.22±2.939a	96.67±0.000a
	5×10^5	0.00±0.000a	0.00±0.000a	0.00±0.000a	14.45±4.005a	44.44±4.841ab	86.67±5.774a	95.55±2.223a
	7×10^5	0.00±0.000a	6.67±1.925a	26.67±5.093a	52.22±6.758a	83.34±6.667a	91.11±5.557a	100.00±0.000a
	1×10^6	0.00±0.000a	2.22±2.223a	17.78±2.223a	56.67±6.938a	95.55±2.223a	100.00±0.000a	100.00±0.000a
Ocimum basilicum	3×10^5	0.00±0.000a	3.33±0.000a	18.89±6.758a	60.00±11.547ab	97.78±1.110a	100.00±0.000a	100.00±0.000a
	5×10^5	0.00±0.000a	0.00±0.000a	3.33±1.925a	21.11±7.776a	44.45±11.759ab	85.56±9.492a	97.78±1.110a
	7×10^5	1.11±1.110a	7.78±2.939a	23.33±3.333a	37.78±1.110a	62.22±4.005a	94.45±2.223a	98.89±1.110a
	1×10^6	0.00±0.000a	2.22±2.223a	20.00±3.851a	46.67±7.699a	93.33±1.925a	94.44±1.113a	97.78±2.223a
Control	-	0.00±0.000a	0.00±0.000a	0.00±0.000a	0.00±0.000a	0.00±0.000b	0.00±0.000b	0.00±0.000b

* Means followed by the same letter in each column are not significantly different (P > 0.05)

Table 6

Mortality of Monomorium pharaonis *after contacted with various concentrations of three plant extracts in repellence bioassay*

Treatment	Concentration (ppm)	Mortality (%)						
		1 h	3 h	6 h	12 h	24 h	48 h	72 h
Citrus hystrix	3×10^5	-	2.22±2.223a	2.22±2.223a	4.44±4.443a	42.22±16.024a	100.00±0.000a	100.00±0.000a
	5×10^5	-	0.00±0.000a	1.11±1.110a	2.22±1.110a	4.44±1.113ab	6.67±1.925b	7.78±2.939b
	7×10^5	-	0.00±0.000a	0.00±0.000a	0.00±0.000a	5.56±2.940ab	42.22±14.573ab	87.78±12.223a
	1×10^6	-	0.00±0.000a	1.11±1.110a	2.22±1.110a	5.55±4.005ab	5.55±4.005b	15.55±6.187b
Mentha piperita	3×10^5	-	0.00±0.000a	1.11±1.110a	1.11±1.110a	1.11±1.110ab	1.11±1.110b	3.33±1.925b
	5×10^5	-	2.22±1.110a	2.22±1.110a	10.00±1.923a	27.78±11.600ab	75.56±22.799ab	84.44±15.557a
	7×10^5	-	0.00±0.000a	2.22±1.110a	4.44±1.113a	6.67±1.925ab	13.33±3.848b	26.67±8.389b
	1×10^6	-	1.11±1.110a	4.44±4.443a	10.00±8.390a	21.11±8.678ab	32.22±7.777ab	47.78±10.941ab
Ocimum basilicum	3×10^5	-	0.00±0.000a	0.00±0.000a	0.00±0.000a	0.00±0.000b	0.00±0.000b	1.11±1.110b
	5×10^5	-	0.00±0.000a	0.00±0.000a	0.00±0.000a	22.22±11.277ab	65.56±32.792ab	78.89±21.110ab
	7×10^5	-	0.00±0.000a	0.00±0.000a	1.11±1.110a	1.11±1.110b	1.11±1.110b	5.55±2.223b
	1×10^6	-	0.00±0.000a	0.00±0.000a	1.11±1.110a	4.44±2.934b	28.89±7.776ab	70.00±15.275ab
Control	-	-	0.00±0.000a	0.00±0.000a	0.00±0.000a	0.00±0.000b	0.00±0.000b	0.00±0.000b

* Means followed by the same letter in each column are not significantly different ($P > 0.05$)
* '-' No test conducted due to no mortality

Repellence Efficacy Correlates with Ants Mortality. Pearson correlation was conducted to demonstrate how the repellence efficacy related with mortality of ants. Tables 7, 8 and 9 showed that the efficacy of repellence was negatively associated with ant mortality, extract that high in repellence effect, the less likely of causing ants' mortality. Nevertheless, positive Pearson correlation coefficients were shown in some treatments of which the ants were more attracted to the treated side but low mortality.

Table 7

Repellence efficacy of plant extracts correlate with Tapinoma indicum *mortality*

Treatment	Concentration (ppm)	r
Citrus hystrix	3×10^5	-0.726**
	5×10^5	-0.913**
	7×10^5	-0.917**
	1×10^6	-0.817**
Mentha piperita	3×10^5	-0.190
	5×10^5	-0.424
	7×10^5	-0.865**
	1×10^6	-0.832**
Ocimum basilicum	3×10^5	0.288
	5×10^5	-0.206
	7×10^5	-0.870**
	1×10^6	-0.383

** Correlation is significant at $P < 0.01$

Table 8

Repellence efficacy of plant extracts correlate with Pheidole megacephala *mortality*

Treatment	Concentration (ppm)	r
Citrus hystrix	3×10^5	-0.273
	5×10^5	-0.721**
	7×10^5	-0.678**
	1×10^6	-0.851**
Mentha piperita	3×10^5	-0.350
	5×10^5	0.161
	7×10^5	-0.216
	1×10^6	-0.776**
Ocimum basilicum	3×10^5	-0.369
	5×10^5	-0.483*
	7×10^5	-0.595**
	1×10^6	-0.624**

** Correlation is significant at $P < 0.01$
* Correlation is significant at $P < 0.05$

Table 9

Repellence efficacy of plant extracts with Monomorium pharaonis *mortality*

Treatment	Concentration (ppm)	r
Citrus hystrix	3×10^5	-0.754**
	5×10^5	-0.809**
	7×10^5	-0.708**
	1×10^6	-0.275
Mentha piperita	3×10^5	-0.220
	5×10^5	-0.543*
	7×10^5	-0.897**
	1×10^6	-0.910**
Ocimum basilicum	3×10^5	0.102
	5×10^5	-0.963**
	7×10^5	-0.416
	1×10^6	-0.921**

** Correlation is significant at $P < 0.01$
* Correlation is significant at $P < 0.05$

Insecticidal Bioassay

Probit Analysis. Lethal time of causing 50% and 90% of ants' mortality was determined using Probit analysis. The analysis was important in evaluate the efficacy of each of the concentration of tested plant extracts in killing tested ants' species as the shorter time needed in causing 50% and 90% death of the population, indicating the more effective of the plants being used as botanical insecticide. Each tested plant with various concentrations showed varied insecticide effect toward the tested ant species (Tables 10, 11 and 12). The findings showed that the efficiency of plant extracts in killing tested ants was not dose-based, where low concentration could lead to higher ant's mortality than the high concentration.

Kaffir lime extract with concentration, 5 x 10^5 ppm was the most effective treatment for ghost ants with LT_{50} of about 3 hours and 4 minutes (Table 10). On the contrary, big-headed ants reach 50% of mortality within 1 hour and 47 minutes using basil crude extract with concentration, 1 x 10^6 ppm (Table 11). Peppermint extract with concentration, 1 x 10^6 ppm was most usefully used against pharaoh ants with LT_{50} of about 7 hours and 48 minutes (Table 12).

In general, for ghost ants, about 3-9 hours were needed for the tested population to reach 50% mortality in all tested plant extracts (Table 10) while pharaoh ants were about 10 hours (Table 12). Big-headed ants have the lowest LT_{50} of which about 1-7 hours (Table 11). The results again support the previous hypothesis that the big-headed ants are vulnerable and more susceptible than others toward contaminant and toxicant.

Table 10

Time needed to reach 50% and 90% lethal of Tapinoma indicum *inoculated with various concentrations of tested plant extracts*

Treatment	Concentration (ppm)	LT_{50}	95% Confidence limits (hours)		LT_{90}	95% Confidence limits (hours)		X^2	df
			Lower	Upper		Lower	Upper		
Citrus hystrix	3 x 10^5	4.89	3.67	6.38	10.32	7.63	18.73	17.66a	5
	5 x 10^5	3.08	2.23	4.06	9.32	6.69	15.96	12.59a	5
	7 x 10^5	6.38	5.08	7.94	15.76	12.01	24.18	10.85a	5
	1 x 10^6	3.71	3.19	4.27	12.99	10.83	16.25	5.99a	5
Mentha piperita	3 x 10^5	5.97	5.43	6.55	11.53	10.13	13.65	3.72a	5
	5 x 10^5	4.29	3.77	4.86	12.32	10.45	15.12	7.61a	5
	7 x 10^5	5.67	4.53	7.00	15.79	12.10	23.27	8.93a	5
	1 x 10^6	3.69	2.73	4.82	10.67	7.73	17.93	12.65a	5

Table 10 *(Continued)*

Treatment	Concentration (ppm)	LT_{50}	95% Confidence limits (hours)		LT_{90}	95% Confidence limits (hours)		X^2	df
			Lower	Upper		Lower	Upper		
Ocimum basilicum	3 x 10^5	9.78	6.21	15.54	24.75	15.57	73.53	38.12a	5
	5 x 10^5	5.31	3.54	7.72	15.13	9.95	33.97	24.58a	5
	7 x 10^5	3.67	3.20	4.17	10.70	9.05	13.18	3.53a	5
	1 x 10^6	5.60	4.04	7.63	13.74	9.67	26.78	19.89a	5

* Means followed by the same letter in each column are not significantly different (P > 0.05)

Table 11

Time needed to reach 50% and 90% lethal of Pheidole megacephala *inoculated with various concentrations of tested plant extracts*

Treatment	Concentration (ppm)	LT_{50}	95% Confidence limits (hours)		LT_{90}	95% Confidence limits (hours)		X^2	*df*
			Lower	Upper		Lower	Upper		
Citrus hystrix	3 x 10^5	7.38	6.47	8.39	24.48	20.57	30.27	3.48a	5
	5 x 10^5	3.00	2.39	3.68	7.41	5.79	10.74	8.47b	5
	7 x 10^5	2.49	1.84	3.20	6.60	4.93	10.44	11.27b	5
	1 x 10^6	3.48	1.39	6.43	9.01	5.16	58.84	57.06b	5
Mentha piperita	3 x 10^5	6.56	4.78	8.68	37.13	23.64	63.36	8.74a	5
	5 x 10^5	3.72	2.81	4.78	8.82	6.60	14.30	13.56b	5
	7 x 10^5	3.19	2.44	4.06	6.94	5.27	11.11	13.25b	5
	1 x 10^6	3.06	2.48	3.71	7.03	5.57	9.97	8.20b	5
Ocimum basilicum	3 x 10^5	2.78	2.45	3.12	6.50	5.61	7.83	2.26b	5
	5 x 10^5	2.82	2.51	3.15	5.80	5.09	6.85	4.17b	5
	7 x 10^5	2.77	1.07	4.83	6.06	3.70	36.08	53.81b	5
	1 x 10^6	1.79	1.58	2.01	3.65	3.17	4.36	3.46b	5

* Means followed by the same letter in each column are not significantly different (P > 0.05)

Table 12

Time needed to reach 50% and 90% lethal of Monomorium pharaonis *inoculated with various concentrations of tested plant extracts*

Treatment	Concentration (ppm)	LT_{50}	95% Confidence limits (hours)		LT_{90}	95% Confidence limits (hours)		X^2	df
			Lower	Upper		Lower	Upper		
Citrus hystrix	3 x 10^5	12.05	7.45	20.46	26.42	16.63	98.37	48.27a	5
	5 x 10^5	10.84	8.61	13.75	22.64	17.18	36.52	14.86a	5
	7 x 10^5	14.75	10.87	20.12	33.31	23.69	62.61	22.68a	5
	1 x 10^6	13.98	10.52	18.77	28.03	20.50	52.02	22.50a	5

Table 12 *(Continued)*

Treatment	Concentration (ppm)	LT_{50}	95% Confidence limits (hours)		LT_{90}	95% Confidence limits (hours)		X^2	df
			Lower	Upper		Lower	Upper		
Mentha piperita	3 x 10^5	13.73	10.68	17.86	27.43	20.53	47.03	18.50a	5
	5 x 10^5	14.40	10.98	18.97	26.50	19.91	48.30	22.10a	5
	7 x 10^5	17.59	12.24	25.39	42.01	28.42	92.57	29.13a	5
	1 x 10^6	7.80	4.30	13.77	23.17	13.27	95.37	47.66a	5
Ocimum basilicum	3 x 10^5	20.07	11.84	35.35	49.01	29.47	204.36	52.51a	5
	5 x 10^5	14.21	10.04	20.27	34.07	23.24	71.91	26.95a	5
	7 x 10^5	14.99	13.71	16.39	27.63	24.50	32.26	2.14a	5
	1 x 10^6	11.81	3.98	35.16	30.66	15.26	1473.16	105.01b	5

* Means followed by the same letter in each column are not significantly different (P > 0.05)

Bioassay using Gel Bait

Probit Analysis. Gel mixed with various concentrations of plant extracts exhibited slow mortality effect, indicating more time is needed for the ants to be killed considering indirect and delayed contact of ants with the active ingredient within the crude extracts. However, the mortality trend of the tested ant species remained the same as big-headed ants was the first to succumb with LT_{50} of about 10-29 hours (Table 14), followed by ghost ants (14-48 hours) (Table 13) and lastly, pharaoh ants (29-56 hours) (Table 15).

Based on Tables 13-15, the ants' mortality was not dose-related as in some of the low concentration treatments having shorter time in leading to ants' mortality. According to Table 13, basil extract with concentration, 3 x 10^5 ppm was the most effective treatment against ghost ants with LT_{50} of about 14 hours and 40 minutes while the same plant species with concentration, 5 x 10^5 ppm was most useful in against big-headed ants (LT_{50}, 10 hours and 51 minutes) (Table 14). Pharaoh ants were most susceptible toward kaffir lime with concentration, 7 x 10^5 ppm (LT_{50}, 29 hours and 12 minutes) (Table 15).

Table 13

Time needed to reach 50% and 90% lethal of Tapinoma indicum *after contacted with various concentrations of tested plant extracts integrated into gel*

Treatment	Concentration (ppm)	LT_{50}	95% Confidence limits (hours)		LT_{90}	95% Confidence limits (hours)		X^2	df
			Lower	Upper		Lower	Upper		
Citrus hystrix	3 x 10^5	47.98	29.23	134.75	136.54	70.23	-	44.48a	5
	5 x 10^5	33.10	-	-	64.00	-	-	-	5
	7 x 10^5	36.34	24.38	57.36	82.19	55.36	280.10	36.40a	5
	1 x 10^6	26.02	8.03	104.30	50.25	28.43	-	112.08a	5

Table 13 *(Continued)*

Treatment	Concentration (ppm)	LT_{50}	95% Confidence limits (hours)		LT_{90}	95% Confidence limits (hours)		X^2	df
			Lower	Upper		Lower	Upper		
Mentha piperita	3 x 10^5	31.70	-	-	54.53	-	-	-	5
	5 x 10^5	30.73	22.96	41.25	46.10	35.75	90.71	31.60a	5
	7 x 10^5	28.61	24.74	33.12	43.58	36.99	57.56	10.18a	5
	1 x 10^6	25.37	-	-	51.23	-	-	-	5
Ocimum basilicum	3 x 10^5	14.67	9.00	24.66	26.18	17.74	117.60	50.78b	5
	5 x 10^5	15.28	9.93	23.84	39.74	25.16	108.06	36.33b	5
	7 x 10^5	27.04	-	-	41.94	-	-	-	5
	1 x 10^6	25.99	3.96	339.61	45.88	26.87	-	115.64c	5

* '-' Fiducial limits could not be generated

* Same column with same letter is not significantly different (P > 0.05)

Table 14

Time needed to reach 50% and 90% lethal of Pheidole megacephala *after contacted with various* concentrations of tested plant extracts integrated into gel

Treatment	Concentration (ppm)	LT_{50}	95% Confidence limits (hours)		LT_{90}	95% Confidence limits (hours)		χ^2	*df*
			Lower	Upper		Lower	Upper		
Citrus hystrix	3 x 10^5	29.10	19.05	49.31	43.44	31.67	207.31	50.29a	5
	5 x 10^5	24.11	18.42	31.73	42.61	32.26	76.63	24.00a	5
	7 x 10^5	19.15	13.50	27.63	32.83	23.82	80.37	35.13a	5
	1 x 10^6	16.93	13.40	21.56	25.33	20.23	43.70	22.48a	5
Mentha piperita	3 x 10^5	29.16	22.46	38.85	43.53	34.04	85.54	27.05a	5
	5 x 10^5	17.33	16.10	18.64	24.81	22.69	27.97	7.75b	5
	7 x 10^5	18.57	14.47	23.89	34.04	26.03	56.93	19.58a	5
	1 x 10^6	25.36	-	-	48.59	-	-	871.79	5
Ocimum basilicum	3 x 10^5	15.29	-	-	24.97	-	-	1083.45	5
	5 x 10^5	10.85	7.65	15.85	21.96	15.20	52.54	31.49c	5
	7 x 10^5	10.91	8.50	14.19	21.44	16.07	37.36	18.38c	5
	1 x 10^6	11.85	9.87	14.30	23.68	18.86	33.96	10.29c	5

* '-' Fiducial limits could not be generated

* Same column with same letter is not significantly different (P > 0.05)

Table 15

Time needed to reach 50% and 90% lethal of Monomorium pharaonis *after contacted with various concentrations of tested plant extracts integrated into gel*

Treatment	Concentration (ppm)	LT_{50}	95% Confidence limits (hours)		LT_{90}	95% Confidence limits (hours)		X^2	df
			Lower	Upper		Lower	Upper		
Citrus hystrix	3 x 10^5	49.36	34.07	66.42	77.86	60.21	256.67	30.30a	5
	5 x 10^5	32.06	-	-	49.38	-	-	-	5
	7 x 10^5	29.20	19.39	43.43	57.25	39.50	149.75	40.52a	5
	1 x 10^6	31.32	-	-	53.95	-	-	927.81	5
Mentha piperita	3 x 10^5	56.02	42.21	88.88	113.69	76.83	483.61	23.26a	5
	5 x 10^5	39.64	17.10	288.34	104.91	50.94	-	86.19b	5
	7 x 10^5	40.42	15.71	69.29	62.39	44.28	985.64	70.74b	5
	1 x 10^6	32.90	20.06	51.10	51.38	36.93	175.84	55.84b	5
Ocimum basilicum	3 x 10^5	38.71	18.36	127.86	89.72	49.16	-	77.88b	5
	5 x 10^5	29.46	22.81	38.75	44.01	34.58	82.53	26.35b	5
	7 x 10^5	38.35	22.19	58.79	61.59	44.30	242.59	56.66b	5
	1 x 10^6	39.68	36.84	42.53	61.14	56.10	68.33	5.35a	5

* '-' Fiducial limits could not be generated

* Same column with same letter is not significantly different (P > 0.05)

DISCUSSION

Repellence and Insecticide Efficiency of Three Plants

In repellence bioassay, all three tested plant extracts of various concentrations showed repellence effect with all tested ants (ghost ants, big-headed ants and pharaoh ants). Ants already showed repellence against to the lowest concentration which was 3 x 10^5 ppm from each tested plant extract let alone the higher concentrations (5 x 10^5, 7 x 10^5 and 1 x 10^6 ppm) tested.

Ants species that tested in the present study avoid contact with the crude extract (treated side), the tested plants species are commonly produced secondary metabolites such as linalool, citronellol, and β-citronellol by *C. hystrix* (Loh et al., 2011; Nor, 1999), menthone by *M. piperita* as well as trans-anethole, estragole and linalool in *O. basilicum* (Chang et al., 2009), which have repellence (Kumar et al., 2011; Tawatsin et al., 2001) and insecticidal effect (Bakkali et al., 2008; Loh et al., 2011). Ants can also be repelled as they perceive the pungent smell emitted from crude extracts through their olfactory system (Ab Majid et al., 2018).

Ghost ants and pharaoh ants were highly repelled from the plant extracts and had slow mortality. However, big-headed ants were generally attracted to the treated side, followed by high mortality in all treatments. Big-headed ants are considered as predator species or invasive

ants in natural environment (Hoffmann & O'Connor, 2004) and intrinsically tend to constantly extend their colony by producing a large amount of worker ants to compete with other species (Dejean et al., 2007). The aggressive characteristic of this species might be the reason for big-headed ants was less repelled from all tested plant crude extracts. Nonetheless, as this species tends to produce many individual ants in order to be dominant on a particular area, the ants tend to have lower survival rate (Fournier et al., 2009). This phenomenon was shown in all bioassays in the present study of which big-headed ants took lesser time to reach mortality. Therefore, it is assumed that non-aggressive ant species such as ghost ants and pharaoh ants have reduced mortality rates to compensate for their competition weakness in natural environment.

Ant's Mortality

Mortality of ants could be observed throughout the study. This may be due to the ants' intrinsic behaviour including self- and allo-grooming when confronted with contaminant and the contacted active ingredient (Hughes et al., 2002) was accidentally ingested except in the insecticidal bioassay of which the active ingredient was unavoidable. Self-grooming is the individual self-cleaning behaviour while allo-grooming are group-level cleaning behaviours that remove the contaminants from another individual of which commonly happened in ant society considering the ants are capable of detecting the presence of contaminant as well as the risk of infection that occurred either on their own self or on their nestmates (Bos et al., 2012; Morelos-Juárez et al., 2010; Walker & Hughes, 2009). Thus, the frequency of self- and allo-grooming of all tested ants were high as shown in the present study due to this behaviour as a way to remove the contaminant. Although allo-grooming could decrease the contamination surface of ants, this behaviour was not performed as frequent as self-grooming throughout the experiment considering that the contaminated ants were tending to exclude themselves from their broods and perished in isolation reduce the chances of its broods getting contaminated as well (Bos et al., 2012).

Self-grooming can even happen before contamination takes place or when a detrimental substance is detected. This occurrence may indicate that self-cleaning behaviour is more likely intrinsic than a simple pathogens reaction (Morelos-Juárez et al., 2010). However, grooming could be a useful behaviour to be used in insect pest control.

Potential of Botanical Insecticide that used in Toxic Bait

The insecticidal effects of kaffir lime, peppermint and basil showed in the present study were similar and not significantly different from one another ($p < 0.05$). In these three different plant species, similar chemical components of the secondary metabolites could be the primary reason for similarity in insecticide effect. Thus, the different rate of mortality among the tested ants may due to their different susceptibility

level toward the plant extracts. Nevertheless, the supplication of plant extracts directly or indirectly does increase the rate of mortality of all the tested ant species as the ant samples within the control treatment remain alive and active.

With its inclusion into a gel, the potential of kaffir lime, peppermint and basil to be used as botanical insecticide was evaluated. The repellence effect of all the tested plant species was offset by adding sugar as an attractant with insecticidal effect was not impaired. Furthermore, the movement of ants was not affected immediately and there was a minor mortality in the first three hours of each treatment which could meet the requirement of the delayed action of insecticide required in gel bait (Williams et al., 2001). The phenomenon of ' bait-shyness ' (Greaves, 1989) was not observed throughout the experiment and ants were willing to go near to the toxicant contained gel. Results of the gel bait bioassays showed 100% of mortality can be attained within 2-3 days for most replicates in different treatments.

