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JTAS

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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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The *Introduction* explains the scope and objective of the study in the light of current knowledge on the subject; the *Materials and Methods* describes how the study was conducted; the *Results* section reports what was found in the study; and the *Discussion* section explains meaning and significance of the results and provides suggestions for future directions of research. The manuscript must be prepared according to the Journal's **Instructions to Authors**.

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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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2. The chief executive editor sends the article-identifying information having been removed, to three reviewers. Typically, one of these is from the Journal's editorial board. Others are specialists in the subject matter represented by the article. The chief executive editor asks them to complete the review in three weeks.

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3. The chief executive editor, in consultation with the editor-in-chief, examines the reviews and decides whether to reject the manuscript, invite the author(s) to revise and resubmit the manuscript, or seek additional reviews. Final acceptance or rejection rests with the Edito-in-Chief, who reserves 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 chief executive editor 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 chief executive editor in consultation with the editorial board and the editor-in-chief 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 in print 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 on-line.

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Contents

Foreword

Welcome to the Fourth Issue 2018 of 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 and run on a non-profit basis for the benefit of the world-wide science community.

This issue contains 26 articles, out of which one is a case study, two are short communications and the rest 23 are regular articles. The authors of these articles come from different countries, namely Australia, Bangladesh, India, Malaysia, Nigeria, Papua New Guinea, Philippines, Russia and Thailand.

Articles submitted in this issue cover wide range of agricultural science scope including animal production, biotechnology, botany, crop and pasture production, fisheries sciences, food and nutrition development, genetics and molecular biology, microbiology, soil and water sciences, veterinary sciences, and zoology. The most favoured field in this issue is soil and water sciences.

Selected from the scope of animal production is an article entitled "Effect of Palm Kernel Cake and Coconut-based Formulated Diet on Malaysia Village Chicken Growth Performances and Meat Quality" by *Mohd Shahmi Hakimi Mazlishah, Zazali Alias, Wan Syahidah Husain, Wan Khadijah Wan Embong* and *Ramli Bin Abdullah*, fellow researchers from Universiti Malaya (UM) and Makmal Kesihatan Awam Veterinar, Malaysia. The study evaluated the effect of palm kernel cake and coconut-based feeding on the production performance in 400 village chickens (purebred (n = 200) and crossbreed (n = 200) strains. The feed used were Type-A (formulated diet) and Type-B (commercial diet) and they found that the usage of Type-A feed was more beneficial and exhibited prominent values in terms of quality and cost-effectiveness. Details of the study is available on page 1703.

Selected from the scope of soil and water sciences is an article entitled "Impact Assessment of Organic Farming on Soil Nutrients and Heavy Metal Content" by *Azeez, J. O., Ojewande, B. O., Olayinka, O. O.* and *Adesodun, J. K.,* fellow researchers from Federal University of Agriculture, Nigeria. The study assessed chemical properties of souls from an organic farm with different land uses. The soils were analysed for pH, organic carbon, nutrients and heavy metals. They found out that the cumulative concentrations of the heavy metals such as Copper, Pb and Zn were far below the international tolerable limits. Though, they recommended further investigation on the uptake of heavy metals by the organic products. Details of the study is available on page 1811.

Selected from the scope veterinary sciences is an article entitled "Mortality and Repellent Effects of Coffee Extracts on The Workers of Three Household Ant Species" by *Xue Li Yeoh, Hamady Dieng* and *Abdul Hafiz Ab Majid*, from Universiti Sains Malaysia (USM) and Universiti Malaysia Sarawak (UNIMAS), Malaysia. This article presents a study on the impact of coffee extracts on survival and feeding behaviour of three species of ants: *Tapinoma indicum* (ghost ant); *Pheidole megacephala* (big-headed ant); and *Monomorium pharaonis* (Pharaoh ant). Three coffee species (*Coffea arabica*, *Coffea canephora* and *Coffea liberica*) were extracted, diluted and impregnated in gel baits. As a result, *Coffee Arabica* gave the highest mortality on all three ant species with *T. indicum* being the most susceptible species. Details of the article is available on page 1557.

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, 26% were accepted. This seems to be the trend in Pertanika Journals.

We would also like to express our gratitude to all the contributors, namely the authors, reviewers and editors, 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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Riverine Fruit *Dacryodes rostrata* Crude Oil as a Potential Dietary Lipid Source for Malaysian Mahseer, *Tor tambroides*

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ABSTRACT

Riverine fruits, including lipid-rich *Dacryodes rostrata*, are commonly consumed by the omnivorous Malaysian mahseer (*Tor tambroides*) and other tropical riverine cyprinids in their natural habitats. To increase its aquaculture production, feeding the fish with an optimized diet that meets all its nutritional requirements is of crucial importance. This study was performed to investigate the effects of varying levels of *D. rostrata* oil on the growth performance, body composition and fatty acid profile of juvenile Malaysian mahseer. Juveniles (1.81 g ± 0.11) were fed the test diets (0, 1.25, 2.5, 3.75 and 5% *D. rostrata* oil) for 12 weeks in triplicated groups. Crude palm oil (CPO) was used as the control. Fish given 0% *D. rostrata* oil showed the greater growth performance, while juveniles fed 2.5% *D. rostrata* oil demonstrated the highest muscular retention of long-chain polyunsaturated fatty acids (both n-3 and n-6 PUFAs), which have beneficial health effects for human consumers. From the results, it was suggested that *T. tambroides* juveniles be fed with 5% CPO for the grow-out period. A finishing diet containing 2.5% *D. rostrata* oil was suggested for the fish towards the end of its culture period to achieve the highest concentration of long-chain PUFAs (both n-3 and n-6) in the muscle tissue, which is an important criteria for the health of humans as fish consumers.

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INTRODUCTION

Malaysian mahseer (*Tor tambroides* Bleeker) is a riverine cyprinid fish with lots of socio-economic importance in fisheries and conservation. It has also a tremendous potential for freshwater aquaculture (Anon, 2005; Ingram et al., 2005). Similar to other mahseers worldwide, its natural stock has rapidly declined in the last decades (Ingram et al., 2007; Ramezani-Fard et al., 2011). Mahseer catch in Malaysia has dropped by almost 60% from 65,771 kg in 2011 to 28,213 kg in 2015 according to the reports made by Department of Fisheries (Department of Fisheries Malaysia [DOF], 2012, 2013, 2014, 2015, 2016). Since the success of its artificial breeding (Ingram et al., 2005), efforts to culture this species has increased as there is a continuous high demand for this fish. However, its aquaculture production has remained below 22,680 kg a year (DOF, 2012, 2013, 2014, 2015, 2016).

To increase its aquaculture production, feeding the fish with an optimized diet that meets all its nutritional requirements is of crucial importance. Recent studies have shown that *T. tambroides* requires 40-48% dietary protein and 5% dietary lipid (Misieng, Kamarudin, & Musa, 2011; Ng & Andin, 2011; Ramezani-Fard, Kamarudin, Harmin, Saad, & Goh, 2012a). A dietary lipid level of more than 10% suppresses its growth performance and survival (Ramezani-Fard et al., 2012a). Lipids and their constituent fatty acids are the main sources of energy for growth purposes in fish (Tocher, 2003). Fish are the major sources of long-chain polyunsaturated fatty acids of n-3 series (n-3 LC-PUFAs), which are health-beneficial fatty acids for humans as fish consumers (Tocher, 2003). The long-chain polyunsaturated fatty acids with 20 carbons in their chain lengths, especially 20:4n-6 (an n-6 LC-PUFA), have an important role in providing eicosanoids, the cyclic or linear compounds with significant biological activities. Since 18:3n-3 and 18:2n-6 can be converted to n-3 and n-6 LC-PUFAs in freshwater fish and humans, both of them have crucial roles as essential n-3 and n-6 polyunsaturated fatty acids (n-3 and n-6 PUFAs) in their diets (Sargent, Tocher, & Bell, 2002; Tapiero, Nguyen Ba, Couvreur, & Tew, 2002). Therefore, much effort needs to be made to maintain the amounts of these fatty acids in fish tissues. Saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs) are considered to spare n-3 LC-PUFAs in fish tissues (Trushenski, Crouse, & Rombenso, 2015; Turchini, De Smet, & Francis, 2011). Better growth and n-3 LC-PUFA maintenance in the tissues of *T. tambroides* have been reported when fed diets containing low n-3 PUFAs and high SFAs compared to when fed diets including high n-3 PUFAs and low SFAs (Kamarudin, Bami, Arshad, Saad, & Ebrahimi, 2018; Ramezani-Fard, Kamarudin, Harmin, & Saad, 2012b).

Riverine fruits, including oily *Dacryodes rostrata*, are commonly eaten by the Malaysian mahseer and other riverine carps in their natural habitats (Siraj, Daud,

Keong, & Ng, 2007; Tan, 1980). *D. rostrata* is an indigenous fruit of Peninsular Malaysia (Hoe & Siong, 1999; Saw, LaFrankie, Kochummen, & Yap, 1991), Brunei (Tinggal, 1992), Borneo, Thailand, Indo-China, the Philippines, the Indonesian islands of Sumatra and Celebes (Ashton, 1995). This fruit is known as kembayau in Sarawak. The fruit is available from October to February. This white or yellow small fruit turns pinkish and then black when ripe (Saw et al., 1991; Tinggal, 1992). Fresh *D. rostrata* contains the lipid levels of 12.5-14.0%, 11.5-21.3% and 7.9-17.8% in its seeds, pulps and peels, respectively (Kong et al., 2011). Its pulp and seed oils contain 42.2% and 59.5% of 16:0 and 18:0, respectively (Ibrahim, Lim, Salimah, & Mariani, 2007; Tee et al., 2014).

Although this riverine fruit is one of the major food components in the natural diet of Malaysian mahseer and other riverine carps (Misieng, Kamarudin, & Saad, 2015; Siraj et al., 2007; Tan, 1980), its specific roles in the nutrition of riverine cyprinids have not been studied. This study was performed to investigate the effects of dietary *D. rostrata* oil inclusion on growth performance, feed efficiency, body composition, and fatty acid profile of muscle and liver in *T. tambroides* juveniles.

METHOD

Diet Preparation

The *D. rostrata* fruits were procured from a local supplier and transferred to the Fish Nutrition Laboratory, Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia. The fruits were first sun-dried for 1 week to prevent molding. The fruits were then peeled, and about 100 g of the dried peeled fruits (the flesh and seed) was ground using a hammer mill with 600 μm mesh screen. The powder was then moisturized by adding distilled water and using a vertical mixer until about 80% moisture content was achieved. After that, the fruit oil was extracted using the Bligh and Dyer (1959) method.

Five isonitrogenous (40% protein) and isocaloric (17.2 kJ/g) diets containing 5% oil with different levels of *D. rostrata* oil were formulated and prepared (Tables 1 and 2). Crude palm oil (CPO) was used as the control (Bami et al., 2017; Kamarudin, Ramezani-Fard, Saad, & Harmin, 2012; Ramezani-Fard et al., 2012b). Table 3 presents the fatty acid compositions of the oils and lipid of fishmeal and soybean meal. A kitchen mixer was used to mix dry ingredients. A homogeneous mixture was produced after adding distilled water and test oils. A single screw laboratory-scale extruder (Brabender KE-19) was used to extrude the moist mixture through a 2-mm die. The pellets were oven-dried at 45 ˚C for 4 h, cooled to the room temperature, bagged and stored with dehumidifying agents until used.

Juvenile Rearing

T. tambroides juveniles (mean ± SD initial body weight = 1.81 ± 0.11 g) were purchased from a local supplier and transferred to the

Table 1
Feed and chemical compositions of the experimental diets fed to juvenile T. tambroides

	Dietary *D. rostrata* oil (%)				
Diet parameter	0	1.25	2.5	3.75	5
	Ingredient (g/100 g as fed basis)				
Fishmeal[1]	35.8	35.8	35.8	35.8	35.8
Soybean meal	37.8	37.8	37.8	37.8	37.8
Tapioca starch	19.4	19.4	19.4	19.4	19.4
D. rostrata oil	0.0	1.2	2.5	3.8	5.0
CPO[2]	5.0	3.8	2.5	1.2	0.0
Vitamin premix[3]	1.0	1.0	1.0	1.0	1.0
Mineral premix[4]	1.0	1.0	1.0	1.0	1.0
	Proximate composition (% as fed basis)				
Crude protein	39.98 ± 1.64	40.03 ± 1.40	39.95 ± 0.93	39.96 ± 1.43	40.02 ± 1.04
Crude lipid	7.84 ± 0.34	7.76 ± 0.26	7.75 ± 0.35	7.76 ± 0.25	7.79 ± 0.34
Ash	11.89 ± 0.26	11.86 ± 0.40	11.85 ± 0.55	11.87 ± 0.34	11.86 ± 0.19
Carbohydrate[5]	28.61 ± 0.81	28.68 ± 1.03	28.95 ± 1.07	28.76 ± 0.90	28.56 ± 1.34
Gross energy (kJ/g)	17.23 ± 0.29	17.22 ± 0.34	17.20 ± 0.08	17.19 ± 0.60	17.18 ± 0.99
Dry matter	88.32 ± 0.88	88.33 ± 0.46	88.50 ± 0.86	88.35 ± 0.65	88.23 ± 0.56

Note. Mean ± SE (n = 3). [1] Malaysian fishmeal (59.6% crude protein). [2] Crude palm oil supplied by Malaysian Palm Oil Board (MPOB). [3] Vitamin premix (g/kg premix): ascorbic acid, 45; myo-inositol, 5; choline chloride, 75; niacin, 4.5; riboflavin, 1; pyridoxine, 1; thiamin mononitrate, 0.92; Ca-pantothenate, 3; retinyl acetate, 0.6; cholecalciferol, 0.083; vitamin K menadione, 1.67; α-tocopheryl acetate (500 IU/g), 8; biotin, 0.02; folic acid, 0.09; vitamin B_{12}, 0.001; cellulose, 845.11. [4] Mineral premix (g/kg premix): KCl, 90; Kl, 0.04; $CaHPO_4.2H_2O$, 500, NaCl, 40, $CuSO_4.5H_2O$, 3; $ZnSO_4.7H_2O$, 4; $CoSO_4$, 0.02; $FeSO_4.7H_2O$, 20; $MnSO_4.H_2O$, 3; $CaCO_3$, 215; MgOH, 124; Na_2SeO_3, 0.03; NaF, 1. [5] Carbohydrates = Dry matter – [protein + lipid + ash]

Table 2
Fatty acid compositions (% of total fatty acids) of the experimental diets fed to juvenile T. tambroides

	Dietary *D. rostrata* oil (%)				
	0	1.25	2.5	3.75	5
Fatty acid					
14:0	2.15 ± 0.13	2.07 ± 0.12	1.97 ± 0.12	1.81 ± 0.10	1.72 ± 0.10
16:0	38.70 ± 0.34	35.50 ± 1.35	32.56 ± 1.37	28.93 ± 1.40	26.14 ± 1.36
16:1n-7	2.24 ± 0.13	2.24 ± 0.13	2.30 ± 0.13	2.30 ± 0.13	2.36 ± 0.14
18:0	6.22 ± 0.36	8.19 ± 0.47	9.97 ± 0.57	12.74 ± 0.68	13.94 ± 0.80
18:1n-9	32.80 ± 0.19	34.22 ± 1.97	35.50 ± 2.05	36.59 ± 2.11	38.96 ± 2.24
18:2n-6	10.91 ± 0.63	10.68 ± 0.62	10.26 ± 0.59	9.78 ± 0.57	9.29 ± 0.54
18:3n-3	1.29 ± 0.08	1.34 ± 0.08	1.41 ± 0.09	1.36 ± 0.14	1.31 ± 0.08
20:4n-6	1.06 ± 0.06	1.12 ± 0.06	1.18 ± 0.07	1.28 ± 0.08	1.29 ± 0.08
20:5n-3	1.35 ± 0.08	1.34 ± 0.07	1.43 ± 0.08	1.58 ± 0.09	1.50 ± 0.09
22:5n-3	0.57 ± 0.03	0.63 ± 0.03	0.62 ± 0.02	0.65 ± 0.02	0.67 ± 0.04
22:6n-3	2.71 ± 0.14	2.66 ± 0.16	2.81 ± 0.21	2.99 ± 0.17	2.82 ± 0.17
Σ SFA[1]	47.07 ± 0.11	45.77 ± 1.69	44.50 ± 1.82	43.48 ± 1.97	41.80 ± 2.06
Σ MUFA[2]	35.04 ± 0.32	36.46 ± 2.10	37.79 ± 2.18	38.89 ± 2.25	41.12 ± 2.38
Σ n-3 PUFA[3]	5.92 ± 0.26	5.97 ± 0.27	6.27 ± 0.30	6.58 ± 0.36	6.30 ± 0.29
Σ n-6 PUFA[4]	11.97 ± 0.69	11.80 ± 0.68	11.44 ± 0.66	11.06 ± 0.64	10.58 ± 0.61
n-3/n-6	0.49 ± 0.05	0.51 ± 0.05	0.55 ± 0.06	0.59 ± 0.08	0.60 ± 0.07
PUFA/SFA	0.38 ± 0.01	0.39 ± 0.01	0.40 ± 0.01	0.40 ± 0.01	0.40 ± 0.01

Note. Mean ± SE (n = 3). [1] Saturated fatty acids (Sum of 14:0 + 16:0 + 18:0). [2] Monounsaturated fatty acids (Sum of 16:1n-7 + 18:1n-9). [3] The n-3 series of polyunsaturated fatty acids (Sum of 18:3n-3 + 20:5n-3 + 22:5n-3 + 22:6n-3). [4] The n-6 series of polyunsaturated fatty acids (Sum of 18:2n-6 + 20:4n-6)

Table 3
Fatty acid compositions (% of total fatty acids) of different lipid sources

	Type of the lipid source			
Fatty acid	D. rostrata	CPO	Fishmeal[1]	Soybean meal[2]
14:0	0.00	1.53 ± 0.44	4.87 ± 0.06	0.00
16:0	34.00 ± 4.00	43.94 ± 0.32	31.77 ± 0.20	19.51 ± 2.42
16:1n-7	0.00	0.00	5.37 ± 0.25	0.00
18:0	10.79 ± 4.96	4.17 ± 0.56	11.27 ± 0.12	4.69 ± 0.20
18:1n-9	45.63 ± 2.32	39.98 ± 1.18	19.72 ± 0.22	17.07 ± 2.08
18:2n-6	9.36 ± 1.37	10.18 ± 0.21	2.53 ± 0.03	50.85 ± 3.44
18:3n-3	0.23 ± 0.01	0.21 ± 0.06	0.93 ± 0.44	7.90 ± 0.85
20:4n-6	0.00	0.00	3.93 ± 0.13	0.00
20:5n-3	0.00	0.00	5.51 ± 0.02	0.00
22:5n-3	0.00	0.00	2.40 ± 0.05	0.00
22:6n-3	0.00	0.00	11.73 ± 0.16	0.00
Σ SFA[3]	44.79 ± 0.96	49.63 ± 1.33	47.90 ± 0.39	24.19 ± 2.22
Σ MUFA[4]	45.63 ± 2.32	39.98 ± 1.18	25.08 ± 0.04	17.07 ± 2.08
Σ n-3 PUFA[5]	0.23 ± 0.01	0.21 ± 0.06	20.55 ± 0.20	7.90 ± 0.85
Σ n-6 PUFA[6]	9.36 ± 1.37	10.18 ± 0.21	6.47 ± 0.15	50.85 ± 3.44
n-3/n-6	0.02 ± 0.01	0.02 ± 0.01	3.18 ± 0.05	0.16 ± 0.01
PUFA/SFA	0.21 ± 0.03	0.21 ± 0.01	0.57 ± 0.02	2.47 ± 0.40

Note. Mean ± SE (n = 3). [1] 7.9% lipid. [2] 0.6% lipid. [3] Saturated fatty acids (Sum of 14:0 + 16:0 + 18:0). [4] Monounsaturated fatty acids (Sum of 16:1n-7 + 18:1n-9). [5] The n-3 series of polyunsaturated fatty acids (Sum of 18:3n-3 + 20:5n-3 + 22:5n-3 + 22:6n-3). [6] The n-6 series of polyunsaturated fatty acids acids (Sum of 18:2n-6 + 20:4n-6)

Wet Laboratory, Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia and acclimatized in a 1,000-L fiberglass tank for 2 weeks. Juveniles were given a 32% crude protein commercial tilapia starter feed (StarFeed (M) Sdn. Bhd., Malaysia) during the acclimatization period. Fish were then randomly distributed into 15 glass aquaria (60 L) at 20 fish per aquarium. Each aquarium was supplied with continuous aeration. Water quality was monitored every 3 weeks; a thermometer, a portable pH meter (YSI 60, Yellow Springs Instruments) and a DO meter (YSI DO200, Yellow Springs Instruments) were used for the measurement of water temperature, pH and dissolved oxygen. Water temperature ranged between 27.1-29.4 ˚C, and pH averaged approximately 6.5 and 7.4. The ammonia (NH^{3+}) level was retained below 0.2 mg/L. Dissolved oxygen levels in each aquarium was maintained above 7 mg/L. Fish were fed twice daily (0900 and 1600 h) close to visual satiety during the trial. The feeding trial was conducted for 12 weeks.

Fish Sampling

All 20 juveniles from each aquarium were individually weighed at the start and end of the feeding trial as well as every 3 weeks. Weight gain, specific growth rate (SGR), daily feed intake (DFI) and feed conversion ratio (FCR) were measured and estimated at the end of the experiment.

At the start of feeding trial, 20 fish were also sacrificed following anesthetization by MS222 and individually weighed. About 15 of them were kept frozen at -20 ˚C for subsequent whole body composition analysis, and the muscle and liver tissue of other five juveniles were removed and stored at -80 ˚C for fatty acid analysis. At the end of the experiment, 5 fish per aquarium were also sacrificed, individually weighed and dissected for the hepatosomatic index (HSI) and viscera-somatic index (VSI) estimation. The fish were starved for 24 h to facilitate the liver collection and VSI estimation. The dissected fish were then dressed, and muscle from the area between the lateral and dorsal line was removed for fatty acid analyses (Ahlgren, Blomqvist, Boberg, & Gustafsson, 1994). The liver and muscle samples were stored at -80 ˚C instantly for later fatty acid analyses. Another 15 fish per aquarium were sacrificed, weighed individually and stored at -20 ˚C for subsequent whole body composition analysis.

Biochemical Analysis

Before the biochemical analysis, the whole fish (before and after the experiment) were dried in the oven at 40 ˚C for 48 h, and moisture loss was estimated. After that, the samples were ground into fine powder. Moisture contents of experimental diets were estimated using an infrared moisture balance (A&D, AD-4715). The crude protein, crude lipid and crude fiber of experimental diets and the whole body of fish samples were respectively determined by the Kjeldahl method (Foss Kjeltec™ 8000), Soxhlet extraction (Foss Soxtec™ 8000) and hot extraction (Foss Fibertec™ 2010) according to Association of Official Analytical Chemists (AOAC) methods

(1997). The ash content was estimated by cauterizing the dry sample at 600 ˚C for 4 h, and the gross energy was measured by a direct incineration in an adiabatic bomb calorimeter (Leco AC-350).

Lipid from the fishmeal, soybean meal, feed, liver and muscle was extracted using a chloroform : methanol (2:1, v:v) mixture, saponated by KOH and transesterified with methanolic boron trifluoride according to AOAC methods (1997). After that, separation and quantification of fatty acid methyl esters (FAMEs) were performed using a fused silica capillary column (Supelco SP-2330: 30 m × 0.25 mm, film thickness 0.20 µm) in a gas chromatograph (Agilent 7890N) equipped with a split/splitless injector and flame ionization detector. High purity nitrogen was used as the carrier gas with a flow rate of 40 mL/min. The injector temperature was 250˚C, while the detector temperature was 300˚C. The column temperature was programmed at 100˚C for 2 min, warmed up to 170 ˚C at 10 ˚C/min, held for 2 min and then warmed to 220˚C at 7.5 ˚C/min for 20 min to facilitate optimal separation. Fatty acids were identified by comparison of the relative FAME peak retention time with the internal standard, heneicosanoic acid (21:0), obtained from Sigma (St. Louis, MO, USA) and expressed as the area percentage of FAMEs.

Statistical Analysis

All data analyses were performed using one-way analysis of variance (ANOVA). Duncan's multiple range test was conducted if ANOVA indicated there were significant differences among the treatment means. All percentage data were arcsin transformed prior to being analyzed statistically. All analysis was performed using SPSS 18 for Windows (SPSS Inc., Chicago, IL, USA), and the difference was considered significant at $P < 0.05$. Results were reported as mean values ± SE.

RESULTS

Survival, final weight, weight gain, daily feed intake, specific growth rate, feed conversion ratio, viscera-somatic index, hepato-somatic index, protein efficiency ratio and lean percentage of *T. tambroides* fed varying levels of *D. rostrata* oil are shown in Table 4. The dietary *D. rostrata* oil level did not have any significant effects ($P > 0.05$) on the survival of juvenile *T. tambroides*. Juveniles fed 0% *D. rostrata* oil had a significantly higher ($P < 0.05$) growth than those fed 3.75-5% *D. rostrata* oil. Significant differences ($P < 0.05$) were found in FCR, DFI, PER and HSI among the treatments. The highest FCR was observed among fish fed 5% *D.rostrata* oil, while the lowest FCR was found among fish fed 0 and 1.25% *D. rostrata* oil. Fish fed 5% *D. rostrata* oil had the highest DFI and the lowest PER, while those fed 2.5% *D. rostrata* oil had the lowest DFI and the highest PER. Juveniles fed 3.75% *D. rostrata* oil had the highest HSI. VSI was not significantly affected ($P > 0.05$) by various dietary *D. rostrata* oil concentrations.

The *D. rostrata* oil level also did not significantly affect ($P < 0.05$) the body

Table 4
Survival rate, growth performance, feed utilization efficiency and body indices of juvenile T. tambroides *fed varying* D. rostrata *oil levels for 12 weeks*

	Dietary *D. rostrata* oil (%)				
Parameters	0	1.25	2.5	3.75	5
Survival (%)	100.00	100.00	100.00	100.00	100.00
Final Weight (g)	3.98 ± 0.09^{b}	3.76 ± 0.09^{ab}	3.85 ± 0.07^{ab}	3.70 ± 0.09^{a}	3.63 ± 0.05^{a}
Weight Gain (%)	119.89 ± 5.01^{b}	107.55 ± 4.83^{ab}	112.52 ± 3.63^{ab}	104.60 ± 4.78^{a}	100.55 ± 2.55^{a}
SGR[1]	0.87 ± 0.02^{b}	0.81 ± 0.03^{ab}	0.84 ± 0.02^{ab}	0.79 ± 0.02^{a}	0.77 ± 0.01^{a}
FCR[2]	1.89 ± 0.05^{a}	1.98 ± 0.07^{ab}	1.89 ± 0.19^{a}	2.37 ± 0.16^{bc}	2.49 ± 0.08^{c}
DFI[3]	1.57 ± 0.03^{ab}	1.53 ± 0.06^{a}	1.50 ± 0.12^{a}	1.80 ± 0.07^{bc}	1.85 ± 0.07^{c}
PER[4]	1.32 ± 0.04^{c}	1.27 ± 0.04^{bc}	1.35 ± 0.12^{c}	1.07 ± 0.07^{ab}	1.01 ± 0.03^{a}
HSI[5]	2.16 ± 0.14^{ab}	2.14 ± 0.17^{ab}	1.81 ± 0.13^{a}	2.36 ± 0.13^{b}	1.92 ± 0.06^{a}
VSI[6]	7.83 ± 0.23	7.91 ± 0.59	7.65 ± 0.95	8.30 ± 0.20	7.38 ± 0.10
Lean (% wet weight)	60.00 ± 0.64	60.00 ± 0.70	60.00 ± 0.72	60.00 ± 0.17	60.00 ± 0.89

Note. Mean ± SE (n = 3); means within the same row with different superscripts are significantly different at $P < 0.05$. [1] Specific growth rate (%/d) = $\left[\frac{\ln \text{final weight} - \ln \text{initil weight}}{\text{Experimental days}}\right] \times 100$. [2] Feed conversion ratio = $\frac{\text{Total feed consumed (g)}}{\text{Wet weight gain (g)}}$. [3] Daily feed intake (%/d) = $\frac{\text{Total feed consumed}}{[(\text{final weight} + \text{initial weight}) \times (\text{Experimental days}/2)]} \times 100$. [4] Protein efficiency ratio = $\frac{\text{Wet weight gain (g)}}{\text{Total protein intake (g)}}$. [5] Hepatosomatic index (%) = $\frac{\text{liver weight (g)}}{\text{body weight (g)}} \times 100$. [6] Viscerosomatic index (%) = $\frac{\text{visceral weight (g)}}{\text{body weight (g)}} \times 100$

proximate composition of mahseer except fiber and ash (Table 5). Major components in mahseer body were water (67-68.6%), protein (15.1-16.1%) and lipid (12.5-13.4%). Fish fed 3.75% *D. rostrata* oil had a significantly lower ($P < 0.05$) body ash compared to those fed 1.25-5% *D. rostrata* oil. Fish given 0% *D. rostrata* oil had a significantly higher ($P < 0.05$) body fiber content than fish fed 2.5% *D. rostrata* oil. Nevertheless, the fish body fiber remained extremely low (0.01-0.13%).

Juveniles given 5% *D. rostrata* oil had significantly lower ($P < 0.05$) protein and gross energy retention values than those given 0 or 2.5% *D. rostrata* oil (Table 6). Juveniles fed 5% *D. rostrata* oil had also a significantly lower ($P < 0.05$) lipid retention

Table 5
Whole body proximate composition (% wet weight) of juvenile T. tambroides *fed varying* D. rostrata *oil levels for 12 weeks*

		Dietary kembayau oil (%)				
	Initial	0	1.25	2.5	3.75	5
Dry matter	34.33	33.10 ± 0.40	32.97 ± 0.28	32.60 ± 0.11	32.30 ± 0.36	31.40 ± 0.69
Crude protein	14.49	16.15 ± 0.37	15.49 ± 0.72	15.65 ± 0.10	15.36 ± 0.39	15.06 ± 0.52
Crude lipid	11.12	13.40 ± 0.50	13.02 ± 0.57	12.71 ± 0.28	12.95 ± 0.45	12.53 ± 0.26
Ash	4.16	2.83 ± 0.08^{ab}	2.87 ± 0.04^{b}	2.82 ± 0.05^{ab}	2.54 ± 0.07^{a}	2.98 ± 0.15^{b}
Fiber	0.20	0.13 ± 0.06^{b}	0.05 ± 0.01^{ab}	0.01 ± 0.01^{a}	0.06 ± 0.03^{ab}	0.10 ± 0.00^{ab}
NFE[1]	4.35	0.60 ± 0.31	1.55 ± 0.37	1.41 ± 0.22	1.40 ± 0.39	0.72 ± 0.08
Carbohydrate	4.55	0.73 ± 0.37	1.60 ± 0.38	1.42 ± 0.22	1.46 ± 0.42	0.82 ± 0.08
Gross energy (kJ/g)	11.85	8.68 ± 0.08^{b}	8.18 ± 0.16^{a}	8.36 ± 0.25^{ab}	8.49 ± 0.09^{ab}	8.10 ± 0.05^{a}

Note. Mean ± SE (n = 3); means within the same row with different superscript are significantly different at $P < 0.05$. [1] Nitrogen free extract = Dry matter – (protein + lipid + ash + fiber)

than those given 0-2.5% *D. rostrata* oil. Fish fed 0% *D. rostrata* oil showed a significantly higher ($P < 0.05$) energy retention than those given 1.25, 3.75 or 5% *D. rostrata* oil. The results showed that the *D. rostrata* oil level did not have any significant effects ($P > 0.05$) on carbohydrate retention. However, the total carbohydrate amount in the fish was less than the initial amount after the 12-week feeding.

Table 7 presents the effects of *D. rostrata* oil level on the muscle fatty acid composition of *T. tambroides* juveniles. The most dominant fatty acid in the muscle of *T. tambroides* was 18:1n-9 and followed by 16:0, 18:2n-6, 18:0 and 16:1n-7. However, muscle 18:1n-9, 16:0 and 18:2n-6 were not significantly affected ($P > 0.05$) by the dietary *D. rostrata* oil level. The increased dietary *D. rostrata* oil content significantly

Table 6
Estimated protein, lipid, carbohydrate and energy retention (%) of juvenile T. tambroides *fed varying* D. rostrata *oil levels for 12 weeks*

Retention	Dietary *D. rostrata* oil (%)				
	0	1.25	2.5	3.75	5
Crude protein[1]	27.93 ± 2.74^{c}	22.59 ± 3.26^{abc}	25.58 ± 3.26^{bc}	18.30 ± 2.93^{ab}	15.90 ± 1.64^{a}
Crude lipid[2]	124.05 ± 5.39^{c}	102.81 ± 2.00^{bc}	109.89 ± 9.89^{c}	84.28 ± 9.75^{ab}	72.24 ± 2.25^{a}
Carbohydrate[3]	-5.52 ± 1.18	-2.31 ± 1.31	-2.76 ± 0.85	-2.31 ± 1.09	-4.06 ± 0.18
Gross energy[4]	22.27 ± 1.70^{c}	15.24 ± 2.44^{ab}	18.67 ± 2.81^{bc}	13.77 ± 1.68^{ab}	10.32 ± 0.78^{a}

Mean ± SE (n = 3); means within the same row with different superscripts are significantly different at $P < 0.05$

[1] $\text{Protein retention (\%)} = \frac{[(\%\text{ final body crude protein} \times \text{final body weight}) - (\%\text{ initial body crude protein} \times \text{initial body weight})]}{(\text{food intake} \times \%\text{ diet crude protein})} \times 100$

[2] $\text{Lipid retention (\%)} = \frac{[(\%\text{ final body crude lipid} \times \text{final body weight}) - (\%\text{ initial body crude lipid} \times \text{initial body weight})]}{(\text{food intake} \times \%\text{ diet crude lipid})} \times 100$

[3] $\text{Carbohydrate retention (\%)} = \frac{[(\%\text{ final body carbohydrate} \times \text{final body weight}) - (\%\text{ initial body carbohydrate} \times \text{initial body weight})]}{(\text{food intake} \times \%\text{ diet carbohydrate})} \times 100$

[4] $\text{Gross energy retention (\%)} = \frac{[(\text{final body gross energy} \times \text{final body weight}) - (\text{initial body gross energy} \times \text{initial body weight})]}{(\text{food intake} \times \text{diet gross energy})} \times 100$

increased ($P < 0.05$) the 18:0 percentage in the mahseer muscle. Total muscle SFAs, MUFAs were not significantly affected ($P > 0.05$) by dietary *D. rostrata* oil. Although juveniles fed 0% *D. rostrata* oil contained significantly lower ($P < 0.05$) 20:4n-6 than those fed 2.5 and 5% *D. rostrata* oil, total n-6 PUFA content was not significantly affected ($P > 0.05$) by dietary *D. rostrata* oil. Fish fed 0% *D. rostrata* oil had significantly lower ($P < 0.05$) muscle total n-3 PUFA content than those fed 2.5-5% *D. rostrata* oil. Nevertheless, dietary *D. rostrata* oil level did not significantly affect ($P > 0.05$) the n-3/n-6 ratio in the muscle of mahseers. Muscle 18:2n-6, 20:4n-6, 22:5n-3, 22:6n-3 and total n-6 PUFAs were lower at the end of the trial compared to those at the beginning of the trial. The juveniles had higher SFAs and MUFAs in their muscle following the feeding than their initial amounts.

The liver fatty acid compositions of *T. tambroides* juveniles before and after the feeding are shown in Table 8. The most abundant fatty acid in the fish liver was 18:1n-9 and followed by 16:0, 18:0, 16:1n-7 and 18:2n-6. The liver of fish fed 5% *D. rostrata* oil had a significantly lower ($P < 0.05$) 16:1n-7 and a significantly higher ($P < 0.05$) 18:0 than those fed 0% *D. rostrata* oil.

Table 7

Fatty acid composition (% of total fatty acids) of muscle tissue of juvenile T. tambroides *at the beginning and end of the 12-week experimental period*

		Dietary *D. rostrata* oil (%)				
	Initial	0	1.25	2.5	3.75	5
14:0	2.51	2.98 ± 0.17	3.04 ± 0.17	2.70 ± 0.16	2.93 ± 0.17	2.65 ± 0.16
16:0	26.47	33.09 ± 1.91	32.53 ± 1.88	28.87 ± 1.67	29.87 ± 1.73	27.77 ± 1.61
16:1n-7	3.42	5.56 ± 0.32^{ab}	5.42 ± 0.31^{ab}	4.80 ± 0.28^{a}	5.97 ± 0.35^{b}	5.22 ± 0.30^{ab}
18:0	6.99	6.28 ± 0.36^{a}	7.22 ± 0.42^{ab}	7.97 ± 0.46^{bc}	8.18 ± 0.47^{bc}	9.11 ± 0.53^{c}
18:1n-9	33.71	37.44 ± 2.11	37.75 ± 2.08	39.16 ± 1.99	38.52 ± 1.92	39.61 ± 1.88
18:2n-6	18.76	8.91 ± 0.51	8.06 ± 0.47	9.07 ± 0.53	7.65 ± 0.44	7.95 ± 0.46
18:3n-3	1.30	1.14 ± 0.06^{a}	1.28 ± 0.08^{ab}	1.43 ± 0.08^{b}	1.32 ± 0.08^{ab}	1.52 ± 0.09^{b}
20:4n-6	1.86	0.96 ± 0.06^{a}	1.00 ± 0.06^{a}	1.25 ± 0.08^{b}	1.16 ± 0.07^{ab}	1.31 ± 0.08^{c}
20:5n-3	0.54	0.53 ± 0.03	0.53 ± 0.03	0.60 ± 0.03	0.57 ± 0.03	0.63 ± 0.03
22:5n-3	0.45	0.31 ± 0.02^{a}	0.32 ± 0.02^{ab}	0.40 ± 0.02^{c}	0.38 ± 0.02^{bc}	0.34 ± 0.02^{abc}
22:6n-3	4.00	2.79 ± 0.16^{a}	2.86 ± 0.17^{ab}	3.75 ± 0.22^{c}	3.44 ± 0.20^{bc}	3.87 ± 0.23^{c}
Σ SFA[1]	35.96	42.35 ± 2.10	42.79 ± 2.12	39.54 ± 2.97	40.98 ± 2.03	39.53 ± 1.97
Σ MUFA[2]	37.13	43.00 ± 2.44	43.17 ± 2.39	43.96 ± 2.26	44.49 ± 2.26	44.83 ± 2.18
Σ n-3 PUFA[3]	6.30	4.78 ± 0.24^{a}	4.99 ± 0.25^{ab}	6.19 ± 0.31^{c}	5.72 ± 0.28^{bc}	6.37 ± 0.33^{c}
Σ n-6 PUFA[4]	20.61	9.87 ± 0.57	9.06 ± 0.53	10.31 ± 0.60	8.81 ± 0.51	9.26 ± 0.54
n-3/n-6	0.31	0.49 ± 0.05	0.56 ± 0.06	0.61 ± 0.07	0.66 ± 0.07	0.70 ± 0.08
PUFA/SFA	0.75	0.35 ± 0.01^{a}	0.33 ± 0.01^{a}	0.42 ± 0.01^{b}	0.36 ± 0.01^{a}	0.40 ± 0.01^{b}

Note. Mean ± SE (n = 3); means within the same row with different superscripts are significantly different at $P < 0.05$. [1] Saturated fatty acids (Sum of 14:0 + 16:0 + 18:0). [2] Monounsaturated fatty acids (Sum of 16:1n-7 + 18:1n-9). [3] The n-3 series of polyunsaturated fatty acids (Sum of 18:3n-3 + 20:5n-3 + 22:5n-3 + 22:6n-3). [4] The n-6 series of polyunsaturated fatty acids (Sum of 18:2n-6 + 20:4n-6)

Table 8

Fatty acid composition (% of total fatty acids) of liver tissue of juvenile T. tambroides *at the beginning and end of the 12-week experimental period*

		Dietary *D. rostrata* oil (%)				
	Initial	0	1.25	2.5	3.75	5
14:0	2.24	3.21 ± 0.18	3.34 ± 0.19	3.27 ± 0.19	3.36 ± 0.20	3.15 ± 0.18
16:0	26.87	30.49 ± 1.76	29.33 ± 1.69	28.91 ± 1.67	30.86 ± 1.78	27.52 ± 1.59
16:1n-7	4.71	7.21 ± 0.42^{b}	6.73 ± 0.39^{ab}	6.48 ± 0.38^{ab}	6.52 ± 0.38^{ab}	5.81 ± 0.33^{a}
18:0	7.79	6.75 ± 0.39^{a}	8.11 ± 0.47^{ab}	7.89 ± 0.46^{ab}	8.42 ± 0.48^{b}	8.91 ± 0.51^{b}
18:1n-9	31.65	41.11 ± 1.75	41.78 ± 1.74	42.46 ± 1.72	40.99 ± 1.78	42.73 ± 1.74
18:2n-6	14.97	6.16 ± 0.36^{b}	5.72 ± 0.33^{b}	5.79 ± 0.33^{b}	4.60 ± 0.27^{a}	6.21 ± 0.36^{b}
18:3n-3	1.28	1.35 ± 0.08	1.44 ± 0.08	1.51 ± 0.09	1.60 ± 0.09	1.46 ± 0.09
20:4n-6	2.15	0.88 ± 0.05	0.84 ± 0.05	0.85 ± 0.05	0.83 ± 0.05	0.92 ± 0.05
20:5n-3	0.80	0.45 ± 0.03^{a}	0.41 ± 0.02^{a}	0.49 ± 0.03^{a}	0.41 ± 0.02^{a}	0.59 ± 0.03^{b}
22:5n-3	1.08	0.26 ± 0.02	0.24 ± 0.01	0.22 ± 0.01	0.24 ± 0.01	0.24 ± 0.01
22:6n-3	6.47	2.14 ± 0.12	2.07 ± 0.12	2.14 ± 0.12	2.17 ± 0.13	2.46 ± 0.14
Σ SFA[1]	36.89	40.45 ± 1.97	40.77 ± 1.97	40.07 ± 1.93	42.64 ± 2.07	39.58 ± 1.92
Σ MUFA[2]	36.36	48.32 ± 2.17	48.52 ± 2.13	48.94 ± 2.10	47.51 ± 2.15	48.54 ± 2.07
Σ n-3 PUFA[3]	9.63	4.20 ± 0.21	4.16 ± 0.21	4.36 ± 0.23	4.41 ± 0.23	4.75 ± 0.25
Σ n-6 PUFA[4]	17.12	7.03 ± 0.41^{b}	6.56 ± 0.38^{ab}	6.64 ± 0.39^{ab}	5.44 ± 0.31^{a}	7.13 ± 0.41^{b}
n-3/n-6	0.56	0.61 ± 0.06	0.64 ± 0.07	0.66 ± 0.07	0.82 ± 0.09	0.68 ± 0.08
PUFA/SFA	0.73	0.28 ± 0.01^{bc}	0.26 ± 0.01^{b}	0.27 ± 0.01^{bc}	0.23 ± 0.01^{a}	0.30 ± 0.01^{c}

Note. Mean ± SE (n = 3); means within the same row with different superscripts are significantly different at $P < 0.05$. [1] Saturated fatty acids (Sum of 14:0 + 16:0 + 18:0). [2] Monounsaturated fatty acids (Sum of 16:1n-7 + 18:1n-9). [3] The n-3 series of polyunsaturated fatty acids (Sum Of 18:3n-3 + 20:5n-3 + 22:5n-3 + 22:6n-3). [4] The n-6 series of polyunsaturated fatty acids (Sum of 18:2n-6 + 20:4n-6)

Table 9

Estimated fatty acid retention (% dietary respective fatty acids) of muscle of juvenile T. tambroides *fed varying* D. rostrata *oil levels*

	Dietary *D. rostrata* oil (%)				
	0	1.25	2.5	3.75	5
14:0	107.58 ± 5.03	97.75 ± 7.59	90.52 ± 10.60	84.90 ± 5.04	66.54 ± 6.24
16:0	68.91 ± 8.49	61.22 ± 5.99	59.35 ± 9.08	54.89 ± 10.86	45.78 ± 5.29
16:1n-7	217.98 ± 8.32^{c}	180.73 ± 13.12^{b}	158.72 ± 17.59^{b}	163.08 ± 8.27^{b}	116.97 ± 8.05^{a}
18:0	67.37 ± 9.69^{b}	53.05 ± 5.92^{ab}	54.94 ± 8.27^{ab}	34.89 ± 6.81^{a}	32.25 ± 3.34^{a}
18:1n-9	85.80 ± 4.01^{c}	70.05 ± 5.41^{bc}	76.27 ± 8.19^{c}	54.82 ± 3.35^{ab}	47.01 ± 3.20^{a}
18:2n-6	18.45 ± 6.94^{b}	4.66 ± 4.66^{ab}	12.84 ± 7.39^{b}	2.83 ± 2.83^{ab}	0.00^{a}
18:3n-3	57.08 ± 3.31^{a}	55.09 ± 4.89^{a}	67.85 ± 7.81^{b}	46.88 ± 3.06^{a}	53.73 ± 4.44^{a}
20:4n-6	27.15 ± 8.08	19.58 ± 6.99	43.27 ± 9.85	24.84 ± 8.16	28.05 ± 4.68
20:5n-3	27.34 ± 1.35^{b}	22.92 ± 1.87^{ab}	28.13 ± 3.28^{b}	17.80 ± 1.15^{a}	19.16 ± 1.54^{a}
22:5n-3	28.53 ± 4.91^{ab}	21.35 ± 3.52^{ab}	37.51 ± 6.28^{b}	25.17 ± 5.90^{ab}	15.76 ± 2.14^{a}
22:6n-3	53.02 ± 4.47^{a}	45.06 ± 5.13^{a}	79.85 ± 9.88^{b}	48.07 ± 3.70^{a}	54.97 ± 5.61^{a}
Σ SFA[1]	70.47 ± 8.04^{b}	61.41 ± 5.38^{ab}	50.85 ± 5.11^{ab}	45.23 ± 5.42^{ab}	42.12 ± 4.20^{a}
Σ MUFA[2]	94.25 ± 4.26^{c}	76.85 ± 5.88^{bc}	81.28 ± 8.76^{c}	61.22 ± 3.64^{ab}	51.26 ± 3.49^{a}
Σ n-3 PUFA[3]	42.52 ± 2.37^{a}	39.12 ± 3.46^{a}	51.60 ± 6.59^{b}	38.17 ± 2.36^{a}	40.38 ± 3.62^{a}
Σ n-6 PUFA[4]	19.22 ± 7.04	5.43 ± 5.17	15.97 ± 7.61	4.08 ± 4.08	2.38 ± 1.21

Note. Mean ± SE (n = 3); means within the same row with different superscripts are significantly different at $P < 0.05$. Fatty acid retention (%) = $\frac{(\text{g final muscle fatty acid} - \text{g initial muscle fatty acid})}{\text{g total fatty acid fed}} \times 100.$

[1]Saturated fatty acids.

[2]Monounsaturated fatty acids. [3] The n-3 series of polyunsaturated fatty acids.

[4]The n-6 series of polyunsaturated fatty acids.

Total SFAs and MUFAs in the liver were not significantly affected ($P > 0.05$) by dietary *D. rostrata* oil. Although the increment of dietary *D. rostrata* oil concentration significantly lowered ($P < 0.05$) the 20:5n-3 concentration in the liver of mahseer juveniles, total n-3 PUFAs and n-3/n-6 ratios were not significantly affected ($P > 0.05$) in the liver of juveniles fed different dietary *D. rostrata* oil levels. The concentrations of liver SFA and MUFA were higher upon feeding than the initial levels while most of the PUFAs (except 18:3n-3) showed the opposite trends.

High dietary 16:1n-7 retention (>100%) was found among all treatment groups (Table 9). The retention significantly increased ($P < 0.05$) with the increase of *D. rostrata* oil concentration in the diet. Significantly higher ($P < 0.05$) dietary 18:0, 18:1n-9, 18:2n-6, 20:5n-3, total SFA and total MUFA were retained in the muscle of fish fed 0% *D. rostrata* oil than in those fed 5% *D. rostrata* oil. Meanwhile, juveniles fed on 2.5% *D. rostrata* oil showed significantly higher ($P < 0.05$) muscular 22:5n-3 and 22:6n-3 retention compared to those fed on 5% *D. rostrata* oil. The highest 18:3n-3, 22:6n-3 and total n-3 PUFA muscular retention values were found in juveniles given 2.5% *D. rostrata* oil.

DISCUSSION

Lower growth performance and feed efficiency were observed in juveniles given higher than 2.5% *D. rostrata* oil compared to those given less than 2.5% *D. rostrata* oil in spite of similar survival and lean portions. This could be as a result of higher 18:0 content in the former diets than the latter ones. The mentioned fatty acid is considered a low digestible and low absorbable fatty acid in both fish (Francis, Turchini, Jones, & De Silva, 2007; Menoyo, Lopez-Bote, Bautista, & Obach, 2003; Turchini et al., 2005) and mammals (Kritchevsky, 1994), which can influence growth performance. On the other hand, the fatty acids 16:0 and 18:1n-9 are known as predominant energy sources in fish to spare dietary protein for better growth (Henderson, 1996; Lim, Boey, & Ng, 2001; Sargent et al., 2002; Tocher, 2003). MUFAs with a *cis* double bond as well as PUFAs have a strong potential to inhibit other fatty acids to be used in the β-oxidation process during the intramitochondrial NADPH inadequacy (Gurr, Harwood, & Frayn, 2002; Osmundsen & Bjornstad, 1985). However, 16:0 may be even more preferred than 18:1n-9 in β-oxidation as the multi regression results (%WG = -1.34 KEM + 119.63) also demonstrated that higher dietary 16:0 concentration was responsible for the higher growth in juvenile Malaysian mahseer.

Moreover, SFAs and MUFAs have been found to spare PUFAs and to increase the retention of these fatty acids in fish tissues (Mishra & Samantaray, 2004). Feeding fish with high SFA diets produces a readily oxidized lipid source to provide the energy needed and to spare protein for better growth. No protein sparing activity has been observed in the Malaysian mahseer fed a diet with lipid containing low SFA

(Ng, Abdullah, & De Silva, 2008). In contrast, other researchers (Kamarudin et al., 2012; Kamarudin et al., 2018) noted that *T. tambroides* obtains a better growth when palm oil with high SFA is included in its diet than when cod liver oil, linseed oil, sunflower oil, or illipe oil are fed. They demonstrated that the better growth is due to the protein-sparing action facilitated by higher amounts of SFAs. This group of researchers also found higher 22:6n-3 concentration in the liver of juveniles fed on the diet with 50% replacement of cod liver oil with palm oil compared to fish fed on the diets with 50% replacement of cod liver oil with linseed oil or sunflower oil. Ramezani-Fard et al. (2012b) suggested that SFAs spare n-3 PUFAs in the tissue of Malaysian mahseer and provide high retention of n-3 PUFAs as well as a desirable growth performance in this fish species. Therefore, higher protein, lipid and energy retention as well as higher n-3 PUFA retention in juveniles fed 2.5% or less *D. rostrata* oil could be as a result of higher 16:0 contents in their diets than those fed 3.75-5% *D. rostrata* oil. However, some anti-nutritional factors might also be responsible for the lower growth in juveniles fed more than 2.5% *D. rostrata* oil despite higher feed intake in these groups of juveniles. Such factors have been reported in the pulp and the seeds of African plum or safou (*D. edulis*) (Hanson, 2009; Omogbai & Ojeaburu, 2010).

In general, negative carbohydrate, low protein and high lipid retention together with higher tissue concentrations of SFAs and MUFAs at the end of this feeding trial compared to their initial concentrations demonstrated that juvenile *T. tambroides* utilized non-lipid nutrients to provide most of its required energy as well as to convert some of those non-lipid nutrients into body lipid (Hepher, 1988). High dietary SFA and MUFA contents could be another reason of higher tissue concentrations of these fatty acids at the end of the feeding trial than their initial amounts. Lower protein and lipid retention in juveniles given 3.75-5% *D. rostrata* oil compared to those given 2.5% and less *D. rostrata* oil demonstrated the insufficiency of SFAs and MUFAs in diets containing more than 2.5% *D. rostrata* oil to spare protein and n-3 PUFAs in the bodies of these groups of juveniles, which suppressed their growth performance in spite of higher food intake in these groups of fish. The high VSI values showed that *T. tambroides,* in general, had a high tendency to accumulate lipids in its visceral cavity, which had been previously observed in this fish (Bami et al., 2017; Kamarudin et al., 2018; Ng et al., 2008; Ramezani-Fard et al., 2012b).

Ishak, Kamarudin, Ramezani-Fard, Saad and Yusof (2016) have reported *T. tambroides* dietary carbohydrate requirement of about 25%. The SGR (0.9-1.1) and WG (83.2-155.5 %) values obtained by these researchers were higher than those observed in the current research with the diets including 28 % carbohydrates, especially in the fish fed on more than 2.5 % kembayau oil. Actually, these values in the current study were nearer to those fish fed on 30 % carbohydrate obtained by that group of researchers. This could be as the

result of using corn starch in that research which is the best carbohydrate source for *T. tambroides* followed by taro, sago and tapioca starch (Kamarudin et al., 2014). The SGR and WG values obtained in the current research, especially for those juveniles fed 2.5 % or less kembayau oil, were higher than those found in the studies used diets containing lower SFAs and higher PUFAs (Misieng et al., 2011; Ramezani-Fard et al., 2014; Ramezani-Fard et al., 2012b). The reasonably low FCR values obtained in this study, especially for those juveniles fed 2.5 % or less kembayau oil, may demonstrate appropriate feeding schedule, high digestibility of feed ingredients and proper feed utilization. The FCR values calculated in the current study, were comparable to those obtained in other studies (Ishak et al., 2016; Misieng et al., 2011; Ng et al., 2008; Ramezani-Fard et al., 2012a, 2012b; Ramezani-Fard et al., 2014).

Increased activities of proteolytic enzymes, trypsin and chymotrypsin have been observed in juvenile *T. tambroides* feeding on 0.10 % *Spirulina* as a feed additive in its diet (Jalal, Abmbak, Abol, Hassan, & Zahangir Alam, 2005). According to this research, growth performance and body composition of Malaysian mahseer might be promoted by the addition of *Spirulina* in its diet. Moreover, an improvement in the growth performance and body composition of *T. tambroides* fry fed with a phototrophic purple bacteria, *Marichromatium* sp. as a probiotic in their diet has been reported (Chowdhury, Zakaria, Zainal Abidin, & Rahman, 2016). Their study showed that there were trends of increased growth, better survival rate and improved FCR when fed with diet 1 (*Marichromatium* sp.) compared to other diets. There was a significant difference ($P < 0.05$) between the sampling days. The specific growth rate and weight gain of the fish fed with diet 1 were 0.49 % and 4.92 g, respectively, compared to 0.42 % and 4.11 g from the control. This study suggested that purple bacteria could be used in feed formulation as a supplement to promote growth and survival of freshwater fishes in Malaysia.

In addition to different growth performances, food efficiencies, and protein, lipid and energy retention values, different fatty acid compositions were observed in the muscle and liver tissues of juveniles fed on various dietary *D. rostrata* oil levels. The 16:0 in both the muscle and liver of the mahseer were relatively at constant levels (27.52-30.49% and 27.52-30.86%, respectively) despite a wide dietary range of this fatty acid concentration (26.14-38.70%). The findings supported the notion of selective 16:0 retention by *T. tambroides*, which had been previously reported in this fish (Bami et al., 2017; Kamarudin et al., 2018; Ramezani-Fard et al., 2012b). Mahseer has been demonstrated to selectively retain this fatty acid because of its important role in phosphatidylcholine, the significant phospholipid required for the structure and function of biomembranes (Ng, Lim, & Boey, 2003). These findings also explained the significance of a high concentration of 16:0 provided by CPO. Moreover, narrower ranges of 18:0 observed

in juveniles muscle (6.28-9.11%) and liver (6.75- 8.91%) compared to those of the experimental diets (6.22-13.94%) reconfirmed the notion of selective retention of this SFA in *T. tambroides* by other researchers (Bami et al., 2017; Kamarudin et al., 2018; Ramezani-Fard et al., 2012b). Freshwater species have been reported to have a tendency to maintain tissue total SFAs at a constant level regardless of the dietary SFA content (Bahurmiz & Ng, 2007; Greene & Selivonchick, 1990; Ng et al., 2003). Juveniles fed on diets containing higher *D. rostrata* oil accumulated higher 18:0 in their tissues. In general, lesser SFA and higher MUFA concentrations were found in both liver and muscle tissues of all fish compared to their dietary contents. This suggested that *T. tambroides* utilized both SFAs and MUFAs for β-oxidation, and there was a bioconversion of 16:0 and 18:0 to 16:1n-7 and 18:1n-9 by the Δ9 desaturase enzyme in fish tissues. SFAs and MUFAs are preferred over PUFAs for β-oxidation in fish (Lim et al., 2001; Ng et al., 2003). Mishra and Samantaray (2004) observed that vegetable oils had adequate dietary SFA and MUFA amounts to perform a PUFA sparing activity and maintained the levels of PUFAs in the body of some freshwater fish species.

Fish prefer to use 20:5n-3 as an energy source rather than 22:6n-3 as they can readily β-oxidize 20:5n-3 but need to β-oxidize 22:6n-3 proxisomally and mitochondrially (Tocher, 2003). This evidence together with 20:5n-3 bioconversion to 22:6n-3 in fish tissues could be the reason of lower 20:5n-3 than 22:6n-3 in the tissues of juvenile *T. tambroides* of all groups. Less n-3 PUFAs were found in the muscle of fish fed on 0-1.25% *D. rostrata* oil compared to those fed on 2.5% or more *D. rostrata* oil. This could be as a result of slightly lower n-3 PUFA in the diets containing more than 2.5% CPO than the diets including 2.5% or more *D. rostrata* oil. Moreover, as mentioned earlier, 16:0 and 18:1n-9 are preferred over other fatty acids for β-oxidation in fish (Henderson, 1996; Sargent et al., 2002; Tocher, 2003). Although *D. rostrata* oil contained lower 16:0 than CPO, higher 18:0 and 18:1n-9 were found in *D. rostrata* oil. The 18:0, despite having low absorbability and digestibility in animals such as fish (Kritchevsky, 1994), is the precursor for 18:1n-9 production. Therefore, a 2.5:2.5 combination of *D. rostrata* oil and CPO could have resulted in a balance in the dietary concentrations of 16:0, 18:1n-9 and 18:0 to be used as the energy sources, which could have led to a higher retention of n-3 PUFAs in the muscle of juvenile *T. tambroides*. This evidence could also explain that why lower muscular 20:4n-6 content was observed in fish given lower *D. rostrata* oil percentages (0-1.25%) than the other treatments. A similar observation has been reported in our previous studies related to Malaysian mahseer (Bami et al., 2017; Kamarudin et al., 2018).

Ramezani-Fard et al. (2012a) suggested that muscular n-3 PUFA content in *T. tambroides* depends on *de novo* synthesis more than on a direct absorption from the diet. The lower 20:5n-3 and 22:5n-3

observed in the muscle of juveniles fed on test diets compared to their dietary percentages could be as a result of bio-conversion of both 20:5n-3 and 22:5n-3 to 22:6n-3. Nevertheless, the lower concentrations of n-3 LC-PUFAs were observed in the fish muscle when compared to their initial concentrations could also be due to their low dietary contents. Moreover, the higher water temperature in the aquaria (Farkas, 1984), which was about 10 ˚C higher than that of its natural habitat, could also decrease the muscular concentrations of n-3 LC-PUFAs. This notion has been previously suggested by Ramezani-Fard et al. (2012a). Farkas (1984) has also observed a higher PUFA concentration in the tissue of common carp (*Cyprinus carpio*) when reared in a lower water temperature.

The muscular and liver 18:2n-6 percentages in juveniles were in the ranges of 7.65-9.07% and 4.60-6.21%, respectively, despite being fed on diets containing higher levels of 18:2n-6 (9.29-10.91%). Ramezani-Fard et al. (2012b) and made a similar observation and proposed that the selective depletion of this fatty acid in *T. tambroides* tissues demonstrates the high tendency of this fish to mobilize and catabolize the fatty acid. Similar findings have been achieved in other studies on Malaysian mahseer (Bami et al., 2017; Kamarudin et al., 2018).

Lower 20:4n-6 percentages in both liver and muscle of juveniles compared to its initial percentages at the start of the study might also be related to both the decline in PUFA *de novo* synthesis and the preference of desaturase enzymes to use n-3 PUFAs than n-6 PUFAs that could have caused the decrease in 18:2n-6 conversion to 20:4n-6 (Jankowska, Zakęś, Żmijewski, & Szczepkowski, 2010). Moreover, conversion of the dietary 20:4n-6 to the tissue 20:4n-6 is preferred to that of the dietary 18:2n-6 (Jankowska et al., 2010). This could be the reason why tissue 20:4n-6 percentage was as low as its dietary percentage.

Nevertheless, although juveniles fed on various dietary *D. rostrata* oil levels showed muscle n-3/n-6 ratios higher than the 0.1-0.2 ratio recommended by the World Health Organization (Tanamati et al., 2009) at the end of the experiment, the highest retention of n-3 LC-PUFAs was observed in the muscle of juveniles given 2.5% *D. rostrata* oil. A balance between dietary 16:0 and 18:1n-9 concentrations in the mixture might be responsible in sparing PUFAs and maintaining their concentrations in *T. tambroides* body. Moreover, the fish fed on 2.5% *D. rostrata* oil had similar growth and feed efficiency to those fed 0% *D. rostrata* oil. Ramezani-Fard et al. (2012b) suggested that a diet containing 2.5% n-3 PUFA and an SFA/n-3 PUFA ratio of 15.3 could efficiently spare n-3 PUFA concentration in the tissue of *T. tambroides* as well as a satisfactory growth performance of this fish. These researchers added that n-3 PUFA concentrations of less than 2.5% and SFA/n-3 PUFA ratios of higher than 15.3 in the diet of Malaysian mahseer may reduce n-3 PUFA concentration in its tissues. However, they did not suggest the appropriate dietary MUFA level for this fish. No information on the exact dietary

fatty acid requirements of *T. tambroides* has been published. Even by using 2.5% *D. rostrata* oil, a high muscular concentration of 18:0 was found in juveniles with high muscular PUFA concentrations. High 18:1n-9 production from 18:0 as well as a balanced 16:0 concentration in the diet with 2.5% *D. rostrata* oil could be the reason of sparing more n-3 PUFAs in the tissue of juveniles fed on the mentioned diet than juveniles fed on the other test diets. Therefore, there might be a limited dietary requirement of the fatty acid 18:0 for *T. tambroides*. There could be a possibility that the fish given a diet with an 18:0 concentration higher than its nutritional requirement has a high tissue concentration of this fatty acid over a long-term culture period.

CONCLUSIONS

This study showed that *D. rostrata* oil could be used up to 2.5% as a dietary lipid source for mahseer juvenile without affecting the survival, growth, feed efficiency and major body nutrients. From the findings of this study, the use of a grow-out diet containing 5% CPO and a finisher diet containing a combination of 2.5% *D. rostrata* oil and 2.5% CPO were suggested for the mahseer culture to achieve a maximum growth performance and a desired flesh fatty acid profile, especially in the case of n-3 and n-6 LC-PUFAs. The optimal finishing period of juvenile *T. tambroides* needs to be determined. The effects of *D. rostrata* oil on the taste and shelf life of mahseer flesh should also be investigated. Dietary lipid source has been shown to affect the flesh taste, texture and smell in seabream (*Spaurus aurata*) and seabass (*Dicentrarchus labrax*). However, these effects could not be determined in this study as the fish had not reached its minimum commercial size. *D. rostrata* is a seasonal fruit, and no commercial production of the fruit or its oil has been reported. Research on the commercial farming and production of *D. rostrata* fruit and oil should also be conducted as its crude oil showed a high potential as a good source of dietary lipid for the Malaysian mahseer and possibly other highly sought after riverine carps. The addition of *Spirulina* or *Marichromatium* sp. to its diet is also suggested as it may result in an improved growth performance and body composition of this species.

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Mortality and Repellent Effects of Coffee Extracts on The Workers of Three Household Ant Species

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ABSTRACT

Coffee consists of a variety of chemical compounds that has not been documented to have resistance on insects. Hence, this research was conducted to study the impact of coffee extracts impregnated in gel bait towards survival and feeding behaviour of *Tapinoma indicum* (ghost ant), *Pheidole megacephala* (big-headed ant) and *Monomorium pharaonis* (Pharaoh ant) (Hymenoptera: Formicidae). The three coffee species used were *Coffea arabica*, *Coffea canephora* and *Coffea liberica*. The coffee extracts were obtained using Soxhlet extraction method, diluted to 0.01%, 0.05% and 0.10% concentration, and eventually impregnated into two sets of gel bait at with the first set (Set I) sugar solution and the second set (Set II) with distilled water. The overall results indicated that *Coffea arabica* gave highest mortality on all three ant species and higher concentration of extracts showed higher ant mortality in most bioassays. The higher mortality in lower concentration bioassays was probably due to their lower repellency percentages. Furthermore, Set I bioassays had higher mortality as the sugar used act as food attractant. *T. indicum* was the most susceptible species. Owing to the low mortality, the low concentration of coffee used was not effective in killing household ants but it did repel them.

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INTRODUCTION

Ants are known to be an ecologically dominant group and show the highest level of diversity among eusocial insects (Wilson & Hölldobler, 2005). They involve in the interactions with other organisms and also the functional ecosystem processes (Wilson & Hölldobler, 2005). With the exception of Antarctica and Arctic, their dominance is indicated by their worldwide geographical distribution. Each ant species possesses its own particular morphological structures and behaviours, making it distinguishable from other ant species (Lucky, 2009).

Furthermore, ants are one of the most nuisance urban pests when they enter from outdoors to indoors in searching of food and water. Due to their properties of large number appearance, cause contamination of food and hospital sterile equipments, they are considered as nuisance pests and disease organism carriers which make them to be recognized as potential mechanical vectors of human diseases (Beatson, 1972). Some ant species have the ability to cause painful bites or stings with their pincer-like jaws or venomous stings (Marer & Flint, 1991). Household ants can be discovered with a higher frequency in tropical areas compared to temperate areas (Campos-Farinha, 2005; Fowler, Filho, & Bueno, 1993).

In Asia, the pest status of household ants was less significant in the 1990s. However, this situation had soon changed owing to the rise of its pest status (Lee, 2000). In the pest control company of Malaysia, around 10% of the business was constituted by the controlling of ant in 1995 (Na & Lee, 2001). While in United States, the ant control revenue of ant is so high that the ants have attained the top household pest status (Gooch, 1999; Jenkins, 2001; Kaminski, 2000) and ranked the most troublesome pest (Gooch, 1999). From a residential survey, which was carried out in 1995, ants have attained the status of the most important household pests after mosquitoes and cockroaches (Na & Lee, 2001). In Malaysia, there are 23 species of household ants with a total of 15 genera described (Na & Lee, 2001). However, in this research, only three common species of household ants are focused: *Tapinoma indicum* (Forel) (ghost ant), *Pheidole megacephala* (F.) (big-headed ant) and *Monomorium pharaonis* (L.) (Pharaoh ant) (Hymenoptera: Formicidae).

There are several methods to control the household ants. Baiting and residual spraying are the common methods for controlling ants (Lee, 2000), but baiting has served as a more popular method due to its usage safety, target-specific and ability to eliminate or suppress the whole ant colony without the requirement to locate the nest (Suiter, Wu, & Bennett, 1997). Generally, baits are more effective against household ants as many residual contact insecticides used act repellent to ants, especially Pharaoh's ants (Gooch, 1999). Residual insecticide treatment just acts as barriers of preventing ants from entering the houses instead of eliminating the ant population (Klotz, Greenberg, Shorey, & Williams, 1997). Hence, this method is not effective against some species of household ants which reside within the house (Lee, 2000).

Recent studies have shown that plants such as coffee and tea are used as effective biological agent in controlling insects (Ab Majid et al., 2018). Coffee consists of over 1000 chemical compounds (Farah, 2012); while few *Coffea* species are resistant to insect attack naturally (Jaramillo, Borgemeister, & Baker, 2006). Coffee has been utilized to study toxicological effects on several organisms. Caffeine causes damage to the nervous system in bullfrog (Higure & Nohmi, 2002), blocks the fetal development of *Rattus norvegicus* (Smith, McElhatton, & Sullivan, 1987) and inhibits oviposition of shot-hole borer beetle (Hewavitharanage, Karunaratne, & Kumar, 1999). Coffee is known to be a natural repellent to ants at which ants repel when contact with the coffee grounds. Few researches have been reported that coffee is effective in decreasing the mosquitoes' reproductive capacity (Laranja, Manzatto, & de Campos Bicudo, 2003), repelling gravid *Aedes albopictus* female and inhibiting the development of their embryos (Satho et al., 2015).

In this study, three species of coffee: *Coffea arabica* (Arabica coffee), *Coffea canephora* (Robusta coffee) and *Coffea liberica* (Liberian coffee) are extracted by using Soxhlet extraction and impregnated in the gel bait to test their impact in controlling household ants. The coffee species used are roasted type in which they are not mixed with sugar to avoid creating bias towards the attraction of ants. The objectives of this study are, to investigate the effect of the extracts of *C. arabica*, *C. canephora* and *C. liberica* impregnated in gel bait towards survival of the workers of *T. indicum*, *P. megacephala* and *M. pharaonis*.

MATERIALS AND METHODS

Coffee Source

The coffee beans of *C. arabica*, *C. canephora* and *C. liberica* were obtained from Cap Kuda Coffee Company, Sabah, Malaysia. The coffee beans were roasted without adding any sugar compounds. The temperatures used in the roasting process vary from 210°C to 240°C and the roasting time used was about 12 to 30 min. These roasted coffee beans were then ground, packaged and shipped to Universiti Sains Malaysia.

Extract Coffee Using Soxhlet Extraction

A small cotton ball was moistened with water and placed in the chamber of Soxhlet extractor. Fifty grams of each of the roasted coffee (*C. arabica, C. canephora* and *C. liberica)* were weighted and placed separately into the Soxhlet extractor. A volume of 250 ml of methanol that used as the extraction solvent was poured into the flat-bottomed flask. The flat-bottomed flask was then placed on the heating mantle; the Soxhlet extractor together with the reflux condenser was placed atop of the flat-bottomed flask. The Soxhlet extractor was fixed and held by retort stand. Both ends of the reflux condenser were connected to pipes for water in and water out.

When the apparatus was ready, the extraction solvent (methanol) was heated

until its boiling point (64.7 °C) was achieved. Its vapour condensed in the condenser and the condensed extractant dripped into the chamber containing the coffee. When the liquid level in the chamber had risen to the top of the siphon tube, the extract-containing solvent of the Soxhlet chamber were siphoned into the flat-bottomed flask. The whole apparatus was heated for 5 h (Mgbemena, Ebe, Nnadozie, & Ekeanyanwu, 2015).

After 5 h, the entire apparatus was left to be cooled down. The coffee extracts were then collected and poured to a glass petri dish with correct label. The extracts were then placed into drying oven (Memmert GmbH + Co, KG, Western Germany) at 80 °C for evaporation for three days to obtain the coffee extract in solute form. The coffee extracts were then taken out and scraped off by using spatula. The scraped coffee extracts were kept in universal bottle with labeling and then stored in refrigerator for further used.

Collection and Identification of the *T. indicum, P. megacephala* and *M. pharaonis*

Field populations of *T. indicum*, *P. megacephala* and *M. pharaonis* workers were collected from the Minden campus of Univeristi Sains Malaysia, Penang, Malaysia from 7:00 a.m. to 10:00 a.m. The traps were set up by using Eppendorf tubes (with modified holes on the tubes) at which the inner surface of the Eppendorf tubes was coated with a thin layer of Fluon, polytetrafluroethylene suspension (BioQuip Products, Inc., California) to prevent the trapped ants from escaping (Eow, Chong, & Lee, 2004). A minute amount of peanut butter or honey which acts as food attractant to ants was placed on a small piece of paper and inserted it into the Eppendorf tubes. The trapped ants were collected after 1 to 2 h and transferred into container at which the inner surface was coated with fluon. The ants were put in 90% ethanol for identification according to their distinct characteristics based on descriptions by Na and Lee (2001) and Lucky (2009). A brush was used to separate the ant species if more than one ant species were trapped in the same tube.

Preparation of Gel Bait by Using Different Concentration of Coffee Extracts

The coffee extract solution with desired concentration was prepared by mixing coffee extracts and distilled water or 20% of sugar solution. Preparation of coffee concentration. i.e. 0.01%, 0.05% and 0.10% were produced using Arabica, following method of Ab Majid et al. (2018) with slight modification. To allow the coffee extracts to dissolve completely in the solution, the solution was allowed to stir by using magnetic stirrer for 30 to 45 min. After all, the gelling agent, Ferti-plant jelly (Fertiland Trading Co., Malaysia) was added into the prepared solution, allowing it to absorb the solution and expand to its maximum size for 12 h.

Two sets of gel baits were prepared. The first set of gel was the mixture of coffee extract and 20% of sugar solution (Set I).

The scraped coffee extract was diluted to different concentration such that 0.01% (low), 0.05% (medium) and 0.10% (high) by using 100 ml of 20% of sugar solution. The blank bait (control) used for this set contained only 20% of sugar solution. The second set of gel was the mixture of coffee extract and distilled water (Set II). The scraped coffee extract was diluted to different concentration such that 0.01% (low), 0.05% (medium) and 0.10% (high) by using 100 ml of distilled water. The blank bait (control) used consists of only distilled water. All of the gels were made constant mass of 0.50 g by using weighing machine.

Bioassay

A small hole (5 mm in diameter) was made at the center of the petri dish lid by using a hot soldering iron (Williams, 1989). This was to insert the cotton wool moistened with distilled water (without touching the base of petri dish) as moisture for ants (Figure 1)

A 90 mm diameter filter paper was attached to the outer base of petri dish to ease the counting process. The perimeter of the petri dish inner surface was coated with a thin layer of petroleum jelly (Vasline, Unilever Thai, Thailand) (Figure 1). Thirty ant workers were randomly picked and transferred to a petri dish (90 mm in diameter). The first set of gel bait was placed in the petri dish (Set I). Gel bait with only 20% of sugar solutions (without any coffee extracts) was used as the control of the experiment (Figure 2). Parafilm was used to seal up the petri dish to prevent the ants from escaping.

The ants were observed in 30 min, 1 h, 2 h, 4 h, 8 h, 24 h (1 day), 48 h (2 days) and 72 h (3 days) under temperature of 25 ± 2°C and relative humidity of 76 ± 10%. The number of ants which cannot move or respond (ant mortality) were counted and recorded. Their repellency and behaviours were also observed and recorded under dissecting microscope. The number of ants that were not attracted to the region with gel bait during the observed time was considered as repelling.

The above steps were repeated to complete three sets of replicates for each ant species with different concentrations (0.01%, 0.05% and 0.10%) for each coffee species. The bioassay for the second set of gel bait was conducted using the same procedures.

The mean repellency percentage for 30 min, 1 h, 2 h, 4 h, 8 h, 24 h, 48 h and 72 h; and mean mortality were obtained. The significant differences of mortality and repellency were determined using Kruskal-Wallis (KW) analysis of variance by SPSS 22.0 software. The repellency percentage (PR) was calculated using the formula (Abdullah et al., 2015):

$$PR = \frac{NC - NT}{NC + NT} \times 100$$

where,

NC = Number of ants on the region without gel bait

NT = Number of ants on the region with gel bait

RESULTS

Effect of Coffee Extracts Impregnated in Gel Bait Towards Survival of *T. indicum, P. megacephala* And *M. pharaonis*

C. canephora and *C. liberica* showed insignificant result (P>0.05) with P value 0.109 and 0.054 respectively against *T. indicum* between the three concentrations (0.01%, 0.0% and 0.10%) and control in Set I bioassay (Figure 1). *C. arabica* showed significant results (P<0.05) with P value 0.032 against *T. indicum* between the treatments and control in Set I bioassay but no significant differences among the three concentrations (Figure 1). In Set II bioassay, *C. canephora* displayed insignificant results (P>0.05) with P value 0.079 against *T. indicum*; while *C. arabica* and *C. liberica* had significant results (P<0.05) with P values 0.044 and 0.030 respectively against *T. indicum* between treatments and control but no significant differences among the three treatments (Figure 2).

In Set I bioassay, *P. megacephala* displayed the same results with those of *T. indicum* for the similar set. Both *C. canephora* and *C. liberica* showed insignificant result (P>0.05) with P values 0.144 and 0.114 respectively between the concentrations and control (Figure 3). *C. arabica* indicating significant results (P<0.05) with P value 0.030 between the treatments and control but no significant difference among the three concentrations (Figure 3). In Set II bioassay, there were no significant results (P>0.05) for all the three coffee species (*C. arabica* P=0.067; *C. canephora* P=0.127; and *C. liberica* P=0.392) against *P. megacephala*(Figure 4).

For *M. pharaonis*, all three coffee species (*C. arabica* P=0.134; *C. canephora* P=0.144; *C. liberica* P=0.212) had insignificant results (P>0.05) between the three concentrations and control in Set I bioassay (Figure 5). In Set II bioassay, there were insignificant results (P>0.05) for *C. arabica* (P=0.132) and *C. liberica* (P=0.441) between the concentrations and control. On the other hand, *C. canephora* (P=0.048) showed a significant difference (P<0.05). Nevertheless, it did not display significant results among the three concentrations (Figure 6).

At all concentrations in both sets, *C. arabica*, *C. canephora* and *C. liberica* showed insignificant results (P>0.05) among themselves against *T. indicum* with the exception of 0.05% concentration of Set II bioassay (Figures 1 and 2). Instead, its P value of 0.047 displayed a significant difference of the ant mortality among the coffee species at 0.05% at which *C. arabica* and *C. canephora* significantly differed from *C. liberica*. For *P. megacephala*, 0.01% showed insignificant results (P>0.05) among the three coffees with P value 0.141 and 0.100 in Set I and Set II bioassay respectively. However, 0.05% and 0.10% in Set I bioassay indicated significant results (P<0.05) among the coffees with P value 0.042 and 0.048 respectively, at which *C. arabica* differed significantly to both *C. canephora* and *C. liberia* (Figure

3). For Set II bioassay, the result (P=0.088, P>0.05) showed no significant difference at 0.05% among the three *Coffea* spp.; but it showed that *C. arabica* (P=0.034, P<0.05) experienced significant difference to *C. canephora* and *C. liberica* at 0.10% (Figure 4). The analysis for *M. pharaonis* showed that at all concentrations, there were no significant differences among the coffees in both sets of bioassays (Set I: P=0.633 at 0.01%, 0.612 at 0.05% and 0.966 at 0.10%; Set II: P=0.264 at 0.01%, 0.641 at 0.05% and 0.396 at 0.10%) (Figure 5).

There were no significant results (P>0.05) of mean repellency percentage at all observed time intervals (30 min, 1 h, 2 h, 4 h, 8 h, 24 h, 48 h and 72 h) in *T. indicum* between the treatments and control of *C. arabica* and *C. liberica* in both Set I and Set II bioassays.

C. canephora had significant differences at 24 h (P=0.037) and 72 h (P=0.037) in Set I bioassay; and at 48 h (P=0.031) in Set II bioassay between concentrations and control (Tables 1 and 2).

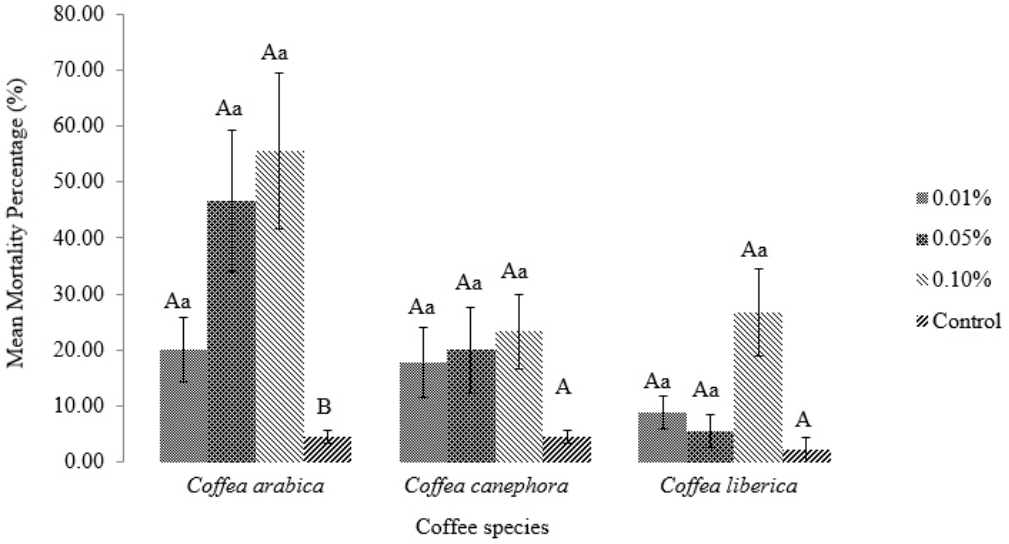

Figure 1. Mean mortality percentage of three different coffees against *T. indicum* in Set I bioassay

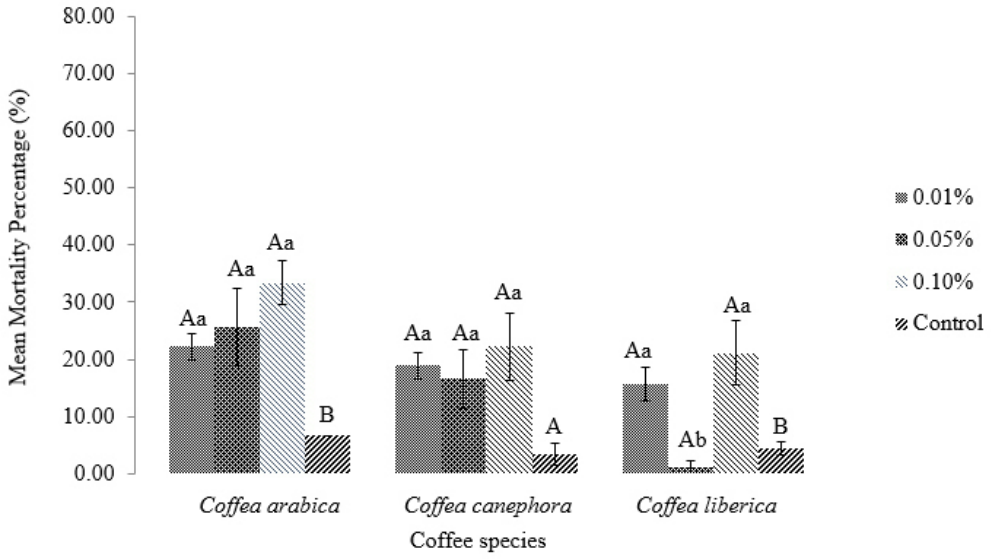

Figure 2. Mean mortality percentage of three different coffees against *T. indicum* in Set II bioassay

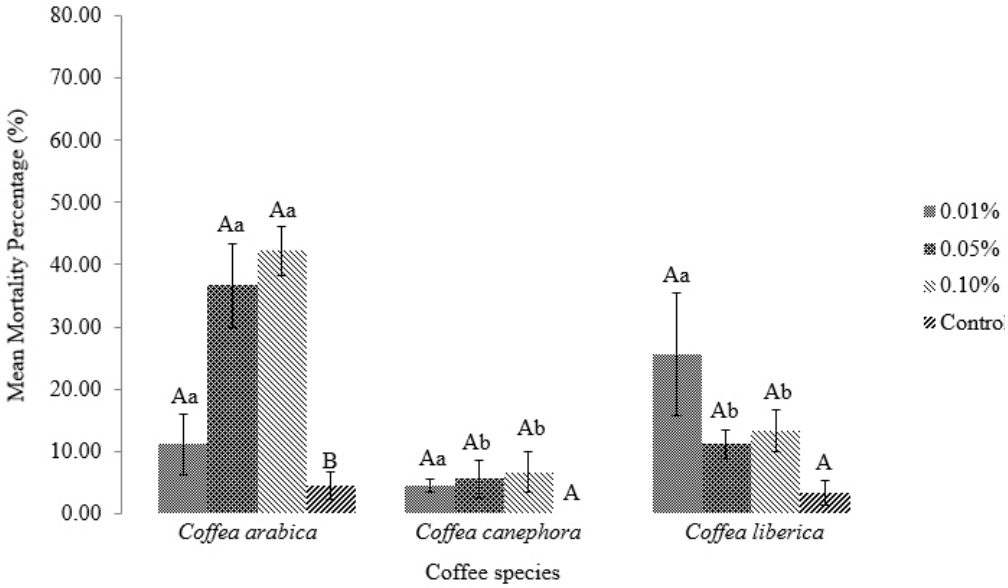

Figure 3. Mean mortality percentage of three different coffees against *P. megacephala* in Set I bioassay

Table 1

Mean mortality percentage and mean repellency percentage of three different coffees against T. indicum *in Set I bioassay*

Coffee species	Concentration (%)	Mean Mortality Percentage (%)[1]	Mean Repellency Percentage (%)[2]							
			30min	1h	2h	4h	8h	24h	48h	72h
Coffea arabica	**0.01%**	20.00 ± 5.77Aa	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	95.55 ± 2.22C	100.00 ± 0.00C
	0.05%	46.67 ± 12.62Aa	100.00 ± 0.00C	95.56 ± 4.44C	93.33 ± 6.67C	100.00 ± 0.00C	97.78 ± 2.22C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C
	0.10%	55.55 ± 13.92Aa	97.78 ± 2.22C	100.00 ± 0.00C	100.00 ± 0.00C	97.78 ± 2.22C	100.00 ± 0.00C	100.00 ± 0.00C	97.78 ± 2.22C	100.00 ± 0.00C
	Control	4.44 ± 1.11B	86.67 ± 10.18C	91.11 ± 8.89C	95.56 ± 4.44C	95.55 ± 2.22C	93.33 ± 3.85C	88.89 ± 5.88C	91.11 ± 5.88C	95.56 ± 4.44C
Coffea canephora	**0.01%**	17.78 ± 6.19Aa	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	97.78 ± 2.22C	100.00 ± 0.00C
	0.05%	20.00 ± 7.70Aa	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	97.78 ± 2.22C	100.00 ± 0.00C
	0.10%	23.33 ± 6.67Aa	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	97.78 ± 2.22C	100.00 ± 0.00C	100.00 ± 0.00C
	Control	4.44 ± 1.11A	95.55 ± 2.22C	93.33 ± 3.85C	97.78 ± 2.22C	93.33 ± 3.85C	95.55 ± 2.22C	88.89 ± 4.44D	86.67 ± 3.85C	88.89 ± 4.44D

Table 1 *(Continue)*

Coffea liberica	0.01%	8.89 ± 2.94Aa	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	95.56 ± 4.44C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C
	0.05%	5.56 ± 2.94Aa	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	91.11 ± 8.89C	97.78 ± 2.22C
	0.10%	26.67 ± 7.70Aa	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	97.78 ± 2.22C	95.55 ± 2.22C	97.78 ± 2.22C
	Control	2.22 ± 2.22A	100.00 ± 0.00C	95.55 ± 2.22C	97.78 ± 2.22C	91.11 ± 4.44C	91.11 ± 5.88C	86.67 ± 6.67C	84.44 ± 4.44C	86.67 ± 7.70C

[1] Mean mortality percentage followed by different letters within the same column are significant different by subjecting to Kruskal-Wallis H Test at $p<0.05$: A & B (comparison of concentration within the same coffee species); a & b (comparison of coffee species within a concentration).

[2] Mean repellency percentage followed by different letters within the same column are significant different by subjecting to Kruskal-Wallis H Test at $p<0.05$: C, D & E (comparison of concentration within the same coffee species).

Table 2

Mean mortality percentage and mean repellency percentage of three different coffees against T. indicum *in Set II bioassay*

Coffee species	Concentration (%)	Mean Mortality Percentage (%)[1]	Mean Repellency Percentage (%)[2]							
			30min	1h	2h	4h	8h	24h	48h	72h
Coffea arabica	0.01%	22.22 ± 2.22Aa	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	97.78 ± 2.22C
	0.05%	25.56 ± 6.76Aa	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	97.78 ± 2.22C	97.78 ± 2.22C	97.78 ± 2.22C	95.55 ± 4.45C	100.00 ± 0.00C
	0.10%	33.33 ± 3.85Aa	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	97.78 ± 2.22C	97.78 ± 2.22C
	Control	6.67 ± 0.00B	97.78 ± 2.22C	95.56 ± 4.44C	100.00 ± 0.00C	95.55 ± 2.22C	95.56 ± 4.44C	91.11 ± 4.44C	95.56 ± 4.44C	84.44 ± 12.37C
Coffea canephora	0.01%	18.89 ± 2.22Aa	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	95.55 ± 2.22C	97.78 ± 2.22C	97.78 ± 2.22C	100.00 ± 0.00C
	0.05%	16.67 ± 5.09Aa	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	97.78 ± 2.22C	97.78 ± 2.22C	100.00 ± 0.00C	100.00 ± 0.00C	91.11 ± 8.89C
	0.10%	22.22 ± 5.88Aa	100.00 ± 0.00C	97.78 ± 2.22C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C
	Control	3.33 ± 1.93A	97.78 ± 2.22C	95.55 ± 2.22C	97.78 ± 2.22C	95.56 ± 4.44C	93.33 ± 0.00C	93.33 ± 6.67C	88.89 ± 2.22D	88.89 ± 5.88C

Table 2 *(Continue)*

Coffea liberica	**0.01%**	15.56 ± 2.94Aa	100.00 ± 0.00	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	97.78 ± 2.22C
	0.05%	1.11 ± 1.11Ab	100.00C ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	97.78 ± 2.22C
	0.10%	21.11 ± 5.56Aa	97.78 ± 2.22C	97.78 ± 2.22C	100.00 ± 0.00C	97.78 ± 2.22C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C
	Control	4.44 ± 1.11B	97.78 ± 2.22C	95.56 ± 4.44C	100.00 ± 0.00C	97.78 ± 2.22C	95.56 ± 4.44C	97.78 ± 2.22C	95.56 ± 4.44C	93.33 ± 3.85C

[1] Mean mortality percentage followed by different letters within the same column are significant different by subjecting to Kruskal-Wallis H Test at $p<0.05$: A & B (comparison of concentration within the same coffee species); a & b (comparison of coffee species within a concentration).

[2] Mean repellency percentage followed by different letters within the same column are significant different by subjecting to Kruskal-Wallis H Test at $p<0.05$: C, D & E (comparison of concentration within the same coffee species).

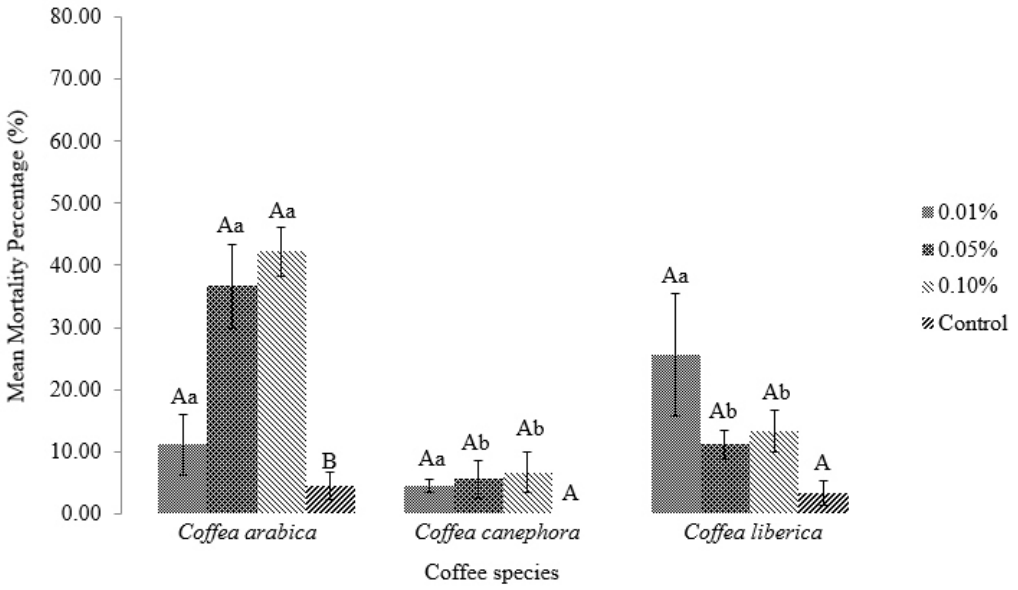

Figure 3. Mean mortality percentage of three different coffees against *P. megacephala* in Set I bioassay

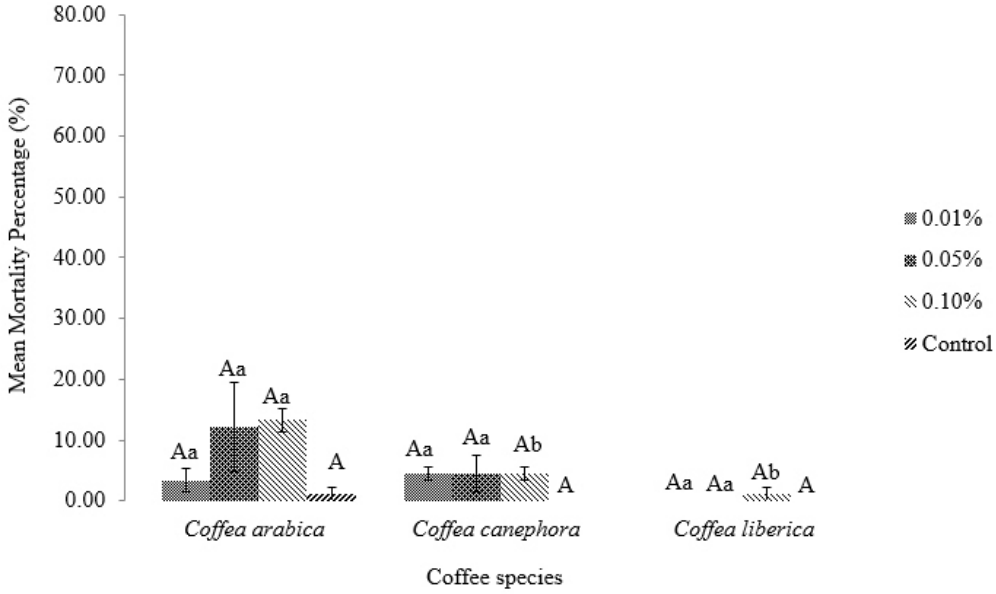

Figure 4. Mean mortality percentage of three different coffees against *P. megacephala* in Set II bioassay

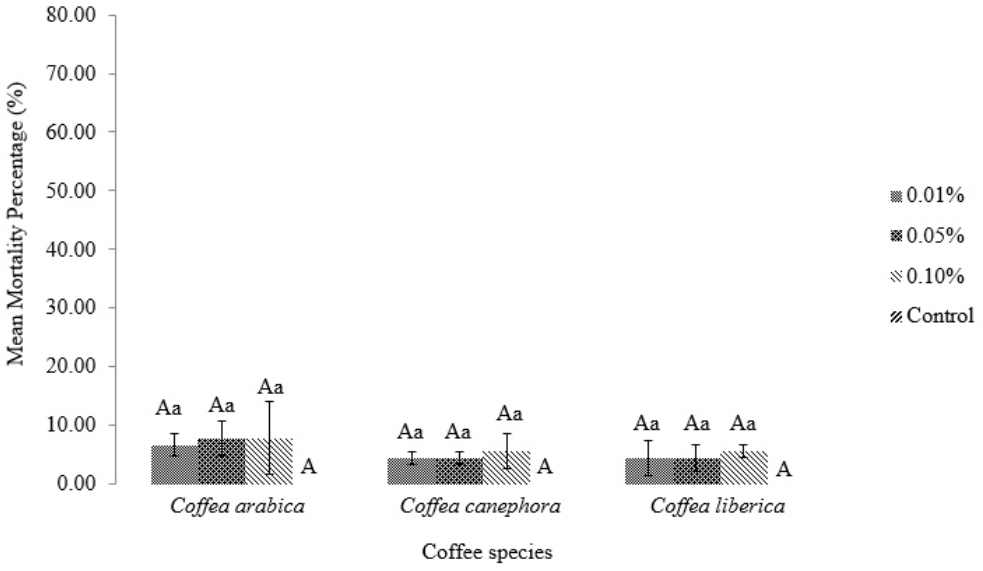

Figure 5. Mean mortality percentage of three different coffees against *M. pharaonis* in Set I bioassay

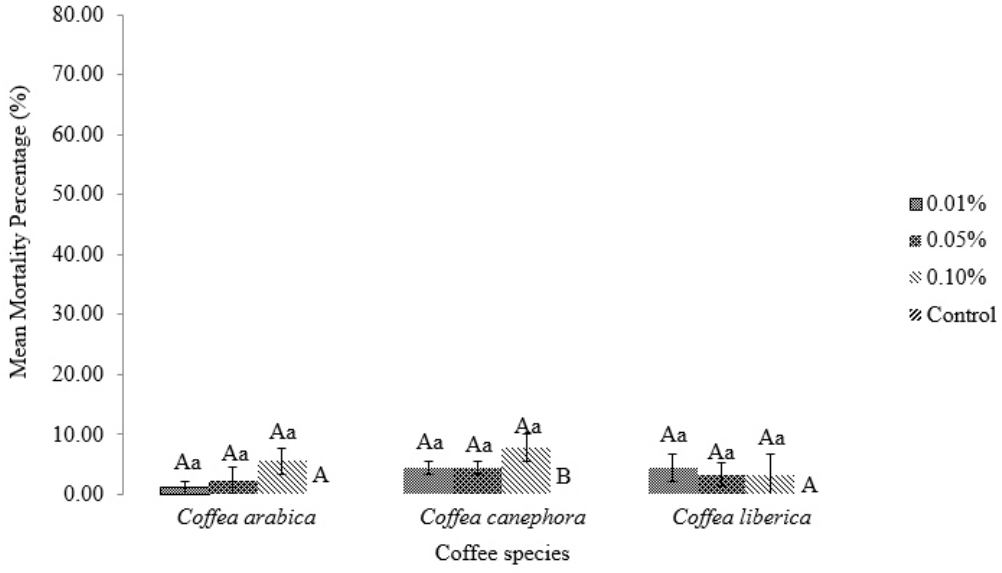

Figure 6. Mean mortality percentage of three different coffees against *M. pharaonis* in Set II bioassay

Effect of Coffee Extracts Impregnated in Gel Bait Towards the Feeding Behaviour of *T. indicum, P. megacephala and M. pharaonis*

In Set I bioassay, *P. megacephala* showed no significant difference (P>0.05) of their repellency behaviour at 30 min (P=0.416), 1 h (P=0.164), 2 h (P=0.382), 24 h (P=0.587), 48 h (P=0.056) and 72 h (P=0.229) between all concentrations of *C. arabica* and the control. At 4 h, the P values 0.038 displayed significant difference among the concentrations and control, at which 0.05% differed to 0.01% (P=0.043), 0.10% (P=0.043) and the control (P=0.046). At 8 h, the P value 0.028 (P<0.05) was a significant result; with 0.01% and 0.05% differed from both 0.10% and control (Table 3). For *C. canephora*, *P. megacephala* showed insignificant results for all time intervals except at 48 h (P=0.030, P<0.05); while there were no significant differences in *C. liberica* at all time intervals between the concentrations and control (Table 3).

