Pertanika Journal of

TROPICAL AGRICULTURAL SCIENCE

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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.

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

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

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

The Journal's peer-review

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

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

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1. The Journal's Chief Executive Editor (CEE) and the Editorial Board Members (EBMs) examine the paper to determine whether it is appropriate for the journal and should be reviewed. If not appropriate, the manuscript is rejected outright and the author is informed.

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

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

3. The CEE, in consultation with the Editor-in-Chief (EiC), examines the reviews and decides whether to reject the manuscript, invites the author(s) to revise and resubmit the manuscript. The CEE may seek additional reviews. Final acceptance or rejection rests with the CEE and EiC, who reserve the right to refuse any material for publication. In rare instances, the manuscript is accepted with almost no revision. Almost without exception, reviewers' comments (to the author) are forwarded to the author. If a revision is indicated, the editor provides guidelines for attending to the reviewers' suggestions and perhaps additional advice about revising the manuscript.

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

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

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

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

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

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ABSTRACTING AND INDEXING OF PERTANIKA JOURNALS

Pertanika is over 40 years old; this accumulated knowledge has resulted in the journals being abstracted and indexed in SCOPUS (Elsevier), Clarivate-Emerging Sources Citation Index [ESCI (Web of Science)], BIOSIS, National Agricultural Science (NAL), Google Scholar, MyCite and ISC.

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Foreword

Welcome to the First Issue of 2020 for the Journal of Tropical Agricultural Science (JTAS)!

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

This issue contains 6 articles; 1 is short communication, 2 are review articles and 3 are regular articles. Articles submitted in this issue cover the scope of algae biotechnology, biotechnology, crop and pasture production, fisheries sciences, microbiology and veterinary sciences.

The first paper presented in the issue is written by Ainatul Nadia Rusnan and her teammates from Universiti Putra Malaysia. They provided an overview of the occurrence of emetic and diarrhoeal food poisoning caused by *Bacillus cereus* in Southeast Asia. It concluded that the food poisoning by *B. cereus* used to be underreported as it had a short duration of illness and the symptoms are usually mild. The further details of the study are found on page 1.

A short communication entitled "The Role of *Nannochloropsis* sp. Methanolic Extract in Reducing Hydrogen Peroxide-induced DNA Damage in L929 Cell Line" discussed on the preventive effect of the phenolic compounds in the methanolic extract of *Nannochloropsis* sp. in protecting L929 normal cells from oxidative DNA damage. It concluded that the compounds in the extract might protect cell from oxidative DNA damage and thus prevent genetic mutation and cancer formation. The detailed information of this article is presented on page 81.

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

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

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

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

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Review Article

Pathogenic *Bacillus cereus*, an Overlooked Food Contaminants in Southeast Asia

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ABSTRACT

Bacillus cereus is a food-borne pathogenic bacterium that can cause infection and intoxication to human beings. Its ability to form spores and produce toxins are significant contributory factors to making it a great health risk for the consumer. This paper aims to provide an overview of the occurrence of emetic and diarrhoeal food poisoning caused by *B. cereus* in Southeast Asia. It concerns foods commonly consumed by Southeast Asia citizens, such as fresh food, beverages and traditional food. Rice is the food most associated with *B. cereus* contamination. Methods used in detecting and quantifying *B. cereus* and enterotoxins as well as cereulides are compiled in this paper. *Bacillus cereus* can be identified using biochemical tests or commercially available kit. The methods used to detect the emetic-producing *B. cereus* are HEp-2 cell vacuole formation, polymerase chain reaction (PCR), commercial kit and High-Performance Liquid Chromatography (HPLC). On the other hand, diarrhoeal-producing *B. cereus* can be detected using a commercial kit and real-time PCR. The food safety laws and regulations implemented in Southeast Asian countries are also included and precautionary steps are suggested. Food poisoning due to *B. cereus* is always overlooked because it has a short duration of illness and the symptoms are usually mild.

Keywords: *Bacillus cereus*, diarrhoeal toxin, emetic toxin, enterotoxin, food poisoning, Southeast Asia

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INTRODUCTION

Food is a basic human need. However, it may be contaminated with microorganisms that can affect one's health. Food-borne illness is defined as any form of an unhealthy condition suffered by the patient after the consumption of food contaminated with pathogens, viruses and parasites (Adley & Ryan, 2016). *Bacillus cereus* is a Gram-positive, spore-forming bacterium that is commonly associated with food-borne illnesses (Ankolekar et al., 2009; Wang et al., 2014). *Bacillus cereus* is currently ranked as the second food-borne pathogen in France and the third in China (Gao et al., 2018). It can be found in many types of foods, such as rice (Sawei & Sani, 2016), meat (Aklilu et al., 2016), vegetables (Seung Kim et al., 2017) and dairy products (Chitov et al., 2008; Lesley et al., 2017). Foods contaminated with *B. cereus* do not usually show signs of spoilage because *B. cereus* does not change the appearance or taste of the food, it can easily go unnoticed until too late (Tewari & Abdullah, 2015).

Bacillus cereus belongs to human pathogen Risk Group 2 (RG2) according to the classification of the Health and Safety Executive (HSE) (2013). Group 2 is classified as a biological agent that can cause diseases to humans and may present hazard to people who work with it (HSE, 2013). This biological agent rarely spreads to the community while treatment and prophylaxis are available in case of infection (ACDP, 2013). According to the World Health Organization (WHO), *B. cereus* is an etiological agent of both emetic and diarrhoeal type of food poisoning (Wang et al., 2014). The emetic type is caused by a cereulide toxin, which is pre-formed inside the contaminated foods; infected persons will experience vomiting and nausea a few hours after ingesting the contaminated foods (Jawad et al., 2015). Contamination is usually related to time and temperature abuse, which includes cooking the food a few hours before consumption or storing the food at room temperature (Bennett et al., 2013). The incubation period of the cereulide toxin of *B. cereus* is 30 minutes to 5 hours (Jawad et al., 2015). The diarrhoeal type of food poisoning caused by an enterotoxin is formed inside the intestine of the host (Toh et al., 2004). The symptoms of the diarrhoeal type infection arise about 12 hours after food consumption, with an intoxication which can last about 24 hours (Jawad et al., 2015). The incubation period of the enterotoxin is between 8 to 16 hours and the symptoms include nausea, abdominal pain and diarrhoea (Kim et al., 2010).

The region of Southeast Asia includes Brunei, Cambodia, Indonesia, Laos, Malaysia, Myanmar, Philippines, Singapore, Thailand and Vietnam. The tropical climate of these countries can contribute to the rapid reproduction of bacteriological contaminants. According to Bennett et al. (2013), food in Asia is commonly associated with *B. cereus* outbreaks (31%). Although *B. cereus* was not among the top 10 food-borne related illnesses in Southeast Asia, it should not be underestimated. *Bacillus cereus* was first identified as a food-borne pathogen responsible for food poisoning

in 1955. Chitov et al. (2008) noted that *B. cereus* gastroenteritis in many countries was not often medically evaluated and hence its incidence was probably underrated. In Southeast Asian countries, official data regarding *B. cereus* food-borne illness has been underreported and this paper attempts to update the current status of *B. cereus* exposure in food. Nevertheless, the degree of the discussion of how the pathogen has affected each country may vary due to limited information from some of those countries.

Bacillus cereus

Bacillus cereus is an aerobic, rod-shaped, Gram-positive bacterium that grows singly or in a chain and can usually be found in the soil and a variety of foods (Aklilu et al., 2016). Six (6) species are categorized under the genus of *Bacillus*: *B. cereus*, *B. mycoides*, *B. thuringiensis*, *B. anthracis*, *B. weihenstephanensis* and *B. pseudomycoides* (Jawad et al., 2015). Table 1 shows some of the tests and expected results to differentiate the members in the *B. cereus* group. Some *B. cereus* species are psychrophiles while others are mesophiles. Physiological and biochemical tests can be conducted to further confirm the identity of the particular *B. cereus*. According to the bacteriological analytical manual of Food and Drug Administration (FDA) (1998), a series of biochemical tests for the identification of *B. cereus* have been suggested. The tests include Gram staining, cell morphology, spore-formation, swollenness of sporangia, motility, nitrate broth, tyrosine agar, lysozyme broth, anaerobic utilization of glucose, Voges-Proskauer (VP) medium, rhizoid growth, hemolysis and crystal toxin production. A commercial identification kit is also available to identify *B. cereus*; namely, the BBL Crystal Identification Systems Gram-Positive ID kit supplied by Becton- Dickson, USA (Lesley et al., 2013).

Table 1
The comparison results of some biochemical test for the identification of Bacillus cereus*,* B. anthracis*,* B. thuringensis*,* B. mycoides*,* B. weihenstephanensis *and* B. pseudomycoides

Biochemical test	*Bacillus cereus*	*Bacillus anthracis*	*Bacillus thuringiensis*	*Bacillus mycoides*	*Bacillus weihenstephanensis*	*Bacillus pseudomycoides*
Colony morphology	White	White	White/grey	Rhizoid	Can be differentiate from other members of *B. cereus* group by ability to growth at < 7˚C but not at 43˚C. *B. weihenstephanensis* can also be detected by using PCR targeting the rDNA or cspA (cold shook protein A).	Have similar physiological and morphological characteristic with *B. mycoides*. Can only be differentiate on fatty acid composition and 16 RNA sequence.
Hemolysis	Positive	Negative	Positive	Positive		
Mobility	Positive	Negative	Positive	Negative		
Susceptible to penicilin	Negative	Positive	Negative	Negative		
Parasporal crystal inclusion	Negative	Negative	Positive	Negative		

Source: Lindback and Granum (2008)

Bacillus cereus can form spores under unfavourable conditions, such as lack of nutrients and unsuitable pH. Unlike other microorganisms that can easily be killed during cooking or the pasteurization process, *B. cereus* can withstand normal cooking temperatures and dry storage. The spore is thermoduric and therefore difficult to kill. Also, it can remain dormant for many years (Lesley et al., 2013). Hence, it is able to survive through various methods of food processing. Once the conditions become favourable again and the germinant receptor detects the presence of nutrients, the process of germination starts and causes *B. cereus* to reactivate and multiply as a vegetative cell (Van der Voort & Abee, 2013). Despite that, in a study of rice in which the maximum *B. cereus* spore level was 10^5 CFU/g, Azanza, and Centeno (2007) claimed that the *B. cereus* spore is moderately heat resistant and that the spore can be reduced by about 90% after heating at 100°C for 5 minutes.

On the farm level, *B. cereus* contamination could be introduced through the plantation soil or the irrigation water. Contamination can also occur during the processing, transport or storage of food.

Exposure to *Bacillus cereus*

Since the symptoms are mild, self-limiting and self-treated, the illness caused by *B. cereus* is often underestimated. This type of food poisoning is also rarely fatal. Most infected persons self-treat instead of seeking medical treatment. However, the elderly and children, with weaker immune systems, might face a higher risk. Currently, there are no reported deaths caused by *B. cereus* food poisoning in Southeast Asian countries. Yet, several fatal cases in children and adults due to mitochondrial toxin have been reported in other parts of the world. In 2003, a child past away in Belgium after eating *B. cereus* contaminated pasta salad (Biesta-Peters et al., 2010). In 1998, three people died in France due to *B. cereus* food poisoning (Lindback & Granum, 2008).

In certain countries of the Southeast Asian region, food safety is not an important concern for either the government or citizens, indicating an indifference probably related to the poverty and economic situation of these nations. Hence, throughout the region, laws concerning the permissible limit of *B. cereus* in foods have not been passed.

The first outbreak of *B. cereus* related food poisoning happened in 1982 at a military camp in Jurong, Singapore (Tay et al., 1982). The source of contamination was believed to be the fried rice for their breakfast. Fourteen army personnel were sick. The symptoms included vomiting (89.5%), abdominal cramps (52.6%), diarrhoea (47.4%), headache (47.4%) and fever (10.5%). The rice was believed to be cooked the evening before and stored inside the rice cooker. Egg and roast pork were then added to the rice and cooked together before being sold.

In September 2017, a food poisoning incident in a school canteen in Singapore had occurred. Fourteen students were ill after eating the fried rice sold in that canteen. The contaminated fried rice was prepared quite similarly to the incident

back in 1982 at the military camp. The fried rice was prepared by firstly the rice being cooked and kept warm for three hours in the rice cooker. After that, the rice was fried along with egg and crab meat. Subsequently, the fried rice was put inside the insulated container for two hours at room temperature before being sold at the school canteen.

The first outbreak in Malaysia was reported in 1984 and occurred in a school hostel in Klang (Rampal et al., 1984). The fried noodles served were contaminated with 2.3×10^6 CFU/g of *B cereus*. Later, on 15 February 2012, another outbreak due to *B. cereus* happened in a primary school canteen at Kota Kinabalu (Jeffree & Mihat, 2016). The source of *B. cereus* food poisoning outbreak was identified as the *nasi kuning* (turmeric rice), which was prepared using untreated water and poor hygienic practices.

The Thailand Bureau of Epidemiology received three reports of *B. cereus* outbreaks in 2008 and 2009, each of which occurred in schools. Moreover, in December 2009, an emetic *B. cereus* food poisoning outbreak associated with sweet stewed egg and pork occurred in a kindergarten located in Bangkok, Thailand (Santayakorn et al., 2012).

Emetic Food Poisoning

The emetic food poisoning is often associated with consumption of cooked or fried rice from a Chinese restaurant and thus it is also known as 'Fried Rice Syndrome' or 'Chinese Restaurant Syndrome' (Sandra et al., 2012; Schoeni & Wong, 2005). Emetic food poisoning is caused by the cereulide toxin. Cereulide is a cyclic dodecadepside, [D-0-Leu-Ala-O-Val-L-Val]3, which is produced by a nonribosomal peptide synthetase (NRPS) that is resistant to heat, pH and proteolysis (Guérin et al., 2017). The symptoms of infection include vomiting and nausea that occur 1 to 5 hours after ingestion (Häggblom et al., 2002). Emetic toxin-producing *B. cereus* usually grows on farinaceous foods such as pasta, noodles and rice, and ingestion of this toxin can lead to fatal liver failure (Mahler et al., 1997). While the mechanism of emetic toxin in the human body is still unknown, researchers believe the cereulide toxin binds to the 5-HT_3 receptor which stimulates the afferent vagus nerve, resulted in vomiting (Agata et al., 1995; Ehling-Schulz et al., 2004). The emetic producing *B. cereus* strain is unable to break down starch or fermenting salicin due to the lack of the hemolysin BL gene (Van der Voort & Abee, 2013). Based on animal studies, the emetic dose is between 9-12 µg/kg (Jeffree & Mihat, 2016). The result was recorded based on the minimum dose of cereulide to cause tested monkeys to vomit (Shinagawa et al., 1995). According to Bilung et al. (2016), the cell dose of *B. cereus* that leads to the emetic syndrome is between 10^5-10^7 cells/g. This is the range of viable cells of *B. cereus* needed for the cereulide to be detectable in the contaminated food.

Method of Detecting the Emetic Toxin. Traditionally, many researchers have utilized a human larynx carcinoma cell (HEp-2)

vacuolation assay to detect the emetic toxin-producing *B. cereus*. This assay depends on the vacuole-forming ability of the cereulide. However, this technique was found to be laborious, subjective and unreliable because the presence of the toxin can easily be missed (Hughes et al., 1988). Researchers have also used an assay in which boar sperm motility is inhibited by damage to the mitochondria caused by the cereulide. Recently, the polymerase chain reaction (PCR) method seems to be preferred by researchers and evidently, PCR detection kit for the emetic toxin is now commercially available. To detect the emetic toxin with the PCR method, the primer is designed to target the NRPS gene sequence since cereulide is produced by the NRPS complex and this gene sequence is only present in the cereulide-producing *B. cereus* (Toh et al., 2004). The primers used were BEF (5' ACT TAG ATG ATG CAA GAC TG-3') and BER (5'- TTC ATA GGA TTG ACG AAT TTT-3') with the amplicon size of 850 bp (Toh et al., 2004).

Currently, two commercial kits for emetic toxic detection are commercially sold. The first kit is Single-path Emetic Tox Mrk, developed by Merck. This is an immunochromatography kit. The sensitivity and specificity of this kit were evaluated by Nakayama et al. (2012), who found that this kit provided the result of high sensitivity and specificity by giving no false-negative result and only three strains of false positive. Another kit, one for detecting cereulide-producing *B. cereus*, is Swiftgene by KAINOS Laboratories, Inc. This kit helps to identify cereulide-producing *B. cereus* by combining the Nucleic Acid Sequence-Based Amplification (NASBA) and Nucleic Acid Chromatography (NAC) method. The NASBA method will amplify the cesPTABCD (ces) mRNA and Internal Control (IC) by using the specially designed primers provided inside the kit. The amplicon will then be determined by a single NAC strip.

In recent years, there has been an increasing interest in the quantification of cereulide. The first attempt to quantify cereulide was made by Häggblom et al. (2002). He used high-performance liquid chromatography (HPLC) connected to ion trap mass spectrometer. This method was attractive because it was sensitive and produced a precise result. The detection limit of cereulide by HPLC-mass spectrophotometry was 10 pg per injection. Ever since this method was first reported, many scientists have shown an interest in developing more sensitive and more rapid methods. Several methods currently exist for the measurement of cereulide including the usage of real-time PCR (Fricker et al., 2007). The micellar electrokinetic chromatography-capillary electrophoresis (MEKC-CE) method was next developed (Oh & Cox, 2010), followed by LCMS, LC-MS^2 and UPLC-ESI-MS/MS (Delbrassinne et al., 2012; Rønning et al., 2015; Zuberovic Muratovic et al., 2014). In most recent studies, cereulide has been successfully measured using the droplet digital PCR (Porcellato et al., 2016). It has been reported that this technique requires a lesser sample volume and has a lower cost of operation.

Diarrhoeal Food Poisoning

Diarrhoeal food poisoning is caused by enterotoxins. Four types of toxins have been identified to be responsible for diarrhoeal food poisoning. The toxins are haemolysin BL (*hblA*, *hblC*, *hblD*), non-haemolytic BL (*nheA*, *nheB*, *nheC*), enterotoxin FM (*entFM*) and cytotoxin K (*cytK*). Unlike the other toxins, the *EntT* toxin is believed to be unrelated to food-borne outbreaks (Lynn et al., 2013). According to Chitov et al. (2008), the most frequently isolated enterotoxin was *nheB* (80.80%) while the least frequently isolated was the enterotoxin gene *nheA* (59.20 %).

The intoxication of 10^5-10^7 cells or spore per gram can lead to diarrhoeal food poisoning (Bilung et al., 2016). These toxins are produced in the intestinal tract of the host and are unstable to heat. Diarrhoeal toxins are produced in conditions of inadequate refrigeration and improper reheating of leftover food. Proteinaceous food has been identified as the main vehicle for enterotoxin-producing *B. cereus*.

Method of Detecting the Diarrhoeal Toxin. Two commercially available kits can be used for the identification of the diarrhoeal toxin. The Tecra *Bacillus* diarrhoeal enterotoxin immunoassay (Tecra-VIA) kit detects the *nheA* gene, while the Oxoid *Bacillus cereus* Enterotoxin-Reversed Passive Latex Agglutination (Oxoid BCET-RPLA) kit can detect the L_2 component from the *hbl* gene. *Hbl* is made up of binding component B and the two lytic component L_1 and L_2 (Häggblom et al., 2002). A study performed to establish the effectiveness of these kits where Beecher and Wong (1994) found that each detected different antigen, as there was a lack of cross-reactivity between the positive controls provided in each kit. However, the Tecra-VIA kit has the capability to identify enterotoxins that have been heat-denatured, and the result is correlated with a Chinese hamster ovary (CHO) cell toxicity assay. The lowest concentration of the L_2 component detected by Oxoid BCET-RPLA kit was 0.6 ng/ml.

Salihah et al. (2018) investigated the effect of two different real-time PCR fluorescence strategies for the detection and quantification of the enterotoxin. The fluorescence methods tested were the ZEN double-quenched probe and improved SYBR Green dye. Of the two, the improved SYBR Green dye provided a much better result.

Prevalence of *Bacillus cereus* in Foods

Rice. *Bacillus cereus* is commonly associated with food poisoning from rice and rice-based foods (Lynn et al., 2013). Rice is a staple food of many Southeast Asian countries, such as Malaysia, Myanmar and the Philippines. At an average, Filipinos consume 4-5 servings of rice every day. *Bacillus cereus* has been identified as normal microflora of rice grain. Thus, it is essential to perform studies to establish that this food is safe for consumption.