CONCLUSION

The tested ants become very susceptible increased rate of mortality after contact with the solution from all the tested plant extracts. Different plant extracts were effective in treated specific species in different bioassays. In insecticidal bioassay, kaffir lime (5×10^5 ppm) was the most efficient in control ghost ants while in gel bait bioassay, basil (3×10^5 ppm) causing the shortest LT_{50}. Big-headed ants were most effectively controlled by basil with concentration, 1×10^6 ppm in insecticidal bioassay while in gel bait bioassay, higher concentration (5×10^5 ppm) of the same species was more useful. Peppermint (1×10^6 ppm) was the most effective in purging pharaoh ants in insecticidal bioassay while kaffir lime (7×10^5 ppm) was the most useful in gel bait bioassay.

ACKNOWLEDGEMENT

The author would like to thank School of Biological Sciences for the facilities. This project was under the support of Fundamental Research Grant (FRGS) 203 / PBIOLOGI / 6711360.

CONFLICT OF INTEREST STATEMENT

The researchers declare that there is no conflict of interest.

REFERENCES

Ab Majid, A. H., Dieng, H., Ellias, S. S., Sabtu, F. S., Rahim, A. H., & Satho, T. (2018). Olfactory behaviour and response of household ants (Hymenoptera) to different types of coffee odor: A coffee-based bait development prospect. *Journal of Asia-Pacific Entomology*, *21*(1), 46-51.

Abdullah, F., Subramanian, P., Ibrahim, H., Malek, S. N. A., Lee, G. S., & Hong, S. L. (2015). Chemical composition, antifeedant, repellent, and toxicity activities of the rhizomes of galangal, *Alpinia galanga* against Asian subterranean termites, *Coptotermes gestroi* and *Coptotermes curvignathus* (Isoptera: Rhinotermitidae). *Journal of Insect Science*, *15*(1), 7.

Adeyemi, M. H. (2010). The potential of secondary metabolites in plant material as deterrents against insect pests: A review. *African Journal of Pure and Applied Chemistry*, *4*(11), 243-246.

Akcay, A. (2013). The calculation of LD50 using probit analysis. *The Federation of American Societies for Experimental Biology Journal*, *27*(1), 1217-1228.

Bakkali, F., Averbeck, S., Averbeck, D., & Idaomar, M. (2008). Biological effects of essential oils – A review. *Food and Chemical Toxicology*, *46*(2), 446-475.

Bos, N., Lefevre, T., Jensen, A. B., & d'Ettorre, P. (2012). Sick ants become unsociable. *Journal of Evolutionary Biology*, *25*(2), 342-351.

Castaño-Quintana, K., Montoya-Lerma, J., & Giraldo-Echeverri, C. (2013). Toxicity of foliage extracts of *Tithonia diversifolia* (Asteraceae) on *Atta cephalotes* (Hymenoptera: Myrmicinae) workers. *Industrial Crops and Products*, *44*, 391-395.

Chang, C. L., Cho, I. K., & Li, Q. X. (2009). Insecticidal activity of basil oil, trans-anethole, estragole, and linalool to adult fruit flies of *Ceratitis capitata*, *Bactrocera dorsalis*, and *Bactrocera cucurbitae. Journal of Economic Entomology*, *102*(1), 203-209.

Dejean, A., Kenne, M., & Moreau, C. S. (2007). Predatory abilities favour the success of the invasive ant *Pheidole megacephala* in an introduced area. *Journal of Applied Entomology*, *131*(9-10), 625-629.

Fournier, D., De Biseau, J. C., & Aron, S. (2009). Genetics, behaviour and chemical recognition of the invading ant *Pheidole megacephala*. *Molecular Ecology*, *18*(2), 186-199.

George, J., Bais, H.P., & Ravishankar, G. A. (2000). Biotechnological production of plant-based insecticides. *Critical Reviews in Biotechnology*, *20*(1), 49-77.

Greaves, J. H. (1989). *Rodent pests and their control in the Near East*. Rome, Italy: Food and Agriculture Organization of the Unite Nations.

Handa, S. S. (2008). An overview of extraction techniques for medicinal and aromatic plants. In S. S. Handa, S. P. S. Khanuja, G. Longo, & D. D. Rakesh (Eds.), *Extraction technologies for medicinal and aromatic plants* (pp. 21-52). Trieste, Italy: International Centre for Science and High Technology.

Hanna, R., Kuate, A. F., Nanga, S. N., Tindo, M., & Nagel, P. (2015). Boric acid for suppression of the ant *Anoplolepis tenella* and effects on an associated scale insect pest *Stictococcus vayssierei* in cassava fields in the Congo Basin. *Crop Protection*, *74*, 131-137.

Hebling, M. J. A., Bueno, O. C., Maroti, P. S., Pagnocca, F. C., & Da Silva, O. A. (2000). Effects of leaves of *Ipomoea batatas* (Convolvulaceae) on nest development and on respiratory metabolism of leaf-cutting ants *Atta sexdens* L. (Hym., Formicidae). *Journal of Applied Entomology*, *124*(5-6), 249-252.

Hinton, P. R., McMurray, I. , & Brownlow, C. (2014). *SPSS explained.* London, United Kingdom: Routledge.

Hoffmann, B. D., & O'Connor, S. (2004). Eradication of two exotic ants from Kakadu National Park. *Ecological Management and Restoration*, *5*(2), 98-105.

Hughes, W. O. H., Eilenberg, J., & Boomsma, J. J. (2002). Trade-offs in group living: Transmission and disease resistance in leaf-cutting ants. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *269*(1502), 1811-1819.

Khater, H. F. (2012). Prospects of botanical biopesticides in insect pest management. *Pharmacologia*, *3*(12), 641-656.

Klotz, J. H., Hansen, L., Pospischil, R., & Rust, M. (2008). *Urban ants of North America and Europe: identification, biology, and management.* Ithaca, NY: Comstock Publishing Associates Cornell University Press.

Kumar, S., Wahab, N., & Warikoo, R. (2011). Bioefficacy of *Mentha piperita* essential oil against dengue fever mosquito *Aedes aegypti. Asian Pacific Journal of Tropical Biomedicine*, *1*(2), 85-88.

Lee, C. Y. (2002). Tropical household ants: Pest status, species diversity, foraging behavior, and baiting studies. *Proceedings of the 4th Interntional Conference on Urban Pests* (pp. 3-18). Blacksburg, USA: Pocahontas Press.

Lee, C. Y., & Tan, E. K. (2004). *Guide to urban pest ants in Singapore*. Singapore: Singapore Pest Management Association.

Loh, F. S., Awang, R. M., Omar, D., & Rahmani, M. (2011). Insecticidal properties of *Citrus hystrix* DC leaves essential oil against *Spodoptera litura* fabricius. *Journal of Medicinal Plants Research*, *5*(16), 3739-3744.

Morelos-Juárez, C., Walker, T. N., Lopes, J. F. S., & Hughes, W. O. H. (2010). Ant farmers practice proactive personal hygiene to protect their fungus crop. *Current Biology*, *20*(13), R553-R554.

Nor, O. M. (1999). Volatile aroma compounds in *Citrus hystrix* oil. *Journal of Tropical Arigculture and Food Science*, *27*(2), 225-229.

Pavela, R., Sajfrtová, M., Sovová, H., Bárnet, M., & Karban, J. (2010). The insecticidal activity of *Tanacetum parthenium* (L.) Schultz Bip. extracts obtained by supercritical fluid extraction and hydrodistillation. *Industrial Crops and Products*, *31*(3), 449-454.

Tawatsin, A., Wratten, S. D., Scott, R. R., Thavara, U., & Techadamrongsin, Y. (2001). Repellency of volatile oils from plants against three mosquito vectors. *Journal of Vector Ecology*, *26*, 76-82.

Walker, T. N., & Hughes, W. O. H. (2009). Adaptive social immunity in leaf-cutting ants. *Biology Letters*, *5*(4), 446-448.

Williams, D. F., Collins, H. L., & Oi, D. H. (2001). The red imported fire ant (Hymenoptera: Formicidae): An historical perspective of treatment programs and the development of chemical baits for control. *American Entomologist*, *47*(3), 146-159.

Pertanika J. Trop. Agric. Sc. 42 (4): 1343 - 1359 (2019)

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Genetic Identification and Micropropagation of Distributed Persimmons (*Diospyros kaki*) in Indonesia

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ABSTRACT

While existing persimmon productivity in Indonesia has been gradually decreasing because of improper cultural practices and environmental conditions, species conservation has become increasingly crucial given its benefit to human health. Genetic identification of the persimmon (*Diospyros kaki*), the type found in Indonesia, would provide valuable genetic information about this persimmon cultivar. Nine selected random amplified polymorphic DNA (RAPD) primers successfully yielded 32 polymorphic bands and 20 monomorphic bands. Amplified bands identified 20 persimmon samples that were derived from one genotype, given their similarity coefficient value of more than 0.76. The genotype was cultured under a controlled environment in order to produce clonal and healthy seedlings over a year. Although persimmon micropropagation had been successfully established at the initiation stage, but the explants growth of each treatment did not significantly differ. However, the highest mean of leaf number was achieved individually on treatment of Woody Plant Medium (WPM) and plant growth regulator (IBA 2 ppm + BAP 2 ppm); this is significant given that the number of formed leaves determines the persimmon multiplication rate. The regenerated shoot must be subcultured

ARTICLE INFO

Article history:
Received: 12 June 2019
Accepted: 18 September 2019
Published: 13 November 2019

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ISSN: 1511-3701
e-ISSN: 2231-8542

on modified culture medium for further persimmon micropropagation stages.

Keywords: Conservation, *Diospyros kaki*, genetic identification, micropropagation

INTRODUCTION

The weather and topographical conditions of Indonesia provide a supportive habitat for the growth of diverse germplasms, and one of the valuable species produced in this diversity is the persimmon. According to Ng (as cited in Santosa et al., 2005, p. 220), persimmons are native to China and were introduced to Indonesia about one hundred years ago. Persimmons that are distributed in Indonesia grow in areas that are at least 1,000 m above sea level, such as Selo (Central Java province) and Magetan (East Java province; Figure 1a). There are many genotypes of persimmon, but *Diospyros kaki* is the most widely distributed species for commercial purposes (Yonemori et al., 2000). Of the two types of persimmons, astringent or non-astringent, those found in Indonesia belong to the astringent type, which requires astringency removal treatment prior to consumption (Figure 1b). Compared with the non-astringent type, it can only be consumed directly when ripe. The fruit and leaves of persimmons are great sources of natural antioxidants and have been studied with regard to human health needs (Butt et al., 2015; Yaqub et al., 2016).

The persimmons distributed in Selo (Central Java province) and Magetan (East Java province) are approximately 80 years old, but unfortunately, fruit production is gradually decreasing because of improper cultural practices and environmental conditions. Therefore, conservation must be conducted in order to protect the particular persimmon genotype of Indonesia, and proper cultivation is required to enhance existing persimmon production. Genetic identification is a beginning step toward conservation establishment; to this end, a molecular marker can be used to identify relationships and genetic variations among

Figure 1. (a) Growth habitat; and (b) persimmon fruit appearance

persimmons, as genetic variation is needed to select desired traits for the next generations. Random amplified polymorphic DNA (RAPD) primers comprise one of the most commonly used methods for DNA marker identification in a manner that is simple and rapid as well as cost- and time-effective. RAPD primers bind randomly on the complementary sequence of the DNA target to amplify large amounts of genomic DNA, and presence or absence of amplified bands in individual lanes is used to determine genetic diversity (William et al., 1990). The genetic diversity among the persimmons in Italy, Japan, Korea, China, Spain and Turkey has been successfully investigated earlier using various molecular markers (Badenes et al., 2003; del Mar Naval et al., 2010; Guo & Luo, 2011; Parfitt et al., 2015; Yamagishi et al., 2005; Yonemori et al., 2008). This research presents the first report of distributed persimmon genetic identification in Indonesia.

Moving a step further toward conservation, micropropagation was performed in order to provide high-quality persimmon seedlings for productivity improvement. Micropropagation is a promising tool for clonal seedling production within a limited space that does not harm the donor plant (Bhojwani & Razdan, 1996; Bonga & Von Aderkas, 1992). The method could produce a healthy plant over the course of a year. The lateral meristem was chosen as an explant because of its higher ability for shoot multiplication. Various persimmon varieties have been successfully cultured on different culture media. A half-strength Murashige Skoog (MS) medium supplemented with various plant growth regulators has successfully induced regeneration of *Diospyros kaki* Thunb. cv Hachiya, *Diospyros kaki* L., *Diospyros kaki* Thunb., *Diospyros japonica*, *Diospyros lotus* and *Diospyros virginiana* (Fukui et al., 1992; Kochanova et al., 2011; Tao & Sugiura, 1992). Alternatively, *D. kaki* Thunb. cv. 'Nishimurawase' was successfully established on Woody Plant Medium (WPM) supplemented with zeatin 10^{-5} M (Fukui et al., 1989). Based on the above results, ½ N MS and WPM supplemented with indole-3-butryric acid (IBA) and 6-benzylaminopurine (BAP) were tested to determine the best culture condition for persimmons in Indonesia.

MATERIALS AND METHODS

Genetic Identification by RAPD Marker

Sample Collection. Persimmon (*D. kaki* L.) leaves were collected from:

- Central Java province, which consisted of three villages:
  - Sened, located at 7°29'51.4"S 110°28'32.1"E and 1,461 m above sea level;
  - Sepandan Lor, located at 7°29'59.4"S 110°29'18.18"E and 1,327 m above sea level; and
  - Gebyog, located at 7°29'57.4"S 110°28'16.1"E and 1,499 m above sea level.
- East Java province, which consisted of:
  - Magetan, located at 7°48'45.97"S 111°2'43.3"E and 1,314 m above sea level.

The distance between the three villages in Central Java province is 1–2 km of each other. The distance of East Java province is 137 km from Central Java province. Five persimmon trees were randomly selected from each village. Leaves were taken from persimmon trees in:

- Sened, coded as S1, S2, S3, S4 and S5;
- Sepandan Lor, coded as SL1, SL2, SL3, SL4 and SL5;
- Gebyog coded as G1, G2, G3, G4 and G5; and
- Magetan coded as M1, M2, M3, M4 and M5.

DNA Extraction. Genomic DNA was extracted from frozen mature persimmon leaves via the cetyltrimethylammonium bromide (CTAB) method described by Doyle and Doyle (1987). A working DNA concentration of 50 ng/µL was prepared and stored at 4°C until use.

RAPD Analysis. Twenty oligonucleotides (Operon Technologies) were initially screened in order to select primers that yielded clear, reproducible bands and polymorphism detection. Selected primers are listed in Table 1. PCR reactions were performed in a volume of 10-µl mix solution of GoTaq® Green Master Mix (Promega) 5 µl, nuclease-free Water 2 µl, primer 0.5 µl and DNA 2.5 µl. PCR conditions were 95°C for 5 min, 40 cycles at [95°C for 45 s, 37°C for 1.45 s, 72°C for 45 s] and 72°C for 7 min. PCR products were electrophoresed on 1.5% agarose gel containing 4 µl DNA stain and visualized on a UV transilluminator. Amplified bands were scored to construct dendrograms using NTSYS 2.02 software analysed by unweighted pair group method with arithmetic average (UPGMA). The similarity coefficient of dendrograms determined the genetic relationship among the persimmons.

Table 1

The bands reproducibility were generated by RAPD primers

No.	Primer	Sequence (5'-3')	Number of amplified bands	Number of polymorphic bands	Polymorphism (%)
1	OPA 5	AGGGGTCTTG	4	1	25.0
2	OPA 18	AGGTGACCGT	4	2	50.0
3	OPC 11	AAAGCTGCGG	4	2	50.0
4	OPD 2	GGACCCAACC	7	5	71.4
5	OPD 8	GTGTGCCCCA	5	4	80.0
6	OPD 11	AGCGCCATTG	7	5	71.4
7	OPD 13	ACGCGCATGT	6	5	83.3
8	OPD 18	GAGAGCCAAC	8	2	25.0
9	OPD 20	ACCCGGTCAC	7	6	85.7
	Total		52	32	61.5

Micropropagation Protocol

Lateral shoots derived from mature persimmon trees in Sened village were used as the explant because they had the highest regenerated explants rate of *in vitro* establishment (Table 2). The cut lateral shoots were cleaned with alcohol 70% and sprayed with a mix solution of fungicide 'Dithane' (1.5 g/100 ml) and bactericide 'Agrept' (1 g/100 ml). The lateral shoots were delivered from the field to the Laboratorium of Plant Physiology and Biotechnology, Agriculture Faculty, Sebelas Maret University.

At the laboratory, lateral shoots were washed under running tap water for 5 minutes and immersed into bactericide 'Agrept' (1.5 g/100 ml) and fungicide 'Dithane' (1.5 g/100 ml) solutions for 20 minutes; they were then rinsed in distilled water five times and placed in laminar air flow (LAF) for further sterilization with sterile distilled water two times. Surface sterilization of the lateral shoots was carried out aseptically by immersion into NaOCl 4% for 4 minutes (Wardani et al., 2019). Afterwards, lateral shoots were cut into single nodes (2-3 cm) and then immersed into ascorbic acid 10% for 20 minutes. Finally, lateral shoots were rinsed again with sterile distilled water three times. Explants were cultured on half-strength (½) Murashige-Skoog Medium (M1) and Woody Plant Medium (M2) supplemented with:

Z1 : IBA 1 ppm + BAP 4 ppm
Z2 : IBA 2 ppm + BAP 2 ppm
Z3 : IBA 3 ppm + BAP 2 ppm

There were six treatments. Due to high contamination and browning, each treatment only consisted of one sample with three replications. Samples were moved to the culture room and maintained at 24°C with a 24-h photoperiod using white fluorescent lighting. Explant growth consisted of callus and leaf formation noted for two months and analysed using SPSS software. Analysis of variance (ANOVA) value indicated no statistical significance for each treatment, so further analysis with Duncan's was not required.

Table 2

The regeneration rate of each persimmon sample

Sample	Browning (dead explants)	Contamination	Regenerated explants
Sened	29.63%	37.04%	33.33%
Sepandan lor	53.70%	46.30%	0%
Gebyog	20.37%	79.63%	0%
Magetan	44.44%	55.56%	0%

RESULT

Genetic Identification by RAPD

The nine selected primers yielded a total of 52 bands ranging from 125 bp to 1.2 kb, and 32 of them were recorded as polymorphic bands (Table 1). The highest number of amplified bands yielded by OPD 18 (8 bands), but only two polymorphic bands were apparent. Its polymorphism rate – 25% – was the same rate generated by OPA 5, while a polymorphism rate of 50% was generated by OPA 18 and OPC 11. The five RAPD primers (OPD 2, OPD 8, OPD 11, OPD 13 and OPD 20) successfully detected more than 50% of polymorphisms among the persimmons; the highest polymorphic bands could be obtained by OPD 20 (Figure 2).

Two main clusters were formed based on the similarity coefficient value (Figure 3). The first main cluster only contains SL5 with a similarity coefficient value of 0.76 to the second main cluster. Nineteen persimmon samples constructed the second main cluster. Genetic mutations may occur on SL5 but were still closely related to the other samples. The second main cluster consisted of two groups: the first group consisted of fifteen persimmon samples from Central Java, and the second group consisted of five persimmon samples from East Java. The similarity coefficient value for both groups was 0.78, indicating that all of the persimmon samples were derived from the same genotype, even though they were located in different provinces. The first group consisted of many subgroups that had similarity coefficient values of more than 0.86; only S1 and S5 were genetically identical samples. The second group also consisted of many subgroups that had a similarity coefficient value of more than 0.886. Both values indicated a close genetic relationship among the persimmons. The genetic variation among the samples was very low, probably because of all persimmons derived from the same genotype.

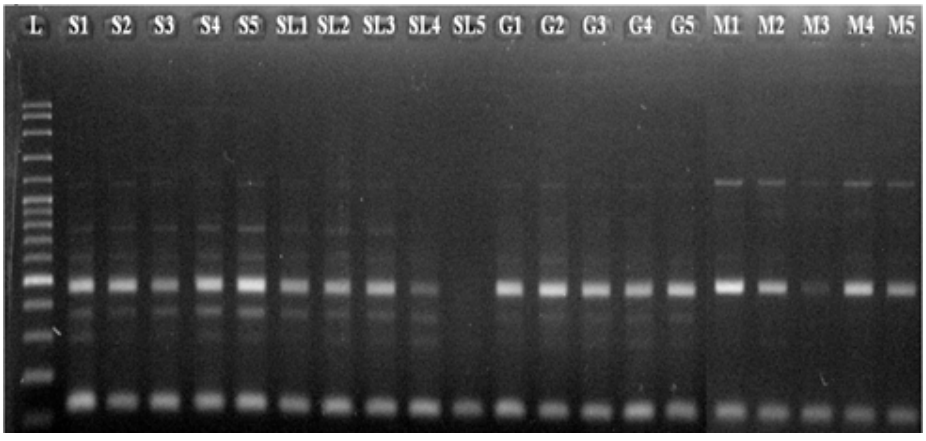


Figure 2. Amplified bands generated by the primer OPD 20. M: 100 bp DNA ladder

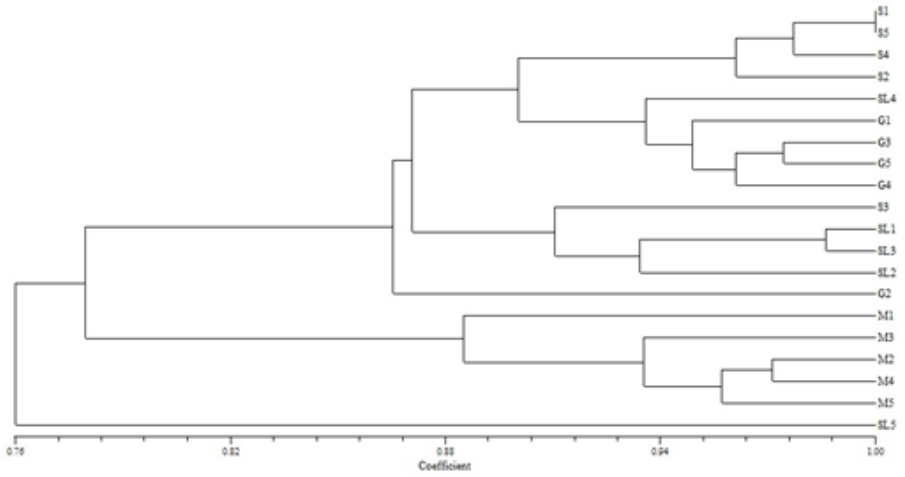


Figure 3. Genetic relationship among the persimmons

Establishment of Persimmon Micropropagation

The micropropagation protocol of persimmon genotype in Indonesia has been established in this study. The lateral shoots of persimmons were successfully cultured on MS (½ N) and WPM supplemented with various combinations of BAP and IBA. Based on ANOVA, there was no interaction between culture medium types containing various plant growth regulators toward explant regeneration (Table 3). In addition, each treatment did not significantly affect explant regeneration. Each explant had an existing bud that formed leaves after more than 50 days of culturing, and leaves induction was obtained without the interference of callus formation (Figure 4a).

A few explants formed callus at the basal stem in response to wounding stress. In addition, all treatments produced non-embryogenic callus. Based on the information in Table 3, the callus emergence of each treatment was not significantly different. The higher percentage of the non-embryogenic callus formation (55%) has no ability to form leaves, resulting in a decrease in the means of leaf number and length of each treatment. However, the highest mean of leaf number (2 leaves) was achieved individually on WPM and plant growth regulator (IBA 2 ppm + BAP 2 ppm), which is noteworthy given that the number of formed leaves determines persimmon multiplication rate.

The highest mean of leaf length was successfully induced on ½ MS of 3.21 mm and plant growth regulator (IBA 2 ppm + BAP 2 ppm) of 3.55 mm. Based on the correlation test, the early emergence of leaves enhanced the number and length of leaves and increasing the number of leaves had a positive correlation with the length of leaves.