In Set II bioassay, *C. arabica* indicated no significant differences of *P. megacephala* feeding behaviour at all time intervals with the exception of 48 h (P=0.023, P<0.05) and 72 h (P=0.025, P<0.05) between concentrations and control. At 48 h, 0.01% (P=0.05), 0.05% (P=0.05) and 0.10% (P=0.046) differed from the control. At 72 h, 0.05% and 0.10% had significant differences with 0.01% and the control (Table 4). *C. canephora* showed significant results at 30 min (P=0.043), 8 h (P=0.025), 24 h (P=0.013), 48 h (P=0.017) and 72 h (P=0.016) between concentrations and control. At 30 min, 8 h and 24 h, the results were significantly differed from the control but not among the three concentrations; while at 48 h and 72 h, the results displayed significant differences among concentrations and also with the control (Table 3). For *C. liberica*, only 48 h (P=0.024) and 72 h (P=0.024) showed significant results, at which they differed among the concentrations and with the control (Table 4).

For *M. pharaonis*, there were no significant results (P>0.05) of mean repellency percentage at all observed time intervals between the concentrations and control of all *Coffea* spp. in Set II bioassay (Table 6). On the other hand, in Set I bioassay, *C. liberica* displayed significant differences at 8 h (P=0.012), 24 h (P=0.013), 48 h (P=0.032) and 72 h (P=0.012); but there were no differences among the concentrations (Table 6).

In Set I bioassay, the mortality of *T. indicum* and *P. megacephala* increased with the increasing concentration (0.01%, 0.05% and 0.10%) of *C. arabica* and *C. canephora* but this trend was not shown in *C. liberica*. Instead, the results showed the lowest mortality at 0.05% of concentration for both the ant species, but still there were no significant differences (*T. indicum* P=0.054; *P. megacephala* P=0.114, P>0.05) among the three concentrations in *C. liberica* (Figures 1 and 5). At 0.05% of *C. liberica*, the mean repellency percentage for *P. megacephala* at 1 h, 2 h, 4 h, 8 h and 24 h are relatively higher as compared to that of 0.01% and 0.10%. These higher percentages indicated *P. megacephala* repelled more

Table 3

Mean mortality percentage and mean repellency percentage of three different coffees against P. megacephala *in Set I bioassay*

Coffee species	Concentration (%)	Mean Mortality Percentage (%)[1]	Mean Repellency Percentage (%)[2]							
			30min	1h	2h	4h	8h	24h	48h	72h
Coffea arabica	0.01%	11.11 ± 4.84Aa	84.45 ± 9.69C	88.89 ± 8.01C	84.44 ± 8.89C	-8.89 ± 4.44C	97.78 ± 2.22C	91.11 ± 5.88C	80.00 ± 7.70C	71.11 ± 8.01C
	0.05%	36.67 ± 6.67Aa	91.11 ± 2.22C	93.33 ± 3.85C	82.22 ± 8.01C	95.56 ± 4.44D	100.00 ± 0.00C	77.8 ± 9.69C	80.00 ± 3.85C	40.00 ± 34.21C
	0.10%	42.22 ± 4.01Aa	84.44 ± 15.56C	86.67 ± 6.67C	55.56 ± 14.57C	15.56 ± 11.11C	-53.33 ± 23.09D	68.89 ± 16.02C	42.22 ± 12.37C	77.78 ± 8.89C
	Control	4.45 ± 2.22B	48.89 ± 22.55C	48.89 ± 15.56C	53.33 ± 25.24C	-26.67 ± 17.64C	-60.00 ± 13.88D	20.00 ± 7.70C	31.11 ± 29.15C	24.22 ± 18.19C
Coffea canephora	0.01%	4.44 ± 1.11Aa	100.00 ± 0.00C	97.78 ± 2.22C	95.56 ± 4.44C	88.89 ± 8.01C	97.78 ± 2.22C	93.33 ± 6.67C	91.11 ± 8.89C	88.89 ± 4.44C
	0.05%	5.56 ± 2.94Ab	88.89 ± 8.01C	82.22 ± 11.11C	100.00 ± 0.00C	97.78 ± 2.22C	100.00 ± 0.00C	100.00 ± 0.00C	-11.11 ± 5.88D	75.56 ± 8.01C
	0.10%	6.66 ± 3.33Ab	100.00 ± 0.00C	93.33 ± 3.85C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	93.33 ± 3.85C	82.22 ± 9.69C
	Control	0.00 ± 0.00A	88.89 ± 5.88C	82.22 ± 9.69C	93.33 ± 6.67C	82.22 ± 2.22C	100.00 ± 0.00C	82.22 ± 11.11C	-26.66 ± 6.67D	42.22 ± 15.56C

Table 3 *(Continue)*

Coffea liberica	**0.01%**	25.56 ± 9.87Aa	97.78 ± 2.22C	97.78 ± 2.22C	100.00 ± 0.00C	93.33 ± 6.67C	100.00 ± 0.00C	93.33 ± 3.85C	33.33 ± 26.94C	35.55 ± 18.99C
	0.05%	11.11 ± 2.22Ab	97.78 ± 2.22C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	77.78 ± 8.89C	95.55 ± 2.22D
	0.10%	13.33 ± 3.33Ab	95.55 ± 2.22C	88.89 ± 11.11C	95.56 ± 4.44C	93.33 ± 3.85C	93.33 ± 3.85C	86.67 ± 10.18C	97.78 ± 2.22D	97.78 ± 2.22D
	Control	3.33 ± 1.93A	82.22 ± 11.11C	86.67 ± 10.18C	93.33 ± 3.85C	93.33 ± 0.00C	82.22 ± 5.88D	55.56 ± 19.75C	24.45 ± 13.52C	26.67 ± 30.06C

[1] Mean mortality percentage followed by different letters within the same column are significant different by subjecting to Kruskal-Wallis H Test at $p<0.05$: A & B (comparison of concentration within the same coffee species); a & b (comparison of coffee species within a concentration).
[2] Mean repellency percentage followed by different letters within the same column are significant different by subjecting to Kruskal-Wallis H Test at $p<0.05$: C, D & E (comparison of concentration within the same coffee species).

Table 4

Mean mortality percentage and mean repellency percentage of three different coffees against P. megacephala *in Set II bioassay*

Coffee species	Concentration (%)	Mean Mortality Percentage (%)[1]	Mean Repellency Percentage (%)[2]							
			30min	1h	2h	4h	8h	24h	48h	72h
Coffea arabica	0.01%	3.33 ± 1.93Aa	80.07 ± 13.81C	71.11 ± 14.57C	53.33 ± 3.85C	-31.11 ± 9.69C	-35.56 ± 27.84C	53.33 ± 11.55C	42.22 ± 25.04C	-42.22 ± 31.35D
	0.05%	12.22 ± 7.29Aa	77.78 ± 5.88C	80.00 ± 11.55C	62.22 ± 9.69C	-24.44 ± 15.55C	-46.66 ± 13.33C	86.67 ± 7.70C	77.78 ± 12.37C	42.22 ± 8.89C
	0.10%	13.33 ± 1.93Aa	84.45 ± 9.69C	68.89 ± 17.78C	57.78 ± 14.57C	73.33 ± 10.18C	42.22 ± 29.14C	68.89 ± 31.11C	95.55 ± 2.22C	80.00 ± 3.85C
	Control	1.11 ± 1.11A	57.78 ± 32.28C	60.00 ± 23.09C	48.89 ± 13.52C	-53.33 ± 37.12C	-62.22 ± 31.35C	46.67 ± 7.70C	-57.78 ± 21.20D	-57.78 ± 9.69D

Table 4 *(Continue)*

Coffea canephora	0.01%	4.44 ± 1.11Aa	91.11 ± 8.89C	60.00 ± 19.24C	91.11 ± 8.89C	97.78 ± 2.22C	100.00 ± 0.00C	100.00 ± 0.00C	97.78 ± 2.22C	62.22 ± 8.01C
	0.05%	4.44 ± 2.94Aa	64.45 ± 9.69C	73.33 ± 17.64C	82.22 ± 8.89C	82.22 ± 11.76C	100.00 ± 0.00C	100.00 ± 0.00C	77.78 ± 5.88D	33.33 ± 7.70C
	0.10%	4.44 ± 1.11Ab	100.00 ± 0.00C	95.56 ± 4.44C	93.33 ± 6.67C	100.00 ± 0.00C	97.78 ± 2.22C	100.00 ± 0.00C	100.00 ± 0.00C	97.78 ± 2.22D
	Control	0.00 ± 0.00A	48.89 ± 13.52D	37.78 ± 19.37C	68.89 ± 19.37C	55.56 ± 37.97C	71.11 ± 5.88D	57.78 ± 13.52D	-33.33 ± 16.78E	-51.11 ± 8.89E
Coffea liberica	0.01%	0.00 ± 0.00Aa	66.67 ± 11.55C	73.33 ± 10.19C	82.22 ± 8.01C	68.89 ± 27/84C	91.11 ± 5.88C	51.11 ± 19.75C	-53.33 ± 20.00C	-77.78 ± 5.88C
	0.05%	0.00 ± 0.00Aa	82.22 ± 8.01C	60.00 ± 30.55C	80.00 ± 10.18C	73.33 ± 23.41C	97.78 ± 2.22C	80.00 ± 20.00C	62.22 ± 5.88D	53.33 ± 3.85D
	0.10%	1.11 ± 1.11Ab	95.55 ± 2.22C	95.55 ± 2.22C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	97.78 ± 2.22E	88.89 ± 11.11E
	Control	0.00 ± 0.00A	57.78 ± 35.55C	71.11 ± 22.22C	71.11 ± 25.63C	51.11 ± 36.38C	84.45 ± 9.69C	26.67 ± 10.19C	-55.55 ± 13.52C	-80.00 ± 10.18C

[1] Mean mortality percentage followed by different letters within the same column are significant different by subjecting to Kruskal-Wallis H Test at $p<0.05$: A & B (comparison of concentration within the same coffee species); a & b (comparison of coffee species within a concentration).

[2] Mean repellency percentage followed by different letters within the same column are significant different by subjecting to Kruskal-Wallis H Test at $p<0.05$: C, D & E (comparison of concentration within the same coffee species).

to the gel and resulted in lowest mortality. The overall results showed that the highest concentration, 0.10% of all three coffee species had the highest mortality against *T. indicum* (Figure 1). However, 0.01% of *C. liberica* showed the highest mortality against *P. megacephala* in the same set of gel bait as *P. megacephala* displayed the lowest mean repellency percentage at 48 h and 72 h (Table 3). For *M. pharaonis*, the mortality also showed a merely increasing trend from 0.01% to 0.10% but the differences were not much noticeable. For instances, 0.05% and 0.10% of *C. arabica* had the same mortality values; 0.01% and 0.05% of both *C. canephora* and *C. liberica* shared the similar mean mortality (Figure 5). This is because the mean repellency percentages for the three concentrations at all hours range from 95.55% to 100.00% (Table 5), indicating a very high repellency behaviour of *M. pharaonis*.

In Set II bioassay, there were increasing trends of *C. arabica* against *T. indicum*, *P. megacephala* and *M. pharaonis* with increasing concentrations (Figures 2 and 6). *C. canephora* and *C. liberica* showed that the lowest mortality on *T. indicum* was at 0.05% (Figure 2). On the other hand, *C. canephora* had the same mean mortality on *P. megacephala* at all concentrations (Figure 4) as the mean repellency percentage had no significant results from 30 min to 24 h (Table 4). The concentration of 0.01% *C. liberica* had a slightly higher mortality on *M. pharaonis* as the mean repellency percentages at 30 min, 1 h, 24 h and 72 h were lower than those of 0.05% and 0.10% (Figure 6 and Table 6). The lower repellency percentage indicated the more ants attracted to the gel and thus fed on the gel.

DISCUSSION

Residual spraying and baiting are common methods in controlling the pest ants. Baiting is considered a more effective measure as it is able to eliminate the entire colony through trophallaxis among the ants (Lee, 2000; Suiter et al., 1997). The uses of commercial and synthetic products have known to create certain issues such as environmental problem. Recent studies have revealed that plants act as potential insecticides, such as essential oil of *Pogostemon cablin* possess the insecticidal and repellence properties against the urban ants (Albuquerque et al., 2013). Plant secondary metabolites such as caffeine (1, 3, 7-trimethylxanthine) have pesticidal activity, anti-feeding properties and potential to be natural pesticide (Magalhães, Fernandes, Demuner, Picanco, & Guedes, 2010).

The overall results indicated *T. indicum* was the most susceptible species as it had the highest mortality among the three ant species. According to Lee, Lim and Yap (1996), the erratic movement of crazy ant allowed it to pick up more insecticidal materials and thus causing a higher mortality. This nature behaviour could also be observed in *T. indicum* at which they move rapidly and erratically, in turn leading to increased foraging activity and higher chance of picking up the toxicant, resulting in higher mortality (Lee et al., 1996). In addition, the very frequent

Table 5
Mean mortality percentage and mean repellency percentage of three different coffees against M. pharaonis *in Set I bioassay*

Coffee species	Concentration (%)	Mean Mortality Percentage (%)[1]	Mean Repellency Percentage (%)[2]							
			30min	1h	2h	4h	8h	24h	48h	72h
Coffea arabica	0.01%	6.67 ± 1.93Aa	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C
	0.05%	7.78 ± 2.94Aa	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	97.78 ± 2.22C
	0.10%	7.78 ± 6.19Aa	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C
	Control	0.00 ± 0.00A	97.78 ± 2.22C	95.56 ± 4.44C	95.55 ± 2.22C	93.33 ± 6.67C	97.78 ± 2.22C	91.11 ± 4.44C	86.67 ± 6.67C	91.11 ± 5.88C
Coffea canephora	0.01%	4.44 ± 1.11Aa	97.78 ± 2.22C	97.78 ± 2.22C	97.78 ± 2.22C	100.00 ± 0.00C	100.00 ± 0.00C	95.55 ± 2.22C	95.56 ± 4.44C	100.00 ± 0.00C
	0.05%	4.44 ± 1.11Aa	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	95.56 ± 4.44C	100.00 ± 0.00C	100.00 ± 0.00C
	0.10%	5.56 ± 5.09Aa	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C
	Control	0.00 ± 0.00A	93.33 ± 6.67C	95.55 ± 2.22C	95.56 ± 4.44C	97.78 ± 2.22C	97.78 ± 2.22C	93.33 ± 3.85C	88.89 ± 5.88C	88.89 ± 5.88C

Table 5 *(Continue)*

Coffea liberica	0.01%	4.44 ± 2.94Aa	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	97.78 ± 2.22C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C
	0.05%	4.45 ± 2.22Aa	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C
	0.10%	5.56 ± 1.11Aa	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	97.78 ± 2.22C	100.00 ± 0.00C
	Control	0.00 ± 0.00A	100.00 ± 0.00C	97.78 ± 2.22C	93.33 ± 6.67C	93.33 ± 6.67C	93.33 ± 0.00D	86.67 ± 3.85D	86.67 ± 3.85D	93.33 ± 0.00D

[1] Mean mortality percentage followed by different letters within the same column are significant different by subjecting to Kruskal-Wallis H Test at $p<0.05$: A & B (comparison of concentration within the same coffee species); a & b (comparison of coffee species within a concentration).
[2] Mean repellency percentage followed by different letters within the same column are significant different by subjecting to Kruskal-Wallis H Test at $p<0.05$: C, D & E (comparison of concentration within the same coffee species).

Table 6

Mean mortality percentage and mean repellency percentage of three different coffees against M. pharaonis *in Set II bioassay*

Coffee species	Concentration (%)	Mean Mortality Percentage (%)[1]	Mean Repellency Percentage (%)[2]							
			30min	1h	2h	4h	8h	24h	48h	72h
Coffea arabica	0.01%	1.11 ± 1.11Aa	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C
	0.05%	2.22 ± 2.22Aa	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C
	0.10%	5.55 ± 2.22Aa	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	91.11 ± 8.89C
	Control	0.00 ± 0.00A	93.33 ± 6.67C	97.78 ± 2.22C	100.00 ± 0.00C	93.33 ± 6.67C	86.67 ± 13.33C	97.78 ± 2.22C	95.56 ± 4.44C	91.11 ± 8.89C

Table 6 *(Continue)*

Coffea canephora	0.01%	4.44 ± 1.93Aa	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	97.78 ± 2.22C	100.00 ± 0.00C
	0.05%	4.44 ± 1.93Aa	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	97.78 ± 2.22C	93.33 ± 3.85C	97.78 ± 2.22C	100.00 ± 0.00C
	0.10%	7.78 ± 2.22Aa	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	95.55 ± 2.22C	100.00 ± 0.00C	100.00 ± 0.00C
	Control	0.00 ± 0.00B	100.00 ± 0.00C	95.56 ± 4.44C	100.00 ± 0.00C	97.78 ± 2.22C	95.56 ± 4.44C	88.89 ± 2.22C	82.22 ± 11.76C	84.45 ± 9.69C
Coffea liberica	0.01%	4.45 ± 2.22Aa	97.78 ± 2.22C	95.56 ± 4.44C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	97.78 ± 2.22C	100.00 ± 0.00C	95.55 ± 2.22C
	0.05%	3.33 ± 1.93Aa	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	97.78 ± 2.22C	97.78 ± 2.22C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C
	0.10%	3.33 ± 3.33Aa	100.00 ± 0.00C	97.78 ± 2.22C	97.78 ± 2.22C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C	100.00 ± 0.00C
	Control	0.00 ± 0.00A	100.00 ± 0.00C	97.78 ± 2.22C	93.33 ± 6.67C	100.00 ± 0.00C	95.56 ± 4.44C	95.56 ± 4.44C	93.33 ± 6.67C	95.55 ± 2.22C

[1] Mean mortality percentage followed by different letters within the same column are significant different by subjecting to Kruskal-Wallis H Test at $p<0.05$: A & B (comparison of concentration within the same coffee species); a & b (comparison of coffee species within a concentration).

[2] Mean repellency percentage followed by different letters within the same column are significant different by subjecting to Kruskal-Wallis H Test at $p<0.05$: C, D & E (comparison of concentration within the same coffee species.

self-grooming of *T. indicum* also contributed to its mortality. Ants perform self-grooming when they detect microbes or materials that endanger themselves (Hughes, Eilenberg, & Boomsma, 2002). They also tend to perform self-grooming for a longer time when encounter with more harmful microbes or materials (Morelos-Juárez, Walker, Lopes, & Hughes, 2010). During self-grooming, the ants may ingest the insecticidal-containing materials. Ants do perform allogrooming, a process of grooming towards other individuals by using their shovel-like mouthparts to remove potential harmful matters from the body surface (Wilson & Hölldobler, 2005). From the observation, *T. indicum* displayed a high frequency of allogrooming among them. This behaviour may cause them to accidentally ingest the toxicant on the body surfaces of other individuals, resulting in the highest mean mortality among the three ant species. On the contrary to *T. indicum*, *M. pharaonis* performed self-grooming and allogrooming less frequently, hence, they experienced the lowest mean mortality.

Trophallaxis is a process of exchanging regurgitated food among the colony members and it is very common to be observed among ants. It also allows the donors and recipients to gain information upon interaction. During trophallaxis, the ants share the food and even the insecticide-impregnated bait among themselves (Lee, 2000). The high frequency of trophallaxis displayed by *T. indicum* might lead to a higher chance for the other members to ingest the toxicant and result in a higher mortality value. Moreover, high performance of antennation among *T. indicum* might also contribute to its highest mortality. Based on the study by Hölldobler (1985) on ponerine ants, antennation functions in social greeting, recruitment and food solicitation. The touching the antennae with the others are known as tactile communication. Both trophallaxis and antennation play important role in food distribution and transmission (Hölldobler, 1985).

The results showed that the mortality increased with the increasing concentration (0.01%, 0.05% and 0.10%) for most but not all bioassays; hence, they were not concentration-dependent. This was in contrast with the previous study of effect of caffeine on tobacco hornworm larvae. The study indicated a dose-dependent effect at which higher concentration of caffeine lead to higher feeding and development inhibition of the larvae (Nathanson, 1984). In the present study, for instance, 0.01% of *C. liberica* showed the highest mortality against *P. megacephala* in Set I bioassay. This might be due to its lowest concentration and lowest mean repellency percentage at 48 h and 72 h indicating the ants were attracting the most to the bait and fed on it, thus causing the highest mortality. Other similar observations had also implied the higher repellency percentages result in lowest mortality of *P. megacephala* in Set I bioassay and *T. indicum* in Set II bioassay at 0.05% of *C. liberica*. Hence, it can be concluded that the repellency percentage is associated with the ant mortality. In addition, Set II *C. canephora* bioassay of the big-headed ants showed almost same or the same mean mortality at all concentrations (Figure 4) although there were fluctuations

of the mean repellency percentage at 48 h and 72 h. This occurrence can be explained by the delay action of the toxicant in the gel bait, which is one of the important characteristics of the gel (Knight & Rust, 1991).

The big-headed ants, *P. megacephala* are known as one of the worst and highly invasive ant species. They have a better ability to discover and exploit food resources than other native ant species (Callan & Majer, 2009). This observation was similar to the results in this present study; at which *P. megacephala* showed higher attraction behaviour towards the bait as they are able to locate the food attractant better. Though they were attracted mostly to the bait when compared to the other two ant species, their ant mortality is not the highest. According to Cokendolpher and Francke, (1985), the ant body size affects their desiccation rate. Ants with smaller body size possess larger surface area to volume ratios tend to desiccate faster (Cokendolpher & Francke, 1985). Both minor and major workers of *P. megacephala* that are larger in size experience a lower desiccation rate and hence lower mortality. However, the smaller size of Pharaoh ant did not show a higher mortality value even though their sizes are much smaller than the big-headed ants. The high repellency behaviour observed in *M. pharaonis* caused the lowest mean mortality. This might be due to the Pharaoh ants showing higher degree of repellency towards the water and sugar solution incorporated in the bait; as according to Fowler et al. (1993), *M. pharaonis* prefer high protein food. In addition, the slow and inactive movement of Pharaoh ant may also explain their lowest mortality.

Almost all Set I bioassays showed higher mortality as compared to Set II bioassay for all three ant species. *T. indicum* displayed feeding preference towards carbohydrate foods but there was no specific preference of carbohydrate foods (Chong & Lee, 2006). According to Lee (2000), most household ants were attracted most to 20% to 30% of sucrose solution. As a proven, *T. indicum* preferred Set I gel bait that consisted of 20% sugar solution and assumed to feed more on the bait, thus resulting in a higher mortality as compared to that of Set II gel bait. Albeit there are food preferences in different ant species, ants do display higher preference towards the sugar solution when compared to water as the carbohydrate providing energy to them (David & Venkatesha, 2013). This explained that most of the results showing higher mortality in Set I bioassay.

Among the three coffee species, *C. arabica* showed the highest ant mortality on *T. indicum*, *P. megacephala* and *M. pharaonis* at almost all concentrations in both Set I and Set II bioassays. According to Itoyama and Bicudo (1992), caffeine reduces the mating frequency, egg-laying capacity, fertility and longevity of *Drosophila prosaltans* (Diptera: Drosophilidae). Further research showed that caffeine suppressed the feeding activity of flies and beetles (Pedronel, Casanova, Ortiz, Henao, & Pelaez, 2007). However, the data obtained was in contrast with those previous studies. From our record on GC-MS analysis (unpublished data), *C.*

arabica contained the lowest composition percentage of caffeine (25.31%) when compared to *C. canephora* (44.70%) and *C. liberica* (47.30%). *C. arabica* occupied the lowest composition percentage of caffeine but it had the highest mortality against the ants. This has suggested caffeine may not be the main compound of *Coffea* spp. to cause ant mortality. From the study on bumblebees, nectar toxins such as caffeine, quinine, nicotine, amygdalin and grayanotoxin do not impede the pollination activity of bumblebees (Tiedeken, Stout, Stevenson, & Wright, 2014). Same to the research on honeybees, low concentrations of the caffeine tend to enhance their visitation frequency to the solution (Hagler & Buchmann, 1993). Moreover, bees show poor acuity and weak sensitivity of detecting plant toxins in sucrose solution (Tiedeken et al., 2014). As both ants and bees are eusocial insects and from the same order of Hymenoptera, it is possible to assume that ants possess the same nature with bees, indicating that caffeine is not the cause of causing mortality.

C. arabica contained undecane, hexadecanamide and tetradecanamide which could not be found in the other two coffee species. Undecane is a volatile hydrocarbon compound and also an alarm pheromone that can be found in the ants (Lenz, Krasnec, & Breed, 2013). Regnier and Wilson (1969) reported that a minute amount of undecane caused some ant species move rapidly. Undecane had also shown attraction and excitement in the workers of the crazy ants (Witte, Attygalle, & Meinwald, 2007). Another outcome had demonstrated this alarm pheromone allow recruitment of workers to the disturbance region. The high volatility of undecane improves the rate of spreading of this compound to the surrounding, increasing the activity of the ants (Lenz et al., 2013). Based on these evidences, undecane is the possible compound in coffee that attracts ants, increases their movement and activities. This can also be proven that the repellency percentages in most *C. arabica* bioassays are lower. However, the possibility of undecane to be the compound in coffee that causes ant mortality is yet to be known.

Many studies revealed the effectiveness of coffee in controlling insects. Caffeine was known to block the larval development of *Aedes aegypti* (Diptera: Culicidae) and cause lethal effect. The effect is dose-dependent as the higher the concentration of the caffeine, the faster the blockade of larval development (Laranja et al., 2003). Caffeine also impedes the oviposition activity and drags the appearance of developmental stages in the life cycle of the tea shot-hole borer beetle, *Euwallacea* (= *Xyleborus*) *fornicates* (Coleoptera: Scolytidae). Nevertheless, there was no observed lethal outcome on the beetle (Hewavitharanage et al., 1999). From a recent study on leaf-cutting ants *Atta sexdens rubropilosa* (Hymenoptera: Formicidae) by Miyashira, Tanigushi, Gugliotta and Santos (2012), caffeine had no significant effect on their survival but decreased the growth rate of the mutualistic fungus of leaf-cutting ants. The fungal growth rate decreased with the increasing

concentration of caffeine. The mutualistic fungus acts as the only food source for the immature stages of leaf-cutting ants at which they require glycogen-rich diet for development. The adults obtained nutrients from the decomposition process of plant tissue by the fungus while the fungus gained benefit from the competition free circumstances with other microorganisms. The symbiotic relationship between the leaf-cutting ants and fungus had demonstrated the ants might be associated with caffeine toxicity to the fungus but not affected directly by the caffeine (Miyashira et al., 2012). According to this study, again, it can be concluded caffeine has little or no direct lethal effect on the ants, therefore matching the data obtained in this research.

Nevertheless, the chemical composition of the coffee compounds may vary dependind on the roasting temperature and time. For instance, the coffee roasted at higher temperatures for a shorter period display higher acidity, more soluble solids and a different volatile profile while comparing with coffee that roasted at a lower temperature with longer period of time (Farah, 2012). Therefore, it is very crucial to have standardized and constant roasting temperature and time to obtain a reliable chemical composition percentage of the compounds while comparing the coffee species.

Basically, the higher concentration of caffeine had led to a higher repellency. Honey bees were less likely to consume the sucrose solution with high dose of caffeine (Mustard, Dews, Brugato, Dey, & Wright, 2012). Nonetheless, this phenomenon was not observed in this study. The concentrations, 0.01%, 0.05% and 0.10% used were based on the study of Miyashira et al. (2012). The repellency behaviours of *T. indicum* and *M. pharaonis* towards the three concentrations were similar. While *P. megacephala* showed some degree of significant results at only 8, 24, 48 and 72 h, at which higher repellency was observed at higher concentration. This is probably due to the highest concentration, 0.10% used in this research was considered low to deter the feeding of ants. It was shown that 0.30% to 10% of caffeine suppressed the feeding activity and growth of tobacco hornworm larvae, *Manduca sexta* (Lepidoptera: Sphingidae) (Nathanson, 1984). From the results obtained and according to these previous studies, coffee that consists of various volatile and non-volatile compounds has the potential to act as a repellent for ants. Minor compounds may act as potent synergists to increase the impact of major compounds. Therefore, future research can be performed to reinforce the caffeine repellency effect on ants by using a higher concentration.

CONCLUSION

In conclusion, all the ant species displayed slightly higher mortality in the bioassay with bait containing sugar attractant. Coffee with low concentration was not effective in killing the household ants because the mortalities obtained after three days did not exceed 50%. Therefore, future researches to test on the lethal effect of coffee can be studied by using higher concentration. Moreover, the potential of coffee to be formulated as ant repellent cannot be ruled out as the overall results showed a great

extent of repellency towards the baits. The natural behaviour of the ant had also contributed to their mortalities. *T. indicum* with higher frequency of trophallaxis, self-grooming, allogrooming and antennation had the highest mortality among the three ant species tested. A further study could be conducted to test the coffee effect on the ant colony instead of only on the ant workers. Furthermore, *C. arabica* had the best impact on the ant mortality. Hence, a focus study on the effect of this coffee species on ants should be carried out in the future.

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Evaluation of Different Arbuscular Mycorrhizal Fungi for Selecting the Best for Inoculating Soybean Cultivars MAUS 2 and MAUS 212

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ABSTRACT

A glasshouse experiment was conducted to screen and select the efficient AM fungi for inoculating two drought susceptible soybean cultivars MAUS 2 and MAUS 212. Screening was done using 10 different species of AM fungi. Plant parameters like plant height, stem diameter, biovolume index, total leaf area, dry biomass, P concentration, and mycorrhizal parameters like root colonization, spore number in the root zone soil were recorded according to the standard procedures. Based on the improvement in plant parameters like biovolume index, total leaf area, shoot and root dry biomass, plant P uptake, pod and seed yield, it was concluded that *Ambispora leptoticha* was the best AM fungus for inoculating both the cultivars MAUS 2 and MAUS 212.

Keywords: *Ambispora leptoticha,* AM fungi, plant growth response, soybean

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INTRODUCTION

Soybean [*Glycine max* (L.) Merr.] is the most important seed legume crop in the world, which contributes to 25 % of the global edible oil, and is the top oilseed crop in India (Agarwal, Billore, Sharma, Dupare, & Srivastava, 2013). In terms of production it has emerged as the most important oilseed crop of India. It stands unique in terms of chemical composition having tocopherol,

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isoflavones, and lecithin besides protein and oil. Soybean protein is called as complete protein due to its amino acids composition and the role of its nutrition value in heart disease and diabetes is well known. In India, soybean is mainly grown as rainfed crop. Its productivity under rainfed conditions is hovering around 1 t/ha despite the yield potential of up to 4 t/ha. The reason for virtually static productivity of soybean is largely due to erratic, uneven and inadequate rainfall and, other abiotic and biotic factors limiting the productivity of soybean.

Application of chemical fertilizers and pesticides to soil is increasing every year to attain maximum yield in crops. In India the use of chemical fertilizers has reached hundred times during the last 5 decades (Food and Agriculture Organization [FAO], 2010). Microbial diversity present in the soil plays a major role in plant growth and conserving the environment. It is well documented that the addition of chemical fertilizers to soil is detrimental to the microbial growth and also deteriorates the soil health and quality. It is therefore essential to reduce the addition of chemical fertilizers by introducing beneficial microbes like mycorrhizal fungi, N fixers, P solubilizers, plant growth promoting rhizomicroorganisms (PGPR) and biocontrol organisms to the soil in order to sustain plant productivity and to maintain soil health (Bollen, 1959). These beneficial microorganisms are applied to crops in order to sustain plant productivity and to maintain soil health.

The role of arbuscular mycorrhizal (AM) fungi on the growth and phosphate nutrition of various plants has been studied extensively (Bagyaraj, Sharma, & Maiti, 2015). All AM fungi are obligate biotrophs and they benefit plants by increasing uptake of diffusion limited nutrients like P, Zn, and Cu, protection from pathogens, tolerance to drought, pathogen protection, beneficial alterations of plant growth regulators and synergistic interactions with beneficial soil microorganisms (Bagyaraj, 2014; Kumar, Ashwin, & Bagyaraj, 2018). Mycorrhizal plants develop extensive root system as compared to non-mycorrhizal plants, which ensures the plant with increased availability of water and nutrient, thereby helping better plant growth and development (Bagyaraj, 2014; Mathimaran, Sharma, Mohan Raju, & Bagyaraj, 2017). Host preference in AM fungi has been reported by earlier workers which enable one to screen and select the best AM fungi for inoculating a particular crop (Chauhan, Bagyaraj, Thilagar, & Ravi, 2012; Srinivasan, Ashwin, & Bagyaraj, 2012).

The two cultivars MAUS 2 and MAUS 212 (drought susceptible) used in the present study were selected based on an earlier field experiment conducted using 25 soybean cultivars obtained from ICAR-Directorate of Soybean Research, Indore and All India Co-ordinated Research Project on Soybean, University of Agricultural Sciences, Bangalore, to investigate their drought adaptive traits. The present investigation was conducted to screen different AM fungi and select the best AM fungi for inoculating

two different drought susceptible cultivars of soybean which yielded more under irrigation compared to other cultivars. The results of the present study will reveal the best AM fungi for inoculating soybean, which will be used to understand the role of the selected AM fungi in enhancing drought tolerance in the two drought susceptible cultivars MAUS 2 and MAUS 212, later.

MATERIAL AND METHODS

The experiment was conducted in a polyhouse at Centre for Natural Biological Resources and Community Development (CNBRCD), Bangalore. The AM fungi cultures used in this experiment were *Funneliformis caledonium, Acaulospora laevis, Rhizophagus fasciculatus, Claroideoglomus etunicatum, Gigaspora margarita, Glomus macrocarpum, G. bagyarajii, F. mosseae, R. intraradices* and *Ambispora leptoticha*. AM fungi used in the study were isolated from various crops by the corresponding author, and some species were procured from various research centres working on AM fungi as detailed in Sreeramulu (1996). All the fungi were maintained in the culture collection of CNBRCD, Bangalore and were selected based on the positive results of earlier studies on other crop plants (Chauhan et al*.,* 2012; Srinivasan et al., 2012, Thilagar & Bagyaraj, 2015). Since AM fungi are obligatory symbionts they were multiplied using traditional "Pot Culture" technique as soil inoculum in pots with Rhodes grass (*Chloris gayana*) as the host using soilrite, perlite and vermiculite in the ratio 1:1:1 (v/v/v basis) under polyhouse condition. After 75 days of growth, shoots of Rhodes grass were cut and the substrate containing spores, hyphae and root bits (cut into about 1 cm pieces) were air dried and used as the inoculum. All the ten AM fungi inocula had infective propagule numbers in the range 1400-1600/ g of substrate (Thilagar, 2015).

Polybags of size of 24 cm x 12 cm with 2.5 kg substrate holding capacity were filled with the sand: soil: compost substrate mixture in 1:1:0.25 (v/v/v). The soil used in this study was collected from an uncultivated field from a depth of 0-15 cm which has been classified as fine, kaolinitic isohypothermic kanhaplustalfs. The substrate had a pH of 6.2 (1:10 soil to water extract ratio), available phosphorus of 5.9 ppm (NH4F + HCl extractable) (Jackson, 1973) and an indigenous AM fungal population of 20 spores/50 g of soil (Jackson, 1973). A planting hole was made in the middle of the polybag up to a depth of 5cm. The polybags were inoculated with 10g of respective AM fungal cultures according to the treatments and were replicated 6 times. Uninoculated control received 10g of soilrite, perlite and vermiculite 1:1:1 (v/v/v basis) with no AM fungi. Two seeds of each cultivar were sown separately per bag in the planting hole and later thinned to leave single plant/ polybag. The polybags were watered whenever necessary.

Sl. No.	Treatments
1	Uninoculated Control
2	Inoculated with *Funneliformis caledonium*
3	Inoculated with *Acaulospora laevis*
4	Inoculated with *Rhizophagus fasciculatus*
5	Inoculated with *Claroideoglomus etunicatum*
6	Inoculated with *Gigaspora margarita*
7	Inoculated with *Glomus macrocarpum*
8	Inoculated with *G. bagyarajii*
9	Inoculated with *F. mosseae*
10	Inoculated with *R. intraradices*
11	Inoculated with *Ambispora leptoticha*

The plants were harvested 90 days after sowing (DAS). At harvest, plant height was recorded from soil surface to the growing tip of the plant using measuring tape and stem diameter was measured 1 cm above the soil surface using digital Vernier Calipers. Biovolume index (BI) (depicts the total volume of a plant) based on its height and stem girth was calculated by the formula given by Hatchell, Berry and Musse (1985). Leaf area per plant was calculated by recording the leaf area in WinDIAS 3 Image Analysis System. Pod and seed weight per plant was calculated by weighing the harvested mature pods from the plant and the separated seeds in a standard weight balance machine.

The plants were harvested 90 days after sowing (DAS). Dry biomass of the shoot and root was determined after drying the plant at 60^0C to a constant weight in a hot air oven. Plant P concentration was estimated colorimetrically following the vanadomolybdate phosphoric acid yellow colour method [9]. AM fungal spore numbers in the root zone soil was estimated by collecting soil samples (50g) from each bag of a treatment and subjecting it to wet sieving and decantation method as outlined by Gerdemann and Nicolson (Gerdemann & Nicolson, 1963). Root bits were stained using trypan blue as outlined by Philips and Hayman (1970) and the per cent mycorrhizal root colonization was estimated by adopting gridline intersect method (Giovannetti & Mosse, 1980). The fungi were ranked for each character and compared pairwise using Duncan's multiple range test at 5% significance level (Gomez & Gomez, 1984).

RESULTS AND DISCUSSION

Host preference among AM fungi has been reported by earlier workers (Soram, Dutta, & Jha, 2012; Srinivasan et al., 2012; Ulfath Jaiba, Balakrishna, Bagyaraj, & Arpana, 2006), hence selecting efficient symbiotic AM fungi that can be used for inoculating different mycotrophic plants has been stressed (Bagyaraj & Kehri, 2012). In the present study, soybean plants showed varied plant growth responses to different AM fungi. In general, AM fungal inoculation resulted in a significant increase in plant height, stem diameter, plant biomass, total leaf area, phosphorus concentration and yield in both the cultivars of soybean (Tables 1 and 3).

Plant height and stem diameter was significantly more in *G. macrocarpum* inoculated plants in MAUS 2 cultivar, and with *G. bagyarajii* inoculation in MAUS 212 cultivar. This was also true for biovolume index (BI) (Tables 1 and 3). The uninoculated control plants had the least BI (Tables 1 and 3). Studies by Meghvansi, Prasad, Harwani and Mahna (2008) on other soybean sp. with three different AM fungi showed significant improvement over plant growth parameters. Improved plant height, stem diameter and plant biomass because of AM fungal inoculation has been reported in other crops like French bean (Chauhan et al., 2012), chilly (Thilagar & Bagyaraj, 2015) and tomato (Pushpa & Lakshman, 2014).

Total leaf area (TLA) was significantly more in *A. leptoticha* inoculated plants in both cultivars MAUS 2 (Table 1) and MAUS 212 (Table 3). TLA is an important parameter which depicts the photosynthetic activity of the plant which in turn shows the yield capability. Hence in this study the TLA results show that inoculation with most of the AM fungi increases the TLA of the plant and thus the photosynthetic activity which in turn will increase the yield (Mondal, Datta, & Mondal, 2017).

In MAUS 2 in general all the 10 AM fungi increased shoot dry biomass but were statistically on par with control treatment whereas in MAUS 212 cultivar inoculation with *A. leptoticha* showed higher shoot dry biomass compared to all other treatments including uninoculated control. In MAUS 2 cultivar, *A. leptoticha* inoculated plants showed significantly higher root dry biomass compared to other inoculated plants but was on par with *F. mosseae* and *C. etunicatum* inoculated plants (Table 1). *A. leptoticha* inoculation to MAUS 212 cultivar also increased the root dry biomass to the maximum extent but was statistically on par with all other AM fungal inoculated plants except those inoculated with *Gi. margarita* (Table 3). Control plants showed least root dry biomass in both cultivars. Total plant dry biomass was also significantly more in *A. leptoticha* (46.48%) which was on par with *F. mosseae* (28.82%), *G. macrocarpum* (27.44%) and *R. intraradices* (21.10%) inoculated plants compared to control treatment in MAUS 2 cultivar (Table 1). In MAUS 212 cultivar *A. leptoticha* inoculation increased total plant dry biomass significantly by 44.64% compared to uninoculated plants, and was statistically on par with the treatments *F. caledonium* (25.60%) and *R. intraradices* (24.97%). Uninoculated control plants had significantly least total plant dry biomass in both the cultivars (Table 3). Similar observation was reported by Gupta and Janarthanan (1991) where inoculation with *G. aggregatum* in Palmarosa enhanced plant dry biomass. This was further confirmed by reports of Gogoi and Singh (2011) which showed inoculation with *A. delicate* increased plant dry biomass of *Piper longum*.

A. leptoticha inoculation to MAUS 2 and MAUS 212 cultivar resulted in highest pod weight and seed weight compared to uninoculated plants which had the least yield (Tables 2 and 4). Inoculation with *A.*

leptoticha to MAUS 2 cultivar increased pod and seed weight by 78.12% and 40.17% respectively. Similarly in MAUS 212 cultivar *A. leptoticha* inoculation increased pod and seed weight by 42.54% and 23.79% respectively. Increased crop yield due to AM fungal inoculation has been reported by earlier workers in several plants like chilly (Thilagar & Bagyaraj, 2015), tomato (Al-Karaki, 2006) and cucumber (Ortas, 2010). This is because of improved nutrient supply by AM fungi to plants, especially in P deficient soils (Berruti, Lumini, Balestrini, & Bianciotto, 2016).

The phosphorus concentration of the plants also increased significantly due to inoculation with all the AM fungi studied compared to uninoculated plants in both the cultivars. Shoot, root and total plant P concentration (excluding pod & seeds) was significantly more in *A. leptoticha* treatment compared to all other AM fungal treatments and the control in both the cultivars. It is well known that AM fungi improve plant growth mainly through enhanced nutrition of diffusion limited nutrients like P. Variation in the plant P status in relation to fungal species is well documented (Rajan, Bagyaraj, & Arpana, 2005; Soram et al., 2012). In the present study plants raised in the presence of *A. leptoticha* showed an increase of 91.29% and 92.00% in total plant phosphorus concentration in MAUS 2 and MAUS 212 cultivars respectively (Table 2 and 4) compared to plants without inoculation. Such an enhanced plant P concentration because of AM fungal inoculation has been reported in other crops (Wang, Pan, Chen, Yan, & Liao, 2011). The high-affinity phosphate transporter (PT) in AM fungal and the nutritional aspects of AM fungal symbiosis have been studied extensively from both physiological and molecular perspectives. AM fungi are capable of significantly improving plant mineral nutrient acquisition by scavenging larger volume of soil, mainly in low-nutrient conditions, and it has clearly been demonstrated that plants possess a symbiotic Pi uptake pathway (Berruti et al., 2016).

In the present study, mycorrhizal parameters, such as extramatrical spores in the root zone soil and percent mycorrhizal root colonization, were considerably higher in all the inoculated treatments compared to the uninoculated control treatment in both the cultivars; however *A. leptoticha* produced significantly more spores in root zone soil of both the cultivars compared to other AM fungal treatments (Tables 2 and 4). The existence of host preference by AM fungi investigated by earlier researchers brought out that the extent of mycorrhizal root colonization and the spore count in the root zone soil varied with different AM fungi and that the host plant responds best to a particular AM fungal symbiont (Bagyaraj, 2011; Helgason et al., 2002; Vandenkoornhuyse, Ridgway, Watson, Fitter, & Young, 2003). The extent of colonization and the spore count varied with different AM fungi. In the present study it can be concluded that the soybean cultivars MAUS 2 and MAUS 212 responded best to inoculation with *A. leptoticha* (which confers

maximum growth benefits) compared to all other fungi used in this study. The cultivars being drought susceptible it is possible that inoculation with the selected AM fungus can confer drought tolerance, which needs further investigation.

Table 1

Influence of different AM fungi on height, stem diameter, biovolume index (BI), total leaf area, and shoot, root and total dry biomass of drought susceptible soybean cultivar MAUS 2

Treatments	Height (cm/ plant)	Stem dia. (mm/ plant)	BI	Total leaf area (cm^2/ plant)	Shoot dry biomass (g/ plant)*	Root dry biomass (g/ plant)	Total plant dry biomass (g/ plant)
Control	90.20d	4.03^{d}	363.51^{bc}	744.82^{e}	5.18	0.71^{c}	5.83^{d}
Funneliformis caledonium	120.50^{ab}	4.28^{bc}	515.62^{ab}	840.11^{cd}	5.72	0.85^{bc}	6.57^{bc}
Acaulospora laevis	101.00^{bc}	4.76^{ab}	479.59^{ab}	770.83^{de}	5.54	0.77^{bc}	6.31^{cd}
Rhizophagus fasciculatus	109.17^{ab}	4.17^{cd}	457.17^{ab}	988.59^{ab}	6.06	0.70^{c}	6.78^{bc}
Claroideoglomus etunicatum	94.17^{cd}	4.77^{ab}	449.19ab	873.49^{bc}	5.64	1.00^{ab}	6.64^{bc}
Gigaspora margarita	119.38^{ab}	4.72^{ab}	563.47^{ab}	897.39^{bc}	5.69	0.76^{bc}	6.45^{bc}
Glomus macrocarpum	123.95^{a}	4.92^{a}	609.834^{a}	1033.00^{ab}	6.50	0.93^{bc}	7.43^{ab}
Glomus bagyarajii	$90.60d^{de}$	4.32^{bc}	391.39^{b}	1112.49^{ab}	6.06	0.84^{bc}	6.90^{bc}
Funneliformis mosseae	99.33^{cd}	4.62^{ab}	460.02^{ab}	1107.26^{ab}	6.39	1.12^{ab}	7.51^{ab}
Rhizophagus intraradices	114.30^{ab}	4.38^{bc}	500.59^{ab}	1005.36^{ab}	6.17	0.90^{bc}	7.06^{ab}
Ambispora leptoticha	106.50^{ab}	4.65^{ab}	492.57^{ab}	1182.46^{a}	7.22	1.32^{a}	8.54^{a}

Note. Means in column with same letters are not significantly different at $P < 0.05$; *Not significant

Table 2

Influence of different AM fungi on pod and seed weight, shoot, root, and total plant P concentration, mycorrhizal spore numbers in root zone soil and mycorrhizal root colonization of drought susceptible soybean cultivar MAUS 2

Treatments	Pod weight (g/ plant)	Seed weight (g/ plant)	Shoot P conc. (%)	Root P conc. (%)	Total plant P conc. (%)	Mycorrhizal spore Nos./ 50 g of dry root zone soil	Mycorrhizal colonization (%)
Control	3.61^{c}	80.09^{c}	0.37^{h}	0.20^{h}	0.57^{h}	105^{f}	66^{d}
Funneliformis caledonium	5.27^{ab}	108.74^{a}	0.61^{c}	0.27^{c}	0.88^{c}	317^{b}	94^{b}
Acaulospora laevis	5.81^{ab}	107.25^{a}	0.50^{f}	0.26^{g}	0.76^{g}	147^{e}	95^{b}
Rhizophagus fasciculatus	5.01^{ab}	101.05^{ab}	0.55^{d}	0.26^{e}	0.81^{e}	138^{ef}	95^{b}
Claroideoglomus etunicatum	4.88^{ab}	108.40^{a}	0.62^{b}	0.26^{c}	0.88^{c}	338^{b}	100^{a}
Gigaspora margarita	4.76^{ab}	98.48^{ab}	0.47^{g}	0.30^{fg}	0.77^{fg}	258^{c}	100^{a}
Glomus macrocarpum	6.07^{ab}	109.25^{a}	0.63^{b}	0.27^{b}	0.90^{b}	270^{c}	100^{a}
Glomus bagyarajii	4.40^{bc}	86.75^{bc}	0.51^{ef}	0.33^{d}	0.84^{d}	185^{d}	95^{b}
Funneliformis mosseae	5.84^{ab}	102.61^{a}	0.52^{e}	0.25^{fg}	0.77^{fg}	130^{ef}	100^{a}
Rhizophagus intraradices	5.55^{ab}	112.17^{a}	0.52^{e}	0.26^{f}	0.78^{f}	197^{d}	100^{a}
Ambispora leptoticha	6.43a	112.26^{a}	0.74^{a}	0.35^{a}	1.09^{a}	371^{a}	100^{a}

Note. Means in column with same letters are not significantly different at $P < 0.05$

Table 3

Influence of different AM fungi on height, stem diameter, biovolume index (BI), total leaf area, and shoot, root and total dry biomass of drought susceptible soybean cultivar MAUS 212

Treatments	Height (cm/ plant)	Stem dia. (mm/ plant)	BI	Total leaf area (cm^2/ plant)	Shoot dry biomass (g/ plant)*	Root dry biomass (g/ plant)	Total plant dry biomass (g/ plant)
Control	60.00^{e}	4.65^{e}	278.97^{e}	1087.23^{c}	7.24^{bc}	0.69^{e}	7.93^{c}
Funneliformis caledonium	73.67^{e}	4.73^{bc}	348.67^{e}	1427.87^{ab}	8.82^{ab}	1.14^{ab}	9.96^{ab}
Acaulospora laevis	62.83^{e}	4.88^{ab}	306.23^{e}	1158.93^{bc}	7.49^{bc}	1.07^{ab}	8.56^{bc}
Rhizophagus fasciculatus	67.50^{e}	4.90^{ab}	331.20^{e}	1341.83^{ab}	8.22^{bc}	1.13^{ab}	9.41^{bc}
Claroideoglomus etunicatum	61.67^{e}	4.92^{ab}	302.82^{e}	1083.52^{c}	6.61^{d}	1.03^{ab}	7.64^{d}
Gigaspora margarita	64.67^{e}	4.82^{ab}	311.65^{e}	1179.37^{bc}	6.77^{cd}	0.93^{b}	7.70^{d}
Glomus macrocarpum	69.33^{e}	4.90^{ab}	340.80^{e}	1281.93^{ab}	7.18^{bc}	1.13^{ab}	8.31^{bc}
Glomus bagyarajii	111.33^{a}	5.35^{a}	595.62^{a}	1364.79^{ab}	8.50^{bc}	1.07^{ab}	9.57^{b}
Funneliformis mosseae	90.00^{b}	5.27^{ab}	476.03^{b}	1444.70^{ab}	8.65^{ab}	1.01^{ab}	9.66^{bc}
Rhizophagus intraradices	69.17^{c}	4.60^{c}	318.00^{c}	1482.32^{ab}	8.94^{ab}	0.97^{ab}	9.91^{ab}
Ambispora leptoticha	95.00^{b}	5.32^{a}	505.40^{b}	1541.44^{a}	10.30^{a}	1.19^{a}	11.47^{a}

Note. Means in column with same letters are not significantly different at $P < 0.05$

Table 4

Influence of different AM fungi on pod and seed weight, shoot, root, and total plant P concentration, mycorrhizal spore numbers in root zone soil and mycorrhizal root colonization of drought susceptible soybean cultivar MAUS 212

Treatments	Pod weight (g/ plant)	Seed weight (g/ plant)	Shoot P conc. (%)	Root P conc. (%)	Total plant P conc. (%)	Mycorrhizal spore Nos./ 50 g of dry root zone soil	Mycorrhizal colonization (%)
Control	3.55^{d}	66.62^{b}	0.17^{e}	0.08^{f}	0.25^{g}	118^{i}	72^{f}
Funneliformis caledonium	4.44^{ab}	71.72^{ab}	0.24^{c}	0.17^{ab}	0.41^{c}	197^{e}	97^{c}
Acaulospora laevis	4.80^{ab}	77.21^{ab}	0.24^{c}	0.15^{cd}	0.39^{d}	185^{g}	96^{d}
Rhizophagus fasciculatus	4.69^{ab}	77.07^{ab}	0.27^{b}	0.13^{e}	0.40^{cd}	153^{h}	100^{a}
Claroideoglomus etunicatum	3.68^{cd}	74.94^{ab}	0.29^{a}	0.15^{cd}	0.44^{b}	212^{d}	100^{a}
Gigaspora margarita	3.69^{cd}	64.72^{b}	0.21^{d}	0.13e	0.34^{f}	186^{g}	98^{b}
Glomus macrocarpum	4.24^{ab}	65.13^{b}	0.26^{b}	0.17^{ab}	0.43^{b}	153^{h}	100^{a}
Glomus bagyarajii	5.02^{a}	81.84^{a}	0.27^{b}	0.16^{bc}	0.43^{b}	294^{b}	90^{e}
Funneliformis mosseae	4.32^{ab}	76.48^{ab}	0.24^{c}	0.13^{e}	0.37^{e}	227^{c}	100^{a}
Rhizophagus intraradices	4.16^{bc}	65.10^{b}	0.23^{c}	0.14^{de}	0.37^{e}	192^{f}	100^{a}
Ambispora leptoticha	5.06^{a}	82.47^{a}	0.30^{a}	0.18^{a}	0.48^{a}	353^{a}	100^{a}

Note. Means in column with same letters are not significantly different at $P < 0.05$

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Phenotypic and Molecular Characterisations of Lactic Acid Bacteria Isolated from Malaysian Fruits

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ABSTRACT

Lactic Acid Bacteria (LAB) are gram-positive, catalase-negative and non-spore forming bacteria known to have many advantages such as starter culture in food fermentation, as antimicrobial agent and plant growth promoter. Limited information on various LAB present in Malaysian fruits hampers further study to explore their potential as autochthonous inoculants in food fermentation, plant disease control and growth promotion. Therefore, the objectives of this study are firstly to isolate and identify LAB from honeydew, ciku, mango and mata kucing by investigating their morphological and biochemical characteristics, secondly to determine the identity of the isolates using 16S rRNA gene sequencing and finally to examine phylogenetic relationship of the LAB present in the fruits. The isolates were subjected to Gram staining, acidity and catalase tests, followed by molecular identification and phylogenetic analysis of the bacteria. Out of 33 isolates, eight isolates were gram- positive, catalase-negative and acid producers, suggesting that they are potentially LAB. 16S rRNA sequencing and NCBI Blast analysis identified the presence of *Lactococcus* sp., *Leuconostoc* sp., *Weissella* sp. and *Aerococcus* sp. in the fruit samples with sequence identity 94-97%. Phylogenetic tree was constructed based on the 16S rRNA sequences using Neighbor-Joining method. This study has assisted in collecting more information about the diversity of LAB in Malaysian fruits, which can be further explored in future for their application as bioinoculant in food fermentation or as biocontrol agent and plant growth promoter in agricultural field.

Keywords: Fruits, lactic acid bacteria, phylogenetic analysis, 16S rRNA sequencing

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INTRODUCTION

Malaysian fruits are highly rich in vitamins and minerals while several of them are known to be helpful in protection against chronic diseases and act as antioxidants (Khoo, Azlan, Kong, & Ismail, 2016). Besides acting as food sources, they also contribute economically to Malaysian agricultural sector. However, excess fruit production and their short shelf- life has led to fruit spoilage, thus prohibiting consumption and increasing waste. Therefore, biopreservation through fermentation process has been one of good ways to preserve fruits and slow down their spoilage (Di Cagno, Coda, De Angelis, & Gobbetti, 2013). Fruits fermentation is mediated by the addition of autochthonous or allochthonous starter culture where Lactic Acid Bacteria (LAB) is usually utilized.

LAB are classified as Gram-positive, non-spore forming, catalase-negative bacteria, which are strictly fermentative and produce lactic acid as a major end product (König, Unden, & Fröhlich, 2017). Some examples of LAB are *Lactobacillus, Lactococcus, Enterococcus and Streptococcus.* They can be grouped into homofermentative or heterofermentative rods and cocci (König et al., 2017). Besides fermentation, LAB also play important roles in food technology where they help to enhance the aroma and texture of food and inhibit the growth of spoilage bacteria (Schleifer et al., 1995). LAB are highly effective against the causal agents of food poisoning and spoilage, thanks to the production of bacteriocin that possesses antimicrobial activites (Zacharof & Lovitt, 2012). In addition, the antibacterial and antifungal substances produced by LAB were reported to be effective in controlling plant pathogens and suppressing pre and post-harvest diseases in crop plants (Belkacem-Hanfi et al., 2014; Hamed, Moustafa, & Abdel-Aziz, 2011; Tsuda et al., 2016).

However, studies on various LAB present in Malaysian fruits are relatively scarce, limiting further strategies to explore their potential as starter culture in food fermentation or as biological control agent for plant diseases. Therefore, this study was carried out to isolate and identify diverse LAB from Malaysian fruits, which can be further studied for their use in industrial and agricultural applications. The isolated LAB can be a great candidate for replacing chemical fertilizers to enhance the uses of biocontrol agents in agriculture besides helping in biopreservation of fruits. Finally, this study is also expected to provide insights into their taxonomic information and distribution in Malaysian fruits.

MATERIALS AND METHODS

Samples Collection and Surface Sterilization

Mango (n=10), mata kucing (n=10), honeydew (n=3) and ciku (n=10) were purchased from Serdang, Selangor. 70% ethanol was sprayed onto the surface of fruits skin to remove epiphytic microorganisms. The fruits were rinsed under running tap water and 70% (v/v) ethanol was sprayed on the fruits surface for 3 minutes followed

by rinsing with sterile distilled water. 10% (v/v) sodium hypochlorite was sprayed and left for 3 minutes followed by thorough rinsing with sterilized distilled water. The fruits were cut into two parts by using sterile knife. The seeds and flesh of the fruits were separated and homogenized in a mortar. 10 ml of sterile distilled water was added into the mortar and mixed well. The seeds and flesh extracts (1 ml) were transferred into universal bottle containing 9 ml of de Man, Rogosa and Sharpe (MRS) broth (Oxoid™). The broths were then incubated for 16 to 24 hours at 37 ºC. The overnight cultures were serially diluted in MRS broth (Oxoid™) from 10^{-1} until 10^{-6}. An aliquot of 100 µl of each dilution factor was spread onto MRS agar (Oxoid™) plates for the growth of bacterial colonies. The plates were incubated for 24-48 hours at 37 ºC.

Isolation of LAB

Bacterial colonies with different morphologies were picked and streaked on MRS agar (Oxoid™) plates. Sub-culturing of the bacterial colonies was made on MRS agar (Oxoid™) to obtain pure colonies. The morphologies of the isolated colonies on MRS agar (Oxoid™) plates were evaluated based on their size, colour, elevation, shape and consistency using a microscope (Leica ICC50).

Biochemical Tests

Gram staining, catalase test and acidity test were performed. Catalase test was conducted by adding one to two drops of 3% hydrogen peroxide (H_2O_2) into a cultured broth and the formation of bubbles was observed (Goyal, Dhingra, Bajpai, & Joshi, 2012). Acidity test was performed according to Sobrun, Bhaw-luximon, Jhurry and Puchooa (2012) with a slight modification by streaking bacterial colonies on MRS agar containing Bromocresol purple dye (MRS+ BCP) at final concentration of 0.004% (w/v) (Sobrun et al., 2012).

Genomic DNA Extraction and Sequencing of the 16S ribosomal RNA Gene

The extraction of genomic DNA of the isolates was carried out by using Wizard® Genomic DNA Purification Kit (Promega Corporation, USA). Amplification of the genomic 16S rRNA region was then performed in 50 µl reaction containing 5 µl of 10X NH_4 reaction buffer, 1.5 µl of $MgCl_2$, 0.25 µl of GoTaq DNA Polymerase (1.25 units), 1 µl of 10 mM dNTPs, 1 µl of 10 µM forward primer 16S 27F (5' –AGA GTT TGA TCC TGG CTC AG -3') and 1492 reverse primer (5' -GGT TAC CTT GTT ACG ACT T -3') (Dai, Li, Wu, & Zhao, 2013), 1 µl of genomic DNA template and 39.25 µl of nuclease-free water. PCR was carried out in Bio-Rad MyCycler Thermal Cycler PCR (Bio Rad Laboratories, Inc) with the following conditions: initial denaturation at 94 ºC for 30 seconds, 30 cycles of denaturation at 94 ºC for 30 seconds, annealing at 56 ºC for 1 min, extension at 72 ºC for 2 minutes and final extension at 72 ºC for 5 minutes. PCR products were analyzed on a 1.0 % (w/v) agarose gel in 1X TAE buffer at 80 V for one hour and purified using the Wizard® SV

Gel and PCR Clean-Up System (Promega Corporation, USA). The purified PCR products were subjected to 1st Base DNA sequencing services and the identity of the isolates was analyzed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS AND DISCUSSIONS

Morphological and Biochemical Test

Out of 200 colonies, only 33 isolates were picked from all plates and further characterised for LAB properties. These isolates were chosen as representatives of isolates with different morphological properties, whereas those with identical morphologies were not selected. Most of the isolated colonies have small and circular shape, creamy and whitish colour. Gram staining result for the 33 isolates showed that all of them were gram-positive and cocci-shaped. Figure 2 shows the Gram staining result for isolate HDS 9 with purple and cocci-shaped colonies. Acidity test revealed that out of the 33 isolates, 20 isolates were able to produce acid, as shown by a color change from purple to yellow on Bromocresol purple agar (Figure 3). *Lactococcus lactis* was used as positive control while *Bacillus cereus* was used as negative control. Catalase test showed that from the 33 isolates, 17 were catalase-negative as indicated by the absence of bubble formation upon H_2O_2 addition, suggesting the absence of catalase enzyme. Most of LAB are catalase-negative, although some of bacteria in this genera may possess this enzyme (Wood & Holzalpfel, 1995). The summary for morphological characteristics and biochemical tests was tabulated in Table 1.

Molecular Identification of Isolates using PCR and 16S rRNA Gene Sequencing

Since only LAB are targeted in this study, out of 33 isolates, only gram-positive, catalase- negative and acid-producing strains were further characterized for molecular identification using 16S rRNA gene sequencing. Table 2 shows 16S rDNA sequencing results of the isolates. The sequencing of 16S rRNA gene revealed the presence of *Lactococcus* sp., *Leuconostoc* sp., *Weissella* sp., *Aerococcus* sp. and two non-LAB species in the fruits samples with sequence similarity of 94-97%. Isolates HDS 1 and HDS 4 were closely related to *Lactococcus lactis* subsp. *lactis* strain UC06 and *Leuconostoc citreum* KM20 with 96% and 94 % similarity respectively. Isolates HDF 8 and HDF 9 showed 95% and 96% sequence similarity with *Weissella cibaria* strain CMS3 and *Weissella confusa* strain A7Gaf respectively. In addition, the 16S rDNA sequence of isolate MS 3A had 96% similarity with *Leuconostoc citreum* KM20 while isolates MS 6 and KS 5 were 94% and 96% similar with *Lactococcus lactis* strain HadRami9. Whereas, isolate KS 2 displayed 97% sequence similarity with *Aerococcus viridans* strain CCUG4311.

Table 1

Morphological and biochemical characteristics of isolates

No	Isolates	Type and Part of fruit	Size and Form	Colour	Margin	Gram stain	Catalase test	Acidity test
1	MS 1	[1]MNG , Seed	Small, circular	Creamy	Entire	Gram-positive , cocci	+	+
2	MS 2	MNG , Seed	Small, circular	Creamy	Entire	Gram-positive , cocci	+	-
3	MS 3A	MNG , Seed	Small, circular	White	Entire	Gram-positive , cocci	-	+
4	MS 3B	MNG , Seed	Small, irregular	White	Undulate	Gram-positive , short rod	-	-
5	MS 4	MNG , Seed	Small, circular	Creamy	Entire	Gram-positive , cocci	+	-
6	MS 6	MNG , Seed	Small, circular	Creamy	Entire	Gram-positive , cocci	-	+
7	MS 7	MNG , Seed	Small, circular	Creamy	Entire	Gram-positive , cocci	-	-
8	MF 1	MNG , Flesh	Small, circular	Creamy	Entire	Gram-positive , cocci	-	-
9	MF 2	MNG , Flesh	Small, circular	Creamy	Entire	Gram-positive , cocci	+	+
10	CS 1	[2]CK , Seed	Small, circular	Creamy	Entire	Gram-positive , cocci	-	+
11	CS 2	CK , Seed	Small, circular	White	Entire	Gram-positive , cocci	+	+
12	CS 3	CK , Seed	Small, circular	Creamy	Entire	Gram-positive , cocci	+	-
13	CS 4	CK , Seed	Small, circular	Creamy	Entire	Gram-positive , cocci	+	+
14	CF 1	CK , Flesh	Small, circular	Creamy	Entire	Gram-positive , cocci	+	+
15	CF 2	CK , Flesh	[a]Mod, circular	White	Entire	Gram-positive , cocci	+	-
16	CF 3	CK , Flesh	Small, circular	White	Entire	Gram-positive , cocci	+	+
17	CF 4	CK , Flesh	Small, circular	Creamy	Entire	Gram-positive , cocci	+	+
18	KS 1	[3]MK , Seed	Small, circular	Creamy	Entire	Gram-positive , cocci	+	-
19	KS 2	MK , Seed	Small, circular	Creamy	Entire	Gram-positive , cocci	-	-
20	KS 4	MK , Seed	Small, circular	Creamy	Entire	Gram-positive , cocci	-	+
21	KS 5	MK , Seed	Small, circular	Creamy	Entire	Gram-positive , cocci	-	+

Table 1 *(Continue)*

22	KF 1	MK , Flesh	Small, circular	White	Entire	Gram-positive, cocci	-	-
23	KF 2	MK , Flesh	Small, circular	Creamy	Entire	Gram-positive, cocci	-	+
24	KF 3	MK , Flesh	Small, circular	Creamy	Entire	Gram-positive, cocci	+	-
25	HDS 1	[4]HD , Seed	Small, circular	Creamy	Entire	Gram-positive, cocci	-	+
26	HDS 2	HD , Seed	Small, circular	White	Undulate	Gram-positive, cocci	+	+
27	HDS 3	HD , Seed	Small, circular	Creamy	Entire	Gram-positive, cocci	+	+
28	HDS 4	HD , Seed	Small, irregular	White	Entire	Gram-positive, cocci	-	+
29	HDS 5	HD , Seed	Small, circular		Entire	Gram-positive, cocci	+	-
30	HDS 6	HD , Seed	Small, circular	White	Entire	Gram-positive, short rod	-	-
31	HDF 2	HD , Flesh	Small, circular	White	Entire	Gram-positive, cocci	-	+
32	HDF 8	HD , Flesh	Small, circular	White	Entire	Gram-positive, cocci	-	+
33	HDF 9	HD , Flesh	Small, circular	White	Entire	Gram-positive, cocci	-	+

Note: [1]MNG indicates mango; [2]CK indicates ciku; [3]MK indicates mata kucing; [4]HD indicates honeydew; [a]Mod indicates moderate size; + indicates positive reaction, - indicates negative reaction

Table 2

16S RNA gene sequencing analysis of isolates

No	Isolates	Closest matches in Genbank	Identity (Base pairs / %)	Accession no.
1	HDS 1	*Lactococcus lactis* subsp. *lactis* strain UC06	1418/1477 bp (96%)	CP015902.1
2	HDS 4	*Leuconostoc citreum* KM20	1204/1286 bp (94%)	DQ489736.1
3	HDF 8	*Weissella cibaria* strain CMS3	1200/1267 bp (95%)	CP013934.1

Table 2 *(Continue)*

4	HDF 9	*Weissella confusa* strain A7Gaf	1021/1067 bp (96%)	KU324936.1
5	MS 3A	*Leuconostoc citreum* KM20	1018/1062 bp (96%)	DQ489736.1
6	MS 6	*Lactococcus lactis* strain HadRami9	1149/1221 bp (94%)	KU324909.1
7	KS 5	*Lactococcus lactis* strain HadRami9	1185/1232 bp (96%	KU324909.1
8	KF 2	*Aerococcus viridans* strain CCUG4311	1425/1473 bp (97%)	CP014164.1

Phylogenetic Analysis

Phylogenetic tree was constructed based on the 16S rRNA gene sequences inferred by the Neighbor-Joining method using Molecular Evolutionary Genetic Analysis 7.0 (MEGA 7.0) in order to identify the relationship of isolates with other different species of LAB according to their ability in producing the antimicrobial substance, bacteriocin. *Klebsiella pneumoniae* strain M5al was used as an out-group. Based on Figure 1, the node placements for all isolates were strongly supported by bootstrap values ranging from 97-100%, indicating reliable placement of the nodes in the phylogenetic tree. Isolates MS3A and HDS 4 were grouped together with *Leuconostoc citreum* KM20 by forming a well-defined cluster at 100% and 98% bootstrap support respectively. *L. citreum* KM20 was previously found predominant in fermented kimchi (Kim et al., 2008). According to Kim et al. (2008), *L. citreum* KM20 can repress the growth of *Bacillus cereus*, *Listeria monocytogenes*, *Micrococcus luteus*, *Pseudomonas aeruginosa* and *Salmonella enterica serovar Typhimurium*. Isolates HDS 4 and MS3A were grouped together with *Leuconostoc mesenteroides* which was reported to be able to produce bacteriocin (Xiraphi et al., 2008). This may suggest that HDS 4 and MS3A also produce bacteriocin, however this needs to be investigated *in vitro* or through whole genome sequencing of the isolates. Other studies have also reported the isolation of other *Leuconostoc* sp. from fruit juices, fresh fruits and vegetables (Emerenini, Afolabi, Okolie, & Akintokun, 2013; Naeem, Haider, Baig, & Saleem, 2012), suggesting that *Leuconostoc* sp. are common in fruits and vegetables.

Isolate HDF 8 and HDF 9 extracted from the flesh of honeydew were both closely related to *Weissella cibaria* and *Weissella confusa* strain A3 respectively. Several studies reported that *Weissella* sp. isolated from fresh fruits and vegetables were able to produce bacteriocin and antimicrobial substances (Goh & Philip, 2015; Papagianni & Papamichael, 2011; Pringsulaka et al., 2012), and therefore are used as biocontrol agent against phytopathogenic bacteria and fungi (Chen, Wu, & Yanagida, 2010; Trias,

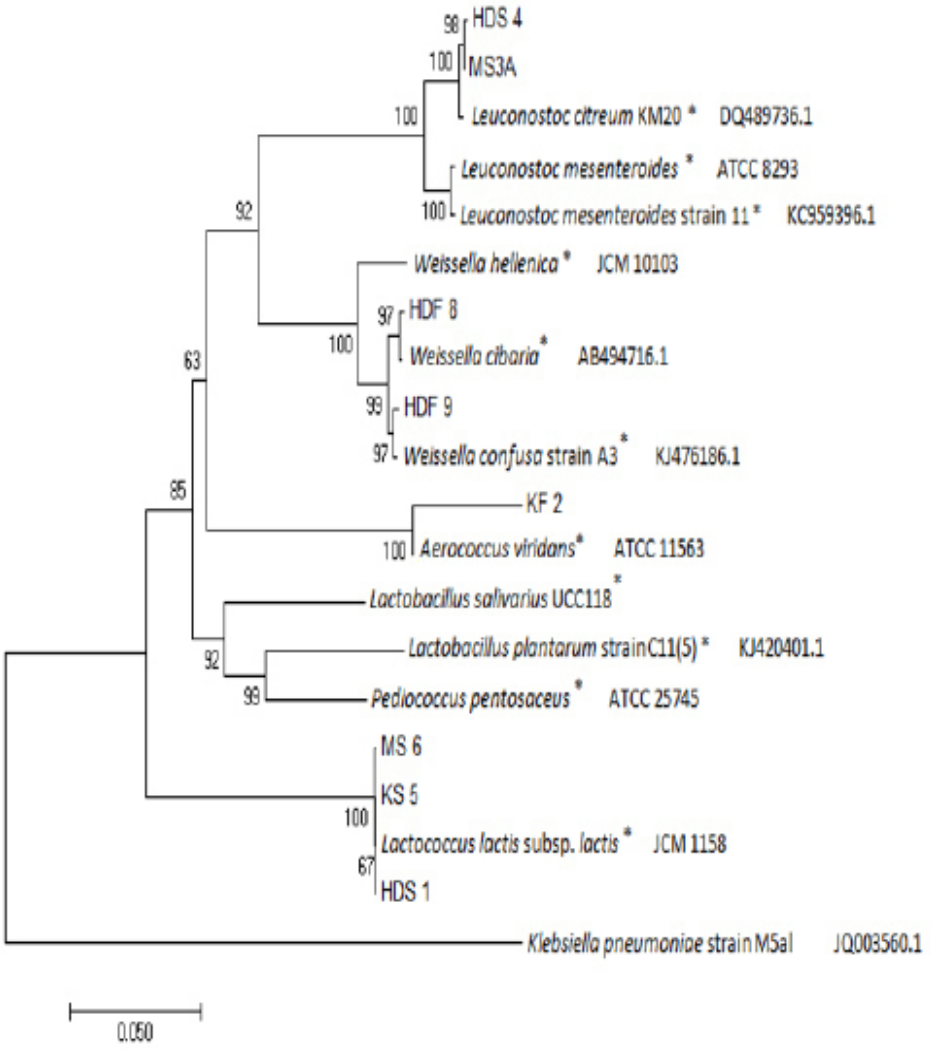

Figure 1. Phylogenetic tree of the selected bacterial isolates using 16S rDNA sequence as phylogenetic marker. Phylogenetic tree showing the relative positions of the isolates as inferred by the neighbor-joining method of 16S rRNA sequences. Branch lengths are proportional to the number of nucleotide substitutions. The bootstrap values for a total of 1000 iterations are shown as percentages at the nodes of the tree. *Klebsiella pneumoniae* strain M5aI was chosen as outgroup. The bar indicates 5% sequence divergence. (Note:* indicates bacteriocin producer)

Bañeras, Badosa, & Montesinos, 2008). *Weissella hellenica* was also reported to be able to be bacteriocinogenic that possesses antimicrobial effect towards food-borne pathogens including yeasts and molds indicating possibility in biopreservation (Chen et al., 2010; Leong et al., 2013). Isolate KF 2 from the flesh of mata kucing was closely related to *Aerococcus viridans* with a strong bootstrap support at 100%.

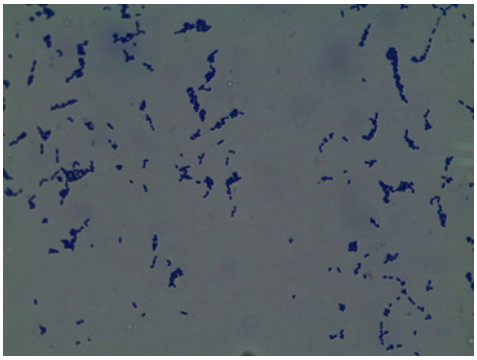

Figure 2. Gram staining of representative isolate HDS 9, with purple and cocci-shaped colonies

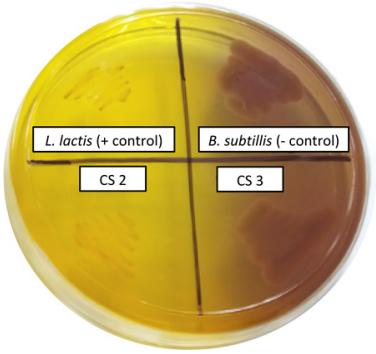

Figure 3. Acidity test for representative isolates CS 2 and CS 3. Isolate CS 2 showed positive result for acidity test where yellow colour formed around the colonies on the agar (MRS agar with addition of bromocresol purple) indicates the ability of the isolate to produce acid whereas isolate CS 3 showed negative result as no colour change observed on the agar and remained purple, indicating the absence of acid production. *L. lactis* and *B. subtillis* were used as positive and negative control respectively

Aerococcus viridans is human pathogen and often associated with endocarditis, urinary tract infection and urosepsis (Gopalachar, Akins, Davis, & Siddiqui, 2004; Jung et al., 2014; Popescu, Benea, Mitache, Piper, & Horstkotte, 2005). This species is found ubiquitous in housing premises, human skin and raw vegetables. The presence of isolate KF 2 similar to this pathogen on the flesh of mata kucing suggested cross-contaminations might have occurred either during bacterial isolation steps or during transport, storage and handling of the fruits from farm to retailers. Therefore, extra caution has to be taken when consuming raw fruits. The *Aerococcus* genus is also associated with bacteriocin production (Ballester, Ballester, & Belaich, 1980). On the other hand, isolates HDS 1, KS 5 and MS 6 isolated from the seeds of mango and mata kucing showed relatedness to *Lactococcus lactis* subsp. *lactis*. *Lactococcus lactis* strains have been isolated from various fresh fruits and vegetables (Laroute et al., 2017). They are also abundant in various sprouted and unsprouted vegetable seeds, while having antagonistic activities against *Listeria monocytogenes*, owing to the presence of bacteriocin (Kelly, Davey, & Ward, 1998). Meanwhile, a study by Park, Itoh, Kikuchi, Niwa and Fujisawa (2003) had reported the ability of *Lactococcus lactis* subsp. *lactis* isolated from kimchi to produce nisin-Z that had antagonistic properties against foodborne pathogens (Park et al., 2003). This may suggest their potential roles in preserving the fruits from food spoilage and also preventing food poisoning in human.

Settanni and Corsetti (2008) had discussed about the uses of bacteriocins as food additives, such as nisin, which is used in kimchi, mashed potatoes, and fresh-cut products. The authors highlighted about bacteriocin-producing LAB as starter culture in fermented and non-fermented vegetables, which may improve microbial

quality, safety and shelf-life of vegetable-based foods (Settanni & Corsetti, 2008). Trias et al. (2008) described the use of Leconostoc, Lactobacillus, Lactococcus and Weissella genera in inhibiting foodborne human pathogens of Iceberg lettuce and Golden apples The addition of bacteriocin-producing LAB in biopreservation has helped to increase the food safety and shelf life (Fhoula et al., 2013; Kasra-Kermanshahi & Mobarak-Qamsari, 2015).

There were also researches carried out in order to study the antibacterial activity of *Lactococcus lactis* and its uses as biocontrol agents and plant growth promoters in agricultural settings (Chen et al., 2010; Trias et al., 2008). The ability of LAB as biocontrol agents has been shown in the control of post-harvest diseases of fruits and vegetables. For instance, LAB was mentioned to be able to protect cucumber roots against pathogen *Pythium ultimum* and increased the germination rate and seedling emergence of tomato seeds (Lutz, Michel, Martinez, & Camps, 2012). Besides that, a study by El-Mabrok, Hassan, Mokhtar, Hussain and Kahar (2012) had also reported the potential of LAB in suppressing the growth of *Colletotrichum capsici* which was the causal agents of anthracnose in chilli (El-Mabrok et al., 2012). In other studies, their application as biocontrol agents has helped in inhibiting plant pathogens and assisted in plant growth promotion such as increasing the rate of seeds germination (El-Mabrok et al., 2012; Hamed et al., 2011; Murthy, Malini, Savitha, & Srinivas, 2012).

Altogether, these results suggested that all of the isolated strains are potentially bacteriocinogenic as they were clustered together with that of bacteriocin-producers, although some bacterial strains were observed to possess different ability of bacteriocin productions even they belong to the same species (Chen et al., 2010). However, further studies need to be carried out to verify the ability of each isolates to secrete bacteriocins and having antimicrobial properties against various pathogens.

CONCLUSION

This study has successfully identified eight isolates belonging to *Lactococcus* sp., *Leuconostoc* sp., *Weissella* sp. and *Aerococcus* sp. with sequence identity 94-97%. Morphological and biochemical analysis showed that they were mostly gram-positive cocci, catalase-negative and acidity-positive. All of the isolates were clustered together with bacteriocin producers, indicating their potential in producing the antimicrobial substance. Further work needs to be done to confirm the ability of these strains to secrete bacteriocin. The work has provided preliminary insights into different Lactic Acid Bacteria in Malaysian fruits, paving the way for further analysis and characterisations to explore their potential as starter culture in food fermentation, as biocontrol agent for pre and post-harvest plant disease control or as plant growth promoters to increase plant growth and crop yields in agriculture.

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Application of *Zea mays* L. Rhizospheric Bacteria as Promising Biocontrol Solution for Rice Sheath Blight

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ABSTRACT

Sheath blight is referred to be a serious soil-borne disease resulting in financial losses towards rice farming. The existing research focused towards examining the potential of *Bacillus subtilis* strain AK38 (GenBank ID: KY458554. 1) and *Pseudomonas fluorescens* strain AK18 (GenBank ID: KY458552. 1), isolated from maize (*Zea mays* L.) rhizosphere, to regulate sheath blight caused by *Rhizoctonia solani* in rice (*Oryza sativa* L.) as well as to examine their impact on plant development. Biocontrol attributes of selected strains, biofilm examination, root colonisation and gnotobiotic examination had been determined. AK38 and AK18 bacterial strains created biofilm effectively and live in rice rhizosphere even after 30 days of the plantation with 5.2×10^5 and 4.8×10^5 CFU/g of root. The quantity of auxin synthesis was registered 31.2µg ml^{-1} in the 72 hr of incubation. Additional plant development attributes i.e. siderophore production, phosphate solubilization, HCN production was confirmed positive with regard to each isolate. The statistical study of data shown significant improvement in root and shoot size 95% and 78.4%, respectively, over control. In addition, 77% decline within disease incidence has been demonstrated *in vivo* trials.

Keywords: Biocontrol, biofilm, rhizosphere, PGP, sheath blight, *Zea mays* L.

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INTRODUCTION

At ongoing annual rate, the entire world population will be expected to expand at 1.2% or even approximately seventy seven million individuals per year**.** As documented by United Nations, the global human

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population is anticipated to rise through 7.6 billion within 2017 to 8.6 billion within 2030, 9.8 billion in 2050 and 11.2 billion in 2100 (Van Bavel, 2013). According to the results of the United Nations 2017 Revision (Food and Agriculture Organization [FAO], 2017), the world's population numbered nearly 7.6 billion as of mid-2017, implying that the world has added approximately one billion inhabitants over the last twelve years. Sixty per cent of the world's people live in Asia (4.5 billion), 17 per cent in Africa (1.3 billion), 10 per cent in Europe (742 million), 9 per cent in Latin America and the Caribbean (646 million), and the remaining 6 per cent in Northern America (361 million) and Oceania (41 million). China (1.4 billion) and India (1.3 billion) remain the two most populous countries of the world, comprising 19 and 18 per cent of the global total, respectively. Irrespective of significant innovations within farming technology over the previous fifty years, substantial volumes of the world's population are still affected from starvation (Table 1) and undernourishment (FAO, 2009). With this particular population increase, it is anticipated that an identical food security challenge may arise with the chances associated with losing farming area due to industrialization and urbanization. Along with existing cultivated fields complications, existing and new plant diseases raise the difficulties for farmers and make it challenging to meet up with the global nutritional requirement for increasing population (Satterthwaite, McGranahan, & Tacoli, 2010).

Rice is an important cereal right after wheat and maize, on which human society largely relies for their nutritional demands (Nadeem et al., 2016). Rice delivers 27 % carbohydrate utilized as dietary energy supply and twenty percent associated with dietary proteins consumption (Muthayya, Sugimoto, Montgomery, & Maberly, 2014). Rice is cultivated through a number of regions and weather conditions. India, China, Pakistan, and Bangladesh are primary producers (Table 2) and consumers associated with rice food. Within India, rice is cultivated under varied environments like rainfed uplands, rainfed shallow, semideep and deepwater lowlands, irrigated lands and hillsides. No other plant varieties are able to cultivate under this kind of broad selection of environmental conditions. On a yearly basis, 148 million hectares (m ha) are sown to rice globally, including 79 m ha (53%) in irrigated environment, 17 m ha (12%) within rainfed uplands 41 m ha (27%) in rainfed lowlands and 11 m ha (8%) in flood prone environment (Haefele, Nelson, & Hijmans, 2014; Singh, McClean, Büker, Hartley, & Hill, 2017).

Sheath blight (ShB) of rice induced by *Rhizoctonia solani* Kuhn (Teleomorph: *Thanatephorus cucumeris* (Frank) Donk) is known as a key biotic concern associated with rice in the majority of the rice cultivating nations of Asian countries (Jisha & Shabanamol, 2014; Zhao et al., 2016). *Rhizoctonia solani* is polyphagous competitive saprophyte and has a broad host selection. Crop deficits usually differ from 0 to 50% based on intensity of the infection

Table 1

Prevalence of undernourishment in the world by region, 2000–2016 (FAO, 2017)

	2000	2005	2010	2011	2012	2013	2014	2015	2016
	Percentage								
WORLD	**14.7**	**14.2**	**11.5**	**11.2**	**11.0**	**10.8**	**10.7**	**10.6**	**11.0**
AFRICA	**24.3**	**20.8**	**18.3**	**17.9**	**17.8**	**17.8**	**18.1**	**18.5**	**20.0**
Northern Africa	**6.8**	**6.3**	**5.1**	**4.8**	**8.5**	**8.4**	**8.3**	**8.3**	**8.3**
Sub-Saharan Africa	**28.1**	**23.7**	**20.6**	**20.2**	**20.0**	**20.0**	**20.4**	**20.8**	**22.7**
Eastern Africa	39.3	34.3	30.9	30.2	30.6	30.6	30.9	31.1	33.9
Middle Africa	37.4	29.4	23.8	23.1	22.5	22.3	24.0	24.4	25.8
Southern Africa	7.1	6.4	6.7	6.3	6.2	6.2	6.5	6.6	8.0
Western Africa	15.1	12.0	10.0	9.9	9.9	9.8	9.8	10.4	11.5
ASIA	**16.7**	**17.0**	**13.2**	**12.8**	**12.5**	**12.2**	**11.9**	**11.6**	**11.7**
Central Asia and Southern Asia	**17.6**	**20.1**	**15.7**	**15.7**	**15.6**	**15.4**	**15.1**	**14.7**	**14.2**
Central Asia	15.7	14.2	10.6	9.9	9.1	8.4	8.2	8.2	8.4
Southern Asia	17.7	20.4	15.9	15.9	15.9	15.7	15.3	14.9	14.4
Eastern Asia and South-Eastern Asia	**16.6**	**15.2**	**11.6**	**10.9**	**10.4**	**9.9**	**9.6**	**9.2**	**9.7**
Eastern Asia	14.6	14.1	11.3	10.7	10.3	9.9	9.5	9.1	9.0
South-Eastern Asia	22.0	18.1	12.4	11.3	10.7	10.0	9.7	9.4	11.5
Western Asia	**11.3**	**10.5**	**9.4**	**9.1**	**8.9**	**8.7**	**8.9**	**9.3**	**10.6**

Table 1 *(Continue)*

LATIN AMERICA AND THE CARIBBEAN	**12.0**	**9.1**	**6.8**	**6.6**	**6.4**	**6.3**	**6.3**	**6.3**	**6.6**
Latin America	**11.1**	**8.0**	**5.9**	**5.7**	**5.5**	**5.4**	**5.4**	**5.5**	**5.9**
Central America	8.1	8.3	7.1	7.2	7.1	7.1	6.9	6.7	6.5
South America	12.2	7.9	5.4	5.1	4.8	4.7	4.8	5.0	5.6
Caribbean	**23.8**	**23.3**	**19.9**	**19.3**	**19.4**	**19.2**	**18.9**	**18.4**	**17.7**
OCEANIA	**5.3**	**5.3**	**5.0**	**5.2**	**5.3**	**5.7**	**6.0**	**6.4**	**6.8**
NORTHERN AMERICA AND EUROPE	**< 2.5**	**< 2.5**	**< 2.5**	**< 2.5**	**< 2.5**	**< 2.5**	**< 2.5**	**< 2.5**	**< 2.5**
Other country group: Western Asia and Northern Africa	9.3	8.7	7.6	7.3	8.7	8.5	8.6	8.8	9.5

Table 2

Top 5 Rice Producing Countries (FAO, 2018)

Rank	Country	Rice Production (metric tonnes)	% of World Total
1	China	206,507,400	27.8%
2	India	157,200,000	21.2%
3	Indonesia	70,846,465	9.5%
4	Bangladesh	52,325,620	7.0%
5	Vietnam	44,974,206	6.0%

as well as the development cycle at which the crop is attacked and environmental situations (Silva, Figueiredo, Andreote, & Cardoso, 2013; Toan et al., 1997). Preliminary indications of sheath blight come in the form associated with spherical, rectangular or ellipsoid, greenish, greyish, water-soaked areas of about 1 cm long that appear on leaf sheaths close to the water line (Toan et al., 1997). The disease develops quickly during flowering when the rice canopy is most dense, forming a microclimate favourable to pathogen growth and spread (Silva et al., 2013). *R. solani* can infect seed to fully mature plant, causing moderate to significant yield losses depending on the plant part affected. Visible plant disease symptoms include formation of lesions, plant lodging, and presence of empty grains. Large lesions formed on infected sheaths of lower rice leaves may lead to softness of the stem thereby initiating stem lodging (Wu et al., 2012). Lodging alters the normal rice canopy design, affecting photosynthetic ability and total biomass production (Silva et al., 2013).

The current research aimed at analyzing the potential of *Bacillus subtilis* strain AK38 (GenBank ID: KY458554. 1) and *Pseudomonas fluorescens* strain AK18 (GenBank ID: KY458552. 1) species to control sheath blight in rice. Various plant development attributes associated with bacterial strains i.e. siderophore production, HCN production, IAA production were also determined.

MATERIALS AND METHODS

Strains

Bacterial isolates intended for current research were isolated and identified from *Zea mays* L. rhizosphere as explained in earlier investigation of Karnwal (2017). *Bacillus subtilis* AK38 (GenBank ID: KY458554. 1) and *Pseudomonas fluorescens* AK18 (GenBank ID: KY458552. 1) were preserved on nutrient agar medium (NAM) at 4°C. Fungal strain, *Rhizoctonia solani* Kuhn, was procured from Indian Agricultural Research Institute (IARI, India) and grown on potato dextrose agar (PDA). The biocontrol potential of bacterial isolates was determined by implementation of dual-culture technique.

Dual Culture

Bacterial isolates were grown in nutrient broth at 150 rpm (3 x *g*) for 24h at 30°C in rotatry shaker incubator. After incubation, bacterial cultures were centrifuged at 6000 rpm (4025 x *g*) for 10min at 4°C. The broth was decanted and bacterial pellets were re-suspended in sterile distilled water. Bacterial cells were counted using a viable plate count and optical density methods on NAM plates, and adjusted to a concentration of 10^8 colony forming unit (CFU) mL^{-1} (OD = 0.5) at 600 nm.

The dual culture / antibiosis assay was performed on PDA in 90 mm diameter petri plates (Khaledi & Taheri, 2016). Fungal pathogen disk of 5mm was placed in the centre of PDA plates whereas 10µL of the bacterial suspensions were uniformly

distributed around the fungal disk at a distance of 20 mm. Dual culture plates were incubated at 28 °C for 48 h. Each combination was replicated 10 times. As negative controls, 5 Petri dishes with PDA were inoculated only with an *R. solani* and 10µL sterilized distilled water at a distance of 20 mm from fungal pathogen.

Bio-film Assay

For biofilm assay AK18 and AK38 bacterial strains were cultured in Luria Bertani (LB) medium and incubated at 37°C for 24 h. Incubated bacterial culture were transferred in four-well polystyrene plates containing casein digest-mannitol medium (Heidarzadeh & Baghaee-Ravari, 2015). These bacteria inoculated polystyrene plates were incubated for 3 days without shaking at 37°C. Three days after, polystyrene plates were rinsed with sterilized distilled water to remove the medium from wells and placed for drying at 37 °C for 30 min. Immediate after drying wells were stained with 1% w/v crystal violet and biofilm development was determined by calculating the OD500 per well using a plate reader. The complete procedure for biofilm formation was repeated three times to reduce the error during experiment.

Biocontrol (Chitinase Assay) and Growth Traits of Bacterial Strains

To access the possibilities of both bacterial isolates as promising biocontrol agent towards fungal pathogen, a substrate depending approach was applied. Bacterial cultures were inoculated into modified LB plate consisting 0. 2% colloidal chitin and 1.5% agar. Modified LB plates were cultivated at 35 °C for 72 h in order to visualize the hollow region of chitin hydrolysis by chitinase enzyme released by bacterial isolates. In addition, plant development traits like auxin formation, siderophore formation, and phosphate solubilization were examined for every single bacterial strain as described by Karnwal (2017).

Seedling Incubation and Inoculation

Selected bacterial strains are potentially amoxicillin resistant (Karnwal, 2017), so for root colonization study each bacterial isolate were grown on amoxicillin amended nutrient broth with 200 µg ml^{-1} of amoxicillin concentration. Rice seedlings were dipped in bacteria inoculated nutrient broth having 10^8 CFU ml^{-1} bacterial cells and incubated in plant growth chamber. After 30 days, bacteria inoculated rice roots were collected and 1 g of root was gently crushed in normal saline and 100 µl serially diluted sample was spreaded on NAM plated having 200 µg ml^{-1} amoxicillin. These plates were incubated at 32°C for 48 h and colony forming unit (CFU) per g of root was calculated as described by Heidarzadeh and Baghaee-Ravari (2015).

In vivo* Antagonism of Tested Bacterial Isolates against *R. solani

To identify the biocontrol potential of bacterial isolates towards experimental phyto-pathogenic fungi *in vivo*, rice

seeds were exposed with bacterial inoculum priory, before exposure to the phyto-pathogenic fungi. Rice seeds were pre-germinated in dark on clean and sterile moist cotton bed in Petri dishes under laminar hood for five days. These seeds were sprayed with bacterial culture having 10^8 cells ml^{-1} of bacterial cell concentration and then germinated in water agar plates for 2 days. After germination, seedlings were shown and incubated along with seven days older fungal culture at 25°C in dark for 2 days. Four replicates of each treatment were carried out to get suitable data regarding statistical evaluation. In control treatment, seeds were exposed to *R. solani* Kuhn and germinated upon water agar plates alone.

Greenhouse Study

Green house study was performed with 14 days old bacterial treated and fungal pathogen contaminated rice seedlings. Seedlings with fungal culture (1:1) were planted in sterilized earthen pots having sterilized sandy loam soil. For increasing bacterial population around root of seedlings, fresh bacterial culture was inoculated around the roots without damaging rice roots. In control (with fungal pathogen, without bacteria treatment) sterilized water was poured for comparison between bacterial treated and non treated trials. These planted pots were kept for 30 days in Greenhouse to report the sheath blight incidence. To achieve the appropriate outcomes, comparison was carried out after 10 and 20 days by using five disease scales mentioned by Chen, Bauske, Musson, Rodriguezkabana and Kloepper (1995): 0 = no disease; 1 = 0-25% of the leaves withered; 2 = 26-50% of the leaves withered; 3 = 61-75% of the leaves withered; 4 = 76-100% of the leaves withered. Disease index was calculated by applying following formula (Heidarzadeh & Baghaee-Ravari, 2015):

$$\Sigma [(P \times DC) \times 100]/(T \times 4),$$

where P = plants per class, DC = disease index and T = total number of plants.

Percent efficacy of disease control was also measured as described by Purkayastha, Saha and Saha (2010)

[(DC of control - DC of bacterial inoculated plants)/DC of control] × 100

Statistical Analysis

Statistical data analysis was performed by using SPSS 16 software for experimental data. To analyze the vital differences among treatments, Fisher's protected LSD was applied with 5% probability level by using Statistical Analysis System software (Karnwal, 2017).

RESULTS

The results of the dual culture study demonstrated that both *Bacillus subtilis* strain AK38 and *Pseudomonas fluorescens* strain AK18 had antagonistic effect on *Rhizoctonia solani* Kuhn. The results produced by antagonists were significantly ($P < 0.05$) different from control as well as within them. *Bacillus subtilis* strain AK38 highly inhibited the growth of test pathogen compared to *Pseudomonas*

fluorescens strain AK18, and the percentage of inhibition increased about two times to AK38 and three times to AK18 from 48 hours to 72 hours incubation (Table 3).