In one of the studies conducted in Selangor, out of 25 samples of local Malaysian varieties of rice grain (*Keladi Halus Wangi*, *Keladi Wangi*, *Kanowit Halus*

Wangi, *Lansam Halus Wangi* and *Bario*), 100% of the samples tested were positive with *B. cereus* (Sandra et al., 2012). Both *Keladi Halus Wangi* and *Lansam Halus Wangi* had a maximum value of more than 1,100 MPN/g. These two varieties were considered too risky for consumption. In the same study, the presence of *B. cereus* was tested in varieties of cooked rice samples (*nasi lemak*, *nasi biryani*, *nasi ayam* and *nasi putih*). The total prevalence of cooked rice samples tested positive was 73.04%. The highest percentage of contamination with *B. cereus* was found in *nasi ayam* (100%), *nasi putih* (76.2%), *nasi lemak* (70.4%) and *nasi biryani* (50%). The cooked rice samples, except *nasi ayam*, had a maximum value of more than 1100 MPN/g. Sandra et al. (2012) used a combination of the most probable number-polymerase chain reaction (MPN-PCR) for the detection of the *gyrB* gene present in *B. cereus*. Using a similar method, another study in Malaysia showed that 85% (17/20) of the local indigenous and 100% (20/20) of imported rice grains tested positive for the presence of *B. cereus*, with a value of more than 1100 MPN/g (Chitov et al., 2008).

Enumeration of *B. cereus* in local Thai rice grain (Happy Rose Quality Thai Fragrant Rice, Liansin Butterfly, Liansin Mr Thai, Liansin Cap Amoi Super Siam, Happy Bamboo Thai, Liansin Beras Pulut Susu and NutriRice Thai Fragrant Brown) and Vietnamese rice grain (Tulip Vietnam Premier Rice, Teana Sargon Super Imported Rice and Lap Padi Emas Premium Quality White Rice) sold in Malaysia showed that all the samples had more than 1100 MPN/g (Tan et al., 2015). These studies showed a high percentage of *B. cereus* on all varieties. However, these studies did not test whether the identified *B. cereus* produced toxins.

Testing of 24 unhusked local rice samples (10 storage samples and 14 fresh samples) from Sarawak, Malaysia showed that in every sample, MPN results were more than 1,100 MPN/g (Bilung et al., 2018). The results indicated that unhusked rice might be the possible source for food-borne illness associated with *B. cereus*. The diarrhoeal syndrome was shown to be caused by 10^3 CFU/g of *B. cereus*, while the emetic syndrome was caused by 10^5 CFU/g (Guinebretière & Brousollee, 2002).

Aside from rice, the ready-to-eat (RTE) cereal distributed and sold in Sarawak was also tested for the presence of toxin-producing *B. cereus* (Lesley et al., 2013). The study found that *B. cereus* was present in 15% of the breakfast cereal (3/19) and 100% of the instant oatmeal (1/1) samples. The contamination was believed to occur due to cross-contamination in the processing line. Cross-contamination can also occur between the equipment used for food preparation and raw food materials. This result suggested that a processed food product might be contaminated with *B. cereus* even when it underwent strict processing conditions and continuous surveillance. The same study also tested a cereal drink, an original cereal, cornflakes and oat cereals, which were found to be free of *B. cereus*. The result of this study might not be significant because the sample size was small, and the amount of *B. cereus* found in the food was not mentioned.

Rice noodles, fresh wheat noodles and dried wheat noodles from Malaysia were found to be 41%, 17.6% and 34 % positive for *B. cereus* respectively (Rusul & Yaacob, 1995). Using the Tecra-VIA and BCET-RPLA, 178 (91.8%) and 164 (84%) of the isolated strains were identified as enterotoxin- producing *B. cereus* respectively.

Fresh Food. A study found that 3.33% (1/30) of the raw and 50% (15/30) of the cooked chicken meat samples in Kota Bharu, Malaysia tested positive for *B. cereus* (Aklilu et al., 2016). *Bacillus cereus* was found to be more prevalent in cooked than in raw chicken meat. It is likely that the contamination was due to the cross-contamination from the cooking utensils, food additives and ingredients used to cook the chicken meat. In this study, the CFU/g was not counted.

The study by Ananchaipattana et al. (2012a) offered the data concerning *B. cereus* contamination in meat and fish or seafood in Thailand. This study found that 2% (1/51) of the tested meat and 35% (13/37) of the fish or seafood were contaminated with *B. cereus*. All the positive samples of meat were obtained from open markets. Meanwhile, for fish or seafood, 7 positive samples were from open markets and the rest were from the supermarket.

Ananchaipattana et al. (2012b) investigated the soybean curd (tofu) in Thailand and found that 41% of the tofu samples were positive for *B. cereus*. Out of the 54 isolates, five were identified as enterotoxin-producing *B. cereus*. *Bacillus* spp. was isolated from 5% (2/38) of the green leafy vegetable samples obtained from independent farms and company farms (Ananchaipattana et al., 2012b). The number of samples contaminated with *Bacillus* spp. was the lowest compared to the other tested microbial contaminants such as *Escherichia coli*, *Pseudomonas* spp., *Staphylococcus* spp. and others.

Beverages. *Bacillus cereus* resides not only in foods but can also contaminate beverages. In Vietnam, common street drinks such as iced tea, sugarcane juice and corn milk were tested for the presence of microorganisms. The result showed that all the drinks contained *B. cereus* along with other pathogenic bacteria. The testing was conducted by Healthplus magazine in collaboration with the Centre for Evaluation and Conformation under the Vietnam Institute of Dietary Supplements (VIDS) of the Ministry of Health.

Additionally, 41.7% (5/12) of formula milk and 3% (6/20) of ultra-high temperature (UHT) milk tested in Malaysia was positive for *B. cereus*. The samples used for formula milk were infant formulas, follow-up formulas and young-child formulas. Of these, only the infant formula samples were negative for *B. cereus* contamination. For the UHT milk, the types of samples tested were whole milk, full cream milk, low-fat milk, skimmed milk and fresh milk. The MPN count showed more than 1100 MPN/g for all these products (Lesley et al., 2013).

Although in Malaysia the food poisoning cases due to milk spoilage had happened a few times, no report indicates the cause to be *B. cereus* contamination. This is probably because the milk normally spoils before *B. cereus* contamination and it is enough to cause gastrointestinal illness (Lesley et al., 2017).

Traditional Food. In Thailand, *B. cereus* has not been documented as a major microbial enteropathogen that causes outbreaks (Adley & Ryan, 2016). However, a study was conducted to investigate the presence of *B. cereus* on pasteurized milk (100%), rice (brown rice and sticky rice; 41.9%), cereal flour (33.3%), noodles (18.7%) and quick-cooked cereal-based meal (62.5%) (Chrun et al., 2017). All samples were contaminated with *B. cereus* at 0.5×10^2 to 1.7×10^3 CFU/g. The enterotoxin gene from the isolated *B. cereus* was profiled using PCR and the result showed that *nheB* (80.8%), *cytK* (70.4%) and *nheC* (69.9%) were the most common enterotoxins.

Another study in Thailand discovered the presence of *B. cereus* in 15% of tested Thai pandan custard, which then increased to 25% and 45% after one–day and two-day storage respectively (Puangburee et al., 2016). The source of the contaminant appeared to be the pandan leaves, 95% of which were positive for *B. cereus* with an average of 3.4 log CFU/g.

Fermented rice noodle is a local food in Myanmar that is commonly consumed during breakfast and as a snack. It was assumed that *B. cereus* contaminated the rice noodle during the fermentation process. Three samples of *B. cereus* isolated from the fermented rice noodle were used to study the enterotoxin gene using molecular assay (Lynn et al., 2013). The result confirmed that the isolates were enterotoxin-producing *B. cereus*.

In Cambodia, the contamination rate of *B. cereus* in local fermented mixed vegetables and fermented single-type vegetables was investigated. The results showed that the fermented single-type vegetables had a higher percentage of *B. cereus* contamination, 35% (17/48), compared to the fermented mixed vegetables, which had 20% (4/20) (Chrun et al., 2017).

Ananchaipattana et al. (2012a) reported that fermented meat and fish in Thailand had higher numbers of *B. cereus* compared to non-fermented meat and fish. Of the fermented meat and fish samples, 82% (9/11) were positive with *B. cereus*. The *B. cereus* likely originated from the cooked rice used as one of the fermentation ingredients.

There is no information on other countries including Malaysian on *B. cereus* food poisoning in traditional foods due to the lack of studies. Further work is needed to determine the prevalence of *B. cereus* in traditional food.

The Potential Use of *Bacillus cereus* as Antimicrobial Bacteriocins. Though responsible for food-borne illness, *B. cereus* can also act as an antimicrobial bacteriocin; this has been demonstrated with *B. cereus* isolated from *budu* in West Sumatra, Indonesia. *Budu* is a local food

made up of fermented fish. It was found that *B. cereus* can inhibit the growth of *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* Typhi, *Bacillus subtilis* and *Listeria monocytogenes* (Yusra et al., 2014). This antimicrobial bacteriocin was found to be stable at a pH range of 2 to 1 and could withstand heat treatment of 121˚C for 15 minutes.

Regulations. *Bacillus cereus* can cause illness only if it is ingested in high concentrations. Different countries have set varying maximum level limits for *B. cereus*. Food Standards Australia New Zealand has set the allowable amount of *B. cereus* in food to be below 10^2 CFU/g, with 10^4 CFU/g being considered risky. However, in the United Kingdom, the limit is 10^3 CFU/g and 10^5 CFU/g are regarded as unsafe (Bilung et al., 2016).

In Malaysia, food must undergo quality control based on the Food Regulations 1985 and the Food Act 1983 (Bilung et al., 2016). Under these regulations, there is no specific microbiological standard for *B. cereus* (Food Act, 1983). Instead, the limit is set at a plate count of less than 10^6 CFU/g at 37˚C for 48 hours. The Ministry of Agriculture and Agro-Based Industry (MOA) and the Ministry of Health (MOH) are the agencies responsible for food safety and hygienic control in Malaysia. MOA oversees the safety and hygienic control of production and primary processing. Imported and processed foods are under the responsibility of the MOH.

The Agri-Food and Veterinary Authority (AVA) of the Ministry of National Development is the administrative body responsible for food standards, safety and hygiene control in Singapore (Hiroaki, 2013). AVA has determined that less than 2.0×10^2 CFU/g for ready-to-eat (RTE) food is considered safe and represents a low risk to the public. Food that exceeds the permissible limit is considered unsafe and must be recalled immediately for further inspection.

The main administrative bodies responsible for food safety, food standards and hygienic control in Thailand are the Ministry of Public Health and the Ministry of Agriculture and Cooperatives (Hiroaki, 2013). The law responsible for protecting the consumers is the Food Act of B. E.2522 (1979) and the government has set the limit of *B. cereus* in food at 100 per 1 g or 1 ml.

Head of the National Agency for Drug and Food Control of the Republic of Indonesia is the government body that is responsible for controlling food contaminant in Indonesia. The government of Indonesia passed Act Number 7 on Food in 1996 (Hiroaki, 2013). Under the Selected Indonesian National Standards (SNI) 2897:2008 for microbial testing in foods such as meat, eggs and milk as well as their by-product, *B. cereus* is not tested. These foods are tested only for total plate count (TPC), coliform, *E. coli*, *Staphylococcus aureus*, *Salmonella* spp., *Campylobacter* spp. and *Listeria monocytogenes*. In 2009, the government made a regulation No. HK.00. 06.1.52. 4011 of 2009 on Maximum

Level of Microbiological and Chemical Contaminants in Foods (Hiroaki, 2013). Though under this legislation, *B. cereus* is also not listed.

Ministry of Health (MOH), Ministry of Agriculture and Rural Development (MARD), as well as the Ministry of Industry and Trade (MIT) are the ministries that are responsible for food safety management in Vietnam (Hiroaki, 2013). The main role for food safety management at the national level falls under the responsibility of the Ministry of Health. As for the Ministry of Agriculture and Rural Development, they work on making policy and managing food safety for the primary production sector. Last but not least, the Ministry of Industry and Trade is responsible for making policy and manage food safety sectors that manufacture products. The government of Vietnam sets the maximum limit of *B. cereus* in powdered nutrition products and special medical-use nutrition products for children up to 12 months old at 5×10^2 CFU/g (Huong & Ward, 2013). The regulation stated that *B. cereus* should not be tested during conformity assessment if the food producers are already taking risk control measures in production (HACCP or GMP). Only if the producer fails to take risk control measures will the standards limit be tested.

Prevention and Control Steps. It is impossible to eliminate *B. cereus* completely from foods since *B. cereus* can form spores and normal cooking temperature is not sufficient to eliminate them (Azanza & Centeno, 2004). Despite that, the bacterial number can be reduced by controlling time and temperature to reduce its potential public health risk.

A common practice in Southeast Asian countries is leaving cooked food at room temperature until the next meal. For example, school children usually bring lunch boxes from home. The food was prepared early in the morning and the kids will eat it only during the recess. Cooking heat will encourage the *B. cereus* to reproduce and leaving the foods at room temperature allows the *B. cereus* to further multiply (Schoeni & Wong, 2005. Reheating the food for a short time does not kill *B. cereus* or neutralizes the toxins it produces. By the time they started eating the food, the population of *B. cereus* might have already exceeded the safety limit. To avoid possible food poisoning, food must be eaten immediately after cooking. The practice of cooling rice and other kinds of food at room temperature must be stopped by bringing an end to the practice of preparing meals several hours before serving.

We should keep our cooked food in the fridge below 4°C within two hours after it is cooked as *B. cereus* has a growth temperature of 4°C to 50°C. Alternatively, the food can be kept warm at 60°C (Sandra et al., 2012).

Furthermore, most food contamination comes from cross-contamination of the raw ingredients, water supply and kitchen utensils. Thus, good food handling procedures need to be practised in the kitchen. At the same time, food handlers must ensure that the water used for preparing

the food is clean. Using contaminated water in the cooking process may also contaminate the food if the microorganisms are not killed during the cooking process.

Bacillus cereus is not only present in raw foods, but also processed foods (Lee et al., 2009). Food with high counts of *B. cereus* poses a health hazard to consumers, so precautions should be taken before consumption. Basic sanitary practices must be enforced throughout the chain of food processing.

Since most outbreaks happen in schools, the school authorities must be stricter when selecting food caterers and kitchen cleanliness needs to be constantly monitored. The most important thing to be done is creating awareness among the public regarding food-borne illness.

CONCLUSIONS

In conclusion, *B. cereus* is commonly detected in foods throughout Southeast Asia, especially in rice. It enters the food cycle likely through cross-contamination from the soil, cooking water and kitchen utensils. The amount of *B. cereus* detected in unhusked, raw and cooked rice counted using the MPN technique were all more than 1000 MPN/g. For other food samples, the levels of contamination ranged from 0.5×10^2 to 1.7×10^3 CFU/g. *Bacillus cereus* is often overlooked as food contaminant in Southeast Asia because of the mild and short duration of illness. Consequently, not many affected persons seek medical treatment which in turn causes food poisoning by *B. cereus* to be underreported.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Review article

House Finch-Associated *Mycoplasma gallisepticum* Responsible for Epizootic Conjunctivitis in Passerines

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ABSTRACT

In 1994, *Mycoplasma gallisepticum* (MG) was reported to be responsible for conjunctivitis outbreak in the North American house finch population. This new course of MG infection in passerine was the result of spillover infections from the poultry strains. In severe cases of the disease, the conjunctival lesions might cause blindness and death, but in the mild form, there is a chance of recovery. The immune system of the recovered birds develops a resistance to the previous strains. However, the incomplete immune responses and the ability of MG to rapidly alter its surface antigens allow the pathogen to evolve new strains that can infect the birds that have already developed immune resistance. Although the rate of mortality decreases as a result of developing resistance, the persistence of the disease continues due to the increase in both virulence and the replication rate of the new strains. Therefore, the morbidity rate has remained steady, and new species of birds become infected as a result of evolutionary adaptation of the new strains. In this regard, the objective of this study is to provide a review of the mycoplasma conjunctivitis in passerine species, notably by looking at it from the host-pathogen interaction point of view.

Keywords: Conjunctivitis outbreak, evolutionary adaptation, house finch, host-pathogen interaction, *Mycoplasma gallisepticum*

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INTRODUCTION

Mycoplasma gallisepticum (MG) is primarily recognized as the causative agent of acute and chronic respiratory disease in birds (Raviv & Ley, 2013). In 1994, MG

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was isolated in the samples collected from the house finch conjunctivitis outbreak and determined as the causative pathogen. This identification gave a new perspective on the pathogenicity of the MG that was not considered a primary pathogen in the wild population of passerine species previously (Ley et al., 1996). Additional analysis of the isolated pathogen revealed that a discrete MG strain (HFMG) was responsible for this outbreak (Dhondt et al., 1998, 2005; Tulman et al., 2012). Further studies showed the possibility of MG infection in a more diverse range of passerine species. Although most of these new cases were detected only through the conventional polymerase chain reaction (PCR) assay, some studies typed the strain of the detected MG in the birds (Allen et al., 2018). This strain typing led to the detection of different MG strains among the susceptible species (Cherry et al., 2006; Hochachka et al., 2013). This discovery has influenced researchers to investigate the fingerprints of these strains in wild birds to address the circulation of the MG in the wild.

The ability to rapidly change its surface antigens leads to fast host adaptation of MG; thus, making it a proper model to study and comprehend enzootic bacterial pathogens. In addition, the probability of spillover infections between the various species of birds is still open to interpretation. The study of the evolutionary developments of the host-pathogen interactions can provide a proper tool to answer such questions. For this concern, this paper describes a review of the *M. gallisepticum* infection associated with house finch and their interaction from various aspects.

Mycoplasma gallisepticum (MG)

Mycoplasmas belongs to the class of Mollicutes. Lack of cell wall, extraordinary reduction of the genome, and diminutive size distinguished this class from other bacteria. Adaptation to distinct hosts along with various tissue tropism plays a crucial role in the metabolism of mycoplasmas and regulates its austerity. Mucosal surfaces of the respiratory tracts, urogenital tracts, and joints are target regions for the colonization of the organism. Remarkable antigenic variation, despite its small genome size, revealed the capability of mycoplasma to survive in immunocompetent hosts. Limited capacity to synthesize the required nutrients and reduced genome have increased the dependency of mycoplasmas of their host cells. Therefore, cell membrane proteins (lipoproteins) play a vital role in the adherence of organism to host cells (Bencina, 2002). So far, 24 avian mycoplasma species have been identified, of which four of them are considered pathogenic to commercial poultry. These include *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, *Mycoplasma meleagridis* and *Mycoplasma iowae*. These 24 species are host-specific pathogens. They can also be commensals in non-host species, but in some birds like psittacine species, mycoplasmas may only be pathogenic (Lierz & Hafez, 2009). *Mycoplasma gallisepticum* is recognized as a multi-host microorganism. Its presence, however, is not always accompanied

by clinical signs. The cytadhesin genes that encode most of the surface proteins (lipoproteins) are involved in attachment to the host cells. The genes are comprised of putative variable protein gene (*pvpA*) and an operon encoding three genes: *mgc1*(*gapA*), *mgc2*, and *mgc3* (Boguslavsky et al., 2000; Yoshida et al., 2000).