After the seventh week of culturing, the white and friable non-embryogenic callus

turned brown and leaves became necrotic (Figure 4b). The culture medium also turned dark brown. This could have resulted from phenolic accumulation causing growth retardation and early senescence. Phenolic secretion was stimulated by cutting on the explant, thereby serving as a plant defence mechanism.

DISCUSSION

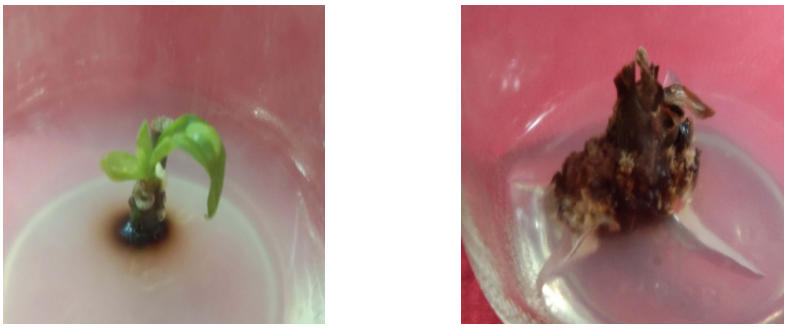
In recent years, pharmacologists have become concerned with the development of natural therapies that pose fewer or no negative effects on humans. Natural therapies are made of medicinal plant extracts, and these species contain certain phytochemicals that have the ability to maintain human health (Ekor, 2014). In general, persimmon could be considered

Table 3

Effect of different culture medium containing various plant growth regulator on persimmon shoot regeneration

Treatments	Leaves emergence (days)	Callus emergence (days)	Leaf number	Leaf length (mm)
Culture medium				
M1	52.11 a	38.67 a	1.11 a	3.21 a
M2	56.59 a	36.33 a	2.00 a	2.90 a
Plant growth Regulator				
Z1	54.38 p	46.50 p	1.50 p	3.07 p
Z2	54.56 p	36.00 p	2.00 p	3.55 p
Z3	54.12 p	30.00 p	1.17 p	2.55 p
Interaction		(-)		

Note. Means in each column followed by the same letter were not significantly different according to Duncan's multiple comparison test ($p \leq 0.05$). (-) no interaction between the two treatments



(a) (b)

Figure 4. Micropropagation of persimmon. (a) Leaves induction; and (b) necrosis plant tissue

as medicinal plant because of its promising phytochemical profile, and specifically, the antioxidant activity of persimmon (*D. kaki*) has been explored to cure various ailments (Butt et al., 2015). Persimmon has become a valuable species of Indonesian germplasms since its introduction one hundred years ago. While the distributed persimmons in Central Java and East Java are approximately 80 years old, fruit production is gradually decreasing because of improper cultural practices and environmental conditions. Therefore, conservation of this species has become crucial in order to preserve and further investigate its promising value.

As the existing persimmon population in Indonesia has not yet been characterized, genetic identification must be pursued in order to provide genetic information concerning this particular persimmon genotype. Nine selected RAPD primers successfully identified a genetic relationship among the persimmons; based on the similarity coefficient value, all of the persimmons were derived from the same genotype, with low genetic variation. Of note, the genetic variation of this persimmon was lower than that found in previous persimmon research (Akbulut et al., 2008; Badenes et al., 2003; Yamagishi et al., 2005; Yyldyz et al., 2007), possibly resulting from narrow persimmon distribution. Lower genetic variation commonly occurs in species with narrow geographical distributions because of genetic drift and inbreeding (Hamrick & Godt, 1990; Li et al., 2012; Willi et al., 2006). Genetic drift leads to heterozygosity reduction, while inbreeding increases homozygosity within populations. These factors have negative consequences on plant reproduction and survival rate (Ellstrand & Elam, 1993; Frankham, 2005). Genetic variation of persimmons has also been caused by plant stress adaptation. According to Boyko and Kovalchuk (2011), stress exposure induces DNA methylation, which consequently results in rearrangements of the genome. Such changes lead to different gene expressions that could be inherited by the progenies.

Using RAPD markers in the current study resulted in an efficient method for identifying this unknown persimmon genotype. Findings revealed that all of the persimmons were derived from same genotype; therefore, higher genetic variation of this persimmon is required to enhance species adaptation to changing environments for productivity improvement. Higher genetic variation could be obtained with the identification of other persimmon samples from other regions. According to Yamagishi et al. (2005), the application of long primer-RAPD (more than 10 base primers) enhance high reliability, reproducibility and polymorphism rate, resulting in a more accurate genetic identification.

Conservation of the persimmon genotype in Indonesia was continued through micropropagation in order to provide healthy and clonal seedlings. Mature lateral shoots were used to culture *in vitro*; the culture of mature tissue is preferred since desired traits at maturity could be determined (Rajeswari & Paliwal, 2007). Persimmon micropropagation was

successfully established using donor plants from Sened village. According to Benson (2000), the difference in regeneration ability also could be influenced by the different developmental stages of plants and by environmental factors. The mature explant is highly related to recalcitrance, a phenomena that refers to inability of plant cells to respond to the culture condition. The low regeneration of persimmon was also influenced by a high percentage of contamination due to a heavy rainy season during explant collection. Roussos and Pontikis (2001) have also reported that explants derived from field-growing trees had lower survival rates than explants derived from greenhouse-growing trees.

This study showed that there was no interaction between culture medium type containing various plant growth regulators toward explant regeneration, and that explant growth of each explant did not significantly differ. However, the highest mean of leaf number was achieved on WPM. The low salt concentration of WPM promotes plant cells regeneration by interacting with osmotic potential that increase water absorption from the medium to the cell (Beauzamy et al., 2015). WPM also gave better shoot length of Japanese persimmon compared to ½ MS. WPM containing BAP has been clarified as an optimum medium for the shoot growth of Japanese persimmon (Fukui et al., 1989). Besides, the highest mean of leaf length was achieved on the a half reduction of salt concentration in MS. The better results of persimmon shoot regeneration were obtained with ½ MS than with MS has been confirmed in previous research (Kochanova et al., 2011). The high salt concentration of culture medium causes lower stem thickening and higher vitrification (Araruna et al., 2017).

The result of this study also showed that plant growth regulator (IBA 2 ppm + BAP 2 ppm) also promoted the highest mean of leaf number and length. The individual application of BAP also successfully induces shoot regeneration of other persimmon cultivars (Belllini & Giordani, 1997; Fukui et al., 1989; Sarathchandra & Burch, 1991), while a combination of BAP and IBA tends to enhance persimmon shoot regeneration (Kochanova et al., 2011). The interaction of auxin and cytokinin has been confirmed for cell division, cell expansion and cell differentiation (Schaller et al., 2015; Su et al., 2011); BAP belongs to the adenine derivatives of the cytokinin class, which are known to be necessary for cell division. BAP also has the ability to induce reinvigoration of mature tissue and natural hormone production (Malik et al., 2005; Zhang et al., 2010). IBA is synthetic auxin that has been known for cell enlargement and as having the greatest stability for root formation (Frick & Strader, 2017; Nordström et al., 1991; Pasternak et al., 2000). The regeneration of this persimmon genotype was lower compared to other persimmon genotypes in the previous studies (Bellini & Giordani, 1997; Fukui et al., 1989, 1992; Kochanova et al., 2011; Palla & Beasley, 2013; Sarathchandra & Burch, 1991; Tao & Sugiura, 1992). This could happen because of different explant

types; different persimmon genotypes have different cell regeneration response to culture condition.

The formation of non-embryogenic callus is an undesired result because it has no organogenesis ability resulting in reduction of leaf number and length means. Callogenesis occurrence depends on genotype because of certain endogenous hormone levels that affect cellular totipotency. According to Jiménez and Bangerth (2001), non-embryogenic callus containing less endogenous indole-3-acetic acid (IAA), abscisic acid (ABA) and gibberellins (GAs), are unable to stimulate embryogenic competence. Callus differentiating ability is promoted by the addition of exogenous plant growth regulator. According to Mohajer et al. (2012), the addition of BAP and IBA successfully influences the balance of endogenous hormones, resulting in embryogenic callus formation of *Onobrychis sativa*. A higher percentage of callus formation (96%) in *O. sativa* was induced by BAP 2 ppm + IBA 3 ppm, while application of BAP 2 ppm + IBA 2 ppm induced 78% callus. The same BAP and IBA concentrations were applied on persimmon culture, but the non-embryogenic callus were highly induced. This different result may have been influenced by the recalcitrance of mature plant tissue. According to Fukui et al. (1989), culture medium containing cytokinin had significant effect on the callus formation at the base of persimmon shoot.

The growth retardation and necrosis of persimmon shoots were observed in this study; this was probably caused by browning. The negative effects of browning were also observed in other persimmon cultivars (Fukui et al. 1989; Sarathchandra & Burch, 1991). The high browning percentage (70%) is highly associated with high secondary metabolites contained in the persimmon (Miller & Murashige, 1976). Browning is triggered by mechanical damage and application of plant growth regulator (North et al., 2012). These factors regenerate reactive oxygen species (ROS) to activate stress-related gene expression (proteinase inhibitor, thionin and secondary metabolites) and hormones synthesis for callogenesis (Ikeuchi et al., 2017; Zhao et al., 2005). The accumulation of ROS causes lipid, protein and carbohydrate peroxidation, resulting in early senescence of plant tissues (Jajic et al., 2015).

Tissue browning is highly associated with increasing PPO activity followed by significant increase of phenolic compound production (Constabel et al., 2000; Thipyapong & Steffens, 1997). The oxidation of phenolic compound creates o-quinones that would be toxic for a plant by metabolism enzymes inactivation (Laukkanen et al., 1999). The deleterious effects of browning caused deterioration of callus regeneration and the insignificant growth of explants toward the culture condition. The results were supported by Chuanjun et al. (2015) that phenolic compound production interfered water and nutrition absorption by the vascular system, giving rise to growth retardation and gradual lethality to the plant.

Browning reduction treatment using ascorbic acid 10% was applied in this research, but the browning percentage was still high. According to Altunkaya and Gokmen (2008) and Nicholas et al. (1994), a higher concentration of ascorbic acid is required to convert the formed quinones into the diphenols and also to act as a PPO inhibitor. According to North et al. (2012), the high phenol content in the medium could be significantly reduced by 53% with the addition of activated charcoal (AC). The very fine pores, along with a large inner surface, of activated charcoal effectively absorb the inhibitory compounds and also promote cell growth (Thomas, 2008). The reduction of polyphenol production in persimmon has been observed earlier. The double layer system of MS containing 2% AC gave better shoots performance (Sarathchandra & Burch, 1991), while precultured persimmon shoot tips in a liquid medium exhibited better shoot growth (Fukui et al., 1989). Determining the most approriate treatment for browning reduction of this persimmon genotype is highly required in order to enhance explant regeneration.

Regeneration of this persimmon could be enhanced with aseptic culture modification. According to Purohit et al. (2011), CO_2-enriched environment, light intensity and modified ventilation promote a high photosynthetic rate and normal anatomical structure (thick cuticular wax, palisade cell, functional stomata), resulting in a higher success of *in vitro* regeneration and acclimatization rate. This modification facilitates low relative humidity, high gaseous exchange and a low ethylene level that enhance tissue vigour. Accelerating explant growth will shorten the plant life cycle, thus reducing the production cost associated with micropropagation.

A successful micropropagation at the initiation stage of the persimmon genotype in Indonesia was established. In this regard, the regenerated shoot must be subcultured on modified culture medium to induce persimmon shoot multiplication. The shoot proliferation rate of *Diospyros kaki* L., *D. kaki* Thun cv. 'Hiratanenashi' and 'Nishimurawase' was successfully induced by certain mediums containing BA or zeatin (Bellini & Giordani, 1997; Fukui et al., 1989; Palla & Beasley, 2013; Sarathchandra & Burch, 1991). Additionally, the multiplied shoots are needed to root in order to accelerate higher survival ability during acclimatization. Many persimmon cultivars have been successfully induced to root *in vitro* (Fukui et al., 1992; Palla & Beasley, 2013; Saratchchandra & Burch, 1991). These micropropagation stages must be attained if a large number of persimmon seedlings is to be provided. Further investigation is still required to determine appropriate treatments for each micropropagation stage of the specific persimmon genotype in Indonesia.

CONCLUSION

The genetic relationship of distributed persimmons in Central Java and East

Java provinces was identified by RAPD markers. Nine primers yielded a total of 32 polymorphic and 20 monomorphic bands. Based on the similarity coefficient value, all of the persimmons are derived from the same genotype. Regeneration of the genotype was conducted through micropropagation. Medium culture supplemented with various plant growth regulators did not significantly affect explant regeneration; the explant growth probably was limited by high browning percentage. As regenerated shoots could be used for further stages of persimmon micropropagation, additional investigation of browning reduction is also required in order to improve persimmon regeneration.

ACKNOWLEDGEMENT

This research was funded by Sebelas Maret University, Indonesia.

REFERENCES

Akbulut, M., Ercisli, S., Yildirim, N., Orhan, E., & Agar., G. (2008). The comparison of persimoon genotypes (*Diospyros kaki* Thunb.) by using RAPD and FAME data. *Roumanian Biotechnological Letters*, *13*(4), 3851-3858.

Altunkaya, A., & Gokmen, V. (2008). Effects of various inhibitors on enzymatic browning, antioxidant activity and total phenol content of fresh lettuce (*Lactuca sativa*). *Food Chemistry*, *107*(3), 1173-1179. doi: 10.1016/j.foodchem.2007.09.046

Araruna, E. Da-C., Ribeiro-oliveira, J. P, Pereira, V. J., Asmar, S. A., & De Melo, B. (2017). Salt concentrations in culture media for the development of *Dipteryx alata in vitro*. *Pesquisa Agropecuaria Brasileira*, *52*(12), 1295-1300. doi: 10.1590/s0100-204x2017001200020

Badenes, M., Garces, A., Romero, C., Romero, M., Clave, J., Rovira, M., & Llacer, G. (2003). Genetic diversity of introduced and local Spanish persimmon cultivars revealed by RAPD markers. *Genetic Resources and Crop Evolution*, *50*(6), 579–585. doi: 10.1023/A:1024474719036

Beauzamy, L., Derr, J., & Boudaoud, A. (2015). Quantifying hydrostatic pressure in plant cells by using indentation with an atomic force microscope. *Biophysical Journal*, *108*(10), 2448-2456. doi: 10.1016/j.bpj.2015.03.035

Bellini, E., & Giordani, E. (1997). *In vitro* culture establishment and shoot elongation of "kaki tipo" (*Diospyros kaki* L.) dormant buds. *Acta Horticulturae*, *436*, 129-134. doi: 10.17660/ActaHortic.1997.436.13

Benson, E. E. (2000). *In vitro* plant recalcitrance: An introduction. *In Vitro Cellular and Developmental Biology*, *36*(3), 141-148. doi: 10.1007/s11627-000-0029-z

Bhojwani, S. S., & Razdan, M. K. (1996). *Plant tissue culture: Theory and practice*. Amsterdam, Netherlands: Elsevier.

Bonga, J. M., & Von Aderkas, P. (1992). In vitro *culture of trees*. Dordrecht, Netherlands: Kluwer Academic Publishers.

Boyko, A., & Kovalchuk, I. (2011). Genetic and epigenetic effects of plant-pathogen interactions: An evolutionary perspective. *Molecular Plant*, *4*(6), 1014-1023. doi: 10.1093/mp/ssr022

Butt, M. S., Sultan, M. T., Aziz, M., Naz, A., Ahmed, W., Kumar, N., & Imran, M. (2015). Persimmon *(Diospyros kaki*) fruit: Hidden phytochemicals and health claims. *EXCLI Journal*, *14*, 542-561. doi: 10.17179/excli2015-159

Chuanjun, X., Zhiwei, R., Ling, L., Biyu, Z., Junmei, H., Wen, H. & Ou, H. (2015). The effects of polyphenol oxidase and cycloheximide on

the early stage of browning in *Phalaenopsis* explants. *Horticultural Plant Journal*, *1*(3), 172–180. doi: 10.16420/j.issn.2095-9885.2015-0030

Constabel, C. P., Yip, L., Patton, J. J., & Christopher, M. E. (2000). Polyphenol oxidase from hybrid poplar: Cloning and expression in response to wounding and herbivory. *Plant Physiology*, *124*(1), 185-196. doi:10.1104/pp.124.1.285

del Mar Naval, M., Zuriaga, E., Pecchioli, S., Llácer, G., Giordani, E., & Badenes, M. L. (2010). Analysis of genetic diversity among persimmon cultivars using microsatellite markers. *Tree Genetics and Genomes*, *6*(5), 677-687. doi: 10.1007/s11295-010-0283-0

Doyle, J. J., & Doyle, J. L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin*, *19*, 11-15.

Ekor, M. (2014). The growing use of herbal medicines: Issues relating to adverse reactions and challenges in monitoring safety. *Frontiers in Pharmacology*, *4*(177), 1-10. doi: 10.3389/fphar.2013.00177

Ellstrand, N. C., & Elam, D. R. (1993). Population genetic consequences of small population size: Implications for plant conservation. *Annual Review of Ecology and Systematics*, *24*(1), 217-242. doi: 10.1146/annurev.es.24.110193.001245

Frankham, R. (2005). Genetics and extinction. *Biological Conservation*, *126*(2), 131-140. doi: 10.1016/j.biocon.2005.05.002

Frick, E. M., & Strader, L. C. (2017). Roles for IBA-derived auxin in plant development. *Journal of Experimental Botany*, *69*(2), 169-177. doi: 10.1093/jxb/erx298

Fukui, H., Nishimoto, K., & Nakamura, M. (1992). Varietal differences in rooting ability of *in vitro* subcultured Japanese persimmon shoots. *Journal of the Japanese Society for Horticultural Science*, *60*(4), 821-825.

Fukui, H., Sugiyama, M., & Nakamura, M. (1989). Shoot tip culture of Japanese (*Diospyros kaki* Thunb.). *Journal of the Japanese Society for Horticultural Science*, *58*(1), 43-47. doi: 10.2503/jjshs.58.43

Guo, D. L., & Luo, Z. R. (2011). Genetic relationships of the Japanese persimmon *Diospyros kaki* (Ebenaceae) and related species revealed by SSR analysis. *Genetics and Molecular Research*, *10*(2), 1060–1068. doi: 10.4238/vol10-2gmr1100.

Hamrick, J. L., & Godt, M. J. W. (1990). Allozyme diversity in plant species. In A. H. D. Brown, M. T. Clegg, A. L. Kahler, B. S. Weir (Eds.), *Plant population genetics, breeding and genetic resources* (pp. 43–46). Sunderland, USA: Sinauer Associates Inc.

Ikeuchi, M., Iwase, A., Rymen, B., Lambolez, A., Kojima, M., Takebayashi, Y., ... Sugimoto, K. (2017). Wounding triggers callus formation via dynamic hormonal and transcriptional changes. *Plant Physiology*, *175*(3), 1158-1174. doi: 10.1104/pp.17.01035

Jajic, I., Sarna, T., & Strzalka, K. (2015). Senescence, stress, and reactive oxygen species. *Plants*, *4*(3), 393-411. doi: 10.3390/plants4030393

Jiménez, V. M., & Bangerth, F. (2001). Hormonal status of maize initial explants and of the embryogenic and non-embryogenic callus cultures derived from them as related to morphogenesis *in vitro*. *Plant Science*, *160*(2), 247-257. doi: 10.1016/S0168-9452(00)00382-4

Kochanova, Z., Brindza, J., & Onus, A. N. (2011). Adventitious shoot regeneration from dormant buds of persimmon (*Diospyros kaki* Thunb.) cv. Hachiya. *Journal of Agrobiology*, *28*(2), 113-118. doi: 10.2478/v10146-011-0012-9

Laukkanen, H., Haggman, H., Kontunen-Soppela, S., & Hohtola, A. (1999). Tissue browning of *in vitro* cultures of Scots pine: Role of peroxidase and polyphenol oxidase. *Physiologia*

Plantarum, *106*(3), 337–343. doi: 10.1034/j.1399-3054.1999.106312.x

Li, Y. Y., Guan, S. M., Yang, S. Z., Luo, Y., & Chen, X. Y. (2012). Genetic decline and inbreeding depression in an extremely rare tree. *Conservation Genetics*, *13*(2), 343–347. doi: 10.1007/s10592-011-0286-x

Malik, S. K., Chaudhury, R., & Kalia, R. K. (2005). Rapid *in vitro* multiplication and conservation of *Garcinia indica*: A tropical medicinal tree species. *Scientia Horticulturae*, *106*(4), 539-553. doi: 10.016/j.scienta.2005.05.002

Miller, L. R., & Murashige, T. (1976). Tissue culture propagation of tropical foliage plants. *In Vitro Cellular and Developmental Biology*, *12*(12), 797–813. doi: 10.1007/BF02796365

Mohajer, S., Taha, R. M., Khorasani, A., & Yaacob, J. S. (2012). Induction of different types of callus and somatic embryogenesis in various explants of sainfoin (*Onobrychis sativa*). *Australian Journal of Crop Science*, *6*(8), 1305-1313.

Nicholas, J. J., Richard, F. C., Goupy, P. M., Amiot, M. J., & Aubert, S. Y. (1994). Enzymatic browning reactions in apple and products. *Critical Reviews in Food Science and Nutrition*, *34*(2), 109-157. doi: 10.1080/10408399409527653

Nordström, A. C., Jacobs, F. A., & Eliasson, L. (1991). Effect of exogenous indole-3-acetic acid and indole-3-butyric acid on internal levels of the respective auxins and their conjugation with aspartic acid during adventitious root formation in pea cuttings. *Plant Physiology*, *96*(3), 856–861. doi: 10.1104/pp.96.3.856

North, J. J., Ndakidemi, P. A., & Laubscher, C. P. (2012). Effects of antioxidants, plant growth regulators and wounding on phenolic compound excretion during micropropagation of *Strelitzia reginae*. *International Journal of The Physical Sciences*, *7*(4), 638-646. doi:10.5897/IJPS11.786

Palla, K. J., & Beasley, R. R. (2013). *In vitro* culture and rooting of *Diospyros virginiana* L. *HortScience*, *48*(6), 747-749. doi: 10.21273/HORTSCI.48.6.747

Parfitt, D. E., Yonemori, K., Honsho, C., Nozaka, M., Kanzaki, S., Sato, A., & Yamada, M. (2015). Relationship among asian persimmon cultivars, astingent and non-astringent types. *Tree Genetics and Genomes*, *11*(24), 1-9. doi: 10.1007/s11295-015-0848-z

Pasternak, T., Miskolczi, P., Ayaydin, F., Meszaros, T., Dudits, D., & Feher, A. (2000). Exogenous auxin and cytokinin dependent activation of CDKs and cell division in leaf protoplast-derived cells of alfalfa. *Plant Growth Regulation*, *32*(2-3), 129–141. doi: 10.1023/A:1010793226030

Purohit, S., D., Teixeira da Silva, J., A., & Habibi, N. (2011). *Current approaches for cheaper and better micropropagation technologies*. Retrieved June 11, 2019, from http://www.globalsciencebooks.info/Online/GSBOnline/images/2011/IJPDB_5(1)/IJPDB_5(1)1-36o.pdf

Rajeswari, V., & Paliwal, K. (2007). *Albizia odoratissima* (L. f.) Benth. micropropagation. In S. M. Jain & H. Haggman (Eds.), *Protocol for micropropagation of woody trees and fruits* (pp. 201-211). Dordrecht, Netherlands: Springer.