Table 3

Effect of AK38 and AK18 bacterial isolate on the radial growth of R. solani *in dual culture method*

Antagonists	Radial growth at 48 hours*		% inhibition	Radial growth at 72 hours*		% inhibition
	Control	Test		Control	Test	
AK38	64.3 mm	45 mm	30.0 %	72 mm	20.8 mm	71.1 %
AK18		49.2 mm	23.5 %		24 mm	66.7 %

* Values are mean of three replicates

Colonisation Potency of Bacterial Isolates

Preliminary experiments confirmed the antifungal activity of *Bacillus subtilis* strain AK38 and *Pseudomonas fluorescens* strain AK18 against *Rhizoctonia solani* Kuhn. Chitinase assay results reported as a clear zone of chitinase activity around bacterial growth. In addition to biocontrol activity, AK38 and AK18 were capable to form biofilm in polystyrene plates. Current study results revealed that AK38 to be more efficient for biofilm formation over AK18 isolate. Colonization studies demonstrated that AK38 and AK18 strains could colonise and live successfully in rice rhizosphere with the density of 5.2×10^5 and 4.8×10^5 CFU/g of root, respectively, after 30 days of treatment.

Phyto-stimulatory Effect of Bacterial Isolates

Both isolates produced detectable IAA concentrations in medium with L-tryptophan (Table 4). At 50 µg ml^{-1} of L-tryptophan AK38, and AK18 released significant concentrations of indole (2.6 µg ml^{-1} and 1.4 µg ml^{-1}, respectively) in contrast to 0 µg ml^{-1} of L-tryptophan concentration. Significant amount of IAA was detected with 100 µg ml^{-1} tryptophan produced by isolates AK38 and AK18 (6.0 µg ml^{-1} and 4.0 µg ml^{-1}, respectively). A considerably higher concentration of IAA synthesis by AK38 and AK18 was noted when 500 µg ml^{-1} L-tryptophan was supplied to the isolates (Table 4).

Accessibility to iron within environment act as a vital limiter for development of living cells, microorganisms, plants, and animals. Many workers (Khaledi & Taheri, 2016; Nadeem et al., 2016) observed that bacterial siderophores could become an effective source to fulfill the need of soluble iron for the host plant and helped in plant growth. In the present study, AK38 and AK18 strains produced orange clear zone around the bacterial growth on CAS agar (Table 4). Bacterial isolate AK38 and AK18 also developed translucent clear zone around the bacterial growth on Pikovskaya's agar plates and confirmed liquefaction of inorganic phosphate by bacteria.

Effectiveness of Antagonists in The Pot Trials in Growth Chamber

In vivo study with two biocontrol agents in rice plant resulted significant decline in various development constraints of rice over controls. Experimental data was statistically analysed through ANOVA by using mean values of four replicates in which treatments

Table 4
Characterisation of IAA and biocontrol traits in antagonistic bacterial isolates

Isolate	L-tryptophan concentration for IAA production (µg ml^{-1})				Biofilm formation	Siderophore production	Chitinase production
	0	50	100	500			
AK38	0.2	2.6	6.0	10.0	+	+	+
AK18	0.2	1.4	4.0	9.9	+	+	+

+: Positive; −: Negative

Table 5

Rice Sheath blight disease control by bacterial isolates

	After 10 day inoculation		After 20 days inoculation	
	Disease index	% efficacy of disease control	**Disease index**	**% efficacy of disease control**
AK38	20.1 ± 1.3	70 ± 1.3	18.2 ± 1.3	76 ± 1.1
AK18	31.2 ± 0.7	55 ± 0.7	29.8 ± 0.9	61 ± 0.5
Control	67.1 ± 1.2	0.0 ± 0.0	73.7 ± 1.6	0.0 ± 0.0

Table 6
Plant growth promotion effect of bacterial isolates under green house condition on rice

Strain	Shoot length (cm)	Root length (cm)	Shoot dry weight. (mg)	Root dry weight. (mg)
AK18	**21.2ab**	**4.1a**	**503.8a**	**76.3a**
AK38	**22.3a**	**3.8b**	**401.0b**	**65.3b**
Zero Control	**12.5c**	**2.1c**	**156.4c**	**17.7c**
LSD value	**1.17**	**0.63**	4.86	**2.64**

Means sharing the same letter(s) within a column did not differ significantly ($P \leq 0.05$)

disease index and disease control efficacy (Table 5). The antagonistic bacteria induced much less greenish grey spots on sheaths with low disease intensity and improved were examined using least significant differences ($p \leq 0.05$). Under greenhouse research, AK38 and AK18 isolates induced shoot growth, root growth and dry weight

significantly (Table 6). Statistical analysis revealed significant increment in root and shoot length with 80.9 and 95% increase in root and 78.4 and 69.6% raise in shoot length with AK38 and AK18, respectively (Table 6). Greenhouse study results proved the beneficial effect of both isolates through a significant increment in shoot fresh weight with AK38 and AK18 against uninoculated controls in presence of fungal pathogen.

DISCUSSION

It was detected by researchers (Ignatova, Brazhnikova, Berzhanova, & Mukasheva, 2015) that varied concentrations of L-tryptophan perform a significant function in determining the concentration of IAA synthesis by microorganisms under trials. Results of the present study supports the earlier published reports (Palacios, Gomez-Anduro, Bashan, & de-Bashan, 2016) regarding the effect of varied L-tryptophan concentrations regulating the biosynthesis of IAA and plant development (Chaiharn & Lumyong, 2011; Karnwal, 2009; Karnwal, 2017). Presence of iron in soil or on root surface encourage competition among soil micro-organisms (Sadeghi et al., 2012). Raupach and Kloepper (1998) reported the impact of iron chelater's (siderophores) produced by rhizospheric bacteria on plant development by increasing the bioavailability of soluble iron in the rhizosphere region. Phosphorus is a macronutrient that is required by all of living organisms. However, plants required this particular macronutrient in an extremely lesser volume although a critically low availability could lead to deficiencies and adverse impact on plant growth (Yasmin, Rahman Bakar, Malik, & Hafeez, 2004). Within soil maximum quantity of phosphorus is existing in solid or powder form that could not be directly utilized by plant. Research workers have documented the usage of soil residing bacteria for liquefaction of mineral phosphates into a plant utilizable form. Soil bacteria synthesized different organic acids for phosphate liquefaction. These types of organic acids ensure the bioavailability of insoluble mineral phosphate into soluble phosphate by acidification process (Zhang et al., 2015). Greenhouse study results are in conformity with other workers study (Balseiro-Romero et al., 2017; Kuan, Othman, Abdul Rahim, & Shamsuddin, 2016) those documented the beneficial effect of indole acetic acid secreted by *Bacillus subtilis*, which often favours plant development by maximizing the amount of root hairs. In order to provide an advantageous impact by PGPR, the colonization associated with bacteria within the plant rhizospheric zone is the most important aspect (Kuan et al., 2016; Zhang et al., 2015). However various other factors i.e. phytohormone formation, eradication of pathogenic microorganisms, phosphate solubilisation, and favouring the inorganic nutrient uptake are also considered to be associated with plant development supported by PGPR (Palacios et al., 2016; Sallam, Riad, Mohamed, & El-Eslam, 2013).

CONCLUSION

This study demonstrates that *Bacillus subtilis* strain AK38 and *Pseudomonas fluorescens* strain AK18 isolated from maize rhizosphere were capable of suppressing the growth of *R. solani in vitro*. The inhibition zones produced by the test isolates of antagonistic bacteria greatly varied. From this result, it could be said that biological control might be effective and alternative in minimizing the incidence of the disease. A significant percent control of sheath blight was observed when seeds soaked with the test bacteria. Both isolates have a great potential as a promising biocontrol agent and offers a good prospect for integrated management of the sheath blight of rice. As well as colonization and phyto-stimulatory study reveal the positive aspect of isolates as promising biofertilizer agents. However, additional research on *Bacillus subtilis* strain AK38 and *Pseudomonas fluorescens* strain AK18 is still needed to proceed further with selected BCA addressing sheath blight disease control under rainfed lowland culture as well as in several areas including i.e. its formulation and applications; repetition of *in vivo* studies with other crops and integration into a production system.

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The Roles of Surface Soil Carbon and Nitrogen in Regulating the Surface Soil pH and Redox Potential of Sulfidic Soil Materials of Acid Sulfate Soils

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ABSTRACT

Acid sulfate soils (ASS) have been described as the "nastiest" soils on earth because of their harmful impacts on various soil and environmental systems. ASS pose no problems unless the sulfidic soil materials are disturbed or exposed and the sulfides are oxidized, leading to production of sulfuric soil materials and generation of sulfuric acidity. In sulfidic soil, unless there is disturbance or exposure in the deep soil, only the surface soil seems to be frequently exposed and disturbed, leading to oxidation as a result of oxygen penetration. We have reported recently the importance of soil carbon and nitrogen in amelioration of ASS but the roles on the surface soil was not clearly established. In this study, the roles of surface soil carbon and nitrogen in regulating the surface soil redox potential (Eh) and pH of sulfidic soil material of ASS was investigated following the addition of different sources of soil carbon and nitrogen. The results showed the mechanisms involved in curtailing of sulfidic soil material oxidation and acidification were dependent on the type of metabolic substrates and the microbial ecology the resources were capable of establishing. Addition of a single nutrient source, e.g. glucose, capable of engaging a few soil microbes, was ineffective in preventing sulfidic soil oxidation, whereas addition of complex metabolic substrates, e.g. organic matter, as a source of multiple resources for microbial metabolism effectively reduced the Eh and highly increased the pH, even under aerobic soil conditions.

Keywords: Acid sulfate soils, Eh, pH, soil carbon and nitrogen, sulfidic soil materials

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INTRODUCTION

Acid sulfate soils are naturally occurring soils or sediments formed under reduced soil conditions (Dent & Pons, 1995; Fanning, 2013; Pons, 1973). The global occurrence of ASS is shown in Figure 1. These soils either contain sulfuric soil materials, sulfuric acid (H_2SO_4) or have the potential to form them, in amounts that can have detrimental impact on soil properties (Baldwin & Fraser, 2009; Ljung, Maley, Cook, & Weinstein, 2009). In general, ASS with sulfuric soil material (pH<4; Isbell, 2002) and that having acidified through oxidation of pyrite are referred to as "*Sulfuric soils*" in accordance with the Australian ASS classification key (Fitzpatrick, Powell, & Marvanek, 2008). ASS with sulfidic soil material (pH>4; Isbell, 2002) that are oxidizable, contains pyrite and have the potential to acidify when exposed to air are referred to as "*Sulfidic soils"* (Fitzpatrick et al., 2008).

Sulfidic soil materials are formed through bacterially-induced formation of iron sulfides, mostly pyrite (FeS_2) in coastal and inland reduced environments (Sammut, White, & Melville, 1996). In an undisturbed state below the water table, the sulfidic soil materials are benign unless exposed due to various natural processes (e.g. drought events) and anthropogenic activities (e.g. excavation) (Dent, 1986; Österholm & Åström, 2004). These processes allow the sulfides present in the sulfidic soil materials to react with oxygen and oxidize the FeS_2. The oxidation processes in turn produces H_2SO_4 which acidifies the surrounding environments (Nordmyr, Åström, & Peltola, 2008). Release of the H_2SO_4 in turn solubilizes soil matrix releasing metals such as iron (Fe^{2+}, Fe^{3+}), aluminum (Al^{3+}) and toxic elements, making them readily available in solution to be dispersed into surrounding environments (Poch et al., 2009; Wilson, White, & Melville, 1999).

The major ecological impacts associated with oxidation of FeS_2 and release of the H_2SO_4, toxic metals and metalloids are loss of natural habitats, degradation of civic infrastructure, loss of crop productivity, and deoxygenation of water bodies (Macdonald et al., 2004; Michael, 2013; Sammut et al., 1996). The major detrimental impact of H_2SO_4 and release of toxic constituents of the soil matric is on redox potential (redox) and pH. These soil properties are regulated by microbial activity, soil oxygen, organic matter and soil water status (McLean, 1982). In turn, redox and pH affect oxidation and reduction of minerals, release and mobility of metals or metalloids, and stability and availability of nutrients to crops (DeLaune & Reddy, 2005). In ASS, an oxidized soil material of high redox values (+300 mV) would mean low soil pH (p<4), and a soil material of high pH (pH>4) would mean reduced soil conditions (-300 mV) (Fiedler, Vepraskas, & Richardson, 2007). Oxidized soil conditions of high redox values (Eh>300 mV) and low pH (pH<4) are characteristics of high concentrations of protons (H^+) and acidic minerals (Fe^{2+}, Fe^{3+}, Al^{3+} etc.), soil condition not suitable for crop production (Ljung et al., 2009; Michael, Fitzpatrick, & Reid, 2017).

In ASS, sulfuric soil material acidity is

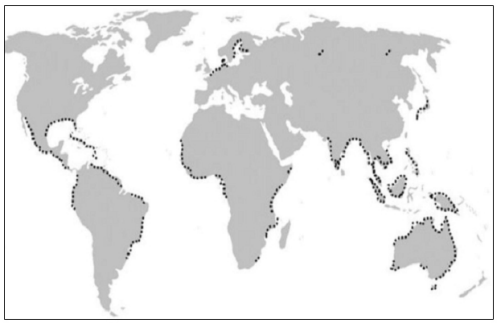

Figure 1. The global distribution of ASS. Of the estimated 17-24 million ha of ASS (Ljung et al., 2009; Poch, Thomas, Fitzpatrick, & Merry, 2009), 6.5 million occur in Asia, 4.5 million in Africa, 3 million in Australia, 3 million in Latin America, 235 000 in Finland and 100 000 in North America, respectively (Simpson & Pedini, 1985)

managed by application of mineral lime and oxidation of sulfidic soil materials by water table management (Melville & White, 2012). We have demonstrated management of soil factors responsible for producing acidity and the oxidation processes as important strategies (Michael, Fitzpatrick, & Reid, 2015, 2016). In one of these studies (Michael et al., 2016), we have reported the importance of surface soil (0−10 mm) carbon and nitrogen in amelioration of sulfuric soil materials under aerobic soil conditions. Based on the results, it became apparent that the importance of soil carbon and nitrogen in regulating the surface soil (0−10 mm) pH and redox of sulfidic soil materials needs to be investigated. Therefore, this study examined the importance of soil carbon and nitrogen on surface soil pH and redox of sulfidic soil materials when maintained under aerobic or anaerobic soil conditions.

MATERIALS AND METHODS

Soils

The sulfidic soil material used in this study collected from a 'sulfuric subaqueous clayey soil' (Fitzpatrick, 2013) at a depth of ca. 1 m in the Finniss River (Figure 2) in Adelaide, South Australia (35°24028.28″S; 138°49054.37″E) was described previously (Michael, Reid, & Fitzpatrick, 2012; Michael et al., 2015). Information on soil classification using the Australian ASS Identification key (Fitzpatrick et al., 2008) and United States Department of Agriculture Natural Resources Conservation Service (NRCS-USDA, 2014) are given in Table 1. When the sample of sulfidic soil material was freshly collected, the pH measured in water 1:5 (pH_w) was 6.7; the water holding capacity was 49% and the residual organic matter content, estimated using the weight loss on ignition method (Schulte & Hopkins, 1996), was 10.6%, respectively. After peroxide treatment (pH_{ox}) (Ahern, McElnea, & Sullivan, 2004), the pH decreased to 1.4.

Treatments

The treatments and the compositions are shown in Table 2. In the experiments involving simple carbon and nitrogen compounds, a plant material amended treatment using lucerne hay as organic matter with high nitrogen content was included to compare the results. All the corresponding control treatments were not amended. The amendments of the three soil treatments were uniformly mixed into the soil, and placed into 70 mL Falcon tubes by

Table 1
Classification of acid sulfate soil materials from the Finniss River used in the study as per Michael et al. (2016)

Soil Type[1]	Previous sampling location reference	Depth (cm bgl)						
			Sulfuric horizon[5]/ *Sulfidic material*[6]	Soil Class[7]	Australian ASS classification key[8]	**Sulfuric horizon**[5]/ *Sulfidic material*[6]	Soil Class[7]	Australian ASS identification key[8]
			Finniss River: Prior to rewetting (2009)			Finniss River: Post rewetting (post 2010)		
Sulfidic soil	FIN26	0-5	Sulfuric	Hydaquentic	**Sulfuric cracking clay soil**	*Sulfidic* **Sulfidic**	**Typic Sulfowassept**	Sulfuric subaqueous clay soil
	M3-4[2]	5-17	Sulfuric	Salfaquept				
	FC10740[3]	17-40	**Sulfuric**			Sulfuric		clay soil
	LF01-B[4]	40-60	**Sulfuric**			Sulfuric		
		60-150	**Sulfidic**			Sulfidic		

[1.] Soil type label used in this paper when this layer of sulfidic material is used to conduct soil organic matter experiments in 70 ml Falcon tubes.
[2.] Sampling location label used in (Fitzpatrick et al., 2009).
[3.] Sampling location label used in (Fitzpatrick, Shand, & Hicks, 2011).
[4.] Sampling location label used in (Bake, Shand, & Fitzpatrick, 2013).
[5.] Acid sulfate soil horizon (NRCS-USDA, 2014).
[6.] Acid sulfate soil material (NRCS-USDA, 2014).
[7.] Currently no subgroup exists in Soil Taxonomy (NRCS-USDA, 2014) that adequately describes these Finniss River soils following their rewetting. They are best described as subaqueous soils with sulfuric horizons or "Sulfuric subaqueous clayey soils" in accordance with the Australian ASS classification key (Fitzpatrick et al., 2008). Consequently, the following new proposal is currently being submitted by Fitzpatrick and Grealish (personal communication) to USDA-NRCS to consider for inclusion in revised versions of the Keys to Soil Taxonomy is: (i) a new suborder, which would be Wassepts, (ii) with the existence of Wassepts suborder, a great group of Sulfowassepts will need to be proposed within the suborder and (iii) with the existence of Sulfowassepts great group, a subgroup of Typic Sulfowassepts will need to be proposed within the great group.
[8.] Australian acid sulfate soil classification (Fitzpatrick et al., 2008).

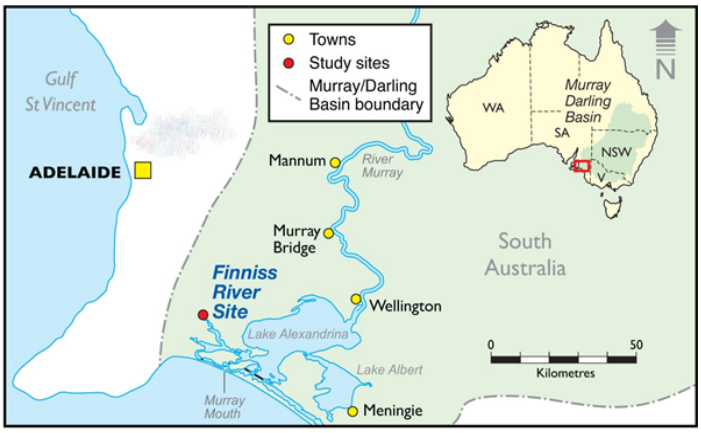

Figure 2. Locality of samples from the Finniss River site at Wally's Landing (Michael et al., 2015)

Table 2

Details of soil treatments with slight modification from Michael et al. (2016)

Amendments	Composition of treatments	
	g/80 g soil	Total N (mg)
Organic matter		
Lucerne hay	1	32
Pea straw	1	12
Wheat straw	1	8
Simple carbon cources		
Glucose	4	
	0.1	
Sodium acetate	4	
Molassess	5	[b]
Simple nitrogen sources		
Sodium nitrate	0.30	50
Ammonium chloride	0.19	50
Urea	0.24	50

[b]Molasses may contain a small amount of nitrogen in the syrup.

weighing. The dried plant materials (hereafter referred to as organic matter) were chopped in an electric blender to pass through a sieve size of 0.5 mm before use. The sulfidic soil material used in the treatments is hereafter referred to as "sulfidic soil" to be consistent with our recent publications (e.g. Michael, 2014, 2017).

On a field-scale, the organic matter applied as amendment (estimated for acre-furrow-slice weighing 1000 tonnes) is between 29.8 (80:1, soil: organic matter) and 149 (16:1) tonnes per ha (Michael et al., 2016). Each treatment was set in triplicates and the tubes arranged in complete randomized design (CRD). Three experiments (Table 2) lasting 6 months were conducted to investigate the changes in soil pH and redox when:

1. Sulfidic soil was mixed with plant materials containing different nitrogen contents and incubated under aerobic and anaerobic conditions.

2. Sulfidic soil was mixed with simple carbon compounds or chopped of lucerne hay and incubated under anaerobic conditions only. The aerobic treatment component was reported as Figure 3 in Michael et al. (2016).

3. Sulfidic soil was mixed with simple nitrogen compounds or chopped lucerne hay and incubated under aerobic and anaerobic soil conditions.

The aerobic and anaerobic treatment conditions were maintained as we described in earlier studies (e.g. Michael, 2015; Michael et al., 2015). The aerobic treatments were maintained under 75% field capacity by adding water on weight basis. The anaerobic treatments were kept flooded (100% field capacity based on initial weight) throughout the study period by adding 50 -100 ml of tap water twice (once in the morning and once in the afternoon) daily as where necessary.

Measurements

Soil pH and Eh were measured within the surface (0−10 mm). Redox was measured using a single Ag/AgCl reference and platinum (Pt) electrode combination using an automated data logger (Michael et al., 2015). The Pt and reference electrodes were inserted into the soil and allowed to equilibrate for 10 min and then Eh measured at 1 min intervals for the next 10 min and averaged (Rabehorst, Hively, & James, 2009). These values were corrected for the reference offset to be relative to the potential of a standard hydrogen electrode by adding 200 mV, and the stability and accuracy of the electrodes were maintained as per Fiedler et al. (2007). Redox conditions of the experimental soils are categorized as: (i) oxidized (≥+300 mV), (ii) moderately reduced (+300 to 0 mV), (iii) reduced (0 - -100 mV) and (iv) highly reduced (-100 - -400 mV) in reference to the change in surface environments shown in Figure 3.

The pH was measured using a 2 g soil (1:5, soil: water w/w) with a pre-calibrated Orion pH meter (720SA model) as described (Michael et al., 2015). The results were compared to the Eh-pH range of surface environment shown in Figure 3. The mechanisms responsible for the changes

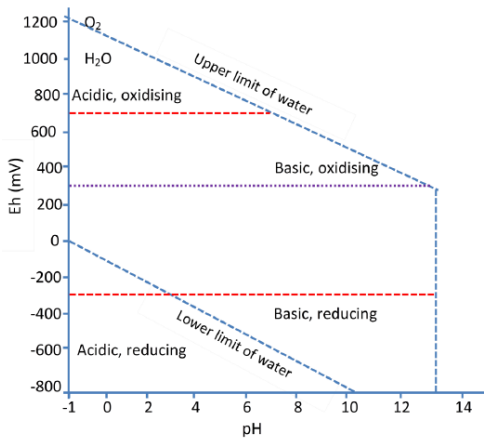

Figure 3. The Eh-pH range in surface environments showing four redox classes: (i) acidic-oxidizing, (ii) basic-oxidizing, (iii) acidic-reducing and, (iv) basic-reducing (adapted with slight modifications from Krauskopf (1967) as per (Delaune & Reddy, 2005; Poch et al., 2009). The lower and upper Eh limits are shown by the red dotted lines. The purple dotted line shows the break between an aerobic and anaerobic condition (Fiedler et al., 2007)

in pH or redox measured are discussed in the last section, based on the observation that the mechanisms involved in inducing the changes in the two soil properties seem to be the same under the two soil moisture regimes. Under aerobic soil conditions, the changes in pH and redox of studies shown in Figures 4 and 6 are inconsistent. This is thought to be caused by the type of microbial ecology that was present in the soil prior to or was established during the study that was capable of utilizing the residual organic matter content (10.6%).

Statistical Analyses

Data from the surface soil (0−10 mm) are presented in this paper. The Eh values obtained over a 10 mins-period were averaged and a treatment average obtained by taking the mean of the three replicates. These values were corrected for the reference offset to be relative to the potential of a standard hydrogen electrode by adding 200 mV (Fiedler et al., 2007). Similarly, treatment average pH was obtained by taking the mean of the three replicates. To compare the treatment means, significant differences ($p<0.05$) between treatments means of profile was determined by two-way ANOVA (Michael et al., 2017) using statistical software JMPIN, AS Institute Inc., SAS Campus Drive, Cary, NC, USA 27513.

RESULTS AND DISCUSSION

Roles of Organic Matter Derived Soil Nitrogen on Sulfidic Soil pH and Redox

The long-term (6 months) effects on sulfidic soil pH and redox measured following the addition of organic matter with varying nitrogen content are shown in Figures 4 and 5. Under aerobic conditions, the unamended sulfidic soil strongly acidified, the pH declining to near 4 (Figure 4). In the amended treatments, lucerne hay and pea straw significantly ($p<0.05$) prevented the soil from acidifying and increased the pH to well over 8, whereas with wheat straw, the pH fell but not as much as in the control. The pH changes were correlated with changes in Eh. Lucerne hay addition significantly reduced the redox to near 100 mV and the other two amended soils were higher but lower than 300 mV. The overall increase in pH caused by addition of organic matter was 1.5, and changes in Eh ranged from between 100 − 300 mV (Figure 4).

Under anaerobic soil conditions, the pH of all the amended soils were increased compared to the initial pH but the increase was lower than 8, similar to the control soil which decreased to near 6 (Figure 5). The effects of the varying nitrogen content of the organic matter on pH were quite similar among the treatments, and not distinct as observed under aerobic conditions. The only dominant effect was the low Eh produced by the amendments (Figure 5). Eh of the control soil under the anaerobic conditions was near 40 mV, compared to the 475 mV under aerobic condition (Figure 4). The overall changes in pH in all the organic matter amended treatments was 0.5, the change in lucerne hay treatment being slightly higher. The reduction in redox caused by organic matter amendment was insignificant except in the lucerne hay amended treatment, ranging from between -29−-14 mV (lucerne hay>pea straw>wheat straw). These results point out that organic matter plays an important role in reducing the redox, thereby buffering sulfidic soil material oxidation.

Roles of Surface Soil Nitrogen in Regulating pH and Redox of Sulfidic Soil

The studies presented in Figures 4 and 5 assessed the role of nitrogen derived from organic matter. Organic matter of plant material origin is complex and is a source of multiple metabolic substrates for soil microbes. The organic matter being a source of multiple nutrients including carbon made the roles of the organic matter derived nitrogen inconclusive. This inconclusiveness was addressed by the studies presented in Figures 6 and 7 following the addition of simple nitrogen sources that contain nitrogen alone, nitrogen and carbon, or nitrogen and other ions. Lucerne hay as organic matter of high nitrogen content was included to compare the results.

The control soil did not strongly acidify under the aerobic condition and the decrease in pH was by 0.7 (Figure 6), contrasting the change in pH of the control soil shown in Figure 4. In the amended soils, urea addition strongly increased the pH to nearly 8, and nitrate and ammonium lowered it to near 6. The changes in pH were correlated with the changes in redox. The control soil was moderately reduced to near 100 mV, agreeing to the circumneutral pH of 6, and the amended soils were reduced to below -50 mV (Figure 6).

The highest reduction in redox was caused by lucerne hay amendment, significantly reducing the Eh to -160 mV. The changes in pH and Eh measured under anaerobic conditions are shown in Figure 7. The control soil pH was stable compared to the initial pH, similar to the small increase of the study shown in Figure 8. In the amended soils, urea increased the pH to 7.8, and nitrate and ammonium moderately lowered the pH to below 6, compared to the changes in the control soil (Figure 7). The changes in pH measured were in agreement with the changes in Eh, which were mainly reduced, ranging from between -50 − 50 mV. These changes are, again, within the range measured in the other studies like

that shown in Figure 5 under the same soil conditions. The overall results show the treatment conditions employed were capable of inducing similar type of changes in the soil properties measured.

The Roles of Surface Soil Carbon in Regulating pH and Redox of Sulfidic Soil

The studies presented in Figures 4 and 5 show the roles of organic matter derived nitrogen in regulating the pH and redox of sulfidic soil. These results were compared to the studies shown in Figures 6 and 7. The results obtained following the addition of the simple nitrogen sources pointed out the roles of nitrogen in regulating soil chemistry but significant changes were induced by the compounds containing both nitrogen and carbon, e.g. urea (Figure 6). So the roles of carbon of the organic matter of plant materials or the simple nitrogen compounds, e.g. urea, were not clear. Therefore, the study shown in Figure 8 was conducted to investigate the roles of soil carbon in regulating pH and redox of sulfidic soil under anaerobic soil conditions. The aerobic component was presented as Figure 3 in Michael et al. (2016).

Under the anaerobic conditions, the control soil pH was raised just above the initial pH (Figure 8). Among the simple carbon compounds, acetate increased the pH above 7 and glucose and molasses strongly lowered it, well below 5. The increase in pH induced by organic matter amendment was high as in the other experiments. Under the anaerobic conditions, all the treatments soils were reduced in agreement with the changes in pH, with glucose and molasses recording the highest Eh values (Figure 8).

The Mechanisms Regulating The Changes in pH and Redox in Sulfidic Soil

As initially pointed out, the changes measured in the two soil properties seem to be regulated by the same mechanism. Under aerobic soil conditions, the control sulfidic soil strongly acidified as a result of sulfidic soil oxidation (Figure 4). This did not happen in the study shown in Figure 6. The explanation for this seems to come from the microbial ecology present that was capable of oxidizing the residual organic carbon content (10.6%) and generate alkalinity. Studies elsewhere show these types of variations in changes in soil chemistry are caused by the type of microbial ecology present capable of generating microbial alkalinity prior to or established following the addition of metabolic substrates (Condron, Stark, O'Callaghan, Clinton, & Huang, 2010; Kuzyakov, Friedel, & Stahr, 2000). The increase in pH caused by organic matter addition was closely correlated with reduction in Eh, consistent with the involvement of sulfur-reducing bacteria (Michael et al., 2016). These bacteria are unable to function under aerobic conditions (Hamilton, 1998), so the reduction in Eh to below 0 mV (e.g. Figure 6) was caused by depletion of oxygen by aerobic bacteria capable of using the organic matter as metabolic substrate (Michael et al., 2015; 2016). Addition of refined carbon sources,

e.g. glucose, did not generate these reducing conditions, even under the anaerobic soil conditions (Figure 8), implying this carbon source was 'recalcitrant' to microbial breakdown (Marschner et al., 2008). When refined nitrogen sources were added, the Eh was reduced but the level of reduction was smaller than the organic matter, both under aerobic (Figure 6) and anaerobic (Figure 7) soil conditions. Some bacteria are able to use acetate and nitrate as the energy sources with the net consumption of protons (Thauer, Zinkhan, & Spormann, 1989). This seems to be the reason nitrate reduced the soil and sustained the pH around circumneutral level under aerobic soil condition (Figure 6).

Under anaerobic conditions, the surface soil often experiences frequent fluctuation in the amount of water that ponds on the surface. In this study, this was prevented from happening by ensuring a sufficient amount of water (5−10 cm) was ponding on the surface by adding 50-100 ml of tap water daily. Consequently, the soil was expected to remain moderately reduced (300−0 mV) to reduced (0 − -100 mV) (Fiedler et al., 2007; Ponnamperuma,1972). In the presence of a suitable metabolic substrate, a certain degree of reduction (-100 − -400 mV) is expected because of microbial oxidation of the resources (e.g. carbon and nitrogen) and reduction reaction of the anaerobic soil condition caused by flooding, sufficient to sustain the alkalinity (Michael, 2014; 2015; Sarwani, Shamshuddin, Ishak, & Husni, 2006). In almost all cases, the control soils remained moderately reduced because of the reducing conditions of flooding (Figures 5, 7 and 8). In the amended soils, glucose and molasses moderately reduced the soil and caused the pH to drop to nearly 3 and 4, respectively (Figure 8). The reduction in pH caused by glucose seemed to have resulted from microbial breakdown of the glucose into fatty acid as observed in an alkaline sodic soil (Chorom, 1996). In the other treatments, the additions reduced the soils (Figure 6), causing the pH to remain near the circumneutral levels (pH 5 − 6).

The differences in the changes in the soil properties measured are significant to deduce that the changes were dependent on the type of microbial ecology the metabolic substrates as resources were capable of establishing. The refined carbon and nitrogen sources, for instance, are simple nutrient sources and are only capable of engaging a simplified microbial ecology capable of causing a smaller change in soil properties. For instance, glucose ($C_6H_{12}O_6$) as a sole carbon source moderately reduced the redox and lowered the pH even under anaerobic soil condition whereas addition of acetate ($C_2H_3NaO_2$) containing carbon and sodium reduced the soil and increased the pH (Figure 8). It was clear too that simple nitrogen compound such as urea (CH_4N_2O) containing both carbon and nitrogen increased the pH under the two soil moisture conditions to 8. These are strong indications that multiple nutrient sources are needed by the soil microbes to generate alkalinity sufficient to induce observable changes in soil chemistry (Michael, 2017).

The organic matter being complex plant materials and source of multiple nutrient employed complex microbial community which highly reduced the soils and increased the pH, even under aerobic soil conditions where sulfides were expected to oxidize and strongly acidify the sulfidic soil materials (Figure 4).

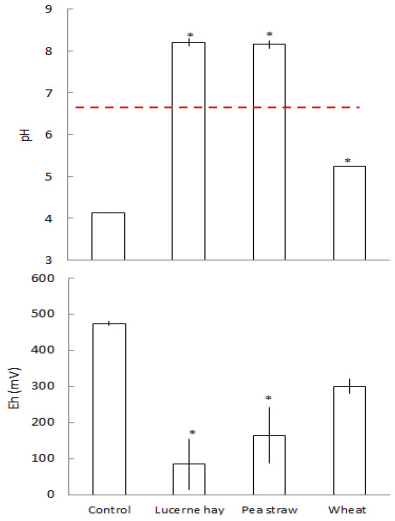

Figure 4. Changes in pH and Eh of sulfidic soil mixed with plant material containing different nitrogen contents. The treatments were incubated under aerobic soil conditions (75% field capacity) for 6 months. Each value is the mean ± s.e. of three measurements. The dotted line is the initial pH. Asterisks indicate significant differences ($p<0.05$) between treatment and control at each depth

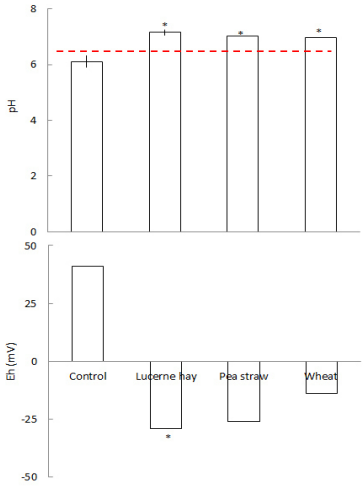

Figure 5. Changes in pH and Eh of sulfidic soil mixed with plant material containing different nitrogen contents. The treatments were incubated under anaerobic (flooded) soil conditions for 6 months. Each value is the mean ± s.e. of three measurements. The dotted line is the initial pH. Asterisks indicate significant differences ($p<0.05$) between treatment and control at each depth

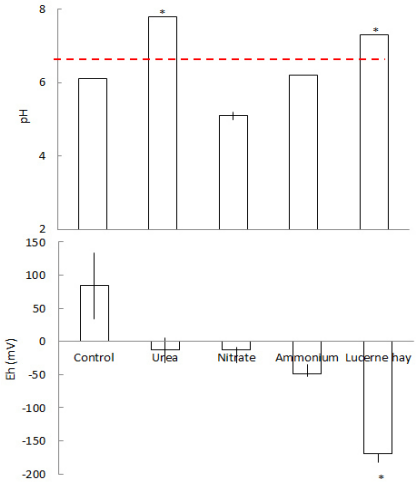

Figure 6. Changes in pH and Eh of sulfidic soil mixed with simple nitrogen compounds or chopped of lucerne hay. The treatments were maintained under aerobic soil conditions (75% field capacity) for 6 months. Each value is the mean ± s.e. of three measurements (n=3). The dotted line is the initial pH. An asterisk indicates significant difference ($p<0.05$) between treatment and control at the same depth

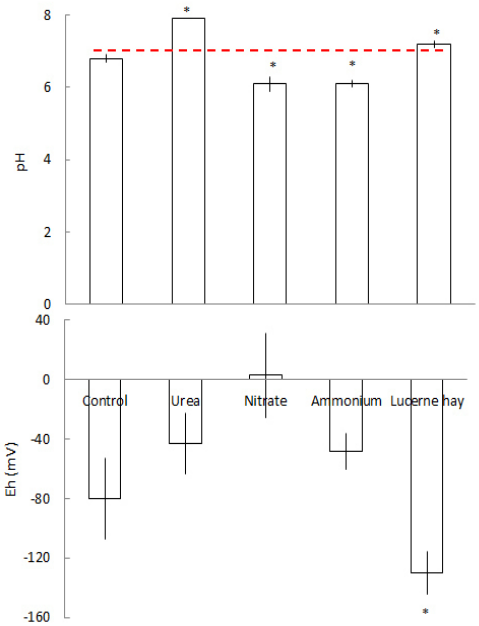

Figure 7. Changes in pH and Eh of sulfidic soil mixed with simple nitrogen compounds or chopped of lucerne hay. The treatments were maintained under anaerobic (flooded) soil conditions for 6 months. Each value is the mean ± s.e. of three measurements (n=3). The dotted line is the initial pH. An asterisk indicates significant difference ($p<0.05$) between treatment and control at the same depth

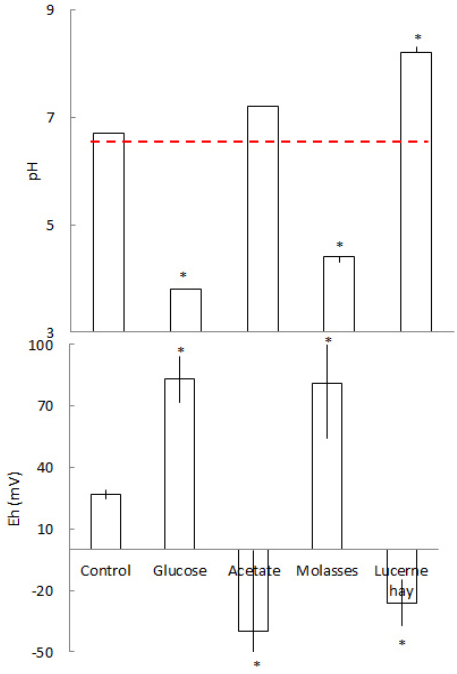

Figure 8. Changes in pH and Eh of sulfidic soil mixed with simple carbon compounds or chopped of lucerne hay. The treatments were maintained under anaerobic (flooded) soil conditions for 6 months. Each value is the mean ± s.e. of three measurements (n=3). The dotted line is the initial pH. An asterisk indicates significant difference ($p<0.05$) between treatment and control at the same depth

CONCLUSIONS

Organic matter as a source of multiple metabolic substrates for soil microbes significantly prevented sulfidic soil oxidation under aerobic condition and sustained the sulfidic soil material alkalinity (pH>4) under anaerobic condition, respectively. Addition of glucose as a source of carbon alone resulted in oxidization of the sulfidic soil even under anaerobic conditions and led to strong acidification (pH<4). Metabolic compounds like acetate and urea containing both carbon and nitrogen had the opposite effects on soil redox and pH. The mechanisms responsible for the changes in soil chemistry measured appear to be dependent on the type of microbial ecology engaged by the metabolic substrates. Organic matter being a source of multiple metabolic substrates effectively established a complex microbial ecology that was capable of generating significant soil alkalinity, reducing the

redox and increasing the sulfidic soil pH. The single metabolic substrate sources had no significant effect on redox and pH, dependent on the types of microbial ecology establishment by these resources being limited to the single nutrients. The findings of this study have implications for management and general use of the surface soils of sulfidic soils under a range of soil moisture regimes.

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Dried *Garcinia atroviridis* Crude Extracts Incorporated with Gum Arabic Coating Controlled Postharvest Anthracnose Diseases in Dragon Fruits

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ABSTRACT

The aim of this study was to examine the antifungal effect of dried *Garcinia atroviridis* fruits extract (GAFE) in combination with gum arabic (GA) against red-fleshed dragon fruits (*Hylocereus polyrhizus*) anthracnose disease caused by *Colletotrichum gloeosporioides* as well as to determine the efficiency of dried GAFE in maintaining the postharvest quality of dragon fruits. *In vitro* results showed that the inhibition in mycelial and conidial growth was dose-dependent and greatest inhibition was recorded at 15 mg/mL dried GAFE+10% GA. Such concentration also conferred fungistatic effects to fungal growth and spore germination *in vitro*. Concentration at 10 mg/mL dried GAFE+10% GA exhibited similar effectiveness comparable to commercial fungicide (Mancozeb at 3.2 mg/mL) to control anthracnose disease in dragon fruits. In addition, fruits treated with 10 mg/mL dried GAFE+10% GA markedly suppress disease incidence and severity as well as effectively maintained SSC and TA level throughout cold storage. On the other hand, lower dried GAFE concentrations (1 mg/mL and 4 mg/mL dried GAFE+10% GA) suppressed weight loss and retained tissue firmness throughout shelf life of dragon fruits. Coating application at highest concentration of 15 mg/mL dried GAFE+10% GA impaired physicochemical quality by displaying phytotoxic effect on the dragon fruits.

Keywords: *Colletotrichum gloeosporioides,* dried *Garcinia atroviridis*, gum arabic, *Hylocereus polyrhizus*, postharvest quality

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INTRODUCTION

The red-fleshed dragon fruits (*Hylocereus polyrhizus*) is cultivated in Southeast Asian countries including Malaysia (Nguyen, 2006). This rare fruits attracted worldwide attention due to the their prominent purple-red colour, economic importance as a food source and antioxidant properties associated with its rich betacyanin source (Le Bellec, Vaillant, & Inbert, 2006). Initially introduced at the end of the 1990's in Perak, the cultivation has expanded enormously around 927.4 ha in 2006 with a grand total of produce about 2534.2 tons (Masyahit, Sijam, Awang, & Mohd Satar, 2009).

Besides abiotic conditions such as chilling injury, mechanical injury and moisture loss, pests and diseases infestation can also affect dragon fruits cultivation and storage (Cheah & Zulkarnain, 2008). Recently, *Colletotrichum gloeosporioides* has caused a severe threat to the cultivation of the fruits due to suitable environmental condition for the growth of the fungus (Masyahit et al., 2009). The symptomatic characterizations of anthracnose on dragon fruits are reddish-brown spots and chlorotic haloes that appear on the fruit and stem, which coalesced to rot (Masyahit et al., 2009). This pervasive fungus contributed to more than 50% postharvest losses in commodities (Paull, Nishijima, Reyes, & Cavaletto, 1997).

Gum arabic (GA), a dried adhesive exudate from the stems and branches of *Acacia* species has been developed into edible coatings and used as carriers of antimicrobial constituents through their incorporation with antimicrobial natural products to delay ripening and extend the shelf life of fruits such as papaya and banana (Ali, Cheong, Zahid, 2014; Maqbool et al., 2011). Additionally, this natural biopolymer provides a physical and chemical barrier against the growth of microbes on the fruit surface (Ali, Maqbool, Ramachandran, & Alderson, 2010).

Traditionally, synthetic fungicides such as Propineb and Difenoconazole are used to control anthracnose caused by *Colletotrichum* species on several fruits and vegetables (Hoa, 2008). Nevertheless, consumers' trend of a healthier lifestyle demands the fresh produce to be free from synthetic fungicide. Applying single fungicide continuously on fresh fruits and vegetables could develop resistant fungal strains and decrease the effectiveness of fungicide against the target organisms (Edirisinghe, Ali, Maqbool, & Alderson, 2014; Maqbool et al., 2011).

Additionally, some microbial growth on fresh produce has been controlled by using various chemicals-based washing and sanitizing agents such as chlorine (Ali, Goh, & Yeoh, 2016). Despite their antimicrobial effect on fruits, its use has been restricted due to their carcinogenicity, long degradation time and high residual toxicity which cause serious effect to human health and ecosystem (Zahid, Maqbool, Siddiqui, Manickam, & Ali, 2015). Alternative approach using natural products such as plant parts (fruit, leaf, seed, root and pulp) which confer antimicrobial properties due

to their bioactive secondary metabolites are used to control several phytopathogens (Tripathy & Dubey, 2004).

Garcinia atroviridis Griff. ex T. Anders, a medium-sized tree, which is common in Peninsular Malaysia especially in the northern states is one medicinal plants with significant antimicrobial activity. Apart from its culinary use, the sun-dried slices of the fruits are also used in folkloric medicine such as a post-partum medication and weight management on a short-term basis, and also rich in antioxidants and other secondary metabolites such as xanthone, benzophenones, flavonoids, biflavanoids (Minami, Takahashi, Kodama, & Fukuyama, 1996; Waterman & Hussain, 1983) and lactones and phenolic acid (Joseph, Jayaprakasha, Selvi, Jena, & Sakariah, 2005) which are beneficial for human health (Nursakinah et al., 2012).

Additionally, i*n vitro* studies of dried *G. atroviridis* showed their wide antibacterial effects against *Bacillus subtilis* B28 & B29, MRSA, *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* as well as antifungal properties against phytopathogen *Clasdosporium herbarum* (Mackeen, Ali, & Lajis, 2000) while being non-cytotoxic in mammalian cells (Mackeen et al., 2012). However, the antifungal effect of dried *G. atroviridis* to control postharvest anthracnose in dragon fruits caused by plant pathogen *C. gloeosporioides* has not been reported.

Considering the antifungal properties of *G. atroviridis* and the fact that it is generally regarded as safe (GRAS), biodegradable and environmentally friendly, the study examined the antifungal effect of dried *G. atroviridis* fruits extract (GAFE) in combination with GA against dragon fruits anthracnose disease caused by *C. gloeosporioides*. Finally, the efficacy of dried GAFE in maintaining the postharvest qualities of dragon fruits was further evaluated.

MATERIALS AND METHODS

Materials

Red-fleshed dragon fruits were purchased from a local supplier in Semenyih, Selangor, Malaysia. Fruits were chosen and assorted to be unvarying in size, colour, and shape, free from diseases, blemishes and physical injury. Fruits were washed with distilled water and air-dried at ambient temperature (25±1 °C) before coating treatment. Sun-dried *Garcinia atroviridis* was obtained from a local supplier in Ipoh, Perak, Malaysia while gum arabic powder was purchased from Sigma-Aldrich, USA.

Isolation and Identification of *C. gloeosporioides* from Dragon Fruits

Fungal Isolation and Preparation of Cultures on PDA. *C. gloeosporioides* was isolated from infected dragon fruits exhibiting definite anthracnose symptoms. Small slices of the infected fruit with disease lesions were excised using a scalpel. Surface sterilization was conducted by dipping excised tissue in 1% sodium hypochlorite solution and 70% ethanol for 2 min each, followed by 3x washing in distilled water.

The washed tissue was dried on a tissue paper, plated on Potato Dextrose Agar (PDA) and incubated at 25±1 °C for one week. The mycelial growth was observed and the colonies were re-isolated on fresh PDA plates to obtain pure cultures. The isolates were identified as *C. gloeosporioides* based on their morphological and cultural features (Barnett & Hunter, 1972) and preserved for further use.

Preparation of Crude *Garcinia atroviridis* Dried Fruits Extract

The crude extract was obtained by maceration using methanol as suggested by Mackeen et al. (2000) with slight modifications. Sun-dried sample was oven dried at 45 °C for 72 hours to remove moisture completely prior to grinding the materials into powdered form. The sample was pulverized using rotor beater mill. Approximately 500 g of the pulverized sample was macerated in 800 mL methanol for three days. Three times extraction was performed until the powerderd sample looked pale or white. The extract solution was pooled after 3x extraction before filtration. The filtered pooled solution was concentrated by evaporation under reduced pressure at 45 °C in water bath using a rotary evaporator (BUCHI Rotavapor R-200) to obtain crude extract. The extract was then kept at 4 °C until further use.

Preparation of Gum Arabic Solution

Gum arabic (10%) was prepared by dissolving 100 g of gum arabic powder in 1 L distilled water. The gum arabic solution was stirred for 60 min at 40 °C. The solution was then filtered through three layers of muslin cloth to remove undissolved impurities.

In vitro Antifungal Assay of Dried GAFE+10% GA Against *C. gloeosporioides* Antifungal Assay

The antifungal efficacy of crude extracts was determined by poisoned food technique based on inhibiton of radial mycelial growth and conidial germination of *C. gloeosporioides* (Balamurugan, 2014). PDA was amended with different concentrations of dried GAFE incorporated with 10% GA - 1 mg/mL, 4 mg/mL, 10 mg/mL and 15 mg/mL. The 10% GA and PDA was prepared separately in the ratio of 1:7. The amended PDA media was allowed to solidify prior to antifungal assay. Three control lines were used: PDA plates with commercial fungicide Mancozeb (3.2 mg/mL), PDA plates with 10% gum arabic as positive control and plates containing solely PDA as negative control. Mycelial plugs (7 mm) of *C. gloeosporioides* were placed at the centre of PDA plates and were incubated at 25±1 °C. *In vitro* assays were carried out in three replicates for each treatments including control.

Radial Mycelial Growth. Radial mycelial growth was measured daily until the control dishes reached the edge of the plate. The percentage inhibition in radial growth (PIRG) was recorded after 8 days of incubation according to the formula described by Sivakumar, Hewarathgamagae,

Wijeratnam and Wijesundera (2002).

Inhibition of Conidial Germination. Conidial germination inhibition test was carried out using the cavity slide technique (Cronin, Yohalem, Harris, & Andrews, 1996). An aliquot of 100 µl of the spore suspension was pipetted onto each treatment plates including of controls (diluted to 10^5/mL conidia concentration using a hemocytometer). After 48 hours of incubation, the conidia were killed by adding 10 µl 2% sodium azide to each cavity. Approximately, 100 conidia per replicate were observed for germination in each treatment. The conidium was presumed to be germinated when the germ tube was partial or more than the length of the conidium. The percentage of inhibition in germination was calculated:

% germination inhibition = 1- (Gr/Gc) x 100 [1]

where,

Gr = Number of spore germination in the treatment;

Gc = Number of spore germination in the control

Scanning electron microscopy on spore and mycelial structure

Four spores and mycelial samples (1.5 mm^3) were taken from all the treatments including control and mounted on aluminium stubs and viewed and photographed under the scanning electron microscope (Model Quanta 400 FESEM, FEI Company, USA).

In vivo Antifungal Assay of Dried GAFE+10% GA Against *C. gloeosporioides*

Preparation and Coating Application on Fruits. A stock solution of GAFE crude extracts was prepared at 33.9 mg/mL by dissolving the extracts with 10% GA. The working concentrations (1 mg/mL, 4 mg/mL, 10 mg/mL and 15 mg/mL) were prepared by adding 10% GA to the stock solution until a final volume of 1.5 L (volume enough to completely immerse the fruit). The working concentrations were homogenized for 30 min prior to use. Fruits were washed with 0.01% sodium hypochlorite, rinsed with distilled water and left to dry at 25±1 °C. Dragon fruits were immersed for 1 min in *C. gloeosporioides* spore suspension (10^5 spore/mL) and air-dried.

Subsequently, fruits were immersed in working concentrations (1 mg/mL, 4 mg/mL, 10 mg/mL, 15 mg/mL) for 1 min and air-dried fully. For negative and positive controls, fruits were immersed in spore suspension (10^5 spores/mL) and commercialized fungicide solution Mancozeb (3.2 mg/mL concentration) for 1 min and air-dried fully. Prior to cold storage, dragon fruits were packed in cardboard carton boxes and stored at 10±1 °C, 80±5% RH.

Disease Incidence and Disease Severity. The disease incidence (DI) and disease severity (DS) was measured at 7 days interval for 21 days. Data for DI were determined based on the percentage of fruits exhibiting anthracnose out of the

total amount of fruits per treatment (Cooke, 2006). DS was scored using a scale as per the method of Sivakumar et al. (2002) with some modification.

A scale of 0 was given when there was no sign of disease on the fruit surface. Scales 1, 2, 3 and 4 were given when the surface of the fruits were infected up to 25, 50, 75 and >75% with symptoms of anthracnose. Experiments were conducted in three replicates with 5 fruits per treatment (n=15).

Postharvest Physicochemical Fruit Quality Assessment

A digital balance (Model GF-6100, Japan) was used to weigh fruits from each treatment at day 0 and at 7 days intervals for 21 days. Total weight loss during storage intervals was measured by subtracting the initial and final fruit weight. Weight loss was expressed as percentage weight loss. The firmness of the fruit was measured using an Instron texture analyzer with an 8 mm plunger tip (Instron 5540, USA). The fruit was subjected to a puncture test at a constant speed of 20 mm/min on three points along the surface of the fruit.

Dragon fruits were cut into small pieces and the pulp was homogenized in a kitchen blender by grinding 20 g of the dragon fruit pieces in 80 mL distilled water. After blending, the filtrate (juice) was filtered using a muslin cloth. Soluble solid content (SSC) of dragon fruit pulp was analyzed using a Palettle Digital Refractometer (Model PR-32α; Atago Co, Ltd. Japan). An aliquot of 100 µl of the filtrate was pipetted onto the prism glass of the refractometer to obtain the reading. The refractometer was calibrated with purified water before analysis. The readings were multiplied by the dilution factor to obtain the SSC of the dragon fruits pulp.

Titratable acidity (TA) was measured using the remaining filtrate of SSC. A 5 ml aliquot of filtrate was titrated with 0.1 N NaOH until the pH reached an endpoint pink 8.1. The results were expressed as the percentage citric acid per 100 g of fresh weight. Physical and biochemical assessments were conducted in three replicates with 5 fruits per treatment (n=15).

Statistical Analysis

Experiments were conducted in a completely randomized design. Experimental data were analyzed using the analysis of variance (ANOVA) procedure in Genstat 16 version (VSN International Ltd, UK). The means separation was done using Fisher's unprotected LSD at $p<0.05$.

RESULTS

In vitro* Antifungal Assay of Dried GAFE+10% GA Against *C. gloeosporioides

The efficacy of dried GAFE to control mycelial and conidial growth was dose dependent. Plates with combined treatment of 15 mg/mL dried GAFE+10% GA exhibited the greatest fungistatic inhibition effect against *C. gloeosporioides* radial mycelial growth (Figure 1). Dried GAFE at 15 mg/mL, 10 mg/mL, 4 mg/mL and Mancozeb treated plates showed significant

fungistatic effect (p<0.05) of up to 70.26, 61.29, 30.16 and 55.5% respectively compared to negative control (only PDA) after 10 days. Based on Figure 1, the inhibitory effect on mycelial growth was not significant in the positive control of PDA (only 10% GA) indicating that GA does not confer antifungal effect.

The effect of the dried GAFE in inhibiting conidia germination was also studied. Spores from negative control (PDA only) and positive control plates (PDA+10% GA) germinated and grew to form long hyphae (germ tube) (Figure 2a). In contrast, the crude extract markedly inhibited conidial germination after 48 hours of incubation in dark (Figures 2b and 2c). The efficacy of the extract to inhibit conidial germination was also dose dependent and was highest effects were at 15 and 10 mg/mL dried GAFE+10% GA.

Based on the conidial germination assay, scanning electron microscopy (SEM) was performed to examine the mode of action of dried GAFE on morphology of *C. gloeosporioides* fungal hyphae. Figure 3a showed that fungal hyphae in the control plates appeared elongated and normal meanwhile fungal hyphae in GAFE-treated plates were shrunk, wrinkled and distorted as compared to the control (Figure 3b).

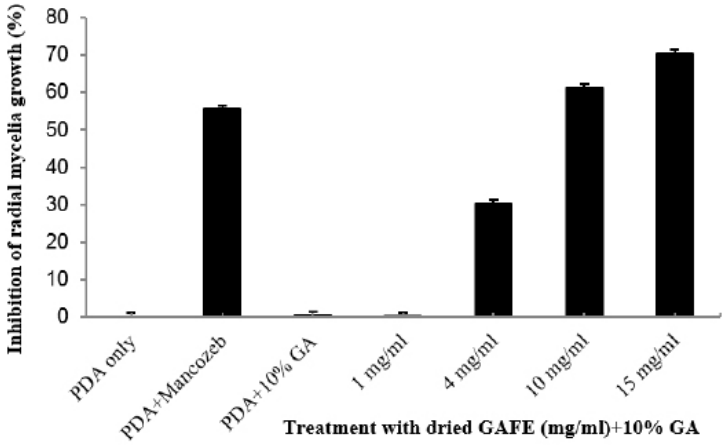

Figure 1. Inhibition percentage of dried *Garcinia atroviridis* fruit extract (GAFE) incorporated with 10% gum arabic (GA) on *C. gloeosporioides* mycelial growth with respect to the negative control after 8 days at ambient temperature (25±1 °C). The vertical bars represent standard error of means for three replicates

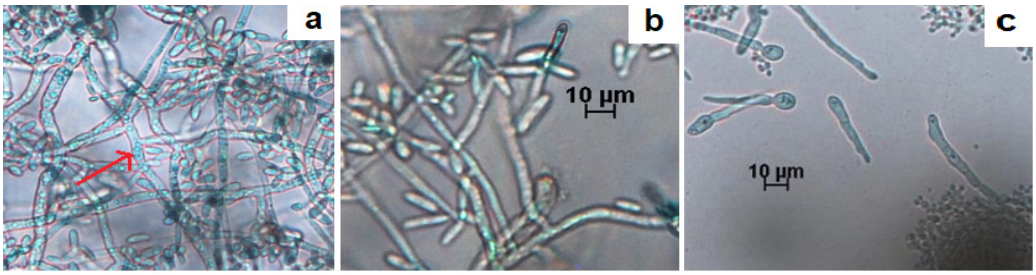

Figure 2. Inhibition of conidia germination (a) in the Potato Dextrose Agar (PDA) control and PDA+10% gum arabic (GA) plates (b) 1 mg/mL+10% GA and 4 mg/mL+10% GA (c) 10 mg/mL+10% GA and 15 mg/mL+10% GA after 48 hours incubation in the dark. Red arrow indicates elongated form of germ tub

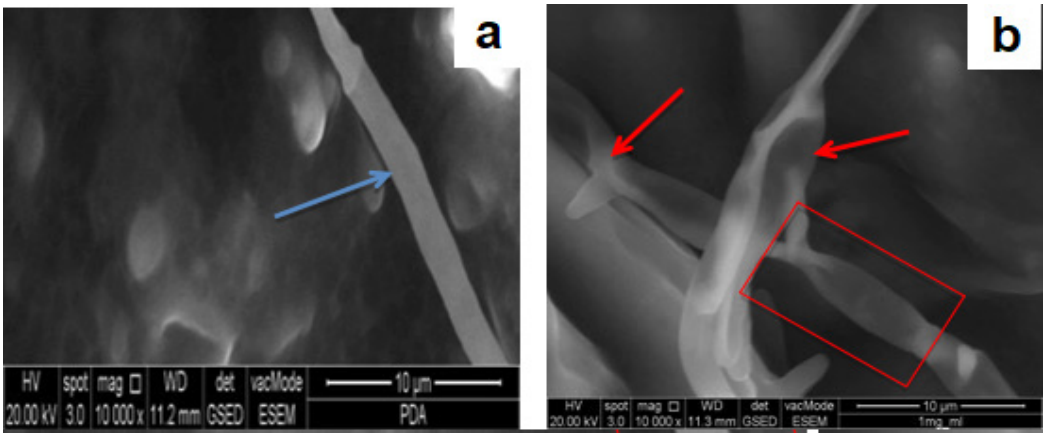

Figure 3. Scanning electron micrographs (SEM) of *C. gloeosporioides* spore and mycelial structure. Fungal hyphae in (a) control plates and (b) plates treated with dried GAFE+10% GA. Blue arrow indicates normal, elongated and healthy hyphae. Red arrows indicate shrunken, wrinkled and swollen hyphae while red box shows distortion of hyphae

Disease Incidence and Disease Severity

There were no significant effects (p>0.05) of dried GAFE treatments on disease incidence (DI) and disease severity (DS) after 21 days of cold storage. Irrespective of treatments, DI and DS increased significantly throughout storage, however DI and DS were the lowest at 10 mg/mL dried GAFE+10% GA as compared to the control (Figure 4a and 4b). The efficacy of 10 mg/mL dried GAFE+10% GA was also comparable to commercialized fungicide (Mancozeb) in controlling anthracnose disease in dragon fruits.

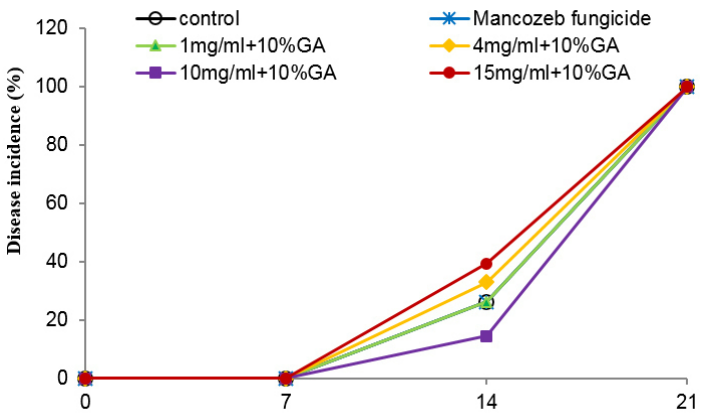

Figure 4(a). Effect of dried GAFE in combination with 10% GA on disease incidence during 21 days of cold storage at 10±1 °C. Vertical bars indicate standard error of means for three replicates

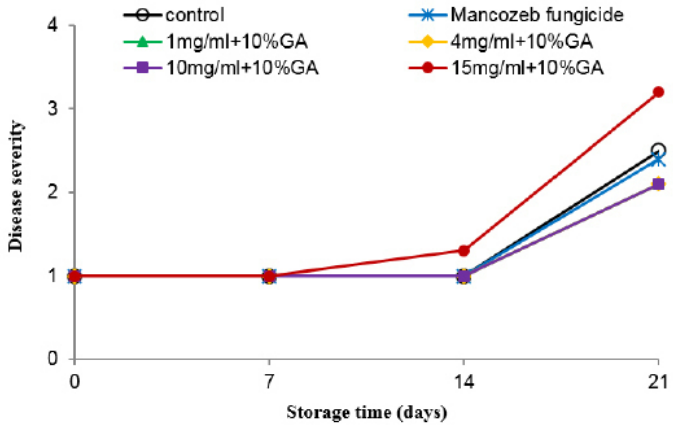

Figure 4(b). Effect of dried GAFE in combination with 10% GA on disease severity of dragon fruits caused by *C. gloeosporioides* during 21 days of cold storage at 10±1 °C. Vertical bars indicate standard error of means for three replicates

Postharvest Physicochemical Fruit Quality Assessment

Weight loss of dragon fruit was significantly affected ($p<0.05$) by GAFE and GA treatments throughout cold storage (Figure 5a). The lowest weight losses were observed in control fruits (4.5%) on final day of storage. Meanwhile, highest doses of GAFE at 15 and 10 mg/mL+10% GA experienced highest weight loss of 5.9% and 5.7% respectively (Figure 5a). There were no significance differences ($p>0.05$) between

all dried GAFE the treatments on weight loss and regardless the treatment, the weight loss increased significantly (p<0.05) until the end of storage periods.

The firmness of dragon fruit declined gradually throughout cold storage (Figure 5b). Fruits treated with 15 and 10 mg/mL dried GAFE+10% GA experienced lowest firmness loss with 25% and 24% respectively at the end of storage. In contrast, higher firmness loss of 29% and 27% were recorded in control fruits and 1 mg/mL dried GAFE+10% GA treated fruits respectively (Figure 5b). Nevertheless, there was no significance difference (p>0.05) amongst dried GAFE treatments on tissue firmness throughout cold storage.

SSC of dragon fruits stored at cold storage were slightly reduced but there were no significance difference (p>0.05) between control and GAFE treated-fruits (Figure 5c). Fruits treated with 10 mg/mL dried GAFE+10% GA maintained higher SSC compared to other treatments throughout storage. As ripening takes place, an increment in SSC was coincident with TA decline. Fruits treated with extract at 10 mg/mL incorporated with 10% GA recorded highest acidity value compared to the control (Figure 5d). However there was no significant difference (p>0.05) amongst GAFE treatments throughout storage.

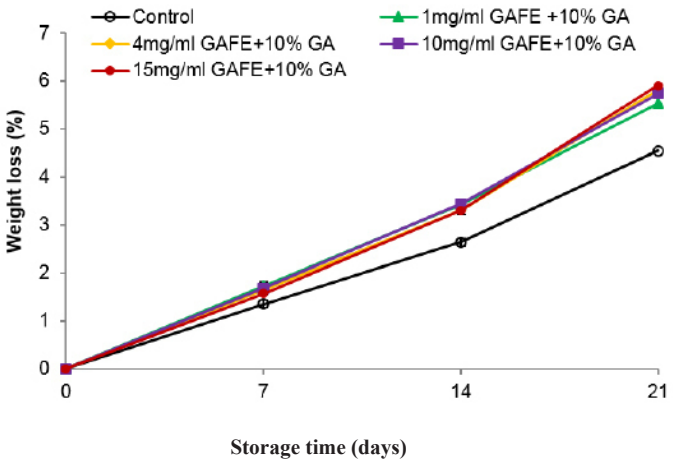

Figure 5. Effect of the dried GAFE incorporated with 10% GA on weight loss of dragon fruits during 21 days of cold storage at 10±1 °C. Vertical bars indicate standard error of means for three replicates

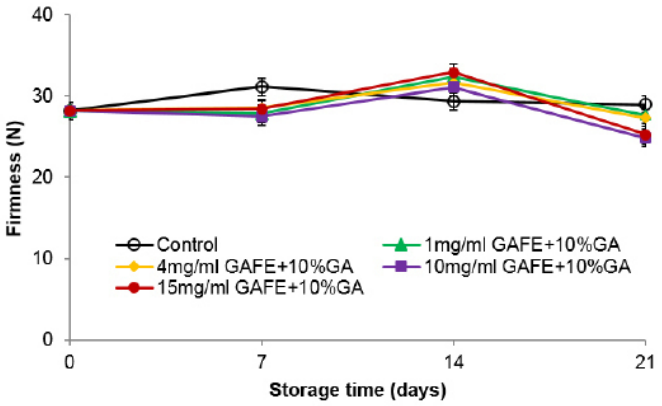

Figure 5. Effect of the dried GAFE incorporated with 10% GA on tissue firmness of dragon fruits during 21 days of cold storage at 10±1 °C. Vertical bars indicate standard error of means for three replicates

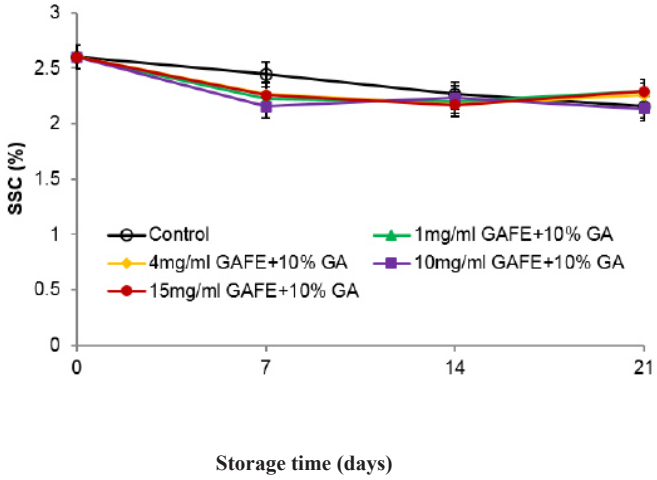

Figure 5. Effect of the dried GAFE incorporated with 10% GA on soluble solid content of dragon fruits during 21 days of cold storage at 10±1 °C. Vertical bars indicate standard error of means for three replicates

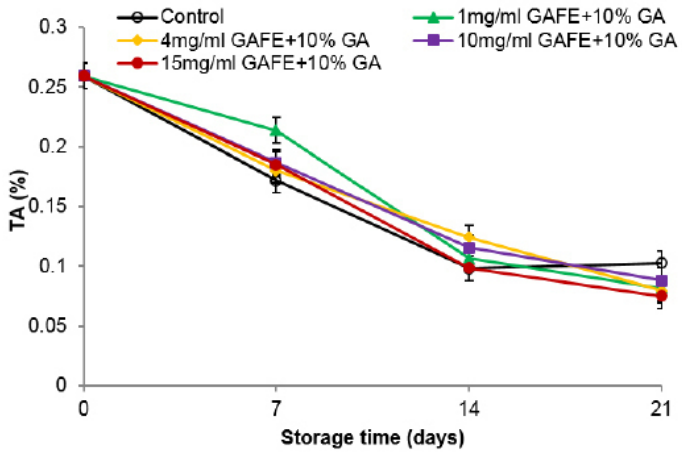

Figure 5. Effect of the dried GAFE incorporated with 10% GA on titrable acidicity (TA) of dragon fruits during 21 days of cold storage at 10±1 °C. Vertical bars indicate standard error of means for three replicates

DISCUSSIONS

Mycelial growth and conidial germination were clearly affected at higher concentrations of dried GAFE (15>10> 4>1 mg/mL)+10% GA. Among all the treatments tested, the 15 mg/mL of GAFE+10% GA was found to be most effective in controlling fungal growth and spore germination *in vitro*. Dried GAFE significantly inhibited *C. gloeosporioides* growth and germination and that the inhibition was due to the presence of bioactive compounds which strongly exerted antifungal properties. The higher crude extract concentrations may contain higher accumulation of secondary metabolites thus resulting in stronger effects of fungal inhibition *in vitro*. Dose dependent antifungal effect of natural products against *C. gloeosporioides* has been reported in earlier literatures (Ali, Chow, Zahid, & Ong, 2013; Ali et al., 2014; Rahman, Mahmud, Kadir, Abdul Rahman, & Begum, 2008).

Based on the SEM results, it can be observed that the GAFE extract caused distortion, swelling and shrivelling of fungal hyphae. Crude extract of natural products may result in structural alteration of the hyphal morphology and interference of plasma membrane of fungi (Ait Barka, Eullaffroy, Clément, & Vernet, 2004). The actual mode of action of GAFE extract is still unclear however the extract might have caused disruptions of the mycelial growth and an inactivation of the spore germination due to its high antifungal properties.

On the other hand, findings with dried GAFE extract were not apparent in the *in vivo* study. DI and DS in dragon fruits were prevalent at highest concentration (15 mg/mL) in combination with 10% GA

probably due to phytotoxicity arising from the concentrated extract. The phytotoxic effect might render some host cells more vulnerable to fungal pathogen attack resulting in tissue damage, thus may reduce disease resistance to anthracnose (Yulia, Shipton, & Coventry, 2006).

However, lower concentrations such as 4 and 1 mg/mL dried GAFE+10% GA may have insufficient amount of bioactive compound to inhibit the fungal growth, therefore compounded the DI in dragon fruits. Meanwhile, at concentration of 10 mg/mL extract markedly inhibited progression of DI and DS. This could be due to fact that at such concentration when combining with 10% GA might be the optimum concentration to reduce the development of anthracnose caused by *C. gloeosporioides*. Treatment with dried GAFE not only provides antifungal effects but the synergistic interaction between GA coatings by limiting respiration rate in fruit which might reduce the incidence of anthracnose.

Fruit weight loss is closely related to respiration rate and moisture evaporation through the skin (Hernández-Muñoz, Almenar, Valle, Velez, & Gavara, 2008). Higher weight loss was observed in fruits coated with higher concentrations of dried GAFE (15 and 10 mg/mL)+10% GA. This study revealed that low concentrations of GAFE extract (1 and 4mg/mL)+10% GA was more effective compared to higher concentrations, explaining higher composite coating may resulted higher weight loss in the fruit due to anaerobic respiration and subsequently leading to respiratory heat. Studies by Ghasemnezhad, Shiri and Sanavi (2010) and Zahid, Ali, Siddiqui and Maqbool (2013) also reported higher concentration of natural products showed higher weight loss in apricot and dragon fruits respectively.

Softening is commonly associated with progressive activity of hydrolase enzymes acting on plant cell wall during ripening onset (Ali, Chin, Marimuthu, & Lazan, 2004). Reduced tissue firmness was observed in all fruits regardless of treatments. However, firmness loss was more apparent in fruit treated with 15 and 10 mg/mL GAFE extract+10%GA compared to lower concentrations. A plausible explanation might be due to the decreased cohesion between fruit and the extract as the concentration of dried GAFE increases, which in turn prevent the change in internal atmosphere of the fruit resulting in the loss of textural firmness (Zahid et al., 2013).

It was well documented that respiration process requires breakdown of carbohydrate into sugar and use of organic acids as an energy source respectively (El-Anany, Hassan, & Rehab Ali, 2009). In all cases, SSC increment and TA reduction in dragon fruits were observed during storage. Coating with 10 mg/mL GAFE+10% GA was found to be the most effective by decelerating metabolic activities compared to other treatments. This is probably because of modification of the internal atmosphere that suppressed respiration rate of the fruit which also might slowed down SSC increment and TA reduction throughout storage period.

CONCLUSIONS

In conclusion, 15 mg/mL dried GAFE incorporated with 10% GA exhibited maximum inhibitory effect on mycelial and conidial growth. At such concentration, the extract has significant antifungal effect on dragon fruits' anthracnose *in vitro.* In addition, 10 mg/mL dried GAFE plus 10% GA was as efficient as the commercial fungicide during *in vitro*, markedly controlled DI and DS progression and maintained higher SSC and acidity value throughout storage period. Albeit its outstanding antifungal effectiveness *in vitro,* the dried GAFE at higher concentrations (15 and 10 mg/mL+10% GA) resulted undesirable outcomes in postharvest quality such as higher weight loss and hastened tissue firmness. Nevertheless, coating application of natural extract from dried GAFE incorporated with 10% GA can be considered as environmentally friendly alternatives that provide protection from postharvest anthracnose disease and also prolonging the shelf life of dragon fruits.

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Length-weight and Length-length Relationship Models of Four Carangid Fishes from The Matang Mangrove Estuaries, Perak, Malaysia

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ABSTRACT

Four species of Carangid fishes from Matang Mangrove Estuaries, Perak, Peninsular Malaysia were selected for the study of length-weight relationships (LWR) and length-length relationships (LLR). A total of 308 individuals were collected from September 2015 to August 2016 by push net (mesh sizes ranges from 2.5 to 5.0 cm). Overall, the growth coefficient (b) values were 2.005, 3.014, 3.452 and 3.194 for *Carangoides malabricus*, *Atule mate*, *Decapterus macrosoma* and *Selaroides leptolepis*, respectively. Growth coefficient of three species (*A. mate*, *D. macrosoma* and *S. leptolepis*) in the present study were within expected range of 2.5 – 3.5 and this indicated that those species have normal growth pattern. This research serves as the first record of LWR and LLR data for four species of Carangid fishes in the Matang Mangrove Estuaries and surrounding ecosystem, Malaysia.

Keywords: Carangid, length-weight relationship, Matang mangrove, Malaysia

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INTRODUCTION

The Carangid fishes are important component of fisheries throughout tropical regions. They also form important portion of income for the majority of commercial and subsistence fishermen (Alongi, Chong, Dixon, Sasekumar, & Tirendi, 2003; Chong,2006). These fishes had been

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exploited as food production where they considered as high-quality food fish in some regions and are cooked or conserved by a variety of methods (Kiso & Mahyam, 2003; Affendy & Chong, 2006). They comprise about 30 genera worldwide that can be subdivided into approximately 140 species (Qamar, Panhwar, & Brouwer, 2016). The fishes can be found abundantly in the Indo-Pacific region from east Africa in the west to Hawaii in the east, extending north to Japan and south to Australia (Ambak, Isa, Zakaria, & Ghaffar, 2012). Copepods, small fish and invertebrate are some of common example of their staple diet (Thabet, Mansour, Al Omar, & Tlig-Zouari, 2016). The range of their maximum length size are within 25 – 100 cm, depend mainly on the species (Bray & Justine, 2013).

The length-weight relationship (LWR) and length-length relationship (LLR) are among the fundamental analysis in observing fisheries status as well as estimating and comparing life histories of fishes among different study sites (Chew, Chong, & Hanamura, 2007; Chong, 2005; Faunce & Serafy, 2006; Laegdsgaard & Johnson, 2001). For LWR, the researchers utilize the graph of total length (TL) versus body weight (BW) in order to obtain the value of a (intercept), b (growth slope or coefficient) and r^2 (regression coefficient). The b value is the most important for LWR analysis as it is an indicator for the type of growth of fishes; $b < 3.0$ (negative allometric growth), $b = 3.0$ (isometric growth) and $b > 3.0$ (positive allometric growth) (Froese, 2006). Meanwhile, the analysis of LLR is essential where one length type is preferable in comparative growth studies (Quinn & Deriso, 1999).

Matang Mangrove Estuaries which are situated in Malaysia is one good example of a specific single location where several studies have been carried out to elucidate its importance in various field of fisheries (Chew & Chong, 2011; Chong, 2006; Ooi & Chong, 2011), yet those previous studies showed little about the importance of LWR of dominant fish except the study by Chong (2005). This is unfortunate because a complete understanding of the stock status of fish and their dependence on the study area is not possible without a complete finding of their distribution and morphometric statistics. Thus, the analysis of LWR and LLR in several parts of study area would reveal partially the population model and stock status for the targeted fishes that are resided over there. The main objective of this study was to establish the LWRs and LLRs of four Carangid fishes from Matang Mangrove Estuaries, Peninsular Malaysia as well as to share the information with respected authorities and researchers for management purposes and extending population studies.

MATERIALS AND METHODS

This study was conducted in the estuarine waters of Matang Mangrove Forest Reserve (MMFR), Perak, Peninsular Malaysia and sampling activities were carried out monthly between September 2015 and August 2016. Fish samples were collected from landing sites of three main rivers ('Sungai' in

Malay Language) in MMFR areas, namely 'Sungai Tiram', 'Sungai Tinggi' and 'Sungai Sepetang'. The coordinates of sampling stations were ranged from N 04º 55' to N 04º 40' for latitude and E 100º 25' to E 100º 40' for longitude. These geographic locations were considered sufficient enough in covering the fish species that available in the study area.

Fish samples were obtained from local fishermen who operated push net boat in MMFR areas for their main source of income. This medium size of motorized boat is operated and trawl net is attached at the front side of the boat, with the net specification is 14.0 – 15.0 m in length, 2.0 – 5.0 m in width, and 2.5 – 5.0 cm mesh sizes. At each station, around 4 kg of unsorted fish samples were obtained and they were immediately preserved in ice chest. The species identification was done by using the identification key of Ambak et al. (2012). The total length (TL) and standard length (SL) of specimens were measured using a digital caliper to the nearest 0.1 mm while the electronic balance of up to 0.01 g accuracy were utilized in measuring the wet weight of specimens. Both types of measurements of the specimens were directly conducted in the field.

The commonly used relationship $W = aL^b$ was applied in order to establish the length-weight relationship (Ricker, 1975; Quinn & Deriso, 1999) where W is the weight (g), L is the total length (cm), 'a' is the intercept and 'b' is the growth slope (growth coefficient). The parameters of 'a' and 'b' were estimated by least squares linear regression from log–log transformed data: $\log_{10} W = \log_{10} a + b \log_{10} L$. The coefficient of determination (r^2) was used as an indicator of the quality of the linear regression (Pauly, 1980). Additionally, the statistical significance level of r^2 and 95% confident limits of the parameters 'a' and 'b' were estimated. Total length and standard length relationships were estimated by using linear regression analysis of TL = a + b SL.

RESULTS AND DISCUSSION

A total of 308 individuals representing four different fish species of Carangid were analyzed in this study. The LWR parameters of four Carangid fishes are presented in Table 1 while the LLR regressions are given in Table 2. From Table 1, two species (*D. macrosoma* and *S. leptolepis*) showed positive allometric growth ($b > 3$) with only *C. malabricus* showed negative allometric growth ($b < 3$). In contrast, *A. mate* was the only species in this study that showed isometric growth ($b = 3$). All LLRs were highly correlated ($P < 0.05$) with $r^2 \geq 0.984$ (Table 2). Additionally, supplemental data that contained spatial and monthly data of water parameters in the study area were also shown in Table A and Table B. Both tables showed the specific water parameters that had significant variation ($P < 0.05$) amongst different sampling stations and months in this study.

The results of LWRs and LLRs in this study were varied to each species might be due to several factors that were not considered earlier such as feeding

Table 1

Statisctical description and LWR parameters obtained for four Carangid fishes in the Matang Mangrove Estuaries, Malaysia from September 2015 to August 2016

		TL (cm)		BW (g)		Regression parameters				
Species	**n**	**Min**	**Max**	**Min**	**Max**	***a***	**95% CI of *a***	***b***	**95% CI of *b***	**r^2**
Atule mate	57	5.5	27.0	2.0	228.0	0.0115	0.0101 – 0.0132	3.014	2.92 – 3.14	0.99
Carangoides malabricus	117	3.0	10.0	0.5	20.0	0.0699	0.0665- 0.0742	2.005	1.91 – 2.12	0.76
Decapterus macrosoma	70	6.5	19.0	0.5	20.0	0.0026	0.0016 – 0.0042	3.452	3.23 – 3.65	0.94
Selaroides leptolepis	64	9.0	17.5	6.0	60.0	0.0070	0.0049 – 0.0113	3.194	3.09 – 3.32	0.95

n, number of specimens sampled; TL, total length; BW, body weight; a, intercept; b, slope/growth coefficient ; CI, confidence interval; r^2, coefficient of determination.

Table 2

Length-length relationship parameters comparison of total length (TL) and standard length (SL) of four Carangid fishes from Matang Mangrove Estuaries, Malaysia between September 2015 and August 2016

Species	n	Equation	a	b	**95% CI of *b***	r^2
Atule mate	57	TL = *a* + *b* SL	0.751	1.047	0.995-1.010	0.997
		SL = *a* + *b* TL	1.322	0.952	0.904-1.000	
Carangoides malabricus	117	TL = *a* + *b* SL	0.835	1.043	0.991-1.010	0.993
		SL = *a* + *b* TL	1.200	0.951	0.903-0.999	
Decapterus macrosoma	70	TL = *a* + *b* SL	0.736	1.057	1.004-1.110	0.984
		SL = *a* + *b* TL	1.379	0.930	0.884-0.977	
Selaroides leptolepis	64	TL = *a* + *b* SL	0.683	1.074	1.020-1.128	0.988
		SL = *a* + *b* TL	1.464	0.920	0.874-0.966	

n, sample size; *a*, intercept; *b*, slope; r^2, coefficient of determination; CI, confidence interval

habit, seasonal affect, sexual maturity and environmental condition (water parameters) (Zain et al., 2018). For instance, it is likely that Carangid fish that had positive growth rate in this study (*D. macrosoma* and *S. leptolepis*) were more tolerable toward variation and fluctuation of water parameters especially in the estuarine area, thus, enabled them to survive and grow better. Furthermore, the b values of *D. macrosoma* and *S. leptolepis* in the present study were 3.452 and 3.194, respectively, and these were in agreement with other studies of other Carangid fishes (*Rastrelliger kanagurta* and *Atule mate*) in Malaysia by Amin, Mohd Azim, Fatinah, Arshad and Rahman (2014) and Mohd Azim, Amin, Romano, Arshad and Yusoff (2017) that yielded the b value = 3.215 and 3.148, respectively. It can be assumed that the body of these Carangid fishes grows faster in relation to their body size and this indicates good environmental condition and food availability in the habitats. The slope (b) values of LWR of three Carangid fishes (*A. mate*, *D. macrosoma* and *S. leptolepis*) in the present study were within expected range of 2.5 – 3.5 as suggested by Froese (2006). The intercept (a) values showed a fusiform body shape in these three species and comparable with other Carangid fishes, based on the Bayesian length-weight predictions available in FishBase website (Froese & Pauly, 2016). Only one species, *Carangoides malabricus*, was below the expected range of b value (2.005) and this was maybe due to very low yield of sufficient variable in sample size.

CONCLUSION

Overall, *C. malabricus* showed negative allometric growth (b = 2.005) and *A. mate* indicated isometric growth (b = 3.014) whereas *D. macrosoma* (b = 3.452) and *S. leptolepis* (b = 3.194) shown positive allometric growth. This baseline study on LWR and LLR of Carangid fishes could be part of fishery management in Matang Mangrove Estuaries.

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APPENDIX

Table A

Spatial variation among three different stations of different water parameters in the estuarine waters of Matang Mangrove Estuaries, Malaysia from September 2015 to August 2016

Parameters	Sg. Tiram	Sg. Tinggi	Sg. Sepetang
*Temp. (°C)	30.59	30.85	29.76
DO (mgL^{-1})	3.86	4.74	3.99
*Salinity (psu)	27.49	27.02	14.78
pH	7.41	7.48	7.13
*Con. ($mS\ cm^{-1}$)	44.06	43.50	24.78

Table B

Monthly variation of different water parameters in the estuarine waters of Matang Mangrove Estuaries, Malaysia from September 2015 to August 2016

Month / Year	***Temp. (^0C)**	***DO (mgL^{-1})**	***Salinity (psu)**	***pH**	***Cond. ($mS\ cm^{-1}$)**
September2015	21.29	2.75	16.24	6.85	28.53
October 2015	29.14	3.15	18.52	7.02	29.68
November2015	29.26	3.36	20.29	7.08	32.46
December2015	29.32	3.49	21.13	7.22	33.78
January 2016	29.61	3.76	22.59	7.29	37.44
February 2016	30.13	3.93	23.69	7.31	38.89
March 2016	30.15	3.97	24.64	7.44	39.34
April 2016	30.46	3.99	25.04	7.45	40.16
May 2016	30.97	4.13	25.55	7.52	40.31
June 2016	31.26	4.60	25.73	7.53	40.41
July 2016	31.75	5.34	25.84	7.60	43.54
August 2016	32.51	7.88	27.89	7.76	44.80

Legend: Temp. = Temperature; DO = Dissolved Oxygen; Cond. = Conductivity

Note: *The mean difference is significant at 5% level ($P < 0.05$).

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The Rice-Growing Cycle Influences Diversity and Species Assemblages of Birds in the Paddy Field Ecosystem in East Peninsular Malaysia

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ABSTRACT

The paddy field ecosystem is an important habitat for water birds, as well as some migratory species due to the abundance of food resources. We want to determine which factors influence bird species abundance, diversity, and composition in different paddy field sites with different rice growing stages. Rapid assessments of birds were conducted in three paddy fields located on the east coast of Peninsular Malaysia: Pahang (Pekan), Terengganu (Besut) and Kelantan (Melor). The survey method involved point count sampling. From the survey, Pekan recorded 1,141 individuals from 17 species, Melor with 992 individuals from 11 species, and Besut, with 348 individuals from eight species. The Ardeidae family was the most dominant, at 71%, at all study areas, followed by the Rallidae (21.43%), Columbidae (14.29%) and Halcyonidae (14.29%). The species richness and assemblages were found to correlate with the rice-growing cycle, where the post-harvest (land preparation) and seedling (vegetative) stages were associated with the highest species incidence, as demonstrated in Pekan and Melor, due to these sites being inundated or flooded. Species abundance was statistically significant for different feeding guild groups based on Welch's $F(4, 4.095) = 68.027$, $p<0.05$. Carnivorous birds were most common during the post-harvest and seedling stage, in contrast to insectivorous/granivorous birds, which were most common during the flowering stage. This study could aid in pest management in relation to bird communities in the paddy field ecosystem through the application of biological control practices instead of chemicals, without compromising rice yield.

Keywords: Birds, bird pest, feeding guild, paddy field, rice growing cycle

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INTRODUCTION

Paddy, which is the world's most widely cultivated crop cover about 1.55 million km^2 of earth's land surface (Donald, 2004). Until the year 2016, world rice (*Oryza sativa*), production has reached 741 million tonnes from only 568 million tonnes, 20 years ago (Food and Agriculture Organization [FAO], 2018). Rice is a staple food of Malaysia, and it has become the main gastronomic resource there. Thus, rice growing has become a major agricultural activity in Malaysia. Rice growing is particularly intense around the north coast of Peninsular Malaysia due to its topographical aspects, specifically being less mountainous and having more fertile soil.

Donald (2004) stated that most wetlands had been converted into paddy fields to increase rice production. In addition, many forested areas have been converted for agricultural uses, such as oil palm stands, rubber plantations and paddy fields (Aratrakorn, Thunhikorn, & Donald, 2006). This conversion leads to habitat loss for many organisms that have inhabited these forests for thousands of years, including birds. The number of bird species in wetland rice fields less than that in forested areas (Wood et al., 2010) because paddy fields are poorer breeding sites for resident birds (Fanslow, 2006). Birds are important and diverse consumers in the paddy ecosystem, residing in either the secondary or top trophic level. Birds, as compared to arthropods, and aquatic organism, are more often thought as pests in the paddy fields (Elphick, 2008). According to Zakaria and Rajpar (2013), water birds rely entirely on wetlands to perform their daily activities, such as nesting, foraging, loafing and moulting, while terrestrial birds visit wetland areas for shelter and food and also to forage. Paddy fields serve as a unique wetland landscape that creates feeding and nursery grounds for birds (Mojiol, Hassan, Maluda, & Immit, 2008), especially water birds, raptors, and sparrows. The presence of such animals in the paddy field environment is mainly due to human activities, such as ploughing, planting and seasonal flooding in that such activities attract many kinds of bird species for foraging and resting (Stafford, Kaminski, & Reinecke, 2010). Sightings of small prey near the water surface lure birds to feed (Acosta et al., 2010). In addition to birds, other organisms inhabiting paddy field landscape, includes weedy plants, plankton, various types of bacteria, aquatic insects, mice and water snakes (Lu, Watanabe, & Kimura, 2002). The interaction of these organisms creates a balanced ecosystem, thus making paddy fields a good habitat for many species (Deb, 2009).

According to Wells (1999), the abundance of birds in paddy fields is also influenced by bird migration during the migratory season, which occurs from September to March. Paddy fields cater a large number of migratory water birds as well as raptors (King, Elphick, Guadagnin, Taft, & Amano, 2010) due to the large number of food resources available, such as crustaceans, molluscs and polychaetes (Stafford et al., 2010). However, most paddy fields are now affected by development

and reclamation (Munira et al., 2014). Therefore, it is necessary to manage the habitat sustainably because birds play many roles in the ecosystem, such as acting as predators, pollinators, scavengers, seed dispersers, seed predators and ecosystem engineers (Whelan, Wenny, & Marquis, 2008). For instance, the Asian open bill (*Anastomus oscitans*), a vagrant stork species in Peninsular Malaysia, has been using paddy fields for feeding and roosting grounds. It was also found to be a biological control agent for the golden apple snail (*Pomacea caniculata*), a common pest in the paddy field landscape (Zainul-Abidin, Mohd-Taib, & Md-Nor, 2017).

Studies of bird diversity and assemblages in paddy fields have been restricted to the west coast of Peninsular Malaysia (Munira et al., 2014; Shah et al., 2008). Not many studies explore factors that shape the abundance and diversity of birds on the east coast of Peninsular Malaysia. The aim of the study was to document the diversity and assemblages of bird species in different paddy field sites in east Peninsular Malaysia, as well as how these correlate with the rice-growing cycle. Several paddy fields in various rice-growing stages were chosen on the east coast of Peninsular Malaysia. We hypothesized that the ecological traits of birds, such as feeding guild, would correspond to the rice-growing stage. This information is vital for conservation efforts targeting birds in the paddy fields, particularly those that become natural biological control agents for certain pests. Specific practices can also be implemented to control bird pest species in the paddy fields and thus enhance rice yield.

MATERIALS AND METHODS

Study Site

The study was conducted in three states on the east coast of Peninsular Malaysia: Pahang (Pekan) (03°01.587' N, 102°36.343' E), Terengganu (Besut) (05°93.542' N, 102°34.376' E) and Kelantan (Melor) (05°70.824' N, 102°53.403' E) (Figure 1).

The study was conducted between September and November of 2015, alternating from site to site. Observation was made for a week at each site in each month, and repeated for the subsequent months. At the time of sampling, Melor, Besut and Pekan were in the seedling (vegetative), flowering (reproductive) and post-harvesting (land preparation) stages, respectively (International Rice Research Institute [IRRI], 2017). The vegetative stage refers to the germination to panicle initiation. At this stage, farmers plant rice seeds in flooded plots. These inundated plots are very important because they also allow fishes and amphibians to be seen and eaten by predators such as raptors. The reproductive stage refers to everything from panicle initiation to flowering. In this stage, the paddy plots are dense with paddy plants, thus limiting food detection by predators. The land preparation stage refers to the ploughing activity that takes place prior to the transplanting stage, after the harvesting season. At this stage, ploughing activity was

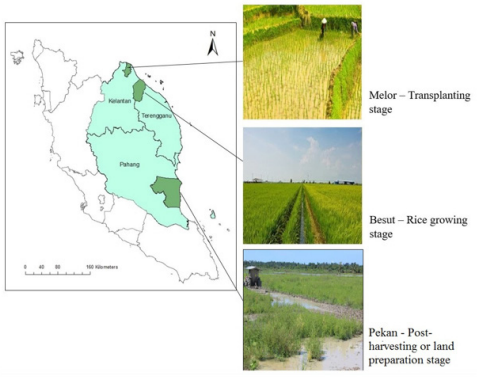

Figure 1. Location of the study sites on the east coast of Peninsular Malaysia and their respective rice growing phases

been performed to fertilise the soil prior to the seedling stage. Such activities also cause aquatic insects and fishes to move freely and thus be seen by predators.

Sampling

Birds were observed by performing point count method, with the aid of 10x42 binoculars. A total of 20 points were randomly chosen in respective paddy plots. At each point, observation was conducted for 10 minutes. Two observers were involved in the survey (FSMT and HAK). The data recorded were species abundance. Migratory species were also census as it indicates the role of paddy field ecosystem to harbour migratory species. The observation began at 0700 hour until 1900 hour, with 45 minutes time interval and 15 minutes breaks to avoid continuous data collection. Only birds observed within 30 meters distance from both sides of the points were counted, and points selected were at least 200 meters apart to avoid double counting (Gregory, Gibbons, & Donald, 2004). We tried to avoid double counting by using a careful observation especially on flying birds over the census area. However, same individual birds recorded at subsequent points will be treated as different individuals. Each species is identified as a member of a specific feeding guild, following Zakaria and Rajpar (2010).

Data Analysis

To compare species compositions between habitats, we used a Euclidean distance matrix to determine the degree of similarity in terms of species composition between various sites. Principal component analysis (PCA) was conducted to determine the species assemblages inhabiting various paddy sites by visualizing the similarities or differences between factors involved. The index for PC1 explains the most variation in the data. Both analyses were performed using MVSP (Multivariate Statistical Package), Version 3.13b. A one-way ANOVA was conducted to determine whether species abundance differed for groups with different rice-growing stages and feeding guilds. These data were analysed using SPSS, Version 23.

RESULTS

The Ardeidae family was the most common family overall, at 35.71% across all study areas. This family is followed by the Rallidae, at 21.43% with 94 individuals from three species, as well as the Columbidae and Halcyonidae, which were both represented at a rate of 14.29%, with two species and 120 and 44 individuals, respectively. Other

Table 1

Euclidean distance matrix for species distribution in various paddy field sites

	Pekan	Besut	Melor
Pekan	0		
Besut	334.657	0	
Melor	204.203	356.679	0

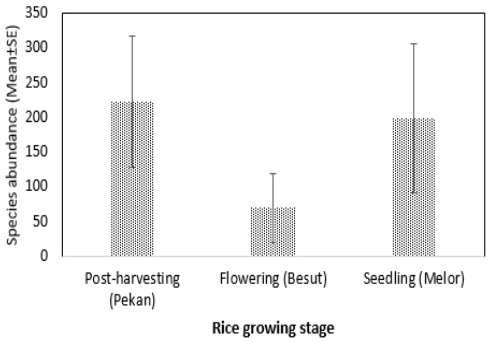

Figure 2. Mean ± SE of species abundance in various rice-growing stages

families were represented by only one species each. Among all of the species recorded across the three study sites, Pekan shows the highest species richness, with 17 species from 12 families. The species commonly found in Pekan were the great egret (*Ardea alba*), cattle egret (*Bubulcus ibis*), jungle myna (*Acridotheres fuscus*), Eurasian tree sparrow (*Passer montanus*), Pacific swallow (*Hirundo tahitica*) and little egret (*Egretta garzetta*). In comparison, Melor recorded a fairly high number of species, 12, and the most common were similar to species found in Pekan. The lowest number of species was found in Besut, with only eight species, which were dominated by the jungle myna and Eurasian tree sparrow. The species assemblages in Besut stand very much in contrast to those of Pekan and Melor because of the absence of any water birds (Ardeidae). A Euclidean distance matrix shows a high similarity between Pekan and Melor, with Euclidean distance 204.2 (Table 1). Besut shows a low similarity to both Pekan and Melor, with distances of 334.7 and 356.7, respectively.

The post-harvesting and seedling stages show a similarly high abundance of birds, as illustrated in Figure 2. A one-way ANOVA was conducted to determine whether the species abundance was different according to rice-growing stages. The data were normally distributed for each group, as assessed via Shapiro-Wilk test ($p>0.05$). There was homogeneity of variances, as assessed by Levene's test for the equality of variance ($p=0.189$). However, there were no significant differences in species abundance among different rice-growing stages, $F(2, 12) = 0.889$, $p=0.436$. Species abundance was highest in the post-harvest stage in Pekan (222.4±94.56), followed by the seedling stage in Melor (198.4±106.95). The lowest species abundance was seen at the flowering stage in Besut (69.6±49.04). Species abundance differed significantly between post-harvesting (Pekan) and flowering stage (Besut) ($p<0.05$).