MG Outbreak in House Finches

In February 1994, several cases of conjunctivitis in house finches (*Haemorhous mexicanus*) were reported that involved hundreds of infected birds at feeders and rescue centers (Doster, 1994). Conjunctival lesions were either unilateral or bilateral and ranged from mild to severe. The lesions were accompanied by serous to mucopurulent drainage and nasal discharge. Ley et al. (1996) attempted to identify the infectious agent. In this regard, a total of 25 conjunctival and infraorbital sinus swabs were collected from songbirds, especially house finches, and directly submerged in Frey's broth with 15% swine serum (Frey, 1968). Direct immunofluorescence (IF) was applied to identify the species. Of all these 25 samples, eleven isolates were detected positive using both direct IF and PCR. One of the two samples collected from blue jays was also detected positive to MG. Ultimately, researchers concluded that owing to the pathogenicity of mycoplasmosis in poultry; there may be subclinical long-term carriers within the wild population of songbirds (Ley et al., 1996). These findings introduced a new facet of the disease epidemiology in poultry and susceptible wild bird species like songbirds. Since then, conjunctivitis has been recognized as one of the typical clinical signs of MG infection in songbirds. This conjunctivitis outbreak was then spread readily to the eastern population of house finches inhabiting in Minnesota, Iowa, Missouri, Tennessee, and Mississippi states (Dhondt et al., 1998). The expansion of the disease was observed by experienced observers who participated in the ongoing Project Feeder Watch (Laboratory of Ornithology, Cornell University, Ithaca, New York, USA) from November 1994 to March 1997. An average of 24864 observations was recorded monthly. Based on this information, researchers were able to notice the spread of the disease and estimate the monthly prevalence of the infection. Data showed the rapid expansion of MG to eastern populations of passerine species. Irregular patterns of rising and fall were observed in the prevalence of the disease with the highest prevalence in the autumn due to dispersing juveniles. The high prevalence of MG infection among the breeding population had a devastating impact on the winter population of house finches in the eastern region of the US. For this concern, projects such as the feeder watch were established to monitor the status of conjunctivitis in finches. Data from the feeder watch project also indicated the occurrence of conjunctivitis in other species of songbirds. Hence, Hartup et al. (2000) tried to confirm the MG infection in selected songbird species that displayed conjunctivitis using culture,

PCR, and serology tests. Bird feeders were equipped with traps and nets year-round. Trapped birds were marked by applying a numbered aluminum leg band. Any signs of conjunctivitis such as swelling, erythema, exudation, or epiphora in eyelid were considered as conjunctivitis. Eye swabs were collected from the affected eye(s). Body condition score, wind chord, gender, and seasonal variation were considered as the confounding parameters and analyzed using logistic regression. In total, 1243 observations were made. Three species of songbirds, including house finch, goldfinch, and purple finch showed conjunctivitis and were found to be positive in the MG culture, PCR, or serology tests. In addition, two brown-headed cowbirds and four tufted titmice were positive in the plate agglutination test. None of the samples taken from these two birds was subjected to culture or PCR assay. Researchers concluded that the prevalence of mycoplasma conjunctivitis in this study area was lower than the northeast wintering house finch populations. Before 2003, the geographical distribution of the mycoplasmal conjunctivitis was primarily confined to the eastern populations of house finches. From then on, the disease was spread to the western populations (Figure 1).

House Finch-Associated MG in Other Wild Birds

Mycoplasma gallisepticum can be readily spread through horizontal transmission. Therefore, there is always a chance of disease transmission between different species of birds. In the case of mycoplasmal conjunctivitis, house finch was suspected to be responsible for the spread of MG infection in other passerine species in

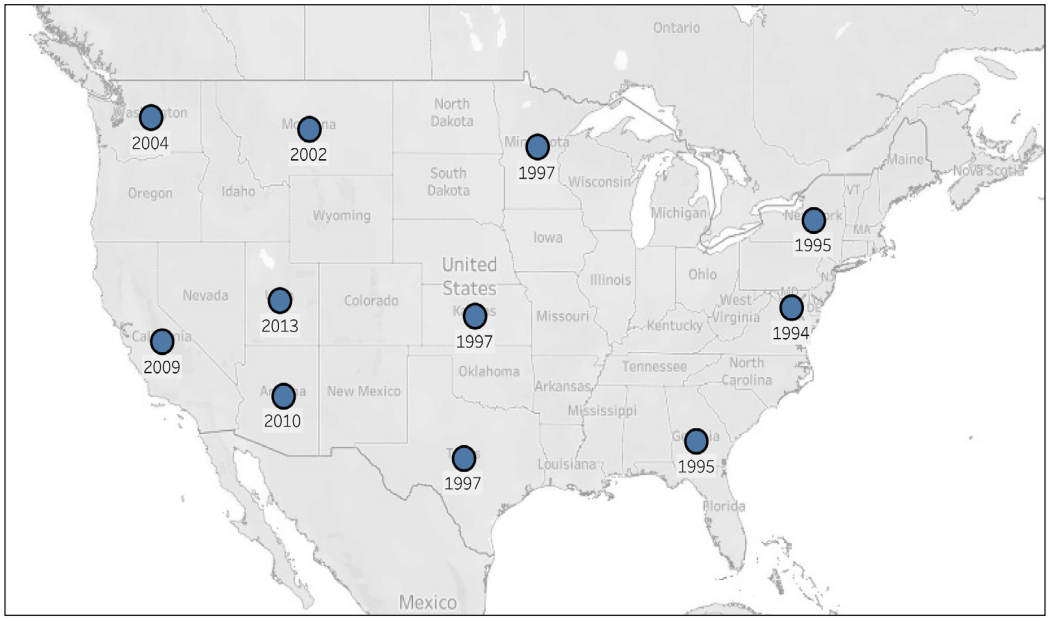

Figure 1. Geographical distribution of the HFMG in North America

North America. Therefore, further studies were conducted to reveal the role of house finches in the sustainability of the disease. To illustrate, in the study carried out by Luttrell et al. (2001), the prevalence of MG infection among passerine species that were living in poultry and non-poultry sites were measured and compared. The birds were captured using a mist net and assessed initially based on clinical signs and serum plate agglutination (SPA) test. Birds that were positive to the SPA test and had MG clinical signs were euthanatized and assessed further by culture, serology, PCR, and histopathology tests.

MG was only isolated from the house finches, and only the samples of house finches and tufted titmouse were positive for MG by PCR. However, histopathological lesions were observed in six house finches, five tufted titmice, three northern cardinals, one white-throated sparrow, and one yellow-rumped warbler. Ultimately, the higher prevalence of MG infection among the birds at poultry sites was statistically significant. These findings pointed towards the potential role of a house finch and tufted titmouse in the transmission of the disease. The MG infection in other songbirds was hypothesized to be the product of spillover infections that originated from the infected populations of house finches. In other words, the sporadic accidental infections in songbirds, excluding house finch, cannot break the transmission barriers between different species (Hartup et al., 2001). Therefore, after the exclusion of the regions with the historical presence of conjunctivitis (n=546), a total of 29266 observational data that were collected within four years were analyzed to test the hypothesis. The presence of the disease was confirmed when two wildlife veterinarians reported the signs of conjunctivitis. Bird species were divided into the target and non-target species based on the previous knowledge of susceptible species that was acquired from previous studies. A total of 297 cases of conjunctivitis from 27 non-target species recorded. A total of 187 of these cases occurred in American goldfinch, and 23 cases were reported in the Northern cardinal population. Logistic regression models were developed to determine the likelihood of conjunctivitis in birds by intervening in the occurrence of conjunctivitis in house finches on that particular temporospatial point. Results showed that the odds of observing conjunctivitis in both target and non-target species increased by the presence of house finches, especially during winters. Finally, the authors concluded that there is an epidemiological association between conjunctivitis in the population of American goldfinch, purple finch, and house sparrows with epizootics in house finch population, but the confirmation of the disease in other bird species required further investigations (Hartup et al., 2001). Therefore, Dhondt et al. (2014) aimed to investigate the risk factors involved in MG infection in wild songbirds. These factors included feeder usage, migration, and seasonal variation. For this concern, researchers set traps to catch birds at feeder and non-feeder sites between January 2007 and June 2010. Using culture,

PCR, and SPA tests, researchers were able to detect a broader range of hosts compared to the previous studies.

Eleven species of passerines were positive based on the findings from both SPA and PCR assays. Like the earlier study by Hartup et al. (2001), the occurrence of MG infection in wild bird species was shown to be directly associated with the incidence of MG infection in house finches. Moreover, it was found that seasonal change and migration, as the risk factors, influenced the occurrence of the disease as using feeder did not (Dhondt et al., 2014). Elaborate projects and facilities like the Feeder Watch Project and exclusive rehabilitation centers provided researchers with the proper tools to monitor the ecology and evolution of infectious diseases. This led to the identification of new MG susceptible passerine species (Ley et al., 2016). Rogers et al. (2019) were able to isolate and detect MG from conjunctival lesions of California scrub-jays (*Aphelocoma californica*) that were housed together with house finches at a rehabilitation center. The isolation of MG in the samples that were collected from these house finches and scrub jays were concurrent with the isolation of *M. synoviae*.

Impacts of *Mycoplasma gallisepticum* on House Finch Population

One of the early studies that assessed the impacts of mycoplasmal conjunctivitis on house finch populations was the study conducted by Hartup and Kollias (1999). Briefly, a total of 39 eggs and 110 nestling samples were assessed for the presence of MG using both culture and PCR. There was no MG isolated, and only two pooled choanal and conjunctival samples that were collected from two different broods of house finches tested positive for MG by PCR. These findings indicated that there is no evidence of vertical transmission in house finches. Therefore, direct contact with infected parents through brooding behavior, preening, or feeding was deemed as the possible route of infection in nestlings. To further examine the vertical transmission of MG in house finches, the reproductive success rate and the prevalence of MG among nestling were investigated by Nolan et al. (2004). A total of 280 nest boxes were used for sample collection and to check the presence of any clinical signs. Like the previous study, no evidence of vertical transmission was observed, and there was no significant impact on the hatchability rate. However, infected chicks had a smaller body size and relatively smaller tarsi that made them more accessible to predators. The contradiction between the new and initial course of the disease in terms of mortality and morbidity was the result of decreased virulence of MG strains, natural selection of the resistance house finches or the combination of both.

As a result of infection, the production of glucocorticoid, a stress hormone, can be incited and cause suppression of the host immune system (Dhabhar, 2009; Weidenfeld et al., 1995). Corticosterone, the main glucocorticoid in birds, was reported to be increased in house finches with conjunctivitis (Lindström et al., 2005).

It was also discovered that the pre-infection concentration of corticosterone has a negative correlation with the severity of clinical signs and sickness behaviors in house finches infected with MG (Adelman et al., 2015). Love et al. (2016) found a prolonged and late increase in the corticosterone level of the blood samples collected from house finches that were experimentally inoculated with low virulence MG strain. A higher concentration of corticosterone was also found to have a direct relationship with the severity of conjunctiva. This finding can be explained by the stimulation of cytokine activity due to the increase of corticosterone levels. These studies revealed the degree at which stress hormones can affect and alter the response of a house finch population to the MG infection.

Molecular Characterization of House Finch MG Isolates

Since the first report of mycoplasmal conjunctivitis (house finch eye disease), house finch disease survey (HFDS) has started to monitor the populations of house finches. The collected data were used for different reasons, such as measuring the mortality rate among the house finch populations. For instance, in the study conducted by Hochachka and Dhondt (2000), a high mortality rate (>50%) was reported from 1994 to 1999. The reports of high mortality rate and reduced reproductive success in house finches by Nolan et al. (2004) emphasized the need for an evolutionary study of the house finch-associated MG. Initially, the isolates were assessed by random amplification of polymorphic DNA (RAPD) test in which the banding patterns among the house finch MG strains (HFMG) were similar. However, different patterns of bands were observed between HFMG and three vaccine strains, including F, 6/85, and ts-11 (Hartup et al., 2000). Hong et al. (2005) used different techniques to discriminate different MG strains, including HFMG strains: K4997 and K4409. These techniques included sequencing the direct repeat (DR) region of *mgc2* gene amplified by PCR, RAPD, and amplified fragment length polymorphism (AFLP). The results showed that the gene target sequencing using *mgc2* DR region could differentiate these strains into seven groups. RAPD analysis of MG strains showed different 11 groups of strains highly similar to AFLP. However, AFLP is considered as a DNA typing method that can be linked to the database of the AFLP patterns, which allows researchers to use previous studies results. In all these techniques, three house finch isolates were classified as a unique group (Hong et al., 2005). In subsequent years, new technologies were employed to fingerprint the HFMG strains. For instance, Allen et al. (2018) developed a qPCR technique specific to HFMG strains. They compared the genome sequence of HFMG reference strains with those of low-passage R, F, S6, TS-11, 6/85, and A5969 strains to develop a primer specific to HFMG. Eight MG isolates from two American goldfinches, one purple finch, one house finch, two lesser goldfinches, one western scrub-jay,

and one American crow were assayed by the validated qPCR protocol and subjected to subsequent RAPD fingerprinting. The results have shown that all the isolates except that of American crow were favorable to the qPCR and demonstrated similar patterns.

The studies above indicated the circulation of one predominant MG strain in wild passerine populations. Some studies reported the presence of various HFMG strains. To illustrate, Liu et al. (2001) evaluated the sensibility of a molecular technique in which the *pvpA* gene was separated and amplified to distinguish between different MG strains, including house finch strains. PCR restriction fragment length polymorphism (PCR-RFLP) assay was conducted using PCR along with the restriction enzymes, including *Pvu*II, *Acc*I and *Scr*FI. PCR using selective primers for the gene of interest produced amplicons ranging from 266 to 497 bp. Four HFMG isolates displayed a 437 bp PCR product size and were placed in the F group of RFLP. One HFMG strain, however, showed a 266 bp product size and categorized as group H among RFLP groupings (Liu et al., 2001).

Further studies utilized more advanced techniques for phylogenetic analysis of HFMG. Cherry et al. (2006) designed a study to evaluate genomic variability between house finch isolates using both RAPD and AFLP. Samples were inclusive of 10 HFMG cultures isolated from different songbirds (one blue jay, one American goldfinch, and eight house finches) as well as six vaccine and reference strains isolated from poultry. The RAPD procedure was adapted from Ley et al. (1997), and primer sets were based on previous studies (Fan et al., 1995; Geary et al., 1994). AFLP fingerprinting technique was conducted based on the procedure described in a previous study (Kokotovic et al., 1999) in which the combination of *Bgl-II* and *Mfe-I* restriction enzymes were employed. The method that employed two sets of primers yielded at least two unique banding patterns of RAPD. The results of AFLP showed similar patterns among eight house finch isolates with the linkage level of 87%, indicating that these AFLP fragment patterns possibly belonged to one strain.

On the other hand, the AFLP pattern of one sample was unambiguously distinct from other songbird isolates with less than 78% linkage level. From the results of these two studies, it can be inferred that MG infection in songbirds might initially have a single point origin and the emergence of different strains of HFMG is the result of a molecular evolution after the first introduction and the following expansion of the infection. However, the clades or progenies from which this single point HFMG was evolved or originated were unclear until Hochachka et al. (2013) aimed to answer such questions by analyzing 107 isolates from poultry, house finches and other songbirds. Briefly, all the isolates were identified by direct immunofluorescence and then were subjected to molecular characterization based on partial genome sequencing. To make a comparison between these isolates, 13 sets of primers were employed to amplify the 8399 nucleotides of the whole genome in total. The result of

sequencing indicated two haplotypes among the isolates. One of these two haplotypes were from domesticated poultry, while the other one from house finch. This finding and the identical sequencing pattern between house finch and other songbirds indicated the primary interaction between poultry MG strains and the first HFMG strain. In other words, field strains of MG have been exchanged continuously between poultry and songbirds, especially house finches. Two aspects of host-pathogen interaction are involved here: 1) introduction into a new host, and 2) subsequent adaptation to new hosts that leads to minimal diversion among haplotypes. In an earlier study by Tulman et al. (2012), phylogenetic analysis of various MG isolates by using whole-genome sequencing indicated that HFMG strains were progenies of the poultry strains, notably between index isolate of MG in house finch (VA94) and F strain based on *vlhA* family group genes. In addition, significant divergence among HFMG strains implies the multi-points origin of the infection in the house finch. This indicates the existence of different primary lineages or earlier circulation of source strain in the wild before 1994. The identification of new hosts in subsequent years supported these results (Ley et al., 2016).

By considering all the findings reported from phylogenetic studies, it can be concluded that various MG strains isolated from house finches were progenies of poultry and/or ancestral wild strains (Tulman et al., 2012; Hochachka et al., 2013). Contradictory perspectives on the origin of HFMG might arise from the lack of sufficient index isolates. These findings emphasized the need for recognizing the potential of MG host among bird species through more elaborate trials. To illustrate, the potency of American goldfinches as a competent host for HFMG has been reported (Dhondt et al., 2013). However, such studies in different species of the Corvidae family are lacking. Conjunctivitis and identification of MG in blue jays had been reported since the first isolation of MG from house finches (Ley et al., 1996). In a recent study by Allen et al. (2018), the western scrub-jay was also reported positive of MG. These imply the potential role of the Corvidae family in sustainability and spillover infections of MG. A similar study revealed the susceptibility of tufted titmice to HFMG by displaying the significantly higher number of infected birds at poultry sites (Luttrell et al., 2001). However, there is a paucity of phylogenetic studies to comprehend what is the role of these birds in HFMG enzootics.

Experimentally Induced House Finch-Associated MG Infection

Inoculations of the HFMG were made for multiple reasons. For instance, Farmer et al. (2005) assessed the susceptibility of new hosts reported in previous studies (American goldfinches, eastern tufted titmice, house sparrows, pine siskins, chipping sparrows, purple finch, zebra finches, and budgerigars) by inoculation of an infective dose of a specific HFMG strain. Except for chipping sparrow, MG was detected in all species

using PCR. However, the clinical signs were not developed in house sparrows, zebra finches, and budgerigars. Another reason for conducting the experimental studies was to investigate the ways that MG can infect house finches. For this reason, Dhondt et al. (2007) designed a study to investigate whether fomites could transfer HFMG. They found that although HFMG strain was viable for 24 hours on the feeder's port tube and retained its infectious capability, the severity of the clinical signs was reduced when HFMG was inoculated to healthy house finches. Testing the theory of spillover infection and host jump events as the main reason behind the circulation of HFMG within wild passerine species was another reason to design the experimental studies. For instance, it was reported that the goldfinches are more susceptible than house sparrows to certain house finch MG strains; therefore, they are more infectious to house finches (Dhondt et al., 2008). This can be due to the different course of MG infection in goldfinches in which less severe clinical signs, faster improvement of conjunctivitis, and persistence of the pathogen in the conjunctiva were found. The consideration of American goldfinch as a competent host was assessed further and ultimately American goldfinch deemed as a competent host with more capability to spread HFMG, mostly because of its long-distance migration (Dhondt et al., 2013). As the infection spreads through enzootics, the MG will get the chance to endure and become enzootic in new areas by the introduction to new hosts. The virulence of MG will increase gradually, especially when birds infected with lower virulent strains are exposed to strains of higher virulence. High virulent strains will cause more severe signs; thus, they will shed copiously (Hawley et al., 2010, 2013; Williams et al., 2014). For further assessment, an experimental study was designed to find out whether the increase in virulence as a dynamic of enzootic disease is independent of the previous infection caused by a heterologous strain (Dhondt et al., 2017). The results of the study showed that the response to the re-infection was highly dependent on the pathogenicity of the former and new strain, regardless of whether the causative strains were heterologous or not. In other words, a high virulent strain can cause a more severe infection in house finches without any previous exposure compared to those with previous exposure. These findings were consistent with the primary expansion of the infection in new areas, especially during the fall and winter (Altizer et al., 2004; Dhondt et al., 2006; Hartup et al., 2001), and the subsequent decline in the number of infected house finches in enzootic regions due to the fact that juveniles recovered from previous infections became more resistance, regardless of the pathogenicity of the first strain (Hosseini et al., 2004). Drawing a comparison between exposed and non-exposed populations of house finches in terms of genetic variation and immune response can be very useful to understand how host resistance naturally influences bacterial virulence and replication rates. For instance, it was shown that the eastern

(the first exposed population) and western population of house finches had similar gene expression to MG inoculation until 2000. After this period, those house finches of the eastern population that were exposed to MG and survived, evolved genetic resistance by gaining the ability to mount a protective cell-mediated immune response (Bonneaud et al., 2011, 2012). In a recent study, the course of experimentally induced MG infection was compared between house finches with and without previous exposure to the pathogen. The results indicated that MG had become more virulent since recent strains caused more severe infections. In addition, as a result of immune adaptation, the possibility of developing lethal clinical signs in a previously exposed population would be reduced (Bonneaud et al., 2018). However, owing to the imperfect nature of the host immune memory, most of the responses to the infection were incomplete. These incomplete responses can provide a favorable condition for the evolution of more virulent strains (Fleming-Davies et al., 2018). These findings were consistent with a more elaborate study indicating that the initial spread of resistance to HFMG infection among house finches was the primary driver of the increasing virulence rather than the replication rate (Tardy et al., 2019). It was also observed that there is a direct association between the virulence of the HFMG and the expression of pro-inflammatory cytokines (Vinkler et al., 2018). In a recent immunological pathway study of both virulent and attenuated MG strains, a significant increase in the expression of the genes encoding proteins associated with pro-inflammatory responses was observed in the virulent strains. This indicates that the increase in the virulence of the pathogen leads to maladaptive and dysregulated immune responses of the host (Beaudet et al., 2019).

From these results and reports, it can be inferred that the recurrent induction of MG from the old host into the new host will ultimately end up with the adaptation to the new host. The host immune system plays a crucial role in this process, and MG infection in house finches is the result of the inefficiency of the immune system, especially the non-specific immune system, in the detection and elimination of the pathogen. By developing the immunity against the enzootic MG strains, through the prevention of the induced immune suppression instead of preventing the establishment of the pathogen, the proportion of infected house finches will drop, and the transmission of disease will be harder. On the other hand, the ability to alter the surface components, along with the predisposing environmental factors that affect the host immune system, can provide a suitable situation for MG to invade to new hosts and evade the immune system. This highlights the significance of monitoring programs in large scales such as the Feeder Watch Project for the fast identification of new competent hosts. From the studies investigating the effects of MG on house finch populations, it can be inferred that the higher rate of reproduction after high mortality and morbidity of the primary outbreaks was a result of the

adaptation of MG to its hosts whereby it can spread more readily by infecting the nestlings and even other species such as cowbirds. This interpretation is consistent with the general increase in virulence and evolving more virulent strains. To achieve this conclusion, however, direct examination of genital organs of adult house finches will be required to eliminate the possibility of vertical transmission of MG in house finches.