Roussos, P. A., & Pontikis, C. A. (2001). Oxidative browning in 'Koroneiki' olive explants as influenced by oxidative enzyme activities and endogenous phenolic compounds. *The Journal of Horticultural Science and Biotechnology*, *76*(4), 441-446. doi: 10.1080/14620316.2001.11511391

Santosa, E., Sugiyama, N., Nakata, M., Kawabata, S., & Kubota, N. (2005). Cultivation of the Japanese persimmon in West Java, Indonesia. *Japanese Journal of Tropical Agriculture*, *49*(3), 220-226.

Sarathcahandra, S. U., & Burch, G. (1991). Micropropagation of Japanese persimmon (*Diospyros kaki* Thun.) cv. 'Hiratanenashi'. *New Zealand Journal of Crop and Horticultural Science*, *19*(2), 113-120. doi: 10.1080/01140671.1991.10421788

Schaller, G. E., Bishopp, A., & Kieber, J. J. (2015). The yin-yang of hormones: Cytokinin and auxin interactions in plant development. *Plant Cell*, *27*(1), 44-63. doi: 10.1105/tpc.114.133595

Su, Y-H., Liu, Y-B., & Zhang, X-S. (2011). Auxin-cytokinin interaction regulates meristem development. *Molecular Plant*, *4*(4), 616-625. doi: 10.1093/mp/ssr007

Tao, R., & Sugiura, A. (1992). Adventitious bud formation from callus cultures of Japanese persimmon. *HortScience*, *27*(3), 259-261.

Thipyapong, P., & Steffens, J. C. (1997). Tomato polyphenol oxidase (differential response of the polyphenol oxidase F promoter to injuries and wound signal). *Plant Physiology*, *115*(2), 409-418.

Thomas, T. D. (2008). The role of activated charcoal in plant tissue culture. *Biotechnology Advances*, *26*(6), 618-631. doi: 10.1016/j.biotechadv.2008.08.003

Wardani, N., C., Yuniastuti, E., & Sulandjari. (2019). Micropropagation protocol of juvenile buds derived from mature persimmon (*Diospyros kaki* L.) tree. *AIP Conference Proceedings*, *2120*, 030005-1-030005-5. doi: 10.1063/1.5115609

Willi, Y., Van Buskirk, J., & Hoffmann, A. A. (2006). Limits to the adaptive potential of small populations. *Annual Review of Ecology, Evolution, and Systematics*, *37*, 433–458. doi: 10.1146/annurev.ecolsys.37.091305.110145

Williams, J. G. K., Kubelik, A. R., Lival, K. J., Rafalski, J. A., & Tingey, S. V. (1990). DNA polymorphisms amplified by arbitary primers are useful as genetic markers. *Nucleic Acid Research*, *18*(22), 6531-6535. doi: 10.1093/nar/18.22.6531

Yamagishi, M., Matsumoto, S., Nakatuska, A., & Itamura, H. (2005). Identification of persimmon (*Diospyros kaki*) cultivars and phenetic relationships between *Diospyros* species by more effective RAPD analysis. *Scientia Horticulturae*, *105*(2), 283–290. doi:10.1016/j.scienta.2005.01.020

Yaqub, S., Farooq, U., Shafi, A., Akram, K., Murtazam, M. A., Kausar, T., & Siddique, F. (2016). *Chemistry and functionality of bioactive compounds present in persimmon*. Retrieved June 11, 2019, from http://downloads.hindawi.com/journals/jchem/2016/3424025.pdf

Yonemori, K., Honsho, C., Kitajima, A., Aradhya, M., Giordani, E., Bellini, E., & Parfitt, D. E. (2008). Relationship of European persimmon (*Diospyros kaki* Thunb) cultivars to Asian cultivars, characterized using AFLPs. *Genetic Resources and Crop Evolution*, *55*(1), 81–89. doi: 10.1007/s10722-007-9216-7

Yonemori, K., Sugiura, A., & Yamada, M. (2000). Persimmon genetics and breeding. *Plant Breeding Reviews*, *19*, 191–225. doi: 10.1002/9780470650172.ch6

Yyldyz, M., Bayazit, S., Cebesoy, S., & Aras, S. (2007). Molecular diversity in persimmon (*Diospyros kaki* L.) cultivars growing around hatay province in Turkey. *African Journal of Biotechnology*, *6*(20), 2393-2399. doi: 10.5897/AJB2007.000-2375

Zhang, H., Horgan, K. J., Reynolds, P. H. S., & Jameson, P. E. (2010). 6-benzyladenine metabolism during reinvigoration of mature *Pinus radiata* buds *in vitro*. *Tree Physiology*, *30*(4), 514-526. doi: 10.1093/treephys/tppl30

Zhao, J., Davis, L. C., & Verpoorte, R. (2005). Elicitor signal transduction leading to production of plant secondary metabolites. *Biotechnology Advances*, *23*(4), 283-333. doi: 10.1016/j.biotechadv.2005.01.003

Pertanika J. Trop. Agric. Sc. 42 (4): 1361 - 1373 (2019)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Spider Assemblage (Arachnida: Araneae) in A Riparian Firefly Sanctuary of Sungai Chukai, Terengganu, Malaysia

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ABSTRACT

Sungai Chukai is a riparian sanctuary for synchronize firefly population. In riparian ecosystem, spider acts as top-invertebrate predator influencing the food-web. A survey of spider was conducted in Sungai Chukai, Terengganu, Malaysia. The objective of this study was to identify the assemblage of spider in the riparian area. Samples were collected in six sampling occasions from December 2017 to May 2018 using sweep net at both day and night. Collection of samples was done at 20 sampling points along the riverbanks with an averaged interval of 200 meter between each point. A total of 149 spider individuals from seven families (Araneidae, Clubionidae, Oxyopidae, Salticidae, Sparassidae, Tetragnathidae and Thomisidae) and 26 morphospecies were successfully collected. The most abundant family was Thomisidae (50 individuals; 33.56%) followed by Sparassidae (49 individuals; 30.87%) and Salticidae (24 individuals; 16.11%). There is no significant difference ($p>0.05$) for spider population between night and day. Three spider guilds had been identified according to its foraging pattern which are the orb-weaver, running hunter and ambush hunter. There is strong positive correlation between Araneidae and Dysderidae, Oxyopidae and Salticidae while Dysderidae is negatively correlated

ARTICLE INFO

Article history:
Received: 17 June 2019
Accepted: 03 September 2019
Published: 13 November 2019

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ISSN: 1511-3701
e-ISSN: 2231-8542

with Thomisidae and Sparassidae. This study adds to the inventories of spiders in Peninsular Malaysia.

Keywords: Firefly sanctuary, Peninsular Malaysia, riparian, spider

INTRODUCTION

Spiders are an important trophic group in ecosystems as they prey on smaller insects and directly stabilise ecological food webs (Sharma et al., 2010). This group of invertebrates feeds on smaller insects and becomes the food of larger vertebrates, such as birds and bats. Currently, there are 48,127 described spider species worldwide, from 117 families and 4150 genera (World Spider Catalog, 2019). Although the spider fauna of South East Asia has been recorded extensively, records are scarce for spiders in Peninsular Malaysia. The checklist of spiders in Peninsular Malaysia done by Norma-Rashid and Li (2009) includes 425 species from 42 families and 238 genera. The effort to inventory the spider fauna has included different habitats, such as mangroves (Norma-Rashid et al., 2009), forests, and agricultural plantations (Nasir et al., 2014).

While inventory of spiders in different habitats of Peninsular Malaysia is ongoing, records of the spider fauna in riparian habitats are limited. The riparian zone is an ecotone or interface between terrestrial and aquatic zones in ecosystems (Gregory et al., 1991). Riparian areas are constantly challenged by land-use change; human activity takes place immediately adjacent to the river edge, and there are multiple uses of the land in the riparian buffer. Agricultural practices such as oil palm growing and livestock farming often disregard the need for an appropriate distance from the riverbank (Lokman, 2016; Yunus et al., 2004).

The riparian area of Sungai Chukai is an important habitat for the synchronously flashing firefly, *Pteroptyx tener*. Fireflies are well known for their bioluminescent signalling, which is used for species recognition and mate choice (Lewis & Cratsley, 2008). Signalling is also likely to influence firefly interactions with potential predators such as spiders. In our ongoing study of fireflies at Sungai Chukai, we have observed spiders in our samples. According to Long et al. (2012), jumping spiders are highly likely to interact with fireflies, with bioluminescent signalling increasing the risk of predation of fireflies by the spiders. A predator–prey relationship between spiders and fireflies has never been recorded in Malaysia. In addition, spider assemblages, particularly those in riparian habitats that act as firefly sanctuaries, have never been documented before.

The restoration of riparian ecosystems is crucial because they can be refuges for wildlife. It is important to have baseline ecological data for comprehensive assessment and area management for restoring riparian habitats. Therefore, the aim of this study was to record preliminary data on the spider assemblage in a firefly sanctuary area, Sungai Chukai, and to provide a checklist of spiders in the riparian area. Quantification of spider functional

diversity will serve as a basis for uncovering the relationships between spiders and fireflies in Malaysia. The results of this study will provide background knowledge for management of the Sungai Chukai riparian firefly sanctuary, as well as contribute to the inventory of spiders in Malaysia.

MATERIALS AND METHOD

Sample Collection

Samplings were conducted in 20 sampling points along riverbank of Sungai Chukai, Kemaman, Terengganu (4° 18' 27.36'' N, 103° 22' 21'' E) (Figure 1). Sungai Chukai located at east coast of Peninsular Malaysia. This riparian area is noted as habitat for congregating firefly, *Pteroptyx tener* as the display tree of firefly, *Sonneratia caseolaris* can be found at the riverbanks (Cheng et al., 2019; Muhammad Mahmod et al., 2018). Twenty riparian trees along the river with an averaged distance of 200 m were selected as sampling points. The sampling points were assembled at both sides of the riverbanks from downstream towards upstream (Figure 2). Samples of spider were collected in six occasions for six consecutive months starting from December 2017 to May 2018 using sweep net for one minute at each of the sampling points. Sampling was conducted at daytime and nightime. At night, sampling was conducted from 8 p.m. until 10 p.m. while during the day, the sampling was conducted from 9 a.m. to 11 a.m. During each occasion, net was swept randomly on the riparian trees at a height of

Figure 1. Location of study site in Peninsular Malaysia

approximately three to four meters from the ground. The total sampling effort was total up to 240 (6 months x 20 sampling points x 2 times). Spider samples were placed in bottles containing ethyl acetate which then brought to laboratory for identification. Samples were identified using the morphospecies approach to the lowest taxa level wherever possible using the identification keys with the aid of illustrations notably by Dippenaar-Schoeman and Jocque (1997), Koh (1989), and Murphy and Murphy (2000). Classification of spiders into guild was done by referring to Rodrigues and Mendonça (2012) and Uetz et al. (1999). Voucher specimens were stored in Center of Insect Systematics, Universiti Kebangsaan Malaysia. The riparian host tree of spider were collected and identified.

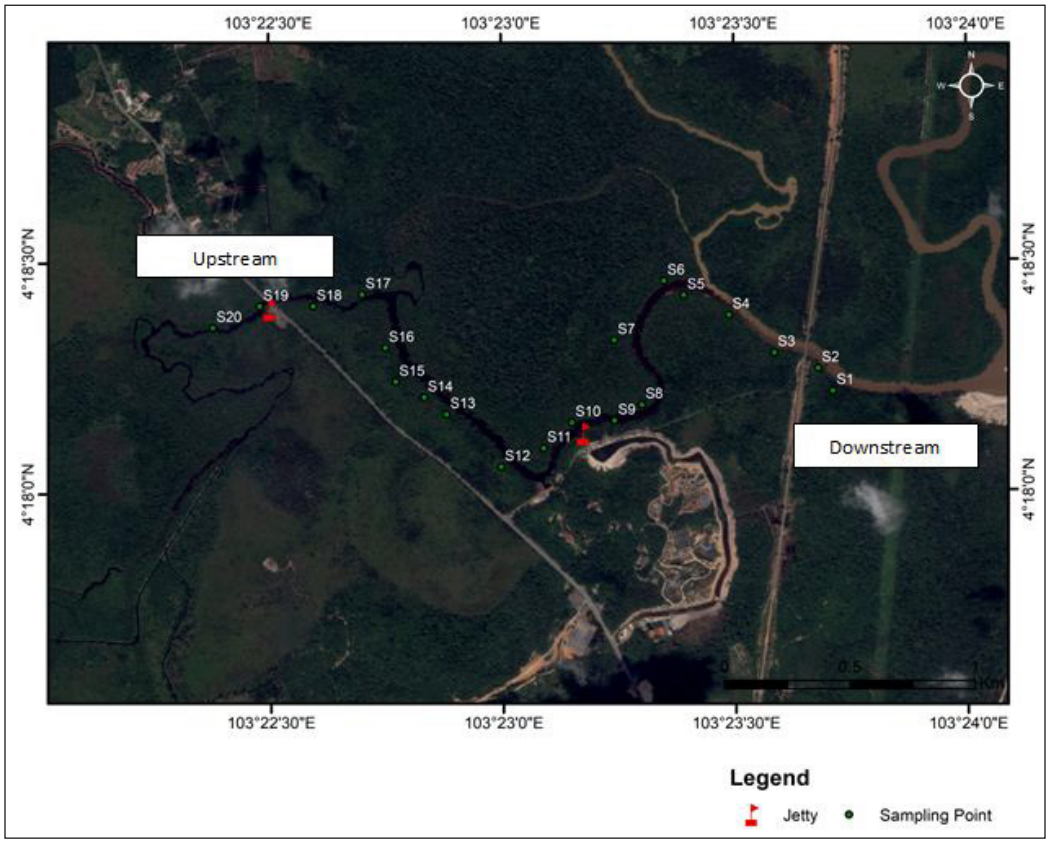

Figure 2. Sampling points in Sungai Chukai, Terengganu, Malaysia

Data Analysis

Spider diversity and riparian vegetation diversity were expressed by the Shannon-Wiener diversity index (H'). The Shannon-Wiener diversity index incorporates species richness as well as relative abundances of species. The indices were generated using PAST software version 2.17 (Hammer et al. 2001). In order to test for any significant difference between

populations of spider at different time of the day (day and night), Mann-Whitney test was conducted using Minitab software version 17.1. The adequacy of sampling effort within an area may be indicated by species accumulation curve (Chao et al., 2009). The species accumulation curve was generated after 100 randomization of sample order with nonparametric estimator, Chao 1 and Jackknife using EstimateS software version 9.1 (Colwell, 2009). Chao 2 and Jacknife was selected as the normality assumption was not satisfied. Cluster analysis was conducted to find structure of spider community in the riparian area with software PCORD 5.0 using Euclidean and Ward's distance matrices. Correlation network plot was analyzed and generated using package 'spaa' in R software version 3.5.3. The correlation network plot helps to determine the interaction between taxon (Zhang, 2011).

RESULTS AND DISCUSSION

A total of 149 spider individuals representing 26 species in seven families (Araneidae, Clubionidae, Oxyopidae, Salticidae, Sparassidae, Tetragnathidae and Thomisidae) (Table 1). The most abundant family was the Thomisidae (50 individuals; 33.56%) followed by the Sparassidae (49 individuals; 32.89%) and Salticidae (24 individuals; 16.11%) (Figure 3). The species accumulation curve generated using Chao 1 and Jacknife estimator shows an ascending trend, indicating that more spider species are yet to be discovered (Figure 4).

The Mann–Whitney *U* test showed no significant differences ($p>0.05$) between daytime and night-time spider populations, suggesting that resource partitioning occurred spatially rather than temporally. Nevertheless, results showed that spider abundance was higher at night than during the day (Table 1). It is important to note that the diel activity of spiders is highly influenced by the circadian rhythms of their prey (Krol et al., 2018). The higher abundance of spiders at night suggests that most of their invertebrate prey in Sungai Chukai were active at night-time. In addition, larger-bodied spiders tend to be more active at night to avoid diurnal predators such as birds (Krumpalova & Turf, 2013; van Berkum, 1982). This explains the higher abundance of the larger Sparassidae at night than during the day. The genus *Heteropoda* from this family was also nocturnal, as it was found to hunt actively at night (Zhang et al., 2015, 2018). On the other hand, we found that small-bodied spiders such as the Salticidae tended to be more active during the day. The Salticidae use visual cues to hunt for their prey and being active during daytime allow these spiders to make full use of their ability to see in fine detail (Jackson & Cross, 2011).

The spiders collected in this study are common in Malaysia. Since records of spiders are scarce for Peninsular Malaysia, we could compare our results only to those of Nasir et al. (2014), Norma-Rashid and Li (2009), and Norma-Rashid et al. (2009). Orb-weaver spiders such as the Tetragnathidae (*Tegranatha* sp.) and Araneidae (*Neoscona*

Table 1
Checklist of spider in Sungai Chukai, Terengganu, Malaysia

Family	Morphospecies	No. of Individuals		Total
		Day	Night	
Araneidae (A)	*Neoscona* sp.	1	1	2
Clubionidae (C)	*Araneus* sp.	0	4	4
	Clubiona sp. 1	0	2	2
	Clubiona sp. 2	0	1	1
	Clubionidae sp. 1	5	0	5
Oxyopidae (O)	*Oxyopes* sp. 1	0	1	1
	Oxyopes sp. 2	3	1	4
Salticidae (SA)	*Icius* sp. 1	4	0	4
	Icius sp. 2	3	1	4
	Myrmarachne sp.	1	0	1
	Salticidae sp. 1	3	1	4
	Spartaeus sp. 1	4	5	9
	Spartaeus sp. 2	2	0	2
Sparassidae (SP)	*Heteropoda* sp. 1	8	37	45
	Heteropoda sp. 2	0	1	1
	Heteropoda sp. 3	1	2	3
Tetragnathidae (TE)	*Tetragnatha* sp.	2	1	3
	Tetragnathidae sp. 1	0	4	4
Thomisidae (TH)	*Ebrechtella* sp.	0	5	5
	Mastira sp. 1	4	5	9
	Mastira sp. 2	1	0	1
	Mechapesa sp.	0	4	4
	Thomisidae sp. 1	2	0	2
	Thomisidae sp. 2	0	1	1
	Thomisidae sp. 3	1	2	3
	Thomisidae sp. 4	11	14	25
	TOTAL	**56**	**93**	**149**

sp.) have been recorded in the west coast of Peninsular Malaysia by Nasir et al. (2014). Long-jawed orb weavers, *Tegranatha* sp. have been found in mangrove areas of Morib and Tioman (Norma-Rasyid et al., 2009) while sac spider, Clubionidae (*Clubiona* sp.) has been found before in Tioman (Norma-Rasyid et al., 2009). These genera have been confirmed to be non-endemic to Peninsular Malaysia as they have also been recorded in Sarawak (Koh et al., 2013) and Sabah (Dzulhelmi et al., 2014), the Malaysian states in the island of Borneo. The lynx spider, Oxyopidae (*Oxyopes* sp.) was also recorded in Sarawak, but not in Sabah, while in Peninsular Malaysia it had

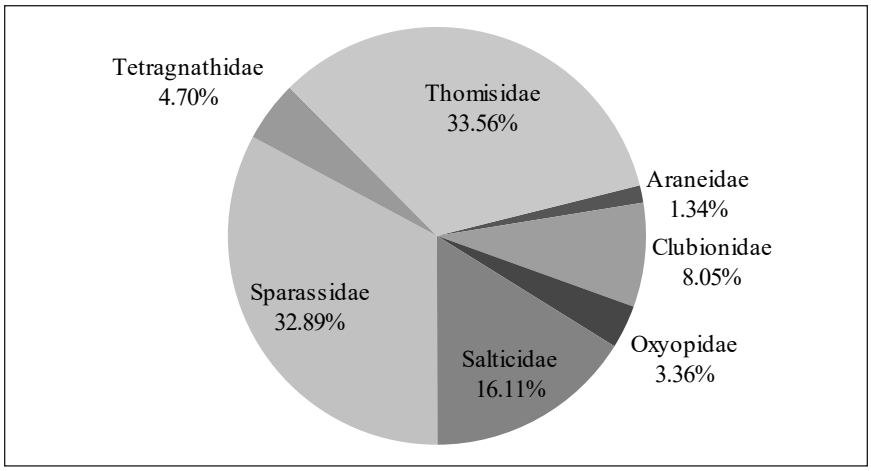

Figure 3. Assemblages of spider in Sungai Chukai, Terengganu, Malaysia

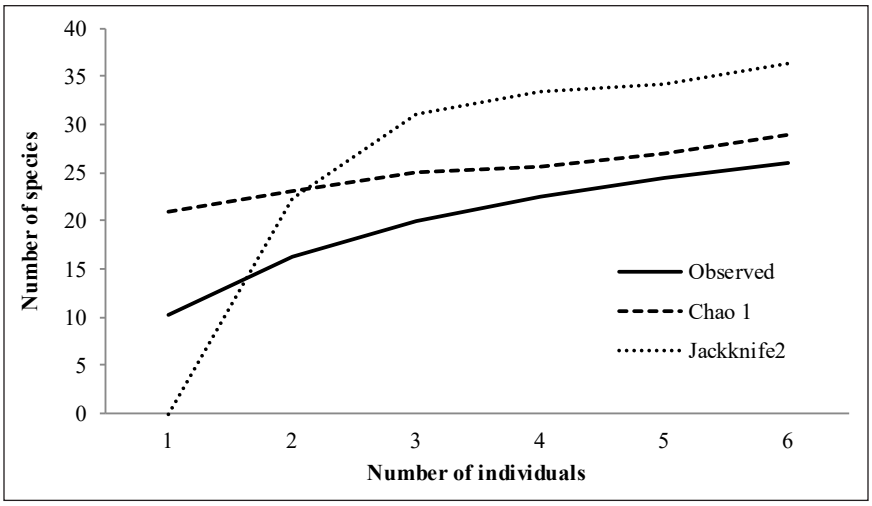

Figure 4. Species accumulation curve using Chao and Jackknife Estimator for spider species found in Sungai Chukai, Terengganu, Malaysia

been found in Penang, Tioman and Morib (Norma-Rayid et al., 2009; Norma-Rasyid & Li, 2009). Both genera of Salticidae, *Icius* and *Myrmarachne*, collected in this study had been recorded in Peninsular Malaysia (Norma-Rasyid & Li, 2009). However, *Icius* has never been recorded from Sabah and Sarawak. The huntsman spider, Sparassidae (*Heteropoda* sp.) and crab spider, Thomisidae (*Mastira* sp.) are widespread in Peninsular Malaysia, Sabah and Sarawak (Koh et al., 2013; Nasir et al., 2014; Norma-Rasyid & Li, 2009). On the other hand, *Ebrechtella* is a new record from Peninsular Malaysia, although it has been recorded in Sabah and neighbouring Thailand (Deeleman-Reinhold & Floren, 2008).

In this study, four riparian plant species i.e. *Barringtonia racemosa*, *Hibiscus tilaceus*, *Nypa fruticans* and *Sonneratia caseolaris* were observed to host spiders. Spider diversity was different between these species. The Shannon diversity index was the highest (*H*' = 1.782) for spiders on *Hibiscus tilaceus* and the lowest for spiders on *N. fruticans* (*H*' = 0.6315). The diversity index for spiders on *B. racemosa* and *S. caseolaris* was *H*' = 1.723 and *H*' = 1.563, respectively. The difference in spider diversity was hypothesised to be caused by differences in plant architecture. In comparison to the other three riparian genera, *H. tilaceus* has denser foliage, which may provide greater and more structurally complex support for web building, thereby resulting in the highest spider diversity. In contrast, the palm *N. fruticans* with its simple arrangement of pinnate leaves and linear leaflets, held the lowest spider diversity. These results are supported by a study by Hatley and Macmahon (1980) which found that spider diversity increased with shrub foliage volume and density. Highly dense foliage may hold a greater number of smaller invertebrates as prey options, as well providing substrates for web attachment. This demonstrates that vegetation composition and structure may play a significant role in controlling spider diversity in this area.