Based on the PCA analysis (Figure 3), the four species with the highest scores in PC1 were the Great egret, Cattle egret, Jungle myna, and Eurasian tree sparrow. PC1, with

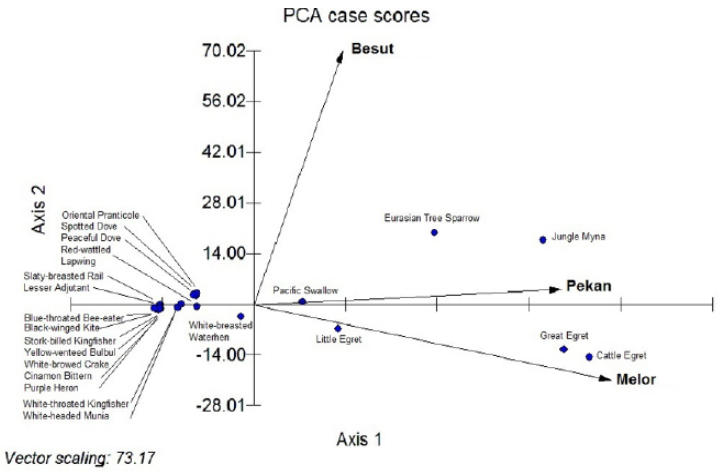

Figure 3. Scree plot of PCA loading abundance for each species and for various paddy field sites

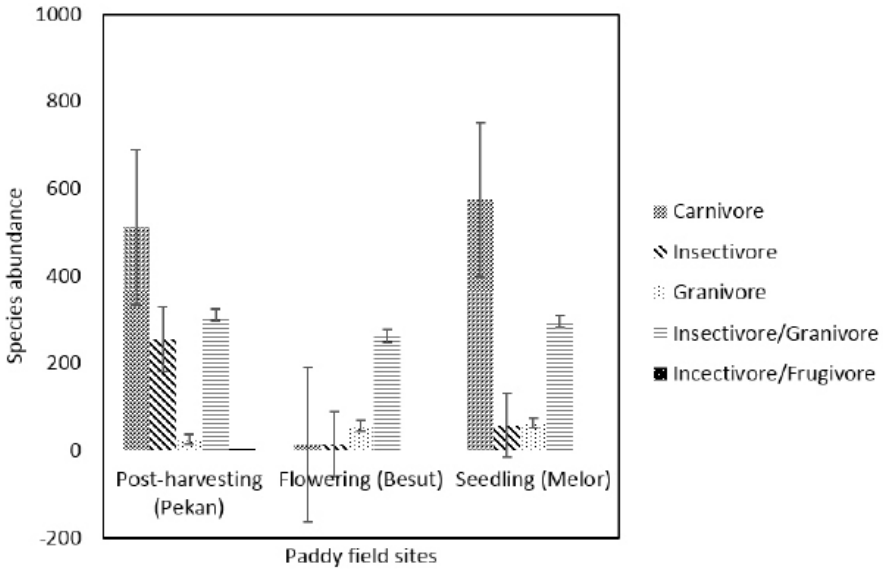

Figure 4. Species abundance for birds of various feeding guilds by rice-growing stage

its eigenvalue percentage 83.2%, could be explained by the high flood level during the post-harvesting and seedling stages, whereby PC2, with a percentage of 9.5% could be explained by the flowering, or reproductive, stage. The positive PCA loading for both axes, which represent the jungle myna and Eurasian tree sparrow, shows an abundance of these species during high flood levels as well as the flowering stage. This is proven with the high abundance of these species in all rice-growing stages, specifically the

species assemblages in Besut during the flowering stage. The Pacific swallow shows a direction parallel with that of Pekan, confirming that this species was restricted to Pekan and was thus represented during the high flood levels in the post-harvest stage. The positive loading for PC1 and negative loading for PC2, on the other hand, represent the great egret, cattle egret and little egret. These species were highly abundant during the high flood levels, especially during the post-harvest and seedling stages in Pekan and Melor. The negative loading for PC1 and the positive loading for PC2 represent several species, such as the spotted dove (*Spilopelia chinensis)*, peaceful dove (*Geopelia placida)* and oriental pranticole (*Glareola maldivarum),* which explain these species were present at the site with low flood levels during the flowering stage. The weak negative PCA loading for the white-breasted waterhen (*Amaurornis pheonicurus)* indicates that this species is affected by neither flood level nor flowering stage. Nevertheless, it was only found in fairly high abundance in Pekan and Melor. The remaining species generally had negative PCA loadings for PC1 and loadings of 0 at PC2, which indicates that they were less influenced by flood level and flowering stage. These species were generally from different feeding guilds and were present only during the post-harvesting stage in Pekan, for instance, the white-throated kingfisher (*Halcyon smyrnensis)*, purple heron (*Ardea purpurea)*, yellow-vented bulbul (*Pycnonotus goiavier)* and cinnamon bittern (*Ixobrychus cinnamomeus)*.

Based on feeding guild, birds were categorized into five groups: carnivore, insectivore, granivore, insectivore/granivore (I/G) and insectivore/frugivore (I/F). A one-way Welch ANOVA was conducted to determine whether species abundance differed by feeding guild in various rice-growing stages (Figure 4). Data were normally distributed for each group, as assessed by Shapiro-Wilk test ($p>0.05$). The assumption of the homogeneity of variances was violated, as assessed by Levene's test of the homogeneity of variances ($p=0.001$). Species abundance was statistically significant for various feeding guild groups, Welch's $F(4, 4.095) = 68.027$, $p<0.05$. Species abundance was highest in carnivores (367.33±177.6), followed by I/G (289.67±14.5) and insectivores (109.67±74.2) and was lowest in granivores and I/F with (48.67±11.5) and (2±1.75), respectively. The abundance of the carnivore feeding guild was high during both the seedling and post-harvest stages, but this guild type was not present during the flowering stage. These species refer to birds from the Ardeidae family, particularly the great egret, cattle egret, and little egret, which are highly abundant during both the seedling and post-harvesting stages. The I/G feeding guild was about equally distributed across the various rice growing stage, though it was somewhat more common during the flowering stage. Insectivores were significantly more common in the post-harvest stage as compared to the seedling and flowering stages.

DISCUSSION

Bird Assemblages in Various Rice-growing Stages

The study has shown that the Pekan paddy field has the highest species richness and diversity of birds, followed by Melor and, finally, Besut. Apart from that, species assemblages also differed in relation to rice-growing stages, which was itself influenced by landscape matrix type. This result is in parallel with other research projects on birds in paddy fields (Munira et al. 2014; Paliwal & Bhandarkar 2014). The paddy field ecosystem provides abundant food sources. Thus, the presence of diverse food types in different parts of the rice-growing cycle also significantly affect species assemblages.

Based on this study, Pekan and Melor sites had greater abundances of water bird species, mainly the carnivorous feeding guild, such as the great egret, little egret and cattle egret. Water birds from the Ardeidae family were the most dominant species in the rice field, particularly during the inundated periods of the seedling and post-harvest phases. Pekan, which was undergoing ploughing activities in the land preparation period, attracted many carnivorous birds because these activities allow for the openness of plots. Thus, prey such as aquatic insects, small vertebrates, fishes, tadpoles, snakes and rodents can be easily seen and caught by predators (Stafford et al., 2010). This is a benefit to carnivorous birds because they have many food resources (Paliwal & Bhandarkar, 2014). In addition, Pekan also recorded other water birds species, such as the lesser adjutant, purple heron, and cinnamon bittern, in lower numbers. Lourenço (2006) demonstrated that the abundance of carnivorous birds increased with the increase in numbers of prey, such as shrews, snakes and rats. The noise and territorial behaviours of shrews cause them to be vulnerable to these predators (Munira et al., 2014). These carnivore species can become good biological control agents for pest populations, particularly rats and snails.

Insectivorous bird species are also categorized as beneficial species in paddy fields due to their ability to control insect pests. Insectivorous birds can be found in paddy fields due to the abundance of insects during all rice-growing stages, as shown in the similar distributions of insectivorous species across all paddy field sites. Herbivorous insects from the Homoptera, Orthoptera, Hymenoptera, Lepidoptera, Hemiptera and Coleoptera families constitute the main pests in paddy fields, particularly the sap-feeders (Norela, Anizan, Ismail, & Maimon, 2013). Insects choose to breed and complete their lifecycles in paddy fields because they like moist conditions and dense foliage (Chettri, Deb, Sharma, & Jackson, 2005). Thus, the abundant insects in the paddy fields attract insectivorous bird species. Therefore, the presence of insectivorous species such as the house sparrow and myna (Rajashekara & Venkatesha, 2014), which were found abundantly in all paddy field sites, could potentially control these insect pests. In this study, the abundance of insectivorous birds is correlated with the abundance of insects in various rice-growing stages. According

to Norela et al. (2013), insect populations decreased in tandem with the rice-growing stages in that they peaked during the early vegetative stage but declined at the end of the maturation stage of the paddy. Therefore, this supports this study's finding that there was an increase in the abundance of insectivorous birds, particularly the Pacific swallow and blue-tailed bee-eater, which were only present during the post-harvest stage, as well as the Oriental pratincole, which was present during both the post-harvesting and seedling stages. This is due to the abundance of insects during the post-harvesting stages. These species, on the other hand, were generally absent or declined during the flowering stage.

Compared to carnivorous and insectivorous species, granivorous species are commonly found in high abundance due to their efficient reproductive capacity in agricultural habitats (Dhindsa & Saini, 1994), thus contributing to economic losses in a variety of agricultural sectors. In paddy fields, prior to the harvesting season, the rice ripens, thus attracting granivorous species (Maeda, 2001) such as the white-headed munia, spotted dove and peaceful dove. The flowering stage in Besut in particular recorded a high number of granivorous and insectivore/granivore species due to the emergence of rice buds and seeds. These species are thought of as pests in the paddy field environment because they reduce the rice yield (Sridhara, Subramanyam, & Krishnamoorthy, 1983). Sridhara et al. (1983) also found a similar pattern of maximum species density among granivorous and insectivorous species during the ripening stage in Bangalore, India, due to low abundance of predators during the breeding season. Breeding activities among grain eaters involve the shifting of their diet from grain to insects to feed their nestlings (Mathew, Narendran, & Zacharias, 1980; Simwat, 1977), thus increasing the abundance of these species during the flowering stage. Several methods have been implemented to control for these pests in paddy fields, including the use of physical and chemical techniques (Dhindsa & Saini, 1994). The use of pesticides, however, has indiscriminately killed some endangered birds (Dhindsa, 1986). Therefore, precautions should be taken when using this method. The black-winged kite species, a raptor found in Besut, on the other hand, could control smaller pest birds, particularly granivorous birds (Sridhara et al., 1983).

Conservation of Birds in Paddy Fields Ecosystem

Paddy fields serve as a great habitat and nursery grounds for birds (Mojiol et al., 2008). There is plenty of food available in the paddy fields such as grains, paddy seeds, insects, fishes, tadpoles, snakes, and even small mammals such as rats. The lesser adjutant, a species categorised as vulnerable in the IUCN Red data list was present in the Pekan site, as were other migrant species namely the Oriental pratincole, and blue-tailed bee-eater (*Merops philippinus)*. Migration could influence the species richness of paddy fields because migrant

birds need food and shelter to survive weather fluctuations (Wells, 1999) and there is a large number of food resources in paddy fields (King et al., 2010) such as crustaceans, mollusc and polychaetes (Stafford et al., 2010). However, the number of migrants species found in this study was very low compared to that found in paddy field in northwestern Peninsular Malaysia by Munira et al. (2014) who recorded migratory waders from the Scolopacidae family. The west coast region contained more wetland, shoreline, marshes, mangrove and agriculture than the east coast of Peninsular Malaysia (Zainul-Abidin et al., 2017), thus explaining the remarkably low diversity of birds in this study. Furthermore, the west coast region is also an important pathway for waterbird species as they travel through Peninsular Malaysia (Li, Yeap, & Kumar, 2007).

Pesticides have been widely used in the agricultural industry including rice production, which subsequently reduces biodiversity in the ecosystem (Samharinto, Abadi, Rahardjo, & Halim, 2012; Wood et al., 2010). This, in turn, shrinks the food web in the rice fields via the elimination of organism at various trophic levels such as raptors and other predators, thus resulting in population built-ups of insect, rodent and bird pest species (Dhindsa & Saini, 1994). Very few raptor species were found during the survey which indicates an insufficient use of natural biological control agents in these paddy fields. Plantations of trees surrounding the rice fields for perching and roosting are also essential in promoting certain populations of birds in the paddy field particularly raptors, insectivorous and frugivorous species which could help control pests in the paddy field.

CONCLUSIONS

Due to our relatively small sample size (sampling did not cover the whole rice growing stages in different paddy field sites), future study should incorporate bird survey in a complete cycle of rice growing stages, in different paddy field sites. Nevertheless, this study provided some valuable insight, that paddy fields in the east coast of Peninsular Malaysia contain many carnivorous and insectivorous species that could serve as good biological control agents against pest. Understanding the bird assemblages in different rice growing cycle could enlighten farmers on when best to control for bird pest in particular. More studies must be carried out to investigate how biological control application particularly among bird communities can enhance the effectiveness of pest control in rice fields without compromising rice yield.

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APPENDIX

List of bird species and their relative abundance in each study sites

Family	Common Name	Taxa	Pahang	Terengganu	Kelantan	Residency status	Protection status	Guild type
Ciconiidae	Lesser adjutant	*Leptoptilos javanicus*	7 (0.6)	0 (0.0)	0 (0.0)	R	V	C
Ardeidae	Purple heron	*Ardea purpurea*	6 (0.5)	0 (0.0)	0 (0.0)	R&M	LC	C
	Cinnamon bittern	*Ixobrychus cinnamomeus*	5 (0.4)	0 (0.0)	0 (0.0)	R&M	LC	C
	Great egret	*Ardea alba*	194 (17.0)	0 (0.0)	218 (22.0)	R&M	LC	C
	Little egret	*Egretta garzetta*	79 (6.9)	0 (0.0)	105 (10.6)	R&M	LC	C
	Cattle egret	*Bubulcus ibis*	182 (16.0)	0 (0.0)	252 (25.4)	R&M	LC	C
Accipitridae	Black-winged kite	*Elanus caeruleus*	0 (0.0)	1 (0.3)	0 (0.0)	R	LC	C
Rallidae	White-breasted waterhen	*Amaurornis pheonicurus*	45 (3.9)	0 (0.0)	43 (4.3)	R&M	LC	I
	White-browed crake	*Amaurornis cinerea*	0 (0.0)	0 (0.0)	2 (0.2)	R	LC	I
	Slaty-breasted rail	*Gallirallus striatus*	0 (0.0)	0 (0.0)	4 (0.4)	R	LC	I
Charariidae	Red-wattled lapwings	*Vanellus indicus*	47 (4.1)	0 (0.0)	0 (0.0)	R	LC	C
Glareolidae	Oriental pranticole	*Glareola maldivarum*	42 (3.7)	15 (4.3)	0 (0.0)	R&M	LC	I

APPENDIX *(Continue)*

Columbidae	Eastern spotted dove	Spilopelia chinensis	0 (0.0)	30 (8.6)	33 (3.3)	R	LC	G
	Peaceful dove	*Geopelia placida*	0 (0.0)	27 (7.8)	30 (3.0)	R	LC	G
Halcyonidae	White-throated kingfisher	*Halcyon smyrnensis*	17 (1.5)	8 (2.3)	9 (0.9)	R	LC	C
	Stork-billed kingfisher	*Pelargopsis capensis*	5 (0.4)	5 (1.4)	0 (0.0)	R	LC	C
Meropidae	Blue-tailed bee eater	*Merops philippinus*	7 (0.6)	0 (0.0)	0 (0.0)	R&M	LC	I
Hirundinidae	Pacific swallow	*Hirundo tahitica*	162 (14.2)	0 (0.0)	0 (0.0)	R	LC	I
Pycnonotidae	Yellow-vented bulbul	*Pycnonotus goiavier*	6 (0.5)	0 (0.0)	0 (0.0)	R	LC	I/F
Sturnidae	Jungle myna	*Acridotheres fuscus*	164 (14.4)	138 (40.0)	190 (19.2)	R	LC	I/G
Passeridae	Eurasian tree sparrow	*Passer montanus*	147 (12.9)	124 (35.6)	106 (10.7)	R	LC	I/G
Estrildidae	White-headed munia	*Lonchura maja*	26 (2.3)	0 (0.0)	0 (0.0)	R	LC	G
Total (Individual)			1141	348	993			

Note: Residensy status: R=resident, R&M=resident and migrant, Protection status: V=vulnerable, LC=least concern, Feeding guild: C=carnivorous, I=insectivorous, I/F=Insectivorous and frugivorous, G=granivorous, I/G=insectivorous and granivorous.

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Effects of Solvent System, Drying and Storage on the Total Phenolic Content and Antioxidant Activities of *Clinacanthus nutans* Lindau (Sabah Snake Grass)

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ABSTRACT

The effects of extraction solvent system (boiling water, water at room temperature, 50% aqueous methanol and 100% methanol), drying (oven drying and sun drying) and storage (refrigeration) on the total phenolic content (TPC) and antioxidant activities of the extracts from *Clinacanthus nutans* Lindau (Sabah Snake Grass) leaves were studied. TPC was determined using Folin-Ciocalteau method. Antioxidant activities were evaluated using three different methods, namely i) 2,2- diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, ii) ferric reducing antioxidant power (FRAP) assay, and iii) beta-carotene bleaching (BCB) assay. Boiling water extraction of fresh leaves resulted in the highest TPC and DPPH and FRAP activities. However, methanolic (100% methanol) extract from the fresh leaves showed the highest antioxidant activity in BCB. Both oven drying and sun drying caused a significant decrease in the antioxidant capacity with sun drying resulting in a lower TPC than oven drying. Cold storage (at 4 ± 2°C) for three weeks after drying resulted in a decrease in TPC. A significant decrease was observed in DPPH, FRAP and BCB activities after three weeks of refrigeration storage. TPC was strongly correlated to DPPH, $1/EC_{50}$ ($r^2 = 0.969$, $p < 0.05$) and FRAP ($r^2 = 0.991$, $p < 0.01$) activities. However, there was no correlation between TPC and BCB. In conclusion, boiling water extraction of fresh *C. nutans* leaves resulted in the highest TPC and antioxidant activities. Drying and storage resulted in deterioration of the TPC and antioxidant activities of *C. nutans* leaves.

Keywords: Antioxidant, *Clinacanthus nutans* Lindau, drying, phenolic content, solvent, storage

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INTRODUCTION

Non-communicable diseases such as cancer, stroke and cardiovascular disease have become a major cause of human mortality. According to World Health Organization [WHO] (2013), an estimated of 36 million deaths (or 63%) of the 57 million deaths globally in 2008 were due to non-communicable diseases. Cancer claims about 7.6 million lives worldwide each year (Union for International Cancer Control [UICC], 2013). One of the causes of non-communicable diseases is the exposure to free radicals. Free radicals are often associated with many diseases including cancer, cataracts, stroke, gastrointestinal disease, and arteriosclerosis (Fu et al., 2011; Greenly, 2004).

Antioxidants are chemical compounds that are able to bind and neutralize free radicals thus preventing the free radicals from causing cell damage (Devasagayam et al., 2004). Antioxidants are important in health care because of their desirable biological effects, such as anti-inflammatory, anti-carcinogenic and anti-atherosclerotic effects (Krishnaiah, Sarbatly, & Nithyanandam, 2011). Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been widely used in the food industry as a food preservative to prevent the oxidation of lipids that leads to altered taste and smell, especially in high-fat foods (Williams, Iatropoulos, & Whysner, 1999). BHT, however, has been reported to enhance tumour development in the liver, lung and gastro-intestinal tract (Witschi, 1986). This has resulted in consumers concerns on the potential harmful effects of synthetic antioxidants on human health (Maisuthisakul, Suttajit, & Pongsawatmanit, 2007). Natural antioxidants have gained increasing attention and interest because they are regarded as safe for human consumption (Qader et al., 2011). Natural antioxidants are readily available in vegetables, fruits and plants (Fu et al., 2011). Antioxidants from plants have received much attention recently due to their effectiveness in scavenging free radicals. Besides, epidemiological studies have found a close relationship between the intake of certain plant extracts and the reduced risks of chronic diseases such as atherosclerosis and cancer (Podsędek, 2007). This is due to the high content of antioxidant compounds found in certain plants. Certain plants have been used as traditional medicine since ancient times and continue to provide treatment for different diseases (Krishnaiah et al., 2011). *Clinacanthus nutans* Lindau (CN), also known as Sabah snake grass, is a traditional herb from the Acanthaceae family. It is used in Thailand as traditional medicine to treat inflammation, viral infections, herpes infections, snake bites, and varicella zoster virus (Sittiso et al., 2010)

In the present study, the effects of extraction solvent system, drying and storage on the total phenolic content (TPC) and antioxidant activities of the extracts from CN leaves were investigated. Phenolic compounds were extracted from CN leaves using four extraction solvent systems, namely i) boiling water, ii) water at room

temperature, iii) 50% aqueous methanol, and iv) 100% methanol. Three different types of CN leaf samples were studied, namely i) fresh, ii) dried, and iii) stored. Sun drying and oven drying were studied for their drying effects. Subsequent storage study after drying was carried out under refrigeration conditions for three weeks. The TPC and the antioxidant activities of the different extracts obtained were determined.

MATERIALS AND METHODS

Plant Materials

Fresh, harvested *C. nutans* plants were obtained from a local supplier in the Sunday morning market in Gaya Street in Kota Kinabalu, Sabah. The plants were in the maturity stage of around three to ten weeks. The leaves were separated from the plant stems and the selection was based on colour, size and physical appearance to ensure uniformity. Small and discoloured leaves were discarded. Those with white and black spots and holes were also excluded.

Sample Preparation

All fresh leaf samples were briefly cleaned with tap water. For drying study, oven drying and sun drying were carried out. Oven drying was carried out in an oven dryer (Thermoline) for 24 hr at 50 ± 2°C. For sun drying, the fresh leaf sample was placed in a drying rack under the sun for two consecutive days. The sun exposure was 7-9 hr a day depending on the weather conditions. For storage study, the dried sample was kept in a sealed container and stored in a refrigerator (4 ± 2°C) for three weeks. Antioxidant analyses were carried out weekly during the storage period. Moisture contents of the samples were determined to allow all results to be reported in dry weight (DW) basis.

Sample Extraction

Prior to extraction, all leaf samples were homogenized with a food blender (MX-337, Panasonic). Four extraction solvent systems were used, namely boiling water, water at room temperature, 50% (v/v) aqueous methanol, and 100% methanol. For boiling water extraction, the homogenized sample (5 g) was boiled in distilled water (100 mL) at 90-100°C for 10 min. For the other extraction solvent systems, the homogenized sample (5 g) was added with the respective solvent (100 mL) in a conical flask and the mixture was placed in a shaker incubator (WiseCube WIS-20, Witeg) at room temperature for 24 hr.

Determination of Total Phenolic Content (TPC)

The TPC of the plant extracts was determined using Folin-Ciocalteau's reagent according to the method described by Surveswaran, Cai, Corke and Sun (2007) with slight modifications. Plant extract (200 µL; 1 mg/mL for fresh and dried samples, 5 mg/mL for stored samples) and Folin-Ciocalteu (750 µL; 10-fold dilution) were added to a test tube. Higher concentration was used for the stored samples due to their

lower TPC after storage. The mixture was allowed to react for 5 min at room temperature before the addition of 300 µL sodium carbonate (75 g/L) to neutralize the reaction. The mixture was left for 30 min in dark conditions at room temperature to allow complete reaction to form a blue complex. The absorption of the sample was measured at 765 nm against a blank sample using a UV-Vis spectrophotometer (Lambda 35, Perkin Elmer). The TPC of the sample was determined based on the calibration graph (absorbance versus concentration; $y = 0.3135x$, $r^2 = 0.9932$) of gallic acid constructed using 0-5 mg/mL gallic acid. TPC was determined based on the equation below and expressed as mg gallic acid equivalents/100 g dry weight (mg GAE/100 g DW).

$$\text{TPC (mg GAE/100 g DW)} = C \times \left[\frac{V}{m\,(100\% - k)}\right] \times 100000$$

where,

C is the gallic acid concentration obtained from the gallic acid calibration graph (mg/mL),

V is the volume of the extract (mL),
m is the weight of the extract (mg),
k is the moisture content (%).

Determination of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity

The radical scavenging activity of the plant extracts was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical as described by Kchaou, Abbes, Blecker, Attia and Besbes (2013) and Yang, Gadi, Paulino and Thomson (2010). The plant extract (0.5 mL) of 0.5 mg/mL concentration was added to 1.0 mL DPPH (0.1 mM) in methanol. The mixture was kept for 30 min in dark conditions at room temperature. The absorption of the sample was measured against a blank sample at 517 nm with a UV-Vis spectrophotometer (Lambda 35, Perkin Elmer). The procedure was repeated 6-8 times using the same plant extract and the same volume (0.5 mL) but different plant extract concentrations (up to 3.0 mg/mL). This was to obtain a radical scavenging activity (%) curve as a function of plant extract concentration. The radical scavenging activity (%) of each plant extract concentration was determined according to the equation below. Based on the equation of the linear regression line obtained, the EC_{50} of the plant extract was determined. EC_{50} is defined as the concentration of the extract that reduces the initial concentration of DPPH radical initiator by 50%. Ascorbic acid was used as positive control for DPPH analysis. The procedure for the determination of the EC_{50} of ascorbic acid was similar to that of the plant extract except that ascorbic acid was used instead of the plant extract.

$$\text{Radical scavenging activity (\%)} = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100$$

where,

A_o is the absorption of the control sample (blank, without extract),

A_1 is the absorption of the sample extract.

Determination of Ferric Ion Reducing Antioxidant Power (FRAP)

FRAP was determined according to the method described by Bakar, Mohamed, Rahmat and Fry (2009). FRAP reagent was prepared by mixing 300 mM acetate buffer solution at pH 3.6 (adjusted with sodium hydroxide), 10 mM solution of 2,4,6-tripyridyl-s-traizine (TPTZ) in 40 mM hydrochloric acid (HCl) and 20 mM ferric chloride hexahydrate ($FeCl_3.6H_2O$) in the volume ratio of 10: 1: 1. The reagent was placed in a water bath for 4 min at 37°C.

FRAP reagent (3 mL) was mixed with 0.1 mL plant extract (1 mg/mL for the fresh and the dried samples; 5 mg/mL for the stored samples) and incubated for 30 min in a water bath (37°C). Absorbance was measured with a UV-Vis spectrophotometer (Lambda 35, Perkin Elmer) at 593 nm. Based on the absorbance, the ferric ion reducing power (FRAP) of the plant extract was determined based on the calibration graph (absorbance versus concentration; $y = 0.5847$, $r^2 = 0.9927$) of ferrous sulphate constructed using 0-2 mmol ferrous sulfate. FRAP was calculated based on the equation below and expressed as mmol ferrous sulfate per 100 g dry weight (mmol $Fe_2SO_4.7H_2O$/100 g DW).
FRAP (mmol Fe2SO4.7H2O/ 100 g DW) =C × [(V)/(m (100%-k))]×100000

where,

C is the concentration of ferrous sulfate obtained from the ferrous sulfate calibration graph (mmol),

V is the volume of the extract (mL),
m is the weight of the extract (mg),
k is the moisture content (%).

Determination of β-Carotene Bleaching (BCB)

BCB assay was carried out according to the method described by Velioglu, Mazza, Gao and Oomah (1998) with some modifications. β-carotene (1 mL) of 0.2 mg/mL in chloroform was added to a 50 mL round bottom flask containing 20 µL linoleic acid and 200 µL Tween 20. The mixture was evaporated using a rotary evaporator (Laborota 400, Heidolph) for 10 min at 40°C under reduced pressure conditions. Thereafter, distilled water (100 mL) was added and the mixture was subjected to vigorous shaking for one min to form an emulsion. The emulsion (5 mL) was added to 200 µL plant extract (1 mg/mL for fresh and dried samples; 5 mg/mL for stored samples) and the mixture was left in dark conditions at 40°C for 120 min. The absorbance of the mixture at 470 nm was measured at t = 0, 20, 40, 60, 80, 100 and 120 min with a UV-Vis spectrophotometer (Lambda 35, Perkin Elmer). Butylated hydroxyanisole, BHA (1 mg/mL in methanol) and methanol were used as standard and negative control, respectively. The amount (%) of inhibition was calculated using the two equations below (Al-Saikhan, Howard, & Miller, 1995).

Rate of bleaching, $R = [\ln (A_0/A_t)]/t$

where,

A_0 = absorbance of the mixture at t = 0 min

A_t = absorbance of the mixture at t = 20, 40, 60, 80, 100 and 120 t = min

$$\text{Inhibition (\%)} = \left[\frac{(R_{control} - R_{sample})}{R_{control}}\right] \times 100$$

where,

$R_{control}$ is the rate of bleaching in the control sample,

R_{sample} is the rate of bleaching in sample extract.

Statistical Analysis

All analyses were conducted in triplicate and the results (in dry weight (DW) basis) are expressed as mean ± standard deviation. Statistical analysis of data was carried out using SPSS (Statistical Package for Social Sciences version 21.0., IBM SPSS Statistics, USA). One-way ANOVA (analysis of variance) was carried out. Comparison of the different extraction solvent systems (i.e. boiling water, water at room temperature, 50% methanol and 100% methanol) and the different types of samples (i.e. fresh, oven-dried and sun-dried) was carried out using Duncan's post hoc multiple comparisons test. Bivariate Pearson's correlation test was used to determine the correlations between the TPC and the antioxidant activities (DPPH, FRAP, BCB). All analyses were carried out at a significance level of $p < 0.05$.

RESULTS AND DISCUSSION

Effect of Solvent System

The TPC and the antioxidant activities (DPPH, FRAP and BCB) of the extracts obtained using different solvent systems from the fresh and the dried leaf samples are shown in Table 1 and Table 2, respectively. For all three samples (fresh, oven-dried and sun-dried), boiling water extraction (474.89 ± 40 to 1453.64 ± 205 mg GAE/100 g DW) resulted in the highest TPC, followed by water at room temperature (300.42 ± 92 to 1040.17 ± 121 mg GAE/100 g DW), 50% methanol (197.68 ± 55 to 807.10 ± 89 mg GAE/100 g DW) and 100% methanol (117.05 ± 19 to 516.51 ± 63 mg GAE/100 g DW) extractions. Water is a very polar solvent. Methanol is also a polar solvent but is less polar than water. The results suggest that most of the phenolic compounds present in all three samples were of high polarity. Boiling water extraction resulted in a higher TPC than water extraction at room temperature because the high temperature of boiling water can liberate the phenolic compounds that are bonded covalently to the cell wall (Lattanzio, Lattanzio, & Cardinali, 2006). Such phenolic compounds may not be extracted by water extraction at room temperature.

Based on the results shown in Table 1 and Table 2, it was noted that high TPC resulted in high DPPH and FRAP activities for all three samples regardless of solvent system. The results suggest all phenolic compounds present in all three samples contribute significantly to DPPH

Table 1
TPC in fresh and dry leaves of Clinacanthus nutans *Lindau*

Solvent system	TPC		
	mg GAE/100 g DW		
	Fresh	Oven drying	Sun drying
Boiling water	1453.64 ± 205^{a1}	967.11 ± 82^{a2} (-33.5%)	474.89 ± 40^{a3} (-67.3%)
Water	1040.17 ± 121^{b1}	719.09 ± 89^{b2} (-30.9%)	300.42 ± 92^{b3} (-71.1%)
50% Methanol	807.10 ± 89^{b1}	567.30 ± 129^{b2} (-29.8%)	197.68 ± 55^{bc3} (-66.2%)
100% Methanol	516.51 ± 63^{c1}	339.03 ± 65^{c2} (-34.4%)	117.05 ± 19^{c3} (-77.4%)

For each column in each assay, values with different letter superscripts (a-d) denote significant differences ($p < 0.05$).
For each row in each assay, values with different number superscripts (1-3) denote significant differences ($p < 0.05$).
Values in parentheses are the changes (%) compared to the fresh sample where the signs '+' and '-' refer to an increase and a decrease, respectively.

and FRAP activities. Although the TPC of the 50% methanol and the 100% methanol extractions were lower, these TPC resulted in a higher BCB antioxidant activity when compared to those obtained through boiling water and water at room temperature extractions regardless of the type of sample. This suggests that these relatively less polar phenolic compounds obtained through 50% methanol and 100% methanol extractions contribute more significantly to BCB activity than those more polar phenolic compounds obtained through aqueous (boiling water and water at room temperature) extractions. The antioxidant mechanism based on hydrogen atom transfer (HAT) of BCB is different from those of DPPH and FRAP which are based on single electron transfer (SET) (Apak et al., 2013). In all three samples, the relatively less polar phenolic compounds seem to be more in favour of the HAT mechanism in their antioxidant activities than those more polar phenolic compounds.

Effect of Drying

For all four solvent systems, fresh sample (516.51 ± 63 to 1453.64 ± 205 mg GAE/100 g DW) had the highest TPC, followed by oven-dried sample (339.03 ± 65 to 967.11 ± 82 mg GAE/100 g DW) and sun-dried sample (117.05 ± 19 to 474.89 ± 40 mg GAE/100 g DW) (Table 1). Both oven drying and sun drying resulted in the reduction of TPC. When compared to fresh sample, sun drying (-66.2 to -77.4%) caused a higher loss of TPC than oven drying

Table 2

DPPH, FRAP and BCB activities in fresh and dry leaves of Clinacanthus nutans *Lindau*

Solvent system	DPPH			FRAP			BCB		
	EC_{50} (mg/mL)			mmol $Fe_2SO_4.7H_2O$/100 g DW			Inhibition (%)		
	Fresh	Oven drying	Sun drying	Fresh	Oven drying	Sun drying	Fresh	Oven drying	Sun drying
Boiling water	0.26 ± 0.05^{a1}	1.10 ± 0.05^{a2} (+323.1%)	1.61 ± 0.17^{a3} (+519.2%)	348.56 ± 32^{a1}	264.59 ± 45^{a2} (-24.1%)	165.15 ± 18^{a3} (-52.6%)	80.83 ± 0.41^{a1}	73.68 ± 0.94^{a2} (-8.8%)	64.60 ± 2.73^{a3} (-20.1%)
Water	0.51 ± 0.15^{b1}	2.07 ± 0.50^{b2} (+305.9%)	2.94 ± 0.24^{b3} (+476.5%)	269.36 ± 42^{b1}	166.30 ± 35^{b2} (-38.3%)	95.91 ± 13^{b3} (-64.4%)	76.38 ± 1.41^{b1}	64.45 ± 2.13^{b2} (-15.6%)	60.89 ± 4.74^{a2} (-20.3%)
50% Methanol	0.87 ± 0.03^{c1}	3.79 ± 0.72^{c2} (+335.6%)	4.73 ± 0.19^{c3} (+443.7%)	185.23 ± 52^{c1}	113.79 ± 18^{b2} (-38.6%)	65.97 ± 25^{b2} (-64.4%)	88.33 ± 0.87^{c1}	82.25 ± 1.53^{c2} (-6.9%)	73.20 ± 2.72^{b3} (-17.1%)
100% Methanol	1.22 ± 0.06^{d1}	4.79 ± 0.60^{d2} (+292.6%)	5.54 ± 0.23^{d3} (+354.1%)	101.27 ± 17^{d1}	53.17 ± 10^{c2} (-47.5%)	23.12 ± 4^{c3} (-77.2%)	92.12 ± 0.71^{d1}	86.87 ± 1.79^{d2} (-5.7%)	81.78 ± 1.23^{c3} (-11.2%)
Standard	Ascorbic acid: 0.0260 ± 0.0001^{e}						Butylated hydroxyanisole (BHA): 98.09 ± 0.39^{e}		

For each column in each assay, values with different letter superscripts (a-d) denote significant differences ($p < 0.05$).

For each row in each assay, values with different number superscripts (1-3) denote significant differences ($p < 0.05$).

Values in parentheses are the changes (%) compared to fresh sample where the signs '+' and '-' refer to an increase and a decrease, respectively.

(-29.8 to -34.4%). Phenolic compounds are thermally degraded due to their high heat sensitivity and susceptibility to oxidation (Lim & Murtijaya, 2007). For both oven drying and sun drying, the TPC obtained through 50% methanol (-29.8% and -66.2%, respectively) had the lowest reduction after drying, followed by boiling water (-33.5% and -67.3%, respectively), water (-30.9% and -71.7%, respectively) and 100% methanol (-34.4% and -77.4%, respectively). This suggests the phenolic compounds extracted with 50% methanol had the highest thermal stability thus experiencing the least thermal degradation during drying.

The loss of TPC after drying may be due to the enzymatic reactions involving polyphenol oxidase during drying (Cavalcanti, Resende, Carvalho, Silveira, & Oliveira, 2006). Polyphenol oxidase acting as an enzyme catalyst in the hydroxylation reaction in the presence of oxygen has the ability to degrade and destroy phenolic compounds. The lower TPC for sun drying may be due to the slower deactivation of the degradative enzyme when compared to oven drying. More phenolic compounds were degraded in sun drying because of the slower drying process due to the uneven heat distribution across the leaf sample and the temperature fluctuation. On the other hand, oven drying involved placing the leaf sample in a preheated oven dryer at 50°C causing thermal shock and eventually deactivation of the enzymes (Jaiswal, DerMarderosian, & Porter, 2010). Besides, oven drying had a more uniform and consistent heat transfer than sun drying causing the heat-labile enzymes to be degraded more rapidly. However, some enzymatic reactions may have taken place in the early stages of the drying process which resulted in some degradation of phenolic compounds (Lim & Murtijaya, 2007). Jaiswal et al. (2010) also reported a higher reduction of anthocyanin (a phenolic compound) in pomegranate in sun drying when compared to oven drying. It was found that a higher deterioration rate of polyphenol oxidase during drying resulted in a lower reduction rate of the anthocyanin. A 61% loss of anthocyanin for oven drying was observed when the polyphenol oxidase deterioration was 68%. When the polyphenol oxidase deterioration was lower at 45% for sun drying, a higher anthocyanin loss at 83% was observed.

The reduction in TPC after drying caused similar effect on the DPPH, FRAP and BCB activities. A decrease in the DPPH antioxidant activity is reflected by an increase in the EC_{50} value. Oven drying resulted in lower FRAP reduction (-24.1 to -47.5%) when compared to sun drying (-52.6 to -77.2%) (Table 2). The polar phenolic compounds obtained through boiling water extraction recorded the lowest reduction in FRAP antioxidant activity. Similar to FRAP, a lower reduction in BCB antioxidant activity was observed for oven drying (-5.7 to -15.6%) when compared to sun drying (-11.2 to -20.3%). In contrast to FRAP, the relatively less polar phenolic compounds obtained through 50% methanol and 100% methanol extractions had a lower reduction in BCB antioxidant activity.

Effect of Storage

The TPC and the antioxidant activities (DPPH, FRAP and BCB) of the extracts from the dried leaf samples during the 3-week cold storage period are shown in Table 3 and Table 4, respectively. For both oven drying and sun drying, the TPC and the antioxidant activities decreased with storage time regardless of the solvent system. Boiling water extraction resulted in the highest TPC regardless of the storage time (Table 3). Similar to the fresh sample (Table 1), the amount of phenolic compounds extracted by the four solvent systems from the dried samples (both oven drying and sun drying) was in the order of boiling water > water at room temperature > 50% methanol > 100% methanol. A similar order was observed for the stored samples regardless of the storage time. The reduction in phenolic compounds after one, two and three weeks of storage was -4.0 to -12.2%, -6.6 to -14.1%, and -11.3 to -29.2%, respectively, depending on the solvent system. For all four solvent systems, the reduction of phenolic compounds increased with increasing storage time. Upon cold storage for three weeks, the total reduction in phenolic compounds in the oven-dried sample and the sun-dried sample was -11.3 to -25.5% and -19.1 to -29.2%, respectively, depending on the solvent system. The rates of reduction in phenolic compounds due to storage (-11.3 to -29.2%) (Table 3) were lower than those caused by drying (-29.8 to -77.4%) (Table 1). This may be due to the phenolic compounds of high susceptibility to oxidation being degraded during the drying process. The residual phenolic compounds after the drying process were those of higher stability and therefore experienced less oxidation during the storage (Srivastava, 2006). In addition, the low temperature during cold storage presented less oxidative stress when compared to the high temperature applied during drying. For both oven drying and sun drying, the final reduction rate of phenolic compounds at the end of the 3-week storage decreased with increasing polarity of the solvent (Table 3). This indicates the more polar phenolic compounds (obtained through more polar solvents) were more stable and therefore experienced less degradation during storage when compared to the relatively less polar compounds (obtained through relatively less polar solvents). According to Kevers et al. (2007), the stability of phenolic compounds in plants during storage varies greatly depending on the type of plant and the type of polyphenol compounds. The TPC in broccoli was found to increase during the early stages of 27-day cold-storage before declining sharply. For 8-day cold storage, the decrease in TPC was 30% on the second day for lettuce and 50% on the fifth day for celery. The decrease of the TPC in both vegetables may be caused by the deterioration of flavonoids, a group of polyphenol compounds.

For both oven drying and sun drying, DPPH, FRAP and BCB activities decreased with increasing storage time regardless of

Table 3

TPC in dry leaves of Clinacanthus nutans *Lindau after storage*

Solvent system	TPC		
	mg GAE/100 g DW		
	Week 1	Week 2	Week 3
Boiling water (Oven drying)	973.33 ± 16^{a12} (-4.0%)	953.56 ± 17^{a2} (-6.0%)	899.16 ± 11^{a3} (-11.3%)
	Week 0: 1013.99 ± 11^{a1}		
Water (Oven drying)	728.01 ± 20^{b12} (-5.4%)	711.67 ± 21^{b2} (-7.5%)	677.47 ± 34^{b3} (-11.9%)
	Week 0: 769.22 ± 27^{b1}		
50% Methanol (Oven drying)	468.69 ± 23^{c12} (-5.0%)	454.93 ± 22^{c2} (-7.8%)	394.07 ± 20^{c3} (-20.1%)
	Week 0: 493.41 ± 26^{c1}		
100% Methanol (Oven drying)	354.42 ± 26^{d12} (-5.4%)	335.86 ± 24^{d2} (-10.4%)	274.84 ± 16^{d3} (-25.6%)
	Week 0: 374.77 ± 30^{d1}		
Boiling water (Sun drying)	466.63 ± 14^{c12} (-6.1%)	453.55 ± 11^{c2} (-8.7%)	401.84 ± 11^{c3} (-19.1%)
	Week 0: 496.94 ± 16^{c1}		
Water (Sun drying)	320.31 ± 72^{d1} (-9.43%)	306.58 ± 73^{d2} (-10.5%)	268.24 ± 63^{d3} (-21.8%)
	Week 0: 342.67 ± 79^{d1}		
50% Methanol (Sun drying)	194.23 ± 53^{e1} (-11.6%)	188.71 ± 51^{e12} (-14.1%)	157.71 ± 40^{e2} (-28.2%)
	Week 0: 219.74 ± 57^{e1}		
100% Methanol (Sun drying)	111.61 ± 10^{f12} (-12.2%)	102.47 ± 10^{f2} (-19.4%)	90.00 ± 10^{e3} (-29.2%)
	Week 0: 127.08 ± 11^{f1}		

For each column in each assay, values with different letter superscripts (a-f) denote significant differences ($p < 0.05$).

For each row in each assay, values with different number superscripts (1-3) denote significant differences ($p < 0.05$).

Values in parentheses are the changes (%) compared to Week 0 where the signs '+' and '-' refer to an ncrease and a decrease, respectively.

Table 4

DPPH, FRAP and BCB activities in dry leaves of Clinacanthus nutans *Lindau after storage*

Solvent system	DPPH			FRAP			BCB		
	EC_{50} (mg/mL)			mmol $Fe_2SO_4.7H_2O$/100 g DW			Inhibition (%)		
	Week 1	Week 2	Week 3	Week 1	Week 2	Week 3	Week 1	Week 2	Week 3
Boiling water (Oven drying)	1.12 ± 0.04^{a12} (+4.7%)	1.20 ± 0.40^{a23} (+12.1%)	1.28 ± 0.04^{a3} (+19.6%)	228.59 ± 20^{a12} (-4.9%)	216.89 ± 15^{a2} (-9.7%)	182.11 ± 12^{a3} (-24.2%)	69.33 ± 1.06^{a2} (-6.6%)	61.87 ± 0.47^{a3} (-16.6%)	55.67 ± 0.50^{a4} (-25.0%)
	Week 0: 1.07 ± 0.02^{a1}			Week 0: 240.29 ± 22^{a1}			Week 0: 74.21 ± 0.36^{a1}		
Water (Oven drying)	1.92 ± 0.10^{b12} (+7.3%)	2.07 ± 0.11^{b2} (+15.6%)	2.24 ± 0.15^{b2} (+25.1%)	137.09 ± 11^{b12} (-6.5%)	131.10 ± 12^{b2} (-10.6%)	110.34 ± 10^{b3} (-24.7%)	61.48 ± 0.20^{b2} (-6.4%)	55.37 ± 0.53^{b3} (-15.7%)	49.73 ± 1.04^{b4} (-24.3%)
	Week 0: 1.79 ± 0.08^{b1}			Week 0: 146.61 ± 12^{b1}			Week 0: 65.67 ± 0.53^{b1}		
50% Methanol (Oven drying)	3.71 ± 0.24^{c12} (+9.4%)	4.07 ± 0.26^{c2} (+20.1%)	4.45 ± 0.29^{c2} (+31.3%)	98.29 ± 16^{c1} (-7.1%)	93.56 ± 16^{c2} (-11.6%)	75.97 ± 12^{c3} (-25.4%)	78.34 ± 2.01^{c1} (-5.6%)	71.96 ± 1.90^{c2} (-13.3%)	63.75 ± 2.36^{c3} (-23.2%)
	Week 0: 3.39 ± 0.27^{c1}			Week 0: 105.83 ± 17^{c1}			Week 0: 82.98 ± 1.20^{c1}		

Table 4 *(Continue)*

Table 4

DPPH, FRAP and BCB activities in dry leaves of Clinacanthus nutans *Lindau after storage*

Solvent system	DPPH			FRAP			BCB		
	EC_{50} (mg/mL)			mmol $Fe_2SO_4.7H_2O$/100 g DW			Inhibition (%)		
	Week 1	Week 2	Week 3	Week 1	Week 2	Week 3	Week 1	Week 2	Week 3
100% Methanol (Oven drying)	5.15 ± 0.85^{d12} (+12.0%)	5.74 ± 0.93^{d23} (+24.8%)	6.28 ± 1.05^{d3} (+36.5%)	44.75 ± 8^{d12} (-8.3%)	42.80 ± 8^{d2} (-12.3%)	33.91 ± 6^{d2} (-30.5%)	83.89 ± 2.31^{d1} (-4.4%)	76.92 ± 3.18^{d2} (-12.3%)	70.76 ± 2.64^{d2} (-19.3%)
	Week 0: 4.60 ± 0.70^{d1}			Week 0: 48.78 ± 9^{d1}			Week 0: 87.72 ± 1.44^{d1}		
Boiling water (Sun drying)	1.59 ± 0.09^{b12} (+4.6%)	1.70 ± 0.12^{b23} (+11.8%)	1.88 ± 0.11^{b3} (+23.7%)	164.94 ± 13^{b12} (-5.0%)	160.13 ± 11^{b2} (-7.8%)	128.94 ± 11^{b3} (-25.7%)	59.14 ± 0.32^{b2} (-10.6%)	54.34 ± 0.41^{b3} (-17.9%)	48.91 ± 0.95^{b4} (-26.1%)
	Week 0: 1.52 ± 0.08^{b1}			Week 0: 173.63 ± 14^{b1}			Week 0: 66.15 ± 0.69^{b1}		
Water (Sun drying)	3.29 ± 0.27^{c12} (+8.6%)	3.53 ± 0.26^{c2} (+16.5%)	3.82 ± 0.26^{c2} (+26.1%)	91.60 ± 11^{c12} (-9.4%)	82.04 ± 12^{c2} (-18.9%)	71.50 ± 10^{c3} (-29.3%)	57.26 ± 1.57^{b2} (-9.8%)	53.20 ± 1.43^{b2} (-16.2%)	48.42 ± 0.60^{b3} (-23.7%)
	Week 0: 3.03 ± 0.26^{c1}			Week 0: 101.14 ± 13^{c1}			Week 0: 63.46 ± 2.29^{b1}		

Table 4 *(Continue)*

Solvent system	DPPH			FRAP			BCB		
	EC_{50} (mg/mL)			mmol $Fe_2SO_4.7H_2O$/100 g DW			Inhibition (%)		
	Week 1	Week 2	Week 3	Week 1	Week 2	Week 3	Week 1	Week 2	Week 3
50% Methanol (Sun drying)	5.11 ± 0.18^{d12} (+9.7%)	5.65 ± 0.18^{d23} (+21.2%)	6.16 ± 0.25^{d3} (+32.2%)	68.66 ± 14^{d12} (-11.9%)	61.81 ± 12^{d2} (-20.7%)	48.66 ± 10^{d3} (-37.6%)	67.94 ± 3.07^{a12} (-6.9%)	62.07 ± 3.01^{a23} (-14.9%)	57.34 ± 2.22^{a3} (-21.4%)
	Week 0: 4.66 ± 0.21^{d1}			Week 0: 77.92 ± 19^{d1}			Week 0: 72.94 ± 3.79^{a1}		
100% Methanol (Sun drying)	6.21 ± 0.24^{e12} (+11.1%)	7.00 ± 0.35^{e23} (+25.2%)	7.76 ± 0.33^{e3} (+38.8%)	21.79 ± 2^{e12} (-13.1%)	19.44 ± 3^{e2} (-22.5%)	16.10 ± 2^{e2} (-35.8%)	77.89 ± 2.12^{c1} (-5.0%)	70.66 ± 1.78^{c2} (-13.8%)	65.65 ± 2.04^{c2} (-19.9%)
	Week 0: 5.59 ± 0.30^{e1}			Week 0: 25.08 ± 3^{e1}			Week 0: 81.95 ± 1.69^{c1}		
Standard	Ascorbic acid: 0.026 ± 0.0001^{f}						Butylated hydroxyanisole (BHA): 98.09 ± 0.39^{e}		

For each column in each assay, values with different letter superscripts (a-f) denote significant differences ($p < 0.05$).

For each row in each assay, values with different number superscripts (1-3) denote significant differences ($p < 0.05$).

Values in parentheses are the changes (%) compared to Week 0 where the signs '+' and '-' refer to an increase and a decrease, respectively.

solvent system (Table 4). The reduction in the antioxidant activities may be explained by the reduction in TPC. The total reduction of FRAP and BCB activities at the end of the 3-week storage was -24.2 to -37.6% and -19.3 to -26.1%, respectively, depending on the solvent system. The EC_{50} of DPPH increased (indicating a decrease in DPPH activity) by 19.6-38.8% after 3 weeks of storage depending on the solvent system.

Correlation

The total phenolic content extracted from the fresh leaf sample was positively correlated with DPPH activity, $1/EC_{50}$ ($r^2 = 0.969$, $p < 0.05$) and FRAP activity ($r^2 = 0.991$, $p < 0.01$). The antioxidant activities of DPPH and FRAP are based on the single electron transfer (SET) mechanism. SET-based mechanism measures the ability of the antioxidant in donating electrons to reduce (stabilize) the free radicals (Prior, Wu, & Schaich, 2005). The high positive r^2 values (close to 1) suggest the phenolic compounds extracted contribute significantly to DPPH and FRAP activities. This may indicate that most of the phenolic compounds favour the SET mechanism in their antioxidant activities. However, there was no significant correlation between TPC and BCB activity ($r^2 = -0.760$, $p > 0.05$). The BCB antioxidant activity is based on the hydrogen atom transfer (HAT) mechanism. HAT-based mechanism measures the antioxidant capability in preventing free radical chain activities by donating a hydrogen atom (Apak et al., 2007). The lack of correlation between TPC and BCB activity may suggest that most of the phenolic compounds extracted do not proceed through the HAT mechanism in their antioxidant reactions.

CONCLUSION

The extracts obtained from fresh leaf sample of *Clinacanthus nutans* Lindau had the highest TPC and antioxidant activities (DPPH, FRAP and BCB) when compared to the extracts from dried leaf samples. Drying (oven drying and sun drying) reduced the TPC and antioxidant activities with sun drying causing higher reduction effect. Cold storage for three weeks also resulted in a reduction in TPC and antioxidant activities with increasing reduction level with increasing storage time. In term of extraction of TPC, boiling water recorded the highest yield, followed by water at room temperature, 50% methanol and 100% methanol. The TPC of the fresh leaf sample was positively correlated with the DPPH and FRAP activities. No significant correlation was found between the TPC and the BCB activity.

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Effect of Palm Kernel Cake and Coconut-based Formulated Diet on Malaysia Village Chicken Growth Performances and Meat Quality

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ABSTRACT

The potential of the usage of palm kernel cake (PKC)-coconut based feeds has been considered as an alternative to commercial feed due to their low cost and abundance in availability and accessibility in Malaysia. However, previous studies have shown that the use of high fiber feed such as PKC resulted in poor performance of poultry due to low digestibility and palatability. In this study, a total of 400 village chickens from purebred and crossbred strains were reared to evaluate the effects of PKC and coconut-based feeding on their production performance. Body weight (BW), carcass evaluation, abdominal fat, meat conversion percentage, proximate analysis, and amino acid profile analyses were recorded in the study. The results showed that the Type A feed that contained higher fiber level resulted in poorer BW and carcass weight for both strains. It could be suggested that inconsistent size of fiber particles could influence the chicken's digestibility. However, it was also shown that the quality of the meat of the village chicken and Type A feed were better in terms of having lower abdominal fat and crude fat contents. It also resulted in higher CP in crossbred strain meat which is correlated significantly with meat conversion percentage. Type B-fed

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chicken meat recorded low meat conversion percentage indicating higher formation in bone mass and feather, which is supported by the data of the ash in crossbred strain, indicating higher formation of mineral build-up such as bone mass. Furthermore, there were 11 amino acids that were recorded to be significantly higher in Type A-fed chicken meat compared with Type-B fed chicken meat, indicating a higher meat quality. In conclusion, the usage of Type-A feed as daily feed for village chicken was beneficial and exhibited prominent values in terms of quality and cost-effectiveness. However, more studies should be done to improve the digestibility and palatability of Type A feed to improve in their overall performances so that it can be used widely in poultry, particularly in village chicken farming.

Keywords: PKC-coconut-based, proximate analysis, purebred, crossbred, village chicken

INTRODUCTION

The usage of PKC-based feed that contains higher fiber content for chicken farming is considered less efficient in terms of digestibility and palatability, especially in the chicken industry. It was studied that by increasing the fiber content in the diet, it could help in the development of production of enzyme, improve digestibility, and organs development in poultry (Mateos, Jiménez-Moreno, Serrano, & Lázaro, 2012; Rodriquez & Preston, 1997). As for broiler production, the usage of high fiber content diet has been limited. However, in the village chicken industry, it is seen to be an option for the farmers in order to decrease the feed cost. This is also due to the fact that village chicken grows slower (Azahan, Azlina Azma, & Noraziah, 2011), thus taking longer time before it can be marketed, as well as requiring higher maintenance and feed costs.

In Malaysia, palm kernel cake is highly available due to the importance of oil palm industry to the country. The palm kernel cake is usually used for ruminant animals, such as goat and cattle, as supplementary diet. However, in poultry, it was stated by Alimon (2004) that the usage of high PKC content might result in toxicity to the chicken due the high fiber contents in the diets. Furthermore, author also suggested that an inclusion of a maximum of 20% of PKC in poultry feed is adequate (Alimon, 2004). Meanwhile, coconut-based feed is also considered as a feed ingredient that exhibits good palatability for chicken. A previous study has shown that feeding chicken with extracted coconut meat did not gave any adverse effects to the chicken egg production as well as results in an increment of feed consumption (Moorthy & Viswanathan, 2010).

It is known that purebred village chicken grows slower compared to commercial broiler, thus resulting in slower expected weight for market. Crossbred village chicken is the product of crossbreeding the purebred village chicken with broilers to improve their growth rate and production of eggs.

The slow growth of village chicken is due to many factors, such as genetics, environment, nutrition, parasites, young mortality, and diseases (Cumming, 1991). Furthermore, the growth of chicken is governed by the ability to digest feed that contains crude protein. It was suggested that 90% of amino acid content inside poultry meat to be an indicator for actual crude protein content in the meat (Hunton, 1995). Furthermore, the quality of protein within any meat is largely influence by the amino acid concentration and it is also responsible for human wellness and health (Schaafsma, 2000).

The objectives of this research were to investigate the effects of local feed ingredients that were based on palm kernel cake and coconut-based feed towards the growth performance of village chicken as well as to identify the quality of the meat by observing proximate and amino acid data of the meat of chicken fed using the 2 types of diets and using 2 types of village chicken, which were purebred and crossbred village chicken in Malaysia.

MATERIALS AND METHODS

A total of 400 of 1-day old village chickens with cumulative weight 38 grams, from purebred (n = 200) and crossbred (n = 200) strains, were reared in Livestock Science Centre, Institute of Biological Sciences, University of Malaya from April 2016 to February 2017. The study was approved by University of Malaya Institutional Animal Care and Use Committee (UMIACUC) with ethics number I/02022017/03112016-01/R.

Table 1

The composition and dry matter percentage of formulated feed ingredients (Type B) (Mazlishah, Zazali, Wan Khadijiah, Wan Syahidah, & Abdullah, in press)

Ingredients	Amount (Kg)	Dry matter (DM, %)
Corn	29.8	87.0
Rice Bran	11.9	91.0
Palm Kernel Cake	7.4	90.6
Coconut Meat	14.1	43.0
Soybean Meal	26.0	91.0
Vitamin Premix	4.5	-
Mono-Dicalcium Phosphate (MDCP)	4.5	-
Salt	0.9	-
Limestone	0.9	-
TOTAL	100	-

**The composition of vitamin premix for the study was included Vitamin A, D3, E and C. The specific amounts were disclosed due to the company privacy.*

Table 2

Comparison of proximate analysis between Type-A diet and Type-B diet (Mazlishah et al., in press)

Nutrients composition	Type A	Type B
Dry matter (%, DM)	81.16	89.05
Crude protein (%, DM)	14.95	13.40
Crude fat (%, DM)	3.78	3.02
Crude fiber (%, DM)	12.22	3.12
Ash (%, DM)	5.18	4.43
Gross energy (MJ/kg DM)	17.13	16.30
Metabolizable energy (MJ/kg DM)	9.84	9.36

There were 2 types of diets used in this study. Commercial diet was labelled as Type-B and the formulated diet was labelled as Type-A feed. The diets were given to the chicken after Week 3 of rearing. There were 4 groups with 100 chicks in each group (purebred: type A, purebred: type B, crossbred: type A, and crossbred: type B). Each group consisting 100 chicks were divided into 5 pens where each pen consisting 20 chicks. At one time, 2 groups from same strains were reared due to limited space. Table 1 shows the composition of the formulated diet used in the study based on (Mazlishah et al., 2018). There were 2 local ingredients used in the study which were palm kernel cake (PKC) and coconut meat (milk extracted coconut meat). The chemical composition is shown in Table 1 and the proximate analysis of the feed is shown in Table 2. The body weight (BW) at Weeks 8 and 12 were recorded for crossbred strains, while BW at Week 12 and Week 16 were recorded for purebred strain. The age of slaughtering for purebred and crossbred strains were different due to each strain reaching the required weight for market (1.5 kg) at different times. Carcass performances (carcass weight, abdominal fat, and meat conversion percentage), feed conversion ratio (FCR), and proximate analysis on chicken meat were also recorded. Carcass evaluation and live weight were carried out to identify the maximum weight gain for the village chicken given the two types of diet. Eviscerated chicken carcasses were separated from the bone for meat quantity percentage and weight. Then, fresh meats were dried inside an oven at 98°C for 48 hours. The carcass quality and meat conversion of the chicken were compared among the diets and strains.

Proximate and amino acid profile analyses were carried out using the dried meat at the Veterinary Public Health Laboratory, Bandar Baru Salak Tinggi, Sepang, Selangor. The parameters measured for proximate analysis were dry matter (DM), crude protein (CP), crude fat (CF), crude fiber (CFb), and total ash. The amino acid profile analyses were conducted using Ultra Performance Liquid Chromatography with Photodiode Array (UPLC - PDA) Triplicates for each sample were tested using the dried meat in crossbred village chicken to identify the meat's amino acid content for the village

chickens that were fed with Type-B and Type-A. The meat to bone percentage was calculated based on the following formula:

$$Meat - Bone\ percentage\ (\%) = \frac{Deboning\ meat\ weight}{Carcass\ weight} \times 100$$

Statistical analysis: The data were analyzed using Statistical Package for the Social Science (SPSS) software T-test for each slaughtered age (Crossbred: W8 and W12) and (Purebred: W12 and W16) with diets as treatments for live body weight, carcass performances, proximate analysis, and amino acid profile comparison between Type-A and Type-B. ANOVA was used to calculate the mean differences in meat conversion percentage. ANOVA could not be used to differentiate the significance of means between strains due to difference in age of slaughtering.

RESULTS AND DISCUSSION

The body weight for village chicken for purebred was significantly heavier for Type B at Week 12, while no significant differences were seen in Week 16 for both Type-B and Type-A. Meanwhile, carcass weight for purebred showed similar trends with the body weight. The abdominal fat content for purebred was significantly higher for Type-B chickens at Week 16 of age. The meat to bone percentage showed that Type-A chickens produced more meat than Type-B chickens at Week 12 and 16 of ages. However, the FCR for Type-A fed purebred village chickens were obviously higher compared to Type-B for the same strain as shown in Table 3.

The usage of high fiber feed ingredients such as PKC in poultry diets is one of the alternative feed ingredients to minimize the cost of the feed. However, the limitation of the PKC amount that could be utilized by poultry is only up to 20-30% in the diet. Higher levels of PKC, more than 30%, could cause a reduction in energy due to the high level of fiber content in the feed (Zahari & Alimon, 2005). Type-A feed that was used in this study had higher crude fiber (12.22%) compared with Type-B (3.12%). This high percentage of crude fiber inside Type-A feed might have been contributed by the PKC amount in the diet despite the amount used was well below the maximum recommended PKC level (Alimon, 2004), which was 7.4% from the total formulated diet. The relatively high crude fiber content in Type-A diet might be due to the uneven manual mixing and surface area of the PKC particles grounded using the machines, which were non-similar and structurally inconsistent inside the mixture of the feed. Furthermore, it was also stated in a previous study that PKC had abundance of insoluble fiber as well as non-starch polysaccharides (NSPs) (Alimon, 2004; Fransech & Brufau, 2004; Sundu & Dingle, 2003). Besides, the level of PKC in Type-A diet that was high compared with Type-B might also affect the physiology of the intestinal villi, which could lead to the decrease in width, height, and surface area of the intestinal villi. This could result in poor utilization and digestibility of the feed that would also affect the body weight for Type-A fed chicken for both strains (Kalmendal, Elwinger, Holm, & Tauson,

Table 3

Body weight, carcass weight, abdominal fat, meat to bone percentage and feed conversion ratio for Purebred village chicken at Week 12 and Week 16

	Purebred				
	Week 12			Week 16	
	Type B	Type A		Type B	Type A
Body Weight, (g)	2359.0±92.3**	1846.0±100.9*	Body Weight, (g)	2295.0±196.2*	2303.0±121.0*
Carcass Weight, (g)	1761.0±67.2**	1332.0±73.6*	Carcass Weight, (g)	1837.0±165.9*	1754.0±105.9*
Abdominal fat, (g)	4.5±1.7*	5.4±1.6*	Abdominal fat, (g)	24.1±6.6**	7.5±1.7*
Meat-bone percentage, (%)	39.2	47.4	Meat-bone percentage, (%)	52.4	60.0
Feed conversion ratio (FCR) (Weeks 3–12)				2.9±0.6*	5.2±1.6*

* indicates no significant difference in same row using T-test ($p>0.05$)

** indicates no significant difference in same row using T-test ($p<0.05$)

Table 4

Body weight, carcass weight, abdominal fat, meat to bone percentage and feed conversion ratio for crossbred village chicken at Week 8 and Week 12

	Crossbred				
	Week 8			Week 12	
	Type B	Type A		Type B	Type A
Body Weight, (g)	1718.0±47.4**	1408.0±28.0*	Body Weight, (g)	2791.0±149.9**	1951.0±116.8*
Carcass Weight, (g)	1321.0±85.6**	1020.0±25.9*	Carcass Weight, (g)	2146.0±109.6**	1818.0±83.0*
Abdominal fat, (g)	15.6±4.5**	3.9±1.8*	Abdominal fat, (g)	31.0±7.0**	9.9±9.5*
Meat-bone percentage, (%)	48.6	50.1	Meat-bone percentage, (%)	45.0	48.0
Feed conversion ratio (FCR) (Weeks 3–12)				2.4±0.4*	2.6±0.4*

* indicates no significant difference in same row using T-test ($p>0.05$)

** indicates no significant difference in same row using T-test ($p<0.05$)

2011; Moharrery & Mohammadpour, 2005). The physical nature of the high fiber ingredients such as PKC is coarse and ground which is harder to grind and digest, thus could result in accumulation of fiber particles inside the poultry gizzard (Hetland, Svihus, & Choct, 2005).

Meanwhile, the CP recorded for Type-A was slightly higher compared to Type-B diet as shown Table 2. This might be due the higher dietary fiber content for Type-A diet, which is correlated with accumulation of fiber in gizzard. This would result in the increase of antiperistaltic movements inside the gastrointestinal tract (GIT) that could help in the secretion of pancreatic enzymes due to the increase of cholecystokinin release. This will further increase the GIT activity as well as increase the CP and other dietary components' digestibility as shown in Table 2 (González-Alvarado, Jiménez-Moreno, Lázaro, & Mateos, 2007; Hetland et al., 2005; Jiménez-Moreno, González-Alvarado, Lázaro, & Mateos, 2009; Svihus, Juvik, Hetland, & Krogdahl, 2004). Type-B fed crossbred village chicken recorded significantly higher body weight, carcass weight, and abdominal fat compared to Type A. Meanwhile, Type-A recorded higher percentage of meat to bone ratio compared to Type-B. However, the FCR for Type-A fed crossbred village chicken was slightly higher compared to Type-B fed chicken as shown in Table 4. The meat conversion percentage of CVC fed with Type A feed was significantly higher for both male and female chicken compared with Type B fed CVC as shown in Table 5.

Table 5

Percentage of meat conversion for purebred village chicken (PVC) and crossbred village chicken (CVC) fed on Type-A and Type-B diets based on genders

Strains	Type B (Mean ± SEM)		Type A (Mean ± SEM)	
	Male	Female	Male	Female
PVC (Week 12), (%)	27.8±0.6^{a}	37.8±0.3^{b}	32.1±0.7^{ab}	34.2±0.9^{b}
CVC (Week 8), (%)	31.8±0.4^{a}	31.8±0.3^{a}	31.0±0.9^{a}	32.0±0.6^{a}
PVC (Week 16), (%)	37.9±0.5^{a}	41.5±0.7^{b}	39.0±0.5^{a}	36.7±0.9^{a}
CVC (Week 12), (%)	33.6±0.5^{b}	27.9±0.6^{a}	43.6±0.8^{c}	36.1±0.03^{b}

^{a,b,c} Means with the same superscript letter in same row shows no significant difference using ANOVA ($P>0.05$).

The high crude fiber in the feed affected the growth performances recorded in the experiment, where the live weight recorded for the village chicken in both strains were significantly heavier in chickens that were fed on Type-B compared with Type-A at their first slaughtering ages, respectively. However, there was an improvement in body weight for purebred village chicken as it recorded no significant differences between chicken fed with Type-B (2295.0±196.2 g) compared with Type-A fed chicken (2303.0±121.0 g). Despite that, in crossbred village chicken, the body weight for Type-B (2791.0±149.9 g) fed chickens were still significantly heavier than Type-A fed chickens (1951.0±116.8 g). This might be due to the fact that crossbred village chickens grow faster, thus exhibiting the capability of crossbred village chickens to utilize feed better compared with purebred village chickens. Similar trend was also observed for carcass weight for both strains (Table 3).

One of the main advantages in the village chicken from the consumers' perspective is the preference for low fat content in the meat and carcass of village chicken or indigenous chicken (Ahn, Park, Kwon, & Sung, 1997; Musa, Chen, Cheng, & Mekki, 2006). Purebred and crossbred village chickens showed highly significant differences in abdominal fat content fed on Type B (24.1±6.6 g, 31.0±7.0 g) compared to Type-A (7.5±1.7 g, 9.9±9.5 g) at Week 16 for purebred and Week 12 of age for crossbred village chicken, respectively. The differences in abdominal fat content in chickens fed on Type-B and Type A might be due to the differences in the difference of CP content between Type-B (13.40%) and Type-A (14.95%), which was mentioned in a previous research done by Summers and Leeson (1979). They stated that the similarity in energy content in the diet and differences in crude protein content in the diet could influence the deposition of fat in poultry. Moreover, the data for proximate analysis on village chicken meat for crude fat also showed high-fat deposition in Type-B fed chicken meat (5.5±0.1%) compared to Type-A (3.3±0.8%) for crossbred village chicken. However, the data was contradicted by a previous research that recorded high-fat depots in chickens that were on feed based on palm oil source diet compared with Type-A fed chicken meat in terms of crude fat content (Velasco et al., 2010).

Meanwhile, the FCR recorded was different between the purebred and crossbred village chickens, where the FCR for purebred fed on Type-B and Type-A were 2.9 and 5.2respectively. Meanwhile, crossbred village chicken recorded FCR 2.4 and 2.6 for Type B and Type A fed chickens respectively. FCR for crossbred strains were lower with no significant differences between Type-B and Type-A fed chickens. This might be due to genetics reason where crossbred village chickens could grow faster and bigger as they have higher utilization ability for the feed compared to purebred village chickens. Furthermore, the genetics factor of purebred village chickens could also influence the nutritional factor, where the usage of high fiber contents feed such

as PKC could also result in reduction of body weight, as well as lowering the FCR value due to the low utilization capability of the feed (Pushpakumara, Priyankarage, Nayananjalie, Ranathunge, & Dissanayake, 2017; Sharmila, Alimon, Azhar, Noor, & Samsudin, 2014). In addition, the fat content recorded in crossbred village chickens were higher compared with purebred village chickens. This might be due to the low protein catabolism in the crossbred strain that was used to fulfill the requirement for growth and muscle mass, which was higher compared to slow growing chickens (Dransfield & Sosnicki, 1999).

Table 6

Proximate analysis of crossbred village chicken meat using 2 types of diets

Composition	Type A	Type B	T-test significance
Dry matter, DM (%)	97.9±0.3	97.5±0.04	ns
Crude Protein, CP (%)	84.5±1.1	78.4±2.5	**
Crude Fat, CF (%)	5.3±0.6	4.9±0.9	ns
Crude Fiber, CFb (%)	3.3±3.3	1.8±1.3	ns
Ash, (%)	5.3±0.4	9.4±1.6	**

* indicates no significant difference in same row using T-test ($p>0.05$)

** indicates no significant difference in same row using T-test ($p<0.05$)

Table 7

*Proximate analysis of purebred village chicken meat using 2 types of diet*s

Composition	Type A	Type B	T-test significance
Dry matter, DM (%)	97.6±0.2	97.2±0.4	ns
Crude Protein, CP (%)	89.3±2.2	87.7±0.3	ns
Crude Fat, CF (%)	3.3±0.8	5.5±0.1	**
Crude Fiber, CFb (%)	5.2±3.1	2.4±1.8	**
Ash, (%)	4.9±0.3	6.8±0.3	ns

* indicates no significant difference in same row using T-test ($p>0.05$)

** indicates no significant difference in same row using T-test ($p<0.05$)

The meat conversion percentage showed significant differences in male and female Type-A fed chickens (43.6±0.8% vs. 36.1±0.03%), especially in crossbred village chickens compared with Type-B fed chickens (33.6±0.5% vs. 27.9±0.6%). This indicates higher potential of useful development aspect in chicken such as meat as has been shown in chicken fed with Type-A compared with Type-B fed feed. This could mean that Type-B feed could result in the formation of more bone and feather than meat (Table 5). This could be supported by the data from proximate analysis, where CP in meat for Type-A (84.5±1.1%) fed chicken meat were significantly higher compared CP in Type-B (78.4±2.5%) fed chicken meat for crossbred village chicken as shown in Tables 6 and 7. Purebred village chicken showed no significant differences in CP content in the meat despite apparent high CP recorded for Type-A (89.3±2.2%) compared with Type-B (87.7±0.3%) fed purebred meat. In addition, the low percentage of meat conversion in Type-B reflects high ash content in Type-B (9.4±1.6%), where it was significantly higher than in Type-A (5.3±0.4%) chicken meat, especially in crossbred village chicken. This indicates that the formation of bone mass in Type-B fed chickens were higher than Type-A fed chickens. Significant data on crude fiber in purebred village chicken meat fed on Type-A was recorded (5.2±3.1%) compared in Type-B fed chicken meat (2.4±1.8%), while no significant differences recorded in crossbred village chicken. The high crude fiber in purebred strain meat was higher than recorded by Ogunmole, Taiwo and Ayankoso (2013) that used exotic chickens. Furthermore, higher crude fiber in purebred strain might be related to the tenderization of the meat, where high proteolytic activity could increase tenderization of meat due to the weakening of muscle fiber inside the meat (Dransfield & Sosnicki, 1999).

The amino acid profile analysis in crossbred village chicken meat fed on Type B and Type-A diets showed that the 11 amino acids were significantly higher in Type-A fed chicken meat compared with Type-B chicken meat as shown in Table 8. The 11 amino acids were serine, arginine, proline, lysine, threonine, aspartate, alanine, methionine, leucine, tyrosine, and phenylalanine. Some of the amino acids are responsible for the village chickens' unique characteristics preferred by consumers, such as glutamate that is responsible for village chicken flavor. The study of both Type-B and Type-A fed chicken meats recorded high glutamate content compared with the commercial broiler as reported by previous researchers (Choe et al., 2010; Shahidi, 2012; Wattanachant, 2008). The optimum level of essential amino acids in the meat is important to provide the required nutritive value to the meat. It was previously stated that 90% of amino acid comprised the amount of crude protein present in the meat (Hunton, 1995).

Table 8

Amino acid profile in crossbred village chicken meat fed on Type-A and Type-B diets

Amino Acid	Amount (Mean±SEM)		T-test significance
	Type A (n=10) (%)	Type B (n=10) (%)	
Histidine	6.89±2.13	5.15±2.10	Ns
Serine	9.35±4.10	4.91±3.00	**
Glycine	6.89±2.13	5.17±0.66	Ns
Arginine	8.61±0.80	5.62±0.31	**
Proline	5.41±0.41	3.66±0.47	**
Cysteine	14.31±1.39	11.19±1.34	ns
Lysine	2.41±0.62	0.16±0.03	**
Valine	2.52±0.33	1.87±0.25	ns
Aspartate	13.52±0.92	8.33±0.83	**
Glutamate	15.92±1.92	13.52±1.55	ns
Threonine	6.04±0.57	4.14±0.50	**
Alanine	8.24±0.62	4.77±0.36	**
Methionine	4.79±0.32	3.52±0.51	**
Tyrosine	10.59±0.79	5.09±0.58	**
Isoleucine	8.99±0.60	7.88±0.52	ns
Leucine	10.50±1.39	3.89±0.27	**
Phenyalanine	6.19±0.60	3.09±0.18	**

* indicates no significant difference in same row using T-test ($p>0.05$)

** indicates no significant difference in same row using T-test ($p<0.05$)

CONCLUSION

From the findings of the present study, it could be summarized that the inclusion of PKC and milk extracted coconut meat into the feed formulation in the diet could directly affect the growth performance and the carcass quality of the village chicken. Type-A feed resulted in lower body weight and carcass quality in village chickens in both strains due to digestibility and palatability of the feed, which could affect the ability of the village chicken to utilize the feed to its full potential. This might be due to higher fiber content compared to Type-B diet. However, the high fiber content inside the Type-A diet might have also caused other dietary components to improve. Despite the low growth rate and low carcass quality, the village chickens are continually in high demand due to its unique preferences by the consumers

who believe that village chicken meat has medicinal properties, preferred taste, and healthier to consume. In the present research, proximate analysis showed that Type-A feed gave low abdominal fat, crude fat, and is higher in terms of crude protein in the meat and meat conversion percentage. Furthermore, the unique characteristics of village chickens such as flavor and tenderization could be observed more in Type-A meat by observing the amino acid contents and crude fiber in both strains, especially in crossbred village chicken meat compared with Type-B meat. Thus, it could be said based on the data that Type-A could increase the quality of the meat produced by the village chicken by observing the amino acid content where most of Type-A fed chicken produced highly significant data compared to Type-B fed chicken's meat. However, more studies should be conducted to improve the digestibility and palatability of Type-A feed, to ensure the growth and sustainability of village farming in the country.

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Effect of Cooking on Antioxidant and Enzymes Activity Linked to Carbohydrate Metabolism and Lipid Peroxidation of Eggplant (*Solanum melongena*)

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ABSTRACT

The present study is aimed at investigating the influence of cooking on antioxidant and the inhibitory ability of eggplant on enzymes linked to hyperglycemia and induced lipid peroxidation in rat's kidney (*in vitro*). The total phenolic content (TPC), total flavonoid content (TFC), total antioxidant capacity (TAC), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity reducing power, lipid peroxidation inhibition and α-glucosidase and amylase enzymes were assayed to evaluate the effect. The results showed that cooking ($P < 0.05$) significantly increased TPC and TFC contents thereby enhancing DPPH scavenging activity and reducing power. Also α-amylase, α-glucosidase and Fe^{2+} induced lipid peroxidation inhibition in rat's kidney are enhanced significantly ($P < 0.05$) upon cooking. These results showed that cooking enhanced the ability of the eggplant to inhibit enzymes linked to diabetic mellitus and lipid peroxidation in kidney of rats *in vitro*.

Keywords: α-amylase, α-glucosidase, cooking, lipid peroxidation

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INTRODUCTION

Diabetes mellitus is a carbohydrate metabolic disorder characterized by hyperglycemia. The prevalence of diabetes mellitus has been on a steady rise in recent time and it is of concern in global public health. Not only is it difficult to treat, it has also being a major contributor to organ(s) damage, notable among them is kidney. According to National Institutes of Health (NIH), diabetes

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mellitus is one of the prominent causes of kidney damage, causing nearly twenty-five percent of kidney ailments (Afkarian et al., 2016). Studies have shown that high glucose level in the blood enhances reactive oxygen species production in mesangial cells of the kidney glomerular (Brownlee, 2005). The overproduction of these reactive oxygen species has been implicated as one of the main causes of damage to kidney (Nishikawa et al., 2000). Also, it has been well proven that oxidation of lipid is enhanced during diabetes mellitus which can lead to tissue damage (Nourooz-Zadehet al., 1997). Therefore antioxidant may be essential for the prevention and management of this diabetic complication. Hyperglycemia, an abnormal condition of excessive increase in blood level of glucose after a meal has been associated with the onset of non-insulin dependent diabetes mellitus (NIDDM) and its associated complications (Di Carli, Janises, Grunberger, & Ager, 2003). Increased postprandial blood glucose level probably occurs due to excessive activity of pancreatic enzymes; glucosidase and amylase. Evidences have been on the increase that foods possess inhibitory properties against these enzymes (Oboh et al., 2012). It is assumed that when these enzymes are inhibited, there is a significantly reduction in the postprandial elevation of blood glucose level thereby preventing hyperglycemia and hyperglycemia-induced oxidative stress. Hence it can be a therapeutic target in the management diabetic kidney disease.

Food processing such as cooking in water is usually done to improve or enhance the final qualities of food. It does not only improve flavour and taste of foods, it also enhances the bioavailability of nutrients by suppressing growth inhibitors and antinutrients in foods (Chau, Cheung, & Wong, 1997). It may also influence the total phenolics and flavonoids of food samples which may eventually affect the biological activities of such food samples (Luthria & Mukhopadhyay, 2006).

Eggplant (*Solanum melongena*) is an important agronomical plant belonging to the family Solanaceae and widely distributed throughout the temperate and tropical regions (Eun-Ju, Myung-Suk, Eun-Kyung, Young-Hong, & Seung-Cheol, 2011). It is a well consumed fruits in sub-Saharan Africa. It has many varieties across regions which usually come in different colours and shapes. It is usually eaten uncooked as snack or cooked for making stew. The eggplant is rich in polyphenol compounds and some essential vitamins (Hanson et al., 2006).

A lot of studies has shown that cooking affects the nutrients and antioxidant capacity of fruits and vegetables (Dewanto, Wu, Adom, & Liu, 2002; Chuah et al., 2008; Kao, Chiu, & Chiang, 2014). Also, many studies have reported the amylase and glucosidase inhibitory ability of eggplant extracts (Kwon, Apostolidis, & Shetty, 2008; Nwanna, Ibukun, & Oboh, 2013). The effects of processing on the physicochemical and antioxidant properties of this fruit have also been reported (Arkoub-djermoune et

al., 2016), but no study has been done to evaluate the effect of cooking on amylase, glucosidase and induced lipid peroxidation inhibitory ability of eggplant. Therefore the aim of this study is to investigate the effect of cooking eggplant on antioxidants and inhibition of enzymes linked to carbohydrate and induced lipid peroxidation in rat's kidney (*in vitro*).

MATERIALS AND METHODS

The fruits of eggplant (*Solanum melongena*) used in the work were gotten from local market (Bodija), Ibadan and identified and in the Botany Department, University of Ibadan. They were at full commercial maturity stage and eating quality. They were fresh and without infection, washed with distilled water and used for the research. The white variety usually comes in whitish yellow colouration while the green variety is usually green in colour (Figure 1).

Preparation of Cooked Sample

Fresh whole *Solanum melongena* fruit was cooked in the 500 ml-boiling water for 10 minutes. The beaker was covered to prevent water and heat loss due to evaporation. After cooking, the cooked samples were drained off. Fifty grams (50 g) of cooked sample was blended with 500 ml of distilled water using electric blender, the blended sample was filtered using muslin cloth and filtrate centrifuged at 3000 rpm (988 x *g*) for 10minutes. The supernatant was used for the chemical assays.

Preparation of Uncooked Sample

Fifty grams (50 g) of the fresh whole *Solanum melongena* fruit and was blended with 500 ml of distilled water using electric blender. The blended sample was filtered using muslin cloth and the filtrate centrifuged at 3000 rpm (988 x *g*) for 10 minutes. The supernatant was used for the chemical assays.

Figure 1. Varieties of eggplant used

Determination of Total Phenolic Content (TPC)

TPC of cooked and uncooked *Solanum melongena* was done using the (Kim, Jeong, & Lee, 2003) method with slight modifications. One millitre (1.0 ml) of the sample was mixed with 1.0 ml (10 %) of Folin-ciocalteu phenol reagent. After 5 mins, 5.0 ml (instead of 10.0) of 7 % Na_2CO_3 was added followed immediately with by addition of 5.0 ml (instead of 13.0 ml) of distilled water and shaken thoroughly. The mixture was kept in the dark for 90 minutes at room temperature. The absorbance was read at 750 nm and the TPC was evaluated from gallic acid standard curve and expressed as gallic acid equivalent (mg GAE/100g of fresh weight).

Determination of Total Flavonoid Content (TFC)

TFC of the cooked and uncooked *Solanum melongena* was done using the (Park et al., 2008) method. Sample (0.3ml) was mixed with 3.4 ml (30 %) of methanol, 0.15 ml (0.5 M) of $NaNO_2$ and 0.15 ml (0.3 M) of $AlCl_36H_20$ consecutively. After 5 minutes, 1 ml of 1 M NaOH was added and mixed well. The absorbance read at 506 nm and the flavonoid content evaluated from quercetin standard curve and expressed as quercetin equivalent (mg QUE/100g fresh weight).

Determination of 1, 1-diphenyl-2-picrylhydrazyl Scavenging Activity (DPPH)

DPPH radical scavenging activity of cooked and uncooked *Solanum melongena* was done using the (Gyamfi, Yonamine, & Aniya, 1999) method with slight modification. Appropriate dilutions of sample were added to 4 ml (instead of 3.9 ml) of DPPH solution (30 mg/l) prepared in methanol. The samples were mixed thoroughly and left in the dark for 30 minutes. The absorbance was read at 520 nm. The inhibition percentage was calculated as

Inhibition percentage of DPPH = {(Abs control- Abs Sample)/ (Abs Control)} × 100

DPPH solution without sample served as control.

Determination of Reducing Power (RP)

RP of cooked and uncooked *Solanum melongena* was done using the method of Oyaizu (1986). Appropriate dilutions of sample were mixed with 1 ml phosphate buffer (0.2 M, pH 6.6) followed by 1 ml of potassium ferricyanide (1 %) and incubated for 20 minutes at 50 ^{0}C. The reaction was terminated by 1 ml trichloroacetic acid (10 %). One millitre (1ml) of the upper portion was taken, mixed with 1 ml of distilled water and followed by 1 ml ferric chloride (0.1 %). The reaction mixture was thoroughly mixed and the absorbance was read at 700 nm. Higher absorbance indicates the higher reducing power of the sample.

Amylase Inhibition Assay (AI)

AI of the cooked and uncooked *Solanum melongena* was done using the (Worthington, 1993) method. Appropriate dilutions of sample and 500 µl (0.02 M) of sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing 0.5 mg/ml α-amylase solution

were incubated at room temperature for 10 min. Thereafter, addition of 500µl (1 %) of starch solution prepared with 0.02 M sodium phosphate buffer (0.006 M sodium chloride, pH 6.9) was followed. The reaction mixtures were then incubated at room temperature for 10 min. The reaction was halted with 1.0 ml (96 mM) of dinitrosalicylic acid. The reaction mixtures were then heated in boiling water for 5 minutes and allowed to cool. The absorbance was read at 540 nm. The inhibition percentage was calculated:

Inhibition percentage = {(Abs Control-Abs Sample)/ (Abs Control)} × 100

Glucosidase Inhibition Assay (GI)

GI of the cooked and uncooked *Solanum melongena* was done using (Apostolidis, Kwon, & Shetty, 2007) method. Appropriate dilutions of sample were suspended in 1000 µl α-glucosidase solution (1.0U/L) prepared in of 0.1 M sodium phosphate buffer (pH 6.9) and incubated at room temperature for 10 min. After incubation, the reaction mixture was mixed with 500 µl (5 mM) of nitrophenyl-glucopyranoside solution prepared in 0.1 M sodium phosphate buffer (pH 6.9). The reaction mixtures were left at room temperature for 5 minutes. The absorbance was read at 405 nm and inhibition percentage calculated as follows

Inhibition percentage = {(Abs control-Abs Sample)/ (Abs Control)} × 100

Inhibition of Lipid Peroxidation (LP)

Experimental animals: - Healthy Wistar albino rats (four) of weight between 100-120 g were bought from Animal House of Physiology Department, University of Ibadan. The rats were given fed (Ladokun feed) and water *ad libitum*. They were kept under a constant 12-h light and dark cycle and acclimatized for 1-week before used for the experiment. The experimental procedures were conducted in line with procedure approved by University of Ibadan Animal Care Use and Research Ethics Committee for care and use of experimental animals.

Preparation of kidney homogenate**: -** The preparation of homogenate was done by the method of Akinyemi, Ademiluyi and Oboh (2013). The rats were sacrificed under sodium pentobarbitone anesthesia and the kidney was removed, placed on ice and weighed. Kidney was homogenized immediately in cold normal saline water (1:4 w/v of fresh weight). The kidney homogenate was centrifuged at 3000 rpm (988 x *g*) for 10 minutes. The debris was discarded and lipid-rich supernatant obtained was used for lipid peroxidation assay

LP assay**: -** LP of cooked and uncooked *Solanum melongena* was done using (Ohkawa, Ohishi, & Yagi, 1979) method of with slight modifications. The reaction mixture was made up of 200 µl of the tissue homogenate, 30 µl of 0.1M Tris-HCl buffer (pH 7.4), sample solution ((0.1 - 0.4ml) and 30 µl of the freshly prepared pro-oxidant solution (5mM Sodium nitroprusside). The reaction mixture was incubated at 37^0 C for 2 h. The chromophore was developed by adding 300 µl (8.1%) Sodium dodecyl sulphate, 600 µl (pH 3.4) of acetic acid and

600 µl (0.8%) of TBA consecutively to the reaction mixture, thereafter the reaction mixtures were incubated for 1 hour at 100 ^{0}C. The absorbance of TBA-adduct (Malondialdehyde) formed was monitored at 532 nm. Inhibition of Malondialdehyde (MDA) production was calculated by the method of Banerjee (2005).

Statistical Analysis

Data are expressed as the mean ± SD of three measurements. The significance of the differences between the means of the samples were established by the analysis of variance using (SPSS 20) least significant difference $P< 0.05$, charts were drawn with graph pad prism 5 and Pearson correlation test was conducted to determine the correlation between antioxidant activities and enzymes inhibition. Significant levels were established using $P< 0.05$.

RESULTS

The effect of cooking on TPC and TFC of *Solanum melongena* was showed in Table 1. The TPC of uncooked white and green varieties of *Solanum melongena* were 72.50 mgGAE/100gfw and 70.2 mg GAE/100gfw respectively while TFC were 37.00 mgQUE/100gfw and 42.50 mgQUE/100gfw for white and green varieties respectively. On cooking, the total phenolics and total flavonoids increased significantly. TPC increased to 86.80 mg GAE/100gfw (white) and 86.20 mg QUE/100gfw (green) while TFC increased to 129.00 mg QUE/100gfw (white) and 177.00 mg QUE/100gfw (green). Cooking also significantly enhanced the capacity of the *Solanum melongena* to scavenge DPPH to Diphenylpicrylhydrazine with the loss of its violet colour (Figure 2). The uncooked samples were found to have an IC_{50} of 0.940 ml and 0.854 ml for white and green varieties respectively. Upon cooking the IC_{50} were found to decrease 0.386 ml and 0.321 ml respectively. Also reducing ability of the *Solanum melongena* was significantly enhanced in both varieties upon cooking in water (Figure 3).

The lipid peroxidation assay showed that the capacity of the *Solanum melongena* samples to inhibit the production of MDA in rat's kidney (*in vitro*) increased (Figure 4). The uncooked samples had the maximum inhibition of 63.34 % (white) and 63.70 % (green). Upon cooking, the inhibition increased to 78.25 % (white) and 82.84% (green). The increase in percentage inhibition of MDA produced was statistically significantly between the cooked and uncooked *Solanum melongena* samples. Also similar trend was observed in α-glucosidase and α-amylase inhibition assays. For α-glucosidase and α-amylase inhibitions (Figures 5 and 6), the uncooked *Solanum melongena* had maximum inhibition of 36.35% (white) and 37.85% (green), upon cooking, it significantly increased to 40.2% (white) and 45.85% (green). Cooking also significantly increased inhibition of α-glucosidase, the uncooked *Solanum melongena* had maximum inhibition of 22.71% (white) and 25.04% (green), while the cooked *Solanum melongena* had 28.24 % (white) and 31.53% (green) (Tables 2 and 3).

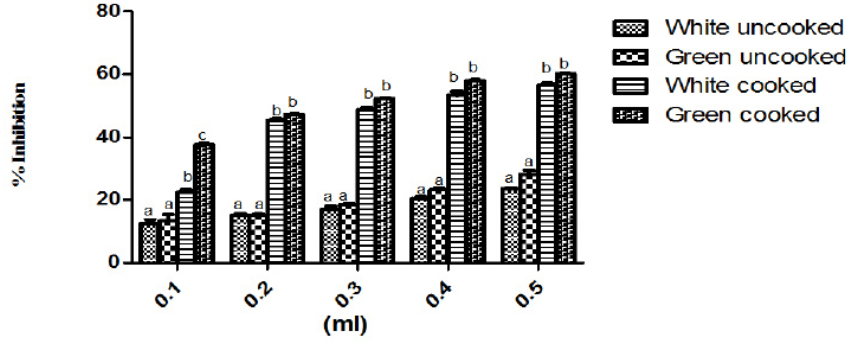

Figure 2. DPPH scavenging activity of cooked and uncooked *Solanum melongena*. Values were presented as Mean ± SD of triplicate reading. Values with the same superscript letter on grouped bars are not different significantly ($P < 0.05$)

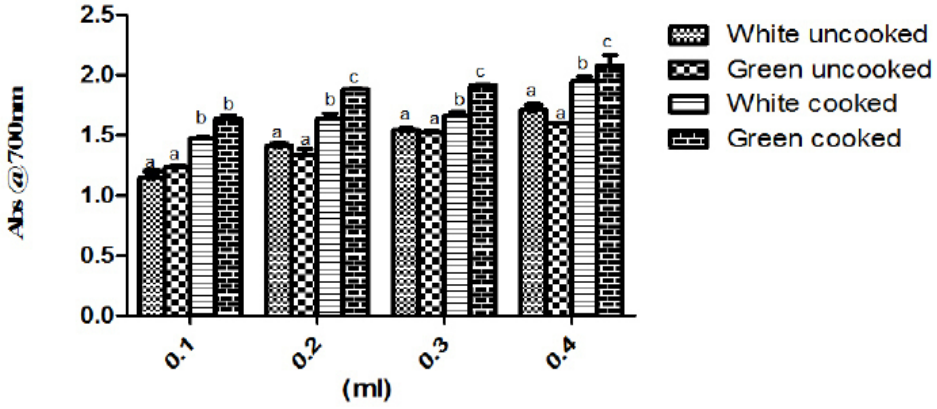

Figure 3. Reducing ability of cooked and uncooked *Solanum melongena*. Values were presented as Mean ± SD of triplicate reading. Values with the same superscript letter on grouped bars are not different significantly ($P < 0.05$)

Values followed by similar letters under the same column are not significantly different at p = 0.05 according to Duncan's multiple range test.

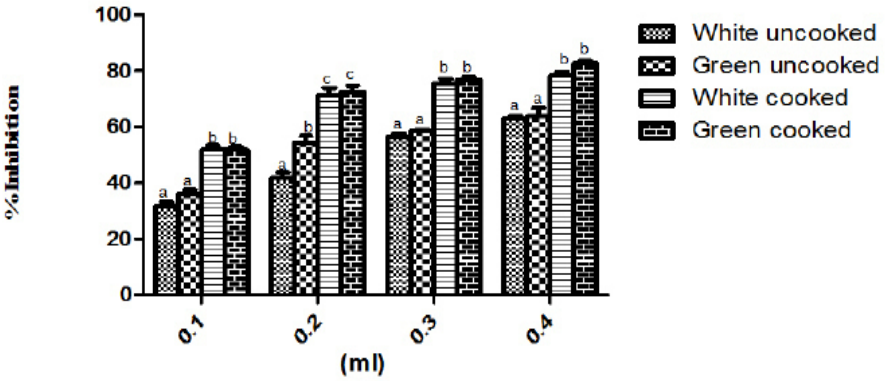

Figure 4. Inhibition of Fe^{2+} induced MDA production in Kidney by cooked and uncooked *Solanum melongena*. Values were presented as Mean ± SD of triplicate reading. Values with the same superscript letter on grouped bars are not different significantly ($P < 0.05$)

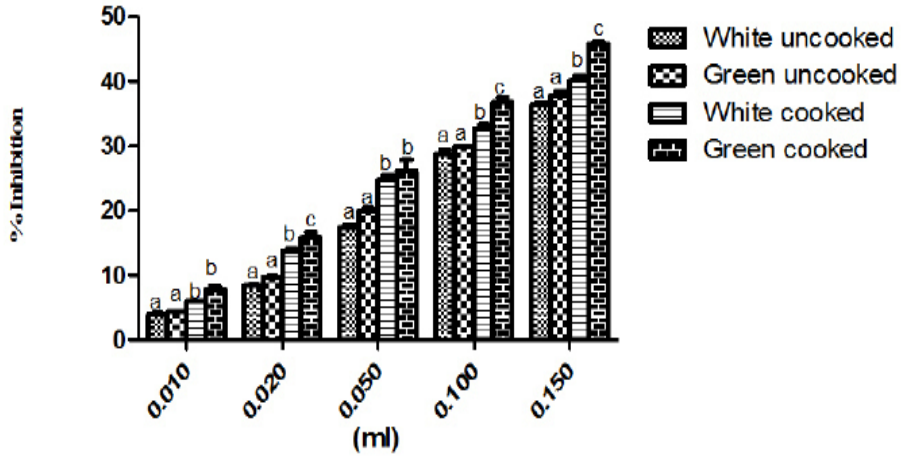

Figure 5.α-glucosidase inhibition ability of cooked and uncooked *Solanum melongena*. Values were presented as Mean ± SD of triplicate reading. Values with the same superscript letter on grouped bars are not different significantly ($P < 0.05$)

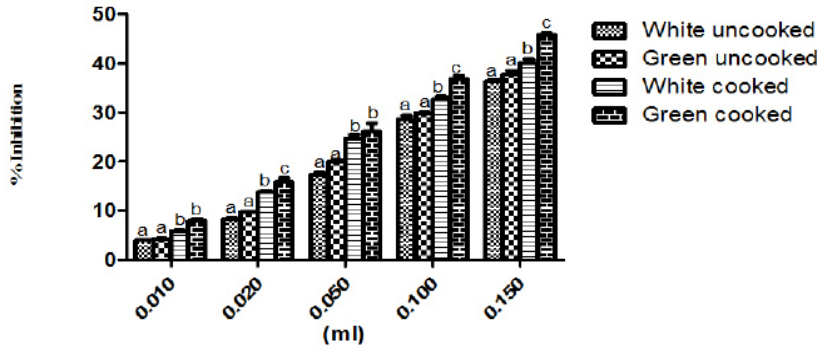

Figure 6.α-amylase inhibition ability of cooked and uncooked *Solanum melongena*. Values were presented as Mean ± SD of triplicate reading. Values with the same superscript letters on grouped bars are not different significantly ($P < 0.05$)

Table 1

Effect of cooking on TPC and TFC of cooked and uncooked Solanum melongena

	WU	GU	WC	GC
TPC	72.50±0.06^{a}	70.20±0.05^{a}	86.80±0.04^{b}	86.20±0.04^{b}
TFC	37.00±0.02^{a}	42.50±0.08^{b}	129.00±0.04^{c}	177.00±0.06^{d}

TPC: Total phenolic content (mg GAE/100 g fw), TFC; Total flavonoid content (mg QUE/100 g fw). WU; white uncooked, GU; Green uncooked, WC; White cooked, GC; Green cooked. Values were presented as Mean

Table 2

IC_{50} (ml) of uncooked and cooked Solanum melongena *on DPPH, α-amylase, α-glucosidase and MDA production*

	WU	GU	WC	GC
DPPH scavenging	0.94±0.06^{c}	0.85±0.04^{c}	0.39±0.03^{b}	0.32±0.03^{a}
α-amylase activity	0.49±0.04^{c}	0.41± 0.05^{b}	0.32±0.05^{a}	0.29±0.04^{a}
α-glucosidase activity	0.43±0.04^{c}	0.40±0.03^{b}	0.37±0.03^{b}	0.21±0.02^{a}
MDA	0.28±0.02^{d}	0.19±0.02^{c}	0.16±0.01^{b}	0.10±0.01^{a}

WU; white uncooked, GU; Green uncooked, WC; White cooked, GC; Green cooked. Values were presented as Mean ± SD of triplicate reading. Values with similar letter across the row are not different significantly ($P < 0.05$).