CONCLUSION

The mycoplasmal conjunctivitis outbreak caused by *Mycoplasma gallisepticum* revealed a new feature of the mycoplasmosis in birds. In this new course of MG infection, the increase in virulence as a result of spillover infections was observed. The spillover infections might lead to the evolution of new strains that might be capable of infecting the population of birds that evolved resistance to the ancestral strains. This emphasizes the role of free-living birds in the circulation of the pathogen. While mycoplasmal conjunctivitis mostly occurs in the house finches, almost every species of passerine birds can be the carrier of the pathogen, which makes the eradication of the disease from the wild more difficult. Therefore, the application of strict biosecurity in poultry farms to reduce the pathogen transmission between the commercial poultry and wild population of birds is highly advisable.

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Chemical Constituents of Water Extract of *Acmella uliginosa* (Sw.) Cass. Flowers, Leaves, Stems and Roots from Malaysia

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ABSTRACT

A few reports on the phytochemical analysis of the organic solvent extract of *Acmella uliginosa* (Sw.) Cass. (*A. uliginosa*) have been published previously. Water extract is preferable depending on the intended final applications of the extract, such as those for the food and pharmaceutical industries. Thus, this current study focused on the phytochemical study of water extract from various parts of *A. uliginosa* which was collected from Kelantan, Malaysia. The sample of *A. uliginosa* flowers, leaves, stems and roots were converted into powder form and extracted with water macerated extraction procedures. The crude extracts were freeze-dried, and the extracts obtained were analysed using gas chromatography mass spectrometry. More than 80 chemical compounds were identified from the flowers, leaves, stems and roots of water extracts of *A. uliginosa*. The bioactive compounds that were identified include alkaloids, terpenoids, phenolic compounds and steroids. This study also revealed that the most abundant compound in both flowers and leaves were spilanthol, whereas linoleic acid and 2-Furancarboxaldehyde, 5-methyl- were identified as the highest compound for stems and roots; respectively. This finding suggested that water extracts of various parts of *A. uliginosa* are good sources of many bioactive compounds that might have pharmacological potential and high therapeutic value. Further experiments are needed to prove that all parts of the water extract of *A. uliginosa* do have therapeutic value.

Keywords: *Acmella uliginosa* (Sw.) Cass., gas chromatography mass spectrometry, water extract

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INTRODUCTION

Acmella uliginosa (Sw.) Cass. (*A. uliginosa*) or commonly known as "subang nenek" in Malaysia is a species of flowering plant which is indigenous and widely distributed in the tropics and sub-tropics regions especially in the West Indies, Venezuela,

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Brazil, Africa, Indonesia and Malaysia. "Subang nenek" does not specifically refer to *A. uliginosa*, but it is referred to a genus *acmella*. Genus *acmella* is commonly used by the Malay community in Malaysia to relieve pain which often associated with mouth ulcers, toothache, sore throat and stomachache. The alkaloid compound known as spilanthol or its IUPAC name is N-Isobutyl-2(E),6(Z),8(E)-decatrienamide was reported to be responsible for this property. The plant is known as the toothache plant due to the analgaesic effect of spilanthol. Spilanthol can be found not just in genus *acmella* such as *A. uliginosa*, *Acmella oleracea*, *Acmella ciliate* and *Acmella paniculata* but also in other plants such as *Heliopsis longipes* (Barbosa et al., 2016).

The use of genus *acmella* is not only limited to pain relief. It is also traditionally used to treat tuberculosis (Storey & Salem, 1997) and leucorrhoea (Hossan et al., 2010). In the present study, we have focused on *A. uliginosa*. Previous studies of *A. uliginosa* showed that this plant had various pharmacological properties such as antioxidant (Maimulyanti et al., 2016), anti-inflammatory (Paul et al., 2016) and antimicrobial properties (Lagnika et al., 2016). However, most of the previously reported studies focused on *A. uliginosa* flowers that were obtained using organic solvent including methanol, hexane, and ethyl acetate (Maimulyanti et al., 2016; Modak et al., 2017; Ong et al., 2011). The study of the other parts of *A. uliginosa* such as its stems and roots, however, is still lacking. The *in vitro* study of the methanolic extract of *A. uliginosa* flowers collected from West Bengal showed anti-inflammatory effect (Modak et al., 2017). The *in vivo* study of the methanolic extract of *A. uliginosa* flowers collected from Pahang, an East Coast state in Malaysia showed antinociceptive activity in chemical and thermal-induced nociception mice models (Ong et al., 2011). On the other hand, an *in vivo* study of water extract of *A. uliginosa* flowers revealed that there were anti-inflammatory and anti-arthritic properties in arthritic rats that ingested the extract (Paul et al., 2016). Furthermore, a previous study of oral toxicity had shown that water extract of *A. uliginosa* leaves had low oral toxicity compared to dichloromethane and methanol extracts of it on rats (Lagnika et al., 2016).

Water extract is preferable depending on the intended final applications of the extract, especially in pharmaceutical and food industries. In the present study, we report on the chemical constituents of the water extract from the flowers, leaves, stems and roots of *A. uliginosa* from Malaysia. As far as we know, it is the first report about the phytochemical analysis of the water extract of *A. uliginosa*.

MATERIALS AND METHODS

Sample of *Acmella uliginosa*

Acmella uliginosa sample was collected from Kelantan, Malaysia. The plant was confirmed by a botanist from Forest Research Institute Malaysia (FRIM), Dr. Fadzureena Jamaludin. A voucher specimen was then deposited in the Herbarium of FRIM, under code sample SBID 035/19.

Preparation of Water Extract of *Acmella uliginosa*

Water extract of *A. uliginosa* was prepared based on the methods described by Lagnika et. al (2016) with some modification. The whole parts of *A. uliginosa* were cleaned, separated and dried in an oven (40°C) for a day and were ground into a fine powder. The extraction was performed under mechanical agitation with distilled water (3:7 w/v) in a glass bottle for one day. The mixture was filtered and dried using a freeze dryer. The dried extract was stored in amber glass and was kept in the freezer (-20°C).

GC-MS Conditions and Parameters

The phytochemical analyses of various parts of water extracts of *A. uliginosa* were carried out using Hewlett Packard 6890 Gas Chromatograph equipped with 5973N Mass Selective Detector (Agilent Technologies, USA). The column was fused silica capillary, HP-5 column (30 m x 0.25 mm i.d x 0.25 µm film thickness). The carrier gas was helium at a flow rate of 1.0 ml/min. The column temperature was programmed from 50°C (held for 2 min) to 280°C (held for 10 min) at a rate of 20°C/min. The injection and interface temperatures were set at 250°C and 280°C, respectively. The electron ionisation was fixed at 70eV. The samples (1 µl) were injected in splitless mode and analysed in MS full scan mode (*m/z* 40-650). Acquisition of data was performed using Chemstation software. Blank solvent (water) was dried and treated similarly as the sample and used as a control.

Identification of Chemical Constituents

The compounds in *A. uliginosa* were identified by matching their mass spectra with the National Institute of Standards and Technology (NIST02) and Wiley275 libraries (≥ 80% matching). The percentage of the compound was calculated from the summation of the peak areas of *A. uliginosa* compounds.

RESULTS AND DISCUSSION

The GC-MS analyses of flowers, leaves, stems and roots of the water extracts of *A. uliginosa* exhibited the presence of various interesting compounds as listed in Table 1. The GC-MS chromatograms obtained are given in Figures 1-4. More than 80 chemical compounds were identified from the water extract of *A. uliginosa*. Generally, GC-MS analyses of water extracts of *A. uliginosa* showed the differences in chemical constituents between the plant's parts. A total of 34 chemical compounds were identified in the flowers and leaves, 30 chemical compounds in the stems and 32 chemical compounds in the roots.

These chemical compounds were grouped into few biologically active classes which were alkaloids, phenolic compounds, terpenoids and steroids. In this study, alkaloids are the major biologically active class of the water extract of *A. uliginosa* was obtained from flowers, leaves and roots. The alkaloids that were found in this study were spilanthol and N-(2-Phenylethyl) (2E,6Z,8E)-decatrienamide. However, spilanthol was the only alkaloid that was identified in all parts of the water extract

Table 1
Chemical compounds of water extracts of Acmella uliginosa *(Sw.) Cass. flowers, leaves, stems and roots*

Class of compounds		Compounds name	Composition (%)			
			Flowers	Leaves	Stems	Roots
Alkaloids	1.	Spilanthol	6.08	8.42	2.74	3.71
	2.	N-(2-Phenylethyl)(2E,6Z,8E)-decatrienamide	1.43			
Phenolics	3.	Benzaldehyde	0.03	0.02		
	4.	Mequinol		0.37		
	5.	4-vinyl-2-methoxy-phenol		1.68		
	6.	Eugenol		0.17		
	7.	Benzeneacetyldehyde			0.86	
	8.	p-Vinylguaiacol			1.65	
Steroids	9.	Cholesterol	0.21			
	10.	Stigmasterol	1.52	2.00	1.66	2.34
	11.	Ergost-5-en-3-ol,(3β)-		0.46		
	12.	Ergost-5-en-3-ol,(3β,5α)-		0.15		
	13.	β-Sitosterol		2.49		
	14.	δ-8(14)-stigmastenol		0.61		
	15.	Campesterol			0.17	
	16	Dihydrochondrillasterol	0.09		0.09	
	17.	γ -Sitosterol				0.75
Terpenoids	18.	Cyperene	0.12			
	19.	Caryophyllene	0.10		1.02	0.42
	20.	β-Cubebene	0.07	0.06		
	21.	Germacrene D	0.20	0.41		
	22.	Caryophyllene oxide	1.69	1.65	0.55	0.97
	23.	Phytol	0.55	1.84		
	24.	Squalene	0.64	0.63		
	25.	α-Amyrin	0.60			0.21
	26.	12-Oleanen -3-yl-acetate(3α)	0.31			
	27.	α- Amyrenyl acetate	0.50			
	28.	Lupenyl acetate	0.24			
	29.	Trans-β-Caryophyllene		1.47		
	30.	Neophytadiene		0.75	0.78	
	31.	α-Cubebene			0.55	0.34
	32.	β-Amyrin			0.10	
	33.	Sitostenone				0.08
Fatty Acids	34.	Lauric acid	0.31	0.28	1.54	0.32
	35.	Myristic acid	0.45		0.93	
	36.	Palmitic acid	2.66	3.33	3.32	4.32
	37.	Linoleic acid	5.80		5.30	
	38.	Stearic acid			1.70	1.80
	39.	Margaric acid				0.39
	40.	Linolenic acid				3.68
	41.	Eicosanoic acid				0.22
	42.	Behenic acid				0.38

Table 1 *(continue)*

Class of compounds		Compounds name	Composition (%)			
			Flowers	Leaves	Stems	Roots
	43.	Vitamin E	0.65	0.12	0.21	0.24
	44.	γ-Tocopherol		0.04		
	45.	β-Monolinolein	3.81	1.72	0.38	0.74
	46.	2-Monolinolenin		3.92		
	47.	Lauric acid, methyl ester	0.11		0.24	
	48.	Palmitic acid, methylester	1.54	2.59	0.91	0.99
	49.	Linoleic acid, methylester	3.75	2.01	0.55	0.58
	50.	9,12,15-Octadecatrienoic acid, methyl ester		3.72		
	51.	Stearic acid, methylester	1.20	4.39		
	52.	Linolenic acid				0.42
	53.	Eicosanoic acid, methyl ester	3.02			
	54.	Tricosanoic acid, methyl ester		0.60		
	55.	2-Cyclopentene-1,4-dione	0.02		0.37	0.06
	56.	Butyrolactone	0.08			0.06
	57.	1(3,6,6-trimethyl-1,6,7,7a-tetrahydrocyclopenta(c) pyran-1-yl ethanone	0.14			
	58.	Looplure	0.89			
	59.	1-Pentadecene	1.63	0.95		1.05
	60.	1,13-Tetradecadiene	0.69			
	61.	Butan-4-olide		0.12		
	62.	1H-Pyrrole-2,5-dione, 3-ethyl-4-methyl-		0.12		
Others	63.	1-Undecanol		0.14		
	64.	9,12,15-Octadecatrien-1-ol		7.05		
	65.	Silikonfett se30 (grevels)		0.31		
	66.	2-Furanmethanol			0.48	0.12
	67.	Benzyl alcohol				0.41
	68.	Butanoic acid, 4-hydroxy			0.19	
	69.	1,2-cyclopentanedione			1.28	
	70.	Cis-7-Dodecen-1-yl acetate			0.74	
	71.	1-Nonadecene			2.88	
	72.	*Spiro*[*2.4*]*heptane, 1,5-dimethyl-6-methylene-*			0.26	
	73.	Tricosanoic acid, methyl ester			0.07	
	74.	Lignoceric acid, methyl ester			0.26	
	75.	Furfural				0.76
	76.	2(3H)-Furanone, 5-methyl-				0.05
	77.	2-Cyclopenten-1-one, 2-hydroxy-				0.13
	78.	2-Furancarboxaldehyde, 5-methyl-				0.40
	79.	2-Furancarboxaldehyde, 5-(hydroxymethyl)-				11.48
	80.	1,11-Dodecadiene				0.66
	81.	2-Tridecanone				0.40
	82.	Cyclononasiloxane, octadecamethyl-				0.95
	83.	Nonanoic acid, 9-(3-hexenylidenecyclopropylidene)-, 2-hydroxy-1-(hydroxymethyl)				0.31

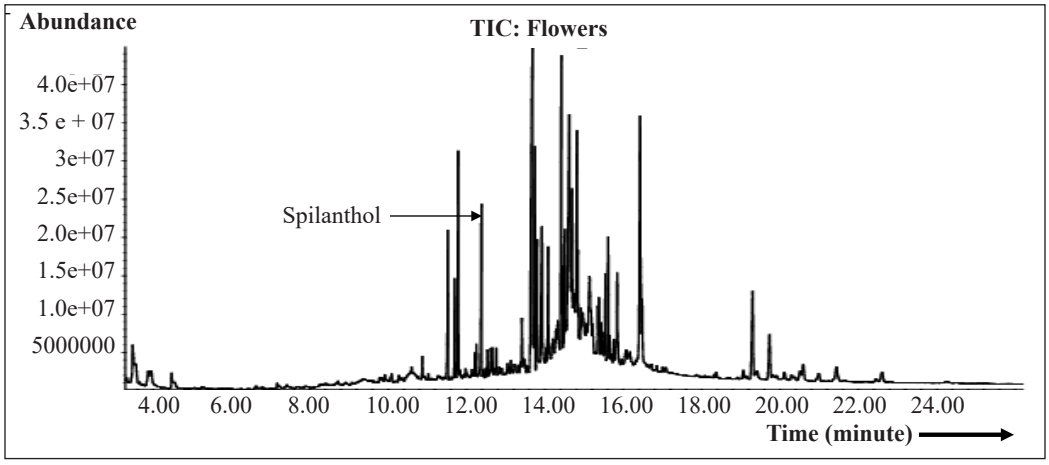

Figure 1. Chromatogram of water extract of *Acmella uliginosa* (Sw.) Cass. flowers

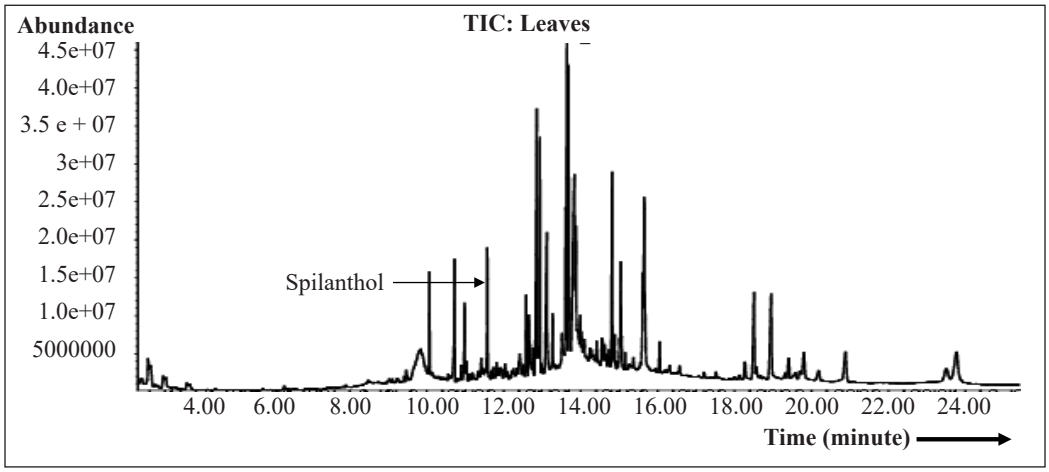

Figure 2. Chromatogram of water extract of *Acmella uliginosa* (Sw.) Cass. leaves

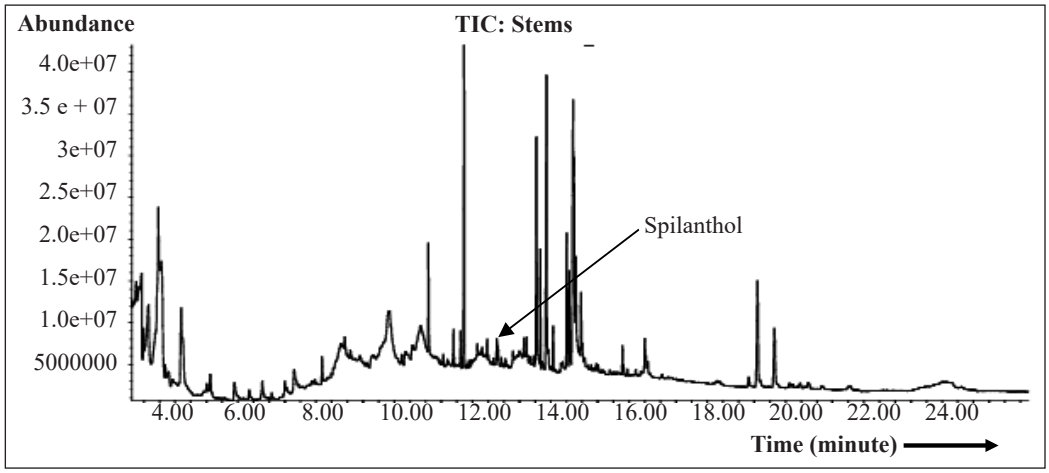

Figure 3. Chromatogram of water extract of *Acmella uliginosa* (Sw.) Cass. stems

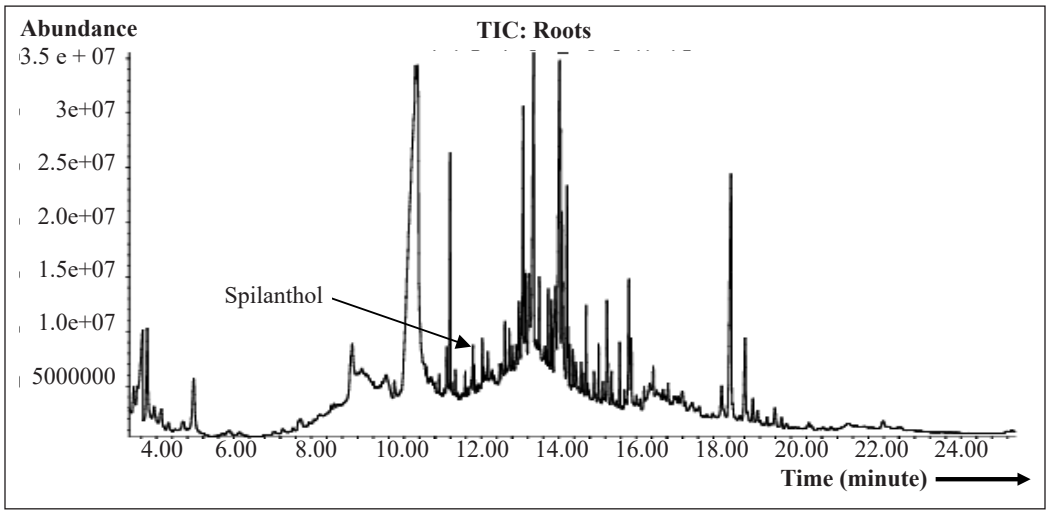

Figure 4. Chromatogram of water extract of *Acmella uliginosa* (Sw.) Cass. roots

of *A. uliginosa*. The highest percentage of alkaloids was found in leaves (8.42%), followed by flowers (6.08%), while lower in the root (3.71%) and stems (2.74%). The terpenoids were the main chemical class that were identified from leaves (6.81%).