The assemblage of spiders in Sungai Chukai plays an important role as predators, although there are differences between different spider groups in their predatory behaviour. Based on cluster analysis, the spiders in Sungai Chukai can be divided into three foraging guilds (Figure 5). Group I consists of orb weavers, from the Araneidae and Tetragnathidae, that use sticky webs, while being stationary, to capture flying insects at all life stages. Orb weavers produce silk as soon as they hatch from the egg sac; as the spider lings grow, the amount of silk they produce increases, and the silk improves in quality, to support a larger body (Sensenig et al., 2011). This may also help the adult spider to capture larger prey. The webs produced by the Aranaidae and Tetragnathidae are bidimensional as opposed to the sheet-web spider's webs that are tridimensional (Ávila et al., 2017). Orb-weaver spiders depend strongly on the presence of structure for web support (Nasir et al., 2017). Riparian habitats in Sungai Chukai apparently provide the structure for web attachment by *Tegragnatha* and *Neoscona*. A complex habitat, particularly

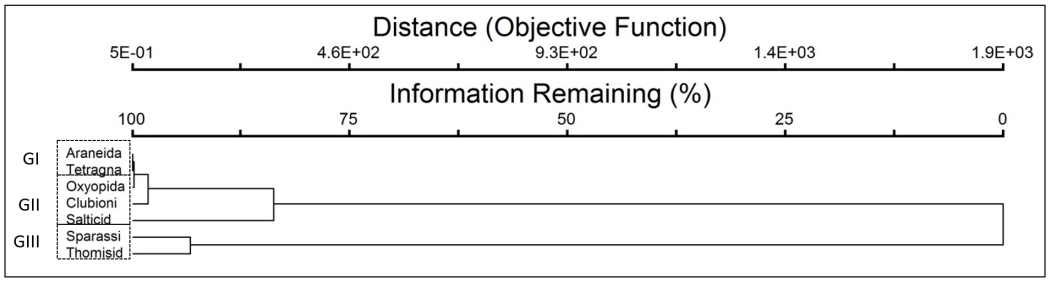

Figure 5. Dendogram for one-way cluster analysis of spider in Sungai Chukai, Terengganu, Malaysia

that with different types of vegetation, will provide more options for orb weavers to attach their webs, and will thus support diverse spider species.

Group II from the cluster analysis is represented by hunting spiders, and is dominated by ambush hunter. Similar to orb weavers, ambush spiders are sit-and-wait predators; however, the members of the Oxyopidae and Salticidae do not build webs (Ávila et al., 2017). They remain motionless until their movement is triggered by the movement of the prey. The Oxyopidae (*Oxyopes* sp.) may prey on calliphorid flies in oil palm plantations (Chin et al., 2008), the tea mosquito bug (*Helopeltis theivora*) in tea plantations (Basnet & Mukhopadhyay, 2014), and the white-backed planthopper (*Sogatella furcifera*) in rice fields (Butt & Xaaceph, 2015). The affinity of this spider for pests of economic importance shows that it has the potential to become a beneficial biocontrol organism. The ant-like salticid spiders (Salticidae, *Myrmarachne* sp.) also prey on various of arthropod prey, as well as being adept at cathing moth (Jackson & Willey, 1994).

Group III includes the families Thomisidae and Sparassidae and are mainly hunting spiders. The two families may be influenced by the same abiotic factors, our analysis consequently clustering them into one group. Therefore, we suggest that it is worthwhile to study how the abiotic factors in the riparian area affect these spider groups. However, the Thomisidae and Sparassidae have different modes of hunting; the former are ambush hunters, whereas the latter consists of both running and ambush hunters (Rodrigues & Mendonça, 2012). The Thomisidae frequently visit flowering plants, and may successfully attack butterflies, dragonflies, and stinging insects, such as bees and wasps (Lovell, 1915). The huntsman spider, Sparassidae, has been reported to be able to hunt on the water surface (Airamé & Sierwald, 2000), making riparian areas particularly suitable habitats for this spider family.

In our study, there is a strong positive correlation ($r>0.58$) between the presence of the Araneidae and the Oxyopidae. The Oxyopidae are also positively correlated ($r>0.58$) with the Salticidae. A strong negative correlation ($r<-0.41$) was exhibited between the Thomisidae and the Salticidae (Figure 6). According to Zhang (2011), a positive correlation may arise as a result of mutualistic or prey–predator interaction, while competition between species may yield a negative correlation. For example, the negative correlation between the presence of the Thomisidae and Salticidae may be evidence of competition for resources. Niche overlap between these two families may occur as both can be found at the same period of time during the day. The Thomisidae and Salticidae also exhibit similar foraging strategies; as ambush hunters, they may compete for the same prey. The positive correlation between the Araneidae and Oxyopidae may be the result of niche differentiation as the Araneidae are located in different niches within the habitat from those of the Oxyopidae. In addition, the types of prey consumed by the orb-

weaving Araneidae may be different from those consumed by the ambush-hunting Oxyopidae. The results for Spearman's correlation between each family are shown in Table 2.

It is also important to note that the spiders in this study coexisted with the congregating firefly populations in the same riparian habitat. The question is whether there is interaction between spiders and fireflies. There is the possibility that spiders' prey on *P. tener* in Sungai Chukai. Both nocturnal and diurnal spiders have been recorded as predators of fireflies (Lloyd, 1973). For example, wolf spiders (Lycosidae) are known to hunt for *Photuris* sp. in the US. A large number of prey items on the web of *Neoscona arabesca*, an orb-weaving spider, consisted of the congregating firefly, *Photinus carolinus* (Lewis et al., 2012). The genus *Neoscona* has also been recorded in our study, although no observation or record was made of its predator–prey relationships. Future research on predation of *P. tener* by spiders is recommended, as currently there are no studies on this subject.

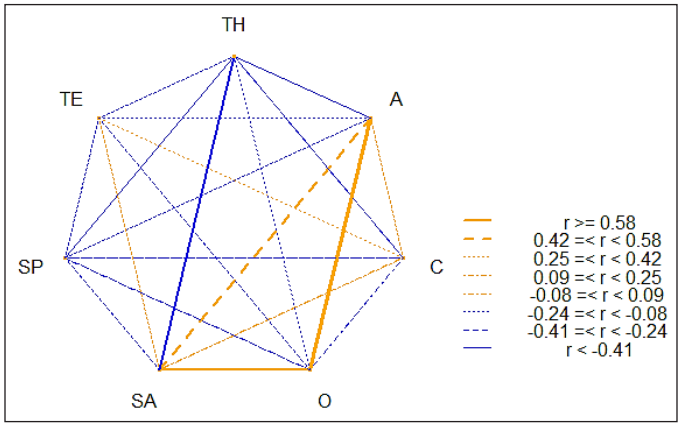

Figure 6. correlation network plot between Arachnids family in Sungai Chukai, Terengganu, Malaysia A=Araneidae, C=Clubionidae, O= Oxyopidae, SA= Salticidae, SP= Sparassidae, TE= Tetragnathidae, TH= Thomisidae

Table 2
Spearman's correlation value between family of Arachnids in Sungai Chukai, Terengganu, Malaysia

	A	C	O	SA	SP	TE	TH
A	1.000	0.333	0.885	0.670	0.000	-0.111	-0.299
C	0.333	1.000	0.156	0.287	0.258	0.333	-0.128
O	0.885	0.156	1.000	0.793	0.000	-0.156	-0.120
SA	0.670	0.287	0.793	1.000	0.124	0.351	-0.282
SP	0.000	0.258	0.000	0.124	1.000	0.000	0.248
TE	-0.111	0.333	-0.156	0.351	0.000	1.000	-0.085
TH	-0.299	-0.128	-0.120	-0.282	0.248	-0.085	1.000

Note. A=Araneidae, C= Clubionidae, O=Oxyopidae, SA= Salticidae, SP= Sparassidae, TE= Tetragnathidae, TH= Thomisidae

CONCLUSION

Malaysia is a tropical country with diverse and fascinating flora and fauna. It is highly feasible that there are more spiders awaiting to be discovered with additional sampling efforts and extension of study periods. The results from this study contribute to biodiversity knowledge of Sungai Chukai, which will help in the conservation of this area of high potential as an ecotourism attraction. A comprehensive inventory of Malaysian spiders is needed to expand our knowledge of spider taxa in the country. Continuing research will contribute to the conservation of both spiders and fireflies, as well as the Sungai Chukai riparian ecosystem as a whole, in the hope that it can be developed as an ecotourism centre and is maintained as an ecological corridor.

ACKNOWLEDGEMENT

We thank Dr. Dzulhelmi Muhammad Nasir, Dr. Nik Ahmad Irwan Izzauddin Nik Him and Juhaida Harun for their assistance in identification of specimen. We are also grateful to Mr. Muhammad Zabidi Yaacob and staff of Zoo Kemaman for their assistance in fieldwork. We thank Nor Shafikah Idris for the map generated in this article. This research was funded by grant from TNB Research Sdn. Bhd. (ST 2017-013).

REFERENCES

Airamé, S., & Sierwald, P. (2000). Hunting and feeding behavior of one Heteropoda species in lowland rainforest on Borneo (Aranae, Sparassidae). *The Journal of Arachnology*, *28*(2), 251-254. doi:10.1636/0161-8202(2000)028[0251:HAFBOO]2.0.CO;2

Ávila, A. C., Stenert, C., Rodrigues, E. N. L., & Maltchik, L. (2017). Habitat structure determines spider diversity in highland ponds. *Ecological Research*, *32*(3), 359-367. doi:10.1007/s11284-017-1442-7

Basnet, K., & Mukhopadhyay, A. (2014). Biocontrol potential of the lynx spider *Oxyopes javanus* (Araneae: Oxyopidae) against the tea mosquito bug, *Helopeltis theivora* (Heteroptera: Miridae). *International Journal of Tropical Insect Science*, *34*(4), 232-238. doi:10.1017/S1742758414000538

Butt, A., & Xaaceph, M. (2015). Functional response of *Oxyopes javanus* (Araneidae: Oxyopidae) to *Sogatella furcifera* (Hemiptera: Delphacidae) in laboratory and mesocosm. *Pakistan Journal of Zoology*, *47*(1), 89-95.

Chao, A., Colwell, R. K., Lin, C. W., & Gotelli, N. J. (2009). Sufficient sampling for asymptotic minimum species richness estimators. *Ecology*, *90*(4), 1125-1133.

Cheng, S., Munian, K., Sek-Aun, T., Faidi, M. A., & Ishak, S. F. (2019). Mitochondrial DNA diversity and gene flow in Southeast Asian populations of the synchronously flashing firefly, *Pteroptyx tener* Olivier (Coleoptera: Lampyridae). *Oriental Insects*. doi: 10.1080/00305316.2019.1600594

Chin, H. C., Marwi, M. A., Jeffery, J., & Omar, B. (2008). Research note on the predation of fly, *Chrysomya rufifacies* (Macquart) by a spider, O*xyopes* sp. Latreille (Oxyopidae). *Tropical Biomedicine*, *25*(1), 93-95.

Colwell, R. K. (2009). *EstimateS: Statistical estimation of species richness and shared species from samples (Version 9.1.0)*. Retrieved April 12, 2019, from http://purl.oclc.org/estimates

Deeleman-Reinhold, C., & Floren, A. (2008). Crab spiders, tree canopy, and biodiversity in tropical

East Asia (Araneae: Thomisidae). In W. Nentwig, M. Entling, & C. Kropf. (Eds.), *Proceedings of the 24th European Congress of Arachnology* (pp. 51-58). Bern, Switzerland: The Natural History Museum Bern.

Dippenaar-Schoeman, A. S., & Jocque, R. (1997). *African spiders: An identification manual*. Pretoria, South Africa: Agricultural Research Council-Plant Protection Research Institute.

Dzulhelmi, M. N., Wong, C. X., Goh, T. G., Juhaida, H., & Faszly, R. (2014). Spider fauna (Arachnida, Araneae) from Sabah, Malaysia. *Journal of Entomology and Zoology Studies*, *2*(5), 335-344.

Gregory, S. V., Swanson, F. J., McKee, W. A., & Cummins, K. W. (1991). An ecosystem perspective of riparian zones. *BioScience*, *41*(8), 540-551. doi: 10.2307/1311607

Hammer, O., Harper, D. A. T., & Ryan, P. D. (2001). PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica*, *4*(1), 1-9.

Hatley, C. L., & Macmahon, J. A. (1980). Spider community organization: Seasonal variation and the role of vegetation architecture. *Environmental Entomology*, *9*(5), 632-639.

Jackson, R. R., & Willey, M. B. (1994). The comparative study of the predatory behaviour of *Myrmarachne*, ant-like jumping spiders (Araneae: Salticidae). *Zoological Journal of the Linnean Society*, *110*(1), 77-102. doi: 10.1111/j.1096-3642.1994.tb01472.x

Jackson, R. R., & Cross, F. R. (2011). Spider cognition. In *Advances in insect physiology* (Vol. 40, pp. 115-174). London, United Kingdom: Academic Press.

Koh, J. K., Koh, Y., Norma-Rashid, Y., & Koh, J. W. B. (2013). A preliminary checklist of Sarawak spiders. *Sarawak Museum Journal*, *92*, 203-254.

Koh, J. K. H. (1989). *A guide to common Singapore spiders*. Singapore: Singapore Science Centre.

Krol, A., Hajdamowicz, I., & Tkaczuk, C. (2018). Diel and seasonal activity of ground dwelling spiders (Araneae) in a sandy grassland habitat. *Turkish Journal of Zoology*, *42*(4), 439-448.

Krumpalova, Z., & Tuf, I. H. (2013). Circadian rhythms of ground living spiders: Mechanisms of coexistence strategy based on the body size. *Polish Journal of Ecology*, *61*(3), 575-586**.**

Lewis, S. M., & Cratsley, C. K. (2008). Flash signal evolution, mate choice, and predation in fireflies. *Annual Review of Entomoogy*, *53*, 293-321.

Lewis, S. M., Faust, L., & De Cock, R. (2012). *The dark side of the light show: Predators of fireflies in the Great Smoky Mountains*. Retrieved April 12, 2019, from http://downloads.hindawi.com/journals/psyche/2012/634027.pdf

Lloyd, J. E. (1973). Firefly parasites and predators. *The Coleopterists Bulletin*, *27*(2), 91-106.

Lokman, T. (2016, May 24). Rivers may get bigger buffer zones. *The News Strait Time*. Retrieved March 22, 2019, from https://www.nst.com.my/news/2016/05/147492/rivers-may-get-bigger-buffer-zones

Long, S. M., Lewis, S., Jean-Louis, L., Ramos, G., Richmond, J., & Jakob, E. M. (2012). Firefly flashing and jumping spider predation. *Animal Behaviour*, *83*(1), 81-86.

Lovell, J. H. (1915). Insects captured by the Thomisidae. *The Canadian Entomologist*, *47*(4), 115-116.

Muhammad Mahmod, A., Sulaiman, A., Asri, L., Abdullah, A., Husin, S. A., Shukor, A. M., ... Sulaiman, N. (2018). Kelimpahan kelip-kelip bersinkroni di Sungai Chukai, Kemaman, Terengganu [The abundance of fireflies is

synchronous in the Chukai River, Kemaman, Terengganu]. *Undergraduate Research Journal for Integrative Biology*, *2*, 117-123.

Murphy, F. M., & Murphy, J. A. (2000). *An introduction to the spiders of South East Asia.* Kuala Lumpur, Malaysia: Malayan Nature Society.

Nasir, D. M., Su, S., Mohamed, Z., & Yusoff, N. C. (2014). New distributional records of spiders (Arachnida: Araneae) from the west coast of peninsular Malaysia. *Pakistan Journal of Zoology*, *46*(6), 1573-1584. doi: 0030-9923/2014/0006-1573

Nasir, D. M., Xing, W. C., Mamat, N. S., Pui, Y. M., & Sulaiman, B. (2017). New records of the spider fauna from Sarawak, Malaysia. *Jurnal Biologi Indonesia*, *12*(2), 307-312.

Norma-Rashid, Y., & Li, D. (2009). A checklist of spiders (Arachnida: Aranae) from Peninsular Malaysia inclusive of twenty new records. *The Rafffles Bulletin of Zoology*, *57*(2), 305-322.

Norma-Rashid, Y., Rahman, N. A., & Li, D. (2009). Mangrove spiders (Araneae) of Peninsular Malaysia. *International Journal of Zoological Research*, *5*(1), 9-15. doi: 10.3923/ijzr.2009.9.15.

Rodrigues, E. N. L., & Mendonça, M. S. (2012). Spider guilds in the tree-shrub strata of riparian forests in southern Brazil. *The Journal of Arachnology*, *40*(1), 39-48. doi: 10.1636/P10-105.1

Sensenig, A. T., Agnarsson, I., & Blackledge, T. A. (2011). Adult spiders use tougher silk: Ontogenetic changes in web architecture and silk biomechanics in the orb-weaver spider. *Journal of Zoology*, *285*(1), 28-38. doi: 10.1111/j.1469-7998.2011.00809.x.

Sharma, S., Vyas, A., & Sharma, R. (2010). Diversity and abundance of spider fauna of Narmada river at Rajghat (Barwani) (Madhya Pradesh) India. *Researcher*, *2*(11), 1-5.

Uetz, G. W., Halaj, J., & Cady, A. B. (1999). Guild structure of spiders in major crops. *Journal of Arachnology*, *27*(1), 270-280.

van Berkum, F. H. (1982). Natural history of a tropical, shrimp-eating spider (Pisauridae). *Journal of Arachnology*, *10*(2), 117-121.

World Spider Catalog. (2019). Retrieved March 21, 2019, from https:// wsc.nmbe.ch/families.

Yunus, A. J. M., Nakagoshi, N., & Ibrahim, A. L. (2004). Riparian land-use and land cover change analysis using GIS in Pinang river watershed, Malaysia. *Tropics*, *13*(4), 235-248. doi: 10.3759/tropics.13.235

Zhang, S., Chen, H. L., Chen, K. Y., Huang, J. J., Chang, C. C., Piorkowski, D., ... Tso, I. M. (2015). A nocturnal cursorial predator attracts flying prey with a visual lure. *Animal Behaviour*, *102*, 119-125.

Zhang, S., Yip, H. Y., Lee, M. Y., Liu, L., Piorkowski, D., Liao, C. P., & Tso, I. M. (2018). Vision-mediated courtship in a nocturnal arthropod. *Animal Behaviour*, *142*, 185-190.

Zhang, W. J. (2011). Constructing ecological interaction networks by correlation analysis: Hints from community sampling. *Network Biology*, *1*(2), 81-98. doi: 10.0000/issn-2220-8879-networkbiology-2011-v1-0008

Pertanika J. Trop. Agric. Sc. 42 (4): 1375 - 1389 (2019)

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Flowering and Reproductive Biology of *Zingiber spectabile*

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ABSTRACT

The species of Zingiberales are sources of globally important spices and ornamental plants, and have long been used in Asian traditional medicine, cuisine and as herbs. Some species have high ornamental value due to their attractive foliage or flowers, including *Zingiber spectabile* Griff. Hybridization has been the major source of genetic variation in flower and ornamental breeding and understanding the flowering season and peaks of flowering is important for flower growers. Stigma receptivity, or the effective pollination period, is one of the important factors determining successful fertilization and has been rarely studied in Zingiberaceae. The objectives of this study were to examine the *Z. spectabile* reproductive biology, to investigate stigma receptivity under several flowering developmental stages, and their reproductive success. The inflorescence development of *Z. spectabile* from the start of the bracts opening to fully open bracts took 13-17 weeks. The ideal time for artificial pollination was between 11:00-13:00 hours, and the anthers dehisced prior to stigma receptivity. Our study demonstrates that *Z. spectabile* is self-compatible and cross-pollination does not increase fruit set and seed set.

Keywords: Flower structure, ornamental traits, pollen viability, stigma receptivity

ARTICLE INFO

Article history:
Received: 13 June 2019
Accepted: 24 September 2019
Published: 13 November 2019

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INTRODUCTION

The ginger family (Zingiberaceae) has over 1500 species, mostly native to Asia and the Pacific (Leong-Skornickova & Gallick, 2010). Members of Zingiberales are sources of globally important spices and ornamental plants, and have long been used in Asian traditional medicine, herbs and culinary. Some species have high ornamental values due to their attractive foliage or flowers,

ISSN: 1511-3701
e-ISSN: 2231-8542

including *Zingiber spectabile* Griff, Alpinia, and Costus that had been commonly used as cut flower. Other Zingiberaceae species such as Globbas, Calatheas, Curcuma and Tapeinochilos are just beginning to be available in the market in Indonesia.

Zingiber spectabile is also known as beehive gingers; its local names include *bihip* (Indonesia), and *tepus tanah* (Malay). Another species of beehive ginger that has been cultivated is *Zingiber olivaceum*. *Zingiber spectabile* is usually 2.5-3 m tall with lots of basal cones, whereas *Z. olivaceum* is 1.5-2 m tall with smaller basal and terminal cones. *Zingiber spectabile* inflorescences have yellow to brown bracts with yellow spots on their true flowers; the inflorescences are popular as cut flowers due to the variable bract colours (Loges et al., 2011) and long-lasting shelf life (Chee & Hoo, 2010).

Zingiber spectabile is a short-day species; it requires at least nine weeks of consecutive short days in order to initiate and develop flowers (Criley, 2011). The inflorescence is terminal and can measure up to 30 cm in height. The bracts vary in colour from white, yellow, orange to red, often darkening as the bracts mature. Zingiberaceae flowers are usually zygomorphic, bisexual with a single functional stamen, and five sterile stamens are transformed into labellum and staminodes (Leong-Skornickova & Gallick, 2010). Flexistyly, a form of heterodichogamy, has been reported to be widespread in Zingiberaceae (Li et al., 2001, Takano et al., 2005); flexistyly can have two floral morphs, i.e. anaflexistylous (protogynous) and cataflexistylous (protandrous) morphs according to the direction of stigma movement and time of pollen release during anthesis (Takano et al., 2005).

Zingiber spectabile foliage ranges from 2-3 m in height. The leaves have been traditionally used to treat various ailments, and their rhizomes are used as a germicide, stimulant and in the treatments of cough and asthma (Sadhu et al., 2007). Sivasothy et al. (2012) evaluated anti-bacterial activities of oils extracted from *Z. spectabile* leaves and rhizomes. Eighty compounds were isolated and identified, and some demonstrated activities against Escherichia and Salmonella. Sivasothy et al. (2013) reported that curcuminoids from the rhizome of *Z. spectabile* had preservative properties with higher antioxidant and antibacterial activities.

Despite their huge economic, cultural and ornamental importance, studies on Zingiberaceae are limited, particularly on *Z. spectabile* flowering biology and hybridization. *Zingiber spectabile* is available in very limited variants, and no information is available on the self -incompatibility of this species. Self-incompatibility has been linked to the reduction in the fitness of the progenies, mainly due to the increased expression of deleterious or lethal genes (Olmstead, 1989). Hybridization has been the major source of genetic variation in flower and ornamental breeding, and half of the flowering plants show self -incompatibility (Gibs, 2014). Therefore, understanding pollination, self-incompatibility, and seed

setting is valuable for breeders in creating new variants. Stigma receptivity refers to the ability of the stigma to support germination of viable and compatible pollens (Yi et al., 2006). Stigma receptivity determines an effective pollination period, i.e. the longevity of the ovule minus the time lag between pollination and fertilization (Dafni & Maues, 1998); it is one of the important factors determining successful fertilization. Knowledge on self-incompatibility provides information for plant breeders to plan breeding programs (Wickramasinghe et al., 2010). The objectives of this study were to examine *Z. spectabile* reproductive biology, to investigate stigma receptivity under several flowering developmental stages, and their reproductive success. The results of this study will allow a better understanding of the flowering of this species, particularly its flowering seasons and peaks of flowering, which is important information for the commercialization of this species, as well as to provide strategies to optimize pollination and increase fruit set.