Table 3

Correlations between DPPH scavenging activity and inhibition of enzymes of carbohydrates metabolism

	Antioxidant	Glucosidase	Amylase
Antioxidant	1		
Glucosidase	0.59*	1	
Amylase	0.57*	0.98*	1

∗Correlation is significant at 0.05 level ($P < 0.05$).

DISCUSSION

Numerous studies have shown that there is positive correlation between sound health and consumption of foods rich in polyphenolic phytochemicals, also cooking or thermal treatments have been shown to alter the polyphenol contents, antioxidants and health functionality of fruits and vegetables (Chuah et al., 2008; Randhir, Kwon, & Shetty, 2008). Therefore, this study was designed to ascertain the effect of water cooking on antioxidant and inhibitory ability of *Solanum melongena* on enzymes linked to hyperglycemia and Fe^{2+} induced lipid peroxidation in kidney of rats (*in vitro*).

Numerous studies have shown conflicting results on effect of cooking in water on the polyphenolic contents, antioxidant ability and health functionality of fruits and vegetables. Although some water soluble or high polarity antioxidants could have been leaked into the water medium but the result obtained in this study showed that cooking in water enhanced the polyphenolic contents, antioxidant and inhibitory ability of eggplant on enzymes linked to hyperglycemia and Fe^{2+} induced lipid peroxidation in kidney of rats (*in vitro*). Polyphenolic are regarded as the major contributors of *in vitro* antioxidant capacity in foods (Li, Pickard, & Beta, 2007). In this study cooking in water enhanced the polyphenolic content of the eggplants used. Hence, the enhanced polyphenolic contents could be due to the mobilization of bound polyphenolic from the breakdown of cellular components and cell walls, other reasons could be that phenolics other than the endogenous ones may be formed as by-products during heat treatment in the eggplants (Randhir et al., 2008), higher extractability of antioxidant components due to matrix softening by cooking (Bernhardt & Schlich, 2006) and liberation of active aglycones from flavonoid conjugates (Miglio, Chiavaro, Visconti, Fogliano, & Pellegrini, 2008; Podsedek, 2007). This result is in agreement with the study of Dewanto et al. (2002) and Turkmen, Sari, &Velioglu (2005) who reported enhanced antioxidant activity in fruits and vegetables upon cooking treatments. Though our study did not extend to chemical profiling of eggplants but previous study by Lo-Scalzo et al. (2010) had reported that cooked eggplant were much richer in phenolic

compounds like caffeic and chlorogenic acids when compared with raw eggplants.

The lipid peroxidation process is induced by the pro-oxidant effect of transition metals. This transition metal mediates the production of lipid peroxides by stimulation of the oxidative machinery ($OH^{\cdot}$) through haber-weiss reaction but the rate constant of this reaction *invivo* is lower than that of the dismutation reaction and would not proceed significantly. However this method has been successfully used to investigate *in vitro* oxidation of lipid since Fenton reported that a mixture of hydrogen peroxide and ferrous salts was an effective oxidant agent (Repetto & Boveris, 2012). Apart from the fact that this procedure can be induced *in vitro*, it has high precision and accuracy. In this study, our results showed that the ability of both varieties of eggplant to inhibit Fe^{2+} induced lipid peroxidation in rat's kidney (*in vitro*) was increased upon cooking in water. This increased inhibition can be attributed to enhanced polyphenolics and antioxidant potential of eggplant upon cooking since several studies have shown that lipid peroxidation can be averted or reduced by antioxidant compounds. Our results showed similar trend with the study of Lo-Scalzo et al. (2010) where cooking enhanced the ability of eggplant to inhibit oxidative burst in human neutrophil (*in vitro*) via enhancement of phenolic compounds. Previous *in vitro* results from Nwanna et al. (2013) and Kwon et al. (2008) have shown that eggplants possess amylase and glucosidase inhibitory activity. In this study, the ability of eggplant to inhibit α-amylase and α-glucosidase enzymes was increased significantly upon treatment by cooking in water. It could be due to the phenolic oxidation, polymerization or changes in phenolic profile due to thermal processing (Randhir et al., 2008). Also our results showed positive correlation between the increased antioxidant capacity and enzymes inhibition which are also in agreement with the study of Randhir et al. (2008) where thermal treatment increased α-amylase inhibition activity in oat and buckwheat grains and increased α-glucosidase inhibition activity in wheat, buckwheat and oats.

CONCLUSION

In conclusion, our findings in this study have shown that the ability of eggplant to inhibit α-amylase, α-glucosidase and Fe^{2+} induced lipid peroxidation in rat's kidney (*in vitro*) is significantly ($P < 0.05$) enhanced by water cooking. This can be attributed to the ability of water cooking to increase bioaccessibility of polyphenol in eggplant.

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Effect of Recipient Surgery Cycles on Survival Rate of Transferred Embryo in Goats

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ABSTRACT

An experiment was carried out to evaluate the effect of recipients physiological condition on pregnancy rate and subsequent kidding in does. Oestrus was synchronised by inserting controlled internal drug release device (CIDR, 0.33 g natural hormone progesterone; EAZI-BREED CIDR, Pharmacia & Upjtohn Limited, NZ) and an i.m. injection of 300 IU equine chorionic gonadotrophin (eCG) (Folligon®; Intervet International B.V., EU). A total of 31 Boer crossbred (Boer × local) recipient does were divided into two treatment groups namely intact group (15 does) and used group (63 does). Intact group may be defined as those goats that had not used for any surgery, while used group may be defined as those goats that had been used for surgery such as laparoscopic ovum pick up, embryo transfer. On Day 7 after CIDR removal, ovaries were checked for corpus luteum (CL) and recipient does with functional CL were only used as recipients. After flushing, recovered embryos were examined under a stereomicroscope and only transferrable embryos (morula and blastocyst) were transferred. Uterine horn was exteriorised over the surface of abdomen with the help of a grasping forceps and a surgical blade. One to 3 fresh embryos along with

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a small amount of flushing medium were transferred into the lumen of the anterior part of the uterine horn. Once completed, the uterine horn was placed back into the peritoneal cavity and the incision was sutured. Pregnancy diagnosis was performed at around 30 to 35 days after embryo transfer by using a real-time ultrasound scanner. The average numbers of embryos transferred per recipient doe were 2.60 and 2.56 for intact and used groups, respectively. The percentage of pregnancy was 26.67 for intact group. No pregnancy was recorded in used group. Average embryo survival rate was calculated based on the number of kids born, which was 7.69. Average gestation length was 148 days. From the findings obtained in this research, it could be concluded that intact does were favourable as recipient in ET programme than repeated surgery does.

Keywords: CIDR, embryo survival rate, goats, oestrus, pregnancy diagnosis, recipient surgery cycle

INTRODUCTION

Assisted Reproductive Technologies (ARTs) such as artificial insemination (AI), oestrus synchronisation, superovulation and embryo transfer (ET) had been introduced to overcome reproductive inefficiencies in goats, and accelerate genetic gain (Nicholas, 1996). Using AI technology, benefit from male germplasm could be optimised via pure-breeding and cross-breeding, however, the female potential remained untouched. Superovulation is traditionally considered to be the main approach to utilise the superior female genetic potential effectively in order to produce high number of quality embryos from each donor at a rapid rate. Superovulation and ET is a systematic programme consists of management of donor and recipient does, oestrus synchronisation of donor and recipient does, superovulation of donor does, natural mating or AI, embryo collection by flushing oviduct, evaluation of collected embryos and subsequently transfer of the embryos to the oviduct of recipient does.

Pregnancy and kidding are the last stages as an acid test of *in vivo* development in which the success of any goat superovulation and ET programme depended on. Generally, high numbers of does were needed for transferring embryos in any superovulation programme. A lot of does used for different ARTs such as laparoscopic ovum pick up (LOPU), superovulation and ET research. Recycling as a recipient of that does used for multiple surgeries could supply the abundant number of recipients in the ET programme. Several researchers had studied the effects of different factors such as the quality of embryos (developmental stage and grade), number of embryos transferred, ovulation rate and progesterone level, ovulation and transfer site and the nutrition of the recipient does on the performance on survival rate of transferred embryo (Armstrong, Pfitzner, Warnes, & Seamark, 1983; Bari, Khalid, Haresign, Murray, & Merrell, 2003; Guignot et al., 2006; Ishwar & Memon, 1996; Mani, Watson & Mckelvey, 1994). However, there was no

data to substantiate whether any difference between intact (intact group may be defined as goats those had not used for any surgery) and repeated surgery recipient does with regard to ET performance. Therefore, the present study was designed to evaluate the effect of recipient surgery cycles on survival rate of transferred embryo in goats.

MATERIALS AND METHODS

A total of 31 Boer crossbred recipient does of 17 to 36 kg BW and 24 to 60 months of age from 2 physiological groups, such as intact recipient does (not used for any surgery before) and non-intact does (used for two times embryo flushing) were synchronised to evaluate the effect of recipient does physical condition on pregnancy rate and subsequent kidding. This experiment was carried out at the Institute of Biological Sciences Mini Farm (at a location of 2° 30′ N, 112° 30′ E), the University of Malaya, Malaysia. This location is 60 m above sea level and has an annual rainfall of 2600 mm. Recipient does of used group were prepared 3 months after any surgery. The experimental does were reared under an intensive management system and received fresh soya waste (20% dry matter) at a rate of 1 kg/head/d. The soya waste contained 27.9% crude protein (CP), 30.5% neutral detergent fibre (NDF) and 5.3% ash. Soya waste was offered to the animal once in the morning (8:00 am), while the Napier grass (*Pennisetum purpureum*) was offered in the morning (9:00 am) and afternoon (4:00 pm). All animals had free access to water and salt lick. All recipient does were synchronised by inserting a controlled internal drug release (CIDR) device for 14 days and an intramuscular (i.m.) injection of 300 IU eCG was carried out on Day 12 (Figure 1).

After CIDR removal, oestrus was observed 3 times in a day (morning at 0800-0900 hr, afternoon at 1300-1400 hr and evening 1900-2000 hr) by placing a buck of proven libido. Extra precaution was taken to prevent mating such as buck was kept in separate individual pen. On Day 7 after CIDR removal, embryo flushing was carried out according to Rahman, Rahman, Wan Khadijah and Abdullah (2014). One day before ET, the recipient does were scanned by using ultrasound scanner to confirm non-pregnant by the absence of pregnancy related structures such as placentomes and amniotic fluid (Figure 2). On the day of embryo flushing, recipient ovaries were checked for CL and does with functional CL were only used as recipients.

After flushing, recovered embryos were examined and graded according to their development stage and morphology under a stereomicroscope and only Grade I & II transferrable embryos (morula and blastocyst) were transferred. Uterine horn was exteriorised over the surface of abdomen with the help of a grasping forceps and a surgical blade. One to 3 fresh embryos along with a small amount of flushing medium (0.1-0.2 ml) were loaded in a 1 ml BD Unopette syringe (BD, 2771 Bristol circle, Oakville, ON L6H 6R5) and were transferred into the lumen of the anterior part of the uterine horn ipsilateral to the ovary

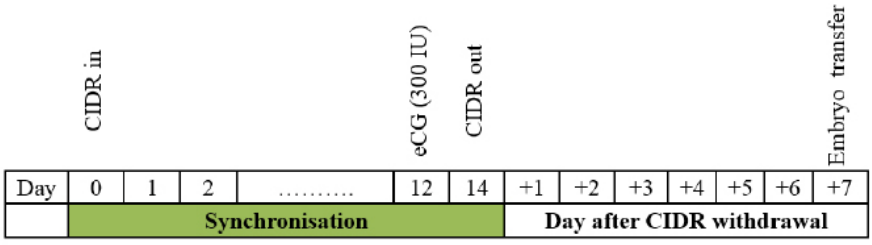

Figure 1. Schematic representation of the oestrus synchronisation protocol for recipient does

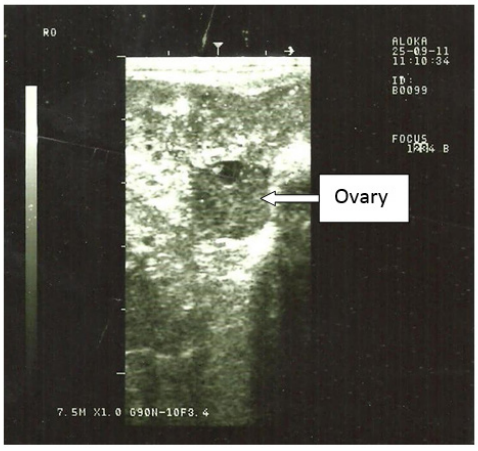

Figure 2. Typical ultrasound image of a recipient doe before embryo transfer

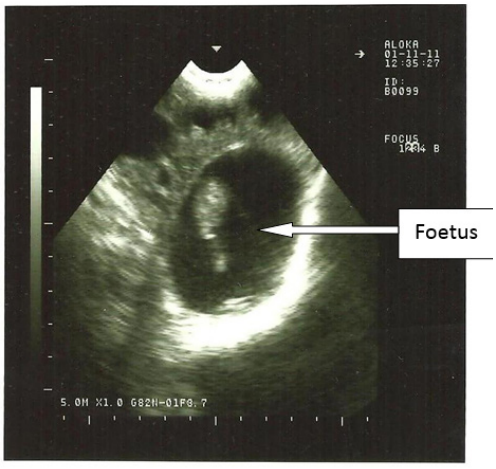

Figure 3. Typical ultrasound image of a pregnant recipient doe after 37 days of embryo transfer

containing a CL through a guided hole prior-punctured using a sterile paper clip. The procedure of embryo transfer was repeated after exteriorising the opposite uterine horn. Once completed, the uterine horn was placed back into the peritoneal cavity and the incision was sutured. Pregnancy diagnosis was performed at around 30 to 35 days after ET by using a real-time ultrasound scanner. Pregnancy rate, kidding and gestation period were calculated. Effect of recipient physical condition on pregnancy was analysed using χ^2 test and results were expressed as the mean ± SEM (standard error of the mean).

RESULTS

Pregnancy rate and subsequent kidding as affected by the physical condition of does i.e. intact does (have not been used for any surgery before) and used does (have been repeatedly used for different surgeries such as LOPU, embryo flushing or ET) are presented in Table 1. A total of 99 does (36 were intact does and 63 were used does) were synchronised, out of that, 31 does (15

Table 1

Effect of recipient surgery cycle on pregnancy rate and kidding

Parameters	Surgery cycle of recipient doe	
	Intact goat	Used goat for different surgery
Number of synchronised recipient does used for embryo transfer	15	16
Total number of transferred embryos	39	41
Average number of embryo transferred/recipient	2.60	2.56
Number of recipient in pregnant	4	0
Pregnancy rate of recipient (%)	26.67[a]	0[b]
Number of recipient delivered kid	2	-
Number of viable young kid	3	-
Embryo survival rate (%)	7.69	-
Average gestation length (day)	148.0±4.0	-

[a,b]Superscripts within rows with different letters differ significantly ($p<0.05$)

intact recipient does and 16 used recipient does) were used. Rest of the recipient was not used due to either have no CL or lack of transferrable embryo. Total 39 and 41 embryos (collected from 47 donors) were transferred to intact and used recipient, respectively. The average numbers of embryos transferred per recipient doe were 2.60 and 2.56 for intact and used groups, respectively. The percentage of pregnancy was 26.67 for intact group. No pregnancy was recorded in used group. In this experiment, only 4 recipient does of produced higher embryo survival rate than earlier stage of embryos in ruminants (Baril et al., 2001; Guignot et al., 2006). However, some earlier researchers found higher survival rates for blastocyst compared to morula in cow (Armstrong & Evans, 1983; Block, Bonilla, & Hansen, 2009; Donaldson, 1985; Hasler, McCauley, Lathrop, & Foote, 1987) and in doe (Li et al., 1990). On the contrary, some other earlier researchers reported that there was no effect of embryo developmental stage on pregnancy rate and subsequent kidding in doe (Gibbons, Cueto,

intact group were pregnant, and out of that 2 does were delivered and another 2 does died before the delivery due to diarrhoea. After ET, pregnancy was confirmed by transabdominal ultrasound scanning at 37 day after ET (Figure 3). The kidding was confirmed by the live born of kids. Average embryo survival rate was calculated based on the number of kids born, which was 7.69. Average gestation length was 148.00±4.00 d.

DISCUSSIONS

Pregnancy and kidding are the last stages which influence the success of any goat superovulation and ET programme. In this experiment, pregnancy rate following fresh embryo transfer was 27% for intact recipient group while no pregnancy was detected for repeated used recipient group. Present result on pregnancy was lower than those of other researchers who reported 79% pregnancy rate in dairy goat (Kiessling, Hughes, & Blankevoort, 1986), 58% (Hong et al., 2007) and 86% (Lehloenya & Greyling, 2010) pregnancy rate in Boer pure bred goat by transferring fresh embryos. On the contrary, present result on pregnancy was in agreement with some other researchers who reported a range of 27 to 60% pregnancy rate following laparoscopic, surgical and non-surgical ET in goat (Bessoudo, Davis, Coonrod, & Kraemer, 1988; Flores-Foxworth, McBride, Kraemer, & Nuti, 1992; Li, Cameron, Batt, & Trounson, 1990). Although only embryos of Grade 1 and II (good grades) were used in the ET program of this study, the reasons of inferior performance of present result were unknown. Many researchers stated that various factors affect on performance including quality of embryos (developmental stage and grade), number of embryos transferred, ovulation rate and progesterone level, ovulation and transfer site and the nutrition of the recipient does (Armstrong et al., 1983; Bari et al., 2003; Guignot et al., 2006; Ishwar & Memon, 1996; Mani et al., 1994).

In the present experiment, number of CL in recipient does was varied from 1 to 4, and the pregnant recipients had only 1 CL on either ovary. However, there were some intact recipients having 1 CL in this experiment did not conceive. Armstrong et al. (1983) reported that there were significant relationships between the number of CL and embryo survivability in recipients does which might be due to increased blood serum progesterone levels. On the contrary, White, Rizzoli and Cumming (1981) and Bari et al. (2003) concluded that recipient CL number in ewe had no effect on embryo survival rate. In normal circumstances, 1 CL is sufficient to secrete progesterone in order to maintain pregnancy in a doe. Therefore, the issue of number of CL affecting pregnancy still cannot be resolved.

In this experiment, morula and blastocyst stage embryos were transferred to the both recipient groups on Day 7 after CIDR removal (Day 6 after oestrus). Previous researchers suggested that morula and blastocysts stages embryos could be transferred into recipient goat on Day 6 after oestrus (Bessoudo et al., 1988; Guignot et al., 2006; Lehloenya & Greyling, 2010) and

& Bonnet, 2011), in ewe (Bari et al., 2003) and in cow (Breuel et al., 1991).

Number of transferred embryos might be a source of variations on pregnancy rate. In this experiment, the average number of embryo transferred per recipient doe was 2.60 and 2.56 for intact and multiple used recipient groups, respectively. Some researchers reported that transferring more than 2 embryos per recipient caused the low survival rate of the transferred embryos (Armstrong et al., 1983; El-Gayar & Holtz, 2005; Ishwar & Memon, 1996; Li et al., 1990). In addition, higher embryo survival rate in dairy goats was obtained after transfer of 2 embryos per recipient (Moore & Eppleston, 1979; Armstrong & Evans, 1983; Tervit, Gold, McKenzie, & Clarkson, 1983). Tervit, Gold and McKenzie (1986) reported that maximum pregnancy rate and kidding could be achieved by transferring 2 blastocysts in each recipient which might be due to the ability of doe to produce multiple kids per pregnancy. On the contrary, Melican and Gavin (2008) had transferred 1, 2 or 3 embryos per recipient during traditional and non-traditional breeding season, and concluded that there was no effect of number of transferred embryo on the pregnancy rate and kidding. They also mentioned that both pregnancy rate and kidding were increased by transferring single embryo to the recipient (Melican & Gavin, 2008).

Embryo survival rate by transferring fresh embryos were 7.69% in this experiment which was much lower than the 36, 37, 47 and 52% reported by earlier researchers by transferring fresh embryo in recipient (Bessoudo et al., 1988; Ishwar & Memon, 1996; Lehloenya & Greyling, 2010; Udy, 1987). The reason of inferior embryo survival rate was not identified, but it might be due to the maternal effect or interaction between transferred embryo and recipient does. All the live born kids were male in this current experiment. Some earlier researchers reported that the observed sex was higher for male kids than female kids. El-Gayar and Holtz (2001) reported that the sex ratio of male and female was 3.5:1 and 2.3:1 for vitrification and conventional freezing, respectively. Melican and Gavin (2008) also reported that the sex ratio of male and female was 2.3:1 and 1.8:1 for fresh embryo transfer during non-traditional and traditional breeding season, respectively. The reason for this result was unknown and more refined research in different aspects of intrinsic and extrinsic factors affecting pregnancy particularly involving male and female embryos/fetuses need to be conducted in future.

The average gestation length in current experiment was 148 days (Day 1 was day of mating and/or AI) which was similar with Lehloenya and Greyling (2010), who reported 148 days of gestation period for Boer goat after transferring fresh embryos. On the other hand, gestation length of doe in this experiment was relatively shorter than the previous finding of Yuswiati and Holtz (1990) who reported 151days of gestation period in Saanen breed following embryo transfer. These observations indicated that small differences in the gestation lengths of different goat breeds due to

external and internal factors such as breeds (gestation period was shorter in lighter breeds of goats) (Mellado, Amaro, Garcia, & Lara, 2000), season of kidding (Ruvuna, Cartwright, Blackburn, Okeyo, & Chema, 1988; Deshpande & Mehta, 1992), litter size (Deshpande & Mehta, 1992; Ruvuna et al., 1988), litter weight and parity (Mellado et al., 2000), but all within an acceptable range. In this experiment, recipient of the repeatedly used goat group was not found as pregnant which might be explained by resistance against gonadotrophin (Baril et al., 1993; Beckers et al., 1990; Remy et al., 1991), surgical trauma during surgery and formation of post-operative adhesions (Ishwar & Memon, 1996; Mckelvey, Robinson & Aitken, 1985; Pereira, Sohnrey, & Holtz, 1998; Suyadi & Holtz, 2000) or some other unknown factors yet to be studied. Moreover, the pregnancy rate and kidding rate from the intact group was rather low, thus there is a possibility that the outcome of this study may be also influenced by the technical skill of ET and animal husbandry and not only due to the repeated used of the recipient for other surgery that impacts their reproductive system.

CONCLUSION

From the findings obtained in this research, it could be concluded that intact does were favourable as recipient in ET programme than repeated surgery does but further detailed research on the latter to enhance their pregnancy efficiency is needed.

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Parasite Contamination of Freshly Harvested Vegetables from Selected Organic and Conventional Farms in The Philippines

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ABSTRACT

Vegetables are considered as an important part of a healthy diet. However, there have been reports showing contamination of vegetables with parasites. This study aimed to assess parasite contamination of freshly harvested vegetables from selected organic and conventional farms in the Philippines. A total of 252 freshly harvested vegetables were collected from 20 farms through systematic random sampling and were processed by means of sedimentation technique. Positive samples were subjected to molecular analysis for further identification of species. Results showed that 58 out of 252 (23.02%) vegetable samples were contaminated with parasites eggs/cysts/oocysts. The parasites found were *Ancylostoma ceylanicum*, *Toxocara* sp., *Trichuris trichiura, Ascaris suum, Hymenolepis* sp., unknown trematode egg, *Isospora*, *Balantidium, Giardia intestinalis* and *Cryptosporidium*. *Ascaris suum* had the highest contamination rate in organic and conventional farms at 13.09% and 8.33%, respectively. *Cryptosporidium* (≥800 oocysts/kg) and *Giardia intestinalis* (≥240 cysts/kg) had the highest mean density in both farms. Also, lettuce showed the highest contamination rate among the sampled vegetables in both types of farms. Furthermore, results revealed that texture of vegetables, distance to the soil substrate, and farming practices could possibly contribute to the parasite contamination of vegetables in this study. These findings have important implications on public health that may aid regulatory agencies for prevention and control strategies for food safety.

Keywords: Farming practices, food-borne parasites, food safety, Philippines, vegetables

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INTRODUCTION

Philippines is a largely agriculture-based country where 13M hectares are devoted to agriculture, 6.1 M hectares of which are highly suitable for cultivation (Carating, Fernando, Abrina, & Tejada, 2010). There are two types of farming systems that are mainly practiced in the Philippines: organic and conventional farming. Though there have been differences in these farming practices, both farm types aim to produce large quantities of crops, such as vegetables. However, there have been reports showing vegetable contaminated with parasites that maybe associated with farming practices (Abe, Ajah, Ayuba, Mogaji, & Ekpo, 2016; Matini, Shamsi-Ehsan, & Maghsood, 2016; Mohamed, Siddig, Elaagip, Edris, & Nasr, 2016).

The aforementioned gap led the researchers to assess the parasite contamination of vegetables and to associate this with the different farming practices from selected organic and conventional farms in the Philippines.

MATERIALS AND METHODS

Study Sites

The study sites were two provinces in the Northern and Southern Luzon, Philippines, which produced and supply large quantities of fresh produce within the regions. The present study includes 20 farms, ranging from 1 to 2.5 hectares; 14 of which are from Northern Luzon (7 organic and 7 conventional farms) and six are from Southern Luzon (3 organic and 3 conventional farms).

Sampling Method

A total of 252 vegetable samples were freshly harvested from organic and conventional farms of the selected farms in Northern and Southern Luzon Provinces. The collection period in both types of farms was done during dry season (November to March). Systematic random sampling was used in harvesting vegetable samples. One sample unit of vegetables was equivalent to 250g. Samples were placed in individual plastic bags, labeled and placed in a cooler box (4 ° C). The samples were transported to the laboratory for immediate processing.

Recovery and Identification of Parasites from Vegetables

Pooled samples were processed through sedimentation technique as described by Nazemi, Raei, Ameri and Chaman (2011). Approximately 100µl of the sediment from each sample was examined for parasites' eggs/cysts/oocysts under a compound microscope (Olympus, Tokyo, 100x – 400x magnification) (Uga et al., 2009). For positive samples, the number and developmental stage of detected parasite were recorded. Another 100µl from the sediment was also used for immunofluorescence assay in examining *Cryptosporidium* and *Giardia* using *Crypto/Giardia* detection kit (Cellabs Pty. Ltd., Brookvale, New South Wales, Australia), following manufacturer's instructions. Prepared slides were scanned at 400x and 1000x under an epiflourescence microscope.

Molecular Characterization of Parasites

The vegetable samples that were positive with *Ascaris, Trichuris,* hookworm, *Giardia,* and *Cryptosporidium* were subjected to molecular identification. Different protocols and gene markers were used for each parasite. For all PCR reactions, positive and negative controls (distilled water) were included. Confirmation of parasites identification at the species level is important to provide information on the source of contamination whether from animal or human feces.

DNA Extraction

The eluate (approximately 200µl) from samples positive for *Ascaris, Trichuris,* hookworm, *Giardia,* and *Cryptosporidium* were extracted using Powersoil ® DNA Isolation Kit (Mo Bio Laboratories, Inc) following manufacturer's instructions.

Multiplex PCR for the detection of soil-transmitted helminths

The protocol of Phuphisut et al. (2014) were modified to obtain the target genes for *Ascaris, Trichuris* and hookworm. The target gene for *Ascaris* is COI (Fw: 5' GGSGGTTTTTGGGTCTTTGG 3'; Rw: 5' CCAAACAAGGTAGCCAACCA 3') which amplified 192 bp; 18S (Fw: 5' CTGCGAGGATTGACAGATCA 3' ; Rw: 5' GTACAAAGGGCAGGGACGTA) amplified 498 bp for *Trichuris* and; ITS1 (Fw: 5' ATGCTTGGCAAGAGTCGTTT 3' ; Rw: 5' TGTTGGCGTCCACACATATT 3') amplified 330bp for hookworm. The primary reaction (20 µl) consisted of the following: 10 µl Premix (X-Prime Taq Premix 2x), 1 µl for each primer, 1µl of dH_2O and 3 µl of DNA template. The PCR thermocycling program was modified having 94°C for 5 minutes, followed by 30 cycles of denaturing for 30 sec at 94°C, annealing for 30 sec at 55°C and extension for 30 at 72°C, followed by a final extension at 72°C for 7 minutes.

Semi-nested PCR for Hookworm Speciation

The semi-nested protocol of Ngui, Lee, Tan, Roslan and Lim (2012) for hookworm speciation was modified to obtain the ITS region. All the positive eluate samples for hookworm in multiplex were subjected to semi-nested PCR for hookworm speciation. The primary PCR reaction used the forward primer, NC1 (5' ACG TCT GGT TCA GGG TTC TT-3') and reverse primer, NC2 (5'-TTA GTT TCT TTT CCT CCG CT-3'), which amplified 310 and 420 bp amplicons. The primary reaction (20 µl) consisted of the following: 10 µl Premix (X-Prime Taq Premix 2x), 2 µl for each primer, 3µl of dH_2O and 3 µl of DNA template. The PCR thermocycling program was as follows: 94°C for 5 minutes, followed by 30 cycles of denaturing for 30 sec at 94°C, annealing for 30 sec at 55°C and extension for 30 sec at 72°C, followed by a final extension at 72°C for 7 minutes.

The samples that produced the 310 and/or 420 bp amplicons were subjected to a second amplification. An amplicon of 250 bp for *Necator* and 130 bp for *Ancylostoma* were amplified using the forward primer NA (5' ATG TGC ACG TTA TTC ACT-3') for *Necator americanus* and AD1 (5'-

CGA CTT TAG AAC GTT TCG GC-3') for *Ancylostoma* sp. NC2 was used as the common reverse primer for both species. The secondary reaction (20 µl) consisted of the following: 10µl of Premix (X-Prime Taq Premix 2x), 2 µl for each primer, 1µl of dH_2O and 3 µl of the first PCR product. The thermocycling program for the secondary PCR were as follows: 94°C for 5 mins, followed by 40 cycles of denaturing for 1 min at 94°C, annealing for 1 min at 55°C and extension for 1 min at 72°C, followed by a final extension at 72°C for 7 minutes.

Nested PCR for the Detection of *Giardia* Species

Detection of *Giardia* was carried out targeting the TPI gene by the protocol used by Sulaiman et al., (2003). The forward and reverse primers used were AL3543 (5'- AAATIATGCCTGCTCGTCG- 3') and AL3546 (5'- CAAACCTTITCCGCAAACC 3'), which amplified the 605 bp amplicon. *T*he primary reaction (20 µl) consisted of 10 µl of Premix (SolGent™ 2x *h-Taq* PCR Smart Mix), 1 µl for each primer, 5 µl of dH_2O and 3 µl of the DNA template. The PCR thermocycling program were: 94°C for 5 minutes, followed by 40 cycles of denaturing for 45 sec at 94°C, annealing for 45 sec at 50°C and a final extension at 72°C for 10 minutes.

For the secondary reaction, an amplicon of 530 bp was amplified using the forward and reverse primers: AL3544 (5'- CCCTTCATCGGIGGTAACTT 3') and AL3545 (5'-GTGGCCACCACICCCGTGCC 3'), respectively. The secondary reaction (20 µl) consisted of 10 µl of Premix (SolGent™ 2x *h-Taq* PCR Smart Mix), 1 µl for each primer, 5 µl of dH_2O and 3 µl of the first PCR product. The secondary PCR thermocycling program used the same modification with the primary PCR reaction.

Nested PCR for the Detection of *Cryptosporidium* Species

The protocol used by Nichols, Campbell and Smith (2003) was modified to obtain the SSU rRNA gene that was the target gene for the nested PCR protocol in detecting *Cryptosporidium*. The forward and reverse primers used were N-DIAGF2 (5'-CAA TTG GAG GGC AAG TCT GGT GCC AGC-3') and N-DIAGR2 (5'-CCT TCC TAT GTC TGG ACC TGG TGA GT-3'), which amplified the 655 to 677 bp amplicons. The primary reaction (20 µl) consisted of 10 µl of Premix (X-Prime Taq Premix 2x), 1 µl for each primer, 5 µl of dH_2O and 3 µl of the DNA template. PCR thermocycling was performed as follows: 95°C for 5 mins, followed by 35 cycles of denaturing for 30 sec at 94°C, annealing for 1 minute at 68°C and extension for 30 sec at 72°C, followed by a final extension at 72°C for 10 minutes.

Meanwhile the protocol of Johnson, Pieniazek, Griffin, Misener and Rose (1995) was modified for the secondary reaction, wherein an amplicon of 435 bp was amplified using the forward and reverse primers: CPB-DIAGF (5'-AAG CTC GTA GTA GTT GGA TTC TG-3') and CPB-DIAGR (5'TAA GGT GCT GAA GGA GTA AGG-3'), respectively. The

secondary reaction (20 µl) consisted of 10 µl of Premix (X-Prime Taq Premix 2x), 1 µl for each primer, 3 µl of dH_2O and 5 µl of the first PCR product. PCR thermocycling was performed as follows: 95°C for 5 minutes, followed by 35 cycles of denaturing for 30 sec at 94°C, annealing for 1 min at 60°C and extension for 30 sec at 72°C, followed by a final extension at 72°C for 10 minutes.

Sequencing and Analysis of Sequence Data

The primary and secondary amplifications were incubated in the MyCycler thermal cycler (Bio-Rad, Hercules, USA). PCR amplicons were then analyzed by 2% agarose gel-electrophoresis at 100V for 50 minutes (multiplex for STH), 90V for 35 minutes (hookworm speciation and *Giardia* species) and 100V for 40 minutes (*Cryptosporidium* species). Positive bands were excised from the gel and purified using QIAquick Gel Extraction Kit (Qiagen, Germany), according to the manufacturer's instructions, and sent to Genomics BioScience and Technology Co., Ltd. Malaysia for bidirectional sequencing. Sequences were aligned and checked manually using BioEdit (version 7.1.11) (Applied Biosystem, UK).

Documentation of Farm Practices and Hygiene

Farm practices were documented through survey interviews of farmers and farm owners during the sample collection of vegetables. The purpose of this documentation was to determine the farming practices that might affect the contamination rate of parasites on vegetables. Informed consent was taken from the respondents before the survey was conducted.

Statistical Analysis

Contamination rate (%) was calculated as the number of positive samples divided by the number of total vegetables sampled multiplied to 100. Mean density was calculated as the total number of parasites divided by the total number of positive samples. The density of parasite's eggs/cysts/oocysts in contaminated vegetables were converted to per kg. For instance, one parasite egg/cyst/oocyst in 0.1ml (1 drop) of the concentrate would have 20 parasite eggs/cysts/oocysts in 2 ml (from a 250g pooled vegetable sample unit) and 80 parasite eggs/cysts/oocysts per kg vegetable. Independent sample t-test was used to assess the difference between the contamination rates of vegetable samples from organic and conventional farms; Moreover, the association between contamination rates and farming practices were analyzed using Point-Biserial correlation. All statistical analyses were done at 95% level of significance.

RESULTS AND DISCUSSION

Identification of Parasites from Vegetables

Parasites were detected in 58 out of 252 (23.02%) vegetable samples collected from selected organic and conventional farms in Northern and Southern Luzon Provinces. The parasites observed microscopically were hookworm, *Toxocara* sp., *Trichuris* sp., *Ascaris* sp., *Hymenolepis diminuta*,

unknown trematode egg, *Isospora* sp., *Balantidium coli, Giardia* sp. and *Cryptosporidium* sp. (Figure 1).

The samples that were positive for parasites were subjected to molecular analyses. Out of 58 vegetable samples that have undergone DNA extraction and PCR amplification, 16 showed the expected bands. BLAST results of the obtained sequences for parasites are summarized in Table 1. Species of parasites confirmed via molecular techniques include *Ancylostoma ceylanicum, Trichuris trichiura, Ascaris suum* and *Giardia intestinalis*. *Cryptosporidium* was not identified to species level which could be due to the low *Cryptosporidium* DNA concentration from the samples.

Parasite Contamination Rates in Vegetables between Organic and Conventional Farms

Among the parasites identified, *Ascaris suum* had the highest contamination rate in both organic and conventional farms at 13.10% and 8.33%, respectively. On the other hand, *Cryptosporidium* sp. and *Giardia intestinalis* have the highest mean density with ≥ 800 oocysts and ≥240 cysts per kg in both farms respectively (Table 2).

Ascaris suum showed the highest contamination rate in organic and conventional farms which could be attributed to the presence of pigs in the farm. According to the farmers, pig manure is commonly used in organic farms while chicken manure is a widely used supplement with chemical fertilizers in conventional farms. Abe et al. (2016), Adamu, Adamu and Mohammed (2012), Elom, Eze, Nworie and

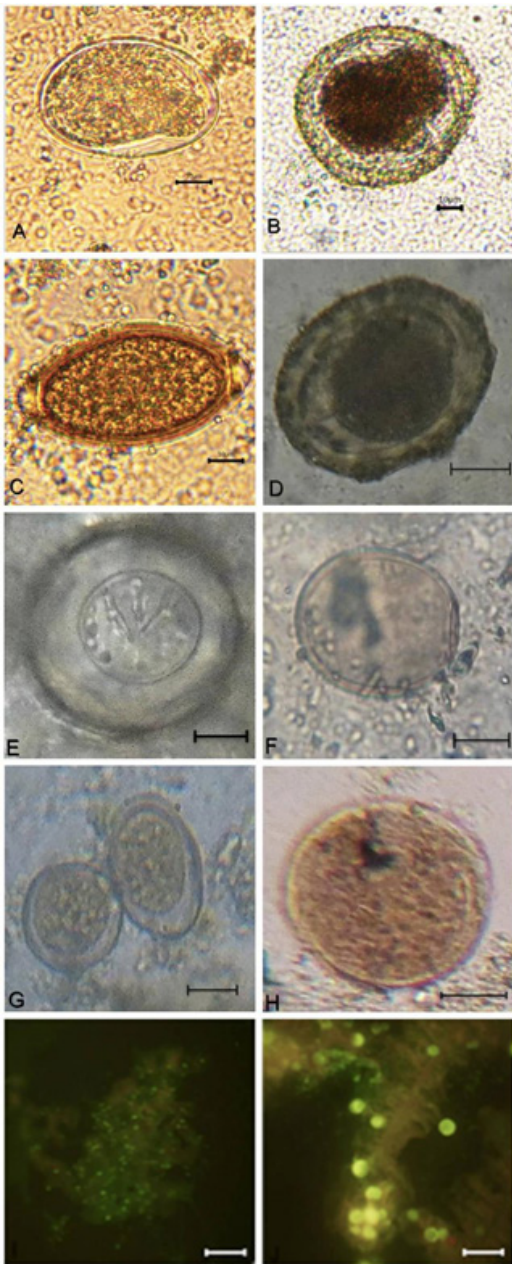

Figure 1. Eggs and cysts recovered from vegetables in organic and conventional farms (A) Hookworm/ strongylid (B) *Toxocara* sp. (C) *Trichuris* sp. (D). *Ascaris* sp. (E) *Hymenolepis* sp (F) Fluke (G) *Isospora* (H) *Balantidium* (I) *Cryptosporidium* (J) *Giardia* (scale bar: 10μm)

Table 1

BLAST results of sequences of parasites recovered in positive vegetable samples

Vegetable Samples Code	Closest Match		Identity (%)
	Accession No. From Genbank	Species Name	
CGI6	JN120881.1	Ancylostoma ceylanicum	100
LT14-GI3	KF279134.1	Ancylostoma ceylanicum	100
HW	JN120871.1	*Ancylostoma ceylanicum*	98
LT21-GI2	LC036567.1	*Ancylostoma ceylanicum*	98
CLR1B	KF279134.1	*Ancylostoma ceylanicum*	98
LT10-GI3a	HQ452519.1	*Ancylostoma ceylanicum*	98
CLRIA	KC632568.1	*Ancylostoma ceylanicum*	98
LT10-GI2a	JQ673421.1	*Ancylostoma ceylanicum*	97
LT10-GI4	LC036567.1	*Ancylostoma ceylanicum*	97
LT14-GI4	LC036567.1	*Ancylostoma ceylanicum*	97
LT1-GI1	KR075936.1	*Giardia intestinalis*	99
LT10-DR	JQ863257.1	*Giardia intestinalis*	99
LT10-GI1	KR075936.1	*Giardia intestinalis*	98
CGI4	X54253.1	*Ascaris suum*	99
CLR2	AB591802.1	*Ascaris suum*	99
CGI2	AB699090.1	Trichuris trichiura	99

Akpotomi (2012) and Klapec and Borecka (2012) observed the same results, having *Ascaris* as the most prevalent parasite in their studies. *Ascaris* species is known for its thick shell that allows it to withstand the adverse environmental conditions and thus, can survive longer in the environment.

Meanwhile, *Toxocara* was found to be present only in organic farms while high density of hookworm (*Ancylostoma ceylanicum*) was also recorded in organic farms, where presence of stray dogs and cats was a common sight. Previous studies showed that *T. canis* and *T. cati* were the most prevalent parasites in stray dogs (69.5%) and cats (24.2%), respectively (Borecka, 2005; Klapec & Borecka 2012). Contamination of vegetables were also linked to the presence of stray dogs and cats in the West of Iran (0.8%) and in Mexico, City (33.3%) (Matini et al., 2016; Vázquez, Martinez-Barbabosa, Tay Zavala, Ruiz Hernandez & Perez Torres, 1997).

Table 2

Contamination rate and mean density of parasites recovered from vegetables in organic and conventional farms according to types of parasites

Parasites	Organic Farms			Conventional Farms		
	Contamination Rate (%)	Mean Density (eggs/cysts/ oocysts per kg)	SD	Contamination Rate (%)	Mean Density (eggs/cysts/ oocysts per kg)	SD
Protozoans						
Balantidium coli[1]	2.98	80	40	-	-	-
Isospora sp.[1]	3.57	160	0	1.19	400	
Giardia intestinalis[1]	0.59	~240	-	2.38	~240	113.14
Cryptosporidium sp.[2]	1.19	~800	56.57	5.95	~800	230.94
Cestodes						
Hymenolepis diminuta[3]	0.59	240	-	1.19	80	-
Trematodes[3]	0.59	80	-	-	-	-
Nematodes						
Ascaris suum[3]	13.10	80	55.21	8.33	160	92.38
Ancylostoma ceylanicum[3]	9.52	160	120.44	2.38	80	0
Toxocara sp.[3]	2.98	320	0	-	-	-
Trichuris trichiura[3]	0.59	160	-	-	-	-

~ estimation (too many to count) [1]cysts [2]oocysts [3]eggs

- no SD value, as parasites were only found in only one sample

The presence of parasites such as *Giardia intestinalis* and *Cryptosporidium* may be due to the contaminated water source for the crops. Some of the farms were located near sewers which could be an ideal source of parasitic protozoans. The use of contaminated water for watering crops could be a potential source of contamination with these parasites. Hajjami, Ennaji, Fouad, Oubrim and Cohen (2013) reported that untreated and treated wastewater with even just one helminth egg/L must not be used in irrigations for green leafy vegetables because these are commonly eaten raw.

Hymenolepis diminuta (organic farms: 0.59%; conventional farms: 1.19%) and *T. trichiura* (organic farms: 0.59%: conventional farms: 0%) have the lowest contamination rate among the detected parasites. The presence of *H. diminuta* in some farms could be due to the sporadic occurrence of rats, as affirmed by the farmers. While the presence of *Trichuris* in organic farms might be due to the fertilizers from pig's feces that the farmers were using. Humans are the principal host of *T. trichiura* though pigs have also been reported to be infected (Stephenson, Holland & Cooper, 2000).

Meanwhile, among the different types of vegetables, deep red lettuce was found to have the highest contamination rate among the vegetables. The current data concurred with the study in Sudan, Benha, Egypt and Alexandria, Egypt (Eraky, Rashed, Nasr, El-Hamshary & El-Ghannam, 2014; Mohamed et al., 2016; Said, 2012). Texture and distance of the vegetable in soil may have a great impact on the contamination of vegetables. Rough, highly textured surfaces with deep crevices would be more likely to harbour soil, with the possible consequence of increased numbers of parasitic organisms. Also, vegetables closer to the ground could be more susceptible to contamination with helminth eggs especially during heavy rains and floods (Mohamed et al., 2016; Omoyawe & Falola 2012; Said, 2012).

In addition, this study revealed that organic farms (23.81%) showed higher contamination rate than conventional farms (21.43%), albeit not significantly different (p >0.05) (Table 3). Gharavi, Jahani and Rokni (2002) also reported that in Tehran, there was a relatively low parasitic contamination in farms that uses chemical fertilizers. Conversely, a study in Poland showed that conventional farms had higher contamination rate than organic farms (Klapec & Borecka, 2012). Most of the organic farms in the current study have fences around their vegetable plots to avoid the entrance of stray animals, however, the use of animal manure as fertilizer could be potential source of contamination. On the other hand, most of the conventional farms in this study were smaller in scale than the organic farms, making it easier for the former to manage and control possible contamination.

Table 3

Contamination rate of parasites in organic and conventional farms according to types of vegetables

Vegetables	Organic Farms			Vegetables	Conventional Farms		
	Samples Examined	No. Positive	%		Samples Examined	No. Positive	%
Leafy vegetables							
Broccoli	8	0	0	Broccoli	8	0	0
Cabbage	8	0	0	Cabbage	-	-	-
Camote tops	4	1	25	Camote tops	-	-	-
Kangkong	4	0	0	Kangkong	-	-	-
Lettuce varieties							
Deep Red	12	9	75	Deep Red	8	4	50
Green Ice	24	11	46	Green Ice	12	4	33
Ice Berg	16	6	38	Ice Berg	-	-	-
Romaine	16	7	44	Romaine	12	3	25
Singkang	12	0	0	Singkang	4	0	0
Onion Leaves	8	0	0	Onion Leaves	4	0	0
Parsley	8	1	13	Parsley	-	-	-
Polonchai/ Pechay	4	1	25	Polonchai/ Pechay	4	0	0
Spinach	4	0	0	Spinach	-	-	-
Taro	4	1	25	Taro	-	-	-
Fruit vegetables							
Eggplant	8	0	0	Eggplant	-	-	-
Luffa	8	2	25	Luffa	8	2	25
Okra	4	0	0	Okra	4	1	25

Table 3 *(Continue)*

Vegetables	Organic Farms			Vegetables	Conventional Farms		
	Samples Examined	No. Positive	%		Samples Examined	No. Positive	%
Fruit vegetables							
Squash	-	-	0	Squash	4	0	0
Strawberries	4	1	25	Strawberries	4	1	25
String beans	-	-	-	String beans	4	1	25
Tomato	4	0	0	Tomato	4	0	0
Root crop							
Carrot	8	0	0	Carrot	4	2	50
Total	**168**	**40**	**23.81**	**Total**	**84**	**18**	**21.43**

Association between Farming Practices and Parasite Contamination in Vegetables

Farming practices observed in both organic and conventional farms were associated with the parasite contamination rates of vegetables. Statistical analysis revealed that only the presence of toilet facilities (p = 0.020), deworming of farmers and farm animals (p = 0.026) and presence of animals in or near the farm (p = 0.025) showed significant relationship with the presence of parasites on vegetables (Table 4).

It was observed that some farms have clean toilets with available water, while some farms have toilets but do not have enough access to water for cleaning, some others do not have toilets in the farms. There was also no proper sewage system observed in most of the sampled farms, increasing the chance of contamination. It was stated that infectious organisms may be present in human or animal by-products and if these were not properly disposed, it might become a source of contamination, especially to crops that are planted near to it (McLaughlin, 2012; Nazemi et al., 2011).

Improper handling of farm produce by farmers could also be a source of contamination. Farmers are commonly using gloves only during application of chemical fertilizers. Also, some farmers were observed washing their hands in water buckets used for watering crops. Meanwhile, deworming of animals also showed significant relationship with vegetable contamination in farms (p =0.026). Since it is not a habitual practice to deworm farm

and companion animals, it is likely that these may increase the risk for parasite contamination in the environment. Several studies also revealed that animal feces was a common cause of contamination of vegetables by intestinal parasites (Idahosa, 2011; McLaughlin, 2002).

Table 4

Association of farming practices with organic and conventional farms in northern and southern Luzon provinces

Farming Practices	Northern (N=14)		Southern (N=6)		***p* value***
	Organic (n=7)	Conventional (n=7)	Organic (n=3)	Conventional (n=3)	
Fertilizers used					
Processed manure with inoculants	7	7	2	3	0.452
pure manure	0	0	1	0	
Water sources					
district tap water	7	7	3	1	0.441
mountain spring	0	0	0	2	
Hygiene					
Toilet facilities					
Present	3	1	2	1	0.020
Absent	4	6	1	2	
Using gloves during planting, harvesting, treating of soil					
Yes	2	1	2	2	
No	5	6	1	1	0.071

Table 4 *(Continue)*

Farming Practices	Northern (N=14)		Southern (N=6)		***p* value***
	Organic (n=7)	Conventional (n=7)	Organic (n=3)	Conventional (n=3)	
Hygiene					
Washing of hands before and after eating, planting, harvesting, treating of soil					
Yes	7	7	1	0	
No	0	0	2	3	0.410
Deworming of farmers and farm animals					
Yes	0	0	1	0	
No	7	7	2	3	0.026
Farm animals in or near the farm					
Present	4	0	3	3	
Absent	3	7	0	0	0.025

*p > 0.05 not significant

Raising farm animals inside or near the farm could also contribute to differences in levels of contamination between farms ($p = 0.025$). Animal feces maybe converted as fertilizers or may directly contaminate the soils in farms. Heavy rains and flooding may also aggravate the contamination of vegetables in the farms. Also, the farmers themselves could step on the feces and might bring soiled shoes or slippers into the planting area.

CONCLUSION

This study revealed parasite contamination of freshly harvested vegetables in both organic and conventional farms. Thus, consumers should be conscientious with food preparation, such as thorough washing of freshly harvested vegetables and fruits from farms. Also, the role of various farming practices underscores the dynamics of parasite transmission and contamination in agricultural setting. Hence, measures should be taken to mitigate the impacts of foodborne parasites from the source to the consumers. Measures should also be emphasized regarding health and well-being of all those who work in food supply systems and of the animals and plants destined for human consumption. It is essential that there are standards by which food can be produced in a safe manner. As these guidelines and regulatory documents are developed and revised, food producers should pay attention to the best scientific information that reduces risks for parasites in food.

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Effect of Green Manure Application on Cassava (M*anihot esculenta* Crantz) Growth, Yield Quantity and Quality in Degraded Alfisols

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ABSTRACT

The use of green manures as alternatives to minimize the usage of chemical fertilizers that are very expensive and also environmentally unfriendly is considered as a good agronomic practice. However, the effect of each green manure on soil properties and crop yield depends on its chemical composition. Hence field experiments were conducted in sandy soil during 2015/2016 and 2016/2017 to compare impacts of green manures (GM) and N15:P15:K15 fertilizer on soil properties, growth, root yield, mineral, starch and hydrocyanic acid (HCN) contents of cassava. The GM from leaves of: Neem (*Azadirachta indica* A. Juss.), Moringa (*Moringa oleifera* Lam.), Gliricidia (*Gliricidia sepium* (Jacq.) Kunth ex Walp.) and Leucaena (*Leucaena leucocephala* (Lam.) de Wit) were applied at 5 t ha^{-1}, and the NPK fertilizer was applied at 400 kg ha^{-1}. There was a no fertilizer (control). Application of GMs reduced soil bulk density and increased soil organic matter (OM), N, P, K, Ca, Mg, growth and fresh root yield of cassava compared with the control. The NPK fertilizer had no effect on soil bulk density and soil OM, but increased soil N, P, K, Ca, Mg, growth and fresh root yield of cassava compared with the control. Gliricidia increased growth and fresh root yield of cassava compared with other GMs and NPK fertilizer. In the first year (2015/2016) and compared with control; Gliricidia, Moringa, Leucaena, Neem and NPK fertilizer increased fresh root yield of cassava by 53, 33, 30, 29 and 28%, respectively. In the second year (2016/2017), these treatments increased fresh root yield of cassava by 85, 65, 61, 60 and 36%, respectively. The GMs and NPK fertilizer increased mineral and starch contents and reduced HCN content in the

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cassava tuber roots compared with the control. Use of GM has potential to improve soil properties, and growth, fresh root yield and nutritional contents of cassava than does NPK fertilizer. The Gliricidia treatment best improved soil properties and cassava productivity as indicated by the benefit:cost ratio. For those growing cassava for fresh root quantity Gliricidia is recommended as green manure. Moringa is recommended as green manure for those that desire fresh root quality.

Keywords: Azadirachta indica, cassava, *Gliricidia sepium*, growth, *Leucaena leucocephala, Moringa oleifera,* quality, yield

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is known for its inherent ability to produce reasonably good yields on eroded and degraded soils, and under adverse climatic and soil conditions, where other crops would fail (Agiriga & Iwe, 2016; Howeler, 2002, 2014), increasingly more marginal and fragile soils are being used for its production in Africa, Asia and Latin America (Cadavid, El-Sharkawy, Acosta, & Sanchez, 1998; El-Sharkawy, 1993; Howeler, 1994, 2014).

According to Howeler (1991b, 2012, 2014), cassava is often believed as a 'scavenger crop', that is, highly efficient in nutrient absorption from a low-nutrient soil, leaving that soil even poorer than before. Thus it is often concluded that cassava extracts more nutrients from soil than most other crops, resulting in nutrient depletion and a decline in soil fertility. For instance, for a target fresh root yield of 35.7 t ha^{-1}, cassava removes 55, 13.2 and 112 kg ha^{-1} of N, P and K, respectively from soils (Howeler, 1991b, 2014). This plainly shows the need to restore and maintain soil nutrient status during cultivation through the use of sound and appropriate nutrient management practices.

Additional nutrient in form of organic manure and inorganic fertilizer (especially NPK fertilizer) must be applied to restore soil fertility, and therefore sustain cassava productivity. However, use of chemical fertilizers is limited due to its high cost and scarcity during planting, and its prolong use leads to decline in soil organic matter content, acidification, nutrient imbalance and degradation of soil properties. Therefore, one way of restoring fertility, and increasing soil OM content of tropical soils, is with use of green manure (Ali, 1999; Adekiya, Agbede, Aboyeji, Dunsin, & Ugbe, 2017). Use of green manure as a source of soil fertility is not a common practice among cassava growers in the tropics. Green manure application can increase production of cassava under tropical conditions by reducing soil density, enhancing soil fertility and productivity. Green manure plants are non-polluting, less dangerous and biodegradable with no unsafe deposits in soil, water and air. They are environmentally friendly, edaphologically suitable, and by and large do not leave buildup in stored food product (Adekiya et al., 2017).

Neem (*Azadirachta indica* A. Juss.), Moringa (*Moringa oleifera* Lam.), Gliricidia (*Gliricidia sepium* (Jacq.) Kunth ex Walp.) and Leucaena (*Leucaena leucocephala* (Lam.) de Wit) can be used as green manure, but use of their leaves for cassava production has not been investigated. It was reported that application of fresh neem leaves at 5 t ha^{-1} or dry neem leaves at 1.25 t ha^{-1}, used in conjunction with urea, resulted in higher N recovery percent and N response ratio and gave increased grain yield compared to the yield obtained due to the application of urea alone (Santhi & Palaniappan, 1986). Moyin-Jesu (2014) also reported that mixture of water extracts of neem leaf and wood ash gave the best growth and yield parameters of plantain and this was due to its nutrients superiority compared to the other treatments. In contrast, Moringa is an underutilized tropical crops. Incorporation of Moringa shoots as green manure increased fertility level of agricultural soils (Ekene, Ezeaku, & Ndubaku, 2014; Fahey, 2005). Gliricidia has been used to improve quality and as a potential source of N (Makumba et al., 2006; Srinivasarao Rao et al., 2011). Incorporation of tender twigs of Leucaena has been found beneficial for meeting N requirement and improving productivity of maize; and there are significant residual effects on soil fertility and productivity of the following crops (Kebede, 2016; Mureithi, Tayler, & Thorpe, 1994).

No field study has been conducted in Nigeria to determine the effects of green manures and NPK fertilizer on soil properties, and cassava yield's quality and quantity. In Nigeria, cassava yields have declined in recent years, particularly in Alfisols that are very low in organic matter content and nutrients (Salami & Sangoyomi, 2013). Such soils are used intensively, receiving little or no fertilizer, and are rarely fallowed (Ayoola & Makinde, 2007; Salami & Sangoyomi, 2013). The working hypothesis in this study was that application of green manures would significantly improve soil properties, and cassava yield's quality and quantity in comparison with application of NPK fertilizer. Thus, the objectives of this study were to determine the effects of green manures and NPK fertilizer on soil properties, growth, yield, mineral, starch and hydrocyanic acid content of cassava.

MATERIALS AND METHODS

Site Description and Treatments

Field experiments were conducted at the Teaching and Research Farm, Rufus Giwa Polytechnic, Owo, Ondo State, Nigeria, in April 2015 and 2016. Rufus Giwa Polytechnic, Owo, lies between Lat 7°12`N and long 5°35'E and is located in the forest-savanna transition zone of Nigeria. The soil at Owo is in the Okemesi Series and is an Alfisol classified as Oxic Tropuldalf or Luvisol (Soil Survey Staff, 2014) derived from quartzite, gneiss and schist. Rainfall is bimodal, averaging about 1400 mm per year, with most of it occurring during March to July and mid-August to November. Mean annual temperature is about 32°C. The trial was established in a field left fallowed for a year after it had been cropped with

yam, maize, cassava, melon, cocoyam and tomatoes, respectively, during the previous 6 years and had not received fertilizer application. The predominant weeds at the site were Siam weed (*Chromolaena odorata* L. King and Robinson), Haemorrhage plant (*Aspilia africana* Pers. Adams) and goat weed (*Ageratum conyzoides* L.).

The experiment consisted of green manure from leaves of: Leucaena, Neem, Moringa, Gliricidia, and N15: P15: K15 fertilizer and a control with no green manure or fertilizer. The treatments were arranged in a randomized complete block design with 4 replications. Each block comprised 6 plots, each of which measured 7 × 5 m^2. Blocks were 2 m apart, and plots were 1 m apart. The same site was used in both years.

Land Preparation and Crop Establishment

The site was manually cleared and weeds removed. Thereafter, the soil was plowed and disked to a 20 cm depth followed by ridging at a spacing of 1 m apart. The green manure plant leaves used for the experiments were harvested from nearby sites and comprised green tender stems and leaves of the plants. Plant leaves were chopped to 5 mm and incorporated at 5 t ha^{-1} to a depth of about 10 cm using hoes. The NPK and control treatments plots were prepared the same way as those of green manures plots, but without incorporation of green manure. Plots were left for 1 week before planting.

Healthy mature stems of cassava (TMS 30572) obtained from the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria were cut at 25 cm length, with 5 to 8 nodes. One stem cutting was planted per hill on the crest of ridges on 6 April 2015 and 10 April 2016 at a spacing of 1 m x 1 m, providing 35 plants per plot which is equivalent to a plant population of 10,000 plants per hectare. 400 kg ha^{-1} of NPK fertilizer (15 N, 15 P_2O_5, 15 K_2O) was applied manually by ring method in two split applications, the first at one month after planting (1 MAP) and the second at 3 months (3 MAP) after planting. Four manual weeding regimes were carried out at 3, 7, 12 and 17 weeks after planting (WAP) to control weeds.

Determination of Growth and Yield Parameters

Ten plants per plot were randomly selected from which growth and yield data were obtained. Growth parameters measured were plant height, number of leaves and leaf area index (LAI = LA/a, where LA = leaf area and a = land area) and these were determined at 6 months after planting (MAP). At harvest (12 months after planting), fresh storage root yields were determined.

Soil Sampling and Analysis

Prior to the start of the experiment in 2015, soil samples were collected from 0-15, 15-30 and 30-45 cm depths of a profile pit dug in 10 different points selected randomly from the experimental site. Undisturbed samples were collected from the centre of the depth intervals using steel coring tubes (4 cm diameter, 15 cm high) and were

put in an oven set at 100°C for 24 h for determination of bulk density (Campbell & Henshall, 1991). Particle-size analysis was done using the hydrometer method (Gee & Or, 2002). Textural class was determined using a textural triangle (Brady & Weil, 1999; Hunt & Gilkes, 1992). Before ridging, composite soil samples were collected from the three depths and analysed for chemical properties. Disturbed soil samples were also collected per plot at harvest of cassava from 0-15 cm depth in 2015/2016 (first year) and 2016/2017 (second year) and similarly analysed for chemical analysis. The soil samples collected were air dried, ground, and passed through a 2 mm sieve. The sieved soil samples were taken to the laboratory for chemical analysis as described by Carter and Gregorich (2007). Soil organic carbon was determined by the procedure of Walkley and Black using the dichromate wet oxidation method (Nelson & Sommers, 1996). Organic matter (OM) was deduced by multiplying carbon (C) by 1.724. Total N was determined by micro-Kjeldahl digestion (Bartels, 1996) and distillation techniques; available P was extracted using Bray-1 solution and determined by molybdenum blue colorimetry (Frank, Beegle, & Denning, 1998). Exchangeable bases (K, Ca and Mg) were extracted with 1 N NH_4OAC buffered at pH 7.0 (Van Reeuwijk, 2002). Thereafter, K was determined using a flame photometer, and Ca and Mg were determined by the EDTA titration method (Hendershot, Lalande, & Duquette, 2008). Soil pH was determined in 1:2 soil-water medium using glass electrode pH meter (Ibitoye, 2006).

Determination of Soil Physical Properties

Two months after incorporation of green manure leaves, determination of bulk density in all plots was started and repeated at 4, 6, 8, 10 and 12 months after green manure incorporation. Five soil samples were collected at 0-15 cm depth from the centre of each plot and 10 cm away from each cassava plant using steel coring tubes and were used to evaluate bulk density as described above (Campbell & Henshall, 1991).

Analysis of Green Manure Leaves and Fresh Storage Roots of Cassava

Prior to incorporation of green manure leaves to plots, leaf samples were collected randomly from each green manure, oven-dried for 24 h at 80°C and ground in a Willey mill. The samples were analyzed for leaf N, P, K, Ca and Mg as described by Tel and Hagarty (1984). Leaf N was determined by the micro-Kjeldahl digestion method. Ground samples were digested with nitric-perchloric-sulphuric acid mixture for determination of P, K, Ca and Mg. Phosphorus was determined colorimetrically using the vanadomolybdate method, K was determined using a flame photometer and Ca and Mg were determined by the EDTA titration method. The percentage of organic carbon (OC) in the green manure leaves was determined by the Walkley and Black procedure using the dichromate wet oxidation method.

At 12 months after planting, the 10 central plants from each plot were harvested. From all the roots harvested per plot, five

of them were selected randomly. Selected roots were washed and peeled. From the proximal, central and distal sections of each root, a slice was taken. Samples from each root were mixed together and chopped into small pieces. Resulting chips were properly mixed to obtain a uniform sample of root tissue from the five original roots. A 100 g sample was then taken and oven-dried at 60^{o}C for 24 h. Dried samples were then ground in a mill with a stainless steel grinding tool and stored in air-tight containers prior to chemical analysis. Hydrocyanic acid (HCN) in the cassava root were determined, using the enzymatic assays developed by Cooke (1978) and O'Brien, Taylor and Poulter (1991). Mineral elements of cassava roots were determined according to methods recommended by the Association of Official Analytical Chemists (AOAC) (2012). The samples were digested using nitric-perchloric-sulphuric acid mixture. Phosphorus was determined by the molybdenum blue colorimetric technique. K, Ca, Fe and Zn contents were determined by the atomic absorption spectrophotometer. Peeled cassava tubers 100 g each were macerated and passed through 15 µm pore size sieve, using tap water. The extracted starch in water was allowed to stand for 6-12 h, after which excess water was decanted, and the starch was dried to a constant weight in an oven set at 80^{o}C.

Statistical Analysis

Data collected from each experiment were subjected to analysis of variance (ANOVA) using the Genstat statistical package (Genstat, 2005). If the treatment by year interaction was significant it was used to explain the results. If the interaction was not significant, treatments means were separated using Duncan's multiple range test.

A cost: benefit analysis was done to determine relative economic returns on treatments using 2016 and 2017 annual market prices. Total yield and cost benefit analyses were determined using the harvest from the central bed (1 m^2) of each plot. Costs of farm services were from Emure-Ile market in Owo Local Government Area of Ondo State, Nigeria.

RESULTS AND DISCUSSION

The physical characteristics and chemical properties of the experimental site before the commencement of the experiment in 2015 are shown in Table 1. The soil was sandy loamy in texture, slightly acidic and high in bulk density. The clay content increased progressively down the profiles, which was attributable to clay lessivation at the soil surface, whereas the sand and silt contents decreased with depth. Also, the value of soil organic matter (OM), total N, available P, exchangeable K, Ca and Mg decreased with depth. This was adduced to the fact that more decomposition occurs on the upper layers of soil profile because more organic matter was added through litter fall. All of the soil nutrients in the soil profile clearly showed that the nutrient status of the soil was low, and below the critical levels recommended for cassava cultivation (Howeler, 1998). Therefore, it was expected

that the crops should response to fertilizer application. The poor soil fertility status could be adduced to nature and continuous cultivation over the years without addition of manure or fertilizer inputs.

The chemical analysis of the green manures indicated considerable amount of nutrients which were expected to benefit the soil and cassava crop (Table 2). The green manures have high amounts of nutrients and lower C-to-N ratios. Gliricidia had the highest K and N values, and Moringa had the highest Ca value. Neem was highest in P and Mg, while Leucaena had higher N value similar to that of Gliricidia (Table 2).

Table 1

Soil physical and chemical properties of the experimental site before experimentation in 2015

Soil property	0-15 cm depth	15-30 cm depth	30-45 cm depth
Sand (g kg^{-1})	757 ± 3.5	749 ± 2.5	735 ± 3.2
Silt (g kg^{-1})	125 ± 1.5	118 ± 1.8	112 ± 1.4
Clay (g kg^{-1})	118 ± 0.6	133 ± 0.5	153 ± 0.4
Textural class	Sandy loam	Sandy loam	Sandy loam
Bulk density (Mg m^{-3})	1.65 ± 0.02	1.68 ± 0.03	1.71 ± 0.04
pH (water)	5.8 ± 0.05	5.7 ± 0.06	5.8 ± 0.04
Organic matter (%)	1.75 ± 0.01	1.53 ± 0.02	1.24 ± 0.02
Total N (%)	0.14 ± 0.01	0.11 ± 0.01	0.09 ± 0.01
Available P (mg kg^{-1})	7.1 ± 0.4	6.8 ± 0.3	5.9 ± 0.2
Exchangeable K (cmol kg^{-1})	0.11 ± 0.01	0.09 ± 0.01	0.07 ± 0.01
Exchangeable Ca (cmol kg^{-1})	1.62 ± 0.02	1.42 ± 0.02	1.26 ± 0.03
Exchangeable Mg (cmol kg^{-1})	0.33 ± 0.01	0.27 ± 0.01	0.23 ± 0.01

Table 2

Chemical composition of various green manures

Green manure	OC (%)	N (%)	C:N	P (%)	K (%)	Ca (%)	Mg (%)
Neem leaves	40a	1.30c	30.8a	0.83a	1.67c	0.77d	0.75a
Leucaena leaves	39a	3.23a	12.1c	0.23c	1.68c	1.24b	0.35c
Moringa leaves	36b	2.56b	14.1b	0.43b	2.04b	2.62a	0.56b
Gliricidia leaves	41a	3.26a	12.6c	0.41b	2.76a	1.08c	0.36c

Values followed by similar letters under the same column are not significantly different at p = 0.05 according to Duncan's multiple range test.

Incorporation of green manures and NPK fertilizer influenced soil bulk density and chemical properties in both years (2015/2016 and 2016/2017) (Table 3). When studied as individual factors, year (Y) was not significant for bulk density, but fertilizer (F) was significant for bulk density (Table 3). The Y × F interaction was not significant for bulk density. The second year (2016/2017) reduced bulk density compared with the first year (2015/2016) (Table 3). In both years, incorporation of green manure leaves significantly reduced soil bulk density compared to NPK fertilizer and the control (Table 3). This could be attributed to increased soil OM from decomposed green manures. Presence of green manures should have increased activities of beneficial soil fauna in organic matter decomposition which could lead to enhanced soil porosity and reduced soil bulk density (Salahin, Alam, Islam, Naher, & Majid, 2013). In both years, all treatments had similar trend for bulk density. In both years, there were no significant differences in bulk density between Moringa, Leucaena, Gliricidia and Neem leaves used as green manure (Table 3). Application of NPK fertilizer did not reduce soil bulk density compared with green manures. Averaged over cropping seasons, the green manures (Moringa, Leucaena, Gliricidia and Neem) reduced soil bulk density by 23% compared with NPK fertilizer and the control.

When studied as individual factors, year (Y) was significant for soil pH and N, but not significant for OM, P, K, Ca and Mg; fertilizer (F) was significant for soil pH, OM, N, P, K, Ca and Mg (Table 3). The interaction of Y × F were not significant for soil pH, OM, N, P, K, Ca and Mg. In both first year (2015/2016) and second year (2016/2017), incorporation of green manures increased soil OM, N, P, K, Ca and Mg compared to the control (Table 3). In both years, the treatments had similar trend for soil chemical properties. The second year (2016/2017) had higher concentrations of soil chemical properties compared to the first year (2015/2016). The high concentrations of OM, N, P, K, Ca and Mg in the second cropping season than the first cropping season could be attributed to improved soil organic matter status of the organic materials, since soil organic matter is a natural source of nutrients and cation exchange capacity. It also implies that the values of organic materials as fertilizers are cumulative and extend considerably beyond the year of application. This might be due to the slow release patterns of their nutrients. Adeleye and Ayeni, (2010) reported that the cumulative agronomic values of some organic wastes applied to agricultural soils could be more than five times greater in the post application period than the values realized during the year of application. There was no significant difference in pH between the first year (2015/2016) and the second year (2016/2017). In both years, among fertilizers, incorporation of green manures increased soil OM, N, P, K, Ca and Mg compared with the control. This indicates that nutrients in the plant tissues are released into the soil. NPK fertilizer increased soil N, P, K, Ca and Mg compared

with the control. The OM of NPK fertilizer treated soil was similar to the control. NPK fertilizer did not increase soil OM because it did not contain organic matter. All the various green manures had similar pH, but pH of NPK fertilizer was significantly lower compared with other treatments. The significant decrease in pH of the plots treated with NPK fertilizer compared with organic manures and the control could be due to its acidic nature. In both years, Gliricidia leaves incorporated as green manure had the highest soil N, K and Mg compared with other green manures (Table 3). NPK fertilizer and the control decreased soil nutrient status over time, whereas incorporation of leaves of green manures increased soil nutrient status over time.

When studied as individual factors, year (Y) affected fresh root yield of cassava, but not plant height, number of leaves and leaf area index of cassava; fertilizer (F) did affect number of leaves, plant height, leaf area index and fresh root yield of cassava (Table 4). The interaction of Y × F did affect fresh root yield of cassava, but not plant height, number of leaves and leaf area index of cassava. In both years, incorporation of green manures and NPK fertilizer influenced growth and fresh root yield of cassava (Table 4). Year 2016/2017 (second crop) significantly increased number of leaves, leaf area index and fresh root yield of cassava compared with year 2015/2016 (first crop). The improved growth performance of cassava crops in the second year compared with the first year could be attributed in part to the increased plant nutrients availability due to residual concentration from the first cropping season and the subsequent application of the organic manure in the second year. Plant height in both years did not differ from each other. In both years, plant height, number of leaves, leaf area index and fresh root yield of cassava in green manured plots and NPK fertilizer treated plots were significantly greater than the control plots (Table 4). In the second year (2016/2017) of cropping, Gliricidia and other green manures significantly increased growth, and cassava fresh root yield (Figure 1) compared with NPK fertilizer. Across similar individual treatment over the 2 years, growth and cassava fresh root yield of control plots and NPK fertilizer plots decreased gradually over the years, whereas growth and cassava fresh root yield of green manured treated plots increased gradually over the years. In both years, and in all cases of growth (Table 4) and fresh root yield (Figure 1, Table 4) decreasing order was: Gliricidia > Moringa = Leucaena = Neem > NPK fertilizer > control. In the first year (2015/2016) and compared with control; Gliricidia, Moringa, Leucaena, Neem and NPK fertilizer increased fresh root yield of cassava by 53, 33, 30, 29 and 28%, respectively. In the second year (2016/2017), these treatments increased fresh root yield of cassava by 85, 65, 61, 60 and 36%, respectively. In the first year and compared with NPK fertilizer; Gliricidia, Moringa, Leucaena and Neem increased fresh root yield of cassava by 19, 4, 1 and 0.7%, respectively, whereas in the second year, the same treatments increased

fresh root yield of cassava by 36, 22, 19 and 18%, respectively. Averaged across similar individual treatment, second year (2016/2017) Moringa, Leucaena and Neem increased cassava fresh root yield by 13, 13, 10 and 13%, respectively, compared to the first year (2015/2016).

Table 3

Effect of various green manures and NPK fertilizer on soil bulk density, and soil chemical properties

Year/ season	Fertilizer	Bulk density (Mg m^{-3})	pH (water)	OM (%)	N (%)	P (mg kg^{-1})	K (cmol kg^{-1})	Ca (cmol kg^{-1})	Mg (cmol kg^{-1})
2015/2016	Control	1.56a	5.75a	1.48c	0.12e	6.4d	0.10e	1.37d	0.28d
	Moringa leaves	1.28b	5.65ab	2.84b	0.17c	11.4b	0.27b	2.73a	0.47b
	Leucaena leaves	1.29b	5.54bc	3.17a	0.20b	9.2c	0.24c	2.37b	0.48b
	Gliricidia leaves	1.28b	5.54bc	3.12a	0.22a	11.2b	0.32a	2.71a	0.61a
	Neem leaves	1.30b	5.55bc	2.83b	0.17c	12.7a	0.23c	2.34b	0.50b
	NPK 15-15-15 fertilizer	1.54a	5.34d	1.51c	0.16d	10.3c	0.22d	1.86c	0.39c
2016/2017	Control	1.51a	5.73a	1.42c	0.10e	6.0d	0.08e	1.31d	0.26d
	Moringa leaves	1.17b	5.63ab	3.24b	0.19c	13.6b	0.33b	3.13a	0.57b
	Leucaena leaves	1.19b	5.52bc	3.57a	0.22b	11.4c	0.30c	2.77b	0.54b
	Gliricidia leaves	1.18b	5.52bc	3.52a	0.24a	13.4b	0.38a	3.11a	0.67a
	Neem leaves	1.20b	5.53bc	3.23b	0.19c	14.9a	0.29c	2.74b	0.56b
	NPK 15-15-15 fertilizer	1.51a	5.32d	1.45c	0.14d	9.9c	0.20d	1.80c	0.37c
P									
Year (Y)		0.120	0.002	0.388	0.010	0.461	0.224	0.369	0.630
Fertilizer (F)		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Y x F		1.000	0.999	0.998	0.735	0.995	0.895	0.995	0.993

p – Probability of F statistic from ANOVA; means in column for each effect followed by similar letter are not significantly different at p = 0.05 according to Duncan's multiple range test.

Table 4

Effect of various green manures and NPK fertilizer on growth and fresh root yield of cassava

Year/ season	Fertilizer	Fresh root yield (t ha^{-1})	Plant height (m)	Number of leaves per plant	Leaf area Index (LAI)
2015/2016	Control	21.5c	1.05d	70c	1.57d
	Moringa leaves	28.6b	1.33bc	91b	2.32b
	Leucaena leaves	27.9b	1.46a	89b	2.24b
	Gliricidia leaves	32.8a	1.40ab	97a	2.61a
	Neem leaves	27.7b	1.31bc	86b	2.23b
	NPK 15-15-15 fertilizer	27.5b	1.35bc	87b	2.07c
2016/2017	Control	19.6d	0.99d	62e	1.43d
	Moringa leaves	32.4b	1.35bc	99bc	2.72b
	Leucaena leaves	31.6b	1.48a	105b	2.64b
	Gliricidia leaves	36.3a	1.42ab	113a	3.01a
	Neem leaves	31.3b	1.33bc	102b	2.63b
	NPK 15-15-15 fertilizer	26.6c	1.29c	79d	1.95c
P					
Year (Y)		0.005	0.840	0.690	0.494
Fertilizer (F)		0.000	0.000	0.000	0.000
Y x F		0.024	0.845	0.975	0.992

p – probability of F statistic from ANOVA; means in column for each effect followed by similar letter are not significantly different at p = 0.05 according to Duncan's multiple range test-

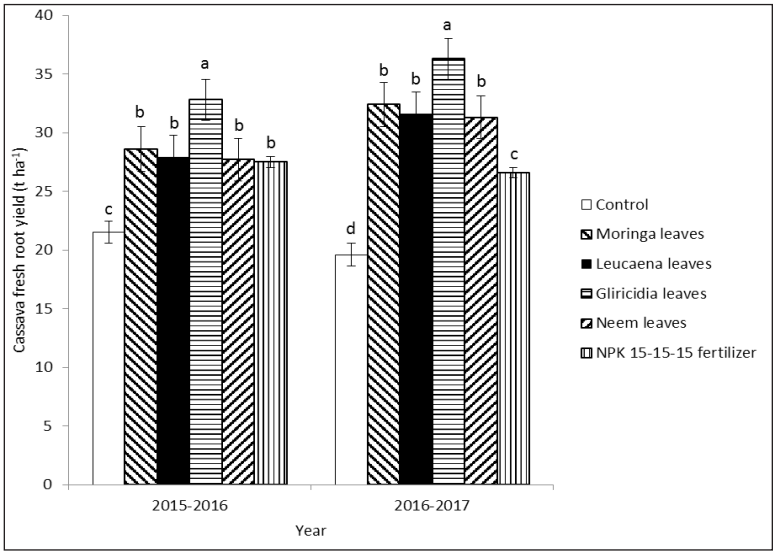

Figure 1. Effect of various green manures and NPK 15-15-15 fertilizer on fresh root yield of cassava in 2015-2016 and 2016-2017

In both years, incorporation of green manures and NPK fertilizer increased growth and fresh root yield of cassava compared with the control. The increase in the performance of cassava as a result of green manure could be due to reduced soil bulk density and increased availability of soil OM, N, P, K, Ca and Mg concentrations from the manures. The increase in the performance of cassava as a result of green manure compared with the NPK fertilizer could be due to reduced soil bulk density and increased availability of soil OM, N, P, K, Ca and Mg concentrations from the manures. Lampurlanes and Cantero-Martinez (2003) found that reduced soil bulk density enhances root growth and water and nutrient uptake and yield. The better performance of cassava under NPK fertilizer plots compared with the control might be due to availability of essential nutrient elements especially (K and N) from the inorganic fertilizer which were absorbed by the cassava plants. In both years, incorporation of Gliricidia increased performance of cassava compared with NPK fertilizer and other green manures due to availability of soil N and especially soil K (Table 3).

According to Howeler (2011) and Uwah, Effa, Ekpenyong and Akpan (2013), the two major elements that are particularly critical in production of cassava are K and N. Nitrogen plays a dominant role in promoting vegetative growth, including leaf development, and K being important in tuber initiation and bulking. Potassium is required in large amount which has a special role in carbohydrate synthesis and translocation. Abundant K supply favours the primary processes of photosynthesis. It also regulates the balance between assimilation and respiration in a way that improves net assimilation. This is a prerequisite for vigorous growth and the formation of reserve assimilates (Imas & John, 2013; Jansson, 1980). Potassium application not only enhances tuber yield, but also improves tuber quality (Imas & John, 2013; Nair & Aiyer, 1986). Soil K was greatly increased by green manure incorporation after the second year, irrespective of green manure treatments. The soil K increased by 13% in the second year of cropping due to incorporation of green manure, indicating that the incorporation of plant residues rich in K was advantageous. Cassava is known to accumulate more than 60% of the absorbed K in its storage roots (Howeler, 1991a; Pellet & El-Sharkawy, 1997), pointing to the need to replenish soil K in poor soils under continuous cassava cultivation. The results suggested that K was an important limiting factor (the main limiting nutrient) for storage root yield of cassava. Soil K level of 0.09 cmol kg^{-1} recorded under the control treatment was much below the critical level recommended for cassava (Cadavid et al., 1998; Howeler, 2002), hence poor growth and yield.

In both years, soil Ca and Mg were also greatly enhanced by incorporation of green manure leaves compared with NPK fertilizer and the control. For example, these two elements under the control plots

depleted rapidly by continuous cassava cultivation. These findings indicate that incorporation of green manure leaves into poor sandy soils are advantageous in supplying adequate levels of Ca and Mg. The lower performance of cassava under NPK fertilizer compared with Gliricidia, Moringa, Leucaena and Neem leaves might partly be due to its quick release of nutrients within a short time that may not benefit long gestation crops like cassava, and also losses due to leaching and erosion.

When studied as individual factors, year (Y) was not significant for P, K, Ca, Fe, Zn, starch and HCN contents; fertilizer (F) did significant for P, K, Ca, Fe, Zn, starch and HCN contents (Table 5). The interaction of Y × F were not significant for P, K, Ca, Fe, Zn, starch and HCN contents. The second year (2016/2017) significantly increased P,

Table 5

Effect of various green manures and NPK fertilizer on mineral composition, starch and HCN contents of cassava roots

Year/ Season	Fertilizer	P	K	Ca (mg 100^{-1} g)	Fe	Zn	Starch (%[1])	HCN (mg kg^{-1})
2015/2016	Control	20.5d	189.4d	10.7d	0.12e	0.23d	19.7c	64.2a
	Moringa leaves	56.6a	348.8a	21.4a	0.27a	0.48a	29.3a	44.5d
	Leucaena leaves	28.4c	220.5c	13.7c	0.20c	0.44b	28.1a	49.9c
	Gliricidia leaves	43.1b	311.1b	14.0c	0.19cd	0.33c	28.6a	51.0c
	Neem leaves	44.7b	306.3b	17.4b	0.22b	0.44b	28.2a	50.6c
	NPK 15-15-15 fertilizer	43.0b	223.3c	13.5c	0.22b	0.32c	24.9b	56.0b
2016/2017	Control	18.1d	179.8d	8.9d	0.11e	0.21d	17.9c	63.4a
	Moringa leaves	65.8a	381.2a	23.6a	0.29a	0.52a	33.9a	38.9d
	Leucaena leaves	35.2c	252.9c	15.9c	0.22c	0.48b	32.7b	44.3c
	Gliricidia leaves	49.9b	343.5b	16.2c	0.21cd	0.37c	33.2b	45.4c
	Neem leaves	51.5b	338.5b	19.8b	0.24b	0.48b	32.8b	45.0c
	NPK 15-15-15 fertilizer	49.8b	253.7c	15.7c	0.24b	0.36c	29.5c	50.4b
P								
Year (Y)		0.283	0.383	0.423	0.449	0.798	0.994	0.206
Fertilizer (F)		0.000	0.000	0.000	0.000	0.000	0.000	0.000
Y x F		1.000	0.995	0.996	0.996	0.871	0.358	1.000

p – probability of F statistic from ANOVA; means in column for each effect followed by similar letter are not significantly different at p = 0.05 according to Duncan's multiple range test

K, Ca, Fe, Zn and starch composition and reduced hydrocyanic acid (HCN) content compared with the first year (2015/2016) (Table 5). In the first year (2015/2016) and second year (2016/2017), the various green manures and the NPK fertilizer increased P, K, Ca, Fe, Zn and starch composition and reduced HCN content in cassava root tubers significantly compared with the control (Table 5). This could be adduced to nutrient availability in soil because of the mineralization of the manures/fertilizer leading to take-up by cassava plants. In both years, Moringa had the highest P, K, Ca, Fe and Zn composition and lowest HCN content compared with other treatments. In the first year (2015/2016) and second year (2016/2017), there were no differences in starch content between Moringa, Leucaena, Gliricidia and Neem. In both years, Moringa, Leucaena, Gliricidia, and Neem had higher starch content and lower content of HCN than NPK fertilizer. This could be attributed to their higher soil K levels and their subsequent absorption by cassava roots compared with NPK fertilizer. Organic manure reduced nutrient leaching due to its binding nature in building large and stable aggregates in most soils compared to chemical fertilizers which are susceptible to leaching of nutrients. Howeler (1985) found that application of fertilizer, especially K fertilizer, influenced the quality of tuberous roots resulting in an increase in dry matter and starch content and a decrease in cyanogenic potential. A marked decrease in total cyanogens (HCN) in fresh cassava roots was noted following the application of K fertilizer and this was attributed to increases in starch content and dry matter of the tuberous roots rather than a decrease in synthesis of cyanogens (Bokanga, Ekanayake, Dixon, & Porto, 1994; El-Sharkawy & Cadavid, 2000). Cadavid et al. (1998) found that application of NPK fertilizer resulted in a decrease in the concentration of total cyanogens in cassava roots, along with an increase in root biomass. Results of a long term fertilizer experiment carried out in India revealed that cassava plants supplied with ash and crop residues, which are known to have a high content of potassium, had low root cyanide content (Susan John, Ravindran, & George, 2005).

The correlation coefficient between soil properties and mineral composition, starch and HCN contents and growth and yield of cassava showed that in 2015/2016, cassava yield was more dependent on soil chemical properties (Table 6), whereas in 2016/2017 cassava yield, growth and mineral composition, starch and HCN contents were dependent on both soil chemical properties and soil bulk density (Table 7). The significant correlation of bulk density and growth, yield and mineral composition, starch and HCN contents in 2016/2017 could be related to the residual effects of the manure in the

Table 6

Correlation between soil properties and mineral composition, starch and HCN contents and growth and yield of cassava in 2015/2016

	P	K	Ca	Fe	Zn	Starch	HCN	Yield	Plant height	Number of leaves	LAI
Bulk density	-0.493 (0.321)	-0.729 (0.100)	-0.608 (0.200)	-0.578 (0.228)	-0.785 (0.065)	-0.927 (0.008)	0.877 (0.022)	-0.759 (0.008)	-0.741 (0.094)	-0.741 (0.092)	-0.860 (0.028)
pH	-0.297 (0.567)	0.042 (0.937)	0.072 (0.891)	-0.344 (0.504)	-0.078 (0.883)	-0.315 (0.543)	0.135 (0.799)	-0.482 (0.332)	-0.607 (0.192)	-0.518 (0.292)	-0.397 (0.436)
OM	0.362 (0.480)	0.635 (0.175)	0.502 (0.310)	0.471 (0.346)	0.726 (0.103)	0.874 (0.023)	-0.815 (0.048)	0.717 (0.109)	0.725 (0.103)	0.685 (0.133)	0.820 (0.046)
N	0.431 (0.393)	0.513 (0.298)	0.249 (0.634)	0.408 (0.421)	0.418 (0.410)	0.823 (0.044)	-0.655 (0.158)	0.954 (0.003)	0.866 (0.026)	0.929 (0.007)	0.950 (0.004)
P	0.847 (0.033)	0.821 (0.045)	0.730 (0.099)	0.791 (0.061)	0.704 (0.118)	0.841 (0.036)	-0.760 (0.080)	0.744 (0.090)	0.606 (0.202)	0.751 (0.085)	0.779 (0.068)
K	0.702 (0.12)	0.743 (0.090)	0.549 (0.259)	0.667 (0.148)	0.625 (0.185)	0.944 (0.005)	-0.833 (0.040)	0.976 (0.001)	0.856 (0.030)	0.984 (0.000)	0.994 (0.000)
Ca	0.734 (0.097)	0.849 (0.033)	0.716 (0.110)	0.733 (0.098)	0.768 (0.075)	0.975 (0.001)	-0.934 (0.006)	0.887 (0.018)	0.776 (0.070)	0.906 (0.013)	0.949 (0.004)
Mg	0.546 (0.263)	0.728 (0.101)	0.429 (0.396)	0.459 (0.360)	0.523 (0.287)	0.884 (0.019)	-0.728 (0.101)	0.948 (0.004)	0.746 (0.088)	0.892 (0.017)	0.973 (0.001)

Table 7

Correlation between soil properties and mineral composition, starch and HCN contents and growth and yield of cassava in 2016/2017

	P	K	Ca	Fe	Zn	Starch	HCN	Yield	Plant height	Number of leaves	LAI
Bulk density	-0.825 (0.028)	-0.717 (0.041)	-0.778 (0.013)	-0.868 (0.024)	-0.876 (0.040)	-0.883 (0.035)	0.818 (0.042)	-0.882 (0.020)	-0.852 (0.035)	-0.926 (0.008)	-0.939 (0.006)
pH	-0.361 (0.482)	-0.511 (0.301)	-0.179 (0.735)	-0.435 (0.388)	-0.216 (0.681)	-0.467 (0.351)	0.292 (0.574)	-0.285 (0.587)	-0.501 (0.311)	-0.234 (0.655)	-0.176 (0.739)
OM	0.462 (0.357)	0.364 (0.478)	0.615 (0.193)	0.535 (0.274)	0.765 (0.077)	0.785 (0.034)	-0.800 (0.026)	0.886 (0.019)	0.798 (0.037)	0.946 (0.004)	0.938 (0.006)
N	0.563 (0.245)	0.536 (0.273)	0.576 (0.231)	0.572 (0.235)	0.676 (0.140)	0.868 (0.025)	-0.822 (0.043)	0.979 (0.001)	0.906 (0.013)	0.978 (0.001)	0.973 (0.001)
P	0.813 (0.049)	0.772 (0.072)	0.866 (0.026)	0.805 (0.053)	0.822 (0.045)	0.917 (0.010)	-0.898 (0.015)	0.896 (0.016)	0.757 (0.031)	0.884 (0.020)	0.911 (0.012)
K	0.670 (0.146)	0.627 (0.183)	0.688 (0.130)	0.658 (0.155)	0.735 (0.096)	0.903 (0.014)	-0.882 (0.020)	0.993 (0.000)	0.886 (0.019)	0.974 (0.001)	0.993 (0.000)
Ca	0.771 (0.113)	0.628 (0.182)	0.790 (0.062)	0.715 (0.110)	0.819 (0.046)	0.894 (0.016)	-0.920 (0.009)	0.957 (0.003)	0.829 (0.041)	0.946 (0.004)	0.983 (0.000)
Mg	0.667 (0.148)	0.623 (0.187)	0.693 (0.127)	0.624 (0.186)	0.704 (0.118)	0.873 (0.023)	-0.853 (0.031)	0.986 (0.000)	0.822 (0.042)	0.972 (0.001)	0.994 (0.000)

second year of application which would have increased soil OM decomposition which led to enhancement of soil porosity and reduction in bulk density. Reduction in soil bulk density is known to increase root penetration and therefore water and nutrient uptake (Adekiya et al., 2017; Lampurlanes & Cantero-Martinez, 2003) which will consequently increase growth and yield.

Cost of purchasing of NPK fertilizer was higher than the cost of cutting and transporting each green manure treatment (Table 8). Use of Gliricidia produced the highest gross return (₦532,840 ha^{-1}) and net return (₦200,600 ha^{-1}) followed by Moringa treatment with a gross return of ₦469,700 ha^{-1} and net return of ₦137,460 ha^{-1}; the gross return and net return produced by Moringa was not significantly greater than that of Leucaena and Neem treatments. The lowest gross return (₦317,240 ha^{-1}) was produced by the control. All green manures, and the NPK fertilizer, produced higher net profit than the control. The economic returns and net benefits from all green manures were higher than for the NPK fertilizer treatment. The Gliricidia treatment was more cost effective and profitable in production of cassava than all other treatments, as indicated by its high return rate, or value/cost ratio of 13.37.

Table 8

Economics of producing cassava under green manures and NPK fertilizer tested in the first year (2015/2016) and second year (2016/2017)

Treatment	Monetary gain (₦ ha^{-1})	Production increase value (₦ ha^{-1})	Production increase (%)	Cost of cutting and transporting of green manures/ cost of NPK fertilizer (₦ ha^{-1})	Net return over each fertilization (₦ ha^{-1})	Return rate or value/cost ratio of each fertilization
Control	317,240	-		-	-	-
Moringa leaves	469,700	152,460	48.06	15,000	137,460	9.16
Leucaena leaves	458,920	141,680	44.66	15,000	126,680	8.45
Gliricidia leaves	532,840	215,600	67.96	15,000	200,600	13.37
Neem leaves	454,300	137,060	43.20	15,000	122,060	8.14
NPK 15-15-15 fertilizer	417,340	100,100	31.55	80,000	20,100	0.25

Notes: In the first year of 2015/2016, the price of fresh root yield of cassava was ₦15,400 per hectare; NPK 15-15-15 fertilizer was ₦9,800 per 50 kg bag. In the second year of 2016/2017, the price of fresh root yield of cassava was ₦15,400 per hectare; NPK 15-15-15 fertilizer was ₦9,800 per 50 kg bag.

This present study indicated that Gliricidia, Moringa, Leucaena and Neem could be used as green manure on degraded soils for sustainable soil and cassava productivity. The results of this investigation confirmed that these locally available plant species can be utilized as green manure to enhance soil and crop quality, and can serve as an alternative replacement for scarce and expensive chemical fertilizer.

CONCLUSIONS

Green manure serves as an important resource not only to supply nutrients for crops but also to replenish the soil organic matter content of most cultivated soils. For those that desire increased yield Gliricidia is recommended as green manure**.** For those desiring to grow cassava for root quality Moringa is recommended as green manure. Green manures can serve as an alternative replacement for inorganic fertilizers for optimum cassava yield and quality.

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Effect of Foliar Application of Zinc on the Yield, Quality and Storability of Potato in Tista Meander Floodplain Soil

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ABSTRACT

An experiment was conducted at Breeder Seed Production Centre (BSPC), Debiganj, Panchagarh under AEZ-3 (Tista MeanderFloodplain Soil) during the Rabi season of 2013-14 and 2014-15 to study the effect of foliar application of zinc on the yield and quality of potato. The treatments comprised foliar application with six different concentration of zinc such as: T1 (0 ppm Zn), T2 (140 ppm Zn), T3 (280 ppm Zn), T4 (420 ppm Zn), T5 (560 ppm Zn) and T6 (700 ppm Zn). The experiment was laid out in a randomized complete block design (RCBD) with three replications. The tuber yield of potato was significantly influenced by the foliar application of different concentration of zinc. The maximum yield (37.2 and 36.7 t ha^{-1} for 2013-14 and 2014-15, respectively) was found in 560 ppm Zn application. The highest dry matter content of potato tuber was found in this treatment. The highest gross margin and marginal benefit cost ratio were recorded in the same treatment. T5 (560 ppm Zn) also showed the minimum weight and rottage loss of potato tuber. Therefore, foliar application of 560 ppm Zn can be recommended for quality potato production.