Out of 83 identified chemical compounds, only nine chemical compounds were present in the water extracts of all parts of the plant which were spilanthol, stigmasterol, caryophyllene oxide, lauric acid, vitamin E, β-monolinolein, palmitic acid, palmitic acid methyl ester and linoleic acid methyl ester. All these chemical compounds are known to have a range of biological properties. Spilanthol is known to possess local analgaesic effect and various biological properties such as neuroprotective, antioxidant, antimutagenic, anticancer, antimicrobial and anti-inflammatory activities (Barbosa et al., 2016). A recent study of stigmasterol indicated that this steroid has antiangiogenic (Michelini et al., 2016) and antinociceptive properties (Walker et al., 2017). While the study of caryophyllene oxide showed that this terpenoid compound had anticancer and analgaesic properties (Fidyt et al., 2016).

Flowers

GC-MS analysis of water extract of *A. uliginosa* flowers showed that 34 chemical compounds were identified, and the main biologically active classes were alkaloids (7.51%) and terpenoids (5.02%). This finding was in agreement with a previous study of n-hexane extract of *A. uliginosa* flowers obtained from Indonesia (Maimulyanti & Prihadi, 2016). Both studies indicated that alkaloids and terpenoids were the main biologically active classes identified in *A. uliginosa* flowers. Interestingly, the major compound identified from *A. uliginosa* flowers in both regions was spilanthol. However, a study by Maimulyanti and Prihadi (2016) found that the percentage of spilanthol was 37.80%, whereby the percentage of the spilanthol in the

current study was only 6.08%. Other than spilanthol, N-(2-Phenylethyl) (2E,6Z,8E)-decatrienamide was also identified in both studies. The main terpenoid identified in the present study was caryophyllene oxide (1.69%), whereby α-Pinene was the main terpenoid identified from the n-hexane extract of *A. uliginosa* flowers (Maimulyanti & Prihadi, 2016). Only two terpenoids were identified from both studies which were caryophyllene and β-Cubebene. Other minor biologically active classes found in the present study were steroids (1.82%) and phenolic compounds (0.03%). Steroids were not identified in the n-hexane extract of *A. uliginosa* flowers obtained from Indonesia. Phenolic compounds were found in both studies; however, the compounds differed.

Leaves

The results of water extract of *A. uliginosa* leaves showed that 34 chemical compounds were identified and spilanthol was the main compound (8.42%), followed by 9,12,15-octadecatrien-1-ol (7.05%). Alkaloid (8.42%), terpenoids (6.81%) and steroids (5.71%) were found to be the major biologically active classes in the water extract of *A. uliginosa* leaves. Steroids have been identified to have the highest percentage in the leaves compared to other parts. Similar to the water extract of *A. uliginosa* flowers, alkaloid and terpenoids were the main identified bioactive classes in water extract of *A. uliginosa* leaves. This finding is in agreement with the previously published study on chemical compounds of the n-hexane and ethyl acetate extracts of *A. uliginosa* leaves from Indonesia (Maimulyanti et al., 2016). Study by Maimulyanti et al. (2016) found that alkaloids and terpenoids were the main biologically active classes identified in both organic solvent extracts. Spilanthol was the major alkaloid identified in both studies, whereby caryophyllene oxide was the main terpenoid identified in the current study and caryophyllene epoxide was the main terpenoid identified in the n-hexane and ethyl acetate extracts of *A. uliginosa* leaves (Maimulyanti et al., 2016). This finding contradicted with the methanol extract of *A. uliginosa* leaves from Indonesia (Maimulyanti et al., 2016) and Nigeria (Uraku, 2016). Both phytochemical studies on the methanol extracts of *A. uliginosa* leaves from both regions showed that alkaloids, phenolics and steroids were not found. Terpenoid was found in the methanol extract of *A. uliginosa* from Indonesia but was absent in the methanol extract of *A. uliginosa* from Nigeria. Neophytadiene was the only identified terpenoid in the methanol extract of *A. uliginosa* from Indonesia. In the present study, the following terpenoids β-Cubebene, germacrene D, caryophyllene oxide, phytol, squalene, trans-β-Caryophyllene and neophytadiene were found in the water extracts of *A. uliginosa* leaves. This findings were contradicted with the previous study of phytochemical screening of water extract of *A. uliginosa* leaves from West Africa (Lagnika et al., 2016). A study by Lagnika et al. (2016) found that terpenoids, as well as alkaloids, were not identified in water extract of *A. uliginosa* leaves.

Stems

The water extract of *A. uliginosa* stems revealed the presence of 30 compounds; the main group was fatty acids (12.79%). The main identified compound was linoleic acid (5.30%) and followed by palmitic acid (3.32%). Spilanthol (2.74%) was the least compound identified in the water extract of *A. uliginosa* stems compared to the other parts. Similar to the water extract of *A. uliginosa* leaves and stems, spilanthol was the only alkaloid identified. The other bioactive groups that had been identified were terpenoids (3.00%), phenolic compounds (2.51%) and steroids (1.92%). The major terpenoid identified was caryophyllene (1.02%), followed by neophytadiene (0.78%). The main phenolic compound identified was p-Vinylguaiacol (1.65%), whereby stigmasterol was the main steroid identified in the water extract of *A. uliginosa* stems. Until present, no phytochemical analysis of the *A. uliginosa* stems has been attempted before. However, study on the other species of *Acmella* which is *Acmella oleraceae* Murr. (Asteraceae) showed that terpenoids were not found in the methanolic extract of its stems (Abeysiri et al., 2013).

Roots

The results obtained from GC-MS analysis of water extract of *A. uliginosa* roots showed that 32 compounds were identified. Similar to the water extract of *A. uliginosa* stems, fatty acid was the main identified class (11.11%). The main identified chemical compounds in the water extract of *A. uliginosa* roots was 2-Furancarboxaldehyde, 5-methyl- (11.48%), followed by palmitic acid (4.32%), and spilanthol (3.71%). The 2-Furancarboxaldehyde, 5-methyl- was only identified in water extract of *A. uliginosa* roots. The other bioactive groups that were identified in the water extract of *A. uliginosa* roots were steroids (3.09%) and terpenoids (2.02%). Similar to *A. uliginosa* stems, no previous study on the *A. uliginosa* roots had been done so far. A previous study of the other genus *acmella* which is *Spilanthes acmella* Murr. collected in India showed that the phenolic content was present in the roots (Tanwer et al., 2010). This finding is contradicted with the current study as in our study, the phenolic compound was not identified in the roots.

CONCLUSION

The detection of chemical constituents in the water extracts of various parts of *A. uliginosa* showed the presence of bioactive compounds such as alkaloids, phenolic compounds, steroids and terpenoids. Both flowers and leaves were mostly composed of alkaloids, while the stems and roots were characterised by higher amounts of fatty acids. These compounds are known for their bioactivities. This study revealed that the water extracts of *A. uliginosa* were good sources of many bioactive compounds and it is suggested that all parts of water extract of *A. uliginosa* may have pharmacological potential and high therapeutic value. Further experiments are needed to prove that all parts of water extract of *A. uliginosa* do have therapeutic effects.

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CONFLICT OF INTEREST

Authors declare that there is no conflict of interest regarding the publication of this article.

STATEMENT OF HUMAN AND ANIMAL RIGHTS

This article does not contain any studies with human or animal subjects performed by any of the authors.

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Durian Yield Trends and Distribution Patterns in Peninsular Malaysia

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ABSTRACT

This study mainly aimed at gathering information on durian production trends and distribution in Peninsular Malaysia. Time series data from 2000 to 2017 involving planted areas and their respective production of durian were used for the analysis. The production trends and the magnitude of the slopes for all planted areas were then analyzed using Mann-Kendall and Sen's slope estimator. The results indicated that the hectarage of planted areas showed a downward trend in most parts except for Raub and Rembau districts. Despite the decrease in areas planted with durian, some areas exhibited an upward trend in annual production, particularly in the northern and central regions. This study also revealed that there was a shift in the distribution of durian production throughout the study period. These findings will be useful for policymakers and practitioners to improve durian orchard planning and management. However, future research should be conducted to determine the impact of climate variability on the shifting of durian production in Peninsular Malaysia.

Keywords: Durian production, durian planted area, Mann-Kendall, Sen's slope, variability

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INTRODUCTION

The durian (*Durio zibethinus* L.) also popularly known as "the king of tropical fruits" is one of the most economically important tropical fruits grown in Southeast Asia. Originated from Peninsular Malaysia and Borneo, its distribution has spread to Sri Lanka, Northern Australia, and Hawaii (Honsho et al., 2004). The main producers and exporters of durian are Thailand, Malaysia, and Indonesia while other countries in the region such as the Philippines and Vietnam produce durian for domestic consumption (O'Gara et al., 2004).

There are currently more than 72,391 hectares (ha) of durian planted across Malaysia. However, only 35,178 ha produced good quality durian yielding approximately 210,873 metric tons (MT) (Department of Agriculture [DOA], 2017). DOA (2017) also identified that the main producer states in Peninsular Malaysia were Pahang (Raub and Bentong, which produced about 43,712.7 MT), Perak (Batu Kurau produces 8201.3 MT) and Johor (Muar, Segamat, and Tangkak generated approximately 29,833.9 MT).

In 2016, more than 17,000 MT durians valued at about 17 million USD were exported, mainly to Singapore, Hong Kong, China, and the United States. However, the export volume decreased in 2017 to 14,000 MT with a value of 16 million USD. The fluctuation in the quantity of durian in the export and domestic markets is highly dependent on the season and the associated climatic problems (Ahmad et al., 2018).

Generally, durian fruiting seasons in Peninsular Malaysia are not similar among growing areas as they are influenced by the monsoon, dry spells, and the change from wet to dry weather conditions in a particular place (Chung, 2011). The change from wet and dry spells in monsoon weather systems is caused by solar radiation that triggers changes in land-sea temperatures (Huffman et al., 1997). In the case of durian, the increase and decrease in production trends are strongly related to changes in climatic conditions. Kukal and Irmak (2018) revealed that variations in crop yield could be due to factors such as technology, genetics, soil, field management and practices, fertilizers and climate. Among these variables, yield performance is largely influenced by climatic variability as it is the prominent driver in agricultural production. Unfortunately, unfavorable climatic conditions not only cause erratic production in terms of quantity but also affect the quality of durian (Hariyono et al., 2013).

Over the last two decades, the issue of climate change and its impact on agricultural production has been debated at many global and national forums. In Malaysia, several studies have reported that most crop commodities, particularly rice, are highly vulnerable to climate change (Al-Amin et al., 2011; Alam et al., 2012; Murad et al., 2010). Considering the negative impact of agricultural production due to climate change, many government agencies have developed mitigation and crop adaptation strategies to improve crop productivity.

However, these strategies encompass many scientific unknowns. This is particularly true for durian whereby the causes of durian yield variations due to climatic changes in Malaysia remain debatable. Therefore, studies on the distribution patterns and trend variation of durian in relation to changes in climatic conditions are the first step toward developing comprehensive and scientifically based strategies.

Examination of crop production trends can provide an insight into future crop production and can also be used for planning purposes (Abid et al., 2014). The information from yield trend studies can also help policymakers to propose policies that can improve food sustainability (Malhi & Kiran, 2015). Therefore, to support agricultural management decisions, trend analysis of crop production needs to be explored to include the spatio and temporal trends as a large variation may exist due to diverse climatic conditions (Yue & Hashino, 2003). Furthermore, time-series trends and change point detection analysis have become a popular approach due to the rapid changes in agricultural systems (Anderson, 2011; Jaiswal et al., 2008; Nikiforov & Basseville, 1993).

Trend analysis is an important tool that can extract underlying patterns of behavior and provide useful information on the possibility of tendency variation (Yue & Wang, 2004). Either parametric or non-parametric trend analysis can be used to detect trends and change points in time series data. However, many studies employ the non-parametric trend test as it has no limitation that requires data sets to be independent and normally distributed (Bandyopadhyay et al., 2009; Jaiswal et al., 2008; Shadmani et al., 2012).

The most widely used non-parametric trend test is the Mann-Kendall (MK) test, which evaluates the presence of statistically significant trends in the data. It is not influenced by outliers and is insensitive to the type of distribution (Ahmad et al., 2015, 2017; Chen et al., 2007; Hui-Mean et al., 2018). This method has been applied in a wide range of applications including climatological, hydrological, and agricultural studies.

The MK test evaluation is based on the correlation between the observed ranks and time sequences (Ahmad et al., 2017; Hamed, 2009; Yusof et al., 2012, 2014). The application of the MK test can be found in a report by Jain and Kumar (2012). They identified an increasing trend in temperature, rainfall, and a number of rainy days in India. Meanwhile, Yusof et al. (2012) produced a map that described the drought occurrence trend in Peninsular Malaysia using a rainfall dataset that was categorized based on the standardized precipitation index (SPI) and further verified according to the MK test. The map showed that major parts of Peninsular Malaysia experienced an increased period of dry spells.

Previous studies have used the MK test for climatic parameters including rainfall, wind speed, humidity, maximum and minimum temperature, and even the number of rainy days and occurrences of drought. However, the application of the MK test in

agricultural production studies has not been emphasized as compared to climatic and hydrological studies. Interestingly, Poudel and Shaw (2016) investigated the seasonal and annual trends of climate variables as well as yields of rice, maize, millet, wheat, and barley using the MK test and the magnitude was quantified using Sen's slope. The findings were further correlated to observe the climate-crop yield relationship.

Therefore, it is important to examine the trend analysis of durian production in Malaysia. This study statistically analyzes the spatial and temporal trends in durian production as well as planted area variations. Hence, the objectives of this study are: (1) to identify the trend of planted areas and durian production in Peninsular Malaysia using the MK test, (2) to estimate the magnitude of changes using Sen's slope estimator, and (3) to display the results of trend analysis using a geostatistical method.

Study Region and Data Collection

In this study, crop data, including cultivated area and production quantity of durian in Peninsular Malaysia from 2000 to 2017, were collected from the Department of Agriculture, Malaysia. These data were taken from 88 districts in the 11 states namely, Terengganu, Selangor, Pulau Pinang, Perlis, Perak, Pahang, Negeri Sembilan, Melaka, Kelantan, Kedah and Johor as shown in Appendix 3.

Durian crop yield was measured in metric tons. The areas with the highest production for the total period were the Segamat and Muar districts in Johor, which produced from 419,000 to 465,000 metric tons of durian. This was followed by Raub, Batu Pahat and Pontian districts with a production of 368,000, 261,000 and 231,000 metric tons, respectively (DOA, 2017). Figure 1 is a map of total durian production for Peninsular Malaysia for the period.

A preliminary analysis was performed using basic descriptive statistics to obtain an early understanding of the data. A total of four areas with a very small or no cultivated area of durian namely Kuala Nerus (Terengganu), Mualim, Bagan Datoh, and Kampar (Perak) were identified and these were excluded from the study.

MATERIALS AND METHODS

Mann-Kendall Trend Test

The MK trend test (Kendall, 1975; Mann, 1945) is an applicable technique for identifying and interpreting the trend patterns in time series data, especially nonlinear trends (Zhai & Feng, 2009). The test is evaluated based on the correlation between the observed ranks and the order of time.

The application trend is expressed as

$$S = \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} sign(x_j - x_i) \quad (1)$$

$$sign(x_{j-} x_i) = \begin{cases} 1;\ x_i < x_j \\ 0;\ x_i = x_j \\ -1;\ x_i > x_j \end{cases} \quad (2)$$

$$V(S) = \frac{n(n-1)(2n+5)}{18} \quad (3)$$

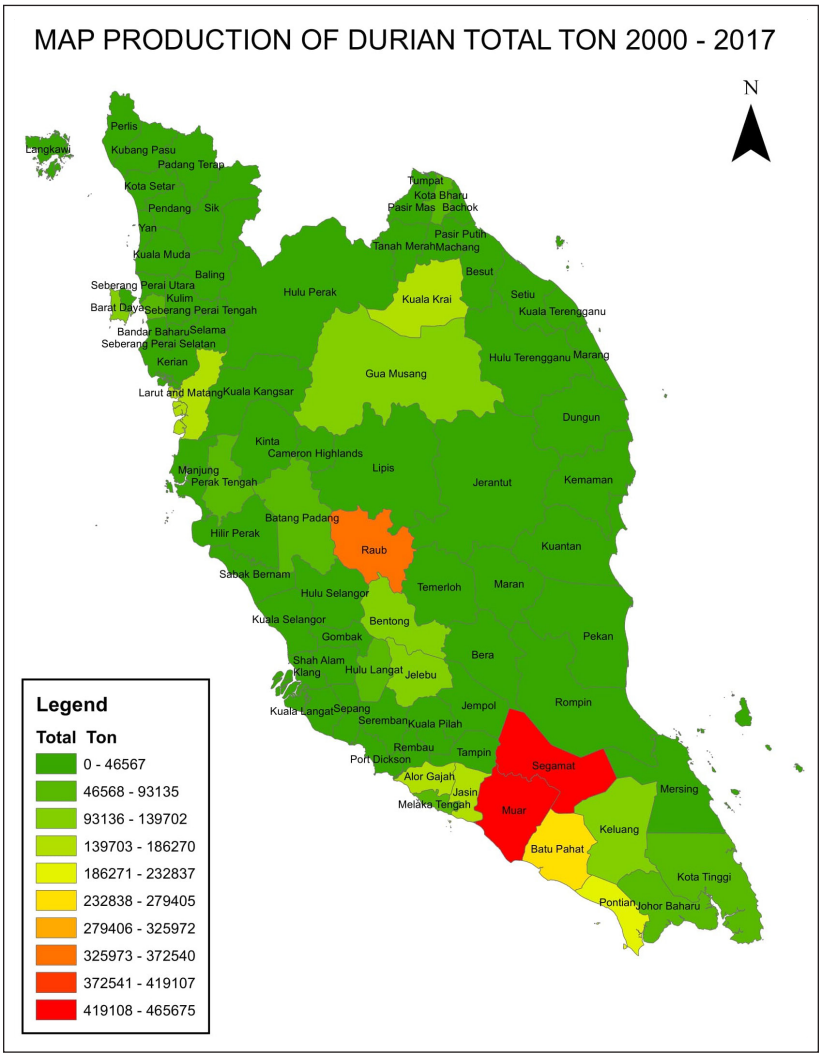


Figure 1. Total production (Metric Ton) of durian in Peninsular Malaysia (2000 to 2017)

$$Z = \begin{cases} \dfrac{(S-1)}{\sqrt{V(S)}}; S > 0 \\ 0; S = 0 \\ \dfrac{(S+1)}{\sqrt{V(S)}}; S < 0 \end{cases} \quad (4)$$

where $\{x_t : t = 1,2,\ldots,n\}$ is a time series for n sample size.

Positive values of Z indicate an increasing trend while negative values indicate a decreasing trend. The test hypothesis for this MK test is described as follows:

H_0: There is no trend
H_a: A monotonic trend exists

At the significance level $\alpha = 0.1$, if $|Z| > Z_{1-(\frac{\alpha}{2})}$, then H_0 is rejected and there is a significant trend in the time series (Partal & Kahya, 2006).

Sen's Slope Estimator

The direction and magnitude of the trend in the time series data were determined using Sen's slope (Sen, 1968). Sen's slope b is calculated using

$$b_i = \frac{x_j - x_i}{j - i}, i = 1,2,\dots,N, \qquad j < i \tag{5}$$

The Sen's estimator of the slope is the median of these N values of b_i. The sign of b reflects the direction of the trend data while the value represents the magnitude. The trend analysis was conducted using the R Foundation for Statistical Computing Platform Version 3.4.0.

Map of Durian Production and Planted Area Trends

The map of these parameters was produced using ArcGIS 10 Software. All spatial data created were standardized using local projection Kertau RSO Malaya meters (EPSG:3168). To produce thematic maps, layer symbology was used to differentiate each unique value using districts as designated boundaries with appropriate color schemes to differentiate the level of intensity.