MATERIALS AND METHODS

The experiments were conducted at the Ornamental Plant Research Station of Indonesian Ornamental Crops Research Institute (IOCRI), at Segunung, Cianjur, West Java, Indonesia (6.7°S, 107.0°W GPS) between January to September 2010. The research station is located on the highland of ± 1100 m above sea level. The plants were grown in a net house which transmitted ± 55% of natural light intensity. The relative humidity inside the net house ranged from 70-90%, with average day/night temperatures of 24-26°C/18-20°C.

Three-year-old plants of the *Z. spectabile* collection of IOCRI were used for this study. The plants were grown on 18 plots of 1 m x 8 m, using a mixture of soil: manures: rice husk media with ratio 1:1:1 (v/v/v). The plants began flowering 8 months after planting and continuously produced flowers. The inflorescence of *Z. spectabile* has 1-3 flowers open at a time acropetally, thus every floret in one inflorescence exhibits a range of different developmental stages.

Description of the Flower Stage, Inflorescence Structure and Floral Biology

The growth stages of *Z. spectabile* was based on the principal growth stage described in Biologische Bundesanstalt, Bundessortenamt and Chemical industry (BBCH) scale (Meier, 1997) and growth stage of *Etlingera elatior* determined by Choon et al. (2016). Morphological changes of the shoot apices were observed daily to study the onset of floral initiation. Ten (10) flower buds were tagged to record the duration of flower opening, temporal and spatial separation of pollen shedding and stigma receptivity. The stigma was considered as receptive when extended papillae and exudates were visible on the stigmatic surface. Morphological characteristics such as length and diameter of the bracts, length and diameter of the stem, bract colour, and percentage of open bracts were measured weekly starting at bud stage, i.e. 10% of bract opening, to

full opening, i.e. >80% of bract opening, or when no more florets developed. Petal and labellum length and width, petal colour, length of the pistil and pollen, number of ovules, and number of pollens per anther were measured at anthesis on ten flowers from randomly selected plants. Bract and petal colours were determined using the Royal Standard Colour Chart as reference. All data were recorded between 8:00 and 16:00 hours daily between March-August 2010. The flower structure and number of ovules per ovary were observed from five flowers from different plants under the dissecting microscope (Nikon SMZ1000) at 40x magnification.

Pollen Viability

Five (5) nearly open flowers were collected hourly between 08:00-14:00 hours from the inflorescences of randomly selected plants, and the pollens were collected to study pollen germination. Each inflorescence had 1-3 opened flowers on the same day. Flowers were immediately put in a cool box after collection and brought to the laboratory for further study. The anthers of five randomly picked open flowers were transferred to a single concave microscope slide containing Brewbaker and Kwack (1963) medium for germination study. The Brewbaker and Kwack medium was prepared by dissolving 100 mg of H_3BO_3, 300 mg of Ca $(NO_3)_2.4H_2O$, 200 mg of $MgSO4.7H_2O$ and 100 mg of KNO_3 in 100 mL of double-distilled water as stock solution. The pollen germinating medium was prepared by adding 90 mL of distilled water to 10 mL stock solution. The pollens were incubated in the medium for one hour prior to germination count under the light microscope (Olympus BX51) at 100x magnification. Pollen viability, i.e. those having the pollen tube no less than the pollen diameter, was calculated as percentage of germinated pollen.

The number of pollens of five flowers was counted using a hemacytometer. Pollen from the two-lobes anther was extracted, mixed with 1 ml distilled water, stirred thoroughly and counted on the hemacytometer.

Time and Duration of Stigma Receptivity

Stigma receptivity was evaluated by monitoring the secretion on the stigmatic surface and style extension. Stigma receptivity was also recorded based on fruit set and seed set from artificial pollinations conducted hourly between 08:00 to 14:00 hours on five flowers and replicated three times.

The time of stigma receptivity was determined based on the time of anthesis, position of the stigma and style, maximum stigma secretion, and the time when fruit set, and seed set were highest following hand pollination.

Fifteen flowers were emasculated early on the day of anthesis but before anther dehiscence prior to a hand pollination which was conducted hourly between 08:00-14:00. Pollination was conducted using a tweezer with pollens from flowers of different plants. The stigma was covered with a

plastic covering until the following day to avoid pollination by insect pollinators. Fruit set was calculated as the percentage of pollinated flowers that developed into fruits, and the seed set was calculated as the percentage of ovules within an ovary that developed into viable seeds.

Mating System

To study the mating system, fifteen flowers each were self and cross hand pollinated and another fifteen flowers were left for open pollination as control. For self pollination, flowers were emasculated early on the day of anthesis and then pollinated with the pollen of the same flower, whereas for cross pollination, flowers were pollinated with the pollens from flowers of different plants. For open pollination, flowers were left to natural (insect) pollination. Pollination was conducted between 08:00 to 12:00 hours to avoid the high temperature during midday. The percentage of fruit set, and seed set were calculated from different pollination types and reproductive success was calculated as described by Wiens et al. (1987).

RESULTS

Description of the Flower Stage and Inflorescence Structure

The flowering of *Z. spectabile* in the study location began between January and February (during the rainy season) and the seeds matured during June or July. The spikes grew directly from the rhizome. The flower structure was visible when a tiny spike with fully differentiated bractea developed from the terminal growth apex of the shoot, as comparable to stage 30 of *E. elatior* (Choon et al., 2016) of the principal growth stage 3 or peduncle elongation (Meier, 1997). It took 7 days from the first visible bud to spike emergence of about 20 mm above ground. The emerged spikes took about 3 weeks to fully develop; this stage is comparable to stage 39 of *E. elatior* (Choon et al., 2016), and another 2 weeks to reach bracts opening (a total of about 6 weeks), or comparable to stage 50 of *E. elatior* (Choon et al., 2016) of the principal growth stage 5 or inflorescence emergence according to Meier (1997) (Figure 1A - stage 1). The spike consisted of a stem and inflorescence that comprised of bracts. The flowers were born from the ovate bracts.

Based on its value as cut flower, the morphological development of the *Z. spectabile* inflorescence was classified into 5 stages as follows: 1) bracts showed about 10% opening (3.97 ± 2.07cm long) and were pale yellow in colour (Figure 1A); 2) bracts were elongated (7.07±2.47 cm long) and their colour changed to yellow; 20-30 bracts were open (Figure 1B); 3) flowers emerged from the bracts (30-40 bracts), starting from the lowest position along the inflorescence (Figure 1C); 4) bracts' colour changed to reddish and inflorescence continued to grow until reaching its maximum size (13.47±2.06 cm long) (Figure 1D); 5) bracts' colour changed to bright red indicating the end of flowering in the inflorescence. Flowers rarely emerged from the distal part of the inflorescence (Figure 1E). The morphological characteristics of the inflorescence development in *Z. spectabile* are described in Table 1.

(A) (B) (C) (D) (E)

Figure 1. Five stages of inflorescence development of *Zingiber spectabile*. (A) Stage 1: inflorescence with pale yellow bracts at 6 weeks after spike emergence. (B) Stage 2: inflorescence with yellow bracts at 2-3 weeks after stage 1 (20-30 bracts). (C) Stage 3: inflorescence with reddish bracts, beginning of flower emergence, 4 weeks after stage 2. (D) Stage 4: inflorescence with pale red bracts, flower emergence reached the distal part of the inflorescence, 4-5 weeks after stage 3. (E) Stage 5: inflorescence with bright red bracts, the end of flower emergence, 3-5 weeks after stage 4. Red arrows show opened flowers on each bract

Table 1
Inflorescence development of Zingiber spectabile

Stage	Characteristics	Time	Inflorescence size* (mm)		Notes
			Length	Diameter	
1	Bracts started opening	6 weeks after spike emergence	39.7±20.7	36.6±18.5	Bract colour was pale yellow
2	More opening of bracts	2-3 weeks after stage 1	70.7±24.7	62.0±9.8	Bract colour changed to yellow
3	Bracts fully opened	4 weeks after stage 2	101.9±21.2	70.9±5.7	Around March; first flower opened
4	Bracts' colour changed to reddish	4-5 weeks after stage 3	134.7±20.6	74.9±4.1	Opened flowers up to the middle of the bracts
5	Bracts' colour changed to bright red	3-5 weeks after stage 4	134.7±20.6	75.2±3.9	No more flowers opened

*Values indicates averages ± S.E. (n =15)

Stage 1 of the inflorescence development in this study occurred at the end of January to early February and lasted ± 2-3 weeks before the inflorescence reached stage 2. Stage 3 occurred ± 4 weeks after stage 2 (when the bracts were fully open) by the end of March. The whole development of the inflorescence from stage 1 to stage 5 lasted about 13-17 weeks. The development of the spike from emergence to bract opening took ± 6 weeks, so the actual inflorescence development took about 19-23 weeks. This study was conducted in a location with high elevation in the mountain area of West Java, Indonesia. Flowering phenology changes with elevation gradients. Plants at lower elevation and warmer temperatures typically flower earlier than plants of the same species growing at higher elevation and cooler temperatures (Ziello et al., 2009). Therefore if *Z. spectabile* is to be grown in a lower elevation, this species is likely to flower

earlier. The longer growing period at higher elevation produces larger inflorescences and more marketable cut flowers.

The *Z. spectabile* inflorescence is suitable as cut flower at all stages of its development. Stages 3, 4 and 5, however, display the most attractive bract colour and good- sized flowers for floral arrangement. The selection of inflorescence sizes depends on its purpose of use in a floral arrangement. Thus, the main production of *Z. spectabile* as cut flower lasts between May-July. However, a limited number of inflorescences is available almost all the time provided the plants are maintained carefully. Lessa et al. (2015) reported that the postharvest longevity of *Z. spectabile* in different vase solutions was about 9 days, and that tap water was sufficient to maintain the quality and longevity of *Z. spectabile* inflorescences. Understanding the time of flowering, flower development and flowering period is useful for commercial purposes.

Flower Structure

Zingiber spectabile flowers comprise three petals, one purple labellum with yellow spots, a pistil and one fertile stamen. The *Z. spectabile* flower development began with the appearance of flower buds enclosed by transparent sepals from the bracts around 08:00-09:00 hours (Figure 2A). The sepals splitted open around 10:00 hours (Figure 2B). Hence, the petals splayed while the style was still curving, followed by the appearance of a curved style around 11:00 hours (Figure 2C). At around 12:00 hours the flower was fully blooming, indicated by straightened styles (Figure 2D). The stamen consists of a short undeveloped filament and an anther; the anther consists of two lobes and is positioned at the lower part of the style (Figure 2E). The style is long with the hairy stigma at the tip (Figure 2F). The whole structure of the pistil, i.e. ovary, style and stigma, is longer than the stamens (Table 2) so the stigma is located at a higher position than the anthers at anthesis.

Anthesis occurred at 11:00 hours and the opened flowers began to wilt by 16:00 hours of the same day, so the flowers only last for one day. Every day, one to three flowers open in an inflorescence during the flowering period until all the flowers of the inflorescence have opened. The ovary comprises 3 locules and bears 43.8±10.7 ovules (Table 2). The average number of ovules in an ovary determines the potential number of seeds in a fruit (capsule),

Table 2
Floral characteristics of Zingiber spectabile

Floral parts	Measurement (average±S.E.)
Flower length (cm)	6.80±0.35
Labellum length (cm)	3.17±0.24
Labellum width (cm)	1.36±0.10
Corolla length (cm)	3.23±0.15
Corolla width (cm)	0.72±0.05
Corolla colour	yellow group 10C – 14D*
Pistil length (cm)	5.84±0.25
Anther length (cm)	1.16±0.23
Ovules number in an ovary	43.8±10.7
Pollen number in an anther	143,360±94,979

*Colour classification was based on Royal Standard Colour Chart

although the number of pollens in the anther (143,360±94,979) was considerably higher than the number of ovules. The high number of pollens produced by a flower could serve as pollen stock for other flowers.

Anther dehiscence started around 09:00 hours before flower blooming, during which time the secretion on the stigmatic surface was still absent. However, pollen germination was low, indicating that some pollen was still at maturation stage when the anther had dehisced. The pollen matured 3 hrs after anther dehiscence as it reached highest germination (Table 3). Flower opening (anthesis) started to occur at 11:00 hours at which time secretion started to appear on the stigma and the style was still curving. Full bloom occurred around 12:00 hours, accompanied by the straightening of the style, and higher secretion appeared on the stigmatic surface that lasted until 13:00 hours. The high secretion coincided with high pollen germination (Table 3), however, spatially the stigma was at the farthest position from the anther, and so voluntary self pollination was unfeasible. The temporal separation of anther dehiscence and stigma receptivity is common in Zingiberaceae

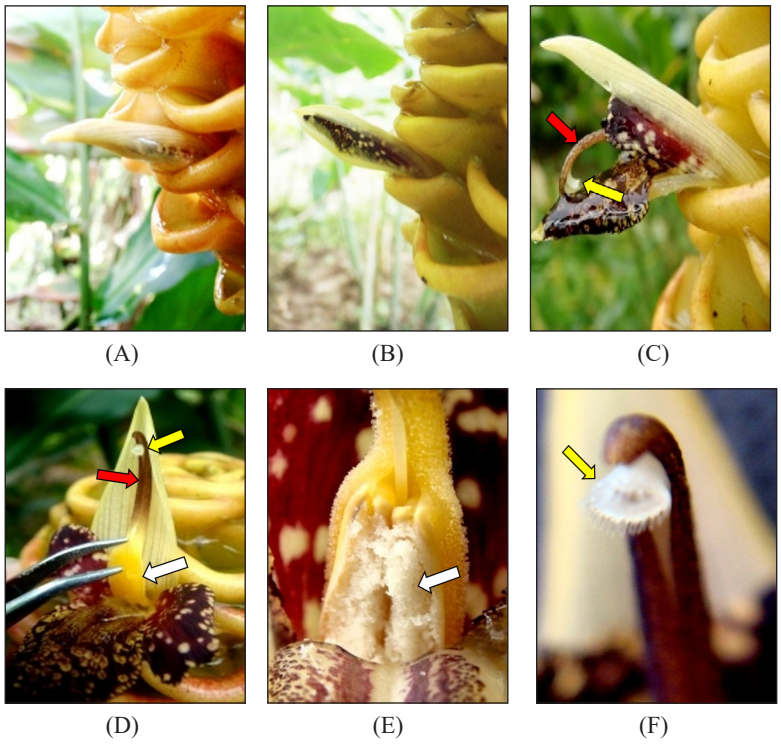


Figure 2. Stages of *Zingiber spectabile* flower development: (A) Flower bud enclosed by yellowish sepals appears from the bract, 08:00-09:00 hours. (B) The sepals split open showing the petals, 10:00 hours. (C) Flower starts to bloom, petals splay, and the style is still curving (red arrow) with the stigma still folded (yellow arrow), 11:00 hours. (D) Fully blooming flower, the style has straightened (red arrow), the stigma is exposed (yellow arrow) and the anther is at the lower part of the style (white arrow), 12:00 hours. (E) The dehisced anther (white arrow). (F) The stigma with stigmatic hairs (yellow arrow) at the tip of the style

although the overlapping between the two events varies among species. *Curcumorpha longiflora* anthers dehisce one day before the stigma becomes receptive (a two-day flower) (Gao et al., 2004). In *Curcuma aeruginosa* the difference is a matter of several hours (Aswani & Sabu, 2017) whereas it is only around 20 minutes in *Zingiber officinale* (Melati et al., 2015). In this case the flower of *Z. spectabile* cannot be categorized as flexistyly because the flower's function as protogynous or protandrous was determined temporally and was not according to the direction of stigma movement. The period of high pollen germination and high stigmatic secretion suggests that pollination is best performed between 12:00-13:00 hrs. Information on the time and duration of stigma receptivity is crucial for successful breeding or artificial pollination as it determines the success of pollination, i.e. higher fruit set and seed set (Dafni & Maues, 1998).

Based on the flower development hand cross pollination was carried out from 08:00 (before anther dehiscence) to 14:00 hours (secretion from the stigmatic surface had started diminishing). Hand cross pollination showed that the fruit set, and seed set did not follow the pattern of pollen germination and stigmatic secretion (Table 4). Fruit set (60-93.3%) and seed set (45.7-72.4%) were not significantly different during the day of anthesis. Although the anther dehisced in the morning, pollen germination was low, and the stigma was somehow unexposed for pollination, the fruit set, and seed set were equally high as with any other time of the day. It is possible that the maturing pollen that arrived onto the unreceptive stigma synchronized so that the recognition process between the pollen and stigmatic surfaces occurred at the prime time and resulted in fertilization, hence yielding fruit set and seed set. It was surprising that the higher pollen germination and the receptive stigma (high secretion) at 12:00 hours (Table 3) did not result in a higher fruit set and seed set than the earlier time of the day when the pollen germination was low, and the stigma was not receptive yet. The higher temperature during the daytime (24-26^{o}C) might be harmful to the pollen and reduce its viability rapidly, thus, lowering the fruit

Table 3
Morphological changes in anther and stigma during anthesis of Zingiber spectabile

Time	Flower stage	Anther	Pollen germination (%) *	Style	Stigmatic surface
08.00	Flower bud	Intact	20.95^{b}	curving	No secretion
09.00	Flower bud	Dehiscence started	14.60^{b}	curving	No secretion
10.00	Flower bud	Dehiscence	34.15^{b}	curving	Secretion noticeable
11.00	Start to bloom	Dehiscence	36.58^{b}	curving	More secretion
12.00	Fully blooming	Dehiscence	62.67^{a}	straightening	High secretion
13.00	Fully blooming	Start withering	32.46^{b}	straightened	High secretion
14.00	Fully blooming	Withered	35.88^{b}	straightened	Less secretion

*Numbers followed by the same letter are not significantly different based on DMRT at 0.05

Table 4
Time of pollination, fruit set, seed set and reproductive success of Zingiber spectabile

No	Time of pollination	Fruit set (%)	Number of seeds per fruit (seed set %) *	Reproductive success (%) **
1	08.00	14 (93.3)	29.5 (67.4)	62.9
2	09.00	14 (93.3)	26.9 (61.4)	57.3
3	10.00	13 (86.7)	31.7 (72.4)	62.8
4	11.00	11 (73.3)	23.5 (53.7)	39.4
5	12.00	14 (93.3)	27.5 (62.8)	58.6
6	13.00	9 (60.0)	20.0 (45.7)	27.4
7	14.00	14 (93.3)	24.6 (56.2)	52.4

*Seed set was calculated based on number of ovules per ovary (43.8) (Table 2) on 15 pollinated flowers
** Reproductive success was calculated according to the method described by Wiens et al. (1987)

set and seed set. The data indicated that despite dichogamy, pollination success was comparable throughout the day of anthesis. So, it was concluded that the optimum time for hand pollination was between 11:00-13:00 hours. Technically, hand pollination is much easier when the flower has started to bloom as the stigma is more exposed and accessible. Pollination after midday tends to produce fewer seeds per fruit which could be due to the deterioration of the entire flower making it unfit for pollination and fertilization.

The pre-emergence reproductive success indicates the proportion of the ovules that developed into viable seeds. Our study demonstrated that the reproductive success following cross pollination was relatively high, ranging between 27.4 – 62.9% (Table 4), which meant 274-629 out of 1000 ovules produced by a *Z. spectabile* could potentially develop into viable seeds.

The number of ovules in an ovary averaged 43.8 (Table 2), but the number of seeds per fruit ranged from 20.0-31.7 (45.6-72.4%), indicating that not all ovules could develop into viable seeds following cross pollination. Our observation also revealed that not all bracts could produce viable flowers. Most of the blooming flowers were those positioned at the two third midsection of the inflorescence. IOCRI reported that the average number of bracts per inflorescence of *Z. spectabile* was 159 (IOCRI/balithi.litbang.pertanian.go.id/leaflet-download-08-zingiber.pdf). Hence the potential for seed yield is relatively high and gives breeders considerable opportunity to accomplish hybridization.

Fruit set from self pollination was not significantly different from that of cross pollination, ranging from 74.3-80% (Table 5), whereas open pollination did not set any fruit. Similarly, the seed set from self pollination was similar to that from cross pollination, ranging from 15-36 seeds per fruit (34.2-82.2%). These data imply that *Z. spectabile* is self-compatible. This finding is rather unexpected. So, we reckon that the temporal and spatial separation of the reproductive organs (stigma is positioned higher than the anther and anthers dehisce

Table 5
Zingiber spectabile *fruit set and seed set following different types of pollination*

Types of pollination	Flower	Fruit set (%)	Number of seeds per locus			Number seeds per fruit
			Side locule	Middle locule	Site locule	
Self	1	100	13±1.7	7.3±2.5	11.7±2.5	32±5.6
	2	100	11.3±3.1	8.3±3.8	10.3±1.5	30±7.9
	3	33.3	14±0	8 ±0	14±0	36
	4	66.7	7±9.9	4±5.7	5±7.1	16±22.6
	5	100	10.3±2.3	8±3.6	10.7±1.2	29±4.6
	Average	80.0				
Cross	1	66.7	5±7	3.7±4.7	6.3±7.1	15±18.7
	2	66.7	11±1.4	3.5±5.0	9	23.5±3.5
	3	100	12.3±4.5	5	15±3.5	32.3±7.6
	4	33.3	11	7	6	24
	5	100	11.7±4.2	8±4.4	10.7±3.5	30.3±11.4
	Average	73.3				
Open	1	0	0	0	0	0
	2	0	0	0	0	0
	3	0	0	0	0	0
	4	0	0	0	0	0
	5	0	0	0	0	0

before the stigma is receptive), is the mechanism of the species to prevent self pollination. Gao et al. (2004) reported that self-compatibility was also found in *Curcumorpha longiflora* (Zingiberaceae) as indicated by the similar rate of germinated pollen and pollen tube length following self and cross pollination.

The consequence of self-compatibility is that emasculation will have to be done for hybridization which will make the crossing tasks more tedious. However, emasculation might not be necessary in *Z. spectabile* because the anther is embedded at the proximal end of the pistil (near the ovary), whereas the stigma is at the distal end, and so voluntary self pollination is not feasible. Therefore, restriction of insect visits is necessary to avoid unwanted hybridization when emasculation is not implemented.

The diameter of the ovaries of the self and cross-pollinated flowers increased significantly and reached its maximum size during the first four weeks after pollination/ WAP (Figure 3), and was larger than that of open pollination, merely due to the number of seeds developed within (Table 5). The ovary diameter did not show more growth until the fruits and seeds matured, indicating that the seeds were morphologically mature at four weeks after pollination when the embryo had been formed. The accumulation of storage reserve in the seeds likely occurred between 4-10 WAP until the seeds were physiologically mature.