Keywords: dry matter, foliar application, potato storage, yield, zinc

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INTRODUCTION

Potato is one of the most important vegetables in Bangladesh. Besides, it acts as main vegetables and supplies necessary nutrients to the poor people (Hossain & Miah, 2012; Islam, Zaman, Hossain, & Hossain, 2009). Potato is one of the main tubers and a nutritious crop. Studies have shown that utilization of micronutrients increase the yield and quality of tubers. The national average yield of potato in Bangladesh is 19.5 tons per hectare and it is lower than the major potato growing countries (Bangladesh Bureau of Statistics [BBS], 2015). The causes of lower yield might be due to application of imbalanced fertilizers and lower zinc (Zn) and boron (B) content in the soils (Keller, 2013). That is why, the crop yield and production are being seriously hampered owing to micronutrients deficiencies (Bose & Tripathi, 1996; Mousa, 2009). In plant, micronutrients are needed in smaller quantities which are very essential for growth and development. (Al-Jobori & Al-Hadithy, 2014; Benepal, 1967). There are many evidences that it is impossible to achieve maximum yield from other nutrients except micronutrients (Banerjee et al., 2016; Trehan & Grewal, 1997). The micronutrient use requires a thorough knowledge about the soil capacity to supply micronutrients and plant requirement. No adhoc recommendations on their use can be made because of the antagonism between different nutrients and the polluting effect that might ensue (Kanwar, 1962). Zinc is a micronutrient necessary for plant growth. It promotes growth hormone biosynthesis, the formation of starch, seed production and maturation (Brady & Weil, 2002). Zinc has many important roles in plant growth and a constant and continuous supply is necessary for optimum growth and maximum yield (Acquaah, 2002). Deficiency of Zn has been found to reduce leaf size and shortened internodes and hence, limited plant growth (Acquaah, 2002; Alloway, 2004). Mousavi, Galavi and Ahmadvand (2007) reported that foliar application of zinc increased all plant characteristics related to yield and quality of potato. Barben et al. (2007) reported that plants grown in the upper level of treatments of zinc generally exhibited rapid growth of leaves, stems and roots. The foliar application of zinc showed optimistic effects on yield attributes, yield, quality components and chemical composition of sweet potato (Abd El-Baky, Ahmed, El-Nemr, & Zaki, 2010). Recently, Osama, El-Nasharty and Badran (2010) reported that spraying potato plants with micronutrients like Zn could improve the physiological performance of plants and nutrient balance of macro and micronutrients. For easy absorption, quick action, reduced toxicity, gathering and stoppage from stabilization, the foliar application of micronutrients such as Zn and B is very effective.

The advantages of foliar application are quick correction of nutrient deficiency and application of lesser rates and thus, reducing toxicity arising from excessive accumulation of elements and preventing nutrients fixation in the soil (Parmar, Nandre, & Pawar, 2016). Nutrient absorption (such as micronutrients) through foliar application is very quick

than the soil application through plant roots (Hashemy, Malakoty, & Tabatabaey, 1998). For sustainable production of potato, micronutrient especially zinc management is very important. However, no data is available regarding foliar application of zinc for potato in Bangladesh. So, the experiment was initiated with the following objectives: i) To evaluate the response of zinc through foliar application, ii) to determine the actual rate of Zn through foliar application to get the highest yield of potato and iii) to find out influence of zinc through foliar application on the quality and storability of potato.

MATERIALS AND METHODS

Experimental Site and Soil Characteristics

The experiment was conducted at high land of Breeder Seed Production Centre (BSPC), Debiganj, Panchagarh during the Rabi season of 2013-14 and 2014-15. The geographical distribution of experimental plot was located at N-26° 12′ and E-88° 76′ in the Tista Meander Floodplain Soil (AEZ-3) of Bangladesh. The texture of soil was sandy loam with acidic in nature where available phosphorus (P) and iron (Fe) were found higher than the critical limit but other nutrients such as total N (0.06%), exchangeable calcium (Ca), magnesium (Mg), sulphur (S), zinc (Zn), and boron (B) were lower than the critical level. The organic matter content was also low. The exchangeable potassium was at par to the critical level (Table 1).

Table 1

The chemical properties of initial soil of the experimental field

Location	Soil texture	pH	O.M%	Ca	Mg	K	Total N%	P	S	B	Fe	Zn
				meq/100g				µg/ml				
BSPC, Debiganj	Sandy loam	5.70	1.10	1.30	0.45	0.14	0.06	28	7.47	0.18	72	0.53
Critical level		-	-	2.0	0.5	0.12	-	7.0	10	0.2	4.0	0.6

Cropping Season

There are three cropping seasons in Bangladesh such as *Rabi* (mid-October to mid-March), *Kharif-I* (mid-March to the end of June) and *Kharif-II* (early July to mid-October). In Bangladesh, potato is cultivated only in winter season from November to February. The highest mean temperature in November was 24.2°C and 25.2°C in 2013 and 2014, respectively, where the coldest month was January. In January 2014, the lowest temperature was observed and it was 15°C. Between two seasons, February was the dry where mean relative humidity was 84.9% and 86.8% in 2014 and 2015, respectively, with 19.3 mm rainfall in February 2014. In 2014-15, December and January were the most humid months with relative humidity of 90.0 and 89.9%, respectively, including a sudden rain flash (38.7 mm) in January 2015. Rest of the months in both seasons was relatively dry.

Experimental Design, Treatment, Fertilizer Application and Intercultural Operation

The experiment was laid out in a randomized complete block design (RCBD) with three replications. There were six treatments comprising different levels of zinc: T1 (0 ppm Zn), T2 (140 ppm Zn), T3 (280 ppm Zn), T4 (420 ppm Zn), T5 (560 ppm Zn) and T6 (700 ppm Zn). Fertilizers were used on soil test basis except zinc (Zn). Urea, TSP, MOP, gypsum, magnesium sulphate, zinc sulphate and boric acid were used as a source of N, P, K, S, Mg, Zn and B, respectively. All the fertilizers but half of urea was applied to the soil before planting tubers. Potato variety *BARI Alu-25* (Asterix) was used as test crop. The unit plot size was 3 m × 3 m. Whole tubers of the potato were planted with a spacing of 60 cm × 25 cm on 15 and 17 November of 2013 and 2014, respectively. The irrigation was applied four times during the growing period. For proper germination, a light irrigation was applied at 7 days after planting (DAP) and second irrigation was done at 30 DAP after earthing up and side dressing of the rest urea. Rest two irrigations were done according to the demand of crops which was at 48 and 63 DAP. Zinc sulphate ($ZnSO_4$) was applied at three stages of plant growth (stolonization, tuberization and bulking) followed by three foliar sprays. In each case of foliar spray, 2.25 L of $ZnSO_4$ solution was applied at 9 m^2 plot (2500 L ha^{-1}). Weeding and mulching were performed as per requirement. Late blight disease was managed by spraying Dithane M45 at the rate of 2gL^{-1}. After maturity, potato was harvested on 25 February 2014 and 30 February 2015, respectively.

Soil Sampling and Chemical Analysis

Soil samples were collected, dried and ground for chemical analysis. Bulk density was determined by core sampler Method (Blake, 1965), soil pH was measured by glass electrode pH meter (1:2.5) and organic carbon by wet oxidation method (Walkley & Black, 1935). Total N content of soil was determined by Kjeldahl method, and available P (Bray-1 method), exchangeable K and available S contents by 0.5M $NaHCO_3$

(pH 8.5), NH_4OAc and $CaCl_2$ extraction methods, respectively as outlined by Page, Miller and Kuny (1989). Available Zn content was determined following DTPA method.

Data Collection

Data were taken on plant height, foliage coverage, stems / hill, tubers / hill, wt. of tubers / hill, tuber yield and tuber dry matter of potato. Tuber size and number were also recorded. Plant height, stem per hill and foliage coverage was assessed at 60 DAP using green method (Groves, Wiltshire, Briddon, & Cunnington, 2005). The yield data were collected at harvest and dry weight was recorded at 7 days after harvest.

Storage Data

The potato tubers were collected after harvest and weight of tubers were recorded. Weighing of tubers started from 30 DASt (days after storing) and continued at 15 days intervals up to 150 days. Rotten tubers were discarded after weighing.

Cost Benefit Analysis

The cost of chemical fertilizers, pesticides (inputs) and outputs (Tuber) were estimated as per prevailing market price. The gross return, net return and return per dollar invested in different nutrient management systems were assessed by computing the cost of the inputs and price of the produce/ output. Economic analysis was performed through partial budgeting followed by marginal benefit cost ratio (MBCR) as suggested by International Maize and Wheat Improvement Center (CIMMYT) (1988).

Statistical Analysis

The analysis of variance (ANOVA) for different crop parameters and dry matter was done following F test. When the F was significant at the $p< 0.05$ level, the means were separated by DMRT (Steel & Torrie, 1960) test. The SAS software (version 9.3) was used to analyze the data.

RESULTS AND DISCUSSIONS

Foliar Application of Zinc on the Yield Contributing Characters of Potato

The plant height (cm), foliage coverage, stem per hill and number of tuber per hill were positively influenced by the application of different doses of foliar zinc but their effects were not significant (Table 2). The weight of tuber per hill was significantly variable among different doses of foliar zinc (Table 1). The highest weight of tubers per hill (0.63 and 0.61kg $hill^{-1}$ for 2013-14 and 2014-15, respectively) was found in T5 (560 ppm Zn) which was followed by T6 (0.62 and 0.60 kg $hill^{-1}$ for 2013-14 and 2014-15, respectively) and T4. The lowest weight of tubers per hill (0.50 and 0.45 kg $hill^{-1}$ for 2013-14 and 2014-15, respectively) was recorded in the control (0 ppm Zn). Mousavi et al. (2007) reported that the weight of tuber was increased with foliar application of Zn at 800 ppm, which is in agreement with the findings of our result.

Foliar Application of Zinc on the Yield and Dry Matter Content of Potato Tuber

The potato tuber yield and dry matter content were significantly influenced by the foliar application of zinc (Table 3). The

highest tuber yield of potato (37.2 and 36.7 t ha^{-1} for 2013-14 and 2014-15, respectively) was found in T5 (560 ppm Zn) which was statistically similar with T6 (700 ppm Zn). The lowest tuber yield (29.3 and 27.9 t ha^{-1} for 2013-14 and 2014-15, respectively) was recorded in the control. It was observed that tuber yield of potato was increased with increase in Zn concentration up to 560 ppm after which yield decreased. Similar results were also observed by Al-Fadhly (2016), Al-Jobori and Al-Hadithy (2014), Mousavi et al. (2007), Sharma and Trehan (1984), Trehan and Grewal (1989) where maximum potato tuber yield was obtained with foliar application of zinc. The dry matter content ranged from 18.7 to 20.7% and 18.8 to 21.1% for 2013-14 and 2014-15, respectively. The highest dry matter content (20.7 and 21.1% for 2013-14 and 2014-15, respectively) was noted in 560 ppm Zn which was followed by 700 ppm Zn and 420 ppm Zn (Table 3).

The minimum dry matter content (18.7 and 18.8% for 2013-14 and 2014-15, respectively) was noted in the control. Mousavi et al. (2007) reported that application of Zn at 800 ppt increased per cent dry matter of up to 5% in comparison to control which corroborated our findings. It was also found that dry matter content was increased with increase in Zn concentration up to 560 ppm beyond that dry matter decreased (Table 3). Romemheld and El-Fouly (1999) reported that foliar application of nutrients had become an efficient way to increase yield and quality of crops. These results are in agreement with those reported by several authors (Barben et al., 2007; Brady & Weil, 2002; Bybordy & Malakoty, 2001; Hashemy et al., 1998; Mousavi et al., 2007; Ranjbar & Malakoty, 2000) who found that zinc application increased all plant characteristics relating to yield and quality of potato.

Zinc plays an important role in protein synthesis, enzyme activation and metabolism of carbohydrate, utilization of fertilizers containing this element increase qualitative and quantitative performance of potato tubers. Due to shortage of Zn, performance and quality of potato decreases (Alloway, 2004).

Table 2
Effect of foliar application of zinc on the yield contributing characters of potato

Treat	Plant height (cm)		Foliage coverage (%)		Stem hill^{-1} (no.)		Tuber hill^{-1} (no.)		Tuber weight hill^{-1} (kg)	
	2013-14	2014-15	2013-14	2014-15	2013-14	2014-15	2013-14	2014-15	2013-14	2014-15
T1	67.6	57.3	95.7	89.7	8.13	7.66	10.5	10.2	0.50c	0.45d
T2	69.9	57.9	95.0	90.0	8.33	7.53	10.5	12.3	0.53bc	0.49cd
T3	66.2	61.1	94.3	93.7	7.73	7.66	10.3	11.8	0.55bc	0.53bc
T4	67.4	59.6	95.0	93.0	7.46	7.60	11.2	11.5	0.58ab	0.57ab
T5	66.8	59.5	95.3	94.7	7.46	8.33	11.6	11.5	0.63a	0.61a
T6	68.2	59.3	96.7	94.7	7.33	8.20	11.5	11.2	0.62a	0.60a
CV (%)	4.31	4.96	2.11	2.48	22.7	13.0	9.97	12.5	3.23	3.00
LSD	5.31	5.34	3.65	4.18	3.16	1.55	1.95	2.61	0.057	0.057

Figure(s) in column having same letter(s) do not differ significantly at 5% level. Note: T1=0 ppm Zn, T2=140 ppm Zn, T3=280 ppm Zn, T4=420 ppm Zn, T5=560 ppm Zn, T6=700 ppm Zn

Table 3

Effect of foliar application of Zinc on the tuber yield and dry matter of potato

Treat	Tuber yield (t/ha)		Dry mater (%)	
	2013-14	2014-15	2013-14	2014-15
T1	29.3 d	27.9 e	18.7 b	18.8 d
T2	30.8 cd	30.2 d	19.7 ab	19.3 cd
T3	32.3 c	32.7 c	20.3 a	19.7 b-d
T4	34.4 b	34.3 bc	20.3 a	20.3 a-c
T5	37.2 a	36.7 a	20.7 a	21.1 a
T6	36.6 a	35.2 ab	20.6 a	20.3 ab
CV (%)	2.57	3.33	3.29	3.35
LSD	1.56	1.57	1.20	1.22

Means followed by the same or no letter in the same column do not differ significantly each other at the 5% level. Note: T1=0 ppm Zn, T2=140 ppm Zn, T3=280 ppm Zn, T4=420 ppm Zn, T5=560 ppm Zn, T6=700 ppm Zn

Several studies reported that utilization of micronutrients increases performance and quality of potato tubers (Mohamadi, 2000; Mousavi et al., 2007; Ranjbar & Malakoty, 2000). Plants supplied with micronutrients during stolonization, tuberization and bulking recorded increased tuber yield and this might be due to the positive effect on mean weight of tuber as well as increased dry matter percentage. Zinc also has a great influence on plant life processes, such as nitrogen metabolism (uptake of nitrogen and protein quality), photosynthesis (chlorophyll synthesis) and carbon anhydrase activity (Al-Fadhly, 2016). Kohnaward, Jalilian and Pirzad (2012) reported that micronutrients increase photosynthesis rate and improved leaf area duration thus resulting in tuber yield increase (Cakmak et al., 1999). Micronutrient elements play a critical role in plants that lead to increased leaf area index and thereby increased light absorption and increased amount of dry matter accumulation and economic yield (Ravi, Channal, Hebsur, Patil, & Dharmatti, 2008).

Effect of Foliar Application of Zinc on Number and Size of Potato Tubers

The application of foliar zinc increased the tuber number of medium and large graded tubers (Table 4). The highest medium size tuber was found (81.6 and 76.2% for 2013-14 and 2014-15, respectively) in T5 followed by T6 and T4. The lowest medium size tuber (72.2 and 70.6% for 2013-14 and 2014-15, respectively) was noted in the control (Table 4). Sharma, Grewal and Trehan (1988) reported that application of zinc increased the number of medium and large graded tubers which are in agreement with the findings of our result.

Cost and Return

The main objective of the marginal analysis is to determine the profit from investment increase as the amount of investment increases (CIMMYT, 1988). The maximum gross margin (US$. 4410.8 and 4356.8 ha^{-1} for 2013-14 and 2014-15, respectively) was found in T5 (560 ppm Zn) (Table 5), which was followed by T6 (US$. 4341.6 and 4168.8 for 2013-14 and 2014-15,

Table 4

Effect of foliar application of Zinc on number and size of potato tubers

Treatment	Grading by No (%)					
	<28mm		28-55 mm		>55m	
	2013-14	2014-15	2013-14	2014-15	2013-14	2014-15
T1	26.89	28.93	72.18	70.65	0.93	0.42
T2	22.92	27.90	76.20	71.41	0.88	0.68
T3	20.10	26.34	78.72	72.47	1.18	1.18
T4	18.91	24.83	78.27	73.74	2.82	1.42
T5	15.32	22.02	81.60	76.20	3.08	1.78
T6	16.83	22.42	80.53	75.90	2.64	1.66
CV (%)	21.8	4.55	5.69	1.63	17.2	18.7
LSD	8.00	2.10	8.07	2.18	0.60	0.41

respectively) and T4. The lowest gross margin (US$. 3517.2 and 3355.2 ha^{-1} for 2013-14 and 2014-15, respectively) was observed in the control. The MBCR (marginal benefit cost ratio) ranged from 2.90 to 16.8 and 4.71 to 18.8 for 2013-14 and 2014-15, respectively. The highest MBCR (16.8 and 18.8 for 2013-14 and 2014-15, respectively) was recorded in T5 followed by T6 and T4. The lowest MBCR (2.90 and 4.71 for 2013-14 and 2014-15, respectively) was observed in T2 (140 ppm Zn) (Table 5). It was observed that MBCR was increased with increase in zinc concentration up to 560 ppm after that it was decreased. Similar results were also reported by Banerjee et al. (2016) where net income and B:C ratio were increased with increase in Zn rate up to 4.5 kg Zn ha^{-1} but both of these parameters decreased with application dose of 6.0 kg Zn ha^{-1}.

Storage Behavior of Potato Tuber

Weight loss

The weight loss was variable among the different zinc concentration and different days after storing (DASt) (Table 6). The minimum weight loss (2.20%) was recorded in T5 followed by T6 (2.38%) at 30 DASt. The highest weight loss (52.8%) was found in the control at 150 DASt, which was statistically identical to T2 (51.7%) and T3 (51.0%) at 150 DASt (Table 5). At the end of the observation (150 DASt), the minimum weight loss was found in T5 (42.7 %) followed by T6 (44.6 %). The results are in line with the findings of Awad, Emam and Shall (2010) where the lowest weight loss percentage was recorded with foliar application of zinc while tubers of untreated plants showed the highest weight loss. Zinc may reduce weight loss by developing flesh

Table 5

Effect of foliar application of zinc on the cost and return for potato

Treat	Tuber yield (t/ha)		Gross Return (UD$/ha)		TVC (UD$/ha)		Gross margin (UD$/ha)		Marginal gross margin UD$/ ha		MBCR	
	2013-14	2014-15	2013-14	2014-15	2013-14	2014-15	2013-14	2014-15	2013-14	2014-15	2013-14	2014-15
T1	29.3	27.9	3517.2	3355.2	0.0	0.0	3517.2	3355.2	-	-	-	-
T2	30.8	30.2	3700.8	3624.0	47.0	47.0	3653.8	3577.0	136.6	221.8	2.90	4.71
T3	32.3	32.7	3876.0	3920.4	49.1	49.1	3826.9	3871.3	309.7	516.1	6.31	10.5
T4	34.4	34.3	4130.4	4112.4	51.1	51.1	4079.3	4061.3	562.1	706.1	11.0	13.8
T5	37.2	36.7	4464.0	4410.0	53.2	53.2	4410.8	4356.8	893.6	1001	16.8	18.8
T6	36.6	35.2	4396.8	4224.0	55.2	55.2	4341.6	4168.8	824.4	813.6	14.9	14.7

Note: T1=0 ppm Zn, T2=140 ppm Zn, T3=280 ppm Zn, T4=420 ppm Zn, T5=560 ppm Zn, T6=700 ppm Zn. TVC = Total Variable Cost, MBCR= Marginal Benefit Cost Ratio

Input prices: Zinc Sulphate monohydrate = US$ 2.04/kg.

Output prices: Potato = UD$ 0.12/kg

Table 6

Weight loss of potato under ambient temperature at different days after storing (DASt) as influenced by organic manure and inorganic fertilizer management

Treat	Cumulative weight loss(%) at different DASt†				
	30	60	90	120	150
T1	4.22 h-j	6.98 hi	15.9 ef	28.4 cd	52.8 a
T2	2.67 h-j	5.97 h-j	17.8 e	31.0 c	51.7 a
T3	3.00 h-j	7.27 gh	14.6 ef	27.3 cd	51.0 a
T4	3.08 h-j	6.52 h-j	15.9 ef	26.7 cd	46.3 b
T5	2.20 j	4.45 h-j	11.9 fg	24.2 d	42.7 b
T6	2.38 ij	6.95 hi	15.4 ef	25.5 d	44.6 b

CV (%) = 14.2 and LSD= 4.65
Means followed by the same or no letter in the same column do not differ significantly each other at the 5% level. Note: T1=0 ppm Zn, T2=140 ppm Zn, T3=280 ppm Zn, T4=420 ppm Zn, T5=560 ppm Zn, T6=700 ppm Zn. DASt†= days after storing

with more combined water which restricts water loss during the early storage periods (Awad et al*.,* 2010; Mohamadi, 2000). Similar results were reported by Saif El-Dein (2005) on sweet potato. Among the four observations, maximum weight loss was found in 150 DASt followed by 120 DASt. 30 DASt showed the minimum weight loss. It was observed that weight loss increased with the increase in days after storing. Similar results were also observed by Kanbi and Bhatnagar (2005) where potato cultivar Kufri Badshah evaluated under integrated nutrient management and the highest weight loss was found in 105 DASt. Weight loss during storage was mainly due to evaporation and contribution of respiratory carbon loss to total weight loss (El-Sayed, El-Morsy, & El-Metwally, 2007; Mehta & Ezekiel, 2010).

Rottage loss

Rottage loss due to *Fusarium* dry rot varied among the treatments and days after storing. After 150 DASt, total rottage loss for *Fusarium* dry rot was the maximum in the control (8.36 %) followed by T2 (8.15 %), T3 (7.27 %) and the minimum loss (5.02 %) was noted in T5. Among days after storing, maximum rottage loss was recorded in 150 DASt followed by 120 DASt while 30 DASt showed the minimum rottage loss (Table 7). Among three observations, Akhter et al. (2014) reported that the maximum rottage loss was noted at 120 DASt followed by 90 DASt which is similar with our result.

Table 7

Rottage loss due to Fusarium dry rot (FDR) of potato under ambient temperature at different DASt

Treat	Rottage loss (%) at different DASt				Total rottage loss (%) at 150 DASt
	30	90	120	150	
T1	1.45 fg	1.88 de	2.21 cd	2.82 a	8.36 a
T2	1.14 gh	1.97 de	2.36 bc	2.68 ab	8.15 a
T3	1.05 hi	1.88 de	1.98 de	2.36 bc	7.27 b
T4	1.01 hi	1.97 de	2.67 ab	0.72 i	6.38 c
T5	0.72 i	0.72 i	1.67 ef	1.91 de	5.02 d
T6	0.72 i	1.45 fg	1.90 de	2.09 cd	6.18 c
CV (%)	12.4				5.40
LSD	0.35				0.68

Means followed by the same or no letter in the same column do not differ significantly each other at the 5% level.

CONCLUSION

Dry matter content and storability are very important for processing purpose and to preserve potato for later use, respectively. More thrust is given to determine the optimum dose of foliar zinc for more dry matter, economics and storage capacity. The tuber yield of potato was significantly influenced by the foliar application of zinc. The highest tuber yield (37.2 and 36.7 t ha^{-1} for 2013-14 and 2014-15, respectively) was found in 560 ppm Zn .The highest dry matter of potato tuber was found in this treatment. The highest gross margin and marginal benefit cost ratio were recorded in the same treatment. In storage behavior, T5 (560 ppm Zn) also showed the minimum weight and rottage loss. Therefore, foliar application of 560 ppm Zn can be recommended for sustainable quality potato production.

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Characterization of Phenazine and Phenazine-1-carboxylic Acid Isolated from *Pseudomonas aeruginosa* UPMP3 and Their Antifungal Activities against *Ganoderma boninense*

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ABSTRACT

The structural characterization of two phenazines, produced simultaneously through biosynthesis by *Pseudomonas aeruginosa* UPMP3 were studied. The phenazine antibiotics play an important role in antimicrobial activities and potentially could be used in biopesticide formulation to control basal stem rot of oil palm. The antibiotics were identified through high-performance liquid chromatography (HPLC) and confirmed using Fourier Transform Infrared (FTIR) spectroscopy. The specific representations in the FTIR spectra of the purified compounds such as absorption peaks at 1353.68 (aromatic nitro compound), 1118.84 and 729.43 cm^{-1} (aromatic ring) were indications of phenazine (PHZ) whereas, absorption peaks at 1717.92 (carboxylic acid), 861.78 and 738.54 cm^{-1} (aromatic ring) were indicatives of phenazine-1-carboxylic acid (PCA). The structures of the compounds were further confirmed by ^{1}H NMR/^{13}C NMR spectroscopy as PHZ ($C_{12}H_8N_2$) and PCA ($C_{13}H_8N_2O_2$). *Ganoderma boninense* was sensitive to purified phenazine antibiotics especially phenazine and a concentration of 1000 ppm completely inhibited the mycelial growth. As far as we know, this is the first study where purified phenazine antibiotics isolated from *P. aeruginosa* UPMP3 were structurally characterised and tested to have positive antagonism against *G. boninense.*

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INTRODUCTION

Ganoderma boninense is the most noxious species of fungal plant pathogen in oil palm plantations. It causes basal stem rot (BSR) on different parts of the same host but lack

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of information on the disease management causes severe economic losses due to this serious disease. The disease can cause losses up to 50% of palm trees and 45% yield from fresh fruit bunch (FFB) in oil palm plantations (Naher, Yusuf, Ismail, Tan, & Mondal, 2013). *Ganoderma* BSR disease currently has no cure because early disease symptoms are not detectable and the resistant stages of mycelium and basidiospores of *G. boninense* inhibit its control.

Previous studies using various control methods such as trench system and replanting techniques had been tried with great potential, while chemical control was the method which showed a significant reduction in BSR incidence when the oil palm trunk was injected with a combination of carboxin and quintozene fungicides or synthetic fungicides such as Anvil® with the active ingredient hexaconazole which was applied directly to the palm base. However, chemical fungicide applications might lead to fungicide resistance in fungus and inhibit the growth of good microbes. Since it is a synthetic formulation, growing concerns about the environment and high cost of chemicals have encouraged researchers to find an alternative way to control BSR. In this context, the development of a natural, effective and safe strategy for controlling *G. boninense* is necessary (Abraham et al., 2015). The use of pathogen-resistant cultivars and biological agents are the biological control measures.

Biological control through the use of antagonistic microbial can be an environmental-friendly and effective approach to control pathogenic fungi. Numerous studies have demonstrated that metabolites including antibiotics, enzymes and volatiles produced by antagonistic bacteria play key roles in the control of various plant pathogens. Natural antibiotics have been a subject of intense research for the past 70 years and natural antibiotics, together with their semi-synthetic derivatives and the formulation of modern antimicrobial therapy. The antibiotic mechanism is well documented and summarized in several review papers (Abraham et al., 2015).

Biological control of *Ganoderma* spp. has achieved notable attention. *In vitro* studies have shown that *Trichoderma* spp*., Aspergillus* spp. and *Penicillum* spp. are antagonistic agents of *G. boninense.* However, these biological agents can only protect the plants at the very early stage of infection, thus, are unable to cure highly infected palms (Abdullah, Ilias, Nelson, Izzati, & Yusuf, 2003). *Pseudomonas aeruginosa* UPMP3, a strain isolated from the rhizosphere of an oil palm plantation was found to have potential to become a biocontrol agent against *G. boninense* (Azadeh & Meon, 2009), being able to produce two types of phenazine antibiotics simultaneously in the culture broth. The antibiotics are phenazine (PHZ) and phenazine-1-carboxylic acid (PCA).

Phenazine is a nitrogen-containing heterocyclic antibiotic agent. More than 6000 phenazine derivatives have been identified and described during the last two centuries. Phenazine antibiotics are well-

known in demonstrating toxicity towards various pathogens by having the potent anti-fungal activity for control of economically important plant pathogens. A previous finding indicated that the isolated phenazine antibiotics exhibited antifungal activity on *G. boninense* (Parvin, Othman, Jaafar, & Wong, 2016).

Phenazines can be produced in two ways, namely biosynthesis and chemosynthesis (Cheluvappa, 2014). Biosynthesis, the natural way of antibiotics production, is achieved *via* phenazine-producing bacteria such as *P. aeruginosa* when the bacteria are incubated in a suitable medium and regulated by nutrient depletion. The high cell density converts the bacterium to biofilm form. Chemosynthesis of antibiotics is through serial oxidation of phenazine core (Nansathit, Phaosiri, Pongdontri, Chanthai, & Ruangviriyachai, 2011). In this present study, phenazine compounds were biosynthesized by an antagonistic bacterium, *P. aeruginosa* UPMP3. The objectives of this research were to characterize phenazine antibiotics produced by *P. aeruginosa* UPMP3 and to evaluate the inhibitory effect of the purified phenazines against *G. boninense in vitro*.

MATERIALS AND METHODS

Chemicals and reagents

Phenazine (PHZ) was purchased from Sigma-Aldrich (USA). Phenazine-1-carboxylic acid (PCA) was purchased from Toronto Research Chemicals Inc. (Canada). The purities of these compounds were more than 99%. Acetonitrile was of HPLC grade and obtained from Friedemann Schmidt Chemical (France). Methanol was of HPLC grade and obtained from RCI Labscan Ltd. (Thailand). Benzene was analytical grade and obtained from R & M Chemicals (UK). Water was triple distilled.

Bacterial and Fungal Cultures

The antagonistic rhizobacterium, *Pseudomonas aeruginosa* UPMP3 was obtained from the Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia that was previously isolated from oil palm rhizosphere, and maintained on King's B agar at room temperature as working culture. *G. boninense* was obtained from Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia and maintained on Potato Dextrose Agar (PDA) at room temperature.

Antibiotics Biosynthesis

P. aeruginosa UPMP3 inoculum on agar was sub-cultivated in Luria Bertani (LB) broth at room temperature for three days in an orbital incubator maintained at 250 r.p.m. The inoculum then was centrifuged to remove all bacterial cells and the supernatant was lyophilized in a vacuum evaporator. The resultant powder form of the supernatant was dissolved in 100 ml distilled water followed by compounds extraction. The PCA and PHZ produced simultaneously in the supernatant were extracted using a previous method (Raio et al., 2011). The oily extract was dissolved in 2 ml methanol and purified by fractionation using analytical HPLC.

Antibiotics Fractionation by HPLC

An analytical reverse phase High Performance Liquid Chromatography (HPLC) column equipped with a fraction collector system (Agilent, USA) was used to fractionate the phenazine-compounds present in the extract. The antibiotics were identified according to a modified method previously described by Fernández and Pizarro (1997). A 150 × 4.6 mm Luna 5 µ C18 (2) 100A column and 30 × 4.6 mm Luna 5 µ 100A guard column (Phenomenex, USA) were used. The injection volume was 20 µL. The mobile phase A was water-trifluoroacetic acid (100:0.04, v/v) and mobile phase B was acetonitrile-water-trifluoroacetic acid (90:10:0.04, v/v/v). The flow rate was 1 mL/min. Chromatograms were developed using a Gold programmable solvent module 126 connected to a diode array detector module 168 (Water, USA). Elution was as follows: Solvent A was maintained for 15 min and then changed to 90% A and 10% B. This mixture was used for 10 min. A linear gradient of 70% A and 30% B in 15 min was then applied. These conditions were maintained for 5 min. Finally, the solvent composition was changed to 64% A and 36% B and maintained until the end of the run (65 min from the starting time). The antibiotics in the effluents of mobile phase were collected automatically by the fraction collection system.

Characterization of Purified Antibiotics

Fourier Transform Infrared (FTIR) spectroscopy was used to study the molecular structure of the antibiotics. One milligram dried antibiotics in amorphous powder was compacted with 0.1 g potassium bromide using a hydraulic pressure. For each spectrum, 21 scans between 400-4000 cm^{-1} were produced at a resolution of 4 cm^{-1}. The FTIR measurement was taken on a Perkin-Elmer spectrophotometer.

The purified antibiotics were characterized using spectroscopic techniques. ^{13}C and ^{1}H NMR spectra were obtained using a Varian Nuclear Magnetic Resonance System VNMRS-500 MHz instrument (Varian Inc., USA). Samples of 5 mg were dissolved in $CDCl_3$ and were analyzed in a liquid state NMR, ^{1}H and ^{13}C NMR spectra were recorded at 500 MHz and 125 MHz, respectively. The same solvent was used as the internal standard.

The Scanning Electron Microscopy (SEM) analysis was carried out. For this purpose, the samples were fixed in a sample holder and covered with a gold layer for 3 min using a sputter coater (Cressington 108, JOEL Inc., Japan). Then, the samples were placed in an ETEC autoscan model JSM-6400 scanning electron microscope, SEM (JEOL Inc., Tokyo, Japan).

***In vitro* Antifungal Activity**

The inhibition activity of purified antibiotics was studied. Treatments of different concentrations (100, 200, 400, 800 and 1000 ppm) of PHZ and PCA were prepared. A dilution of aqueous methanol (80:20 v/v), was used as negative control. Poisonous agar method was applied, after incubation at 28°C for 10 days. The diameter of fungal

mycelia was measured to calculate the inhibition rate. This bioassay was performed with five replications. Inhibition rate was measured using the following formulae.

Percentage inhibition = (C-T)/C x 100

Where,

C = colony diameter (cm) of the control plate

T = colony diameter (cm) of the test plate

Statistical Analysis

The results were analyzed using statistical software JMP (9.3) (SAS Institute, Cary, NC). Data were expressed as means ± SD of replicated samples by one-way analysis of variance (ANOVA) using Tukey's honestly significant difference (HSD) test. Differences were considered significant at $p < 0.05$.

RESULTS

Fractionation of Antibiotics

PCA was collected from a single peak with retention time 51.17 min at 245 nm in the HPLC analysis. Similarly, PHZ was obtained from the peak with retention time 51.77 min at 252 nm. The quantification analysis indicated 685.0 mg of PHZ (Figure 1(a)) and 26.9 mg of PCA (Figure 1(b)) per litre of crude extract. The compounds were collected using a fraction collector and dried at 40°C. Approximately 0.05 mg/ml of yellow oily substance were obtained.

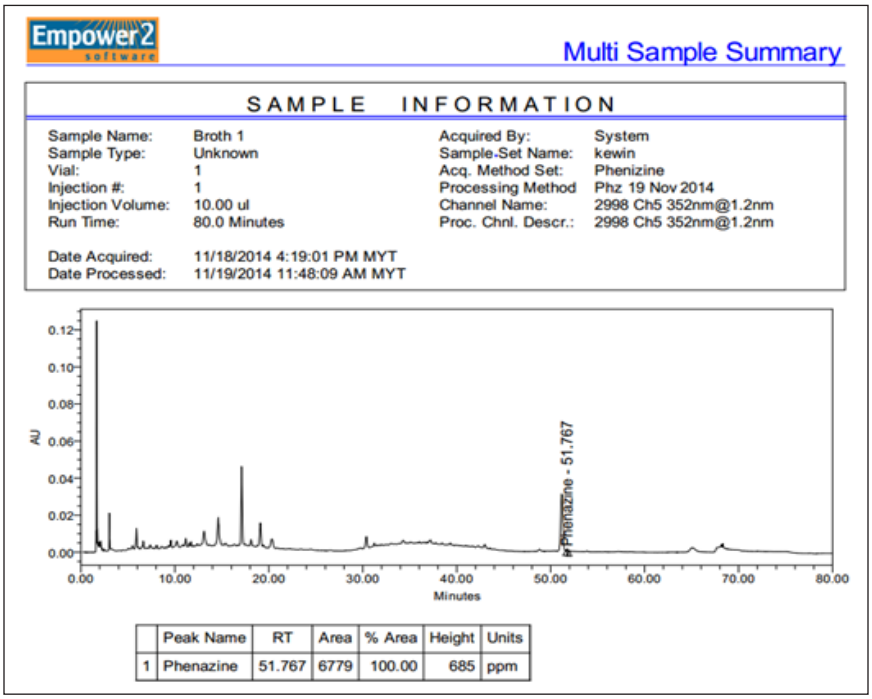


Figure 1(a). HPLC spectrum of PHZ at 252 nm

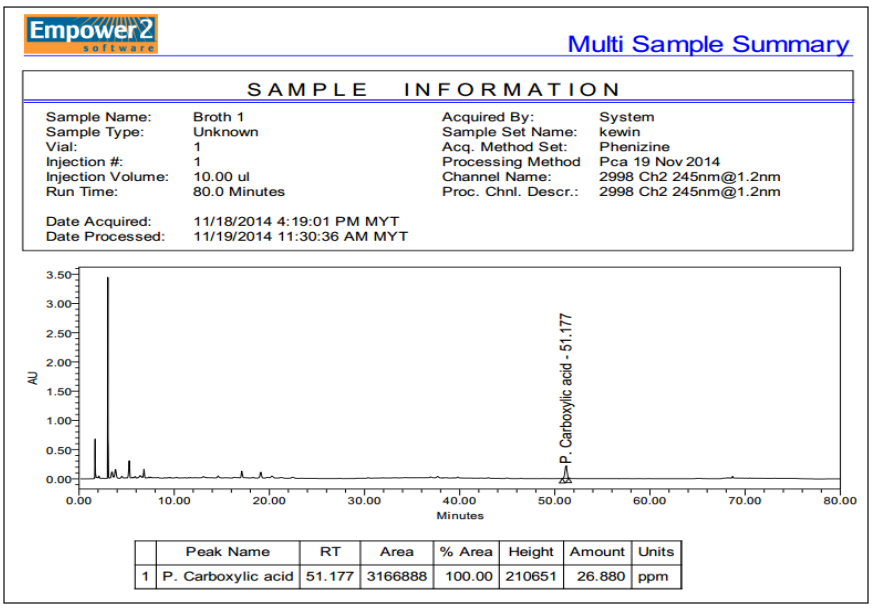


Figure 1(b). HPLC spectrum of PCA at 245 nm

Structure Elucidation of Phenazine

The IR spectrum of purified PHZ as shown in Figure 2(a) exhibited an absorption peak at 1499.23 cm^{-1} for aromatic stretch. Meanwhile, the absorption at 1353.68 cm^{-1} was indicative of an aromatic nitro compound. The absorption bands at 1118.84 cm^{-1} and 729.43 cm^{-1} were from aromatic C-H stretching vibrations.

The IR spectrum of purified PCA as shown in Figure 2(b) exhibited absorption peaks at 1717.92 cm^{-1} which were for the carboxylic acid vibration, 1461.21 cm^{-1} for the aromatic C=C stretch, while 861.78 cm^{-1} and 738.54 cm^{-1} were for aromatic CH stretching vibrations.

The structures of the purified compounds were further confirmed by ^{1}H and ^{13}C NMR spectroscopy. In the ^{1}H NMR spectrum of PHZ (Figure 3(a)) the two doublet peaks at δ7.71 and 8.13 were assigned to the 8 aromatic protons. PHZ being a symmetrical molecule, hence, the 8 aromatic protons only gave 2 overlapped signals in the ^{1}H NMR spectrum. H-2 and H-3 are equivalent to H-7 and H-6 respectively. H-2 and H-3 are coupled to their neighbouring protons H-1 and H-4 respectively resulting in 2 doublets and these signals are at the low field region. Thus, the doublet peak at δ8.13 was assigned to these four protons, H-2, H-3, H-6 and H-7 while the other doublet at δ7.71 was assigned to H-1, H-4, H-5 and H-8. Five well-resolved signals in the ^{13}C NMR spectrum of PHZ (Figure 3(b)) were also observed. The signals at δ143.3, 130.3, and 129.5 consisting of two overlapped peaks each indicated the presence of aromatic carbons in the structure. Looking at the symmetrical characteristic of the PHZ molecule, only 3

carbon signals could be seen in the ^{13}C NMR spectrum. C-5 and C-6 are equivalent to C-8 and C-7 respectively. The lower field carbon signal at 130.3 was hence assigned to C-5 and C-8 while δ129.5 was assigned to C-6 and C-7. Similarly, C-1 being equivalent to C-4 and C-2 to C-3 were assigned peaks at δ130.3 and 129.5 respectively. The

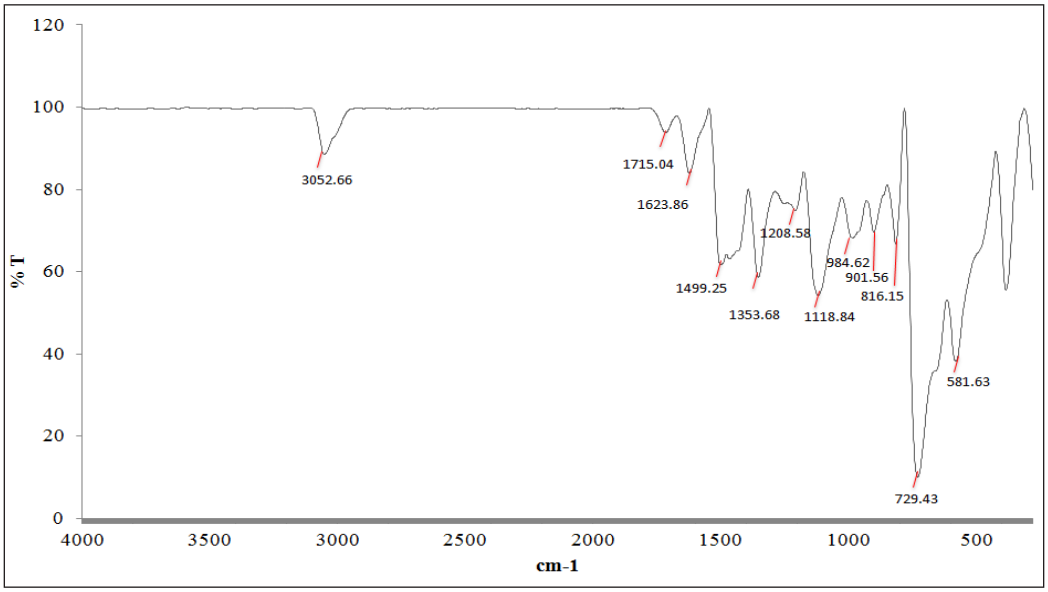


Figure 2(a). FTIR spectrum of PHZ

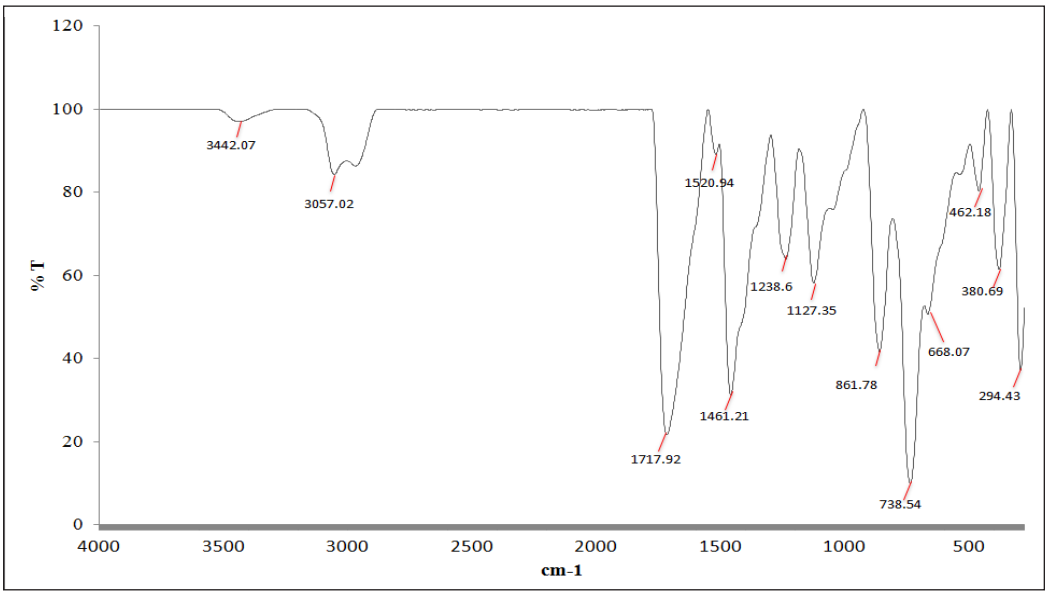


Figure 2(b). FTIR spectrum of PCA

quaternary carbons at position 4(a), and 5(a), 1(a) and 8(a) were duly assigned signals at δ 143.3 since they are all equivalent.

In the ^{1}H NMR spectrum of PCA (Figure 3(c)), seven signals between δ7.77-8.95 were duly assigned to the 8 aromatic protons. The carboxyl proton gave a very low field signal one-proton singlet at δ8.95. The presence of this carboxylic carbon was justified by the ^{13}C NMR signal at δ165.9. The ^{13}C NMR (Figure 3(d)) spectrum also gave 12 signals which agree with the structure of phenazine-1-carboxylic acid. The 4 low field signals at δ143.3, 139.9, 144.0 and 139.7 were assigned to the 4 quaternary carbons C-4a, C-5a, C-8a and C-1a. Seven doublet signals at δ8.50, 8.01, 8.02, 8.50, 8.25, 7.98 and 8.51 were assigned to their respective protons. In Tables 1 and 2, ^{1}H/^{13}C NMR spectral data of purified PHZ and PCA were compared to the previous finding in the literature review to correlate the signals allocation in molecules.

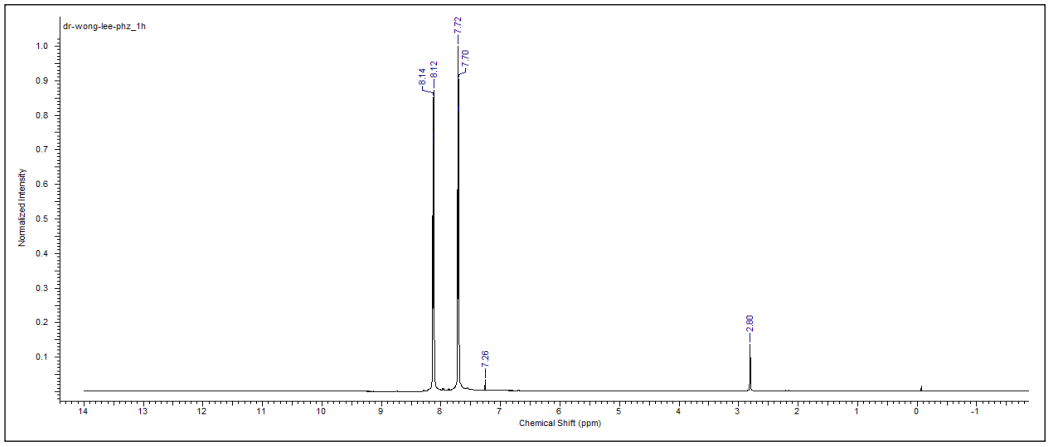


Figure 3(a). PHZ ^{1}H NMR

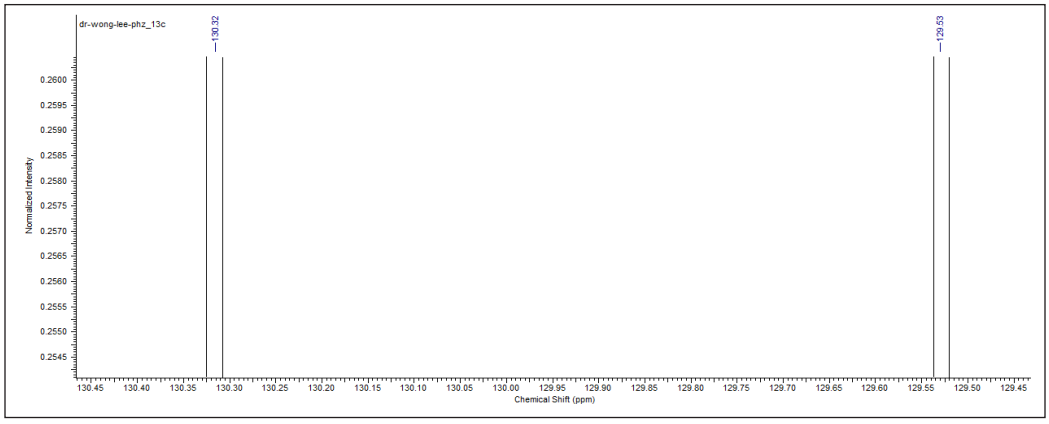


Figure 3(b). PHZ ^{13}C NMR

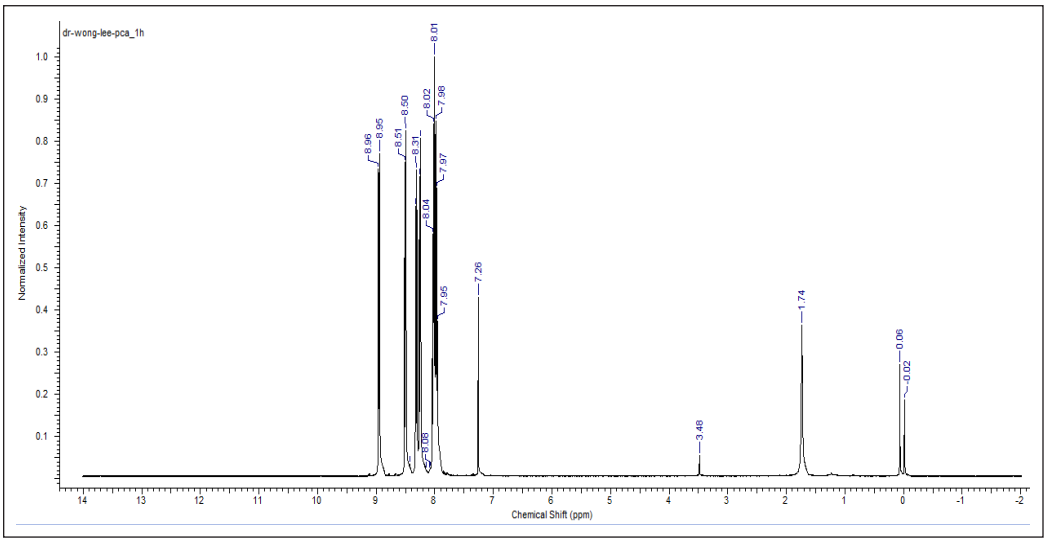


Figure 3(c). PCA ^{1}H NMR

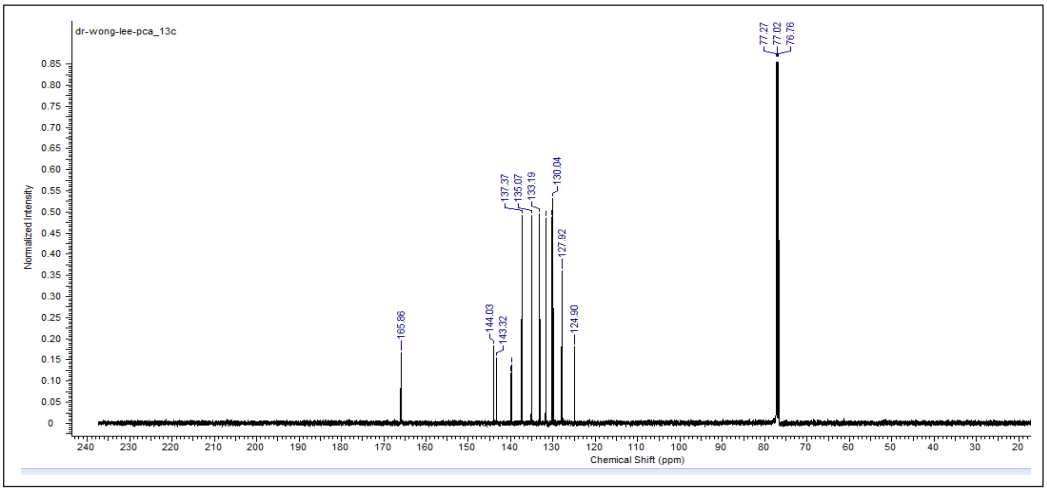


Figure 3(d). PCA ^{13}C NMR

The compounds formed were confirmed by SEM imaging (Figure 4(a)-(b)). The physical structures of purified compounds were demonstrated by electron beams. PHZ and PCA were detected to have crystalline needle-like structure. The PHZ crystalline structure is shown in Figure 4(a) at a magnification of ×7500 and the needle-crystal diameter is less than 1 µm. PCA crystalline structure is best figured at the magnification of ×43 and the size is less than 100 µm (Figure 4(b)).

Table 1
$^{1}H/^{13}C$ NMR spectral data of targeted PHZ purified from Pseudomonas aeruginosa *UPMP3*

Atom position	^{1}H NMR		^{13}C NMR	
	PHZ* (δ)	Purified PHZ (δ)	PHZ** (δ)	Purified PHZ (δ)
1	7.97	7.71, d	130.25	130.3
2	8.26	8.13, d	129.6	129.5
3	8.26	8.13, d	129.6	129.5
4	7.97	7.71, d	130.25	130.3
1a	-	-	143.45	143.3
4a	-	-	143.45	143.3
5a	-	-	143.45	143.3
8a	-	-	143.45	143.3
5	7.97	7.71,d	130.25	130.3
6	8.26	8.13,d	129.6	129.5
7	8.26	8.13,d	129.6	129.5
8	7.97	7.71,d	130.25	130.3

Note. δ in ppm, * (Griesbeck, 2014); ** (Breitmaier & Hollstein, 1976)

Table 2
$^{1}H/^{13}C$ NMR spectral data of targeted PCA purified from Pseudomonas aeruginosa *UPMP3*

Atom position	^{1}H NMR		^{13}C NMR	
	PCA* (δ)	Purified PCA (δ)	PCA* (δ)	Purified PCA (δ)
1	-	-	124,9	127.9
2	8.54	8.51, d	135.1	144.0
3	8.06	8.01, t	130.2	137.3
4	8.99	8.50, d	137.4	135.0
1a	-	-	143.4	144.0
4a	-	-	144.1	143.3
5a	-	-	140.1	139.9
8a	-	-	139.8	139.7
5	8.36	8.50, d	130.1	133.7
6	8.00	7.98, t	131.7	131.7
7	8.02	8.02, t	133.2	130.2
8	8.29	8.51, d	127.9	130.0
COOH	15.61	8.95, s	165.9	165.8

Note. δ in ppm, * (Abraham et al., 2015)

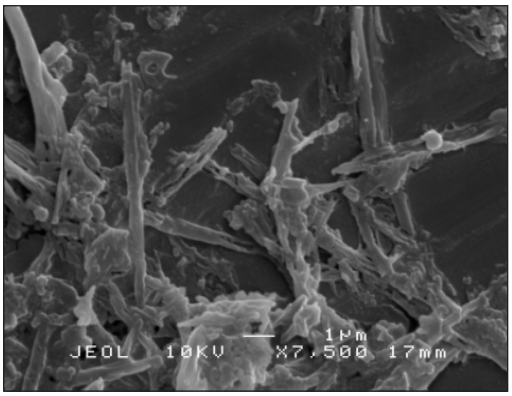


Figure 4(a). Purified PHZ viewed at magnification of × 7500

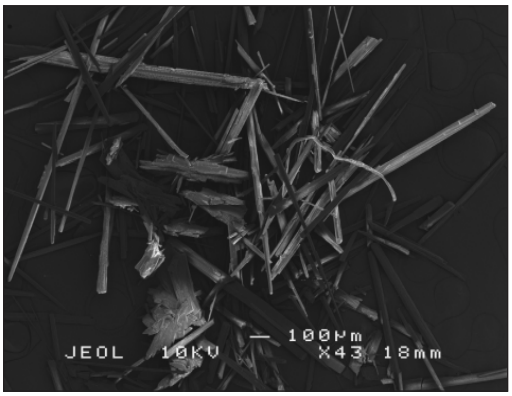


Figure 4(b). Purified PCA viewed at magnification of × 43

In vitro Antifungal Activity

Purified phenazine antibiotics showed antagonistic activity against *G. boninense*. The percentage inhibition of *G. boninense* mycelial growth increased with increasing concentration of purified compounds. Figure 5 indicates that PHZ has a better inhibition rate than PCA. PHZ inhibited 100% mycelial growth of *G. boninense* at 1000 ppm and at 800 ppm the inhibition rate was 92.31% which was more effective than PCA at 1000 ppm.

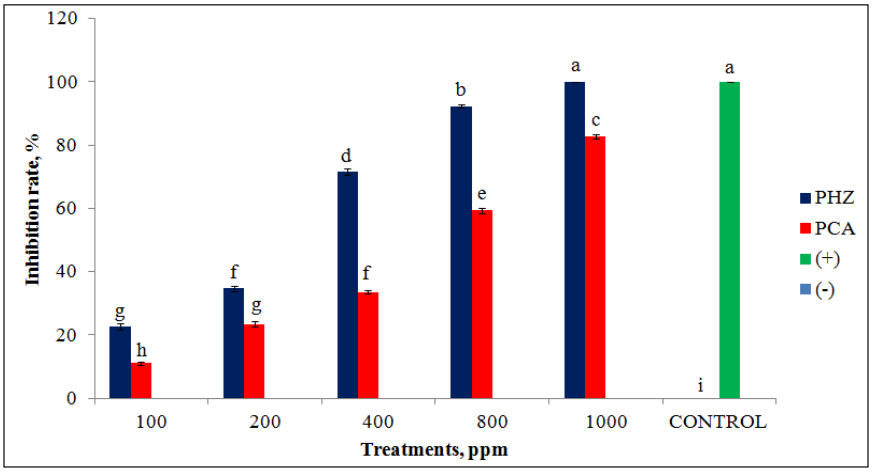


Figure 5. *In vitro* antifungal activities of PHZ and PCA at 100, 200, 400, 800 and 1000 ppm on *Ganoderma boninense* at Day 14

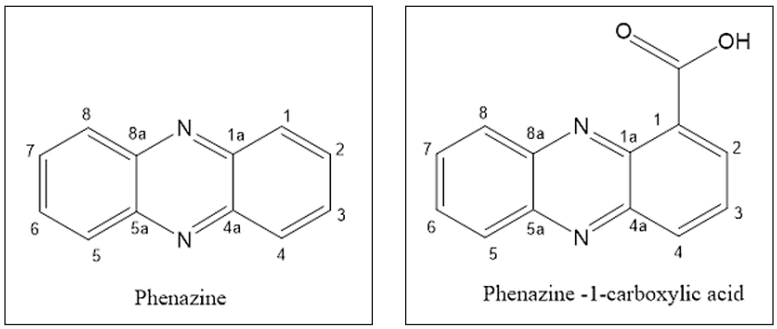
DISCUSSION

Disease caused by the pathogenic fungus *G. boninense* is the greatest threat to the oil palm industry. Increasing concerns over environmental problems due to chemical applications and labour safety supported the need for alternative pathogen control methods. Investigations on microbial

antibiotics are gaining greater momentum in the agrochemical industry as a source for the development of new products. Fungicides of microbial origin have been demonstrated to be not only specifically effective on the target organisms but also inherently biodegradable. Beneficial rhizobacteria have recently been a focus of interest as biocontrol agents and a source of bioactive metabolites (Strobel, Daisy, Castillo, & Harper, 2004). *P. aeruginosa* UPMP3 is indigenous to oil palm plantation rhizosphere, colonizing the tissue locally and systemically. In this study, *P. aeruginosa* UPMP3 was used to produce phenazine antibiotics and these compounds were proven to be the key antagonistic compounds that exhibited maximum inhibition to the growth of *G. boninense*. These compounds were extracted and purified through HPLC downstream processes (Abraham et al., 2015).

Phenazine compounds are structurally identical, which have 3 fused aromatic rings with conjugated dienes and two C-N bonds such as PHZ ($C_{12}H_8N_2$, Figure 6(a)) while PCA ($C_{13}H_8N_2O_2$, Figure 6(b)) has a molecular structure almost identical to PHZ but with a carboxylic side chain (Abraham et al., 2015). Diene is a hydrocarbon that contains two carbon double bonds. The counts of proton and carbon from ^{1}H to ^{13}C NMR spectra revealed the structure of protons and carbons (Figures 3(a)-(b)) which supported the molecular formula of phenazine antibiotics.

The phenazine antibiotics share similarities of having aromatic nitro compounds and several aromatic rings. From the FTIR spectrum of each compound, the presence of aromatic nitro compound was confirmed by the absorption peaks either between 1555 and 1484 cm^{-1} or 1355 and 1320 cm^{-1}. Aromatic C-H was confirmed by the absorption peaks between 1225 and 950 cm^{-1}. C=C-C aromatic ring stretch has peaks between 1510 and 1450 cm^{-1} while aromatic CH vibration have peaks of 900-670 cm^{-1}. The presence of a carboxylic acid moiety in PCA was confirmed by the absorption at 1717.92 cm^{-1}, a carbonyl compound group frequency for carboxylic acid should occur between 1725 and 1700 cm^{-1} (Coates, 2000).



(a) (b)

Figure 6. Structures of (a) phenazine and (b) phenazine-1-carboxylic acid

The indication of peaks in the compound elucidation in FTIR confirmed the presence of functional groups and structure of phenazine compounds.

Phenazines have been recognized for their antibiotic properties for centuries. They inhibit a wide range of plant pathogenic fungi and have a well-characterized mechanism of bacterial plant disease control (Bloemberg & Lugtenberg, 2003). In a medical aspect, pyocyanin caused ciliostasis with epithelial disruption. 1-hydroxyphenazine causes dyskinesia (Wilson et al., 1987) and lung infection of cystic fibrosis. The research showed that pyocyanin could react with glutathione to produce the toxin (Cheluvappa, Shimmon, Dawson, Hilmer, & Le Couteur, 2008). Despite the fact that phenazine antibiotics have the potential to cause infections in human, they are still studied for applications in plant protection.

In 1954, *Pseudomonas aeruginosa* was studied for its antibiotic production of streptomycin and dihydrostreptomycin, two compounds used against *Escherichia coli* (Lightbown, 1954). The pattern of phenazine pigment production by *P. aeruginosa* was studied and the result showed that PCA, pyocyanin, chloraraphin and oxychlororaphin were produced (Kanner, Gerber, & Bartha, 1978). PHZ and pyoluteorin were produced by *Pseudomonas* isolated from green pepper rhizosphere (Liu, Dong, Peng, Zhang, & Xu, 2006). *Pseudomonas aurantiaca* was able to produce HCN, siderophores and homoserine lactones that are antifungal to *Colletotrichum falcatum, Fusarium oxysporium* and *F. lateritium*, important pathogens of sugarcane (Mehnaz, Baig, Jamil, Weselowski, & Lazarovits, 2009). *P. chlororaphis* exhibited biocontrol of cypress canker, caused by the fungi *Lepteutypa cupressi* and *Seiridium unicorne* (Raio et al., 2011). *Pseudomonas* spp. such as *P. borealis, P. chlororaphis, P. fluorescens, P. mandelii, P. marginalis, P. poae, P. putida, P. syringae* and *P. vranovensis* were evaluated for their antifungal activities against root rot of wheat caused by *Rhizoctonia* sp. and *Pythium* sp. (Mavrodi, Walter, Elateek, Taylor, & Okubara, 2012). *P. chlororaphis* strain HT66 was able to inhibit various pathogens by producing antibiotics such as 2-OH-PHZ and PCA. These compounds also possessed potent insecticidal activity (Chen et al., 2015). PCA also showed antagonistic activity against oomycete pathogen *Phytophthora meadii* (Abraham et al., 2015). However, no phenazine derivatives have so far been reported as the active compounds demonstrating antibiosis against *G. boninense*.

The present study reported that PCA gave less inhibition compared to PHZ at the same concentration (Figure 5).

CONCLUSION

Based on the findings, it was concluded that *Pseudomonas aeruginosa* UPMP3 produces phenazine (PHZ) and phenazine-1-carboxylic acid (PCA) as secondary metabolites in the culture broth. Both antibiotics were detected simultaneously under the same HPLC conditions. Characterization of the structure of both

antibiotics by FTIR and NMR supported HPLC identification. Though both PHZ and PCA demonstrated effective inhibition (92-100%) of *G. boninense* mycelial growth *in vitro*, PCA showed lesser inhibition compared to PHZ at the same concentration (1000 ppm). As far as we know, this is the first study where purified phenazine antibiotics isolated from *P. aeruginosa* UPMP3 were structurally characterised and tested to have positive antagonism against *G. boninense*. The results of the present study suggest that phenazine antibiotics isolated from *P. aeruginosa* UPMP3 have the potential to be developed into antifungal formulations in future.

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Impact Assessment of Organic Farming on Soil Nutrients and Heavy Metal Content

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ABSTRACT

Environmental sustainability of recent adoption of organic farming as a wholesome practice in sub-Saharan Africa needs to be investigated, in order to guide agricultural policy makers. Consequently, an assessment of soil chemical properties was done in an organic farm with different land uses. The experiment was 5 × 3 factorial design (five land uses and three soil depths) replicated temporally (two contrasting seasons) and spatially (four replications). Samples collected systematically at 0 - 20, 20 - 40 and 40 - 60 cm depths were analysed for pH, organic carbon, nutrients and heavy metals. Results indicated that manure application limed the soil. Soil pH ranged from 5.1 to 6.3 in grass-land and 5.8 to 6.4 in amaranth farm during dry and wet seasons, respectively. Generally, nutrients and heavy metals were concentrated at 0-20 cm depth. The effect of season on the parameters was erratic. Amounts of mobile, exchangeable and labile Cu were 0.13 - 0.19, 0.07 - 0.15 and 1.39 - 1.74 mg kg^{-1}, respectively while water soluble and mobile fraction Zn ranged from 0.15 - 0.20 and 0.29 – 0.62 mg kg^{-1}. Comparatively, all the metals labile pool was most abundant, while Pb was the most abundant metal. There was no evidence of heavy metal accumulation in the organic system.

Keywords: Heavy metals, land use, organic farming, soil nutrients

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INTRODUCTION

The intensive use of inorganic fertilizers for boosting crop productivity has been linked to rapid decline in soil fertility in the tropics (Babajide, Olabode, Akanbi, Olatunji, & Ewetola, 2008) and also environmental pollution (Dada, Togun, Adediran, &

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Nwilene, 2014). Hence, farm management practices that do not use inorganic fertilizers are being adopted. Organic farming is a management system that maintains soil fertility and at the same time minimizes all form of pollution that might result from agricultural practices. Sustainable farming systems such as organic farming are seen as potential solution to the continued loss of biodiversity and sustainable healthy food production (Muller et al., 2012). The degree to which this is achievable in the African farming system is yet to be widely demonstrated through research.

Land application of farmyard manure is a common practice in many parts of the world as a means of recycling plant nutrients in crop production as well as a method of waste disposal (Kirchmann & Thorvaldsson, 2000; Kumar, Gupta, Baidoo, Chander, & Rosen, 2005). The intensification of animal husbandry has led to the use of feed additives such as heavy metals and veterinary antibiotics to control animal diseases in all classes of livestock including poultry (Chen, Zhang, Luo, & Song, 2012). Most heavy metals are added to animal feeds to prevent disease and increase weight gain and feed conversion, but they are largely expelled in the faeces and urine (Bolan, Adriano, & Mahimairaja, 2004). Nicholson, Smith, Alloway, Carlton-Smith and Chambers (2003) observed that more than 90% of the Cu in animal diets was lost in the faeces. Hence, manures have become an important source of heavy metals application to agricultural land, accounting for approximately 40% of total annual inputs of Zn and Cu and 11% of Cd inputs (Veeken & Hamelers, 2002). The possible environmental consequences of applying livestock manures containing inorganic additives and the residues of organic contaminants derived from animal feedstuffs (Hu, Zhou, & Luo, 2010; Knapp, Dolfing, Ehlert, & Graham, 2010; Lopes, Herva, Franco-Uria, & Roca, 2011) is however, yet to be given the needed full research attention.

Recent global advocacy on the promotion of organic farming has led to the application of livestock manure to soil. The manure contains a significant input of nutrients but also of metals, some of them being toxic e.g. cadmium and lead (Madrid, López, & Cabrera, 2007). Heavy metals in soils may affect the stability and productivity of soil ecosystems, harm plant growth, influence the safety of the human food chain and promote the emergence of micro-organisms with antibiotic resistance genes (Berenguer, Cela, Santiveri, Boixadera, & Lloveras, 2008; Chee-Sanford et al., 2009).

Many researches in sub-Saharan Africa, on the practice of organic farming has centred on crop responses to organic amendments and few has evaluated the net effect of the organic practices on the soil health and quality, at least in the sub Saharan African region. Hence, the justification to hypothesise that organic manures are the single major source of heavy metals to soil under organic farming.

Consequently, the objectives of the study were to:

1. Assess the effect of different agricultural land use types under organic farming on some soil chemical properties and soil copper, lead and zinc fractions.
2. Determine the depth distribution of soil nutrients and heavy metal fractions under different land uses in organic farming.

MATERIALS AND METHODS

Site Description

The experiment was conducted at the organic agriculture skill development plot, Federal University of Agriculture, Abeokuta. This project site is located in the transition zone between tropical sub-humid and savannah climate, characterized by distinct wet and dry seasons (Bimodal rainfall distribution). The wet season begins in April and continues through October. This area has mean annual rainfall of about 1400 mm, mean annual temperature of about 22.2°C and mean annual maximum temperature of about 33.3°C. The study site lies between latitude 7^{o} 12$^{'}$ to 7^{o} 20$^{'}$ N and longitude 3^{o} 20$^{'}$ to 3^{o}28$^{'}$.

History of the Site

The organic farm was established in the year 2007, weed control is mostly done manually, the farming practice involves the use of organic fertilizers like compost, manures and excludes synthetic agrochemicals, fertilizers or any inorganic pesticides. The organic materials mostly used are cured poultry manure and compost (made from poultry manure or cow dung, plant debris, and kitchen waste); the pests are controlled by applying organic bio-pesticides. The soil in the organic farm is classified as Hydraaquentic Humaquent. The details of the fields used for the experiment is presented in Table 1. The farm is partitioned into different land uses as seen in the Table 1. Annual application of 10 tonnes ha^{-1} organic amendments was done to each of the land use, while mono-cropping was done on the plots.

Experimental Design

The experiment was 5 × 3 factorial arrangement repeated in two seasons (dry and rainy seasons) and replicated four times. The factorial experiment consists of five (5) land uses and three (3) soil depths. The five land uses are control, amaranth cropland, celosia cropland, pineapple plantation, plantain plantation while the three soil depths are 0-20, 20-40 and 40-60 cm. The total experimental units are: 5 × 3 × 2 × 4, representing land use, soil depth, seasons and replications. This gives a total of 120 experimental units

Table 1
History of fields used for the experiment

Crop farm	Year under Organic farm	Plot size	Type of manure applied & rate
Vegetable 1 (Amaranth)cropland	3	3 m × 3 m	*Compost at 10 tonnes per hectare
Vegetable 2 (Celosia) cropland	3	3 m × 3 m	Compost at 10 tonnes per hectare
Pineapple plantation	5	3 m × 4 m	**Poultry manure at 10 tonnes per hectare
Plantain plantation	5	3 m × 4 m	Poultry manure at 10 tonnes per hectare
Grassland soils	>8 (control)	4 m × 4 m	None

*Made from poultry manure or cow dung, plant debris, and kitchen waste
**Typically, the manure metal composition is variable but may contain an average of 48, 52.5, 28 and 2.15 mg kg^{-1} of Cu, Zn, Cd and Pb, respectively.

Soil Sampling

Soil samples were collected with the aid of a soil auger from the land use types. Four replicate samples were collected on each land use type. Samples were randomly and systematically collected during dry (December 2014 to February 2015) and rainy (May-July, 2015) seasons at sampling depths of 0-20 cm, 20-40 cm and 40-60 cm. Collected composite soil samples were air-dried passed through a 2 mm sieve and labelled for analyses.

Soil Analyses

Soil pH was determined in 1:2 soil: water suspension (McLean, 1982). Organic carbon was determined using wet oxidation method (Walkley & Black, 1934). Particle size analysis was determined using the hydrometer method (Bouyoucos, 1962). Available phosphorus was determined using Bray 1 method (Bray & Kurtz, 1945). Total Nitrogen was determined by micro Kjeldahl digestion method as described by Bremmer and Mulvaney (1982). The exchangeable bases were extracted using neutral ammonium acetate. The exchangeable Na and K in the filtrates were determined by Flame photometer while the exchangeable Ca and Mg were determined by atomic absorption spectrophotometer. The Effective Cation Exchange Capacity (ECEC) was expressed as the summation of exchangeable cation and exchangeable acidity (Rhoades 1982). Exchangeable acidity was evaluated titrimetrically (Mclean, 1982).

Soil heavy metal fractions were determined according to Akpa and Agbenin (2012) as follows:

1. Mobile fractions: 10 g of air dried 2 mm sieved soil was put in a well labelled sample bottles then 30 ml of 1.0M ammonium nitrate (NH_4NO_3) solution was added.

2. Exchangeable fractions: 10 g of sieved soil was put in a sample bottle then 30 ml of 0.1M calcium chloride ($CaCl_2$) solution was added.
3. Labile fractions: 10 g of well sieved soil (2 mm) was put in a sample bottle then 30 ml of 0.05M etylenediaminetetraacetic acid (EDTA) was added.
4. Water soluble fractions: 10 g of the sieved soil was put in different sample bottle then 30 ml of deionized water was added.

For each fraction, soil + solution mixture was shaken for 2 hrs, centrifuged for 10 minutes then filtered using Whatmann No 42 filter paper to give a clear filtrate. The Zn, Cu and Pb in each filtrate from each fraction were determined using atomic absorption spectrophotometer (AAS). The detection limit for lead, zinc and copper are 0.05, 0.003, 0.003 mg kg^{-1} respectively.

Statistical Analysis

Data collected were subjected to Analysis of Variance at 5% probability level and mean values were separated using Least Significant Difference (GENSTAT). The data obtained were also subjected to Pearson correlation analysis to measure the relationship.

RESULTS

Agricultural land use had highly significant effect at $p \leq 0.05$ on soil pH as shown in Table 2. Amaranth cropland had the highest pH followed by celosia cropland, plantain farm, pineapple farm, while control (grassland) had the least pH. Soil depth also had significant effect on soil pH ($p \leq 0.05$) under different agricultural land uses. Soil depth at 0-20 cm had highest soil pH but not significantly different from the pH at 20-40 cm depth while 40-60 cm had the least soil pH as shown in the Table 2. Season effect was also highly significant on pH ($p \leq 0.01$). The wet season produced significantly higher pH than dry season.

Land use had a highly significant ($p \leq 0.05$) effect on available P. Celosia and amaranth croplands produced significantly higher available phosphorus than other land use types, celosia farm land and control plot had the highest and lowest available P, respectively. Soil depth had significant effect on available P, soil depth at 0-20 cm had the highest available P (17.22 mg kg^{-1}) while the least value was recorded at soil depth of 40-60 cm (7.47 mg kg^{-1}). Available P decreased significantly with increasing soil depths.

The effect of agricultural land use also shown in Table 2, showed a significant effect on soil organic matter ($P \leq 0.05$). Plantain farm had the highest soil organic matter content (14.15 g kg^{-1}) which was significantly different from other agricultural land use types while control had the least value (9.49 g kg^{-1}). Soil organic matter content decreased down the depth with 0-20 cm soil depth having the highest value (17.18 g kg^{-1}) and the least value (8.44 g kg^{-1}) was observed at depth 40-60 cm. There was also significant interaction of the three factors on some of the parameters.

The effects of land use on soil nitrogen content, some exchangeable cations and effective cation exchange capacity are presented in Table 3. Across soil depths and seasons, total nitrogen, exchangeable calcium, Mg and ECEC did not differ significantly ($p < 0.05$) among the different land uses. Land use types had highly significant ($p < 0.05$) effect on both exchangeable Na and K contents. Amaranth and celosia croplands produced significantly higher values (0.54, 0.48 cmol kg^{-1} and 0.31, 0.27 cmol kg^{-1}, respectively) compared with the control which had the least values (0.23 and 0.10 cmol kg^{-1}) of exchangeable Na and K, respectively. Although, the effect was not significant, calcium was the highest among the exchangeable bases followed by Mg, then Na and K. There were higher values in celosia cropland and plantain farm for almost all nutrients except in exchangeable K and Na. Soil depth had significant ($p < 0.05$) effect on soil nutrients under different agricultural land uses. Nutrients are more concentrated at depth of 0-20 cm. The values were significantly reduced at the other depths. Total N and exchangeable cations decreases down the depth, however, the trend shown in Mg was different. No significant difference was recorded in ECEC at different soil depths. Total N, Na, Mg and ECEC varied significantly with season. It was also observed that wet season had significantly higher total N and Mg contents compared with the dry season while there was significant reduction in the ECEC value and exchangeable Na content in dry season and rainy season.

Table 2
The effects of different agricultural land use and soil depth on soil pH, available phosphorus and organic matter in wet and dry seasons

Treatments	pH 1:2 (H_2O)	Avail P (mg kg^{-1})	SOM (g kg^{-1})
Land use (L)			
Control	5.43	6.10	9.49
Pineapple plantation	6.13	8.75	10.14
Plantain plantation	6.16	6.84	14.15
Amaranth cropland	6.36	14.91	12.84
Celosia cropland	6.19	20.34	10.78
LSD (5 %)	0.15**	4.18**	2.32**
Depth (cm) (D)			
0-20	6.18	17.22	17.18
20-40	6.12	9.48	8.82
40-60	5.86	7.47	8.44
LSD (5 %)	0.12**	3.24**	1.80**
Season (S)			
Dry	5.85	11.55	12.00
Wet	6.26	11.23	10.95

Table 2 *(continue)*

Treatments	pH 1:2 (H_2O)	Avail P (mg kg^{-1})	SOM (g kg^{-1})
Season (S)			
LSD (5 %)	0.10**	NS	NS
L × D	0.265**	7.24**	NS
L × S	0.22*	5.91**	3.28**
L × S	0.22*	NS	NS
L × D × S	0.37*	NS	5.68**

*Significant at 5 %. ** Highly significant at 1%. NS means not significant. LSD represent least significant different. SOM signifies soil organic matter

Table 3
Effects of land use and soil depth on total nitrogen, soil exchangeable cations and effective cation exchange capacity in wet and dry seasons

	N	Ca	K	Na	Mg	ECEC
	(g kg^{-1})	----------(cmol kg^{-1})----------				
Land use (L)						
Control	0.55	12.13	0.10	0.23	0.99	23.60
Pineapple plantation	0.42	10.66	0.13	0.28	0.97	28.40
plantain plantation	0.58	12.66	0.20	0.39	1.23	21.10
Amaranth cropland	0.58	11.88	0.31	0.54	1.14	27.60
Celosia cropland	0.68	12.50	0.27	0.48	1.20	18.80
LSD 5%	NS	NS	0.05**	0.08**	NS	NS
Depth (cm) (D)						
0-20	0.74	13.84	0.25	0.45	1.35	28.00
20-40	0.55	13.07	0.18	0.36	0.97	23.10
40-60	0.40	8.98	0.18	0.34	0.99	20.60
LSD 5%	0.19**	1.90**	0.04**	0.06**	0.17**	NS
Season (S)						
Dry	0.40	12.47	0.21	0.43	0.98	34.40
Wet	0.72	11.46	0.20	0.34	1.22	13.40
LSD 5%	0.15**	NS	NS	0.05**	0.14**	6.10**
L × D	NS	NS	NS	NS	ns	NS
L × S	NS	NS	0.07**	0.12**	0.31**	NS
D × S	NS	2.69**	NS	0.09*	NS	NS
L × D × S	NS	NS	NS	NS	NS	NS

*Significant at 5 %. ** Highly significant at 5 %. NS means not significant. LSD represent least significant difference

Agricultural land use had no significant effect ($p < 0.05$) on water soluble Cu and Pb (Table 4). However, effect of land use was significant on water soluble Zn, the highest mean value (0.20 mg kg^{-1}) was recorded in celosia crop land while lowest value (0.15 mg kg^{-1}) was recorded in amaranthus crop land. Soil depth had no significant effect on water soluble heavy metals (Table 4). Seasonal variation had significant influence on water soluble Cu and Zn; while water soluble Cu reduced in value from dry to wet season (0.04 – 0.03 mg kg^{-1}), water soluble Zn increased from dry to wet (0.15 – 0.19 mg kg^{-1}). Season had no effect on water soluble Pb.

Land use had a significant effect ($p < 0.05$) on mobile Cu, Pb and Zn as observed in Table 4. Control and pineapple plantation had significantly higher value of mobile Cu than other land uses; there were no significant differences in the effect of soil depth but decreases were observed in values down the depth. Wet season soil had significantly higher mobile Cu (0.22 mg kg^{-1}) than dry season soil (0.10 mg kg^{-1}). There were no significant differences in the amount of mobile Pb in control, pineapple farm, amaranth and celosia croplands which differ significantly from plantain plantation. No significant difference was observed at different soil depths, there was seasonal variation in amount of mobile Pb in which wet season soil had higher amount (1.48 mg kg^{-1}) than dry season soil (0.10 mg kg^{-1}).

The different organic land use, soil depth and cropping season had highly significant effect ($p < 0.01$) on mobile Zn. Control plot differed significantly from other agricultural land uses, whereas the lowest value was recorded in pineapple and plantain farm. Soil depth at 0-20 cm had significantly higher value of mobile Zn compared to the other depths. The mobile Zn value in rainy season was higher than that of dry season.

The effect of land uses and soil depths shown in Figure 1 had highly significant effect ($p < 0.01$) on water soluble Cu. The water soluble Cu content in the soil as shown in the interaction between land use and depth ranged from 0.01-0.08 mg kg^{-1}. At depth 40-60 cm, pineapple farm had the highest value, compared with other agricultural land uses, while the least value was obtained in control, amaranth cropland and celosia cropland at depth 40-60 cm and 0-20 cm, respectively. The seasonal changes in water soluble Cu content based on different agricultural land use is presented in Figure 2. Water soluble Cu differs significantly under the different land use types during dry season. Amaranth and Celosia croplands had highest and lowest values (0.062, 0.021 mg kg^{-1}), respectively, Pineapple and plantain farm lands had significantly higher water soluble Cu than other land use types that but no significant differences observed between dry and rainy season. Soil depth had a varying effect on water soluble Cu (Figure 3). There were no significant differences in values of water soluble Cu at the 0-20 and 20-40 cm soil depths ~~at~~ in both seasons, depth of 40-60 cm produced significantly highest amount of water soluble Cu during rainy season than in the other depths while depth of 20-40 cm had the least value during the dry season, soil depth of 20-40 cm was higher than 40-60 cm depth which had the least value.

Table 4
Effect of different land uses, soil depths and cropping season on water soluble heavy metals

	Water soluble			Mobile heavy metals		
	Cu	Pb	Zn	Cu	Pb	Zn
	mg kg^{-1}					
Land use (L)						
Control	0.03	0.14	0.17	0.19	0.97	0.62
Pineapple plantation	0.04	0.14	0.19	0.19	0.87	0.29
Plantain plantation	0.04	0.15	0.16	0.14	0.44	0.29
Amaranth cropland	0.04	0.14	0.15	0.13	0.91	0.44
Celosia cropland	0.02	0.14	0.20	0.14	0.96	0.42
LSD	NS	NS	0.04*	0.05*	0.28**	0.19**
Depth (cm) (D)						
0-20	0.03	0.14	0.17	0.17	0.81	0.69
20-40	0.03	0.14	0.19	0.16	0.90	0.29
40-60	0.03	0.14	0.16	0.15	0.79	0.25
LSD	NS	NS	NS	NS	NS	0.15**
Season (S)						
Dry	0.04	0.14	0.15	0.10	0.18	0.27
Wet	0.03	0.14	0.19	0.22	1.48	0.56
LSD	0.01*	NS	0.03*	0.03**	0.175**	0.12**
L × D	0.03**	NS	ns	0.09*	NS	0.33*
L × S	0.03*	NS	0.06*	Ns	0.39**	NS
D× S	0.02**	NS	NS	Ns	NS	NS
L×D×S	NS	NS	0.05**	0.13*	NS	0.47*

*Significant at 5 %. ** Highly significant at 1%. NS means not significant. LSD represent least significant difference.

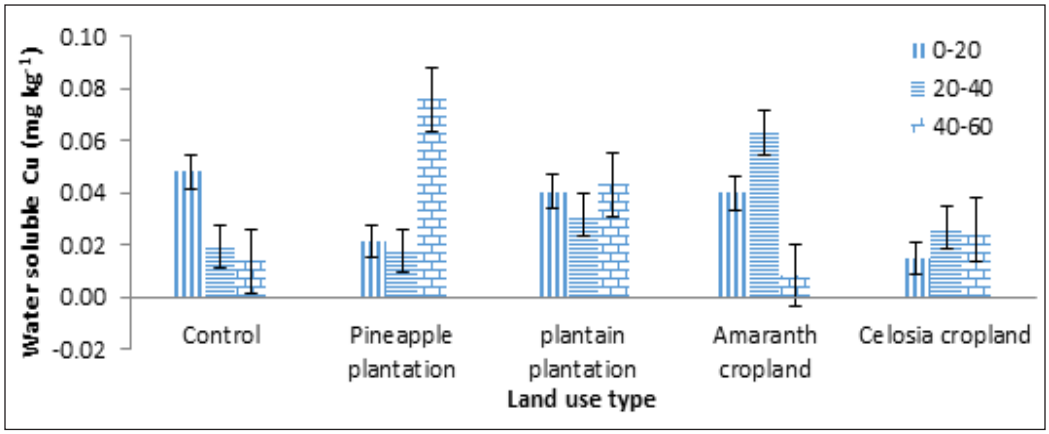

Figure 1. Effect of land uses and soil depths on water soluble Cu; vertical bars indicate standard errors of the mean

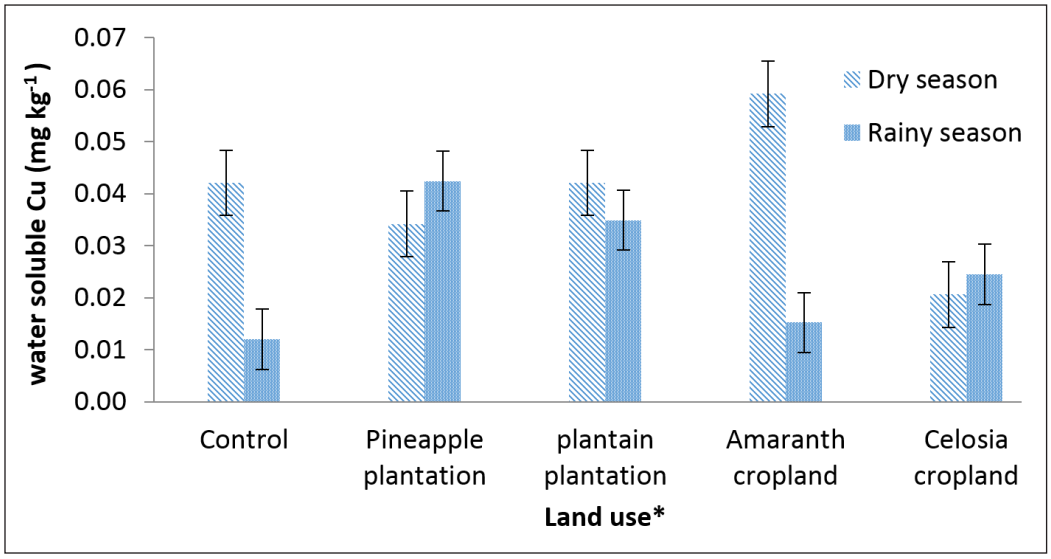

Figure 2. Seasonal water soluble Cu as affected by agricultural land uses
Note: *vertical bars indicate standard errors of the mean

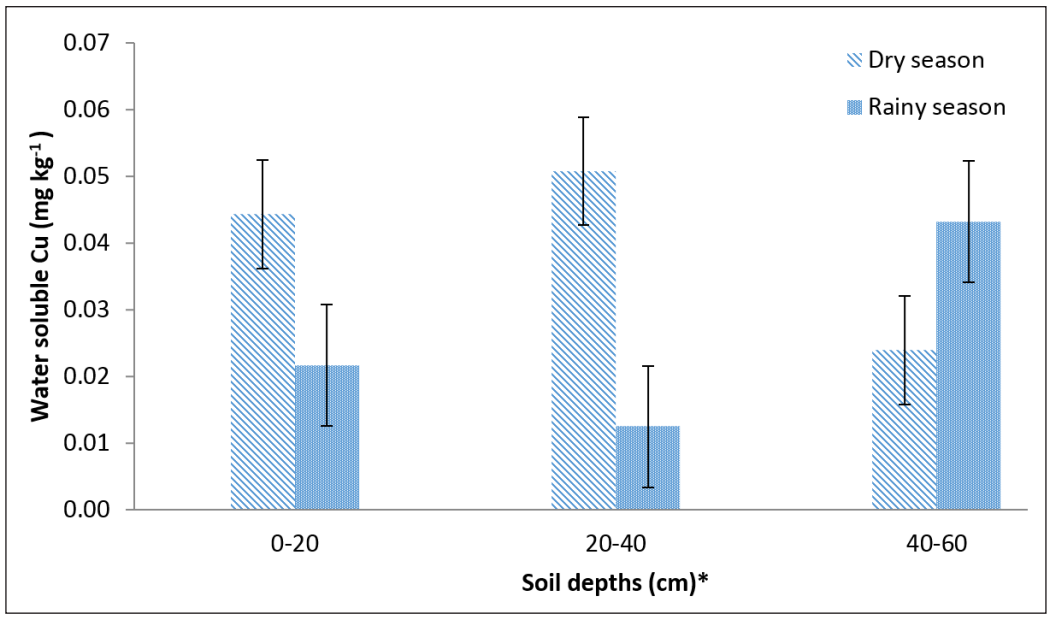

Figure 3. Interaction between soil depth and seasonal changes on water soluble Cu
Note: *vertical bars indicate standard errors of the mean

The effect of different organic land uses, soil depth and seasonal variations on exchangeable heavy metals is presented in Table 5. Organic land use had significant effect ($p < 0.05$) on exchangeable fraction of Cu which ranged from 0.07 - 0.15 mg kg^{-1}. Plantain had the highest value while celosia had the least. The effect of soil depths

Table 5
The Effect of different organic land uses, soil depths, seasonal variations on exchangeable heavy metals

	Exchangeable metals			Labile metals		
	Cu	Pb	Zn	Cu	Pb	Zn
	---------- mg kg^{-1} ----------					
Land uses (L)						
Control	0.10	0.58	0.37	1.74	2.03	2.04
Pineapple plantation	0.11	0.78	0.21	1.59	3.35	1.97
plantain plantation	0.15	0.69	0.19	1.71	2.93	1.85
Amaranth cropland	0.11	1.00	0.35	1.39	3.75	2.27
Celosia cropland	0.07	1.62	0.37	1.39	3.95	2.35
LSD	0.03**	0.66*	NS	0.28*	0.49**	NS
Depth (cm) (D)						
0-20	0.11	0.82	0.33	1.77	3.12	2.90
20-40	0.10	0.90	0.29	1.37	2.89	1.79
40-60	0.12	1.09	0.27	1.54	3.59	1.59
LSD	NS	NS	NS	0.21**	0.38**	0.35**
Season (S)						
Dry	0.11	1.30	0.16	1.61	3.82	1.86
Wet	0.11	0.57	0.43	1.51	2.58	2.33
LSD	NS	0.41**	0.17**	NS	0.312**	0.28**
L × D	NS	NS	NS	0.48*	0.86**	0.77**
L × S	0.05**	NS	NS	0.39*	0.70*	NS
D × S	NS	NS	NS	NS	NS	0.49**
L × D × S	NS	NS	NS	NS	NS	NS

*Significant at 5 %. ** Highly significant at 1%; ns means not significant. LSD represent least significant different.

and cropping seasons were not significant on the exchangeable Cu. Exchangeable Pb was significantly ($p < 0.05$) affected by the ~~on~~ different organic land uses and cropping seasons but soil depth did not show any significant effect on Pb. Celosia cropland had the highest exchangeable Pb value (1.62 mg kg^{-1}) compared to plantain farm with the least value (0.69 mg kg^{-1}). Dry season produced higher exchangeable fraction of Cu which differs significantly from the value obtained in rainy season. The different agricultural land uses and soil depths had no significant ($p < 0.05$) effect on Zn but cropping season had highly significant effect on exchangeable Zn. Wet season highly significantly increased soil exchangeable Zn than dry season. As shown in Table 5, there was a significant effect ($p < 0.05$) of the land use and depth on labile

fraction of Cu while no significant effect of seasonal changes was recorded. Plantain farm had greater value of labile Cu (1.71 mg kg^{-1}) than other agricultural land uses. Soil depth of 0-20 cm had significantly higher labile Cu (1.77 mg kg^{-1}) compared to depth 20-40 cm and 40-60 cm which were slightly different from each other. Land use, soil depth and cropping season had highly significant effects on Labile fraction of Pb. The highest labile Pb occurred in vegetable croplands (amaranth and Celosia) while control had the least value. Soil depth at 20-40 cm had the lowest labile Pb compare to 40-60 cm which had significantly higher value. The effect of seasonal variation was significantly higher in dry season than in the rainy season, higher labile Pb was recorded in dry season than rainy season. There was no significant difference at $p < 0.05$ for agricultural land use on labile fraction of Zn but highly significant ($p < 0.01$) effect was observed in the different soil depths and cropping seasons. The labile fraction of Zn decreases as soil depth increases with 0-20 cm having greater value and depth 40-60 cm having the least value of labile Zn. The amount of labile Zn increased significantly in rainy season than in dry season.

The soil quality index is shown in Table 6, the agricultural land uses had total N, available P and exchangeable K as moderate levels (15-30 mg kg^{-1}, 0.26-1.28 cmol kg^{-1}) for plant growth in amaranth (15 mg kg^{-1}, 0.31 cmol kg^{-1}) and celosia cropland (20.34 mg kg^{-1}, 0.27 cmol kg^{-1}) than other land use that are deficient. In all the agricultural land uses, values of exchangeable Mg were moderate, while the values of exchangeable Ca were extremely high (> 5.00 cmol kg^{-1}) in all agricultural land uses.

The correlations between soil pH, organic matter and the major soil nutrients are presented in Table 7. Soil organic matter, total nitrogen, and the exchangeable cations had significant positive relationship with the pH. Soil organic matter had very significant positive relationship with total N, available P, K, Mg and Na. Similarly, available P positively correlated with K, Mg and Na. Potassium also had significant relationship with Mg and Na contents of the soil while calcium only had relationship with Na.

Table 8 shows the correlations among different fractions of heavy metals and pH of soil under organic farming system. Mobile copper only had significant positive correlation with mobile Zn while it had negative relationship with labile lead, exchangeable lead, and water soluble copper. Mobile Pb correlated positively with mobile Zn and water soluble zinc while it has negative correlation with exchangeable lead, zinc and labile lead. Mobile zinc showed significant positive correlation with other fractions of Zn. There were no correlations of water soluble fractions of the heavy metals with other fractions. Exchangeable Cu positively correlated with fractions of Pb while exchangeable lead only had relationship with labile Pb. Labile copper positively correlated with labile zinc. Other possible correlations were not significant at 5% probability level.

Table 6
Quality Index for selected soil properties of the organic farm

Land use	Total N (g/kg)	Remarks	Avail P	Remarks	K	remarks	Ca	Remarks	Mg	Remarks	Na	Remarks
Control	0.55	Low	6.1	Low	0.1	low	12.13	High	0.99	Moderate	0.23	No adverse effect
Pineapple plantation	0.42	Low	8.75	Low	0.13	low	10.66	High	0.97	Moderate	0.28	No adverse effect
plantain plantation	0.58	Low	6.84	Low	0.2	low	12.66	High	1.23	Moderate	0.39	No adverse effect
Amaranth cropland	0.58	Low	14.91	Moderate	0.31	moderate	11.88	High	1.14	Moderate	0.54	No adverse effect
Celosia cropland	0.68	Low	20.34	Moderate	0.27	moderate	12.5	High	1.2	Moderate	0.48	No adverse effect

Note, Total N(g/kg) > 5 High, 1-5 Moderate, < 1 Low, Avail P (mg/kg^{-1}) from 15 - 30 Moderate, < 15 Low, Exchangeable K (cmolkg^{-1}) > 1.28 High, 0.26-1.28 Moderate, <0.26 Low ,Exchangeable Ca (cmolkg^{-1}) >5.0 High, 0.5-5 Moderate, 0.05-0.5 Low, Exchangeable Mg (cmolkg^{-1}) >4.2 High, 0.42-4.2 Moderate, <0.42 Low, Exchangeable Na (cmolkg^{-1}) > 0.65 High, <0.65 No adverse effect.

DISCUSSION

Sequel to the pressure on land as a result of increasing demand for food, land resources are being over exploited. There is need for more information on of the agricultural production capacity of land and its dynamics in order to promote land management practices that seeks to improve land productivity in a sustainable way (Bindraban, Stoorvogel, Jansen, Vlaming, & Groot, 2000; Lal, 2009). The proposition of organic farming system as a safe and sound ecologically friendly system that would not only boost food production but improve soil productivity was hypothesised. The result of this study implied that the soil pH was improved under different land uses after some years of organic farming. The soil pH improved from being very acidic to slightly acidic after three years of vegetable cropping using organic inputs. The liming effect of the manure application could have been due to the addition of basic cations like Ca, K, Ma from the manure to the soil. These cations will subsequently displace H^+ from the soil exchange complexes. This result is confirming the recent discovery by Olowoboko, Azzez, Olujimi and Babalola (2018), who reported that addition of animal manures increased the soil pH in some tropical soils in Nigeria. Specifically, amaranths and celosia production had the highest soil pH probably because they are short duration crops and their uptake of lime inducing nutrients (Ca and Mg) could have been lower compared with other crops investigated. Hence, the amendments have higher amount of the cations to lime the soil. On the other hand, pineapple and plantain are perennials and heavy feeders that could relatively mine the soil of the basic cations. The duo of the latter crops have been reported to have high amounts of Ca and Mg in their fruits (indicative of high extraction from the soil) by Hossain, Akhtar and Anwar (2015) and Kasa and Yohanis, (2017). There seems to be the accumulation of basic cations, P and organic matter at shallow soil depths. This is reflected in the higher soil pH at 0-40 cm depth. Generally, during rainy season, the soil was more alkaline. In reaction, perhaps the acidity of the soil was diluted by the excess water usually recorded in the organic farm.