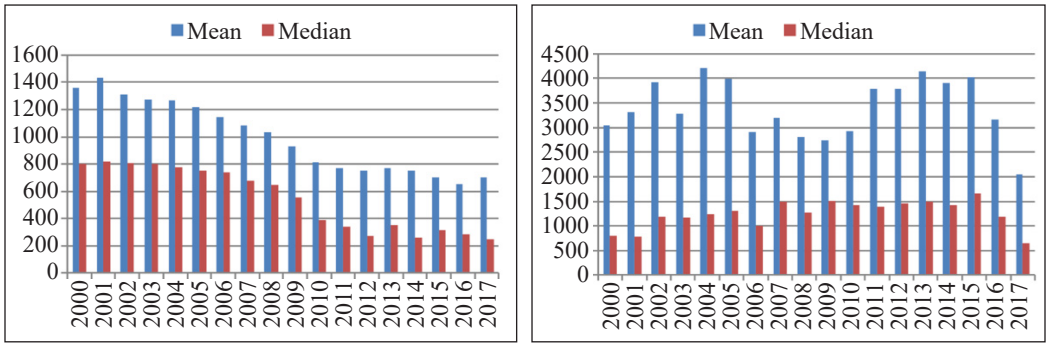
RESULTS AND DISCUSSION

Descriptive Statistics

The descriptive statistics computed the mean and median as a measure of the location while coefficients of variation, skewness, and kurtosis were used as a measure of variation. The yearly descriptive statistics values are summarized in Figure 2 and Figure 3.

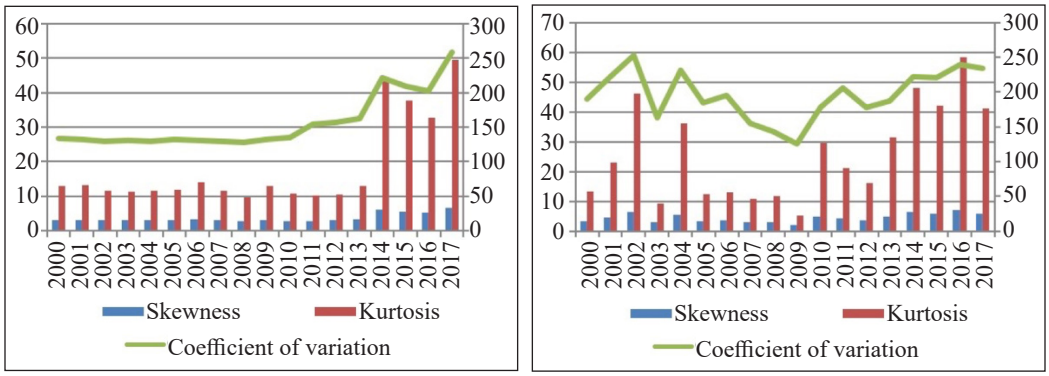
The Measure of Central Tendency

From Figure 2, the average area planted with durian was between 656 ha/year and 1,436 ha/year. Figure 2(a) reveals that the mean value of planted areas decreased annually. The largest area cultivated for durian was in 2001 (1,436 ha) and the smallest area was in 2016 (656 ha). The shrinkage of agricultural land in developing countries, including Malaysia, is generally due to the impact of higher demand for land to accommodate rising population, urban development,



(a) (b)

Figure 2. Mean and Median (a) planted areas and (b) production in Peninsular Malaysia (2000-2017)



(a) (b)

Figure 3. Coefficient of skewness, kurtosis, and variation for (a) planted areas and (b) production in Peninsular Malaysia (2000-2017)

expansion of industrial sectors, and government policy that encourages non-agricultural industries (Masoudi et al., 2017; Tahir & Malek, 2017; Xiao et al., 2006).

The mean of durian production (Figure 1(b)) ranged from 2,052 MT/year to 4,210 MT/year. The highest durian production was recorded in 2004 while the lowest was in 2017. As previously mentioned, the production of durian is greatly influenced by the monsoon and this causes fluctuation in the annual mean production of durian in Malaysia. Durian trees require an average of two to four weeks dry season to induce flowering and wet weather is required during fruit developmental stages for optimal production (Safari et al., 2018).

As can be seen in Figure 2, similar to mean values, the median for both planted areas and production showed similar trends. A higher mean value than the median indicated that the annual distribution of planted areas and production was right-skewed. Under this positively skewed distribution, the extreme values occurred on the right side and with a higher magnitude. That is most of the areas and production were in the lower group, which forms the bulk of the distribution toward the left side. However, some of the areas experienced very high production and larger cultivation areas, as shown by the tail of the distribution, which was extended further to the right side.

Measure of Variability

The coefficient of variation (CV) is a unitless indicator that describes the dispersion of the variables with respect to the mean (Maarof et al., 2012). Thus, the CV for the study period will represent the annual irregularity in durian cultivated areas and production between years. However, our results revealed that the CV of areas planted with durian was rather homogenous and ranged between 128 to 162 throughout the study period, except for the last four years (Figure 2(a)). The smallest CV value (128.2) occurred for 2008 while the highest was in 2017 (257.7). On the other hand, the CV values for annual durian production showed

a large variation between years. The smallest value was recorded in 2009 (125.5) and the largest CV was in 2002 (252.5) (Figure 3(b)).

The coefficient of skewness is normally used to verify the degree of asymmetry of the distribution around the mean. The value of skewness that is near 0 indicates the data is normally distributed. The positive and negative values of skewness indicate the distribution is right or left-skewed, respectively (Maarof et al., 2012). The value of skewness for both planted areas and production was all positive ranging from 2.81 to 6.55 and 2.2 to 7.2, respectively. The results proved that the annual distribution of planted areas and production were right-skewed. The highest values of skewness for planted areas and production were for 2017 and 2016, respectively, indicating a positively skewed distribution with the tail extending to the right (Yusof et al., 2012).

Similarly, the value of kurtosis can be used to determine the peak of the distribution with the kurtosis value of the normal distribution is three. The kurtosis values hectarage and production are all positive in the range of 9.65 to 49.5 and 5.32 to 58.2, respectively (Figure 3). The highest values were in 2017 and 2016 for durian planted areas and production, respectively. This implied a leptokurtic distribution in which the data set tends to have a distinct peak near the mean with a heavy tail (Yusof et al., 2012). Meanwhile, the smallest value of kurtosis (9.65) in 2008 for planted areas and durian production (5.32) in 2009 implied that the data set tended to have a flat peak near the mean.

Trend analysis was conducted for planted areas and production with respect to each district in Peninsular Malaysia across the study period. The results of the MK test for planted areas and production are summarized in Figure 4. The darker regions indicate significant trends while the lighter regions indicate non-significant trends; no changes in trends were detected.

The magnitude of the trends is presented in Appendix 1 and Appendix 2, showing decreasing trends with a 95% significance and no changes in trend except for the circled regions (Figure 4(a)). Raub (PH09 in Pahang) and Rembau (NS05 in Negeri Sembilan) showed a significant upward trend regarding planted areas with magnitudes of 515.70 ha and 18.69 ha per year, respectively. Raub has been recognized as a major durian production area in which 90% of residents are engaged in durian planting. This area is also famous for the Musang King variety of durian ("Discover Raub", 2017). The mountainous terrain with cool night temperatures appears to be suitable for durian cultivation.

A total of 15 out of 84 districts involving four states, namely, Pahang, Kedah, Perak, and Negeri Sembilan, recorded a significant upward trend (95% statistical significance) in durian production for the 17 years (Figure 4(b)). The districts are Bentong, Raub, Pekan, Temerloh (Pahang), Kulim, Sik (Kedah), Larut Matang, Selama, Hulu Perak, Hilir Perak, Bandar Bahru (Perak), Jelebu, Rembau, Seremban, Port Dickson (Negeri Sembilan).

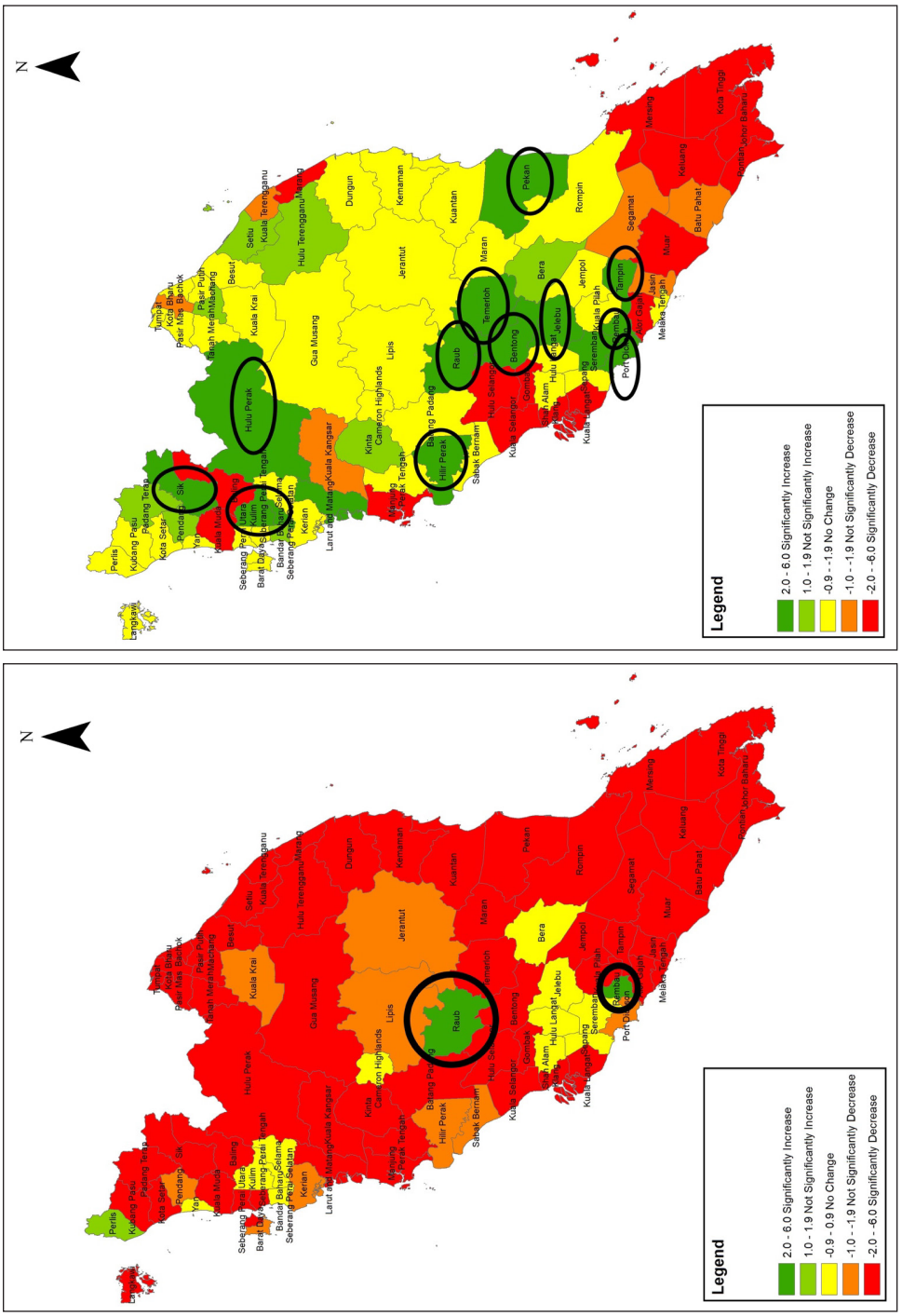


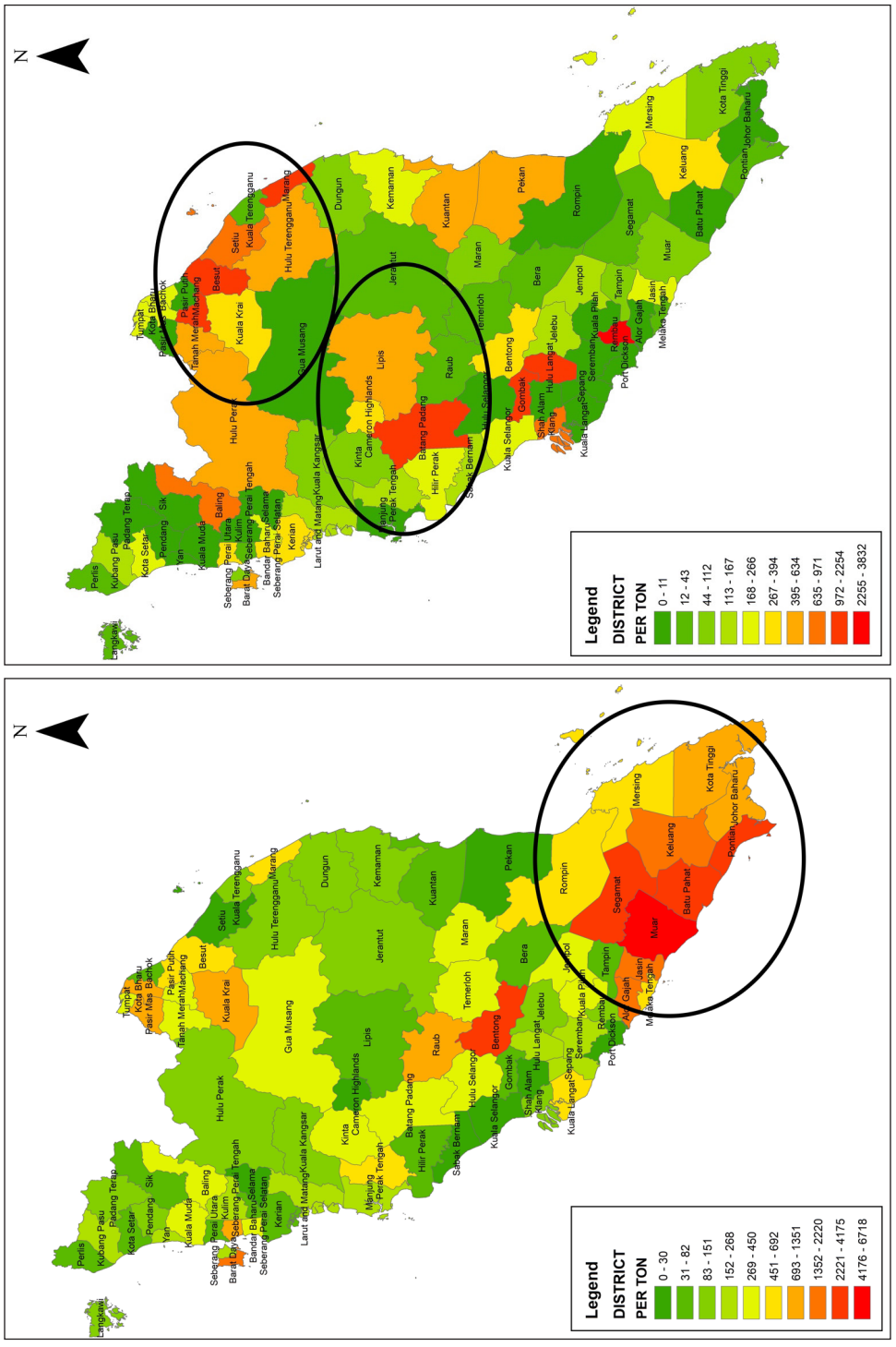
Figure 4. Summary of MK results for (a) planted areas and (b) production in Peninsular Malaysia (2000-2017)

The observed trends for planted area and production for all districts can be further categorized into six cases: (i) increasing trend in planted areas and decreasing trend in production, (ii) decreasing trend in planted areas and increasing trend in production, (iii) increasing trend in both but more significant for production, (iv) decreasing trend in both but more significant for production, (v) increasing trend in both but more significant for planting areas, and (vi) decreasing trend in both but more significant for production. Decreasing the amount of planted areas would lead to reduced production. However, in the 15 districts mentioned above, our results indicated an upward trend in production although hectarage of planted areas showed shrinking trends. We assumed that this is due to more efficient crop management practices by commercial farmers in these areas. We suggest that more attention should be given to improve productivity in some districts of Johor (Muar and Tangkak districts in the southern part of Peninsular Malaysia) that used to be major durian production areas.

Based on the map of total durian production in 2000 and 2017 (Figure 5), the density of durian production is found to have changed and shifted across districts. In 2000, production was relatively higher in the southern parts of Peninsular Malaysia while in 2017, the highest durian production was observed in the central and eastern coastal parts of the region. The higher productivity was assumed to be related to profuse flowering and fruiting seasons in these areas. Some areas experienced more than one fruiting season and would, therefore, have higher production. Furthermore, changing climate patterns, especially the long drought period, could have changed durian production patterns. The occurrence of drought normally triggers the flowering of durian.

A recent study based on drought probability analysis (using the first-order homogenous Markov chain model), suggested that the northwest and middle regions were more susceptible to moderate and severe drought, respectively (Sanusi et al., 2015). Annual extreme and partial duration series analysis revealed that the northern regions have a higher number of dry days compared to more dry spells in the middle regions. Furthermore, according to Hui-Mean et al. (2018), Pahang state has experienced a downward trend in climatic water balance (CWB), in which more dry days occurred. This climatic condition has increased the chances of off-season durian flowering and eventually increased annual production.

Despite climate variability, the shift in durian production pattern in Peninsular Malaysia from the southern to the central region is related to the mountainous demographic of Pahang. Besides the environmental suitability for durian cultivation in Pahang, the hill-grown durian is believed to yield more fruits with great texture of flesh and good taste. Furthermore, to meet the export demand, the Department of Agriculture has initiated durian cluster projects in Pahang and Perak (DOA, 2017). The shift of durian production may also be



(a) (b)

Figure 5. Total Production of durian in (a) 2000 and (b) 2017

due to the changing of other crops after its failure due to soil unsuitability, especially in Johor. Urban and industrial development in Johor also contributes to the decreasing in durian cultivation.

The detected trends and their corresponding magnitudes for planted areas and production in all districts are illustrated in Appendix 1 and Appendix 2, respectively.

CONCLUSION

Trend analysis and distribution patterns for durian production are important to determine the tendency of production in different locations and at different times. The hectarage of durian was also included in the analysis and a decreasing trend was revealed in the hectarage of most durian planted areas in Peninsular Malaysia except for Raub (PH09 in Pahang) and Rembau (NS05 in Negeri Sembilan). However, the annual durian production showed an upward trend, particularly in most of the districts in Pahang, Perak, and Negeri Sembilan. Based on magnitudes obtained from Sen's slope estimator, the area with the highest increase in production was found to be Raub (2,326 MT/year) followed by Larut Matang and Selama (728.67 MT/year) and Bentong (486.25 MT/year). However, the downward trend of annually planted areas suggests decreased production in Peninsular Malaysia, but some areas showed an increase in annual production, especially in the northern and central regions. The present analysis provides valuable information on the distribution patterns of durian production in Peninsular Malaysia. It also provides evidence that there are other external factors such as localized climatic conditions that could shift the distribution of durian production.