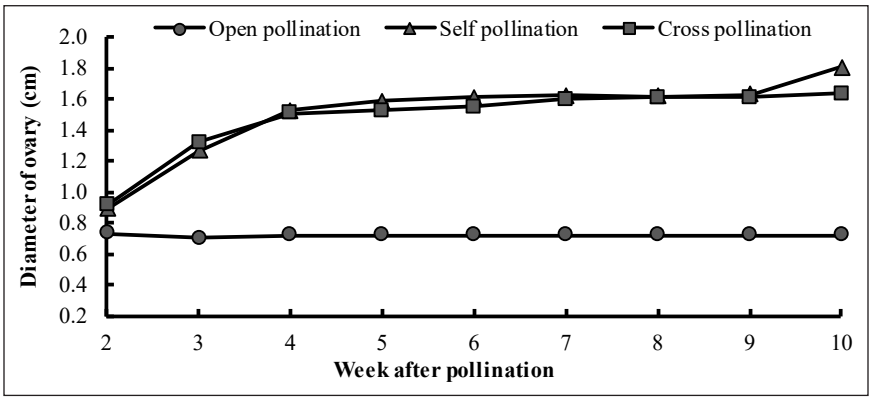


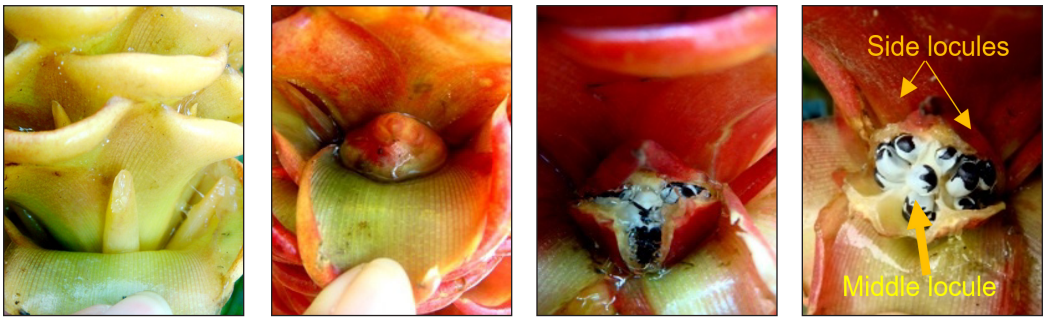
Figure 3. Development of *Zingiber spectabile* ovary diameter following pollination

Open pollination failing to set fruits and seeds was possibly due to the absence of suitable pollinating insects in the area of study. The effective time for insect pollinators to forage the flowers was from morning till early afternoon (08:00-14:00 hours), during which time the insects collected pollens from the dehisced anther and deposited them onto the stigma. Based on our study, very little nectar was available when the flower was fully blooming at 12.:00 hours. So, the primary attractant for the pollinating insect is the colourful petals. This narrows the pollination window considerably. Moreover, the embedded anther at the proximal end of the pistil makes it difficult for the insect to collect the pollens; the delicate structure and position of the stigma make it difficult to be in contact with the insect pollinators.

Phenological flowering stages can have inter-annual variability and large spatial differences (Koch et al., 2009). Variabilities exist between individual plants, and are affected by biotic and abiotic environment, particularly by temperature and precipitation (Koch et al., 2009).

The ovary comprises three locules, and each locule bears several ovules. In most cases the percentage of seed set in the outer/edge locules was higher than that of the inner/middle ones (Table 5). This could be simply because the outer locules are more spacious than the inner locules, allowing the seeds to grow fully. The success of a pollination can be evaluated at 1 WAP, when the ovary appears to be fresh (Figure 4A). The ovary enlarges rapidly within 3 WAP (Figure 4B) and continues to enlarge until it cracks open around 9 WAP (Figure 4C) showing the developing seeds inside. At this stage the seeds are intact in each locule. At 10 WAP the crack gets wider as the seeds continue to develop and become loosely intact (Figure 4D). Harvesting the seeds at this stage is much easier.

Understanding phenology could greatly enhance growers' ability to plan management practices in relation to the events occurring within the plants. Information about the time of flowering, fruit set, and the relationship between these events will allow better planning management of cultural practices at optimum times. The fact that hand



(A) (B) (C) (D)

Figure 4. Development of *Zingiber spectabile* ovary following cross pollination: (A) an ovary after pollination (1 WAP); (B) enlarged ovary (3 WAP); (C) ovary starts to crack (9 WAP), showing the intact seeds; (D) ovary cracks open showing the loosely mature seeds (10 WAP). WAP=week after pollination

pollination successfully set fruits and viable seeds (data not included), the hybridization of *Z. spectabile* will be feasible and more flowers variations can be expected.

CONCLUSION

The inflorescence development of *Zingiber spectabile* from the start of the bracts opening to fully open bracts take 13-17 weeks. The ideal time for artificial pollination is between 11:00-13:00 hours. Anthers dehisce prior to stigma receptivity. *Zingiber spectabile* is self- compatible and cross pollination does not increase fruit set and seed set.

ACKNOWLEDGEMENT

The authors thank IOCRI for the permission to use the *Zingiber spectabile* plant collection for this study, and Professor Peter A. Sopade of the School of Agricultural Sciences, Xichang University, China, for his valuable comments and suggestions on the manuscript.

REFERENCES

Aswani, K., & Sabu, M. (2017). Pollination biology of *Curcuma aeruginosa* (Zingiberaceae): An important medicinal plant. *The International Journal of Plant Reproductive Biology*, *9*(1), 32-36.

Brewbaker, J. L., & Kwack, B. H. (1963). The essential role of calcium ion in pollen germination and pollen tube growth. *American Journal of Botany*, *50*(9), 859-865.

Chee, B. J., & Hoo, L. K. (2010). *The spectacular ginger:* Zingiber spectabile *Griffith*. Retrieved June 12, 2019, from https://www.researchgate.net/publication/248381623_The_Spectacular_Ginger_Zingiber_spectabile_Griffith?enrichId=rgreq-c0b024b22909a63abc5e0f8fc3e18bd6-XXX&enrichSource=Y292ZXJQYWdlOzI0ODM4MTYyMztBUzoxMDE3MzgzMjU0Nzk0NDBAMTQwMTI2NzcxNTY4Mg%3D%3D&el=1_x_2&_esc=publicationCoverPdf

Choon, S. Y., Ding, P., Mahmud, T. M. M., & Shaari, K. (2016). Phenological growth stages of torch ginger (*Etlingera elatior*) inflorescence. *Pertanika Journal of Tropical Agricultural Science*, *39*(1), 77-78.

Criley, R. A. (2011). Response of *Etlingera corneri* and *Zingiber spectabile* to photoperiod. *Heliconia Society Bulletin*, *17*(3), 1-3.

Dafni, A., & Maues, M. M. (1998). A rapid and simple procedure to determine stigma receptivity. *Sex and Plant Reproduction*, *11*(3), 177-180.

Gao, J. Y., Zhang, L., Deng, X. B., Ren, P. Y., Kong, J. J., & Li, Q. J. (2004). The floral biology of *Curcumorpha longiflora* (Zingiberaceae): A ginger with two-day flowers. *American Journal of Botany*, *91*(2), 289-293. doi: 10.3732/ajb.91.2.289.

Gibs, P. E. (2014). Late-acting self-incompatibility – The pariah breeding system in flowering plants. *New Phytologist*, *203*(3), 717–734. doi: 10.1111/nph.12874.

Koch, E., Bruns, E., Chmielewski, F. M., Defila, C., Wolfgang L., & Menzel, A. (2009). Definition of phenology and seasonality. In O. Baddour & H. Kontongomde (Eds.), *Guidelines for plant phenological observations* (pp. 5-13). Geneva, Switzerland: World Meteorological Organization.

Leong-Skornickova, J., & Gallick, D. (2010). What are gingers. In J. Leong-Sknornickova & D. Gallick (Eds.), *The ginger garden* (pp. 5-9). Singapore: National Parks Board Singapore Botanic Garden.

Lessa, M. A., Almeida, E. F. A., Nascimento, A. M. P., Curvelo, I. C. S., Reis, S. N., Nogueira, D. A., … Paiva, P. D. O. (2015). Postharvest conservation of ornamental ginger (*Zingiber spectabile*). *Acta Horticulturae*, *1060*, 307-313. doi:10.17660/ActaHortic.2015.1060.46

Li, Q. J., Xu, Z. F., Kress, W. J., Xia, Y. M., Zhang, L., Deng, X. B., … Bai, Z. L. (2001). Pollination: Flexible style that encourages outcrossing. *Nature*, *410*(260), 432.

Loges, V., da Costa, A. S., Guimaraes, W. N. R., & Teixeira, M. C. F. (2011). Market potential of torch ginger and beehive ginger. *Heliconia Society Bulletin*, *17*(4), 1-4.

Meier, U. (Ed.) (1997). *Growth stages of mono- and dicotyledonous plants: BBCH monograph*. Berlin, Germany: Blackwell Wissenschafts-Verlag.

Melati, Palupi, E. R., & Bermawie, N. (2015). Floral biology of ginger (*Zingiber officinale* Rosc.). *International Journal of Current Research in Bioscience and Plant Biology*, *2*(4), 1-10.

Olmstead, R. G. (1989). The origin and function of self-incompatibility in flowering plants. *Sexual Plant Reproduction*, *2*(3), 127-136.

Sadhu, S. K., Khatun, A., Ohtsuki, T., & Ishibashi, M. (2007). First isolation of sesquiterpenes and flavonoids from *Zingiber spectabile* and identification of zerumbone as the major cell growth inhibitory component. *Natural Product Research*, *Part B: Bioactive Natural Products*, *21*(14), 1242-1247.

Sanzol, J., & Herrero, M. (2001). The effective pollination period in fruit trees. *Scientia Horticulturae*, *90*(1-2), 1–17.

Sivasothy, Y., Sulaiman, S. F., Ooi, K. L., Ibrahim, H., & Awang, K. (2013). Antioxidant and antibacterial activities of flavonoids and curcuminoids from *Zingiber spectabile* Griff. *Food Control*, *30*(2), 714-720.

Sivasothy, Y., Awang, K., Ibrahim, H., Thong, K. L., Fitrah, N., Koh, X. P., & Tan, L. K. (2012). Chemical composition and antibacterial activities of essential oils from *Zingiber spectabile* Griff. *Journal of Essential Oil Research*, *24*(3), 305-313. doi: 10.1080/10412905.2012.676803.

Takano, A., Gisil, J., Yusoff, M., & Tachi, T. (2005). Floral and pollinator behaviour of flexistylous Bornean ginger, *Alpinia nieuwenhuizii* (Zingiberaceae). *Plant Systematics and Evolution*, *252*(3-4), 167–173. doi:10.1007/s00606-004-0258-4.

Wickramasinghe, P., Harrison, D. K., & Johnston, M. E. (2010). Reproductive biology and intergeneric breeding compatibility of ornamental *Portulaca* and *Calandrinia* (Portulacaceae). *Australian Journal of Botany*, *57*(8), 697-707. doi:10.1071/BT0910.

Wiens, D., Calvin, C. L., Wilson, C. A., Davern, C. I., Frank, D., & Seavey, S. R. (1987). Reproductive success, spontaneous embryo abortion, and genetic load in flowering plant. *Oecologia*, *71*(4), 501-509.

Yi, W., Law, S. E., McCoy, D., & Wetzstein, H. Y. (2006). Stigma development and receptivity in almond (*Prunus dulcis*). *Annal Botany*, *97*(1), 57–63. doi:10.1093/aob/mcj013.

Ziello, C., Kostova, M., Koch, E., & Menzel, A. (2009). Influence of altitude on phenology of selected plant species in the Alpine region (1971-2000). *Climate Research*, *39*(3), 227-234.

Pertanika J. Trop. Agric. Sc. 42 (4): 1391 - 1403 (2019)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

The Role of Wood Vinegar in Enhancing the Microbial Activity and Physicochemical Properties of Palm Oil-Based Compost

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ABSTRACT

This paper presents an experimental study of the effect of wood vinegar at different concentrations on the biological and physicochemical properties of the composts by using the solution with the ratio of wood vinegar to distilled water in the range of 1:100 to 1:500 (v/v). The composting process was conducted by in-vessel composting method within 60 days where temperature and pH were recorded daily. The composts were then analyzed on the microbe counts, pH, moisture content, water holding capacity, and nutrient contents. XRF and CHNS analyzers were used to measure the NPK content exist before and after composting process. This research attempted to investigate the effect of wood vinegar concentration towards physiochemical and biological properties of the composts. The results showed that lower concentration of wood vinegar could potentially enhance microbial activity which could accelerate the composting process. However, in terms of physical properties, sample 1:400 (v/v) had recorded the highest reading for water holding capacity and moisture content which were about 2 to 19 % (ml/100g) and 1 to 27 % (w/w) higher than other samples, respectively. Therefore, it can be concluded that sample 1:400 (v/v) shows the best condition where it has

ARTICLE INFO

Article history:
Received: 7 May 2019
Accepted: 24 September 2019
Published: 13 November 2019

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ISSN: 1511-3701
e-ISSN: 2231-8542

achieved well-balanced condition between physicochemical and biological properties.

Keywords: Compost, microbe growth, wood vinegar

INTRODUCTION

Fertilizer plays a major role in agriculture sector. Both organic and inorganic (chemical) fertilizers are widely used in all countries, including Malaysia. The continuous use of chemical fertilizers poses a tremendous risk to the soil and environment. The need of having a new alternative to replace the use of chemical fertilizers has urged the research enthusiasts to discover a new way of benefiting the organic waste. Nowadays, organic farming has become more popular compared to the farming that involves the utilization of chemical fertilizers as it is inexpensive and can be the source of plant nutrients (Yap, 2012).

In Malaysia, palm oil production is currently the most significant agricultural export for the country and has increased due to global demand (Otieno et al., 2016). The increase in the palm oil production has caused pollution to the environment where enormous amounts of palm oil mill effluent (POME) sludge is generated and can be regarded as pollutant and waste material (Kanakaraju et al., 2016). POME sludge is a bio-waste generated from palm oil mills during the processing of the palm fruit for palm oil production (Obi, 2015). According to Rupani et al. (2010), POME sludge has pH of 8.4 with moisture content of 85.0 %. Besides that, POME sludge also contains 3.6 % nitrogen, 0.9 % phosphorus and 2.1 % potassium. Other than that, palm oil mill also produces decanter cake (DC) as waste. DC generates about 4-5 % from the total weight of fresh fruit bunch (FFB) (Dewayanto et al., 2015). DC accounts for 28.5% of the fresh fruit bunch (FFB) and it can be applied as an amendment to soil for the growth of the plant (Embrandiri et al., 2016). DC also has high moisture content, high biodegradability and nutrient-rich contents (Sahad et al., 2014). Research by Yahya et al. (2010) had reported that DC contained about 2.38 % nitrogen, 0.39 % phosphorus and 2.39 % potassium. Meanwhile, rice husk (RH) is an abundant agricultural solid waste which is the result of rice-milling process (Lin et al., 2012). The burning process of RH to generate electricity has produced rice husk ash (RHA). The ash contains 87-97 % silica that makes it a valuable material for agricultural application (Kumar et al., 2012). In agriculture activities, one of the alternatives to reduce the dependence on the use of chemical pesticides and inorganic fertilizers is wood vinegar. Wood vinegar or pyroligneous acid is a kind of crude reddish-brown liquid which is produced from distillation of biomass during pyrolysis process (Zhai et al., 2015). Basically wood vinegar contains acetic acid (34.4 %) propanoic acid (2%) and methanol (5.2 %) as reported by Payamara (2011). In agriculture, wood vinegar is used to promote rooting and germination of seed (Zhai et al., 2015). If applied to the soil in high concentrations, wood vinegar inhibits eelworms and soil diseases. However,

in lower concentrations, it increases the quantity of useful microbes (Kishimoto & Tsuyoshi, 2015).

To overcome the excessive production of POME, the wastes from the palm oil industry can be utilized as organic fertilizer. Although many research has been conducted using POME sludge and DC, there is still lack of data about the effect of these wastes on the composting process. Rice husk and wood vinegar are claimed to be very beneficial to the plant and compost. Thus, the aim of this research is to study the effect of wood vinegar concentrations on biological and physicochemical properties of the composts. The properties include microbe counts, pH, moisture content, water holding capacity, and N, P, K values.

MATERIALS AND METHODS

Sample Preparation

The palm oil mill sludge and decanter cake were collected at FELDA Neram, Terengganu, Malaysia, and the rice husk ash was obtained from Salloma Nursery, Pahang, Malaysia. The wood vinegar was supplied in-kind by ACGE Company, Singapore.

Raw Material Analysis

In order to analyze the raw material, the samples were dried and ground to 0.08 mm before it was tested for physical and chemical properties. For physical properties, samples were analyzed in terms of moisture content, water holding capacity and pH. In order to analyze the moisture content, the samples were weighed before dried in an oven at 105 ± 2°C for 5 h. Then, the samples were taken out from oven and be weighed again. Standard test ASTM D4442-16 was used to calculate the moisture content of sample.

For water holding capacity, the samples were saturated with water by mixing 10 g of sample with 50 mL of water. The weight of the samples with dropped off water were recorded at 30 min intervals until the sample began to dry. After that, the samples were dried at 105°C for 48 h in an over and were weighed again. The water holding capacity was calculated based on standard test ASTM D2980-02. Apart from that, pH of samples was determined by using Takemura DM15 soil pH meter. The pH meter was inserted into the compost and the reading of pH was taken. Meanwhile for chemical properties, analysis of CHNS and XRF were used. For XRF analysis, standard test ASTM E1621 was used to detect the percentage of phosphorus and potassium. The WDXRF X-ray fluorescence spectrometer instrument, model Axios^{mAX} made in Netherlands by PANalytical was used in the investigation. Meanwhile for CHNS analysis, standard test ASTM D5291 was used to detect percentage of nitrogen.

Effect of Wood Vinegar Concentration on the Growth of Microbe

The wood vinegar was diluted in distilled water. The ratio of wood vinegar to distilled water that were prepared are 1:100, 1:200, 1:300, 1:400 and 1:500 (v/v), in which that, 1 mL of wood vinegar was added into the

distilled water of 100 mL, 200 mL, 300 mL, 400 mL and 500 mL for dilution process. The best concentration of wood vinegar in compost mixture towards the microbe growth was chosen based on the highest microbe count (CFU) on nutrient agar after 3 to 5 days of incubation period. Each of the colored dots appeared on the surface of nutrient agar were counted regardless of size and color intensity ("Microbiological examination – Total colony number SCAN-CM 60:02 SCAN-P 81:02", 2002).

Effect of Wood Vinegar on Composting Process

The wood vinegar concentration in the range of 1:100 to 1:500 (v/v) were added to the compost mixture consist of palm oil mill effluent (POME) sludge, decanter cake (DC) and rice husk ash (RHA) with the weight of the compost mixtures was 5 kg. Meanwhile, the mass composition for POME, DC and RHA were based on the previous research reported by Ramli et al. (2016). The process of composting was carried out by using in-vessel composting in a size of 16 cm (H) x 30 cm (L) x 18 cm (W). In order to ensure the samples, reach maturation stage, the temperature and pH of the compost were recorded and monitored daily for 60 days. The composts were turned once a week by using a spade to aid the decomposition process. Apart from that, 200 mL of water was added to the compost once a week in order to maintain the moisture content within the range of 40 to 65 % as suggested by Woods End Research Laboratory (WERL) (2005) and Zakarya et al. (2018). Once the composting process was completed, the samples were then analyzed for physicochemical properties. For this property, the same analysis procedure described in raw material analysis was repeated.

RESULTS AND DISCUSSION

Effect of Wood Vinegar on Microbe Count

The total number of microbes was counted using the colony counts technique. Figure 1 shows the result that was obtained from the experiment. For this experiment, it was expected that the dilution of wood vinegar at ratio 1:300 to 1:500 (v/v) would significantly give a higher count of total microbes (Rui et al., 2014). Based on Figure 1, it shows that the mixture of 1:500 (v/v) of wood vinegar to the sample of POME sludge and rice husk gave the highest colony count with the value of 1.1×10^{18} CFU/mL, while the control sample with absence of wood vinegar solution, only gave the colony counts of 4×10^{17} CFU/mL.

According to Kishimoto and Tsuyoshi (2015), in high concentrations, the wood vinegar is applicable to inhibit the soil diseases. While, at low concentrations, the wood vinegar can be used to improve the quantity of useful microbes. This is because, at high concentrations, the high acidity and the presence of germicidal ingredients such as phenol and methanol in wood vinegar tend to kill the microbes which are weak in acid. However, at low concentration, the population of microbes is significantly increased which is due to the effect of acetic

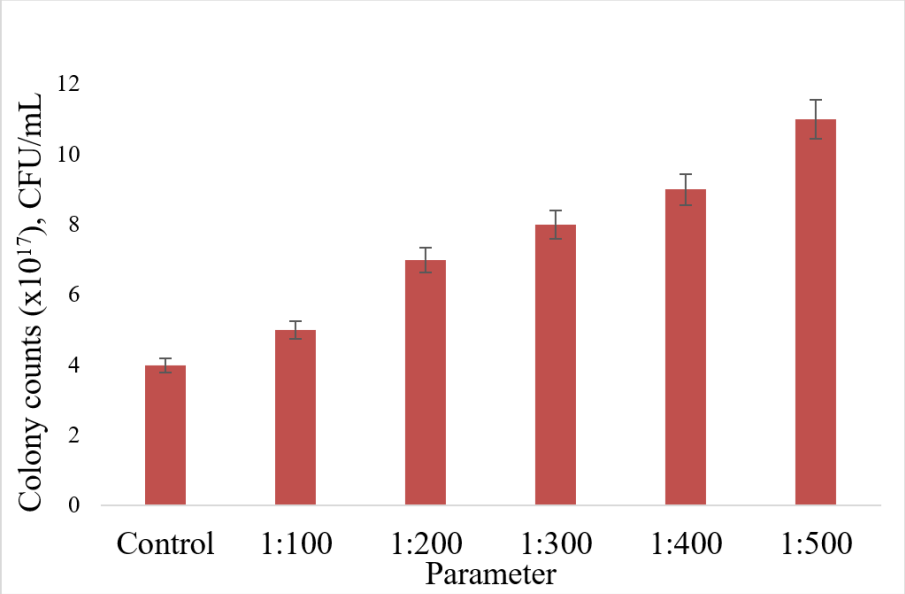

Figure 1. Colony counts of microbes

acids exist in wood vinegar. A substance named as acetyl coenzyme is produced from the presence of acetic acids. The acetyl coenzyme will be further converted into various substances that can facilitate the plants and microbes' growth. However, at a very low concentration of acetic acid, the effect on the growth of microbe would be insignificant as stated by Rui et al. (2014) in their research. Thus, the wood vinegar at 600 fold dilution gave lower microbe count compared to 300 fold and 500 fold dilution of wood vinegar. Therefore, the best concentration of wood vinegar should not be prepared greater than 500 fold dilution to avoid insignificant effect of acetic acid towards the growth of the microbe (Benzon et al., 2015).

Temperature Profile of Compost

The temperature is an important parameter to determine the success of composting process. The heat produced by the compost is a by-product of the microbial breakdown of organic material. In this experimental study, the composts were synthesized using the mixture of palm oil mill effluent (POME) sludge, decanter cake, rice husk and the solutions at different concentrations of wood vinegar. The temperature profile of the compost throughout composting period of 60 days is illustrated in Figure 2.

Based on Figure 2, the temperature of the composts started to rise from day 10 until the temperature reached more than 40 °C. This stage is known as mesophilic stage. The stage when the temperature is maintained at temperature between 41°C to 77°C is known as thermophilic stage of composting. However, for these composts, the temperature obtained by the composts was not high enough to maintain the

thermophilic stage. Some of the composts might not be able to enter this stage. This might be due to the size of plastic container that was too small and leads to the heat loss to the surrounding (Misra et al., 2003). Hence, other external factors such as weather, humidity of surrounding area and thickness of container might also promote the heat loss and caused the compost unable to achieve the temperature above 41°C. However, at this stage the composts still managed to maintain the maximum temperature repeatedly for about 6 days before the temperature dropped to ambient temperature. The similar pattern of temperature profile was also reported by Trisakti et al. (2018). The next stage would be the curing stage. Figure 2 shows the curing stage was between day 40 to day 60, whereby the temperature of compost was slowly dropped to the ambient temperature. At this stage, there is no longer rise in temperature as the compost is already stabilized and the residual substances are fully consumed (Lee, 2016).