Table 7
Correlations among soil nutrients, pH and soil organic matter on organic farming system

	pH	SOM	N	Available P	K	Ca	Mg	Na
SOM	0.234*							
N	0.183*	0.298**						
Available P	0.406**	0.327**	0.101					
K	0.471*	0.316**	0.17	0.591**				
Ca	0.046	0.015	0.125	0.067	0.181*			
Mg	0.262*	0.337**	0.277	0.333**	0.445**	0.259*		
Na	0.386*	0.318**	0.102	0.562**	0.962**	0.228	0.357*	
ECEC	-0.149	0.157	-0.123	0.045	-0.015	0.114	-0.163	0.059

Table 8
Correlations among different fractions of heavy metals and pH in organic farming system

	M- Cu	M-Pb	M- Zn	WS-Cu	WS- Pb	WS- Zn	Exch-Cu	Exch- Pb	Exch-Zn	Lab- Cu	Lab- Zn	Lab- Pb
Mobile Pb	0.47											
Mobile Zn	0.211**	0.373**										
Water soluble Cu	-0.13*	-0.171	0.097									
Water soluble Pb	0.088	-0.072	-0.072	0.133								
Water soluble Zn	0.132	0.256*	-0.018	-0.034	0.007							
Exchangeable Cu	0.159	-0.003	-0.103	0.097	0.099	-0.119						
Exchangeable Pb	-0.209*	-0.206*	-0.17	-0.029	-0.018	0.113	-0.205*					
Exchangeable Zn	0.141	0.351**	0.53**	0.09	-0.028	0.007	-0.069	-0.112				
Labile Cu	0.138	-0.097	0.124	0.112	0	-0.016	0.115	-0.113	-0.042			
Labile Zn	0.186	0.167	0.325**	-0.097	-0.083	-0.05	-0.116	0.02	-0.001	0.207*		
Labile Pb	-0.312**	-0.351**	-0.199	0.154	-0.063	0.049	-0.21*	0.286*	-0.177	0.054	0.053	
pH	-0.009	0.245	0.06	0.024	0.069	0.046	-0.098	0.01	0.075	-0.173	0.15	0.045

Note, M=Mobile;, WS = Water soluble; Exch = Exchangeable; Lab= Labile

Soil organic matter is one of the primary indicators of soil quality (Komatsuzaki & Ohta, 2007). The study revealed significant changes in soil organic matter on different land uses in organic farming system. It was observed that plantain plantation at rainy season had greater soil organic matter than the planted grass land. The values obtained under pineapple plantation were comparable to those on the planted grassland. These differences were due to the general increase in soil organic matter and subsequent improvement in soil fertility on organic farming system. Kapkiyai, Karanja, Qureshi, Smithson and Woomer (1999) and Lemenih, Kaltun and Olsson (2006) reported significant improvement in soil organic matter after long-term applications of organic residues in soils of Kenya and Ethiopia. The accumulation of organic matter in the plantain plantation could also be due to the large amount of biomass of plantain stems, leaves and other plant parts that are usually returned to the soil for decay at harvesting. The study further showed that soil organic matter had significant positive correlations with pH and soil nutrients. This is not surprising as soil organic matter is well known to be a supplier of plant nutrients. Hence farming systems that improve soil organic matter such as the organic farming are desirable.

The distribution of soil nutrients as affected by different agricultural land uses indicated that land maintained for 3 years with organic inputs for celosia cropping significantly accumulated more nitrogen than others. Generally, plantain, celosia, and amaranths are surface feeders and have low biomass. Their life cycles (excluding pineapple) is also short (couple of weeks). This could have resulted in the accumulation of nutrients in the soil. On the contrary, plantain is perennial crop having high nutrients requirement, this leads to the excessive mining of soil nutrients. Similar results were reported by Diacono and Montemurro (2010). Higher total nitrogen in the rainy season is probably due to the leaching of soluble nitrate and other N forms from the environments to the soil at the time of sampling. This could be beneficial for crop growth as most of the cropping activities are done during rainy season. Nitrogen had positive and significant correlations with soil pH and organic matter while the correlation with other soil nutrients were not significant. Sharpley and Smith (1995) also recorded higher nitrogen and phosphorus in soils under the application of organic manure. Calcium decreased considerably as the depth of soil sampling increased from 0-20cm to 40-60 cm. Calcium also varied with season, higher values were obtained at the topsoil and subsoil during dry and rainy seasons, respectively. This could imply that calcium tends to concentrate at the topsoil during dry season but as it rains, the salt gets dissolved and move down the soil profile; the process could be termed calcium leaching. In all, dry season recorded greater values of exchangeable cations than rainy season. The cation exchange capacity which is a measure of the amount of cations a soil can hold; followed a similar trend as the exchangeable cations. Most of the cations

decrease in their concentrations from the topsoil to the subsoil. These are the expected trends and the dilution effect of rains on the salts of these cations could be responsible for these observations. There was a general increase in soil fertility level under the different land use types in the organic farm as there seemed to be no significant change in the effective cation exchange capacity of the soils in the semi-natural grass land compared to those on the land use types where organic farming has been practice for about 8 years. This is in accordance to the findings of Diacono and Montemurro (2010) who observed improvement in soil fertility after long term of using organic amendments.

Using the soil quality indices of Amercher, O' Neill and Perry (2007), the soils are deficient in total N, vegetable farm had moderate P and K level, exchangeable Ca is very high which may later lead to toxicity and exchangeable Mg is also moderate for all plants. Generally, the soil could be rated low in fertility. This could have been due to crop uptake of nutrients.

Agricultural land use with high inputs rates of organic manures could lead to increased risk of soil and water contamination with heavy metals. The result showed general variations among the heavy metals based on land use, soil depth and season though the total concentrations were below the tolerable limit (Kabata-Pendias & Pendias, 2001). There was a considerable difference in the amount of water soluble Cu on the amaranth cropland compared to the Celosia cropland as the latter tends to be significantly lower. There was no significant change in the concentrations of Cu at dry and rainy season.

Changes in Cu concentration at different soil depths and seasons did not follow a particular trend. It was observed that there were no appreciable changes in lead and zinc concentrations with land use, soil depth and season. The low concentrations of these metals could have accounted for this observation. Similarly, there was no considerable accumulation of mobile Cu (ammonium nitrate extractable) Cu, Pb and Zn under the different land use types compared to the semi-natural grassland. The dry season had lower mobile fraction of Zinc compared to the rainy season on the different land uses. Though, below the tolerable limit, topsoil (0-20 cm) accumulated more mobile fractions of Cu, Pb ad Zn. The values obtained were below those reported by Kobierski and Piotrowska (2010). This implied that there was a decrease in mobility of these metals with increasing soil depths. Zhou, Liao, Wu, Zhang and Ren (2008) reported that heavy metals accumulated in the first few centimetres of the topsoil.

The exchangeable fractions (calcium chloride extractable) of heavy metals are those fractions of the metal that could be easily adsorbed on the soil exchange sites. Among the heavy metals, lead had higher concentrations in all the land use types. In order to avoid the risk of future heavy metals contamination or lead poisoning care must be taken in the quality check of organic inputs used in organic farming system. Veeken and Hamelers (2002),

and Nicholson et al. (2003) noted that manures had become an important source of heavy metals applied to agricultural land, accounting for approximately 40% of total annual inputs of Zn and Cu to agricultural land and 11% of Cd inputs.

Plantain plantation showed tendency for increased accumulation of labile fractions (EDTA extractable) of copper and lead. There was a relative increase in the amount of labile Pb in dry season under the vegetable croplands. The variation of these metals appeared to be uniform with soil depth. The amount of these metals were very low (Kabata-Pendias & Pendias, 2001) to pose a threat to both animal and human lives but conscious efforts still need to be made to maintain low levels of lead in the soil.

Furthermore, it was noted that there exist significant positive relationships within and among most of the different fractions of the heavy metals. This implied that increase in one fraction could lead to increased accumulation of the other. It was also observed that there exist poor correlations of water soluble fractions of the heavy metals with other fractions which could be due to the varying degrees of solubility of the metals.

Generally, the arguments on the accumulation of heavy metals in organic systems have not been a conclusive one but most of the scientists are of the opinion that metals found in organic systems are not necessarily due to the additions in the system but aerial depositions from non- point sources (Pandey & Pandey, 2009). However, the use of town refuse in urban agriculture have been reported to contaminate the soil and crops cultivated therein (Pasquini, 2006). It should however, be noted that heavy metal contamination is not necessarily a function of soil loading alone but due to pH, cation exchange capacity and soil nutrient levels are all key factors that determine accumulation of heavy metals in crops. These are myriads of problems the farmer face. Just because people have high expectations form organic farming does not mean they have to be ignored.

Summarily, since the cumulative concentrations of the heavy metals were far below the international tolerable limits, it could be concluded that there is presently no risk of heavy metal contaminations at the organic farm where this research was conducted but attempts should be made to ensure proper quality control of organic inputs used to avoid the risk of future heavy metal contamination which could be harmful to lives. This further demonstrates and confirms organic farming as a safe and sound ecologically friendly system that sustains and improves soil quality, if other likely sources of heavy metals like aerial depositions are kept in check.

CONCLUSION

Manure used in organic farm has liming effect on the soil while soil nutrients are concentrated at surface soil depth. There is the accumulation of soil nutrients in organic farming, irrespective of the land use, cropping seasons and soil depths. Copper, Pb and Zn concentrations in the organic

farm were below the contamination levels. Though, studies on the uptake of heavy metals by organic products is recommended for investigation.

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Species Identification and Molecular Phylogenetics of Processed Sea Cucumbers from Malaysian Market based on 12S Mitochondrial rRNA Gene

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ABSTRACT

Extensive processing of sea cucumber causes body deformation of the marine organism, hence causing difficulties in species identification of processed sea cucumbers. Due to the copresence of cases of unlabelled or mislabelled sea cucumber products in Malaysian markets and worldwide, a study was conducted to determine the species identities of processed sea cucumbers from selected Malaysian markets using non-protein-coding 12S mitochondrial rRNA gene. Phylogenetic analyses based on the distance-based Neighbour Joining method, and the character-based methods i.e. the Maximum Parsimony method, Maximum Likelihood method, and the Bayesian Analysis method of 81 ingroup sequences representing 63 processed sea cucumber specimens, 13 fresh and processed reference samples for species identification, and five fresh additional specimens from Teluk Nipah Beach, Pangkor Archipelago and Manukan Island, Sabah suggested the presence of three main clusters i.e. a *gamat* family cluster consisting of family Stichopodidae (*Stichopus horrens*, *Stichopus vastus*, and *Thelenota anax*) and two clusters of *timun laut* family comprising family Holothuriidae (*Holothuria (Mertensiothuria) leucospilota*, *Holothuria (Metriatyla) scabra*, *Holothuria (Metriatyla) lessoni*, *Holothuria (Halodeima) atra*, and *Holothuria (Halodeima) edulis*) and family Caudinidae (*Acaudina molpadioides*). The outcomes of this study also highlighted the availability of 40 new 12S mitochondrial rRNA gene sequences deposited in the

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GenBank that can be utilised by the enforcement agencies to monitor and overcome the issues of species substitution and product mislabelling of processed sea cucumber products in Malaysian markets.

Keywords: 12S rRNA gene, *gamat*, Malaysian markets, phylogenetic analysis**,** processed sea cucumber, *timun laut*

INTRODUCTION

Species substitution of commercial marine products is one of the critical issues in the seafood industry. Growing international trade, increase in global seafood consumption, fluctuations in the food supply and demand of different marine-dwelling species are among the contributing factors of species substitution or intentional product mislabelling. Species substitution can have serious consequences, which include economic fraud, health hazards, and illegal trade of protected species (Rasmussen & Morrissey, 2008). Wen, Hu, Zhang and Fan (2011) reported the mislabelling of seven samples of commercial sea cucumber products from Guangzhou, China. In fact, China was ranked as the second world's top producer of commercial sea cucumber with 26 commercial species (Choo, 2008). Processed sea cucumbers in the forms of dried and frozen products sold in local markets in Malaysia (Kota Kinabalu, Sabah and at supermarkets in Peninsular Malaysia) were observed as mislabelled with incorrect species name, as well as missing manufacturing or packaging details for some sea cucumber-based products. Malaysia was the fourth world's top producer of commercial sea cucumber with 19 commercial species (Choo, 2008) and the occurrence of product mislabelling and species substitution could cause major economic and health problems as well as conservation issues in Malaysia.

Species identification of sea cucumber was typically done based on the external anatomy e.g. the presence and shape of feeding tentacles and tube feet; the internal anatomy e.g. the types of calcareous rings; and the microscopic observation of ossicle shapes. Among the common shapes of ossicles are perforated plate, anchor, roxette, button, rod, and table. Ossicles are the small pieces of calcified materials in the body tissues of a sea cucumber species considered informative and useful to identify the species of sea cucumbers in any forms e.g. fresh, salted, and dried forms (Toral-Granda, 2005). Nonetheless, different body parts of a particular sea cucumber species may contain different types and sizes of ossicles. Massin, Zulfigar, Hwai and Boss (2002) reported that the table-shaped ossicles in the ventral body part of *Stichopus chloronotus* was larger than the table-shaped ossicles in its dorsal body part. Dabbagh, Keshavarz, Mohammadikia, Afkhami and Nateghi (2012) reported the presence of rod-shaped ossicles in the tentacles, and table- and button-shaped ossicles in the dorsal body wall of *Holothuria (Metriatyla) scabra* from the Persian Gulf, Iran and the presence of uncommon ossicles of *H. scabra* in their specimens i.e. the branched rods. Moreover, Massin et al. (2002) also mentioned that the abundance of rosette-shaped ossicles, and

the shapes of the rod-shaped ossicles from the tube feet and dorsal papillae of *Stichopus herrmanni* were unsuitable to be used as a characteristic to differentiate the curryfish from the other species; thus indicating disadvantages of using ossicles in sea cucumber species identification. Therefore, in order to address the issues of mislabelled products and species substitution; a prompt, reliable, and reproducible molecular method is needed as an identification tool specifically for processed sea cucumbers that underwent shape deformation such as dried, frozen, canned, and pickled products as ossicle shape identification was commonly used for sea cucumber specimens with intact body forms. Approximetely, every cell in the body of a living organism has the same Deoxyribonucleic Acid (DNA). Processed sea cucumbers including the beche-de-mer are available in the forms of dried, frozen, canned, and pickled products worldwide.

Mitochondrial DNA (mtDNA) of animal has been the most preferred model for molecular genetic studies, specifically in the species identification, phylogenetic analyses, and phylogeographical analyses. According to Freeman and Herron (2004), small-subunit rRNA in the mitochondria remained an informative resource for whole-life phylogenies. One of the component of the small subunit of the mitochondrial ribosome is 12S rRNA. In fact, 12S mitochondrial rRNA gene is a non-protein-coding gene, as rRNA only produces polypeptides that are used to make up proteins. In terms of studies related to the non-protein-coding 12S mitochondrial rRNA gene of sea cucumber, only one study by Clouse, Janies and Kerr (2005) has been found to date. In the study, two morphs from the *Bohadschia marmorata* species complex in Micronesia were examined based on the spicules, colour, body size, behavior, and mitochondrial DNA including the 12S mitochondrial rRNA region in order to investigate the taxonomy of the two morphs. Clouse et al. (2005) concluded that *B. marmorata* and *B. bivittata* were not sister species, and *B. bivittata* was genetically closer to *B. argus*, the leopardfish or tigerfish; therefore *B. marmorata* and *B. bivittata* should be regarded as two separate species. The study also suggested the capability of 12S mitochondrial rRNA gene in addressing issues related to species complexity and classification.

Furthermore, there were studies on the use of non-protein-coding 12S mitochondrial rRNA gene to conduct forensics of food products. Wang, Duan and Zhang (2017) used 12S mitochondrial rRNA gene sequences to identify the animal species that were used to produce three types of aquatic and commercial collagens. Liao, Liu, Ku, Liu and Huang (2017) successfully addressed the issue of milk powder adulteration using PCR-based methods of 12S mitochondrial rRNA gene through the detection of cow milk compositions in goat milk powder. Meanwhile, Di Domenico, Di Giuseppe, Wicochea Rodríguez and Cammà (2017) developed and validated Fast real-time PCR TaqMan assays based on 12S mitochondrial rRNA gene and cytochrome b (cytB) gene for species

identification in dairy products. The studies highlighted the need of implementing molecular species identification as analytical checks on commercial and imported food products in order to address issues of species substitution and product mislabelling.

Issues related to species substitution and product mislabelling of sea cucumber-based products can be observed and investigated at some Malaysian markets. As a result, this study is aimed at determining the species identity of processed sea cucumber specimens from selected Malaysian markets by using forensically informative nucleotide sequencing (FINS) technique by Bartlett and Davidson (1992). Online Basic Local Alignment Search Tool program for nucleotide (blastn) was included to resolve the species status of the specimens based on the partial sequences of non-protein-coding 12S mitochondrial rRNA gene. There are scarce studies related to the 12S mitochondrial rRNA gene of sea cucumber that cause lack of 12S mitochondrial rRNA gene sequences of sea cucumber species in the GenBank. Phylogenetic analyses based on the distance-based method with clustering algorithm as the tree building strategy i.e. the Neighbour Joining method, and the character-based methods with optimality criterion as the tree building strategy i.e. the Maximum Parsimony method, Maximum Likelihood method, and the Bayesian Analysis method were incorporated to determine the genetic relationships of the sea cucumber species. The findings of the analyses were compared with the manufacturing or packaging details of the specimens. This study highlights the issues of intentional species substitution and product labelling of processed sea cucumbers in selected Malaysian markets. The information will be useful to tackle issues pertaining sea cucumber-based products in Malaysia.

MATERIALS AND METHODS

Study Site and Sampling

A total of 112 sea cucumber specimens from Pangkor Archipelago, Perak Darul Ridzuan (West Coast region in the northern part of Peninsular Malaysia, n=7); Kuah, Langkawi Archipelago, Kedah Darul Aman (North region of Peninsular Malaysia, n=7); Nilai, Negeri Sembilan Darul Khusus (South region of Peninsular Malaysia, n=8); Kuantan, Pahang Darul Makmur (East Coast region of Peninsular Malaysia, n=27); Kota Kinabalu, Sabah and Kudat, Sabah (East Malaysia, in Borneo Island, n=63, Kamarudin, Mohamed Rehan, & Bahaman, 2017a) were included (Figure 1). Three live and fresh specimens of *Holothuria (Mertensiothuria) leucospilota* from Teluk Nipah Beach, Pangkor Archipelago (HLTNP1-HLTNP3, Kamarudin & Mohamed Rehan, 2015) were collected as reference samples of fresh *timun laut* species, and three live and fresh specimens of *Stichopus horrens* from Pangkor Laut, Pangkor Archipelago (SHP1-SHP3; Kamarudin & Mohamed Rehan, 2015; Kamarudin, Mohamed Rehan, Mohd Noor, Ramly, & Mohamed Rehan, 2017b) were used as reference samples of fresh *gamat* specimens. Besides, seven dried *gamat*-based beche-de-mer specimens

from Kuah, Langkawi Archipelago (North region of Peninsular Malaysia; Figure 1; LKIG1-LKIG7; Kamarudin et al., 2017b; Kamarudin & Mohamed Rehan, 2015) were used as reference samples of processed sea cucumbers. For unlabelled specimens, more information was gained from the salespersons.

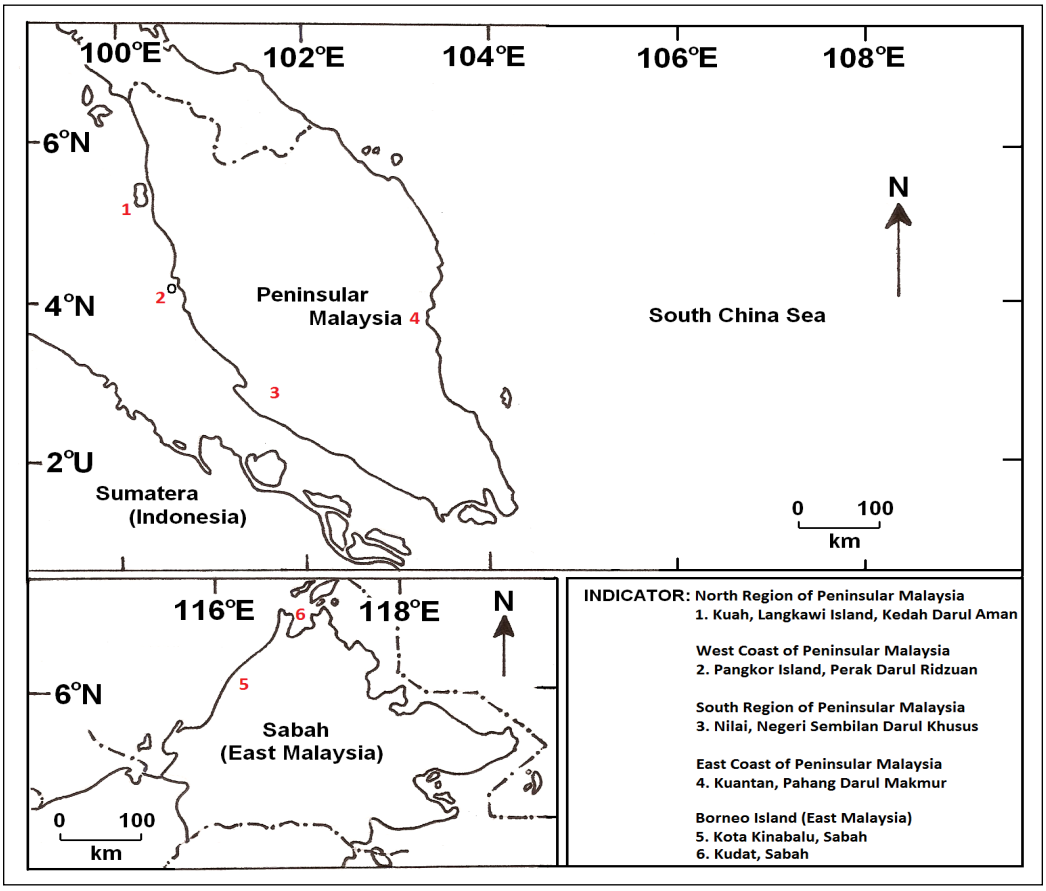

Figure 1. Sampling sites of sea cucumber specimens

Morphological Species Identification

The sea cucumber specimens were morphologically identified by referring to information provided by local residents, experts [Assoc. Prof. Alexander M. Kerr (Marine Laboratory, University of Guam, USA) and the participants of National Science Foundation (NSF) Partnerships for Enhancing Expertise in Taxonomy (PEET) Holothuroid Systematics Workshop held on 7-16 June 2010 at the Marine Laboratory, University of Guam, USA)]; Hashim (2011), Purcell, Samyn and Conand (2012), and the World Register of Marine Species database at http://www.marinespecies.org/index.php.

Total Genomic DNA Extraction

Three methods of total genomic DNA extraction were employed to obtain a better yield of total genomic DNA, i.e. modified cetyl trimethyl ammonium bromide (CTAB) method of Grewe et al. (1993) together with the Geneaid Genomic DNA Mini Kit (Blood/Cultured Cell), total genomic DNA extraction using the DNeasy mericon Food Kit by QIAGEN, and total genomic DNA extraction using the FavorPrep™ Tissue Genomic DNA Extraction Mini Kit. For the second method, homogenised tissue was prepared by using the QIAGEN TissueRuptor whereby ~0.2 g tissue from each specimen was disrupted and homogenised. Furthermore, 1% agarose gel with FloroSafe DNA Stain was used to determine the approximate yield of the total genomic DNA through horizontal gel electrophoresis. The extracts were kept in 4°C chiller for short-term storage prior to the next analysis or -20°C chest freezer for long-term storage.

Polymerase Chain Reaction (PCR)

The non-protein-coding 12S mitochondrial rRNA gene was amplified using two methods:

(a) 25 µl PCR reaction volume using the 2x TopTaq Master Mix Kit by QIAGEN with 1.0 µl of the DNA extract

(b) 50 µl PCR reaction volume containing 33.75 µl of sterilised dH_2O, 5.0 µl of 10X PCR reaction buffer, 3.0 µl of 25 mM magnesium chloride, 2.5 µl of each 5 µM universal primer, 1.0 µl of 10 mM dNTP mix, 2.0 µl of the DNA extract and 0.25 µl of 5 u/µl *Taq* DNA polymerase.

The primer set for non-protein-coding 12S mitochondrial rRNA gene (Palumbi et al. (1991), expected length: ~360 bp)) is as follows:

AB12SA-Lf (forward) 5'- AAA CTG GGA TTA GAT ACC CCA CTA T -3' (25 bases)

AB12SB-Hr (reverse) 5'- GAG GGT GAC GGG CGG TGT GT -3' (20 bases)

Furthermore, two batches of PCR cycle parameters were used:

(a) 2 min at 95°C for initial denaturation, 30 s at 95°C for denaturation, 30 s at optimised temperature for annealing, 45 s at 72°C for extension, repetition of step 2-4 for another 34-39 cycles, 5 min at 72°C for final extension, and forever hold at 4°C.

(b) 5 min at 95°C for initial denaturation, 45 s at 95°C for denaturation, 90 s at optimised temperature for annealing, 1 min 30 s at 72°C (60 s/kb; 29 cycles) for extension, 7 min at 72°C for final extension, and forever hold at 4°C.

PCR Product Purification and DNA Sequencing

The QIAquick PCR Purification Kit by QIAGEN (for direct purification of single

PCR fragment), Geneaid Gel/PCR DNA Fragments Extraction Kit (for direct purification of single PCR fragment), and QIAquick Gel Extraction Kit by QIAGEN (for purification of desired PCR fragment from agarose gel) were used for the PCR product purification. The unpurified PCR products were sent directly to the First BASE Laboratories Sdn Bhd, Seri Kembangan, Selangor Darul Ehsan, Malaysia since the company also provides PCR products clean up service prior to the DNA sequencing.

Phylogenetic Analyses based on DNA Sequences

Chromas program version 2.5.1 (Copyright© 1998-2016 Technelysium Pty Ltd) was used to display all the sequenced PCR products of the non-protein-coding 12S mitochondrial rRNA gene. Each DNA sequence was assigned to a particular sea cucumber species or genus by using the online Basic Local Alignment Search Tool program for nucleotide (blastn). ClustalX program version 2.1 (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997) was used for multiple sequence alignment of forward reaction sequences before the phylogenetic tree reconstruction. Molecular Evolutionary Genetics Analysis version 7.0.14 (MEGA7; Kumar, Stecher, & Tamura, 2016) was subsequently used to reconstruct phylogenetic trees using Neighbour Joining method (a distance-based method with clustering algorithm as the tree building strategy) and Maximum Parsimony method (a character-based method with optimality criterion as the tree building strategy). Prior to the reconstruction of Maximum Likelihood phylogenetic trees, Modeltest (version 3.7) program (Posada & Crandall, 1998) was used to calculate and find the best model for DNA evolution. A number of 56 models of DNA substitution were tested in order to choose the model that fitted the data best. PAUP* (version 4.0b10) program (Swofford, 1998) with 100 bootstrap replicates was then used to reconstruct the Maximum Likelihood phylogenetic tree. Furthermore, the reconstructions of consensus Bayesian Analysis phylogenetic trees were done by using MrBayes (version 3.1.2) program (Huelsenbeck & Ronquist, 2001). The run was stopped when the standard deviation of split frequencies reached below 0.01 (Reference: Tutorial – A Simple Analysis by Huelsenbeck & Ronquist, 2001). The reconstructed phylogenetic trees were displayed and edited by using TreeView (version 1.6.6) program (Page, 1996) and paint.net 4.0.6 (Final 4.6.5693.28) program (Copyright © 2015 dotPDN LLC, Rick Brewster, and contributors).

GenBank Registration and Accession

In terms of GenBank submission, sequence data were prepared by using the submission entry of Sequin version 15.10 program prior to obtaining the accession numbers from the GenBank, National Center for Biotechnology Information (NCBI), U.S. National Library of Medicine.

RESULTS AND DISCUSSION

In the context of product labelling, no specimens of processed sea cucumbers were labelled with species details and manufacturing details were absent for most of the specimens. Intentional species substitution and mislabelling of sea cucumber products have been reported worldwide and have influenced the trading of processed sea cucumbers (Rasmussen & Morrissey, 2008). The identification of *H. atra* species i.e. PFKK6 specimen among the 16 PFKK specimens as *H. edulis* was also identified. Shape deformation of sea cucumber upon extensive processing steps could lead to such situation as the morphologies or physical appearances of all the dried PFKK specimens are similar and difficult to be differentiated from each other. Besides, such difficulty could lead to human handling errors during the packaging process. Similar issue was also observed for the *gamat*-based beche-de-mer LKIG specimens, the reference samples from Kuah, Langkawi Archipelago, Kedah whereby two species i.e. *S. horrens* and *S. vastus* were present instead of one species in a packet (Kamarudin et al., 2017b).

In terms of the number of base substitutions per site from 81 nucleotide sequences of 299 aligned positions of non-protein-coding 12S mitochondrial rRNA gene sequences, the genetic distance values between specimens identified as *S. horrens* ranged from 0 (0%) to 0.0393 (3.93%) with average genetic distance of 0.0114 (1.14%), thus suggesting their status as single morphospecies i.e. morphospecies *S. horrens*. In addition, the genetic distance values between PKSH1 specimen and other *S. horrens* specimens including the reference samples of SHP ranged from 0.0291 (2.91%) to 0.0393 (3.93%). Furthermore, the genetic distance values between KPUI7 specimen and other *S. horrens* specimens including the reference samples of SHP ranged from 0 (0%) to 0.0291 (2.91%). Meanwhile, the genetic distance value between *S. vastus* specimens i.e. PKSO1 and LKIG7 was 0.0256 (2.56%). The genetic distance values between *T. anax* specimens i.e. KKS specimens ranged from 0 (0%) to 0.0094 (0.94%) with average genetic distance of 0.0029, thus suggesting their status as single morphospecies i.e. morphospecies *T. anax*. As for the *timun laut* specimens, the average genetic distance value between the *Holothuria* specimens including the reference samples of HLTNP was 0.0380 (3.8%). The genetic distance values between *H. leucospilota* specimens ranged from 0.0062 (0.62%) to 0.0288 (2.88%) with an average genetic distance of 0.0164 (1.64%). In addition, the genetic distance values between *H. scabra* specimens ranged from 0 (0%) to 0.0128 (1.28%) with an average genetic distance of 0.0038 (0.38%), while the genetic distance values between *H. lessoni* specimen i.e. KPTS1 and *H. scabra* specimens ranged from 0.0063 (0.63%) to 0.0128 (1.28%). As for the *H. atra* specimens, the average genetic distance was 0.0068 (0.68%) ranging from 0 (0%) to 0.0094 (0.94%), with the genetic distance value between PFKK6 specimen and PM3 specimen was 0 (0%). The average genetic

distance between *H. edulis* specimens excluding PFKK6 specimen was 0.0135 (1.35%) ranging from 0 (0%) to 0.1003 (10.03%), thus suggesting their status as single morphospecies i.e. morphospecies *H. edulis*. The genetic distance values between PM1 specimen and PFKK specimens excluding PFKK6 specimen ranged from 0 (0%) to 0.0962 (9.62%). Furthermore, the genetic distance values between *A. molpadioides* specimens i.e. specimens of GK and GN ranged from 0.0062 (0.62%) to 0.0391 (3.91%). Overall, the base frequencies were unequal (i.e. Adenine (A) = 34.14%, Cytosine (C) = 21.12%, Guanine (G) = 18.00%, and Thymine (T) = 26.74%)).

With regard to the availability of the non-protein-coding 12S mitochondrial rRNA gene of sea cucumber species in the GenBank, NCBI, U.S. National Library of Medicine; this study showed the absence of DNA sequences of at least three *timun laut* species i.e. *Holothuria (Halodeima) atra*, *Holothuria (Halodeima) edulis*, and *Holothuria (Metriatyla) lessoni*; and a gamat species i.e. *Stichopus vastus*; thus showing the lack of 12S mitochondrial rRNA gene sequences of some sea cucumber species in the GenBank until May 2017. For example, the *H. edulis* specimens from Kota Kinabalu, Sabah (PFKK) were identified as *Holothuria (Metriatyla) scabra* with 89% identity value (GenBank Accession No. of corresponding matches: KP257577, except for PFKK6 specimen) based on the blastn analyses (Table 1). Meanwhile, the morphospecies *H. atra* specimens from Manukan Island, Kota Kinabalu, Sabah (PM) were identified as *Holothuria (Mertensiothuria) hilla* with 87% identity value (GenBank Accession No. of corresponding match: KX856663, except for PM1 specimen). Moreover, the *H. lessoni* specimen from Kuantan, Pahang (KPTS1) was identified as *H. scabra* with 98% identity value (GenBank Accession No. of corresponding match: KP257577) and the *S. vastus* specimen from Kudat, Sabah (PKSO1) was identified as from genus *Stichopus* with 96% identity value (GenBank Accession No. of corresponding match: HM853683). The partial non-protein-coding 12S mitochondrial rRNA gene sequences obtained from this study were registered with the GenBank, and the deposition of the sequences have contributed to the availability of more DNA sequences of different sea cucumber species in the GenBank database. Therefore, researchers in future who work when using the blastn program especially in the species identification of sea cucumber could obtain higher percentage of identity value.

Even though the Neighbour Joining tree (Figure 2) shows the formation of family Stichopodidae cluster (the *gamat* family) with 83% bootstrap support, the cluster formation of the *timun laut* family was negligible due to the very low bootstrap support i.e. 41%. Thus, the clustering of the specimens of *Acaudina molpadioides* (GN) or the *U-in* specimen from family Caudinidae with the family Holothuriidae cluster is very weak. In contrast, the Maximum Parsimony tree (Figure 3) based on the non-protein-coding 12S mitochondrial rRNA gene sequences show the formation of

Table 1
GenBank accession number, and blastn results based on non-protein-coding 12S mitochondrial rRNA gene of sea cucumber specimens

Location	Type of Sample	Registration - GenBank Accession Number	Blastn Results		Corresponding Match(s) from GenBank
			Query Cover	Identity Value	
Teluk Nipah Beach, Perak	Reference samples of *Holothuria (Mertensiothuria) leucospilota* (live and fresh)	HLTNP1-HLTNP3-KY986419-KY986421	98-99%; 98.67%	98-99%; 98.33%	KX768273
Teluk Nipah Beach, Pangkor Archipelago, Perak	Additional samples (live and fresh)	HL1- KX768273	87%	99%	DQ777101
Manukan Island, Kota Kinabalu, Sabah	Additional samples (live and fresh)	PM1- MF188877 PM3- MF188878 PM4- MF188879 PM5- MF188880	PM1-95% PM3-95% PM4-95% PM5-96%	PM1-88% PM3-87% PM4-87% PM5-87%	PM1- KP257577 PM3- KX856663 PM4- KX856663 PM5- KX856663
Nilai, Negeri Sembilan	Processed samples (frozen)	GN7- MF667552 GN8- MF188876	90%	93.5%	KX856677
Kuantan, Pahang	Processed samples (dried)	KPTS1- MG682240	95%	98%	KP257577
Kuantan, Pahang	Processed samples (frozen)	KPUI7- MG922854 KPUI11- MG682241	95% 88%	99% 86%	HM853683 AY574864
Kuantan, Pahang	Processed samples (frozen)	GK10- MF667553 GK13- MF667554	89%	92%	KX856677
Kota Kinabalu, Sabah	Processed samples (dried)	KKS1-KKS9 - MF188867-MF188875	96.56%	96.11%	KX856681
Kota Kinabalu, Sabah	Processed samples (dried)	PFKK1-PFKK16 - MG682224- MG682239	PFKK1-PFKK5, PFKK7-PFKK16 – 91.13% PFKK6-94%	PFKK1-PFKK5, PFKK7-PFKK16 – 87.8% PFKK6-87%	PFKK1-PFKK5, PFKK7-PFKK16 - KP257577 PFKK6- KX856663

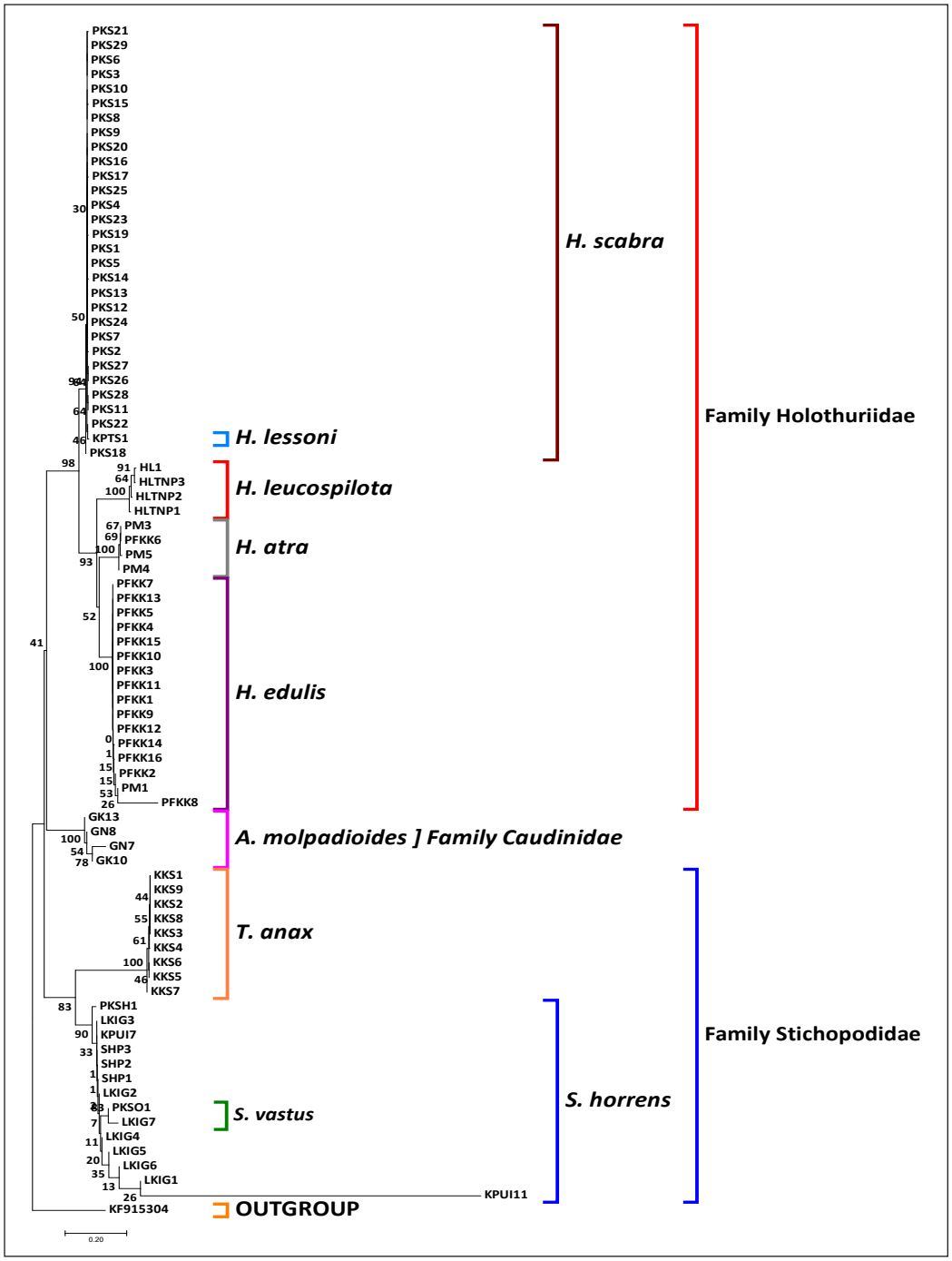

Figure 2. Topology of 50% majority-rule consensus tree of Neighbour Joining of sea cucumber specimens from selected Malaysian markets and other sampling sites including the reference samples and processed specimens inferred from non-protein-coding 12S mitochondrial rRNA gene sequences using MEGA7 program (Kumar et al., 2016) with 1000 bootstrap replicates. Numbers at nodes indicate the bootstrap values in percentage (%)

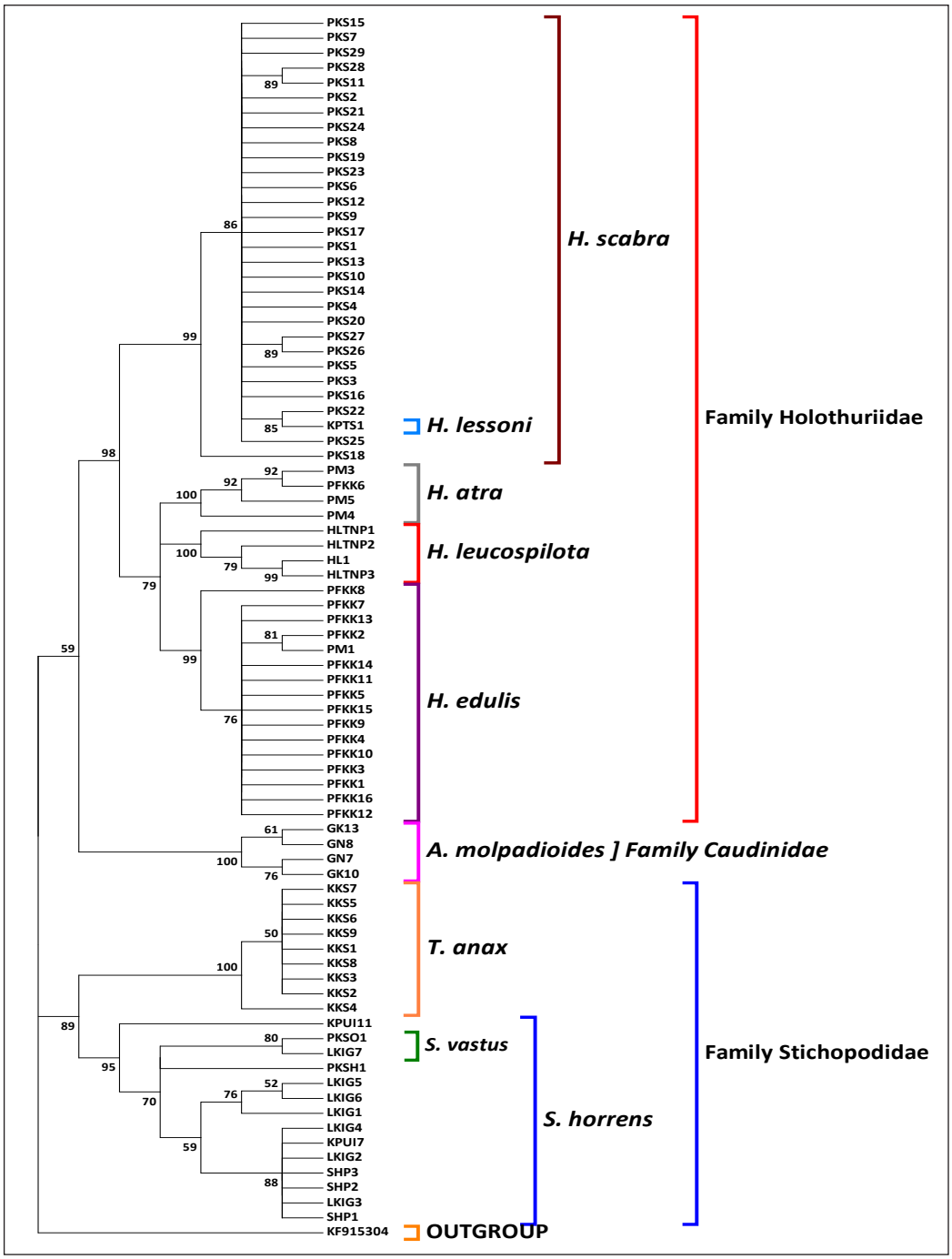

Figure 3. Topology of 50% majority-rule consensus tree of Maximum Parsimony of sea cucumber specimens from selected Malaysian markets and other sampling sites including the reference samples and processed specimens inferred from non-protein-coding 12S mitochondrial rRNA gene sequences using MEGA7 program (Kumar et al., 2016) with 1000 bootstrap replicates. Numbers at nodes indicate the bootstrap values in percentage (%)

two main clusters of the specimens i.e. family Stichopodidae (the *gamat* family) with 89% bootstrap support and the *timun laut* family with 59% bootstrap support. Species from the family Stichopodidae e.g. *S. vastus* and *Thelenota ananas* (the prickly redfish) were known as *gamat* while the term *timun laut* refers to all non-*gamat* species including the species of family Holothuriidae (Kamarudin, Rehan, Hashim, & Usup, 2010; Kamarudin, Usup, Hashim, & Mohamed Rehan, 2015). As for choosing the best model of DNA substitution for the Maximum Likelihood tree reconstruction using the Modeltest program, the model suggested by the Akaike Information Criterion (AIC) was chosen instead of the Hierarchical Likelihod Ratio Tests (hLRTs). According to Posada and Buckley (2004), the AIC and Bayesian Analysis were perceived as a good evaluation of model selection uncertainty, which were capable to compare multiple nested or non-nested models at once, and allowed for the use of all available models for the estimation of phylogenies and model parameters. Regarding the Bayesian Analysis tree reconstruction using the MrBayes (version 3.1.2) program, the standard deviation of split frequencies was 0.009766 at 3 020 000 generations. In contrast to the results of the Neighbour Joining tree (Figure 2) and the Maximum Parsimony tree (Figure 3), the Maximum Likelihood tree (Figure 4) and the Bayesian Analysis tree (Figure 5) show the formation of three main clusters of the specimens i.e. family Stichopodidae (the *gamat* family) and two subclusters of the *timun laut* family i.e. the family Holothuriidae cluster and the family Caudinidae clade/cluster. In other words, both the character-based phylogenetic analyses with optimality criterion suggested the same inference. In conclusion, the phylogenetic analyses suggested the presence of three main clusters of the specimens i.e. family Stichopodidae (the *gamat* family), family Holothuriidae (the *timun laut* family), and family Caudinidae (the *timun laut* family). With regard to the taxonomy, family Holothuriidae and family Stichopodidae are the members of Order Aspidochirotida, and family Caudinidae is from Order Molpadiida.

Regarding the bootstrap support, within the *gamat* family group, the Neighbour Joining method (Figure 2), the Maximum Parsimony method (Figure 3), the Maximum Likelihood tree (Figure 4), and the Bayesian Analysis tree (Figure 5) show the formation of *Thelenota anax* clade with 100% bootstrap values/posterior probability. The strong bootstrap values supported the grouping of the specimens of *T. anax* under the Stichopodidae family i.e. the *gamat* family. In Malaysia, sea cucumbers of the genus *Stichopus* and the genus *Thelenota* were regarded as the members of *gamat* family or the family Stichopodidae (Kamarudin et al., 2009, 2010, 2015). All the phylogenetic trees grouped the KKS specimens from Kota Kinabalu, Sabah with 100% bootstrap value, thus confirming their species status as *T. anax*. Within the genus *Stichopus* cluster, all the phylogenetic trees grouped the specimens of *S. vastus* i.e. LKIG7 specimen

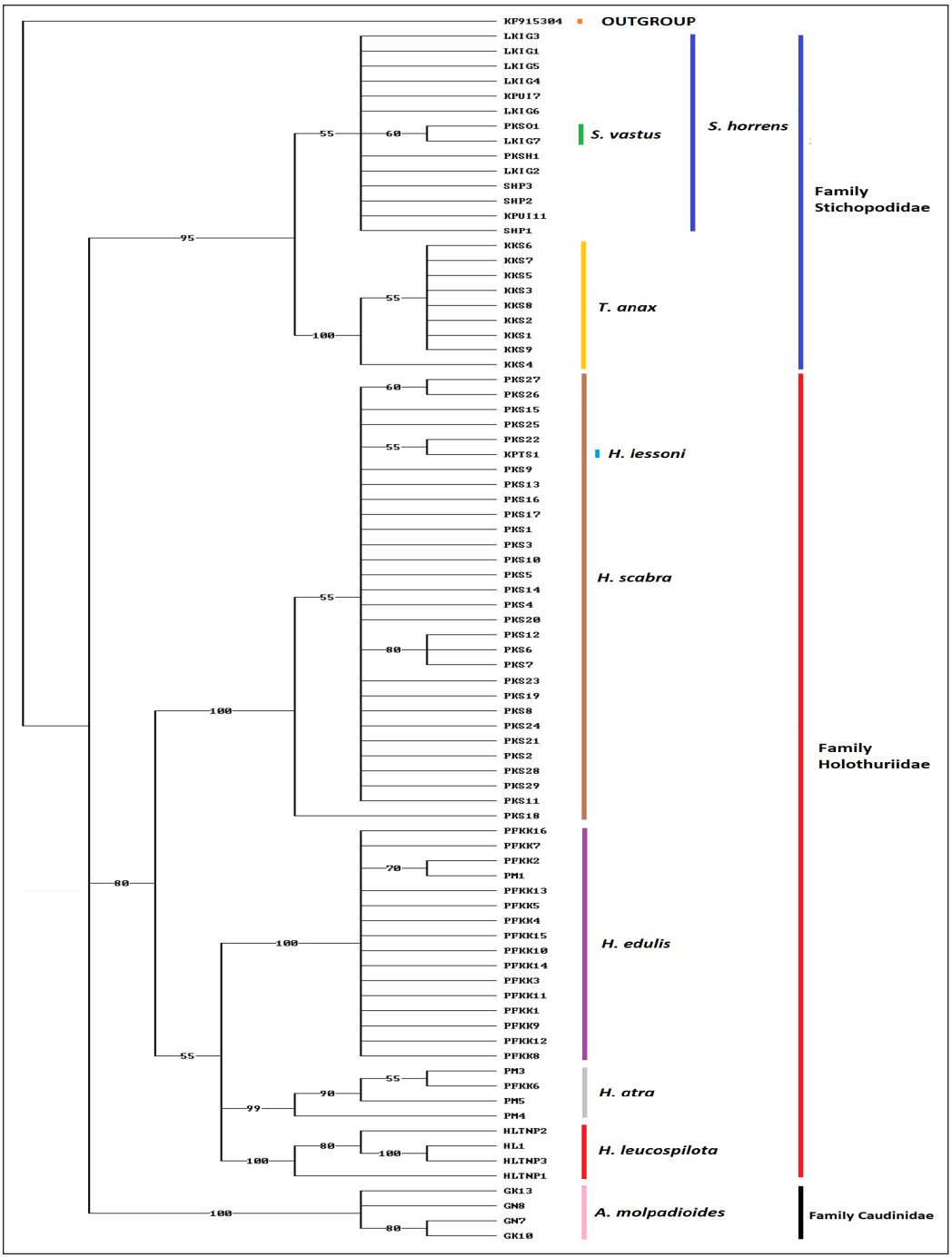

Figure 4. Topology of 50% majority-rule consensus tree of maximum likelihood of sea cucumber specimens from selected Malaysian markets and other sampling sites including the reference samples and processed specimens inferred from non-protein-coding 12S mitochondrial rRNA gene sequences using PAUP* (version 4.0b10) program (Swofford, 1998) with 100 bootstrap replicates. Numbers at nodes indicate the bootstrap values in percentage (%)

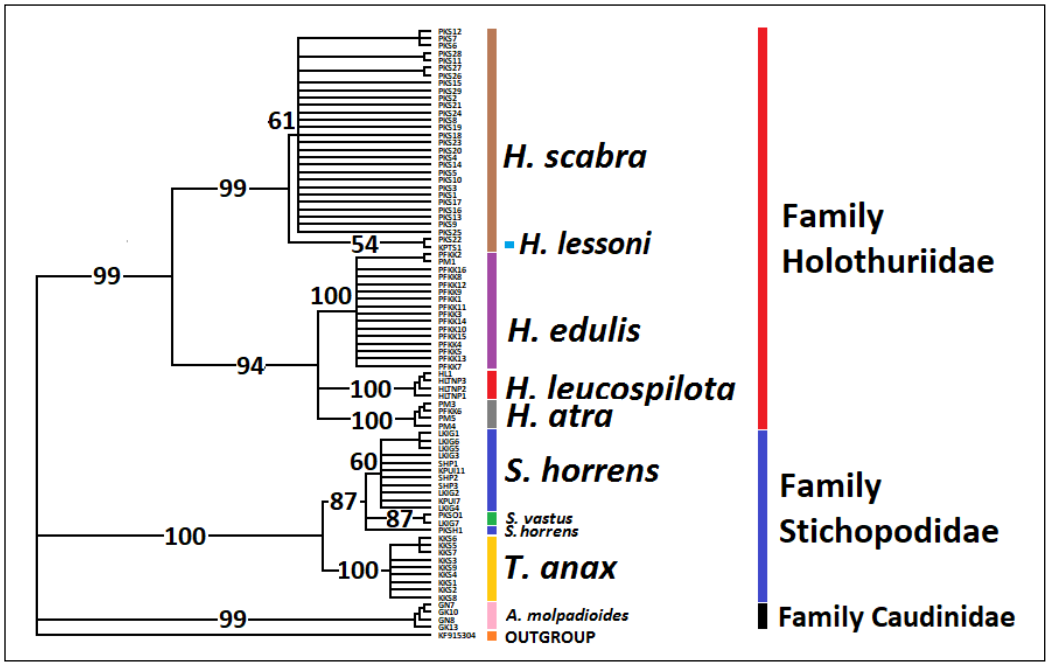

Figure 5. Topology of consensus Bayesian Analysis tree of sea cucumber specimens from selected Malaysian markets and other sampling sites including the reference samples and processed specimens inferred from non-protein-coding 12S mitochondrial rRNA gene sequences using MrBayes (version 3.1.2) program (Huelsenbeck and Ronquist, 2001), with the addition of all compatible groups to the tree. Numbers at nodes indicate the posterior probabilities of clades in percentage (%)

from Langkawi Archipelago, Kedah and PKSO1 specimen from Kudat, Sabah into the *S. horrens* cluster, with bootstrap values ranging from 55% to 90%. This could be due to the very close genetic characteristics between the two *gamat* species, even though their physical appearances are significantly different. Besides, *S. vastus* clusters were supported with bootstrap values ranging from 60% to 87%, as shown in all the phylogenetic trees.

Regarding the *timun laut* family, all the phylogenetic trees supported the formation of Holothuriidae family group with strong bootstrap values ranging from 80% to 99%. The phylogenetic trees also supported the formation of *H. leucospilota* clade and *H. edulis* clade each with 100% bootstrap support, thus confirming their species status. Besides, the Neighbour Joining method (Figure 2), the Maximum Parsimony method (Figure 3), and the Bayesian Analysis tree (Figure 5) supported the formation of *H. atra* clade with 100% bootstrap values/posterior probability, while the Maximum Likelihood tree (Figure 4) supported the formation of *H. atra* cluster with 99% bootstrap values. In addition, all the phylogenetic trees also show the presence of two genera of *timun laut* i.e. genus *Holothuria* and genus *Acaudina*. The specimens of *A. molpadioides* became basal within the *timun laut* family group in the Neighbour Joining tree and the Maximum Parsimony

tree. Such clustering suggested the presence of *timun laut* cluster (family Holothuriidae and family Caudinidae) and *gamat* cluster (family Stichopodidae). Nonetheless, the Maximum Likelihood tree and the Bayesian Analysis tree did not support the basal formation. Interestingly, all the phylogenetic trees show no distinct separation between the specimen of *H. lessoni* (KPTS1) and the specimens of *H. scabra* (PKS) as the specimens were mixed with each other with strong bootstrap values/posterior probability ranging from 94% to 100%. Purcell et al. (2012) recorded a processed *H. lessoni* that is similar to the dried *tip-sum* specimen from Kuantan, Pahang (KPTS1), therefore the specimen was regarded as *H. lessoni*. In addition, *H. scabra* and *H. lessoni* are taxonomically from the subgenus *Metriatyla*. From another point of view, *H. lessoni* was previously known as *Holothuria scabra* var. versicolor (Purcell et al., 2012), thus the clustering of KPTS1 specimen with the *H. scabra* specimens could be due to the close genetic contents between *H. scabra* and *H. lessoni*. Furthermore, only the Neighbour Joining tree (Figure 2) shows that *H. edulis* was genetically closer to *H. atra* with 52% bootstrap value, and the subgenus *Mertensiothuria* represented by the specimens of *H. leucospilota* was genetically closer to the subgenus *Halodeima* represented by the specimens of *H. atra* and *H. edulis* with 93% bootstrap value. *H. edulis* and *H. atra* are from the subgenus *Halodeima*, thus the grouping supported their taxonomic classification.

Overall, nine sea cucumber species were recorded in this study including six *timun laut* species i.e. *H. leucospilota*, *H. atra*, *H. edulis*, *H. scabra*, *H. lessoni*, and *A. molpadioides*; and four *gamat* species i.e. *S. horrens*, *S. vastus*, and *T. anax*. According to Choo (2008), *H. leucospilota*, *H. atra*, *H. edulis*, *H. scabra*, *S. horrens*, *S. vastus*, and *T. anax* were the commercial Malaysian sea cucumber species. Nonetheless, *A. molpadioides* and *H. lessoni* were not listed as commercial Malaysian sea cucumber species. Among the species recorded in this study, two *timun laut* species are included in the International Union for Conservation of Nature (IUCN) Red List for aspidochirotid holothuroids. *H. scabra* and *H. lessoni* were regarded as "endangered, or at a high risk of extinction" based on the IUCN Red List for aspidochirotid holothuroids (Conand et al., 2014). Apart from that, the outcomes of this study provide further information on the level of species substitution and product mislabelling issues of processed sea cucumbers in Malaysian markets. The enforcement agencies can use the information for monitoring and overcoming the issues through the introduction of mtDNA sequencing technique.

CONCLUSION

In conclusion, the phylogenetic trees based on the distance-based method with clustering algorithm as the tree building strategy i.e. the Neighbour Joining method, and the character-based methods with optimality criterion as the tree building

strategy i.e. the Maximum Parsimony method, Maximum Likelihood method, and the Bayesian Analysis method suggested the presence of three main clusters of the specimens i.e. family Stichopodidae (the *gamat* family), family Holothuriidae (the *timun laut* family), and family Caudinidae (the *timun laut* family). Three *gamat* species i.e. *S. horrens*, *S. vastus*, and *T. anax* were clustered under the family Stichopodidae and the specimens of *H. leucospilota*, *H. atra*, *H. edulis*, *H. scabra*, *H. lessoni*, and *A.* molpadioides are the five *timun laut* species grouped under the family Holothuriidae. Moreover, *A. molpadioides* from the family Caudinidae and Order Molpadiida was also present. Issues of intentional species substitution or product mislabeling were recorded due to the observation of unlabelled products in the selected Malaysian markets. The information may assist the enforcement agencies to monitor and address the issues. Pertaining to the export and import transactions, in order to ensure that the right species are utilised in processing and trading matters, the traders and the manufacturers may practise the molecular approach used in this study. Furthermore, the enforcement agencies may apply the mtDNA sequencing technique as an additional identification tool and the non-protein-coding 12S mitochondrial rRNA gene sequences deposited in the GenBank in order to establish facts in legal investigation prior to deciding the charges to the offenders.

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Vegetative Compatibility Groups within *Fusarium* Species Isolates from Tomato in Selangor, Malaysia

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ABSTRACT

Vegetative compatibility provides valuable information on genetic diversity of certain fungal population including *Fusarium* species. *Fusarium* species are capable of causing mass spoilage of perishable vegetable fruits such as tomato either in the field or in storage. A total of 81 *Fusarium* isolates comprising *F. oxysporum* (54 isolates), *F. semitectum* (22 isolates) and *F. subglutinans* (5 isolates) were examined for vegetative compatibility groups (VCGs). *Nit* mutants were generated from minimal medium with chlorate (MMC) and potato dextrose agar with chlorate (PDC) media under varying degrees of chlorate ($KClO_3$) concentrations from 4.5 - 6.0%. Four phenotyping media containing different nitrogen sources (NO_2, NO_3, NH_4 and HX) were used to phenotype the *nit* mutants into different classes: *nit1*, *nit3* and NitM. All heterokaryon self-compatible (HSC) *nit* mutants of *Fusarium* species were paired in all pairwise possible combinations on MM to classify them into VCG. Based on the index and distribution of the VCGs, isolates of *F. oxysporum* demonstrated high genetic diversity where 11 VCGs were recovered. Meanwhile, only three VCGs were recovered in *F. semitectum* isolates. *Fusarium subglutinans* isolates had the least number of VCGs where only two groups were recovered.

Keywords: Chlorate resistant sectors (CRSs), *Fusarium*, *Nit* mutants, vegetative compatibility groups (VCGs)

ITRODUCTION

Fusarium species were reported to cause fruit rot of tomato and have been isolated from infected samples in Malaysia including *F. semitectum, F. oxysporum, F. equiseti, F. subglutinans* and *F. solani* (Abu Bakar, Nur Ain Izzati, & Umi Kalsom, 2013). Realizing

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that the information on genetic diversity of *Fusarium* species is limited in a tropical area, this study examined the genetic diversity of *Fusarium* species isolated from *Fusarium* fruit rot of tomato based on vegetative compatibility (VC).

Vegetative Compatibility Groups (VCGs) provide a crude marker for population genetic studies in numerous underlying genes as well as producing a promising result when two strains are compared. VCGs are employed to provide a means of characterizing a variation based on genetics of the fungus, the origin and relatedness among *Fusarium* strains (Deacon, 2006; Desjardins, Plattner, & Gordon, 2000; Korolev, Katan, & Katan, 2000; Leslie & Summerell, 2006; Pasquali, Dematheis, Gilardi, Gullino, & Garibaldi, 2005). The phenomenon by which two hyphae can anastomose and fuse to form a stable heterokaryon is referred to as VC (Carvalho, & Mendes-Costa, 2011; Somrith, Singburaudom, & Piasai, 2011; Wang, Brubaker, Summerell, Thrall, & Burdon, 2010).

In *Fusarium* and *Neurospora*, VC is defined as the ability of auxotrophic strains to form a prototrophic heterokaryon also known as heterokaryon self-compatible (HSC). Strains for which no prototrophic heterokaryon is formed may be due to vegetative incompatibility or the physical inability of one (or both) of the strains to form heterokaryons with any other strains. Strains that lack of ability to form heterokaryons between mutants derived from the same strain are termed heterokaryon self-incompatible (HSI). Asexually reproducing fungi can only exchange the genetic material through parasexual recombination; therefore, VC becomes a prerequisite for sharing genetic materials between fungi. Heterokaryosis, barrages, and complementation have been recognized in *Fusarium* and other fungal population (Pasquali et al., 2005). The objective of this study was to classify *Fusarium* isolates from post-harvest fruit rot of tomato into respective vegetative compatibility groups (VCG).

MATERIALS AND METHODS

Generation of Nitrate Non-utilizing (*nit*) Mutants

Eighty-one isolates of *Fusarium* species used in this study were obtained from Laboratory of Mycology, Department of Biology, Faculty of Science, UPM according to fungal stock availability. All isolates were previously isolated from infected-post-harvest of tomato at storage areas throughout Selangor, Malaysia. The isolates were identified based on morphological characteristics and translation elongation factor (*tef*) 1-α gene sequence analysis by Abu Bakar et al. (2013) and Murad, Kusai and Zainudin (2016). *Nit* mutants were generated on minimal medium with chlorate (MMC) and potato dextrose with chlorate (PDC) media under varying degrees of chlorate ($KClO_3$) concentrations (4.5 - 6.0%). Four fragments of mycelia (2 mm^2) were placed on MMC and PDC plates incubated at standard incubation condition for 14 days in darkness. Spontaneous chlorate-resistant

sectors (CRSs) usually appeared like fan-shaped with thin transparent mycelium at the edges from the parent colonies were transferred and sub-cultured on minimal medium (MM). Meanwhile, colonies that grew with thin transparent aerial mycelia were identified as *nit* mutants and were used for phenotyping. On the other hand, those germinated with dense non-transparent aerial mycelia (regarded as *crn* mutants) were discarded, as they were representatives of the wild-type or reverted cultures.

Phenotyping of *nit* Mutants

Four phenotyping media containing four different nitrogen source media: MM ($NaNO_3$ medium), nitrite ($NaNO_2$ medium), ammonium (NH_4^+ medium) and hypoxanthine (HX^- medium) were prepared and used to phenotype the *nit* mutants and classify them into different classes (*nit*1, *nit*3 and NitM). A complementation test was conducted on *nit* mutants of the same strain paired on MM in all possible combinations (*nit1* × NitM, *nit3* × NitM and *nit1* × *nit3*) to classify them into HSC and HSI *nit* mutants. Colonies with robust growth of mycelia at the line of intersection were evaluated as HSC strains, whereas those with thin growth of mycelia evenly across the area of the plate were analyzed as HSI strains.

Complementation Test

All HSC *nit* mutants were further paired in all the pairwise possible combinations (*nit1* X NitM, *nit3* x NitM and *nit1* x *nit3*) on MM to classify them into VCGs. Those strains in the same VCGs often depicted a dense aerial mycelium at the line where colonies intersected, whereas strains in the different VCGs showed a thin growth of aerial mycelium. All inter-pairings of the complementation test were repeated at least three times. All HSC *nit* mutants were then labeled with VCGs.

RESULTS

Generation of *nit* Mutants

Spontaneous CRSs were successfully generated from all three *Fusarium* species (*F. oxysporum, F. semitectum* and *F. subglutinans*). The CRSs were produced on MMC and PDC with chlorate concentrations of 4.5 - 6.0%. Single, thin, and transparent CRSs were obtained at the edge of the growing colonies on MMC and PDC plates for most of the isolates. The CRSs kept growing radially like fan-shaped from the center of the colonies with aging. Figure 1 displays the generation of spontaneous CRSs on MMC for isolate of *F. semitectum* (B605T) recovered from Sri Serdang, Selangor. Hence, more than 1000 *crn* mutants were obtained and discarded constituting 75% of *nit* mutants from *F. semitectum* and *F. subglutinans*. A total of 676 *nit* mutants were generated and produced from all *Fusarium* species tested. *Fusarium oxysporum* produced the highest frequency number of *nit* mutants (63%) followed by *F. semitectum* (23%), while the least number was recorded by *F. subglutinans* (14%).

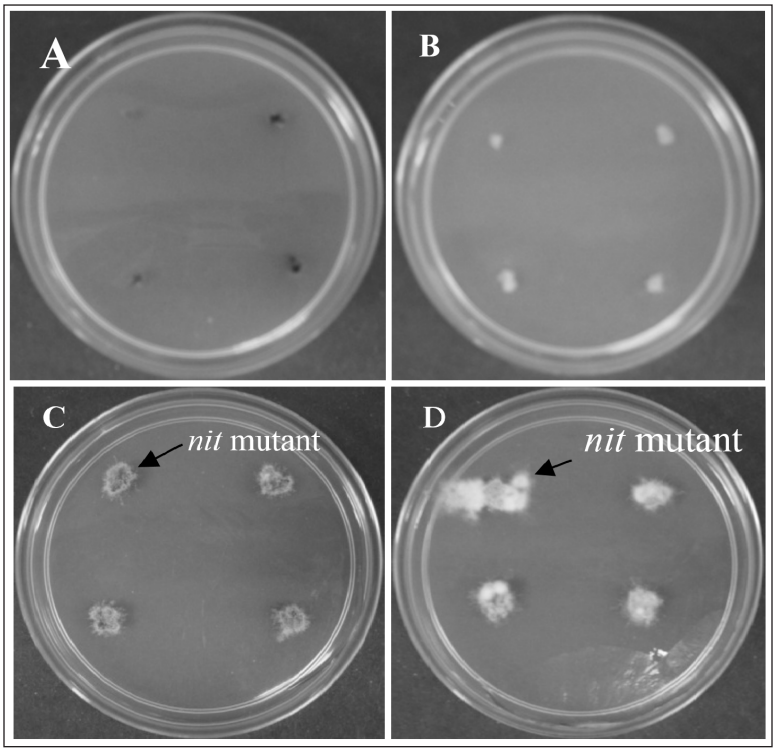

Figure 1. Generation of spontaneous CRSs using different concentrations of chlorate ($KClO_3$) in MMC for *F. semitectum* (Isolate B605T): (A) 4.5% (B) 5.0% (C) 5.5% (D) 6.0%

Nit Mutant Phenotype

The *nit* mutant phenotype of 81 isolates of the *Fusarium* was successfully conducted. *Nit1* mutants produced very thinly mycelia on nitrate medium, but vigorous growth was formed on the nitrite, ammonium, and hypoxanthine media. *Nit3* mutants did not grow well on nitrate and nitrite media while the thick growth of mycelium was formed on the ammonium and hypoxanthine media. NitM mutants did not grow well on nitrate and hypoxanthine media, whereas vigorous growth of mycelium occurred on the nitrite and ammonium media. From the findings, all the *nit* mutants showed different patterns of growth on these media since they possessed different nitrate metabolism pathways. *Nit1* was formed because of mutation in a nitrate reductase holoenzyme while *nit3* was formed due to the mutation at the nitrate-pathway specific regulatory protein. NitM mutants occurred because of mutation of a molybdenum-containing co-factor in the nitrate reductase enzyme. Essentially, these results of phenotyping of *nit* mutants provided a route in which heterokaryosis would be established for all *Fusarium* species. Once the HSC was identified on the isolates, complementation test was then conducted to assign them into VCGs.

The frequencies of *nit* mutants isolated from all three species of *Fusarium* recovered from tomato fruits were obtained. Eighty-five percent (85%) of the total isolates produced *nit* mutants, whereas 15% failed. All classes of the *nit* mutants (*nit1*, *nit3* and NitM) were generated in this study. Table 1 provides a summary of the *nit* mutants recovered from MMC and PDC for all three species. *Fusarium oxysporum, F. semitectum* and *F. subglutinans* generated 427, 152 and 97 *nit* mutants, respectively. Mean of *nit* mutants on both MMC and PDC was considerably higher on *nit1* followed by *nit3* and NitM for all three species of *Fusarium*.

Table 1
Nit mutants recovered on MMC and PDC

Fusarium species	*Nit* mutants		Mean of *nit* mutants					
			MMC			PDC		
	Total	°Mean	*nit1*	*nit3*	NitM	*nit1*	*nit3*	NitM
F. oxysporum	427	8.1	1.6	0.4	0	3.9	1.3	0.9
F. semitectum	152	6.9	0.9	0.3	0	3.1	1.3	1.3
F. subglutinans	97	19.4	4.6	2.4	0.4	8.4	2.4	1.2

º Mean for each isolate

Complementation Test

The complementation test was carried out to classify the isolates into VCGs. *Nit* mutants from the same isolates were firstly paired on MM and if the dense growths of mycelia were formed at the line of intersection of the colonies, then they were confirmed as heterokaryon self-compatible (HSC) *nit* mutants. The isolates that did not produce heterokaryons were considered as heterokaryon self-incompatible (HSI). Interpairings between different HSC isolates were then conducted to assign them into VCGs. The heterokaryon in most of the complementation test is often formed rapidly between *nit1* and NitM or *nit3* and NitM. The pairings between *nit1* and *nit3* in most situations produced weak heterokaryons. A typical complementation test of *F. oxysporum* is illustrated in Figure 2 shows isolates B712T (plate A) and B762T (plate B) formed heterokaryons (HSC). The pairing between isolates B622T and B640T indicated that they belonged to the same VCGs (plate C) while complementation between the isolates B622T and B707T suggested that they belonged to the different VCGs (plate D). Therefore, based on the complementation tests, 10 VCGs were established for 53 HSC isolates of *F. oxysporum* (Table 2). The genetic diversity (number of VCGs/number of isolates) of *F. oxysporum* isolates was 0.187.

Based on the complementation test, three VCGs were established in 22 *F. semitectum* isolates. The ratio of VCGs was 0.136 as depicted in Table 2. Figure 2 shows the complementation test of *F. semitectum* HSC (isolates B745T and B765T). The pairing between B601T and B605T showed

that they belonged to the same VCGs (Figure 2; Plate G), whereas, the pairing between B601T and B767T depicted that they belonged to the different VCGs (Figure 2; Plate H).

Five isolates of *F. subglutinans* were categorized into two VCGs (Table 2). The typical complementation test of HSC on MM for *F. subglutinans* (isolates B658T and B681T) are presented in Figure 2 (Plates I and J). The pairings in plates L and M showed no complementation between vegetatively incompatible isolates, B658T and B679T, as well as B658T and B681T. The genetic diversity of *F. subglutinans* isolates was 0.4.

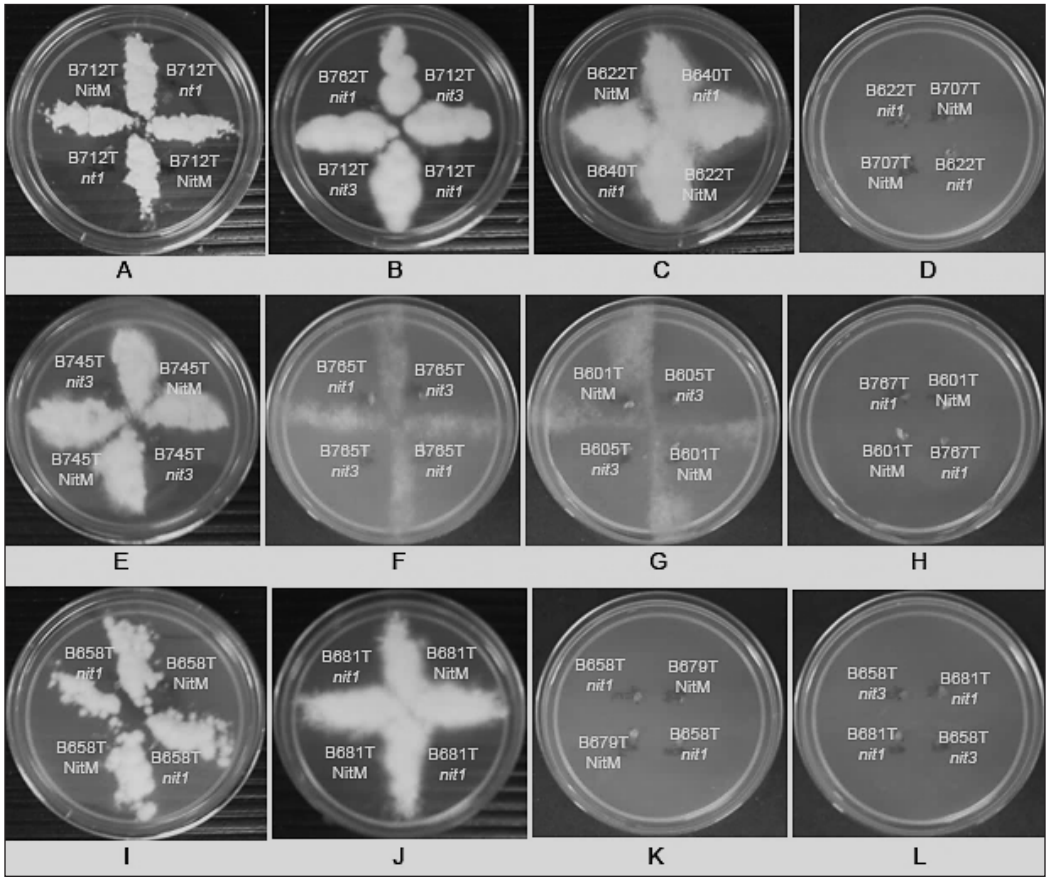

Figure 2. HSC strain of *F. oxysporum* on MM: (A) Isolate B712T (B) Isolate B762T. Complementation test of *F. oxysporum* strains on MMC: (C) Heterokaryon formed between vegetatively compatible isolates B622T and B640T. (D) No heterokaryon occurred between vegetatively incompatible isolates B622T and B707T. HSC strain of *F. semitectum* on MM: (E) Isolate B745T; (F) Isolate B765T. Complementation test of *F. semitectum* on MM: (G) Heterokaryon formed between vegetatively compatible isolates B601T and B605T (H) No complementation occurred between vegetatively incompatible isolates B601T and B767T. HSC strain of *F. subglutinans*: (I) Isolate B658T (J) Isolate B681T. Complementation test of *F. subglutinans* on MM: No complementation was formed between vegetatively incompatible isolates (K) B658T and B679T; and, (L) B658T and B681T

Table 2
Vegetative compatibility groups (VCGs) of Fusarium species isolated from post-harvest fruit rot of tomato in Selangor, Malaysia

VCGs	Strain	Species identification based on VCGs	Location
A01	B622T	*F. oxysporum* f.sp. *lycopersici*	Serdang
	B623T	*F. oxysporum* f.sp. *lycopersici*	Serdang
	B624T	*F. oxysporum* f.sp. *lycopersici*	Serdang
	B625T	*F. oxysporum* f.sp. *lycopersici*	Serdang
	B632T	*F. oxysporum* f.sp. *lycopersici*	Serdang
	B633T	*F. oxysporum* f.sp. *lycopersici*	Serdang
	B635T	*F. oxysporum* f.sp. *lycopersici*	Serdang
	B637T	*F. oxysporum* f.sp. *lycopersici*	Serdang
	B640T	*F. oxysporum* f.sp. *lycopersici*	Serdang
A02	B757T	*F. oxysporum*	Seri Kembangan
	B758T	*F. oxysporum*	Seri Kembangan
	B759T	*F. oxysporum*	Seri Kembangan
	B760T	*F. oxysporum*	Seri Kembangan
	B761T	*F. oxysporum*	Seri Kembangan
	B762T	*F. oxysporum*	Seri Kembangan
	B763T	*F. oxysporum*	Seri Kembangan
	B764T	*F. oxysporum*	Seri Kembangan
A03	B688T	*F. oxysporum*	Kajang
	B689T	*F. oxysporum*	Kajang
	B690T	*F. oxysporum*	Kajang
	B691T	*F. oxysporum*	Kajang
	B692T	*F. oxysporum*	Kajang
	B693T	*F. oxysporum*	Kajang
A04	B711T	*F. oxysporum* f.sp. *lycopersici*	Kajang
	B712T	*F. oxysporum* f.sp. *lycopersici*	Kajang
	B713T	*F. oxysporum* f.sp. *lycopersici*	Kajang
	B714T	*F. oxysporum* f.sp. *lycopersici*	Kajang
	B715T	*F. oxysporum* f.sp. *lycopersici*	Kajang
	B716T	*F. oxysporum* f.sp. *lycopersici*	Kajang
A05	B645T	*F. oxysporum* f.sp. *lycopersici*	Serdang
	B646T	*F. oxysporum* f.sp. *lycopersici*	Serdang
	B654T	*F. oxysporum* f.sp. *lycopersici*	Serdang
	B655T	*F. oxysporum* f.sp. *lycopersici*	Serdang
A06	B695T	*F. oxysporum*	Kajang
	B696T	*F. oxysporum*	Kajang
	B697T	*F. oxysporum*	Kajang
	B699T	*F. oxysporum*	Kajang

Table 2 *(continue)*

VCGs	Strain	Species identification based on VCGs	Location
A07	B703T	*F. oxysporum*	Kajang
	B704T	*F. oxysporum*	Kajang
	B705T	*F. oxysporum*	Kajang
	B706T	*F. oxysporum*	Kajang
A08	B707T	*F. oxysporum*	Kajang
	B708T	*F. oxysporum*	Kajang
	B709T	*F. oxysporum*	Kajang
	B710T	*F. oxysporum*	Kajang
A09	B717T	*F. oxysporum* f.sp. *lycopersici*	Semenyih
	B718T	*F. oxysporum* f.sp. *lycopersici*	Semenyih
	B719T	*F. oxysporum* f.sp. *lycopersici*	Semenyih
	B720T	*F. oxysporum* f.sp. *lycopersici*	Semenyih
A10	B725T	*F. oxysporum*	Puchong
	B726T	*F. oxysporum*	Puchong
	B727T	*F. oxysporum*	Puchong
	B728T	*F. oxysporum*	Puchong
A11	B1358T	*F. oxysporum*	Selayang
B01	B741T	*F. semitectum*	Shah Alam
	B742T	*F. semitectum*	Shah Alam
	B743T	*F. semitectum*	Shah Alam
	B744T	*F. semitectum*	Shah Alam
	B745T	*F. semitectum*	Shah Alam
	B746T	*F. semitectum*	Shah Alam
	B747T	*F. semitectum*	Shah Alam
	B748T	*F. semitectum*	Shah Alam
B02	B765T	*F. semitectum*	Ampang
	B766T	*F. semitectum*	Ampang
	B767T	*F. semitectum*	Ampang
	B768T	*F. semitectum*	Ampang
	B769T	*F. semitectum*	Ampang
	B770T	*F. semitectum*	Ampang
	B771T	*F. semitectum*	Ampang
	B772T	*F. semitectum*	Ampang
B03	B601T	*F. semitectum*	Sri Serdang
	B602T	*F. semitectum*	Sri Serdang
	B603T	*F. semitectum*	Sri Serdang
	B604T	*F. semitectum*	Sri Serdang
	B605T	*F. semitectum*	Sri Serdang
	B606T	*F. semitectum*	Sri Serdang

Table 2 *(continue)*

VCGs	Strain	Species identification based on VCGs	Location
C01	B678T	*F. subglutinans*	Selayang
	B679T	*F. subglutinans*	Selayang
	B680T	*F. subglutinans*	Selayang
	B681T	*F. subglutinans*	Selayang
C02	B658T	*F. subglutinans*	Sri Serdang

DISCUSSION

In this study, chlorate resistant sectors (CRSs) were successfully generated from all three species (*F. oxysporum*, *F. semitectum* and *F. subglutinans*) and using 4.5 - 6.0% $KClO_3$ on both MMC and PDC. A very few CRSs were obtained when 2.5 – 4.0% $KClO_3$ were used. All generated isolates of *F. oxysporum, F. semitectum* and *F. subglutinans* produced thin transparent mycelia on the minimal media (MM) made up of sodium nitrate ($NaNO_3$) as a sole nitrogen source that was required by the nitrate non-utilizing chlorate resistant sectors and the *nit* mutants. More than 1000 *crn* mutants (wild types) were generated in this study with *F. oxysporum* accounted for 50% of the total mutants, followed by *F. semitectum* (30%) and *F. subglutinans* (20%). All these mutants were then discarded.

Based on the phenotyping of the *nit* mutants on each of the four media containing different nitrogen sources, 676 *nit* mutants were generated from 81 *Fusarium* isolates. All *Fusarium* species were able to produce three different classes of *nit* mutants: *nit1, nit3* and NitM. *Fusarium oxysporum* produced 63% of the total *nit* mutants followed by *F. semitectum* (23%) and *F. subglutinans* (14%). Generally, *nit1* mutants were the most abundantly recovered (66%) on both MMC and PDC from all the *Fusarium* species, followed by *nit3* (21%) and NitM (13%). The frequency of *nit1* mutants was significantly higher on PDC than MMC as observed in the previous studies by Carvalho and Mendes-Costa (2011), Desjardins et al. (2000), Wang et al. (2010) and Zainudin et al. (2009). However, a different pattern was observed by Masratul Hawa (2008) of which the highest recovered mutant was *nit3*, contributing approximately 40% of the total mutants. Therefore, this result was inconsistent with the report of the present study and those from previous studies in which *nit1* mutants were obtained at greater frequencies in *F. oxysporum* (66%) (Desjardins et al., 2000; Somrith et al., 2011; Wang et al., 2010) and *F. poe* (63%) (Liu & Sunheim, 1996). Heterokaryons often with dense aerial mycelia were formed after 1 – 2 weeks incubation in the dark. The complementation of NitM between both *nit1* and *nit3* often formed a dense heterokaryon, whereas the pairing of *nit1* and *nit3* formed a weak heterokaryon and required a longer incubation period of 1 – 3 weeks (Carvalho & Mendes-Costa, 2011; Desjardins et al., 2000; Masratul Hawa, 2008; Pasquali et al., 2005).

Based on the results of the present study, all *Fusarium* species were vegetatively compatible but a greater magnitude of heterokaryosis occurred in 11 VCGs of *F. oxysporum* followed by 3 VCGs of *F. semitectum* and 2 VCGs of *F. subglutinans*. Previous studies indicated that heterokaryosis has been recognized in numerous species of *Fusarium* including *F. culmorum* (Balali & Iranpoor, 2006; El-Fadly, El-Kazzaz, Hassan, & El-Kot, 2008), *F. dimerum*, *F. chlamydosporum*, *F. avenaceum*, *F. scirpi*, *F. acuminatum*, *F. equiseti, F. graminearum, F. sambucinum, F. sulfureum, F. lateritium*, *F. graminearum* (El-Fadly et al., 2008), *F. fujikuroi* (Zakaria, Hsuan, & Salleh, 2011)*, F. proliferatum, F. verticillioides* (Zainudin et al., 2009), *F. oxysporum* (Gunn & Summerall, 2002; Leong, Latiffah, & Baharuddin, 2010; Swift, Wickliffe, & Schwartz, 2002), *F. sacchari* (Athman, 2006; Zakaria et al., 2011), *F. solani* (Balali & Iranpoor, 2006; Wang, Brubaker, & Burdon, 2004), *F. semitectum* (Abd-Elsalam, Schniederl, Asran-Amal, Khalil, & Verrett, 2003; Hawa, Salleh, & Latiffah, 2010), and *F. subglutinans* (Desjardins et al., 2000; Zheng & Ploetz, 2002). Based on the results of this study, all species isolates belonged to the same locations were grouped into the same VCGs. Similar observation was noted by Carvalho and Mendes-Costa (2011), Pasquali et al. (2005), Somrith et al. (2011) and Swift et al. (2002).

CONCLUSION

In conclusion, the complementation results indicated that *Fusarium* isolates would fuse to form a stable heterokaryon due to the genetic exchange of their cellular constituents. In the present study, it was evident that *Fusarium* isolates originated from the same geographical areas were clustered into the same VCGs. The results obtained essentially proved that VC is a useful tool for studying the diversity among the isolates of *Fusarium* species, *F. oxysporum, F. semitectum* and *F. subglutinans*. To the best of our knowledge, this was the first report on the classification of *F. oxysporum, F. semitectum* and *F. subglutinans* into VCGs isolated from post-harvest *Fusarium* fruit rot of tomato in Selangor and in the Peninsular Malaysia. Therefore, the findings of the study would possibly draw the attention of the concerned authority to formulate suitable measures to investigate the growth of the pathogens of this important cash crop.

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Use of Path Analysis to Investigate Association between Body Weight and Body Dimensions (Body Metric Traits) in Nigerian Locally Adapted Turkeys

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ABSTRACT

A regression model for estimate of body weight (BW) through body metric traits of Nigerian locally adapted turkeys using path analysis was developed. A total of 150 comprising 86 Lavender and 64 White plumages of both sexes were used. The body weight and five body metric traits which are the thigh length (TH), Keel length (KL), Shank length (SL), Breast girth (BG), Wing length (WL), Wing span (WS) and Body length (BL) were measured and analysed. Sex and genotype had significant effect on the body weight (Lavender: Male 565.26±36.79 g, Female 543.61±53.74 g; White: Male 629.06±46.21 g, Female 394.54±63.40 g) at 8 weeks of age. Pearson's correlations results between body weight and TH, KL, SL, BG, WL, WS as well as BL in both sexes indicated positive and highly correlation. However, Path analysis indicated that BL (path coefficient = 0.560; $p < 0.05$) only had positive and significant direct influence on the male body weights. In female turkeys, direct influence of other biometric traits was not significant on body weight. Also, KL (path coefficient = 0.497; $p < 0.05$) had the highest positive and significant direct influence on the body weight closely trailed by the BL (path coefficient = 0.391; $p < 0.01$). The KL via BL (R^2= 0.18) had the highest influence on the female body weight. The other biometric traits had non-significant direct influence. TH had the utmost input to the body weight of the male turkeys via BL (R^2= 0.065). Thus, selecting and improving BL for males; KL and BL for females will contribute positively to the BW of Nigerian locally adapted turkeys.

Keywords: Biometric traits, locally adapted turkey, Nigerian, path analysis

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INTRODUCTION

Assessing animals visually is a common individual method of judging traits (Abanikannda, Leigh, & Olutogun, 2002; Yakubu, 2010; Yakubu & Mohammed, 2012). Essentially, live body weight is mostly used to appraise body development and conformation features in animals such as turkeys (De Brito Ferreira, Ramos De Carvallo, Nogueria Baros, & De Assis Mello, 2000; Kuzelov, Taskov, Angelkova, Atanasova, & Mladnov, 2011). Association among body features provides valuable information on performance, productivity as well as carcass characteristics of animals. Hence, estimation of genetic parameters in animal genotyping programmes from quantitative determination of size and shapes of animals are necessary in order to improve selection for growth by identifying early and late maturing animals of different sizes (Yakubu & Mohammed, 2012).

In the past, morpho-biometric traits have been used to forecast body weight of animals in which different regression models have been used to exploit the correlation between these traits. These models have aided in ensuring on-farm measurement of body weight to be less tiresome as well as lessen risks related with the use of weighing scale particularly in animals with large body size.

A number of morpho-biometric traits are not only related with the response trait (i.e. body weight) but also among themselves (Sahu, 2013). Therefore, the extent of relationship between response trait and predictor traits determined by correlation analysis does not reflect definite strength of association. This is because other traits from predictor traits might have influenced on both response trait and any predictor trait. Since the predictor traits are not only interrelated with the response trait but also among themselves, there is a need therefore to establish the direct and indirect (via other traits due to interrelationship) effects of each predictor trait on the response trait (Sahu, 2013; Yakubu & Mohammed, 2012). Hence, path analysis which is a quantifiable method of evaluating the relative extent of causal effect of variables represented in a system of presumed causal interactions (Griesemer, 1991) can be adopted. Path analysis is a subset of Structural Equation Modelling and standardized partial regression analysis that involves a closed variables' system which are linearly related (Ullman, 1996). Partitioning of correlation coefficient into parts is permitted by path analysis (Topal & Esenbuga, 2001; Woods, Wynne, Ploeger, & Leonard, 2003). The first part is the path coefficient that measures the direct influence of the predictor trait on the response trait. The second part assesses the indirect influence of the predictor trait on the response trait through other predictor traits (Pfeiffer & Morris, 1994). Ahn (2002) explained path analysis model as an extension of multiple regression model that allowed determination of independent variables that affected mostly the response variable. Hence, it serves as complement method to regression analysis.

However, the use of a multivariate analysis technique such as path analysis

has not been exploited in revealing interrelationship between the body weight and body metric traits of turkeys in Nigeria. The present study therefore investigated the direct and indirect causal influences between body weight and body metric traits of Nigerian locally adapted turkey.

MATERIALS AND METHODS

Experimental Animals and Management

A total of 150 Lavender (male = 41, female =45) and White (male=31, female=33) plumages of locally adapted Nigerian turkeys raised intensively were used for this study. The birds were reared on deep litter in poultry farm house for the management. Water and feed were provided *ad-libitum* to all the turkeys. The turkeys were fed commercial feed from day old until the end of the experiment. This research was carried out at the Poultry Unit, Directorate of University Farm of the Federal University of Agriculture, Abeokuta, Nigeria. All the protocols for this research were approved by the Animal Care and Use Committee of the Federal University of Agriculture, Abeokuta, Nigeria.

Biometric Traits Measured

The traits measured include body weight (BW) and five body metric traits measured at 8 weeks. The body metric traits include: Body length (BL) as distance from the tip of the beak, through the body trunk to the tail; Body girth (BG) as circumference of the breast region; Wing length (WL) as length of the wing from the scapula joints to the last digit of the wing; Shank length (SL) as length of the tarso-metatarsus from the hock joint to the metatarsal pad; Keel length (KL) as length of the meta-sternum. Measurements were done using a tape rule except the body weight which was measured using a balance scale.

Statistical Analysis

Body weight and linear body dimensions averages were computed with their Pearson correlations. In order to have direct appraisal of values that reflect the relative importance of body dimension traits so as to explain variation in the body weight (Seker & Serin, 2004), Standardized partial regression coefficients called path coefficients (beta weights) were also computed.

The path coefficient from an independent variable (X) to a dependent variable (Y) (Mendes, Karabayir, & Pala, 2005) is stated below:

$$P_{yx_i} = \frac{b_i S_{xi}}{S_y}$$

Where:

P_{yxi} = path coefficient from X_i to Y (i= BL, BG, WL, SL, KL),

b_i = partial regression coefficient,

S_{Xi} = standard deviation of X_i,

S_Y = standard deviation of Y

The multiple linear regression model used is given below:

$$Y = \alpha + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_5X_5 + \varepsilon$$

Where:

Y = endogenous variable (body weight),
α = intercept,
b's = regression coefficients,
X's = exogenous variables (BL, BG, WL, SL, KL),
ε = residual error assumed to be normally distributed with null mean and unit variance.

T-test was adopted to verify significance of each path coefficient in the multiple linear regression model as stated by Sangun, Cankaya, Kayaalp and Alkar (2009):

$$t_j = \frac{b_j - \beta_j}{\sqrt{var(b_j)}} \sim t_{\alpha(n-p-1);} \qquad j=1,2,...,p$$

Where:

$var(b_j)$ = the diagonal member of matrix $s^2 (X'X)^{-1}$,

s^2 = Residual mean square gotten from Analysis of variance

The indirect effects of X_i on Y through X_j were calculated as follows:

$$IE_{YXi} = r_{XiXj}P_{Y.Xj}$$

Where

IE_{YXi} = the indirect effect of X_i via X_j on Y,
r_{XiXj} = correlation coefficient between i^{th} and j^{th} independent variables,
$P_{Y.Xj}$ = path coefficient that showed the direct effect of j-biometric traits on the body weight.

Path analysis was used to segregate Coefficient of determination (R^2) into its parts as follows:

$$R^2 = P^2_{Y.X1} + P^2_{Y.X2} + P^2_{Y.X3} + P^2_{Y.X4} + P^2_{Y.X5} + 2r_{X1X2}P_{Y.X1}P_{Y.X2} + 2r_{X1X3}P_{Y.X1}P_{Y.X3} + 2r_{X1X4}P_{Y.X1}P_{Y.X4} + 2r_{X1X5}P_{Y.X1}P_{Y.X5} + 2r_{X2X3}P_{Y.X2}P_{Y.X3} + 2r_{X2X4}P_{Y.X2}P_{Y.X4} + 2r_{X2X5}P_{Y.X2}P_{Y.X5} + 2r_{X3X4}P_{Y.X3}P_{Y.X4} + 2r_{X3X5}P_{Y.X3}P_{Y.X5} + 2r_{X4X5}P_{Y.X4}P_{Y.X5}$$

Where:

$P^2_{Y.X1}$ = direct effects of biometric traits (BL, BG, WL, SL, KL) contributing to the body weight;

$2r_{XiXj}P_{Y.Xi}P_{Y.Xj}$ = combined effects of biometric traits (BL, BG, WL, SL, KL) contributing to the body weight.

The biometric traits were vetted for multicollinearity problems using variance inflation factors (VIF) and tolerance (T) values. All analyses were done in SPSS (2001).

RESULTS

Effect of Interaction between Sex and Genotypes on Body Weight and Biometric Traits of Nigerian Locally Adapted Turkeys

The least square means of the biometric traits studied in the Nigerian locally adapted turkey is shown in Table 1. Genotypes had significant ($P < 0.05$) effect on all traits. The White male turkey was significantly ($p <$

0.05) heavier and had longer biometric traits dimension than its female counterpart, but when compared with the Lavender male and female genotypes, the traits did not differ significantly ($p > 0.05$).

Table 1
Interaction effect of sex and genotype on body weight (g) and biometric (cm) traits of the locally adapted Nigerian turkeys (LSM ± S.E)

	Lavender		White	
Traits	Male	Female	Male	Female
Body weight	565.26±36.79^{ab}	543.61±53.74^{ab}	629.06±46.21^{a}	394.54±63.40^{b}
Thigh length	9.65±0.26^{ab}	9.93±0.42^{ab}	10.53±0.40^{a}	8.79±0.61^{b}
Keel length	11.70±0.33^{ab}	12.02±0.51^{a}	12.76±0.47^{a}	10.16±0.76^{b}
Shank length	6.96±0.20^{ab}	6.92±0.29^{ab}	7.61±0.29^{a}	6.10±0.47^{b}
Breast girth	19.67±0.50^{ab}	19.98±0.83^{a}	21.50±0.77^{a}	17.25±1.15^{b}
Wing length	10.47±0.30^{ab}	10.76±0.45^{ab}	11.77±0.45^{a}	9.35±0.71^{b}
Wing span	23.19±0.64^{ab}	23.52±1.01^{ab}	25.07±0.95^{a}	20.75±1.75^{b}
Body length	18.53±0.63^{ab}	19.50±1.07^{ab}	20.72±0.98^{a}	16.88±1.66^{b}

Means in the same row bearing different superscript differ significantly ($P<0.05$)

Pearson Correlation of the Body Weight and Biometric Traits

Correlations between body weight (BW) and the biometric traits are shown in Table 2. There is high, positive and significant ($p < 0.05$) correlations between the BW and biometric traits. In male turkey, the correlation coefficients values ranged from 0.73 – 0.96, while in female turkey, a range of 0.84 – 0.96 was obtained. The highest

Table 2
Pearson correlation coefficients (with their significant level) of the body weight and body linear dimensions

	BW	TL	KL	SL	BG	WL	WS	BL
BW		0.76***	0.76***	0.73***	0.75***	0.77***	0.76***	0.80***
TL	0.89***		0.94***	0.94***	0.94***	0.92***	0.93***	0.92***
KL	0.93***	0.96***		0.94***	0.93***	0.92***	0.93***	0.92***
SL	0.86***	0.90***	0.92***		0.93***	0.90***	0.91***	0.91***
BG	0.91***	0.93***	0.96***	0.89***		0.92***	0.93***	0.91***
WL	0.84***	0.90***	0.91***	0.87***	0.91***		0.96***	0.92***
WS	0.87***	0.91***	0.92***	0.88***	0.92***	0.95***		0.92***
BL	0.92***	0.92***	0.95***	0.89***	0.92***	0.88***	0.91***	

***: $p<0.001$; Male: Lower diagonal; Female: Upper diagonal

association with body weight in the male turkeys was with body length ($r = 0.80$, $p < 0.05$), while the association between BW and keel length (KL) was the strongest association recorded for the female turkey ($r = 0.93$, $p < 0.05$). The significant ($p < 0.05$) correlation between the biometric traits measured were all high. On the overall, correlation between wing length and wing span (WS) ($r = 0.96$, $p < 0.05$) for male birds and the strongest association in female birds was with keel length having a 96% correlation ($r = 0.96$, $p < 0.05$) with thigh length (TL) and breast girth (BG).