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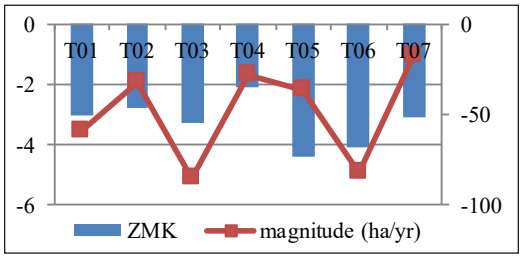
APPENDICES



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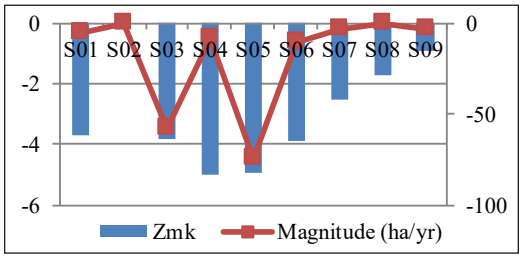

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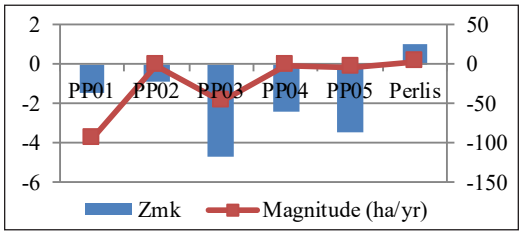

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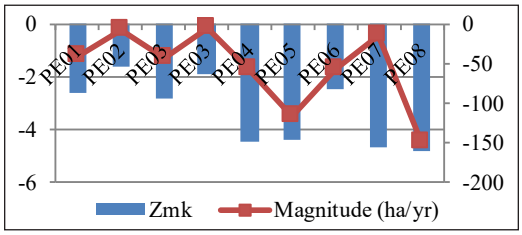

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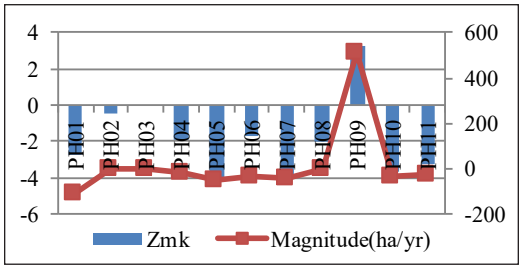

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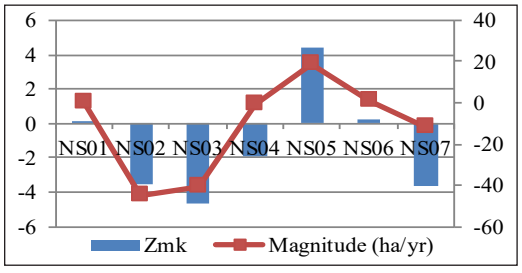

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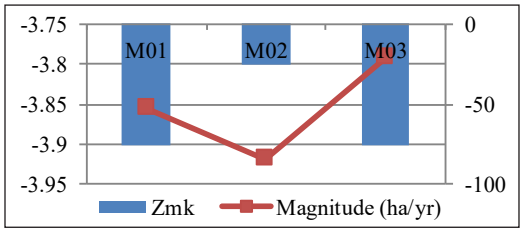

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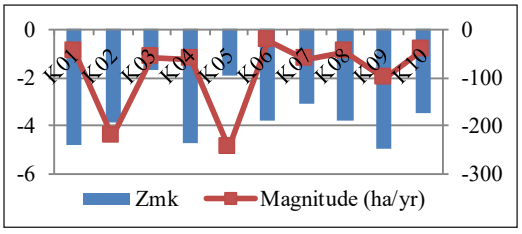

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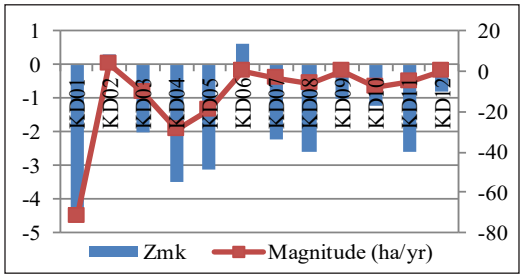

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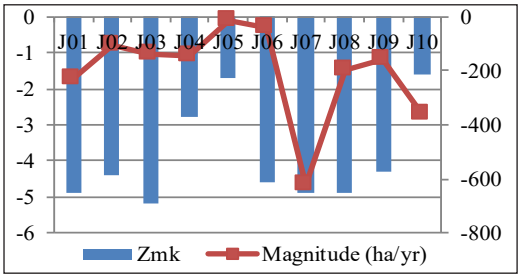
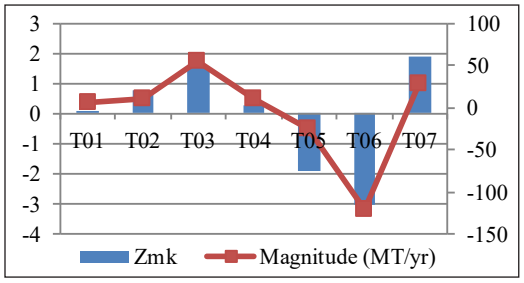


(j) Johor

Appendix 1. Trend and magnitude for durian planted area



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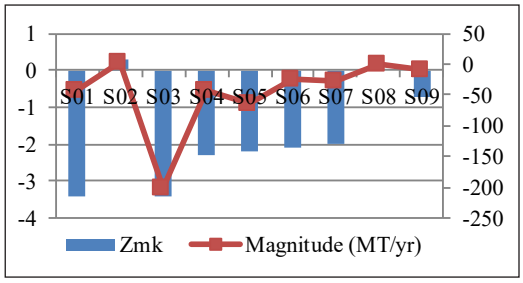

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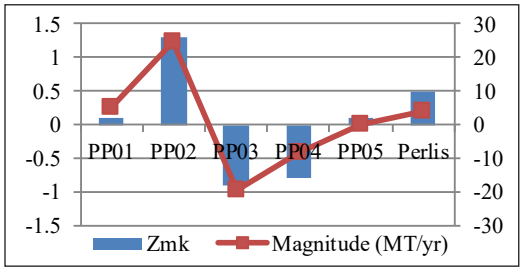

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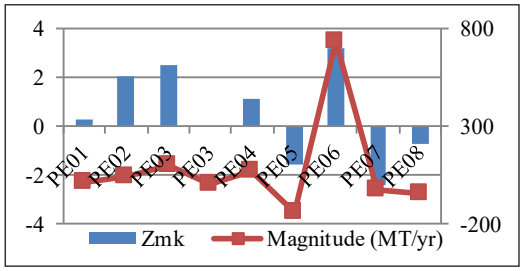

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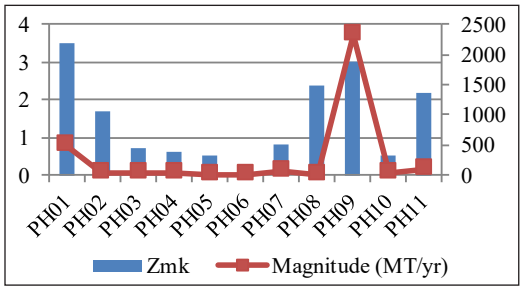

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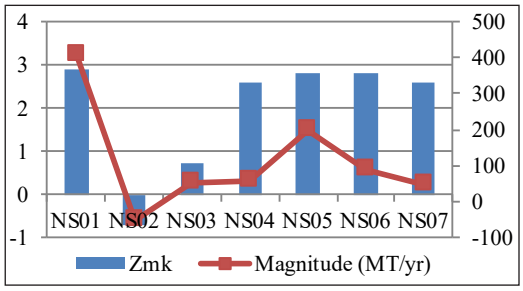
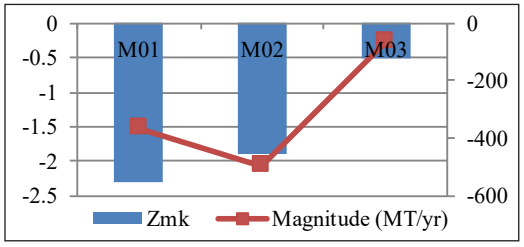
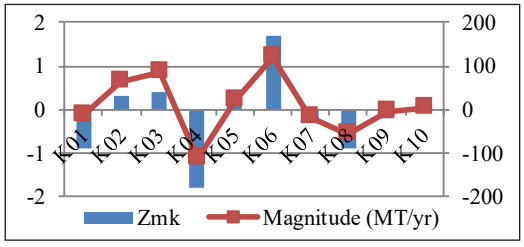


(f) Negeri Sembilan



(g) Melaka



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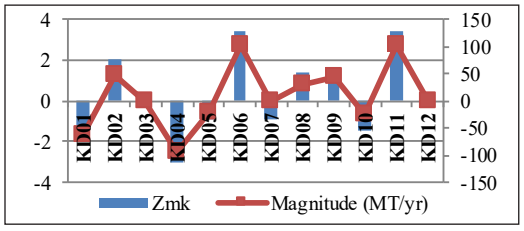

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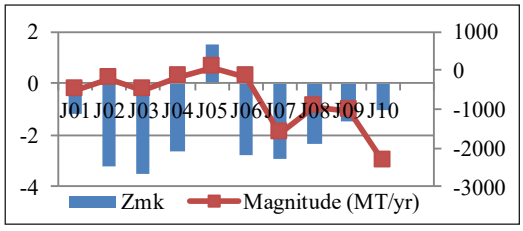

(h) Johor

Appendix 2. Trend and magnitude of durian production

Appendix 3
List of districts involved in this study

	DITRICT	STATE		DITRICT	STATE
T01	BESUT	TERENGGANU	NS01	JELEBU	NEGERI SEMBILAN
T02	DUNGUN		NS02	JEMPOL	
T03	HULU TERENGGANU		NS03	KUALA PILAH	
T04	KEMAMAN		NS04	PORT DICKSON	
T05	KUALA TERENGGANU		NS05	REMBAU	
T06	MARANG		NS06	SEREMBAN	
T07	SETIU		NS07	TAMPIN	
S01	GOMBAK	SELANGOR	M01	ALOR GAJAH	MELAKA
S02	HULU LANGAT		M02	JASIN	
S03	HULU SELANGOR		M03	MELAKA TENGAH	
S04	KLANG		K01	BACHOK	KELANTAN
S05	KUALA LANGAT		K02	GUA MUSANG	
S06	KUALA SELANGOR		K03	JELI	
S07	PETALING		K04	KOTA BAHRU	
S08	SABAK BERNAM		K05	KUALA KRAI	
S09	SEPANG		K06	MACHANG	
PP01	BARAT DAYA	PULAU PINANG	K07	PASIR MAS	
PP02	SEBERANG PERAI SELATAN		K08	PASIR PUTIH	
PP03	SEBERANG PERAI TENGAH		K09	TANAH MERAH	
PP04	SEBERANG PERAI UTARA		K10	TUMPAT	
PP05	TIMUR LAUT		KD01	BALING	KEDAH
Perlis	PERLIS	PERLIS	KD02	BANDAR BAHRU	
PE01	BATANG PADANG	PERAK	KD03	KOTA SETAR	
PE02	HILIR PERAK		KD04	KUALA MUDA	
PE03	HULU PERAK		KD05	KUBANG PASU	
PE03	KERIAN		KD06	KULIM	
PE04	KINTA		KD07	LANGKAWI	
PE05	KUALA KANGSAR		KD08	PADANG TERAP	
PE06	LARUT MATANG SELAMA		KD09	PENDANG	
PE07	MANJUNG		KD10	POKOK SENA	
PE08	PERAK TENGAH		KD11	SIK	
PH01	BENTONG	PAHANG	KD12	YAN	
PH02	BERA		J01	BATU PAHAT	JOHOR
PH03	CAMERON HIGHLAND		J02	JOHOR BAHRU	
PH04	JERANTUT		J03	KLUANG	
PH05	KUANTAN		J04	KOTA TINGGI	
PH06	LIPIS		J05	KULAI	
PH07	MARAN		J06	MERSING	
PH08	PEKAN		J07	MUAR	
PH09	RAUB		J08	PONTIAN	
PH10	ROMPIN		J09	SEGAMAT	
PH11	TEMERLOH		J10	TANGKAK	

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Assessment on Reproductive Biology of Asian Swamp Eel, *Monopterus javanensis* La Cepède 1800 in relation to the Impacts of Paddy Practice Management in Kelantan, Malaysia

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ABSTRACT

In order to understand the life history of the Asian swamp eel, *Monopterus javanensis* found in paddy fields, the time of maturation of its gonads was studied by using the gonadal somatic index. The peak gonadal somatic index was first found during the ploughing and seedling seasons in 2011 and 2012. This was accompanied by the yellowish egg sac observation made in the eels during the ploughing and seedling seasons, which was indicative of the mid- and early maturation stages of the gonads. However, the decline in GSI from the growing until the harvesting seasons indicated the poor development of gonads since differentiating the sex of the eels was hard. This could be due to the heavy application of pesticides and fertilisers during the growing season as cadmium present as impurities in the fertilisers, which slowly accumulated in the gonads. The findings highlighted the availability of Asian swamp eels for local eel collectors as part of their income and the complexity of heavy metal bioaccumulation in their gonads for safe eel consumption. Overall, the habitat of the Asian swamp eels may induce the differences in the maturation timing for the species.

Keywords: Asian swamp eel, Kelantan, Malaysia, paddy seasons, reproductive biology

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INTRODUCTION

Asian swamp eel, *Monopterus javanensis* is widely distributed worldwide and known to be native to Asia (Berra, 2001). It can be found in various aquatic habitats including rivers, lakes, ponds, marshes, swamps, and paddy fields. Due to its unique features, this eel is kept as an aquarium fish (Shafland et al., 2008). The Asian swamp eel's morphological characteristics include a scale-less body, small eyes that are covered by a layer of skin, a cylindrical body with compressed tail tapering to a point, and slate-brown or greenish colour with the ventral portion being lighter in colour and dark spots along the sides (United States Army Corps of Engineers [USACE], 2011). Collins et al. (2002) and Graham (1997) have stated that they are rarely observed as most of the eels are cryptically coloured, active during night time, and often bury themselves in soft sediments (i.e. 80 % muddy and 20 % water areas) (Agromedia, 2000) over a few months. Therefore, chances of their exposure to pollutants are high during paddy seasons. Paddy cultivation consists of several stages, namely, tilling the flooded soils or puddling, transplanting rice, and harvesting (Sahrawat, 2005). The repetitive use of agrochemicals for paddy cultivation has escalated the number of pollutants in its soils. For instance, ploughing the soil can cause pollutants from prior cycles of paddy cultivation to resurface, while the dependency on chemical fertilisers for the seedling and growing seasons adds to the number of pollutants (Sow et al., 2013).

In China, *M. javanensis* has been introduced as one of the rice-fish culture systems in paddy cultivation areas as the system has emerged as an important rural farming system capable of alleviating rural poverty and improving the rural economy (Fang, 2003). Since this species is well-associated with the desirable economic return and thus becomes highly popular among farmers in this study site, the knowledge on gonad development in Asian swamp eels is necessary and required to establish the duration of spawning season, the size and age at their maturity, and the spawning pattern during the paddy seasons. Therefore, this information collectively accounts for eel collectors since this species is vulnerable in Malaysia and may potentially generate income for them. The objective of the present study is to investigate the reproductive biology of Asian swamp eels by using the GSI and basic histological.

MATERIALS AND METHODS

Sampling Location

Monopterus javanensis eels were sampled from paddy fields located at Tumpat, Kelantan (N 06°08.454' E 102°8.430') in Peninsular Malaysia. The paddy plantation has been in operation for a time that is longer compared to this study. A few settlements were located near the study areas, but no heavy industrial activities were reported. In Kelantan, the majority of paddy fields are under the Kemubu Agricultural Development Authority (KADA). A river

nearby the paddy fields, which is known as Jal River, supplies the water used by farmers during a shortage of water during the dry season.

Sample Collection

Asian swamp eels were collected by using the *tukil*. The tool is a semi-closed cylindrical tube made from PVC pipe, which is completely sealed at one end and has a spiny entrance at the other end. The sampling of Asian swamp eels was conducted every month from March to October based on the four paddy seasons in 2011 and 2012. Prior to the collection of the Asian swamp eels, the *tukil* was positioned at a selected spot in the paddy field for a day. A certain amount of bait (particularly cooked fish) was inserted in the *tukil*. The trapping of Asian swamp eel was conducted in the late afternoons as they were active at night time to look for food. The trap was checked for the presence of eels the following morning. The Asian swamp eels collected were placed in polythene plastics bags, brought back to the laboratory, and stored in the freezer at the temperature of -20 ºC until the analysis was commenced.

Sample Digestion

The samples were dried in an air-circulating oven for 3-4 days at 60 °C until a constant weight was achieved. Then, each gonad organ was weighed and placed into a digestion tube with the addition of 10 ml of concentrated HNO_3 (AnalaR grade, BDH 69 %), where the reaction was allowed to proceed at room temperature. Three replicates of the samples were digested for each organ in this study. Next, the tubes were placed in a hot-digester apparatus at 40 ºC for 1 hour and the temperature was gradually increased up to 140 ºC for at least 3 hours. After the completion of the digestion procedure, distilled water was added into the tubes at a certain volume (40 ml) and the extracts were next filtered through a Whatman No. 1 (filter speed: medium) filter paper in a funnel. Subsequently, the filtered solution was collected in an acid-washed polythene bottle (Yap et al., 2002a, 2002b). The prepared samples extracted from the gonads of Asian swamp eels were determined for the presence of Cd and Zinc (Zn) by using an air-acetylene flame atomic absorption spectrophotometer (AAS) PerkinElmer Model AAnalyst™ 800 after the filtration. The data were presented in the form of µg/g (dry weight). Multiple-level calibration standards were analysed to generate the calibration curves against which the sample concentrations were calculated. For the accuracy of the results, the r^2 of the calibration curve was in the range of 0.995-0.998. The quality of the method used as the precision check was the Certified Reference Material for fish (DORM-3) from the National Research Council Canada (NRCC). The agreement between the analytical results for the reference material for each metal shows good and acceptable recovery percentage as tabulated in Table 1.

Table 1
Measured result (µg/g dry weight ± SD) of the Certified Reference Material (CRM) for fish with its certified value for Zn and Cd

Metal	Zn[a]	Cd[b]
Measured	46.744±4.74	2.676±0.33
Certified (CRM)	51.3±3.1	2.11±0.15
Recovery	91.12%	126.82%

Note. a: Certified Reference Material (DORM-3), b: Certified Reference Material (PACS-2); SD: Standard deviation

Gonadosomatic Index

A total of 153 Asian swamp eels were collected in 2011 and 2012 for a GSI study. The total length of each eel was measured to the nearest 0.1 cm with the help of an absolute vernier calliper and a measuring tape for the eels with a longer body length. Body weights were recorded to the nearest 0.1 g by using the triple-beam digital weighing balance. After that, the eels were dissected to take out the gonads. Each of the gonads was weighed with the sensitive electric balance. The mean total length (TL) and body weight (BW) were 45.94 cm and 99.79 g for ploughing, 51.83 cm and 166.41 g for seedling, 43.02 cm and 84.32 g for growing, and 46.64 cm and 103.41 g for harvesting seasons in 2011. For 2012, the mean TL and BW were 42.17 cm and 82.83 g for ploughing, 50.28 cm and 144.80 g for seedling, 43.44 cm and 83.61 g for growing, and 44.79 cm and 106.10 g for harvesting seasons, accordingly. The determination of GSI was calculated by using the following formula as described by Devlaming et al. (1982).

$$Gonadosomatic\ Index = \frac{Weight\ of\ gonads}{Weight\ of\ body} \times 100$$

Gonads Histological

For histological purposes, a total of 37 Asian swamp eels of undifferentiated sexes were collected during the ploughing, seedling, growing, and harvesting seasons in 2013 and then subjected to the histological procedure. To obtain the gonadal histology, two paraffin-based fixatives are generally and widely used, which are 10 % neutral buffered formalin (McCormick et al., 1989) and Bouin's fluid (Willemse & Van den Berg, 1978). Each eel was measured for its TL and BW randomly cut into four parts and fixed in the 10 % buffered formalin solution. The histological procedures were performed by using the paraffin-embedding method, which consisted of fixing the gonadal tissues, paraffin embedding, sectioning and mounting, and haematoxylin and eosin staining (Bell & Lightner, 1988; Drury & Wallington, 1980). An 8 µm thick section was cut on a rotary microtome and the resulting tissue sections were mounted on a glass treated with dibutylphthalate polystyrene xylene (DPX) mounting medium onto the slides before staining with HE (Harris

Modified) and eosin (1 % Eosin Y, aqueous). The staining process comprised of a series of steps, which were: 1) soaking the samples in xylene (I) and xylene (II) for 3 minutes, 2) soaking the samples in absolute alcohol (I) and (II), 95 %, 90 %, 80 %, 70 % alcohol and tap water for 3 minutes. After that, the samples were soaked in haematoxylin for 5 minutes, followed by soaking them in tap water for several times. Next, the samples were soaked in eosin again, followed by tap water and 95% absolute alcohol (I) and (II), xylene (I) and (II) for 3 minutes respectively. Afterward, the stained tissues were analysed by using the light microscopy and digital images obtained.

Statistical Analysis

The findings were reported in the form of mean ± standard deviation (SD) values. The Tukey's Post-Hoc HSD test was carried out to identify the significantly different values upon obtaining a significant ANOVA value. Further statistical analyses were conducted with SPSS Statistic Software 21.0 with $p<0.05$ as the significant level.