According to Sarkar and Chourasia (2017), vigorous microbial activity and rapid degradation of organic matter may increase the compost temperature. From Figure 2, the highest temperature was achieved by sample 1:400 (v/v). However, for the microbe counts, the highest value was obtained by sample 1:500 (v/v) as shown in Figure 1. This contradiction might be due to the insufficient aeration of sample 1:400 (v/v) as manual turning was done on the composts. Previous research by Zhi-Qiang et al. (2017) indicated that the lacked of aeration on the compost had led to the increase of compost temperature, which similarly happened in this case.

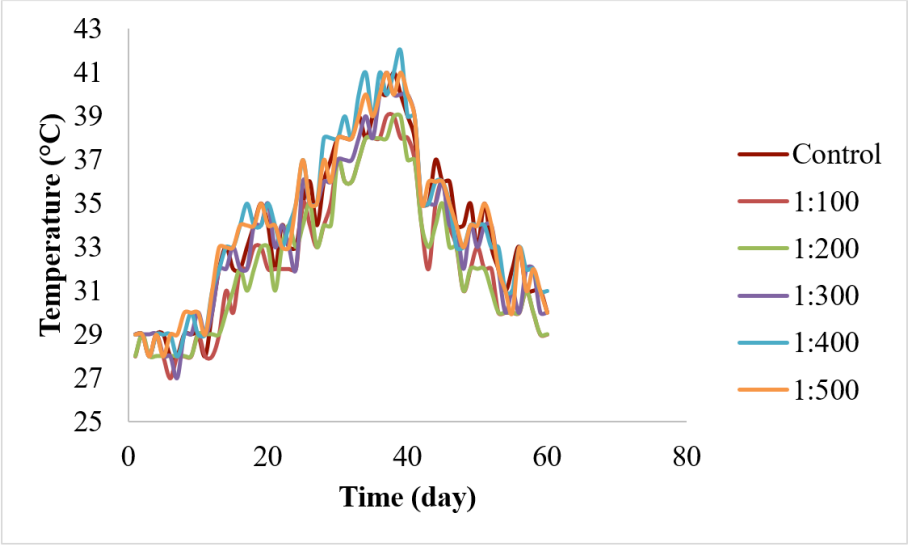

Figure 2. Temperature profile of the compost throughout composting period of 60 days

pH Profile of Compost

The pH of the composts was monitored daily for 60 days. The pH profile for the composting process is presented in Figure 3. Based on the figure, it shows that, at the beginning of the composting process, the pH of the composts was in the range of 3.5 to 5, which was acidic. The lowest pH was recorded by sample 1:100. Later, the pH of the composts was gradually increased on day 15th of composting process and eventually reached the neutral level of 7. Until the 60th day of composting period, the pH profile for all composts tended to be closer to neutral level, which indicated the maturity of the composts.

Based on Figure 3, pH of the composts was acidic at the beginning of the composting process due to the effect of organic acids in raw materials and the presence of acetic acid in wood vinegar. The lowest pH was recorded by sample 1:100 which was likely caused by the presence of high amount of acetic acid as the sample 1:100 contained the highest concentration of wood vinegar. As the time increased, the pH of composts was gradually increased caused by rapid metabolic degradation of organic acid contained inside the composts (Hock et al., 2009). Apart from that, the increase of pH also occurred due to the transformation of nitrogen (N) into ammonia (NH_3) or ammonium (NH_4^+) through ammonification process (Trisakti et al., 2018). At the end of the composting process, the pH of all composts were close to neutral in which the organic acids contained inside the composts were neutralized due to the buffering nature of humic substances (Hock et al., 2009).

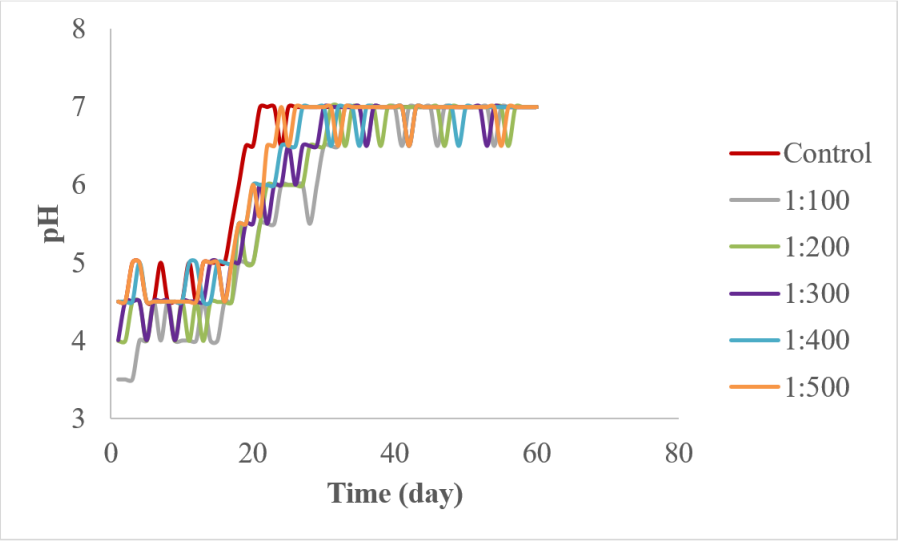

Figure 3. pH profile of the compost throughout composting period of 60 days

Chemical Properties of Compost

The chemical properties of the composts were investigated using XRF and CHNS analysis. By using XRF analysis, the composition of major components which are the phosphorus (P) and potassium (K) contained in each parameter were determined while CHNS analysis was used to determine the composition of nitrogen (N) inside the compost samples. The N, P, K composition in each sample are tabulated in Table 1. For the composition of N and K, it shows that the ratio of 1:500 (v/v) was slightly higher with the value of 8.82 % and 10.70 %, respectively, compared to the other parameters. Meanwhile, for the composition of P, the composts with the ratio of 1:400 and 1:500 (v/v) were slightly higher compared to the other samples with the value of 8.26 %. The lowest N, P, K values were recorded by the control with the value of 5.72 %, 7.75 % and 9.59 %, respectively.

According to Mungkunkamchao et al. (2013), only small amount of N, P, K is contained in wood vinegar. However, the effect of wood vinegar in promoting the growth of root for the plants has enhanced the nutrients intake by the plants and thus, promoting the growth of the plants (Hagner et al., 2013). Previous research by Jeong et al. (2015) also found that the application of wood vinegar had enhanced the N, P, K content in soil. Although it was not significantly increased, it showed that there was a possibility of the N, P, K content would be increased if wood vinegar was added to the compost, compared to the control that did not contain any wood vinegar. The increased of N, P, K content was due to the presence of beneficial microbes inside the composts. The results obtained in Table 1 correlated well with the results of microbial counts in Figure 1 whereby it showed that, as the concentration

Table 1

N, P and K composition of the samples

Materials	Sample	Elements		
		N (%)	P (%)	K (%)
Raw Materials	POME Sludge	5.63	5.23	5.85
	Decanter Cake	2.17	2.39	0.62
	Rice Husk Ash	3.11	0.65	1.44
Compost	Control	5.72	7.75	9.59
	1 to 100	5.93	8.18	10.53
	1 to 200	6.64	8.15	10.63
	1 to 300	7.35	8.17	10.41
	1 to 400	7.61	8.26	10.49
	1 to 500	8.82	8.26	10.70

of wood vinegar decreased from 1:100 to 1:500 (v/v), the microbe count and the N, P, K contents were also increased.

The beneficial microbes play a significant role in boosting the nutrients content, especially the N, P, K, in the composts which are useful for the plant growth (Singh et al., 2017). According to Rashid et al. (2016), the microbes are able to enhance the nutrient availability in the soil through the decomposition of organic matter, N fixation, and P and K mobilization. These nutrients will be converted to the preferred nutrients form for plants, which is in ionic species, such as ammonium, nitrate and phosphate (Jacoby et al., 2017) and potassium ion (Kant et al., 2006).

The presence of nitrogen is beneficial for the plants as it imparts dark-green color in plants, promotes leaves, stem and other vegetative part's growth and development, and also stimulates the root growth. For vegetables, the presence of nitrogen can promote the early growth, improve fruit quality and enhance the leafy vegetables growth (Leghari et al., 2016).

Other than that, the presence of phosphorus is advantageous to plant. According to Razaq et al. (2017), for plant growth, phosphorus is considered as primary nutrient and it is needed by the plants to sustain optimum plant production and quality. Other than that, phosphorus also plays an important role in root branching and lateral root morphology. The combination of nitrogen and phosphorus improves the root surface area, the length of root and root-shoot mass.

According to Prajapati and Modi (2012), potassium has the ability to increase crop yield and improves the quality of the plants. It is also needed by the plant for growth process. The presence of potassium can improve the physical quality, disease resistance and shelf-life of fruits and vegetables.

Physical Properties of Compost

In this section, the physical properties of compost that were measured are pH, moisture content (%), and water holding capacity (mL/100g). The results are tabulated in Table 2.

The pH value of compost is important for the soil as the compost can assist the soil to reach the suitable pH to promote the plant growth. From the data tabulated in Table 2, it shows that the pH for the composts, which were the control and the composts that contained wood vinegar by the ratio 1:100 to 1:500 (v/v), had reached the neutral pH level of 7 after 60 days of composting. The ideal pH of compost should be between the level of pH 6.0 to 7.5, where it should be neutral or slightly acidic (WERL, 2005).

For moisture content of the composts, the value was maintained between 50 to 65 %. The results showed that the compost of 1:400 (v/v) contained a higher value of moisture content with 64.53 % (w/w), which was 1 to 27 % higher than other composts. Meanwhile, the lowest moisture content was recorded by the control with 50.59 % (w/w) of moisture content. Moreover, the result also showed that the compost sample of 1:500 (v/v) had a lower moisture content

Table 2
Physical properties of compost

Materials	Sample	pH	Moisture content (%)	Water holding capacity (ml/100g)
Raw Material	POME sludge	5.5	68.93	60.92
	Decanter cake	5.8	79.06	70.12
	Rice husk ash	6.8	81.20	88.95
	Wood vinegar	2.57	-	-
	Control	7	50.59	68.15
	1:100	7	55.30	71.99
Compost	1:200	7	57.84	75.10
	1:300	7	63.56	79.91
	1:400	7	64.53	81.45
	1:500	7	58.25	77.23

compared to 1:300 (v/v) and 1:400 (v/v). The moisture content of compost sample is directly related to the decomposition process. In this process, the microorganisms will transform the organic materials into carbon dioxide (CO_2), water (H_2O) and other compounds (Gómez et al., 2006). Although the compost of ratio 1:500 (v/v) has the highest count of microbes, the organic materials inside the compost might not be sufficient to the amount of microbes contained in the compost. Thus, it will further reduce the decomposition of organic materials inside the compost and affecting the water produced through the process. In this case, as the production of water by the microbes through decomposition process was reduced, the moisture content of the compost might reduce as well. However, the moisture content of all composts still lies within the recommended range. According to Zakarya et al. (2018), the ideal moisture content that should be possessed by the composts would be in the range of 40 to 60 %. However, for a compost mix, it will require as much as 65 % (w/w) of moisture content to be ideally moistened (WERL, 2005).

For the water holding capacity, Table 2 shows that the water holding capacity in the formulated compost with wood vinegar at ratio 1:400 (v/v) was 2 to 19 % higher than other composts with the value of 81.45 mL/100g of sample. The lowest water holding capacity was recorded by the control with 68.15 mL/100g of sample. The water holding capacity and moisture content was correlated to each other. Thus, the soil with high water holding capacity is expected to maintain a high moisture content as well (Blažka & Fischer, 2014).

CONCLUSION

The mixture of 1:500 (v/v) of wood vinegar

with the sample of POME sludge and rice husk gave the highest colony count with the value of 1.1×10^{18} CFU/mL. The result indicates that the low concentration of wood vinegar promotes the growth of microbes. Besides, the results for chemical properties had shown that the formulated compost of 1:500 (v/v) obtained the highest percentage of nitrogen (N), phosphorus (P) and potassium (K). Other than that, the results for physical properties indicated that the pH of all composts had achieved the neutral level. For percentage of moisture content and water holding capacity, the formulated compost of ratio 1:400 showed the highest value compared to other formulated composts. Even though the formulated compost of 1:500 (v/v) had shown the highest colony count and N, P, K value, the formulated compost of 1:400 (v/v) had shown to give a better performance in terms of physical properties (pH, moisture content, water holding capacity). Therefore, it can be concluded that sample 1:400 (v/v) shows the best condition as it has a well-balanced condition between physical, chemical and biological properties.

ACKNOWLEDGEMENTS

This research work is supported by Universiti Malaysia Pahang under research grant RDU160380.

REFERENCES

Benzon, H. R. L., Rubenecia, M. R. U., Ultra, J. V. U., & Lee, S. C. (2015). Chemical and biological properties of paddy soil treated with herbicides and pyroligneous acid. *Journal of Agricultural Science*, *7*(4), 20–29. doi: 10.5539/jas.v7n4p20

Blažka, P., & Fischer, Z. (2014). Moisture, water holding, drying and wetting in forest soils. *Open Journal of Soil Science*, *4*(5), 174–184. doi: 10.4236/ojss.2014.45021

Dewayanto, N., Husin, M. H. M., & Nordin, M. R. (2015). Solid fuels from decanter cake and other palm oil industry waste. *Jurnal Teknologi (Sciences and Engineering)*, *76*(5), 57–60. doi: 10.11113/jt.v76.5537

Embrandiri, A., Rupani, P. F., Ismail, S. A., Singh, R. P., Ibrahim, M. H., & Abd. Kadir, M. O. (2016). The effect of oil palm decanter cake on the accumulation of nutrients and the stomatal opening of *Solanum melongena* (brinjal) plants. *International Journal of Recycling of Organic Waste in Agriculture*, *5*(2), 141–147. doi: 10.1007/s40093-016-0124-8

Gómez, R. B., Lima, F. V., & Ferrer, A. S. (2006). The use of respiration indices in the composting process: A review. *Waste Management and Research*, *24*(1), 37–47. doi: 10.1177/0734242X06062385

Hagner, M., Penttinen, O., Tiilikkala, K., & Setälä, H. (2013). The effects of biochar, wood vinegar and plants on glyphosate leaching and degradation. *European Journal of Soil Biology*, *58*, 1–7.

Hock, L. S., Baharuddin, A. S., Ahmad, M. N., Hassan, M. A., Shah, U. K. M., Rahman, N. A. A., … Shirai, Y. (2009). Physicochemical changes in windrow co-composting process of oil palm mesocarp fiber and palm oil mill effluent anaerobic sludge. *Australian Journal of Basic and Applied Sciences*, *3*(3), 2809–2816.

Woods End Research Laboratory. (2005). Interpreting waste and compost tests. *Journal of the Woods End Research Laboratory*, *2*(1), 6.

Jacoby, R., Peukert, M., Succurro, A., Koprivova, A., & Kopriva, S. (2017). *The role of soil microorganisms in plant mineral nutrition - Current knowledge and future directions*. Retrieved May 07, 2019, from https://www.

ncbi.nlm.nih.gov/pmc/articles/PMC5610682/pdf/fpls-08-01617.pdf

Jeong, K. W., Kim, B. S., Ultra, V. U., & Lee, S. C. (2015). Effects of rhizosphere microorganisms and wood vinegar mixtures on rice growth and soil properties. *The Korean Journal of Crop Science*, *60*(3), 355–365. doi: 10.7740/kjcs.2015.60.3.355

Kanakaraju, D., Metosen, A. N. S. A., & Nori, H. (2016). Uptake of heavy metals from palm oil mill effluent sludge amended soils in water spinach. *Journal of Sustainability Science and Management*, *11*(1), 113–120.

Kant, S., Kant, P., & Kafkafi, U. (2006). Potassium uptake by higher plants: From field application to membrane transport. *Acta Agronomica Hungarica*, *53*(4), 443–459. doi: 10.1556/aagr.53.2005.4.11

Kishimoto, S., & Tsuyoshi, H. (2015). *Wood vinegar and biochar in agriculture*. Retrieved May 07, 2019, from http://www.warrencc.org.au/wp-content/uploads/2015/12/CharcoalVinegar-with-pictures.pdf

Kumar, A., Mohanta, K., Kumar, D., & Parkash, O. (2012). Properties and industrial applications of rice husk : A review. *International Journal of Emerging Technology and Advanced Engineering*, *2*(10), 86–90.

Lee, Y. (2016). Various microorganisms' roles in composting : A review. *APEC Youth Scientist Journal*, *8*(1), 11–15.

Leghari, S. J., Wahocho, N. A., Laghari, G. M., Laghari, A. H., Bhabhan, G. M., Talpur, K. H., … Lashari, A. A. (2016). Role of nitrogen for plant growth and development: A review. *Advances in Environmental Biology*, *10*(9), 209–218.

Lin, S., Yeong, T., Yih, E., Sim, S., Nie, P., & Clarke, C. (2012). Biotransformation of rice husk into organic fertilizer through vermicomposting. *Ecological Engineering*, *41*, 60–64. doi: 10.1016/j.ecoleng.2012.01.011

Microbiological examination – Total colony number SCAN-CM 60:02 SCAN-P 81:02. (2002). Retrieved May 07, 2019, from https://www.academia.edu/9870218/SCAN-CM_60_02_SCAN-P_81_02

Misra, R. V., Roy, R. N., & Hiraoka, H. (2003). *On-farm composting methods*. Rome, Italy: Food and Agriculture Organization of the United Nations.

Mungkunkamchao, T., Kesmala, T., Pimratch, S., Toomsan, B., & Jothityangkoon, D. (2013). Wood vinegar and fermented bioextracts: Natural products to enhance growth and yield of tomato (*Solanum lycopersicum* L.). *Scientia Horticulturae*, *154*, 66–72. doi: 10.1016/j.scienta.2013.02.020

Obi, O. F. (2015). Evaluation of the effect of palm oil mill sludge on the properties of sawdust briquette. *Renewable and Sustainable Energy Reviews*, *52*, 1749–1758. doi: 10.1016/j.rser.2015.08.001

Otieno, N. E., Dai, X., De Barba, D., Bahman, A., Smedbol, E., Rajeb, M., & Jaton, L. (2016). Palm oil production in Malaysia: An analytical systems model for balancing economic prosperity, forest conservation and social welfare. *Agricultural Sciences*, *7*(2), 55–69.

Payamara, J. (2011). Usage of wood vinegar as new organic substance. *International Journal of ChemTech Research*, *3*(3), 1658–1662.

Prajapati, K., & Modi, H. A. (2012). The importance of potassium in plant growth - A review. *Indian Journal of Plant Sciences*, *1*(2), 177–186.

Ramli, N. H., Hisham, N. E. B., Said, F. M., & Mariyappan, T. (2016). The effect of weight ratio on the physiochemical properties of compost from palm oil mill effluent (POME) sludge and

decanter cake. *Australian Journal of Basic and Applied Sciences*, *10*(17), 34–39.

Rashid, M. I., Mujawar, L. H., Shahzad, T., Almeelbi, T., Ismail, I. M. I., & Oves, M. (2016). Bacteria and fungi can contribute to nutrients bioavailability and aggregate formation in degraded soils. *Microbiological Research*, *183*, 26–41. doi: 10.1016/j.micres.2015.11.007

Razaq, M., Zhang, P., Shen, H., & Salahuddin. (2017). Influence of nitrogen and phosphorous on the growth and root morphology of *Acer mono*. *PLoS ONE*, *12*(2), 1–13. doi: 10.1371/journal.pone.0171321

Rui, Z., Wei, D., Zhibin, Y., Chao, Z., & Xiaojuan, A. (2014). Effects of wood vinegar on the soil microbial characteristics. *Journal of Chemical and Pharmaceutical Research*, *6*(3), 1254–1260.

Rupani, P. F., Singh, R. P., Ibrahim, M. H., & Esa, N. (2010). Review of current palm oil mill effluent (POME) treatment methods: Vermicomposting as a sustainable practice. *World Applied Sciences Journal*, *11*(1), 70–81. doi: 10.5539/jas.v7n4p68

Sahad, N., Som, A. M., Baharuddin, A. S., Mokhtar, N., Busu, Z., & Sulaiman, A. (2014). Physicochemical characterization of oil palm decanter cake (OPDC) for residual oil recovery. *BioResources*, *9*(4), 6361–6372. doi: 10.15376/biores.9.4.6361-6372

Sarkar, P., & Chourasia, R. (2017). Bioconversion of organic solid wastes into biofortified compost using a microbial consortium. *International Journal of Recycling of Organic Waste in Agriculture*, *6*(4), 321–334. doi: 10.1007/s40093-017-0180-8

Singh, S., Singh, V., & Pal, K. (2017). *Importance of microorganisms in agriculture*. Retrieved May 07, 2019, from https://www.researchgate.net/publication/322926367_IMPORTANCE_OF_MICRO_ORGANISMS_IN_AGRICULTURE

Trisakti, B., Mhardela, P., Husaini, T., Irvan, & Daimon, H. (2018). *Production of oil palm empty fruit bunch compost for ornamental plant cultivation*. Retrieved May 07, 2019, from https://iopscience.iop.org/article/10.1088/1757-899X/309/1/012094/pdf

Yahya, A., Sye, C. P., Ishola, T. A., & Suryanto, H. (2010). Effect of adding palm oil mill decanter cake slurry with regular turning operation on the composting process and quality of compost from oil palm empty fruit bunches. *Bioresource Technology*, *101*(22), 8736–8741. doi: 10.1016/j.biortech.2010.05.073

Yap, C. A. (2012). Impact of different fertilization methods on the soil, yield and growth performance of black pepper (*Piper nigrum* L.). *Malaysian Journal of Soil Science*, *16*(1), 71–87.

Zakarya, I. A., Khalib, S. N. B., & Mohd Ramzi, N. (2018). *Effect of pH, temperature and moisture content during composting of rice straw burning at different temperature with food waste and effective microorganisms*. Retrieved May 07, 2019, from https://www.e3s-conferences.org/articles/e3sconf/pdf/2018/09/e3sconf_cenviron2018_02019.pdf

Zhai, M., Shi, G., Wang, Y., Mao, G., Wang, D., & Wang, Z. (2015). Chemical compositions and biological activities of pyroligneous acids from walnut shell. *BioResources*, *10*(1), 1715–1729.

Zhi-Qiang, X., Guo-Xing, W., Zhao-Chen, H., Lei, Y., Ya-Mei, G., Yan-Jie, W., … Wei-Dong, W. (2017). Effect of aeration rates on the composting process and loss of nitrogen during composting. *Applied Environmental Biotechnology*, *2*(1), 1-8. doi: 10.26789/aeb.2015.01.003

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JTAS Vol. 42 (4) Nov. 2019

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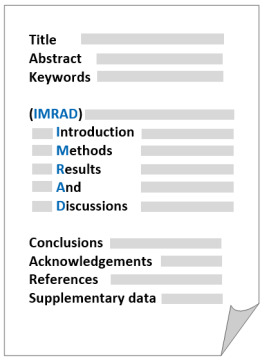

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Horticulture

Soil and Water Sciences

Pertanika Journal of Tropical Agricultural Science
Vol. 42 (4) Nov. 2019

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ISSN 1511-3701
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