Path Coefficient of the Biometric Traits

The path coefficients of the biometric traits are shown in Table 3. In male turkey, the results revealed that body length (BL) had the highest direct influence on BW (path coefficient = 0.560; $p<0.05$). The direct effects of the other biometric traits (TL, KL, BG, WS, shank length, body length (BL) and wing length) were not significant ($p > 0.05$). The non-significant variables except wing length were realized indirectly through body length. In female turkey (Table 4), the highest direct contribution to BW was made by KL (path coefficient = 0.497; $p < 0.05$) and closely followed by BL. The direct effect of the other variables were not significant ($p > 0.05$).

Relative Contribution of the Linear Body Dimensions to Body Weight

Table 5 shows direct and combined influences of biometric traits on the BW difference. In female turkeys, the highest single contributor to the BW difference was KL ($R^2 = 24.69\%$), this was followed closely by BL ($R^2 = 15.30$). Among the interaction of the variable pairs for male turkeys, the combination of thigh length and body length was the highest ($R^2 = 6.50$). The sum of

Table 3
Direct and indirect influences of biometric traits (with their significant level) on body weight of male locally adapted Nigerian turkeys

Variables	Correlation with body weight	Direct Effect	Total indirect effect	Indirect effect						
				TL	KL	SL	BG	WL	WS	BL
TL	0.763***	0.127^{ns}	0.636		0.061	-0.106	-0.006	0.170	0.000	0.517
KL	0.759***	0.065^{ns}	0.694	0.119		-0.106	-0.006	0.169	0.000	0.517
SL	0.735***	-0.113^{ns}	0.847	0.119	0.060		-0.006	0.166	0.000	0.507
BG	0.746***	-0.006^{ns}	0.752	0.119	0.060	-0.105		0.169	0.000	0.509
WL	0.766***	0.184^{ns}	0.581	0.117	0.059	-0.102	-0.006		0.000	0.512
WS	0.763***	0.0004^{ns}	0.762	0.118	0.060	-0.103	-0.006	0.176		0.517
BL	0.797***	0.560***	0.015	0.117	0.060	-0.102	-0.006	-0.054	0.000	

***: $p<0.0001$; **: $p<0.01$; *: $p<0.05$; ^{ns}: $p>0.05$; TL: Thigh Length; KL: Keel Length; SL: Shank Length; BG: Breast Girth; WL: Wing Length; WS: Wing Span; BL: Body Length

Table 4
Direct and indirect influences of biometric traits (with their significant level) on body weight of female of the locally adapted Nigerian turkeys

Variables	Correlation with body weight	Direct Effect	Total indirect effect	Indirect effect						
				TL	KL	SL	BG	WL	WS	BL
TL	0.893***	-0.097^{ns}	0.990		0.478	-0.008	0.234	-0.207	0.133	0.360
KL	0.929***	0.497*	0.432	-0.094		-0.008	0.241	-0.211	0.134	0.370
SL	0.863***	-0.008^{ns}	0.872	-0.087	0.459		0.223	-0.201	0.128	0.349
BG	0.912***	0.251^{ns}	0.662	-0.091	0.476	-0.007		-0.211	0.134	0.361
WL	0.841***	-0.231^{ns}	1.072	-0.087	0.454	-0.007	0.229		0.139	0.346
WS	0.873***	0.146^{ns}	0.728	-0.088	0.456	-0.007	0.230	-0.220		0.357
BL	0.925***	0.391**	0.684	-0.089	0.470	-0.008	0.231	-0.054	0.133	

***: $p<0.0001$; **: $p<0.01$; *: $p<0.05$; ^{ns}: $p>0.05$; TL: Thigh Length; KL: Keel Length; SL: Shank Length; BG: Breast Girth; WL: Wing Length; WS: Wing Span; BL: Body Length

Table 5
Relative contribution of the body metric traits to body weight of the locally adapted Nigerian turkeys

Variable	Partial co-efficient of determination (R^2)	
	Male	Female
Thigh Length	0.02	0.00944
Keel Length	0.00	0.24686
Shank Length	0.01	0.00007
Breast Girth	0.00	0.06296
Wing Length	0.03	0.05349
Wing Span	0.00	0.02126
Body Length	0.31	0.15296
Combined effects		
TL via KL	0.008	-0.05
TL via SL	-0.013	0.00
TL via BG	-0.001	-0.02
TL via WL	0.022	0.02
TL via WS	0.000	-0.01
TL via BL	0.065	-0.03
KL via SL	-0.01	0.00
KL via BG	0.00	0.12
KL via WL	0.01	-0.10
KL via WS	0.00	0.07
KL via BL	0.03	0.18
SL via BG	0.00	0.00
SL via WL	-0.02	0.00

Table 5 *(continue)*

Variable	Partial co-efficient of determination (R^2)	
	Male	Female
SL via WS	0.00	0.00
SL via BL	-0.06	0.00
BG via WL	0.00	-0.05
BG via WS	0.00	0.03
BG via BL	0.00	0.09
WL via WS	0.00	-0.03
WL via BL	0.09	-0.08
WS via BL	0.00	0.05
Total Contribution	64.50	89.10
Residuals	35.50	10.90
Sum Total	100.00	100.00

R^2 was 64.50%, while the determination co-efficient for error was 35.50%. For females, keel length had the highest direct contribution (R^2 = 24.69%) to body weight, this was closely followed by body length (15.30%).

In male turkeys, BL had highest direct contribution to the BW variation (R^2 = 2%). The combined influences of TL and KL were the highest among the variable pairs (R^2 = 31.0%). The sum of R^2 of the independent and interaction of the dependent pairs in the present study for the male turkeys was 64.50%.

DISCUSSION

The morphological differences obtained in this study can be mainly attributed to genetic differences and sexual dimorphism. The differences between the White genotype sexes might be due to variation in rates and strategies of growth as well as metabolic rates. Sexual dimorphism normally results in sex differential hormonal action leading to differential growth rates (Baéza, Williams, Guémené, & Duclos, 2001) in White genotype sexes. BW could be estimated from biometric traits as revealed by positive and strong association between BW and biometric traits. Wolanski, Renema, Robinson, Carney and Fanchert (2006) reported that in the absence of weighing scale, component parts of animal could be used to evaluate animal growth. Hence, biometric traits improvement will invariably result in a resultant improvement in the BW of the locally adapted turkey genotypes. High positive correlation between the traits suggests that they are under the same gene action (Yakubu, 2010).

In the direct effects (path coefficient), only BL of male indigenous turkey was significant while the other body measurements did not meet the significance threshold. The total value of the indirect for BL was small implying that the correlation

between BW and BL was largely due to direct influence. The larger indirect effect observed for the other traits implies that their correlation with BW was realized more indirectly than directly, this is evident from the non-significant effect of their direct effect on body weight. Thus, BL was the only important and useful predictor with predicting body weight in the locally adapted male turkey genotypes. For the female turkey however, KL and BL had significant direct effect on BW. The total value of indirect effect for most of the traits was larger than their direct effects. The large indirect effect obtained indicates that a high percentage of the significant correlation observed between BW and the traits was primarily indirect than direct. Since only KL and BL had significant direct effects on BW, it means they could be used in the estimation of BW in female locally adapted Nigerian turkeys. Thus, selecting and improving BL for males, KL and BL in female turkey will impact positively on the BW of the locally adapted turkey genotypes.

CONCLUSION

In accordance with the result of this study, there were both sex and genotype differences in the morphometric traits of the locally adapted turkey genotypes studied. Pearson's correlations values shown that BW had definite and great association with TL, KL, SL, BG, WL, WS and BL. Path analysis however indicated that BL only had positive and significant direct effect while the TH, KL, WL, WS had positive but non-significant direct effect on the male body weights while the KL had the highest positive and significant direct effect on the female BW followed by the BL with other traits having non-significant direct effect. Thus, the highest contribution to the BW of the male turkeys was by TL via BL while in the female turkeys the KL via BL had the highest contribution.

Therefore, the BW of locally adapted Lavender and White turkey genotypes could be appraised with a high grade of precision using prediction indices like BL for males, KL and BL for females. Also, selection of locally adapted turkeys could be done using biometric traits in order to increase meat production.

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Distribution, Diversity and Abundance of Ferns in A Tropical University Campus

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ABSTRACT

This study assessed the diversity and abundance of ferns species in ten 10 m × 10 m plots which were established each, in less disturbed forest, most-disturbed forest and urbanized areas within the main campus of Universiti Sains Malaysia. Distance of at least 50 m was maintained between plots in each study site. The life forms of the fern species were documented together with their relative abundances. Also, diversity indices of the study sites such as Shannon index, Simpson index, Margalef index and Fisher's alpha were determined. Non-asymptotic rarefaction-extrapolation analysis was carried out to determine the significance differences between the species richness of each study site. One way anova using pairwise permutation test was done to determine the significance differences between the diversity indices in the sites. A total of twenty-three fern species belonging to 14 families were identified. The most abundant ferns are *Lindsaea napaea* (63.4% in less-disturbed forest), *Pyrrosia lanceolata* (36.0% in most-disturbed forest and 47.0% in urbanized area). Urbanized area was observed to have more epiphytic ferns while less-disturbed forest was more popupated by terrestrial ferns. The most accommodating host tree with the highest number of epiphytic fern species is *Samanea saman.* The result of rarefaction-extrapolation analysis showed that less-disturbed forest is significantly richer in species than the other sites while the diversity indices of more-disturbed forest and urbanized area are significantly higher than less-disturbed forest. This was attributed to the common fern species which were found almost in all plots sampled in the more-disturbed sites. The Shannon index in all the sites was less than 2. Therefore,

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Universiti Sains Malaysia campus having a low diversity of ferns could be regarded as a disturbed environment due to the high level of developments in it.

Keywords: Diversity, ferns, Malaysia, *Pyrrosia lanceolate*, *Samanea saman*

INTRODUCTION

Ferns and fern-allies are known as seedless vascular plants in the plant kingdom Pteridophyta. Their body structures are differentiated into roots, stems, fronds and pinnae similar to other vascular plants. These groups of plants are cryptogamic in that they do not produce flowers, seeds and fruits and this differentiate them from higher plants. They are also similar to bryophytes and algae in their mode of reproduction by spores, but differ from them by possessing vascular tissues (Yusuf, 2010). Most ferns are found to occupy lower altitudes of tropical forests whereas they may also colonize the understories of some temperate forests (Rost, Barbour, Stockinga, & Murphy, 2006). About 4400 ferns species have been recorded in South East Asia out of which 1165 species were found from the Malaysian tropical rain forest (Parris & Latiff, 1997; Roos, 1996).

Malaysia, especially some parts of Penang State is well endowed with a wide diversity of ferns because the vegetation lies within tropical rain forests. An activity such as logging which has dominated Malaysia forests has constituted a great threat to the survival of these ferns (Saw & Chung, 2007). This coupled with insufficient information on plant species composition, distribution and their conservation in Malaysia made their study highly imperative. One other aspect that has been neglected in most researches is the economic uses of ferns (Yusuf, 2010). Beside their invaluable impact on the global plant diversity, ferns have been discovered to be good sources of medicine, food, aesthetics and fuel (Akomolafe & Sulaimon, 2018; Benjamin & Manickam, 2007; Jaman & Yusuf, 2010). Ferns also have the ability to clean the environment polluted with various heavy metals through a process called phytoremediation (Akomolafe, Dedeke, & Sirajo, 2013; Oloyede, Akomolafe, & Odiwe, 2013). Over the years, studies have also focused on their taxonomy and mere inventory while researches on using quantitative methods to determine their distributions and diversities are rare in Malaysia probably because of their cryptogamic nature.

In South East Asia, where Malaysia belongs, very few studies have been recorded on using quantitative methods to determine the diversity of ferns (Yusuf, Tan, & Turner, 2003). However, Johnson (1969) was the first recognized to carry out survey on herbaceous plants including ferns using quadrant method. Sato, Saw and Furukawa (2000) also made use of cubic quadrant methods to investigate the diversity of ferns in some man-made and natural forests in Malaysia. The documentation of distribution patterns of Pteridophytes diversities in Malaysia and some other parts of the world is regarded as very deficient compared to angiosperms and other higher plants.

Also, the recent work done by Rahmad, Mansor, Fadzly, Rosely and Mansor (2009) on the status of some forest reserves in Penang, Malaysia also focused more on the diversity of Angiosperms and Gymnosperms with no emphasis on Pteridophytes. The unrealistically low figure of ferns and mosses in most countries reveals the lack of adequate available information on these groups of plants (Mutke & Barthlott, 2005).

Quantitative knowledge of their spread in Penang State using Universiti Sains Malaysia (USM) campus as a case study could provide vital information on conservation threats and ecology which are unsatisfactory at the moment. Therefore, there is a need for greater investment in their taxonomic, geographical distribution and ecological studies in order to broaden the base of plant diversity in Malaysia using USM campus as a case study, hence this study. Conservation plans are needed to be put in place for these neglected and under-utilized plants of Penang, Malaysia.

MATERIALS AND METHODS

Study Area

The study area is the main campus of USM which is the second oldest university in Malaysia established in the year 1969. It is in the tropical belt region of Penang island of Malaysia. This peninsular is described as the floristically richest region of Indomalesian sub-kingdom. The main campus covers an area of 252.7 hectares comprising large canopy trees, lakes and a valley. Extensive land developments have taken place in the campus over the years since inception which has led to loss of some of the native plant species (Asma, Manshhor, Khairun, Mohammed, & Lee, 2009).

Sampling Techniques

Reconnaissance survey was initially conducted in the study area to ascertain the different vegetation types present (Oloyede, Odiwe, & Olujiyan, 2014). The study area was divided into three main sites comprising; the less-disturbed forest, most-disturbed forest and urbanized area based on visual observation of the rate of land developments and human encroachments. The less-disturbed forest is a reserved forest within a valley in the campus with very little human encroachment. The urbanized area is the totality of the areas developed into roads, lecture halls, office and residential complexes whereas most-disturbed forest is the forest occupying an intermediate position between less-disturbed forest and urbanized area. The sampling plots in each site was separated by a minimum distance of 50 m. Ten 10×10 m^2 plots were demarcated in each site, giving a total of 30 plots for the entire study area. This plot area has been stated as the suitable area for diversity study of Pteridophytes in natural and man-made forests (Yusuf et al., 2003). The non-random selective method was adopted where plots were preferentially located to ensure that at least one individual fern is present in each plot (Akinsoji et al., 2016). The geographic coordinates of each study sites were taken using a GPS Garmin eTrex 10 device.

Sample Collection and Identification

In all the plots, the fern species present were recorded and classified as aquatic, terrestrial, epiphytes and lithophytes. Each fern stipe (i.e. the entire frond emanating from the rhizome) is counted as an individual fern as most of the individual fern species usually emerge from a common inseparable underground rhizome thereby forming clumps. Fern specimens were subsequently identified using a taxonomic flora (Piggott, 1988) and the herbarium of USM. The voucher specimens were deposited in the herbarium of USM for references. For the epiphytic ferns, the host trees were also identified.

Statistical Analyses

The fern diversity indices such as Shannon index, Simpson index and Margalef index were quantified for each forest type using PAST software. The relative frequency of each fern species was calculated. A non-asymptotic species richness evaluator called rarefaction-extrapolation analysis using abundance data was carried out for species richness. Significant difference in fern species richness between the forest types was determined by means of confidence intervals, constructed using 50 bootstrap replicates (Addo-Fordhour, Rahmad, & Burham, 2016). This was done using software iNEXT (online version) (Chao, Ma, & Hsieh, 2016). Significant differences in the diversity indices between the forest types were determined using One-way ANOVA in PAST software.

RESULTS AND DISCUSSION

The geographical coordinates of all the study plots and map of the study area are presented in Table 1 and Figure 1. A total of twenty-three (23) ferns species belonging to 14 families were observed in all the sampled sites in USM. Thelipteridaceae and Polypodiaceae had the highest number of ferns species with 5 and 4 species respectively. More ferns species were observed in less-disturbed forests (15 species) than more-disturbed forest (11 sspecies) and urbanized area (11 species). The most abundant fern species in less-disturbed forest, most-disturbed forest and urbanized site are *Lindsaea napaea* (63.4%), *Pyrrosia lanceolata* (36.0%) and *Pyrrosia lanceolata* (47.0%) respectively (Table 2). Ferns have been reported to play very important roles in the ecology of tropical regions as they form a prominent portion of the species composition (Watkins, 2011). It is evident from our study that the study site has a considerable number of fern species. Similar studies carried out in some university campuses in Nigeria, a tropical country have recorded far less number of ferns compared to USM (Akinsoji et al., 2016; Akomolafe & Sulaimon, 2018; Oloyede et al., 2014). In addition, an inventory of ferns carried out in some natural and man-made forests in Johor and Singapore documented not more than eighteen (18) fern species in each forest (Yusuf et al., 2003). The less-disturbed forest is richer in ferns than more-disturbed forest and urbanized area. This is similar to Corbett, Bannister, Bell and Richards (2002) who observed lesser number of

Table 1
Geographical coordinates of study plots at Universiti Sains Malaysia main campus

S/N	LATITUDE (N)	LONGITUDE (E)	ELEVATION (m)
URBANIZED SITE			
1	5° 21' 17.784"	100° 17' 36.816"	21
2	5° 21' 18"	100° 17' 46.176"	11
3	5° 21' 17.892"	100° 17' 48.408"	13
4	5° 21' 18.36"	100° 17' 50.964"	13
5	5° 21' 12.6"	100° 17' 50.388"	18
6	5° 21' 14.688"	100° 18' 1.26"	13
7	5° 21' 18.648"	100° 18' 1.368"	41
8	5° 21' 19.512"	100° 18' 0.684"	10
9	5° 21' 32.796"	100° 18' 8.496"	22
10	5° 21' 29.556"	100° 18' 10.692"	15
MOST-DISTURBED FOREST			
1	5° 21' 15.336"	100° 17' 53.412"	46
2	5° 21' 12.996"	100° 17' 59.064"	14
3	5° 21' 10.188"	100° 18' 2.736"	16
4	5° 21' 17.604"	100° 18' 8.64"	22
5	5° 21' 18.612"	100° 18' 9.144"	10
6	5° 21' 20.988"	100° 18' 9.72"	14
7	5° 21' 21.456"	100° 18' 2.448"	23
8	5° 21' 21.708"	100° 18' 2.772"	17
9	5° 21' 18.468"	100° 18' 12.168"	18
10	5° 21' 16.524"	100° 18' 17.64"	25
LESS-DISTURBED FOREST			
1	5° 21' 37.764"	100° 18' 21.996"	18
2	5° 21' 36.252"	100° 18' 20.592"	16
3	5° 21' 36.612"	100° 18' 20.34"	19
4	5° 21' 37.26"	100° 18' 19.692"	24
5	5° 21' 38.16"	100° 18' 17.82"	16
6	5° 21' 38.592"	100° 18' 17.964"	23
7	5° 21' 38.52"	100° 18' 18"	19
8	5° 21' 38.808"	100° 18' 19.656"	20
9	5° 21' 36.684"	100° 18' 21.348"	15
10	5° 21' 36.72"	100° 18' 21.456"	16

fern species in the highly disturbed forest than less-disturbed one in some parts of Oklahoma USA. However, in terms of abundance, urbanized area seemed to have a larger number of individuals of ferns in each plot, followed by the more-disturbed forest. This is normal as it is expected for less-disturbed forest to have a lesser

Table 2
Distribution of ferns observed in the study area

S/N	Name of fern	Family	Study sites					
			Less-disturbed forest		Most-disturbed forest		Urbanized area	
			Presence/ absence	Relative abundance (%)	Presence/ absence	Relative abundance (%)	Presence/ absence	Relative abundance (%)
1	*Adiantum latifolium* Lam.	Adiantaceae	X	0	X	0	√	0.2
2	*Angiopteris evecta* (Forst.) Hoffm.	Marattiaceae	√	0.4	X	0	X	0
3	*Asplenium nidus* L.	Aspleniaceae	√	2.2	√	1.4	√	2.8
4	*Bolbitis virens* (Wall. Ex Hook. & Grev.) Schott	Lomariopsidaceae	X	0	X	0	√	0.4
5	*Cyathea contaminans* (Hook.) Copel.	Cyatheaceae	X	0	X	0	√	0.3
6	*Cyclosorus ecallosa* Holtt.	Thelipteridaceae	√	1.5	√	0.1	X	0
7	*Davallia denticulata* (Burm.) Mett.	Davalliaceae	√	1.0	√	2.3	√	8.5
8	*Drymoglossum piloselloides* (L.) Presl	Polypodiaceae	√	6.1	√	9.2	√	0.9
9	*Drynaria quercifolia* (L.) J.Sm.	Polypodiaceae	√	0.7	√	32.9	√	35.9
10	*Elaphoglossum callifolium* (Bl.) Moore	Lomariopsidaceae	X	0	√	0.3	X	0
11	*Lindsaea napaea* v.A.v.R.	Lindsaeaceae	√	63.4	X	0	X	0
12	*Lygodium circinnatum* (Burm.) Sw.	Schizaeaceae	√	0.6	X	0	X	0
13	*Merinthosorus drynarioides* (Hook.) Copel.	Polypodiaceae	X	0	√	1.4	X	0
14	*Nephrolepis biserrata* (Sw.) Schott	Nephrolepidaceae	√	3.9	√	2.4	√	1.6
15	*Pronephrium asperum* (Presl) Holtt.	Thelipteridaceae	√	1.1	X	0	X	0
16	*Pronephrium menisciicarpon* (Bl.) Holtt.	Thelipteridaceae	√	4.3	X	0	X	0
17	*Pronephrium salicifolium* (Hook.) Holtt.	Thelipteridaceae	√	2.4	X	0	X	0
18	*Pronephrium triphyllum* (Sw.) Holtt.	Thelipteridaceae	√	3.6	X	0	X	0
19	*Pteris venulosa* Bl.	Pteridaceae	√	0.7	X	0	X	0
20	*Pteris vittata* L.	Pteridaceae	X		X	0	√	0.8
21	*Pyrrosia lanceolata* (L.) Farwell	Polypodiaceae	√	7.7	√	36.0	√	47.0
22	*Salvinia molesta* D.S. Mitchell	Salviniaceae	X	0	√	6.8	X	0
23	*Scleroglossum minus* (Fee) C.Chr.	Grammitidaceae	X	0	√	7.3	√	1.6

KEY: √ means present, X means absent

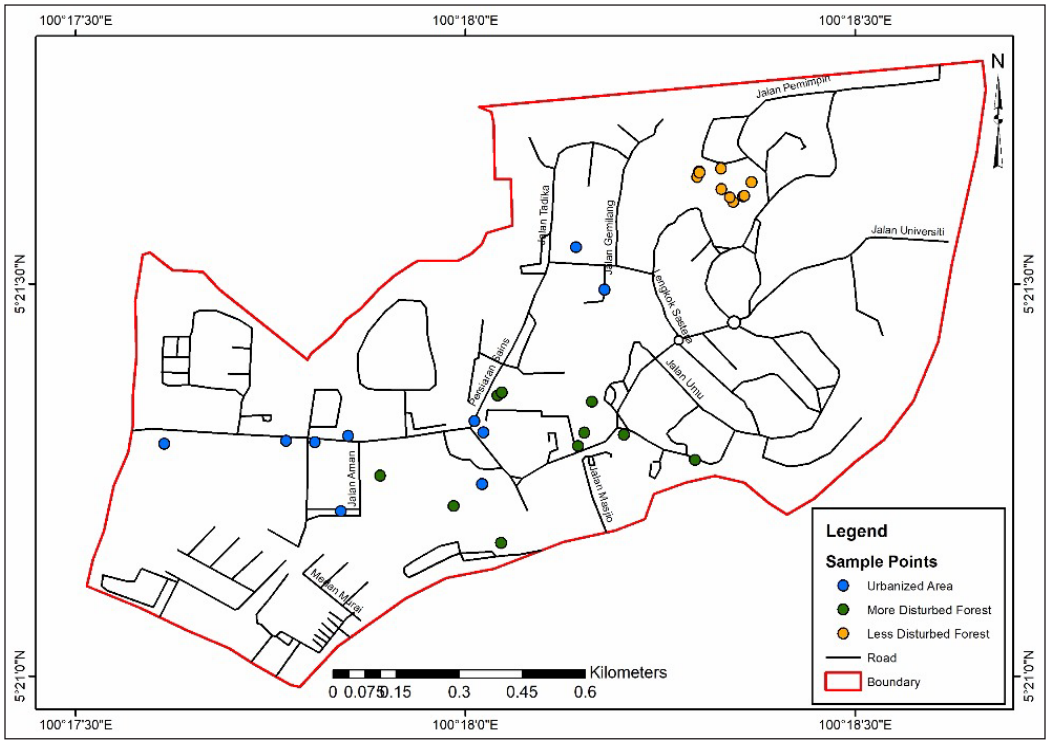


Figure 1. Study area map of Universiti Sains Malaysia main campus

abundance of individual ferns than other study sites. A similar trend was observed in Thailand, where highly disturbed sites had the lowest number of species, but the highest number of individual ferns in each plot than the less-disturbed ones (Sathapattayanon & Boonkerd, 2006).

Most-disturbed forest and urbanized site were discovered to have more epiphytic ferns than the other life forms (Table 3). Some of the species such as *Asplenium nidus, Davalia denticulata* and *Nephrolepis biserrata* were observed to be growing as epiphytes and terrestrial ferns. However, in

Table 3
Life forms of ferns in the study area

S/N	LIFE FORM	NUMBER OF SPECIES		
		Less-disturbed forest	Most-disturbed forest	Urbanized area
1	Aquatic	0	1	0
2	Epiphytic	3	6	7
3	Terrestrial	12	5	3
4	Lithophytic	0	0	3

less-disturbed forest, more terrestrial ferns were observed to be growing there than other life forms. The fact that urbanized area comprises more of epiphytic ferns could be an explanation for the higher abundance of individual ferns observed. Invariably, this is an indication of the suitability of the microenvironment to the growth and abundance of the epiphytic ferns. This also imply that despite the high rate of developments inside the campus over the years, there has been a high level of conservation of tree plants which hosted these ferns. These trees could have been deliberately conserved by the university in order to provide shade and purify the environment. This has a positive impact on the vascular plant diversity and sustainance of diverse animal communities such as birds and reptiles within the campus (Cruz-Angón & Greenberg, 2005; Magrach, Rodríguez-Pérez, Campbell, & Laurance, 2014). *Samanea saman* is the host tree that had the highest number of epiphytic ferns species in both urbanized site and most-disturbed forest (Table 4). These epiphytic ferns could be described as not host specific as they were found growing on more than

Table 4
Hosts of epiphytic ferns in the study area

S/N	Host plant	Epiphytic fern hosted
URBANIZED AREA		
1	Pinus nigra	Davallia denticulata, Pyrrosia lanceolata, Drynaria quercifolia
2	*Cassia fistula*	Asplenium nidus, Davallia denticulata, Drynaria quercifolia, Pyrrosia lanceolata
3	Samanea saman	Drynaria quercifolia, Davallia denticulata, Pyrrosia lanceolata, Drymoglossum piloselloides, Scleroglossum minus
4	*Ficus benjamina*	Davallia denticulata, Drynaria quercifolia, Nephrolepis biserrata, Bolbitis virens
5	*Azadiractha indica*	*Davallia denticulata, Scleroglossum minus*
6	*Tamarindus indica*	*Pyrrosia lanceolata*
7	*Milletia pinnata*	*Pyrrosia lanceolata*
8	*Delonix regia*	*Pyrrosia lanceolata*
9	*Adenanthera pavonina*	*Pyrrosia lanceolata, Drymoglossum piloselloides*
10	*Mimusops elengi*	*Pyrrosia lanceolata*
11	*Peltophorum petrocarpum*	*Pyrrosia lanceolata*
MOST-DISTURBED FOREST		
1	*Samanea saman*	*Drynaria quercifolia, Davallia denticulata, Pyrrosia lanceolata, Drymoglossum piloselloides, Scleroglossum minus*
2	*Azadiractha indica*	*Davallia denticulata, Scleroglossum minus, Pyrrosia lanceolata, Drynaria quercifolia*
3	*Bambusa vulgaris*	*Drynaria quercifolia*
LESS-DISTURBED FOREST		
1	Samanea saman	Drymoglossum piloselloides, Pyrrosia lanceolata, Drynaria quercifolia

one type of host trees. Their predominance in the more-disturbed forest and urbanized area is further supported by Oloyede et al. (2014) who observed similar occurrence of epiphytes in Obafemi Awolowo University campus, Nigeria. Also, the occurrence of *Asplenium nidus* as an epiphyte in this study area agrees with the report of Cummings, Martin and Rogers (2006) that the fern usually creates a special microclimatic conditions in disturbed forests where it is found thereby serving as habitat to various animals.

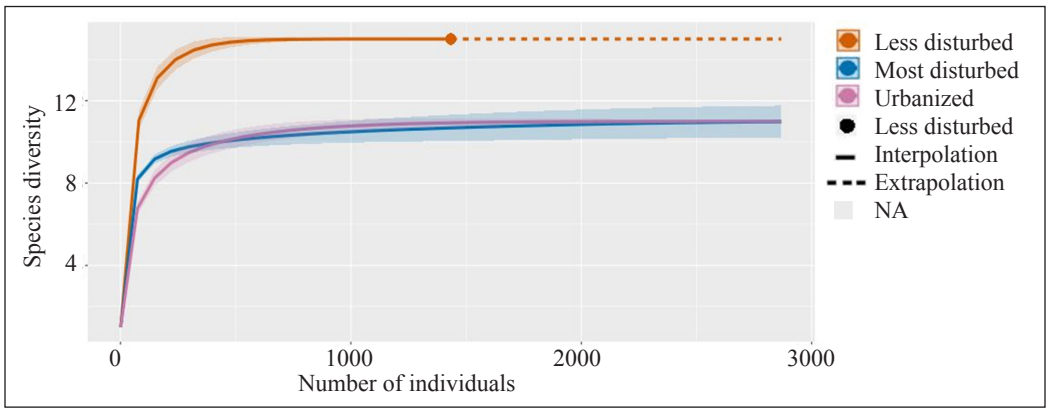
Contrariwise, the fewer number of terrestrial ferns observed in urbanized and more-disturbed forests could serve as justification to the reason why they are disturbed compared with the less-disturbed forest which had a higher number of terrestrial ferns. This agrees with the work of Sathapattayanon and Boonkerd (2006) who investigated the diversity of ferns along a gradient of disturbance in Thailand. They also observed that terrestrial ferns were more in less-disturbed forest than more-disturbed one. It could be assumed that terrestrial ferns are more susceptible to human threats than epiphytic and aquatic ferns in that they can be easily removed alongside with other herbaceous plants. Aquatic fern, *Salvinia molesta* was only found in the most-disturbed forest site, while lithophytic ferns were only observed at the urbanized site.

As shown in Table 5, urbanized area was observed to have the highest total number of individual ferns (3409), followed by more-disturbed forest (3257) and less-disturbed forest (1434). The species richness value of the rarefaction and extrapolation curve for less-disturbed forests was significantly higher than those of more-disturbed forest and urbanized area (Table 5 and Figure 2). There was overlap in the confidence interval of rarefied-extrapolated species richness of more-disturbed forest and urbanized area. The rarefied-extrapolated curves for diversity indices of all the sampled sites

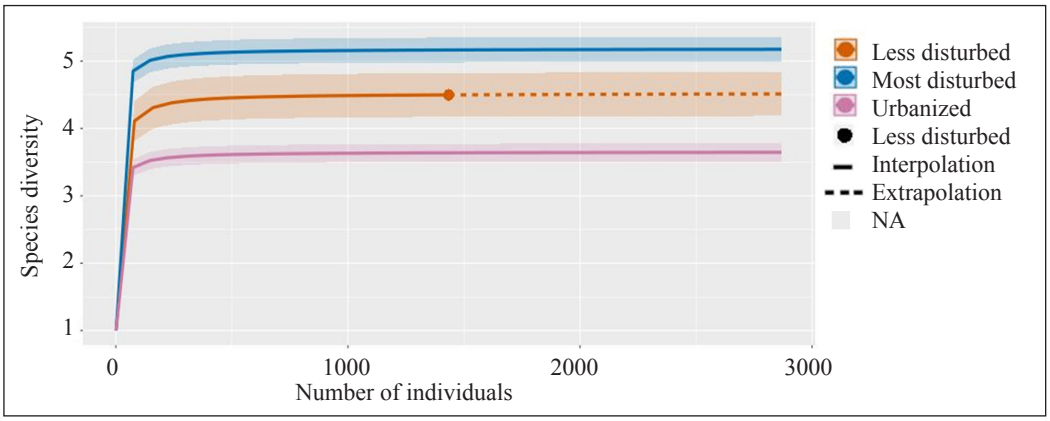
Table 5
Diversity indices of sampled sites in the study area

Diversity indices	Urbanized area	More-disturbed forest	Less-disturbed forest
Observed species richness	11	11	15
Rarefaction and extrapolation species richness*	11^{a}	11^{a}	15^{b}
Number of Individuals	3409	3257	1434
Simpson Index**	0.641^{a}	0.743^{b}	0.582^{c}
Shannon Index**	1.294^{a}	1.644^{b}	1.504^{c}
Species evenness**	0.332^{a}	0.471^{b}	0.299^{c}
Margalef index**	1.229^{a}	1.236^{a}	1.926^{b}
Fisher's alpha**	1.412^{a}	1.422^{a}	2.336^{b}

*Significant difference was determined using confidence interval (95%)
**Significant difference was determined using pairwise permutation test in PAST



(a)



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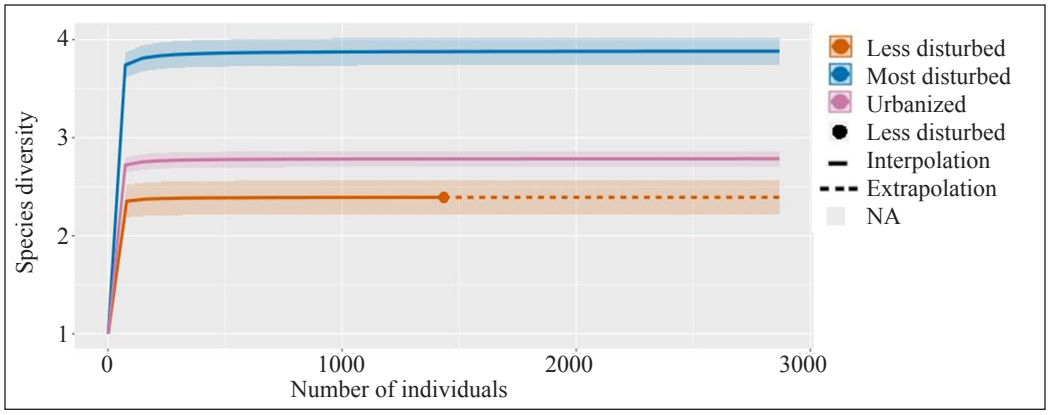

(c)

Figure 2. Individual based rarefaction and extrapolation curves for: (a) Species richness; (b) Shannon index; and (c) Simpson index. Solid lines represent rarefaction curves while dashed lines represent extrapolation curves. Each dot stands for the observed number of individuals

approached asymptote thereby showing the adequacy of sampling of ferns in the study area. The diversity indices such as Simpson index, Shannon index and Species evenness of more-disturbed forest are significantly higher than less-disturbed forest and urbanized area (Table 5). This could be because the common species were found in each plot. The common species observed in these sites are *Davalia enticulata, Drynaria quercifolia* and *Pyrrosia lanceolata.* These species could be regarded as pioneer fern species in most-disturbed areas (Boonkerd, 1996). The lower species evenness index recorded in less-disturbed forest could be attributed to the low number of individual ferns observed and the same species are not found in more than 2 or 3 plots. However, Fisher's alpha and Margalef indices for less-disturbed forests are significantly higher than those of less-disturbed forest and urbanized area ($P \leq 0.05$). According to Barbour, Burk, Pitts, Gillians and Schwartz (1999), a community with Shannon index greater than 2 is regarded as more diverse. It could then be said that all the sampled sites are less diverse in fern species since their Shannon index values are less than 2. This could have a long term negative effect in determining the stability and functionality of this ecosystem. Ecosystems with higher species diversity tend to be more stable and do have higher level of productivity due to the dynamic nature of the species driving them (Seabloom, 2007; Tilman, 1996).

CONCLUSION

Conclusively, the main campus of Universiti Sains Malaysia is a typical example of a disturbed ecosystem. It is obvious that the rate of developments over the years has reduced the diversity and richness of ferns particularly terrestrial ones in the disturbed areas. Efforts should therefore be made in restricting further developments into the less-disturbed forest which had the highest number of terrestrial fern species. This forest has been described as the only green space left in the campus which has been habitat to many native plants and birds (Asma et al., 2009). Therefore, it is recommended for other institutions across the world to maintain a greener environment by conserving the natural tree species within the university campuses. These trees will not only serve as shades and purifiers of the environment, but will also host varieties of birds and epiphytes.

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Light Spectrum Impacts on Early Development of Amphibians (Amphibia: Anura and Caudata)

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ABSTRACT

The aim of the research was to determine the role of color in the early development of three species of amphibians (*Triturus cristatus*, *Rana arvalis* and *Rana temporaria*). The experiments were carried out in laboratory conditions, there were run 4-7 replicates. Standard filter systems were used. We monitored pace and stage of eggs' development, the mortality of eggs, and length of the hatched larvae. The color of illumination did not affect the rate of embryonic development of species with a short period of early development (*R. arvalis* and *R. temporaria*). Mortality at embryonic stages varied in different species. In all species red light negatively affected the survival of developing eggs. The larvae that started active feeding were larger in all three species with green-blue light than with white and, the more red, light.

Keywords: Development, embryos, light spectrum, mortality, *Rana arvalis*, *Rana temporaria*, *Triturus cristatus*

INTRODUCTION

Light is one of the main factors affecting various aspects of amphibians' life. For example, there were studies of the constant and variable illumination effect on the larval development and growth of some anurans (Konstantinov, Vechkanov, Kuznetsov, & Ruchin, 2000; Kuznetsov & Ruchin, 2001; Ruchin, 2000, 2001, 2004a, 2004b), and effect of photoperiod (Bambozzi, Seixas Filho, Thomaz, & Oshiro, 2004; Delgado, Gutiérrez, & Alonso-Bedate, 1987; Kukita et al., 2015). At the same time, extreme lighting conditions such as 24-hour light or 24-hour darkness adversely affected growth and retarded the metamorphosis of *Xenopus laevis* (Delgado et al., 1987). Increasing photophase significantly retards the development of *Discoglossus pictus* larvae (Gutierrez, Delgado, & Alonso-Bedate, 1984). Increasing photophase significantly retards the development of

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Discoglossus pictus larvae (Gutierrez et al., 1984). Larvae reared under longer scotophase developed faster (grew larger and reached further developmental stages) than larvae reared under shorter scotophase. Among other things, light influences behavior, activity time, reproduction of amphibians (Buchanan, 2006; Dastansara, Vaissi, Mosavi, & Sharifi, 2017; Laurila, Pakkasmaa, & Merilä, 2001; Lindgren & Laurila, 2009; Marquis & Miaud, 2008).

The effect of environmental color on animal physiology and behavior is a developing field. As in earlier studies, light spectrum showed both improvement and disruption of animal welfare. These findings are supporting the rising interest to investigate and get a better understanding of the effects of such related rearing conditions on animal performance. As for the color of light impact on the amphibians, there are several studies. For example, we studied the effect of light spectrum on the growth and development of several species of anurans larvae (Ruchin, 2002, 2003, 2004a). Hailman and Jaeger (1974) and Hartman and Hailman (1981) studied some anura species and found some kind of phototaxis for violet, blue, green, yellow, orange and red light, but most species preferred blue and green and were repulsed by violet and red light. The purpose of our research is to determine the role of environmental color in the early development of several species of amphibians.

MATERIAL AND METHODS

In our experiments, we investigated the effect of illumination on the earlier development and mortality of eggs and prolarvae of three species of anurans and caudata, which have different duration of egg development in natural water bodies.

The great crested newt *Triturus cristatus* (Caudata: Salamandridae) is a common species in central Russia. The female lays solitary eggs in a leaf of higher aquatic vegetation and wraps it by the hind legs. The moor frog *Rana arvalis* and the common frog *Rana temporaria* (Anura: Ranidae) belong to the group of brown frogs. These are species with a short embryonic period (5-15 days) and larval development (up to 65 days). Frogspawn of a common frog is always a large cluster, reminding a "mat" that floats on the water's surface to prevent predators and increase the temperature inside. Moor frog, unlike the common one, has more loose egg mass. The investigated species are grown in zooculture. They are necessary for carrying out experimental works (for example, common and moor frogs) in biology, medicine and pharmacology. Larvae and adult amphibians are used for feeding in aquaculture and rearing reptiles. At the same time, their growing use and withdrawal from nature disturbs the balance in biocenoses, reduces their number in the wild, which increases the demand for animals grown in zooculture.

Spawn for the research, was selected in spawning reservoirs after fertilization from one couple of breeders (for one

experiment), which, among other things, facilitated the identification of species. One set of 10-20 eggs from one frogspawn was taken for every case (per Petri dish). The temperature was maintained at 20 + 0.01°C, the oxygen content in water 7.0-7.5 mg/l. The development stages were determined according to the methods indicated earlier (Ruchin, 2018) and recorded every 2-4 h. The developmental rate was calculated as the time necessary for a certain stage of each individual in the experiment. Daily selection of dead eggs was carried out (mortality was taken into account). Mortality was calculated by the relative number of dead individuals to the total number of individuals in the experiment. Water was changed multiply during development stages observations. The experiment was stopped after the larvae started active feeding. The body length of the hatched larvae was measured with an eyepiece micrometer with 0.01 mm accuracy.

All experiments were conducted with four to seven replications. The tabled data were averaged over all experimental series. For illumination, we used luminescent lamps (lamp LB), which do not heat when work, and provide sufficiently strong light flux. During the experiment we scattered light with the help of standard glass (Ruchin, 2004b). Here, one can see that 80-85% of light fall on a narrow zone of spectrum, this zone is a symbol for glass. Light intensity measured on water surface after the passing of light through color filter was 100 lx in all modes. In our experiments light fell at a vertical angle. In this case only 2% of light reflect independently on wavelength. After passing into water depths light was absorbed and diffused, that resulted in the reduction of light intensity depending on spectral structure. If taken into account reflection, absorption and scattering, light intensity on the bottoms of experimental aquariums was: under control lamp – 63.2 lx; by red light – 66.8 lx; by yellow – 64.0 lx; by green – 62.7 lx; by light blue – 62.3 lx; by blue – 58.3 lx. Due to small depth of Petri dishes the decrease of light intensity was insignificant and therefore we can claim that differences in data we got may be explained by spectral structure but not by light intensity.

Data between treatments and sampling times were compared by analysis of variance (ANOVA). The data were statistically processed using a standard method with Student's T Test.

RESULTS

The embryonic development of the crested newt under illumination of the green and blue zones of the spectrum was significantly reduced. In other cases, the rate of development little differed from control group (Table 1). As in other experiments (Ruchin, 2018), mortality of the crested newt was the highest during the period of embryonic development. At the same time, under yellow illumination, it significantly increased by 25.1% ($p < 0.05$), while in the case of blue illumination it decreased by 15.7% ($p < 0.05$) (Table 2). It was also observed that in the yellow light small individuals died and average sizes of surviving larvae became significantly higher

($p < 0.05$) (Table 3). The length of the larvae decreased by 5.5% ($p < 0.05$) in red light, while in the blue illumination it increased, as in the conditions of yellow illumination.

Table 1
Duration of early development in conditions of different colors of illumination (M + SE) (in days from fertilization)

Light spectrum	Beginning of the prolarvae emergence stage			The beginning of the active feeding stage		
	Triturus cristatus	*Rana temporaria*	*Rana arvalis*	*Triturus cristatus*	*Rana temporaria*	*Rana arvalis*
White	15.83±0.10	3.84±0.18	4.55±0.12	15.92±0.18	9.35±0.48	9.86±0.33
Red	15.85±0.11	3.86±0.14	4.51±0.22	16.04±0.14	9.24±0.44	9.80±0.38
Yellow	15.14±0.09	3.80±0.14	4.60±0.18	15.26±0.11	9.34±0.45	9.90±0.34
Green	15.01±0.09*	3.44±0.21	4.07±0.14	15.12±0.13	8.84±0.38	8.90±0.29
Light Blue	14.90±0.11*	3.46±0.11	4.06±0.11	15.08±0.14	8.78±0.36	9.05±0.34
Blue	15.87±0.12	3.55±0.15	4.37±0.17	16.02±0.15	8.94±0.37	9.78±0.25

* - reliable if $p<0.05$

Unlike the crested newt, in common frog, certain monochromatic zones of the spectrum had no significant effect on duration of the early development (Table 1). Some tendency to reduce the development time was traced under green, light-blue and blue light. In contrast to the duration of development, the mortality of embryos and pro-larvae of the common frog significantly increased under red and yellow light. In this case, in the first of these light modes, the length of the larvae decreased by 16.5% ($p < 0.01$). Green light most favorably influenced the early development of the common frog. In this mode mortality decreased and larvae length increased.

Table 2
Mortality rates of amphibian eggs in conditions of different colors of illumination (M±SE)

Light spectrum	Mortality in embryonic stages, %			Mortality in the prolarval stages, %		
	Triturus cristatus	*Rana temporaria*	*Rana arvalis*	*Triturus cristatus*	*Rana temporaria*	*Rana arvalis*
White	36.2±2.1	27.2±2.4	20.4±2.3	6.8±1.0	0	5.3±1.0
Red	38.6±3.8	47.1±6.7*	30.3±5.8	6.4±0.6	12.4±1.2***	4.7±2.3
Yellow	45.3±2.8*	40.5±2.4**	10.7±4.2	4.5±1.2	11.7±1.6***	0
Green	33.6±2.6	40.1±2.8*	9.1±3.6*	4.3±1.1	0	0
Light Blue	30.5±1.8*	33.0±4.6	20.6±2.5	5.1±0.9	0	1.2±0.6*
Blue	34.3±6.5	20.6±3.4	9.8±1.3*	6.3±2.2	0	0

* - reliable if $p<0.05$, ** - reliable if $p<0.01$, *** - reliable if $p<0.001$

As in the experiments on common frog, the duration of early development of the moor frog, differed little under various conditions (Table 1). Mortality at the embryonic stages in experiments with the moor frog has significantly decreased with green and blue light. After hatching, a significant mortality of individuals was observed only in control group and in red light. A considerable increase ($p < 0.05$) in the length of the moor frog larvae was recorded under green and blue light, while in red light this index decreased significantly ($p < 0.01$).

Table 3
Larvae body length in conditions of different colors of illumination (M±SE)

	Larvae body length, mm		
Light spectrum	Triturus cristatus	*Rana temporaria*	Rana arvalis
White	10.23±0.10	8.34±0.09	8.10±0.09
Red	9.67±0.07*	6.96±0.12***	6.90±0.13**
Yellow	10.75±0.09*	8.27±0.08	8.08±0.11
Green	10.45±0.05	7.39±0.08**	7.69±0.09*
Light Blue	10.84±0.11*	8.30±0.07	8.51±0.08*
Blue	10.30±0.04	8.56±0.06*	8.41±0.10

* - reliable if $p<0.05$, ** - reliable if $p<0.01$, *** - reliable if $p<0.001$

DISCUSSION

The species of amphibians we have studied lay their eggs in the upper layers of the water to a depth of no more than 15-20 cm. Considering that the water in the spawning ponds is transparent at this time, the rays of different wavelengths penetrate well into the water and must affect the embryonic development of the species. Amphibian eggs range in color from cream to black. Melanin pigmentation, which imparts the dark color, is typically found on the dorsal hemisphere. Eggs that are hidden from the sun and laid under debris, among leaves, or in foam nests tend to be lighter in color than those that are fully exposed to sunlight. Eggs of aquatic-breeding salamanders tend to be brown, but the eggs and embryos of most species of frogs and toads that breed in open water are nearly black (Wright & Wright, 1949).

Most research focus on the effect of ultraviolet radiation on the eggs of amphibians (Anzalone, Kats, & Gordon, 1998; Blaustein & Belden 2003; Cummins, Greenslade, & McLeod, 1999; Langhelle, Lindell, & Nyström, 1999), and there is practically no evidence on how does visible spectrum part effect the frogspawn. For example, Terentiev (1950) noted that eggs of common frog ceteris paribus developed in the dark and in the light with the same speed. Sytina and Nikol'skaya (1984) had experimentally proven that, despite the temperature increase in in the center of the

spawn, eggs of this species from the upper, most illuminated layers, developed and hatched faster, even though the temperature was lower. In the experiments of Ankley et al. (2000) intense sunlight caused high mortality of developing embryos.

Our experiments showed that environmental color affects the development of amphibians' eggs. However, its influence differs depending on parameters of development, on species, and on embryonic development duration. This is also confirmed by experiments on four similar in biology species of anurans (Ding, Lin, Zhao, Fan, & Wei, 2014). For example, light spectrum doesn't have any significant influence on separate stages of embryogenesis in anurans with short embryonic development (*Rana temporaria* and *Rana arvalis*). On the other hand, eggs of newts, which have four times longer development cycle than the eggs of anurans, developed faster than control group when exposed to green and blue light. Apparently, a certain time is necessary for the manifestation of a stimulating or negative effect of the light spectrum. We obtained reliable information in the analysis of egg survival in different lighting conditions. It turned out that the mortality of embryos and prolarvae increases within the long-wavelength part of the spectrum and decreases with green-blue illumination. Unfortunately, there is very little information about the light spectrum effect on the eggs of amphibians. However, our data are consistent with those obtained in some fish species: eggs development of *Acipenser baerii*, *Dicentrarchus labrax*, *Gadus morhua* and *Scophthalmus maximus* improved when exposed to blue-green spectrum rays (Ruchin, 2016; Sierra-Flores et al., 2016; Villamizar et al., 2011). We assume that the short-wave part of the spectrum (green and blue light) induces the development of amphibian embryos, actively affecting certain stages of development. At the same time, this effect does not become evident immediately, but manifests itself after a certain period of time. It becomes clear from experiments with eggs of anurans, in which the development of eggs is faster than that of caudata.

The data obtained correlate with the results of our previous experiments (Ruchin, 2002, 2003, 2004a), which showed better growth and development of larvae in certain amphibian species under blue-green illumination. Thus, the early development parameters of these species, as well as the prolonged larval development, depended on the environmental color. Obtained results partially agree with Jung's conclusion (Berkovich, 1953) on the negative effect of red light on the growth of tadpoles.

CONCLUSION

Thus, the influence of monochromatic illumination in the visible part of the spectrum for different species is specific. The authors showed that light intensity effect on the growth and development of larvae depends on the species. We showed that the light spectrum does not affect the rate of embryonic development of species with a short period of early development, but affects species with a long embryonic

period. At the same time, different zones of the spectrum differently affect such significant development parameters as the survival rate and size of the larvae that started active feeding. Favorable effect of green-blue light is common for all the three species of amphibians. Unfortunately, our and literary data are still not enough to make assumptions about the effect of illumination color on amphibian eggs. Therefore, for the construction of hypotheses in the future, it is necessary to conduct similar studies on species with other ecological needs.

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Short Communication

Physical Measurement of the Expansion Rate of Anisotropic Tissue Expander in the Skin of the Horse

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ABSTRACT

Tissue expansion technique is one of the most important innovations in skin reconstructive surgery. In this study, anisotropic self-inflating hydrogel tissue expanders were implanted subcutaneously at different anatomical locations; frontal, right shoulder and right forelimb of horses. The resulting skin expansion were observed and recorded daily for the duration of 28 days by manual physical measurements. The rate of height growth and swelling that were recorded are 164.25% at the frontal region of the head, 189.13% at the shoulder region and 122.42% at the forelimb region. The growth of the tissue expander in the three sites could be summarized in three phases; biodegradable in week one, the peak of anisotropic expansion in week two and three, and final degree of swelling in week four.

Keywords: Anisotropic, horse, physical measurement, skin expansion, tissue expander

INTRODUCTION

The clinical use of tissue expander for skin expansion in veterinary field is limited (Bristol, 2005). A study on the use of balloon type tissue expander has been described in three horses and a dog (Madison, Donawick, Johnston, & Orsini, 1989). Tissue expansion enables replacement of lost tissue with similar tissue of matching color, texture and other important local characteristics.

The most common method of tissue expansion is to place balloon expanders near the surgical site. Neumann purposely induced

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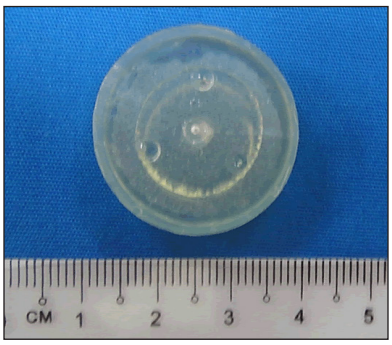
soft tissue growth with a subcutaneously implanted balloon (expander) in an attempt to reconstruct an external ear deformity (Neumann, 1957). However, there are many complications associated with their use, especially with repeated filling through a port. The use of self-inflating tissue expander greatly minimized these complications (Zhu & Czernuszka, 2015).

The purpose of this study is to report the clinical observation and the rate of expansion of anisotropic self inflating hydrogel tissue expander in the skin of the horse upon implantation of the tissue expander subcutaneously at the frontal region of the head, side of shoulder and at the forelimb at the medial cannon. The expansion of the tissue expanders was measured using electronic digital caliper and clinical changes on the skin were observed and recorded daily for the duration of 28 days.

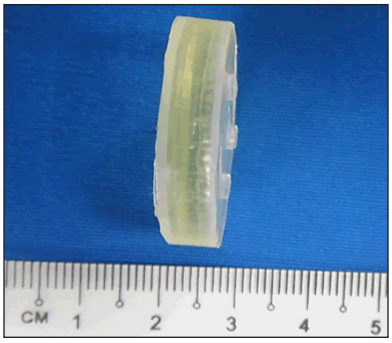
MATERIALS AND METHODS

Tissue Expanders

All the tissue expanders used were Anisotropic, self-inflating (Oxtex)®UK, circular shape devices. Each tissue expander is approximately 6.5mm in height and 27mm in diameter in size (Figure 1(a) - (b)). These surgical devices containing hydrogels were specifically polymerized by Polymeric Sciences Ltd., UK. The gels were prepared by co-polymerization of pharmaceutical grade (ISO 13488) meth methacrylate (MMA) and vinyl-pyrrolidone (VP). This hydrogel (90:10 VP: MMA Hydrogel) was coated with a medical grade impermeable silicone (MED-4211).



(a)



(b)

Figure 1. Oxtex® self-inflating anisotropic hydrogel tissue expanders before expansion. Each tissue expander is approximately (a) 6.5 mm in height and (b) 27 mm in diameter

Surgical Procedure

Implantation of tissue expanders was performed while the horse was standing and sedated with detomidine infusion (100 µg/ml, with constant rate infusion at 70 drops/min). Local block by using 2% lidocaine hydrochloride (5 to 20 ml) was performed on the site of implantations. A total of seven Anisotropic hydrogel tissue

expanders were placed subcutaneously on the rostral part of the frontal region of the horse head, lateral side of right shoulder and the dorsomedial part of the cannon region of the right forelimb (Figure 1-A,B). Three tissue expanders were implanted on the dorsal part of the frontal region of the horse head. Other three tissue expanders were implanted on the right shoulder and one on the dorsomedial part of the cannon region of the right forelimb. Each incision was for one implant material. From the initial incision site, the skin was then undermined with blunt dissection in order to create a subcutaneous tunnel or pocket to insert the tissue expander. Anisotropic tissue expander was gently inserted into the subcutaneous tunnel and was secured in position by placing a few tacking sutures along the subcutaneous tunnel to prevent migration of the tissue expander subcutaneously. The incision wound was then sutured with 2-0/3-0 Ethilon (Ethicon Ltd., Edinburgh, United Kingdom), cross mattress suture pattern. Broad-spectrum antibiotic with combination of procaine penicillin and dihydrostreptomycin sulphate, 22,000 IU/kg was given once daily by intramuscular injection, anti-inflammatory (Flunixin Meglumine 1.1 mg/kg) once daily intravenously for up to three days in order to reduce postoperative secondary bacterial infections and inflammation. The implantation sites were monitored daily by the same observer for any evidence of infection, suture site breakdown, discharge, implant rupture, hematoma and other signs.

Clinical Observation and Physical Measurements

The standard records and physical measurements were taken daily for 28 days period. Physical measurement of the implanted region was taken using electronic digital caliper to determine the height of expanded tissue for every implant and three readings were recorded for each site (Figure 2). The size and growth of the tissue expanders were also monitored weekly using radiography and cast impression techniques.

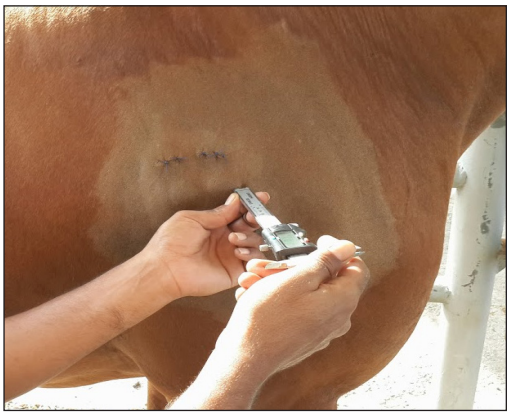


Figure 2. Physical measurement of the implanted tissue expander by using electronic digital caliper

RESULTS AND DISCUSSION

In this study, the physical measurements include measurement of the height of the expanded skin area. The physical measurements were recorded daily throughout the 28 days of post-operative period (Table 1).

Based on the physical measurement data in all horses (n=3), generally, swelling of the tissue expanders and expansion of skin start to increase by the second

day of post-operative implantation of tissue expanders for all sites that were implanted and continue to further expand until around post-operative day 9th -10th. Tissue expansion was considered to increase but at a slower rate after day 10th until day 19th post-operatively as shown in Figure 3.

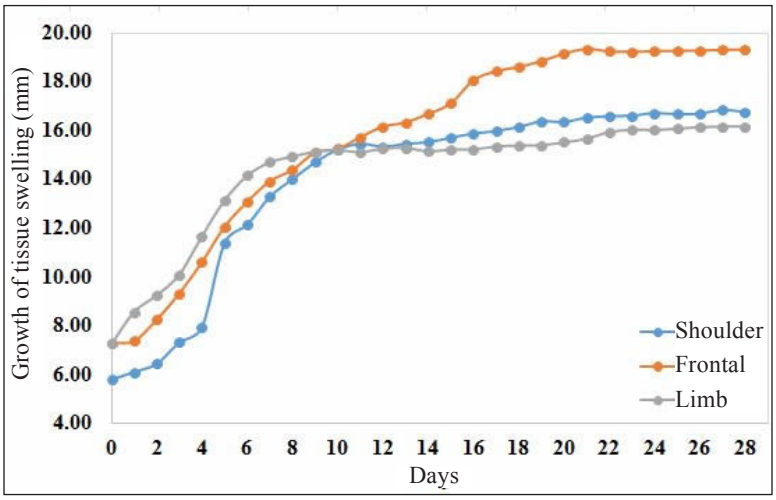


Figure 3. The mean data of physical measurement of tissue expansion of the frontal, forelimb, and shoulder region (n=3), for all horses over the 28 days study period

Data for physical measurement for all horses (n=3) were presented as mean ± s.d. The swelling of the frontal, shoulder and the forelimb at that period were increased. On an average, highest height recorded was 19.31±1 mm (frontal), 16.76±0.53 mm (shoulder) and 16.17±1.85 mm (forelimb) at post-operative day 28th as shown in Table 1.

Table 1
The mean ±s.d of first and last day of the physical measurement of tissue expansion in the frontal, shoulder and forelimb region (n=3)

Skin sites	Measurement mm day1±S.D	Measurement mm day 28±S.D	Difference mm	%
Frontal (n=3)	7.3±0.46	19.31±1	12.01	164.25%
Shoulder (n=3)	5.8±2.0	16.76±0.53	10.97	189.13%
Forelimb (n=3)	7.27±1.18	16.17±1.85	8.90	122.42%

Through the physical measurement in all horses (n=3), the rate of height growth and swelling that were recorded; 164.25% at the frontal region of the head, 189.13% at the shoulder region and 122.42% at the forelimb region (Table 1).

The anisotropic self-inflating tissue expander is efficient for expanding skin tissue in horses (Swan, 2007). This tissue expander has the capacity to absorb fluids, expanded in unidirectional in living tissues as it was expected to. This type of expander can be useful in cases that require anisotropic expansion specifically for delicate anatomical region (Swan, Bucknall, Czernuszka, Pigott, & Goodacre, 2012). The expansion rate of the expander grew at the initial stages at all implantation sites and it was observed in all horses in this study. By the beginning of week three, the size of the expander remained unchanged. Consequently, the growth of the skin is controlled in rate, in contrast to first generation of self-inflating tissue expander, which expands rapidly to its maximal volume only in few days after implantation resulting in complications. Van Damme, Heidbuchel, Kuijpers-Jagtman, Maltha and Freihofer (1992) suggested that surface-area increased by tissue expansion can be because of growth, stretching and recruitment of tissue, or it may be due to a combination of these processes (Van Damme et al., 1992).

In this study, the expansion rate of the tissue expander at different sites are showing different rate of expansion. The expanders at the forehead region were expanding much better than the expander placed at the distal limb region. The difference in growth can be attributed to the anatomical nature of these three sites. The superficial skin of the frontal area in horses is less elastic and tightly attached to the frontal bone allowing space for expansion. (Table 1).

Surgical implantation of all devices was technically straightforward, with no immediate complication seen. All horses made a rapid postoperative recovery and were usually eating and drinking within 1 hours after cessation of anesthesia. In the current experiment, the growth of the tissue expander in the three sites passes in the same three phases; biodegradable in week one, the peak of anisotropic expansion in week two and three, and final degree of swelling in week four.

This result supports the use of self-inflating tissue expander and the effect of implantation time on expansion rate in the three sites. Therefore, to allow for maximum expansion of the self-inflating anisotropic tissue expander, at least a period of three weeks is needed.

CONCLUSION

To conclude, this study will help equine veterinarian to understand the expansion rate of the hydrogel tissue expander in the skin of the horse at the head, shoulder and forelimb regions, for application in advancement skin flap technique and reconstructive of massive skin defects in this species.

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CONFLICT OF INTEREST

None of the authors have any potential conflicts of interest to declare.

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Short Communication

True and Apparent Metabolizable Energy of Crude Glycerin in Betong Chicken

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ABSTRACT

An experiment was conducted to determine the nitrogen-corrected true and apparent metabolizable energy (TMEn and AMEn, respectively) of crude glycerin (CG) for Betong chicken using a precision-fed rooster assay. A total of 15 male Betong chickens were assigned to three groups (0, 10, and 15% CG) of the experimental diet. Based on this experiment, CG supplementation enhanced the GE, TMEn, and AMEn of an experimental diet as the CG level was escalated. The TMEn and AMEn of CG were 3138 and 3046 kcal kg^{-1} at 15% and 2977 and 2896 kcal kg^{-1} at 10%, respectively.

Keywords: Apparent metabolizable energy, Betong chicken, crude glycerin

INTRODUCTION

Thailand, which is the second largest biodiesel producers in Asia, produced approximately 1420 million liters in 2017. The country was expected to increase the biodiesel consumption in the next 4 years to up to 5.97 million liters/day (Sutabutr, 2012). Crude glycerin (CG) is the main by-product of this biodiesel production, derived from approximately 10% of the total feedstock. However, this by-product contains approximately 50 to 90% glycerol and 15 to 35% of impurities such as methanol or ethanol, fatty acids, water, and some chemical compounds generated by transesterification (Dozier et al., 2008; Jung & Batal, 2011). Thus, the demand for CG is still limited because of the impurities and costly process for medium- and small-scale producers to upgrade the quality, although it can be used in the food, pharmaceutical, and animal feed industries (Thompson & He, 2006).

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Literature reports indicate that CG contains various gross energy (GE) of approximately 3173 to 6021 kcal/kg depending on the process and composition (Dozier, Kerr, & Branton, 2011; Thompson & He, 2006). Up to the present time, studies on CG have been primarily oriented towards investigating its effect on poultry performance, meat quality, and egg traits. These studies have revealed that the optimum range of CG inclusion is 5 to 10% of the total feed for broilers, laying hens, and quails (Erol, Yalcin, Midilli, & Yalcin, 2009; McLea, Ball, Kilpatrick, & Elliott, 2011; Németh, Zsédely, & Schmidt, 2013). Recently, CG nitrogen-corrected apparent metabolizable energy (AMEn) content of GE approximates 62.3 to 98.7% for broiler chickens (Dozier et al., 2011) and 97.6% for laying hens (Németh et al., 2013). Other studies indicate that the use of CG in livestock production is determined by the type of animal, age, and amount of glycerin in the diet (Cerrate et al., 2006). As the chickens in the previous studies have been genetically selected, they are assumed to have an improved feed digesting ability. Consequently, the generated energy values might be overestimated for local chickens. However, to the best of our knowledge, this hypothesis has not been reported. Therefore, the aim of this study was to determine the nitrogen-corrected true metabolizable energy (TMEn) and AMEn of CG in Betong chickens.

MATERIALS AND METHODS

Materials and Chemical Analysis

CG was obtained from the Specialized Research and Development Center for Alternative Energy from Palm Oil and Oil Crops, Prince of Songkla University (PSU)**.** Three replicate CG samples were analyzed for moisture, crude protein, crude fat, and ash (Association of Official Agricultural Chemists [AOAC], 2000). Glycerol and the GE were determined using high-performance liquid chromatography (HPLC) (Hu, Luo, Wan, & Li, 2012) and an isoperibol calorimeter (LECO AC500), respectively. Table 1 shows the composition of the CG investigated in this study.

Table 1
Crude glycerin composition in the experimental feed

Item	Value
Moisture, %	15.96
CP, %	0.64
Crude fat, %	15.08
Ash, %	6.34
Gross energy (kcal/kg)	4472
Glycerol (%)	42.88
MONG[1]	34.82

[1]MONG: matter organic non-glycerol. Defined as 100-[glycerol content (%) + water content (%) + ash content (%)]

Birds and Housing

All experimental procedures were approved by the ethical principles of Prince of Songkla University. Fifteen 22-week-old male Betong chickens from the poultry farm of the Faculty of Natural Resources, PSU

were sorted by body weight (1.77 ± 0.11 kg) and randomly placed in individual metabolic cages (50 × 43 × 60 cm dimension) with individual feeders and water bottle nipples inside an evaporative housing system. During the experiment, the temperature was maintained at 25 to 28°C with 16-h lighting. The roosters were adapted to the cage for 7 days prior to the experiment with ad libitum access to water and commercial layer feed.

Feeding Treatment

The experimental diets consisted of three levels of CG supplementation (0, 10, and 15%) in ground corn as the basal diet. The roosters were assigned to three groups with five replications for each treatment. In addition, the true metabolizable energy (TME) was determined using the method of Sibbald (1986). Briefly, all chickens were fasted for 24 h and then the excreta was collected for the next 24 h to calculate the endogenous energy loss. After a 4-day recovery period given ad libitum feed, the chickens were crop intubated with 30 g of the experimental diets following a 24-h fast. Water was provided all the time of the experiments. The excreta was collected using the harness technique and tray under the cages to examine feed regurgitation. The excreta samples were weighed, oven-dried (60°C for 72 h), ground, and pooled for each treatment prior to analyzing the dry matter (DM), total nitrogen (Kjeldahl method), and GE (LECO AC500) using benzoic acid as a standard.

Calculations and Statistical Analysis

The DM digestibility, AMEn, and TMEn of experimental feed were calculated based on the methods of Dozier et al. (2011) and Parsons, Potter and Bliss (1982), respectively. Data from the experiments were analyzed as a completely randomized design. The significant differences subjected to further analysis with Tukey using SPSS version 16.0.

RESULTS AND DISCUSSION

In CG suplemented diet, the values of TMEn, AMEn and GE were increased (Table 2). It is believed to be caused by energy contribution from crude glycerin which carries high gross energy content. The result was found linear with dry matter digestibility which is numerically higher than that of the controlled diet. High absorption rates of glycerin were related to its small molecular weight and passively absorbed in the gut (Guyton, 1991). The enzyme glycerol kinase metabolized glycerol to glyceraldehyde-3-phosphate, was then used for fatty acid synthesis, gluconeogenesis or oxidized via the glycolytic pathway (Robergs & Griffin, 1998). However, such enzyme may be saturated in a high level of CG and as a result, decrease the metabolizable energy value (McLea et al., 2011).

As shown in Table 3, TMEn and AMEn values in 15% level inclusion of CG were higher than those in 10% inclusion. These results indicate that CG metabolizable energy may depend on its level in the diets. Nevertheless, the chickens are able to

Table 2
Dry matter digestibility (%) and energy values (kcal/kg) of experimental feed

Energy value	Crude glycerin			*P value*
	0%	10%	15%	
GE	3994 ± 32[a]	4199 ± 26[b]	4349 ± 62[c]	0.000
AMEn[1]	3214 ± 62[a]	3354 ± 120[a]	3565 ± 105[b]	0.001
TMEn[2]	3320 ± 65[a]	3447 ± 122[a]	3672 ± 104[b]	0.001
DM Digestibility[3] (%)	87.38 ± 0.54	87.49 ± 2.37	88.14 ± 3.58	0.890

[a,b] Means within the same row with different superscripts differ significantly ($p < 0.05$).
[1] AMEn = GE I - GE O - 8.22 NF/FI, where GE I = gross energy intake; GE O = gross energy output; NF = nitrogen retained in the fed bird, FI = feed intake and 8.22 = nitrogen correction factor reported by Hill and Anderson (1958).
[2] TMEn = (FEF - (EEF + 8.22 NF) + (EEU + 8.22 NU)/FI, where FEF = The feed gross energy; EEF = The excreta gross energy from the fed bird; EEU = The excreta gross energy from the fasted bird; NU = Nitrogen retained in the fasted bird; NF and FI as stated above.
[3] Digestibility (%) = (FI-E+E Endogenous/FI) x 100, where E = The fed bird excreta in dry matter; and E Endogenous = The fasted bird excreta in dry matter.

Table 3
Energy values of crude glycerin (kcal/kg)

Energy value	Crude glycerin		*P value*
	10%	15%	
GE	4472 ± 126		
AMEn[1]	2896 ± 104[a]	3046 ± 90[b]	0.040
TMEn[2]	2977 ± 106[a]	3138 ± 89[b]	0.031
% of GE[3]	66.56 ± 2.36[a]	70.18 ± 1.98[b]	0.031

[a,b] Means within the same row with different superscripts differ significantly ($p < 0.05$).
[1] AMEn = Total AMEn intake- Basal AMEn/Glycerin intake
[2] TMEn = Total TMEn intake- Basal TMEn/Glycerin intake
[3] % of GE = TMEn/GE *100%

metabolize CG up to 15%. It is difficult to compare with another study due to limited research in CG utilization for local chicken. Jung and Batal (2011) found that TMEn of CG was varied from 80 to 99% of GE. However, the TMEn content in this study was 66.56 and 70.18%. Meanwhile, the AMEn values of crude glycerin were 64.7 and 68.1% of GE for 10 and 15% levels of inclusion, respectively. The results were close to Dozier et al. (2011) who observed crude glycerin from various sources, including poultry fat (51.54% glycerol) and yellow grease (52.79% glycerol) with 62.29 and 68.68% AMEn values of its gross energy. In contrast, McLea et al. (2011) evaluated crude glycerin (81% glycerol) with broiler chicken. They

found that the AMEn value was 75% of gross energy. Németh et al. (2013) reported a higher AMEn value (97.6% of GE) when conducted the experiment using laying hen and crude glycerin from rapeseed oil (86.8% glycerol).

The differences in the AMEn and TMEn values are believed to be related to different breed, age and sources of CG. Different from Betong chickens, commercial chickens in other studies are genetically selected to optimize the feed utilization. Furthermore, the digestion ability is also influnced by increased ages. This is due to its well-developed gastrointestinal tract. Another factor needs consideration is the quality of CG. Low glycerol level (42.88%) and high crude fat (15.08%) in the present study lead to higher GE (4472 kcal kg^{-1}) than that pure glycerol (4325 kcal kg^{-1}) reported by Dozier et al. (2011). Accordingly, Jung and Batal (2011) found a correlation between crude fat with GE in crude glycerin. The authors concluded that the higher the fat level, the higher GE and the lower glycerol content it would be.

The lower AMEn percentage is due to the representation of the fatty acid in the MONG content. This finding has confirmed the work of Dozier et al. (2011) who reported a reduced AMEn due to its relatively high fatty acid content. Wiseman and Blanch (1994) also found a negative correlation between free fatty acid (FFA) content and AMEn value in young and adult broiler chicken fed with coconut and palm kernel oil. Moreover, inadequate ratio between monoglycerides and FFA which is due to the lack of triglycerides tended to reduce bile secretion. This may result in lower absorption rates compared to oil and fat sources with triglycerides and FFA in the intestine (Sklan, 1979).

CONCLUSION

Based on this study, it could be concluded that the DM digestibility, AMEn and TMEn values of CG were affected by its level in the experimental feed. The CG inclusion in the diet up to 15% was well utilized by the Betong chicken. The TMEn and AMEn of CG were 3138 and 3046 kcal kg^{-1} at 15% and 2977 and 2896 kcal kg^{-1} at 10%, respectively.

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Case Study

Management of An Outbreak of Brucellosis in A Multiple Species Ruminant Farm in Malaysia

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ABSTRACT

This case report describes the management of an outbreak of brucellosis in a mixed ruminant farm in Selangor, central region of Peninsular Malaysia. Two cows with a history of abortion and endometritis were presented to the University Veterinary Hospital, Faculty of Veterinary Medicine, Universiti Putra Malaysia. Based on the history, physical examination and results of Rose Bengal Plate Test (RBPT), brucellosis was highly suspected. This led to the screening of all the animals (n = 384) in the

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affected farm. The Rose Bengal Plate Test (RBPT) revealed that 23 cattle were positive, while no agglutination was observed in goats and deer. A confirmatory test was performed on all the (n = 384) animals using the complement fixation test (CFT) and the results showed that two (2) cows and five (5) deer tested positive. The CFT positive animals were culled. Preventive steps were then taken where the remaining cattle in the herd were vaccinated once using the RB51 vaccine, while the deer and goats were not vaccinated. All animals in the herd were also recommended to be subjected for blood sampling every 4 months and the serum samples to be tested using competitive ELISA to distinguish between serological responses due to vaccination from infection. To overcome outbreak scenario of brucellosis in a farm, a prompt action and a concerted team work among relevant stakeholders are crucial to curb the situation.

Keywords: Brucellosis, multiple species, outbreak, ruminant farm

INTRODUCTION

Cattle, goat and deer are considered as the main ruminant livestocks in Malaysia due to the increased local demand for milk and meat (Department of Veterinary Services [DVS], 2015). However, these livestock are constantly threatened by many infectious diseases and one of such disease is brucellosis. Brucellosis is a serious socio-economic and public health problem in many developing countries (Bamaiyi, Hassan, Khairani-Bejo, & Zainal Abidin, 2014). The prevalence of the disease among livestock in Malaysia is considerably low in relation to other neighboring countries (Bahaman, Joseph, & Khairani-Bejo, 2007; Bamaiyi et al., 2014).

Brucellosis was first confirmed in Malaysia in the 1950s when *B. abortus* was first isolated from large ruminants. However, subsequent studies have reported the presence of brucellosis in small ruminants such as goat and sheep, pets and humans (Sam et al., 2012). For years, the Department of Veterinary Services, Malaysia (DVS) have conducted an active serosurveillance on bovine brucellosis as a part of a serious and committed effort in controlling the disease. The exercise involved culling of infected animals and payment of compensation to owners of the affected farms (Bahaman et al., 2007). However, the status of bovine brucellosis has shifted in the last decade and anecdotal evidence suggests an increase in brucellosis among cattle (Anka et al., 2013), similar pattern was also reported among goats. This case report describes the management of an

outbreak of brucellosis in a multiple species ruminant farm in Malaysia.

Clinical History/Examination

Two cows with a history of abortion and endometritis from a multiple species ruminant farm in Selangor (central Peninsular Malaysia) were presented to the University Veterinary Hospital, Faculty of Veterinary Medicine, Universiti Putra Malaysia. Farm records showed evidence of recent introduction of new cows into the herd. Based on history, physical examination and positive result**s** by Rose Bengal Plate Test (RBPT), brucellosis was suspected. This led to the screening of all the animals (n = 384) in the farm. The decision to screen the whole herd was made due to the farm's proximity to Negeri Sembilan state (central south Peninsular Malaysia), which was reported to have several outbreaks of brucellosis.

Diagnostic Workup

Blood sample collection and Extraction of serum. Approximately 5 mL of blood samples were collected from each animal in a pre-labelled plain vacutainer tube via jugular venipuncture. The blood samples were allowed to clot by keeping the tubes to stand overnight at room temperature. Serum was extracted by centrifugation at 3000 x g for 20 min. The sera were then stored at -20 ºC until used.

Rose Bengal Plate Test (RBPT). Serum samples were screened for brucellosis using the RBPT method described by Swai and Schoonman, (2010). Briefly, 30 µL of RBPT antigen (VLA Weybridge, UK) and 30 µL of test serum samples were placed together on a plate. The plate was then shaken for 4 min and the level of agglutination was evaluated and recorded. The samples were categorized as positive following the presence of agglutination and negative when there was no agglutination. Serum samples that were positive for brucellosis using RBPT were confirmed with CFT.

Clinical Management and Outcome. The result**s** showed that a total of 23 (5.9%) cattle were positive for brucellosis by RBPT. However, none of the goats and deer were positive using RBPT. The Complement Fixation Test (CFT) revealed 2 (0.5%) cattle and 5 (1.3%) deer were positive for brucellosis while none of the tested goats were positive. Following results from the CFT test, which is the confirmatory test for brucellosis, all CFT positive animals (two cattle and five deer) were subjected to mandatory culling as recommended by the Malaysian Veterinary Protocol set by the Department of Veterinary Services of Malaysia. Preventive measures were taken immediately following culling where all cattle in the herd were vaccinated once using the RB51 vaccine, while deer and goats were not vaccinated. All the remaining animals in the herd were recommended to be subjected for repeated blood sampling every 4 months. The serum samples were to be tested using competitive enzyme-linked immunosorbent (ELISA) that differentiates between serological responses due to vaccination from infection until none of the animals were positive for infection.

DISCUSSION

Brucellosis is a major socio-economic problem of the livestock industry. It is one of the most important zoonotic diseases associated with reporductive failures in livestock (Jajere, Atsanda, Bitrus, Hamisu, & Ayo, 2016). The disease is widespread from country to country; however, it is restricted to certain geographical locations. Brucellosis is still considered a major animal health problem affecting the livestock industry in Asia, Africa, Latin American and the Mediterranean (Yahaya, Khairani-Bejo, Zunita, Omar, & Bitrus, 2016). As in 2014, the overall prevalence of bovine brucellosis (*Brucella abortus*) reported in some ASEAN countries were (1%) Thailand, (2%) Indonesia, (4%-5%) in Malaysia (Zamri-Saad & Kamarudin, 2016). In this report, the prevalence was 6% using RBPT as compared to 1.8% with the CFT. The RBPT test is a more sensitive but less specific assay in detecting infected animals, while the CFT test is more specific in detecting animals infected with the disease. The management of the outbreak described in this case report is in accordance with the recommendations by Gürbilek, Tel and Keskin (2017) where the authors reported that successful control programs for brucellosis is based on the combined use of serological tests such as RBPT and CFT. In Malaysia, test-and-slaughter policy is the protocol of choice that was adopted for the control of brucellosis. The protocol was adopted and implemented in 1979 as the national program for "Area-Wise Eradication of Bovine Brucellosis". The test- and- slaughter policy involved culling of all individual animals that tested positive for brucellosis by CFT and corresponding payment of compensation to the affected farm or animal owners. Even though the protocol is considered expensive and recommended only in countries that have efficient surveillance program and excellent laboratory facilities which in this case may not be suitable approach to be practiced in Malaysia. However, the country's cattle population which was estimated to about 800,000 makes the adoption of the program feasible due to less cost incurred on the of execution of the program. Interestingly, the test-and-slaughter policy in Malaysia has achieved tremendous success in reducing the prevalence of brucellosis from 3.3% (1979) to 0.23% (1988). The prevalence however increases from 1% in 1998 to 5% which is the current status of bovine brucellosis in Malaysia. This was attributed to the indifference attitude of the farmers towards the program (Bahaman et al., 2007; Plumeriastuti & Zamri-Saad, 2012; Zamri-Saad & Kamarudin, 2016).

In cattle, the main causative agent of brucellosis is *B. abortus,* infection in animal usually occur as result of contact with the contents of abortion from other infected animals in the farm. Contaminated animal utensils and pasture with aborted materials are probably the most potential sources of infection. Other sources of transmission includes, inhalation, ingestion, skin contamination, conjunctival inoculation and udder inoculation from infected milking cups. Contamination via the colostrum

have also been reported in newborn calves (Blasco & Molina-Flores, 2011; Godfroid et al., 2013). Brucellosis have also been reported in cows that were inseminated with contaminated bull semen (Aparicio, 2013; Xavier, Paixao, Hartigh, Tsolis, & Santos, 2010). In this case report farm history showed evidence of recent introduction of new cow into the farms. Thus, indicating the possible transmission of the disease to other animals in the farm probably due to latent infection in the newly introduced animals.

Greater Yellowstone Ecosystem (GYE) is the classic example and still remains the major reservoirs of B. *abortus* in the United States, where the historical infection in wildlife such as bison (*Bison bison*) and elk (*Cervus elaphus*) are believed to be introduced by domestic cattle in the early 20th century. Recent studies have reported that free-range elks are still serving as reservoirs of infection to livestock (Kamath et al., 2016). Seroprevalence against *B. abortus* recorded in several elk herd units in GYE (Wyoming portion) ranged from 9% to 42% (Scurlock & Edwards, 2010). In some areas of China, the prevalence of brucellosis among deer was 28% (Li, Yao, & Wang, 2007). Medrano et al. (2012) reported 0.9% antibody detection in white-tailed deer (*Odocoileus virginianus*) in northen part of Mexico. Brucellosis is thought to have a role in the list of infectious diseases in deer by the across region distribution and under reported incidence, this eventually could influence the development of deer farming and breeding industry. In Malaysia, as the best of authors knowledge would be the first reporting on seroevident of antibody against *B. abortus* in deer. The deer and other cattle and goat in this farm were not in direct contact, however the roles of indirect transmission such as contaminated fodder, fomites or personnel should not be excluded. In this context, further investigation is needed to establish the status of *Brucella* infection in farmed as well as free-ranging deer in Malaysia with a view to systemizing for prevention and control of the disease among and between wildlife and livestock.

Mixing of animals from different herds or flocks belonging to different owners especially at the markets contribute significantly to the transmission of the disease. Many factors influence the prevalence of brucellosis, which includes contact with wildlife, production systems, management factors, agro-ecological zones and husbandry practices (Godfroid et al., 2013). However, mixing of livestock species is one of the most important factors that contributes to the spread of brucellosis in animals (Godfroid et al., 2013). In this report, the mixing of multiple species in the farm might have contributed to the seropositivity in other species of animals such as deer in this case. Based on the CFT results, it can be suggested that the either the cattle or deer may be the source of infection. It has been reported that mixed farming, especially raising of sheep and/or goats together with cattle to be a risk factor for transmission of brucellosis among different animal species (Ocholi, Kwaga, Ajogi, & Bale, 2004). However, this transmission does not equally occur in both directions.

For deer with cattle, the risk factor for transmission of brucellosis has not been reported or studied yet in Malaysia.

The increase in livestock population and investment along with limited resources have made the prevention and control of brucellosis difficult (Corbel, 2006; Al-Majali, Majok, Amarin, & Al-Rawashdeh, 2007).The re-emergence of brucellosis has been reported in many countries especially in sheep and goats (Blasco & Molina-Flores, 2011). The disease has been eradicated in most technologically advanced countries following years of qualitative investment, vaccination and culling of infected animals. Similar approach was utilized in this case report, the animals that tested positive for CFT were culled, while the remaining seronegative animals were all vaccinated. Zamri-Saad and Kamarudin (2016) stated that vaccination helped to reduce shedding of the organism in the environment and was mostly practiced in areas where the disease was endemic and hence, this move was implemented in this case. Control or eradication strategies for outbreak of brucellosis in a farm needs a prompt action and a concerted team work among relevant agencies to curb the situation as reported in this case report. This will in turn, reduce the burden of brucellosis on human health and the livestock industry (Blasco & Molina-Flores, 2011).

CONCLUSION

This case report describes the seroprevalence of brucellosis in a multiple species farm through the use of two different assays; RBPT and CFT. The CFT used in this case report showed high discriminatory power to brucellosis in deer than RBPT. The main aim of the control program of brucellosis is to reduce the impact of the disease on both human and animal health and the economy.

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(JCU, Australia)

BAU	*- Bangladesh Agricultural University*
BFGI	*- Baba Farid Group of Institutions*
BILS	*- Bhojia Institute of Life Sciences*
BRRI	*- Bangladesh Rice Research Institute*
CNBRCD	*- Centre for Natural Biological Resources and Community Development*
FUNAAB	*- Federal University of Agriculture, Abeokuta*
GBPUA&T	*- Govind Ballabh Pant University of Agriculture & Technology*
GKV	*- Gurukula Kangri Vishwavidyalaya*
IIUM	*- International Islamic University Malaysia*
IPB	*- Bogor Agricultural University*
IPGP	*- Institute of Earth Physics of Paris*
IRRI	*- International Rice Research Institute*
JCU	*- James Cook University*
KU	*- Kasetsart University*
KUK	*- Kurukshetra University*
MARDI	*- Malaysian Agricultural Research and Development Institute*
NUSK	*- Nasarawa State University*
PONDIUNI	*- Pondicherry University*
SACON	*- Salim Ali Centre for Ornithology and Natural History*
TNAU	*- Tamil Nadu Agricultural University*
UGM	*- Gadjah Mada University*
UiTM	*- Universiti Teknologi Mara*
UM	*- Universiti Malaya*
UMK	*- Universiti Kelantan Malaysia*
UMS	*- Universiti Malaysia Sabah*
UMT	*- Universiti Malaysia Terengganu*
UNIBA	*- University of Bari*
UNIGOA	*- Goa Univeristy*
UNIMAS	*- Universiti Malaysia Sarawak*
UNISKA	*- Islamic University of Kalimantan*
UNiSZA	*- Universiti Sultan Zainal Abidin*
UPM	*- Universiti Putra Malaysia*
UPSI	*- Universiti Pendidikan Sultan Idris*
UQ	*- University of Queensland*
USM	*- Universiti Sains Malaysia*
UTAR	*- Universiti Tunku Abdul Rahman (Perak Campus)*
VMC	*- Volusia Mosquito Control, Florida*

While every effort has been made to include a complete list of referees for the period stated above, however if any name(s) have been omitted unintentionally or spelt incorrectly, please notify the Chief Executive Editor, UPM Journals at executive_editor.pertanika@upm.my.

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PERTANIKA JOURNALS

Pertanika Journals

Our goal is to bring high quality research to the widest possible audience

INSTRUCTIONS TO AUTHORS

(Manuscript Preparation & Submission Guide)

Revised: August 2018

Please read the Pertanika guidelines and follow these instructions carefully. Manuscripts not adhering to the instructions will be returned for revision without review. The Chief Executive Editor reserves the right to return manuscripts that are not prepared in accordance with these guidelines.

MANUSCRIPT PREPARATION

Manuscript Types

Pertanika accepts submission of mainly **four** types of manuscripts for peer-review.

1. **Regular article**

Regular articles are full-length original empirical investigations, consisting of introduction, materials and methods, results and discussion, conclusions. Original work must provide references and an explanation on research findings that contain new and significant findings.

Size: Generally, these are expected to be between 6 and 12 journal pages (excluding the abstract, references, tables and/or figures), a maximum of 80 references, and an abstract of 100–200 words.

2. **Review article**

These report critical evaluation of materials about current research that has already been published by organizing, integrating, and evaluating previously published materials. It summarizes the status of knowledge and outline future directions of research within the journal scope. Review articles should aim to provide systemic overviews, evaluations and interpretations of research in a given field. Re-analyses as meta-analysis and systemic reviews are encouraged. The manuscript title must start with "Review Article:".

Size: These articles do not have an expected page limit or maximum number of references, should include appropriate figures and/or tables, and an abstract of 100–200 words. Ideally, a review article should be of 7 to 8 printed pages.

3. **Short communications**

They are timely, peer-reviewed and brief. These are suitable for the publication of significant technical advances and may be used to:

(a) report new developments, significant advances and novel aspects of experimental and theoretical methods and techniques which are relevant for scientific investigations within the journal scope;

(b) report/discuss on significant matters of policy and perspective related to the science of the journal, including 'personal' commentary;

(c) disseminate information and data on topical events of significant scientific and/or social interest within the scope of the journal.

The manuscript title must start with "*Brief Communication*:".

Size: These are usually between 2 and 4 journal pages and have a maximum of three figures and/or tables, from 8 to 20 references, and an abstract length not exceeding 100 words. Information must be in short but complete form and it is not intended to publish preliminary results or to be a reduced version of Regular or Rapid Papers.

4. **Others**

Brief reports, case studies, comments, concept papers, Letters to the Editor, and replies on previously published articles may be considered. PLEASE NOTE: NO EXCEPTIONS WILL BE MADE FOR PAGE LENGTH.

Language Accuracy

Pertanika **emphasizes** on the linguistic accuracy of every manuscript published. Articles must be in **English** and they must be competently written and argued in clear and concise grammatical English. Contributors are strongly advised to have the manuscript checked by a colleague with ample experience in writing English manuscripts or a competent English language editor.

Author(s) **must provide a certificate** confirming that their manuscripts have been adequately edited. A proof from a recognised editing service should be submitted together with the cover letter at the time of submitting a manuscript to Pertanika**. All editing costs must be borne by the author(s)**. This step, taken by authors before submission, will greatly facilitate reviewing, and thus publication if the content is acceptable.

Linguistically hopeless manuscripts will be rejected straightaway (e.g., when the language is so poor that one cannot be sure of what the authors really mean). This process, taken by authors before submission, will greatly facilitate reviewing, and thus publication if the content is acceptable.

MANUSCRIPT FORMAT

The paper should be submitted in one column format with at least 4cm margins and 1.5 line spacing throughout. Authors are advised to use Times New Roman 12-point font and *MS Word* format.

1. Manuscript Structure

Manuscripts in general should be organised in the following order:

Page 1: *Running title*

This page should **only** contain the running title of your paper. The running title is an abbreviated title used as the running head on every page of the manuscript. The running title should not exceed 60 characters, counting letters and spaces.

Page 2: *Author(s) and Corresponding author information*.

This page should contain the **full title** of your paper not exceeding 25 words, with name(s) of all the authors, institutions and corresponding author's name, institution and full address (Street address, telephone number (including extension), hand phone number, and e-mail address) for editorial correspondence. First and corresponding authors must be clearly indicated.

The names of the authors may be abbreviated following the international naming convention.
e.g. Salleh, A. B.[1], Tan, S. G.[2*], and Sapuan, S. M.[3]

Authors' addresses. Multiple authors with different addresses must indicate their respective addresses separately by superscript numbers:

Abu Bakar Salleh[1] and Mohd Zamri Saad[2]

[1]Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

[2]Faculty of Veterinary, Universiti Putra Malaysia, 43400 Serdang, Selangor Malaysia

A **list** of number of **black and white / colour figures and tables** should also be indicated on this page**.** Figures submitted in color will be printed (upon request) in colour. See "*5. Figures & Photographs"* for details.

Page 3: *Abstract*

This page should **repeat** the **full title** of your paper with only the **Abstract** (the abstract should be less than 250 words for a Regular Paper and up to 100 words for a Short Communication), and **Keywords**.

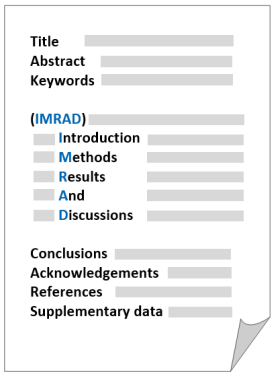
Keywords: Not more than eight keywords in alphabetical order must be provided to describe the contents of the manuscript.

Page 4: *Introduction*

This page should begin with the **Introduction** of your article and followed by the rest of your paper.

2. *Text*

Regular Papers should be prepared with the headings *Introduction, Materials and Methods, Results and Discussion*, *Conclusions, Acknowledgements, References, and Supplementary data* (if avavailble) in this order.



MAKE YOUR ARTICLES AS CONCISE AS POSSIBLE

Most scientific papers are prepared according to a format called IMRAD. The term represents the first letters of the words Introduction, Materials and Methods, Results, And, Discussion. It indicates a pattern or format rather than a complete list of headings or components of research papers; the missing parts of a paper are: Title, Authors, Keywords, Abstract, Conclusions, and References. Additionally, some papers include Acknowledgments and Appendices.

The Introduction explains the scope and objective of the study in the light of current knowledge on the subject; the Materials and Methods describes how the study was conducted; the Results section reports what was found in the study; and the Discussion section explains meaning and significance of the results and provides suggestions for future directions of research. The manuscript must be prepared according to the Journal's instructions to authors.

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These must be set up clearly and should be typed double spaced. Numbers identifying equations should be in square brackets and placed on the right margin of the text.

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All tables should be prepared in a form consistent with recent issues of Pertanika and should be numbered consecutively with Roman numerals. Explanatory material should be given in the table legends and footnotes. Each table should be prepared on a new page, embedded in the manuscript.

When a manuscript is submitted for publication, tables must also be submitted separately as data - .doc, .rtf, Excel or PowerPoint files- because tables submitted as image data cannot be edited for publication and are usually in low-resolution.

5. *Figures & Photographs*

Submit an **original** figure or photograph. Line drawings must be clear, with high black and white contrast. Each figure or photograph should be prepared on a new page, embedded in the manuscript for reviewing to keep the file of the manuscript under 5 MB. These should be numbered consecutively with Roman numerals.

Figures or photographs must also be submitted separately as TIFF, JPEG, or Excel files- because figures or photographs submitted in low-resolution embedded in the manuscript cannot be accepted for publication. For electronic figures, create your figures using applications that are capable of preparing high resolution TIFF files. In general, we require **300 dpi** or higher resolution for **coloured and half-tone artwork**, and **1200 dpi or higher** for **line drawings** are required.

Failure to comply with these specifications will require new figures and delay in publication.
NOTE: Illustrations may be produced in colour at no extra cost at the discretion of the Publisher; the author could be charged Malaysian Ringgit 50 for each colour page.

6. *References*

References begin on their own page and are listed in alphabetical order by the first author's last name. Only references cited within the text should be included. All references should be in 12-point font and double-spaced.

NOTE: When formatting your references, please follow the **APA reference style** (6th Edition). Ensure that the references are strictly in the journal's prescribed style, failing which your article will **not be accepted for peer-review**. You may refer to the *Publication Manual of the American Psychological Association* for further details (http://www.apastyle.org/).

PERTANIKA JOURNALS

7. General Guidelines

Abbreviations: Define alphabetically, other than abbreviations that can be used without definition. Words or phrases that are abbreviated in the introduction and following text should be written out in full the first time that they appear in the text, with each abbreviated form in parenthesis. Include the common name or scientific name, or both, of animal and plant materials.

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Spelling: The journal uses American or British spelling and authors may follow the latest edition of the Oxford Advanced Learner's Dictionary for British spellings.

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   COVER LETTER: All submissions must be accompanied by a cover letter detailing what you are submitting. Papers are accepted for publication in the journal on the understanding that the article is **original** and the content has **not been published** either **in English** or **any other language(s)** or **submitted for publication elsewhere**. The letter should also briefly describe the research you are reporting, why it is important, and why you think the readers of the journal would be interested in it. The cover letter must also contain an acknowledgement that all authors have contributed significantly, and that all authors have approved the paper for release and are in agreement with its content.

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