RESULT

The average concentration of Zn and Cd in the gonads of Asian swamp eels is shown in Table 2. In 2011, the average Zn metal was observed to accumulate the highest in the gonads during the ploughing season (120.00 µg/g), while the average Cd showed higher values during the growing (11.83 µg/g) and harvesting (28.88 µg/g) seasons. Zn showed no significant ($p>0.05$) difference in the gonads across four different paddy seasons in 2012. Similar to 2012, the average Cd was found to be higher in the growing (32.83 µg/g) and harvesting (73.13 µg/g) seasons, and increased threefold from 2011 to 2012. Table 3 shows the observation of Asian swamp eel gonads based on the paddies stages (day) according to four different paddy seasons. In 2011, the GSI peaks of the Asian swamp eels were shown to be higher during the ploughing season (2.28 %), followed by the seedling season (1.94 %). Similarly, the GSI peaks in 2012 were in line with 2011 (Table 3), yielding 1.69 % and 1.58 % for the ploughing and seedling seasons, respectively. The results

Table 2
Zn and Cd concentration in gonads of Monopterus albus *during paddy seasons in 2011 and 2012*

Year	Season	Zn	Cd
2011	Plowing	120.00±88.62[a]	3.97±3.24[b]
	Seedling	68.12±16.00[b]	0.19±0.13[c]
	Growing	71.84±22.54[b]	11.83±10.10[b]
	Harvesting	84.80±13.79[b]	28.88±8.72[a]
2012	Plowing	78.18±14.12[a]	9.93±9.70[b]
	Seedling	82.66±18.49[a]	9.61±7.90[b]
	Growing	86.73±32.65[a]	32.83±23.89[a]
	Harvesting	79.51±30.91[a]	73.13±55.00[a]

Note. n = 153 samples; Objects indicated with different alphabet $p<0.05$, objects indicated with the same alphabet $p>0.05$

Table 3
Gonadal somatic index (GSI) (mean ± S.D) of Asian swamp eels according to four paddy seasons in 2011 and 2012

Year	Season	Paddies' stage (day)	Gonad weight (g)	GSI (%)	Gonads observation
2011	Plowing	0-30	2.41±2.45	2.28±2.08[a]	Eggs sacs present (eggs in yellowish color) and in mid-maturation stage
	Seedling	30-50	2.48±1.95	1.94±2.14[a]	Eggs sacs present (eggs in yellowish color) and in early maturation stage
	Growing	50-90	0.72±0.94	0.76±0.88[b]	Poorly developed; hard to identify Sexes
	Harvesting	100-120	0.24±0.12	0.25±0.10[c]	Poorly developed; hard to identify sexes
2012	Plowing	0-30	1.24±1.37	1.69±1.81[a]	Eggs sacs present (eggs in yellowish color) and in mid-maturation stage
	Seedling	30-50	2.53±2.43	1.58±1.33[a]	Eggs sacs present (eggs in yellowish color) and in early maturation stage
	Growing	50-90	1.00±1.53	0.85±0.99[b]	Poorly developed; hard to identify Sexes
	Harvesting	100-120	1.13±1.32	0.77±0.64[b]	Poorly developed; hard to identify sexes

Note. n = 153 samples; Objects indicated with different alphabet $p<0.05$, objects indicated with the same alphabet $p>0.05$

also demonstrated yellowish egg sacs identified through the dissection of the eels, indicating the peak period of maturity found during the ploughing and seedling seasons. Consequently, it also indicated the availability of mature eels for the eel collectors, which may indirectly increase the source of their income. Nonetheless, an abrupt decline in GSI was seen from 0.76 % during the growing season to 0.25 % during the harvesting season in 2011. For 2012, a gradual decrease in GSI was observed from 0.85 % in the growing season to 0.77 % in the harvesting season. Figure 1

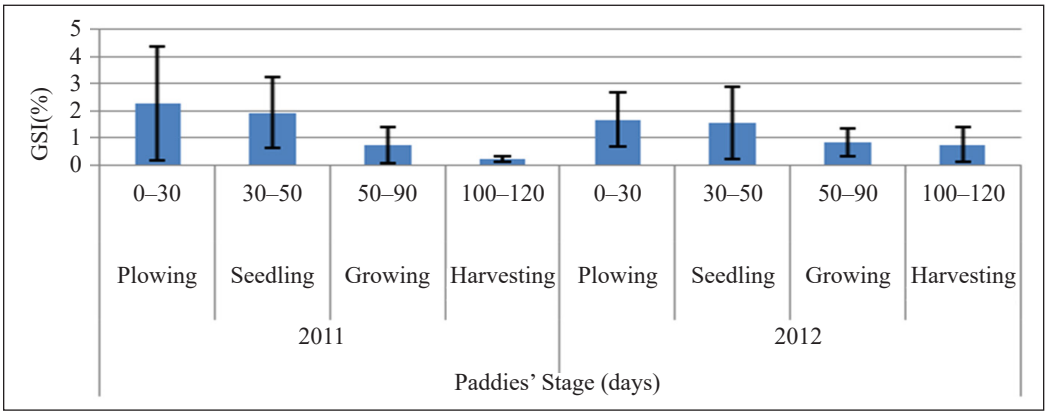

Figure 1. Gonads development based on paddies'stage (days) and GSI (%) in 2011 and 2012

indicates that the maturity of Asian swamp eel gonads begins to decrease from the ploughing until the harvesting season. The error bar in Figure 1 is omitted due to the big variable detected in this study, which is due to the ungrouping of Asian swamp eel sizes according to the BL and BW throughout the four paddy seasons.

DISCUSSION

High or minimal loaded heavy metals have been reported to affect both the quality and quantity of the gametes and endocrine systems, which eventually disrupt the gametogenesis processes (Jezierska & Witeska, 2001). This occurs either indirectly via the accumulation of heavy metals in reproductive organs or directly by attacking the free gametes released into the water system (Ebrahimi & Taherianfard, 2011). Different tissues of the fish species have dissimilar bioaccumulation capacities (Alhashemi et al., 2012), rendering it important to analyse other tissues, such as the gonads (Has-Schön et al., 2008; Jarić et al., 2011). Higher average Cd was found in the gonads of Asian swamp eels in 2011 and 2012 compared to the study conducted by Chi et al. (2007), who reported 0.017 µg/g in *Cyprinus carpio* and 0.010 µg/g in *Cyprinus auratus* from the Taihu Lake in China. The presence of the highest Zn average in the gonads in this study is also supported by the study conducted by Chi et al. (2007). According to Olsson et al. (1989), Zn is an essential element involved in the development of both sperm and ovary.

GSI is one of the important parameters of fish biology, which gives a detailed idea regarding the fish reproduction and reproductive status of the species and helps in ascertaining the breeding period of fish (Mohan & Jhajhria, 2001; Muchlisin et al., 2011; Murua & Saborido-Rey, 2003). In this study, the maturity of Asian swamp eel eggs may be due to the presence of high nutrition as a result of the heavy application of agrochemical fertilisers (Yin et al., 2012) and the abundance of diet (i.e. other smaller living aquatic organisms). It may also be attributable to the irrigation process into paddy fields during the ploughing season, which increases the food availability for these Asian swamp eels located in the paddy field areas. Based on Ali's (1993) study on catfish, *Clarias macrocephalus* on rice fields of Perak, the increase in irrigated water levels in the paddy field and rainfall seemed to be the spawning cues. As stated by Kumar et al. (2013), the presence of humid and waterlogged environments in the rice ecologies causes organic matter decomposition for varied biological organisms (i.e. microorganisms, diatoms, phyto and zooplankton, micro invertebrates and benthic macroinvertebrates). This maintains the soil fertility by releasing nutrients and sediment bio-revolving process. In addition, flooded paddy fields make the nutrients available for farmers and diversify biological organisms that are directly or indirectly performing important ecosystem services (Melo et al., 2015).

A unique characteristic of the Asian swamp eel is its ability to burrow deep into the mud and remain in contact with the water table (Liem & Inger, 1987; Sterba, 1983), which may be another reason for the maturity of gonads. Cheng et al. (2003) and Hill and Watson (2007) had reported that the clay contents of the soils represent the nutritional source for eel's growth. Meanwhile, the timing of maturation for the eel may not be associated with its age or growth, but rather the need to be prepared for certain physiological conditions (Arai & Chino, 2013). They had also reported that the deposition of high Zn in the gonads might play an important physiological role during gonadal maturation. This was observed in this study with the higher Zn accumulation in gonads ranging between 68.12-120 µg/g in 2011 and 78.18-86.73 µg/g for four paddy seasons.

Furthermore, poor development of gonads and difficulties in identifying the sexes were observed in this study, while the GSI decreased during the growing and harvesting seasons for both years. In the current study, the growing stages of paddy were divided into three stages, with the major focus placed on the fertilising process. In stage one, compound fertilisers were introduced, while at stage two, urea fertilisers were used. Meanwhile, urea and compound fertilisers were both used for the growing stage of the paddy cycle. In stage three, foliar fertilisers were applied to the paddies by the farmers. As stated by Yin et al. (2012), heavy application of fertilisers in paddy cultivation areas is done to ensure the quality of rice produced. The presence of heavy metals, especially Cd in the paddy fields through paddy practice management can contribute to the poor development of Asian swamp eel gonads by decreasing the size and number of oocytes in this study. Cd is one of the most toxic heavy metals and a non-essential element widely found in agricultural management practices such as fertilisers and pesticides, otherwise present as impurities (Satarug et al., 2003). El-Ebiary et al. (2013) had concluded that a high level of Cd directly affects the gonad structure through vacuolisation disorders (i.e. abnormally-shaped ova with a high amount of vacuoles and a detached follicular wall). Changes in the ovary of *Heteropneustes fossilis* (stinging catfish) had been observed and reported by Sharma et al. (2011), and of *Carassius gibelio* B. (Prussian carp) by Drag-Kozak et al. (2018). Hwang et al. (2000) had shown that Cd inhibited the synthesis of vitellogenesis in the hepatocytes of the rainbow trout, whereas Szczerbik et al. (2006) had suggested that it acts mainly at the level of the ovary in the Prussian carps. Meanwhile, Brown et al. (1994) had reported that oogenesis was delayed in the brown trout and inhibited in the rainbow trout after exposure to this metal. In addition, Cd has been revealed to disrupt the reproductive function in female fishes by reducing their ovary weight and limiting their development (Szczerbik et al., 2006). Therefore, it can be suggested that Cd affects the quantity and quality of gametes (Annabi et al., 2012).

The histological observation of the female gonads of Asian swamp eel (Figure 2) is done to ascertain the pattern of oocyte

maturation and identify its breeding season. The oocyte maturation can be separated into five stages: 1) oogonia, 2) primary growth or peri-nucleolar stage, 3) cortical alveoli stage, 4) vitellogenesis, and 5) ovulated oocyte, based on the size of oocytes (Table 4). In the present study, it was observed that the Asian swamp eels collected were exclusively in their oogonia and primary growth stage of maturity during the harvesting and seedling seasons, respectively. However, it is not easy to distinguish the newly formed oocytes from oogonia under microscopy (Ravaglia & Maggese, 2002). Therefore, an examination of the histological sections stained with H&E is helpful to distinguish the germinal cells as the cytoplasm becomes basophilic when oogenesis begins (Uribe et al., 2012). As illustrated in Figure 3, the primary oocyte

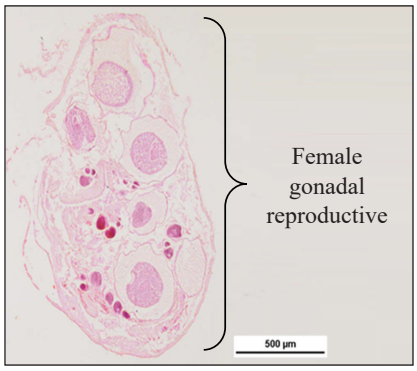

Figure 2. Female gonadal reproductive system with magnification size of 400

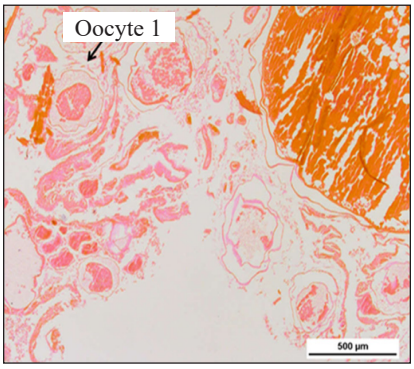

Figure 3. Oocyte 1 (primary growth) of Asian swamp eel with magnification size of 400

Table 4
Counted sizes (µm) of female ovarian development of 37 individuals during different paddy seasons

Season	Oogonia		Oocyte 1		Oocyte 2		Oocyte 3		Oocyte 4	
	No	Size (µm)	No	Size (µm)	No	Size (µm)	No	Size (µm)	No	Size (µm)
Plowing	0	-	0	-	4	728.16-920.37 (809.4)	29	404.77-848 (667.12)	24	667.29-2440.54 (1472.7)
Seedling	0	-	1	214.11	0	-	7	330.37-663.63 (486.68)	15	742.67-2105.34 (1482.1)
Growing	0	-	0	-	0	-	17	375.16-888.85 (655.71)	10	683.27-1692.76 (986.27)
Harvesting	3	16.37-88.78 (53.81)	0	-	2	577.12-623.87 (600.5)	14	312.47-724.23 (539.22)	11	326.39-2030.21 (934.5)

Note. n = 37 samples. Oocyte 1: primary gorwth or perinucleolar stage; oocyte 2: cortical alveoli stage; oocyte 3: vitellogenesis; oocyte 4: ovulated oocyte or matured; value in parentheses (average)

appears slightly irregular in shape with an abundant cytoplasm of diameter about 214.11µm. The irregular shape of the oocyte in the primary growth stage is probably due to the stress generated by the expanding oocytes around them. Oocytes of the cortical alveoli stage then appeared only during the harvesting until ploughing seasons, whereby their diameter increased noticeably compared to those of the primary growth stage. The sizes of the cortical alveoli stage oocytes range from 728.16 to 920.37 µm during the ploughing season (Table 4), which is slightly larger compared to the harvesting season (577.12-623.87 µm). The presence of oocytes in vitellogenesis was not restricted to any season but their quantity was the highest in the ploughing season, followed by the growing season. In vitellogenesis, a clear shrinkage of oocytes is seen (Table 4); this scenario does not parallel results reported in the literature. A histological study of the gonads of Asian spiny eel, *Mastacembelus armatus* has shown an increase in oocyte diameter when it enters the vitellogenesis stage (Ali et al., 2016). Besides, the oocyte diameter of *Synbranchus marmoratus* has increased with correspondence to its ovarian stage (Ravaglia & Maggese, 2002). Meanwhile, the late-maturation stages occur when the oocyte is almost fully grown and ready for ovulation. As illustrated in Figure 4, the matured oocytes are usually recognised by the presence of colour pigments contained within the lipid droplets. Furthermore, at this stage, a decrease in the nucleo-cytoplasmic ratio of the oocytes (Figure 4) is witnessed upon comparison with the primary stage oocytes (Figure 3). The early maturation of male Asian swamp eel gonads is illustrated in Figure 5. During the early maturation stage, testicular maturation begins by elongation due to the mitotic division of both Sertoli cells (SC) and spermatogonia (SG). Moreover, the lobules commonly have discontinuous lumina (Figures 5 and 6), which grows in length during the early maturation stage. A continuous germinal epithelium (GE) is seen within the lobules,

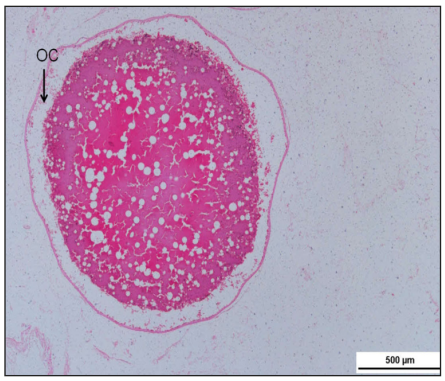

Figure 4. Late maturation of oocytes (OC) 4 (matured) cell of Asian swamp eel with magnification size of 400

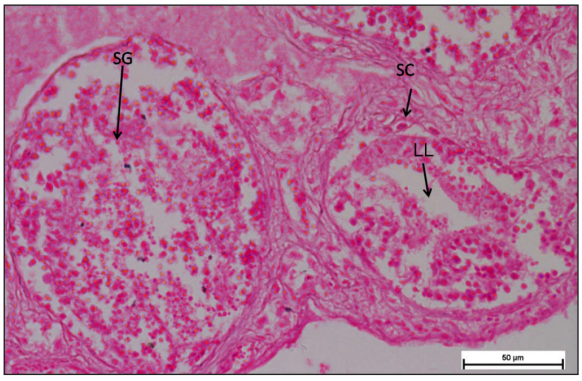

Figure 5. Mid-maturation of male Asian swamp eel with the presence of spermatogonia (SG) and Sertoli cell (SC) with the magnification size of 400. Lobule lumen (LL) is discontinuous

which consists of conspicuous SC where the processes surround a cluster of SG. In contrast, the mid-maturation class of male gonadal reproductive system (Figure 6) is characterised by the presence of GE. A discontinuous GE is composed of the regions of SC associated with germ cells. As mid-maturation progresses, the sperm matures, which is followed by the spermiation (i.e. release of mature sperm into lonular lumina) (Nostro et al., 2003). From the slides observed, sex reversal of Asian swamp eel is identified through the presence of spermatogonia (SG) and mature oocyte (MO) (Figure 7). Sex reversal of Asian swamp eel (Figure 8) is indicated by the presence of vitellogenic oocyte (VOC) and Sertoli cells (SC).

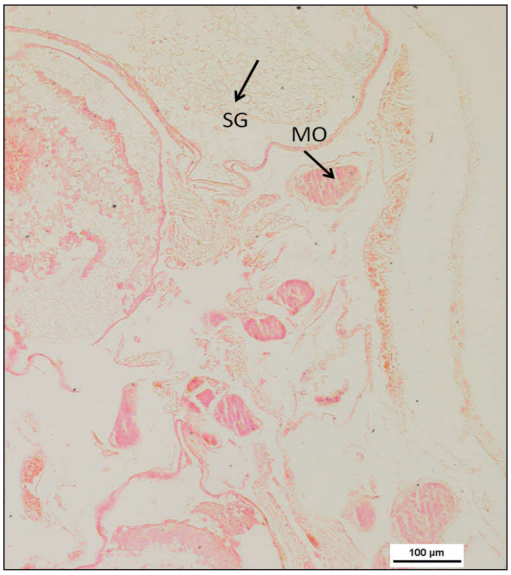

Figure 7. Sex reversal of Asian swamp eelwith magnification of 1000. SG: Spermatogonia, MO: Matured oocyte

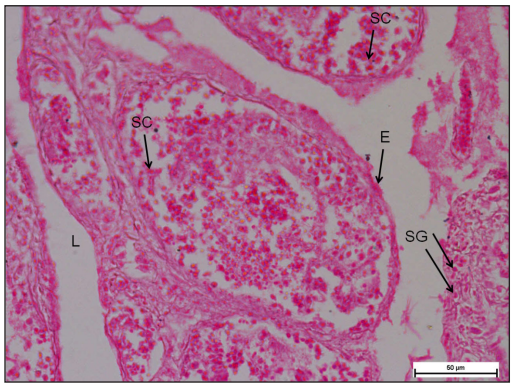

Figure 6. Early-maturation of male Asian swamp eel with the magnification size of 400. A continuous germinal epithelium which consisiting of Sertoli cell (SC) and spermatogonia (SG) within spermatocycsts. Lobules (L) is discontinuous

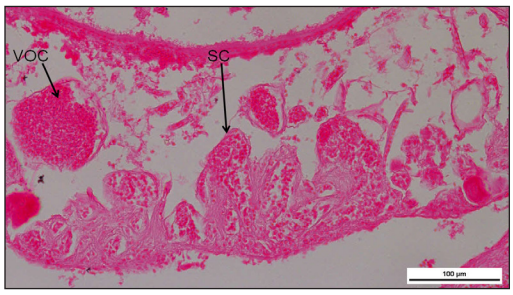

Figure 8. Sex reversal of Asian swamp eel which consisting of vitellgenic oocytes (VOC) and Sertoli cell (SC). The elongation of each testis lobules indicated the sex reversal of Asian swamp eel has begun. Magnification size of 200

CONCLUSION

Although the late-maturation oocytes appeared throughout all four paddy seasons, their number peaked in the ploughing season. This suggests that the ploughing season offers a better environment for the Asian swamp eels to breed. In contrast, the ploughing and seedling seasons revealed bigger-sized late-maturation stage oocytes compared to the late-maturation oocytes formed during the growing and harvesting seasons. The diameter of oocytes at the late-

maturation stage is frequently associated with mortality rate, whereby larger oocytes contain more yolk to enhance the probability of successful hatching. In short, the data collected in Table 4 and GSI calculation, it can be concluded that the ploughing season offers the best environment for Asian swamp eels to breed and a higher chance for the eggs to hatch successfully.

CONFLICT OF INTEREST

Authors declared there is no conflict interest.

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Short Communication

The Role of *Nannochloropsis* sp. Methanolic Extract in Reducing Hydrogen Peroxide-induced DNA Damage in L929 Cell Line

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ABSTRACT

Nannochloropsis sp., is one of the microalgae that produces important antioxidants. We postulate that the presence of phenolic compounds in the methanolic extract of *Nannochloropsis* sp. exhibits preventive effect against DNA damage induced by 100 µM concentration of hydrogen peroxide (H_2O_2) in L929 cells. High performance liquid chromatography (HPLC) analysis revealed that *Nannochloropsis* sp. methanolic extract contained antioxidant compounds such as *p*-coumaric acid, caffeic acid, naringenin and hesperitin. In this study, we utilised comet assay to measure the activity of DNA damage induced by H_2O_2 in L929 cells and the preventive effect of *Nannochloropsis* sp. methanolic extract and caffeic acid. After 24 hours of treatment, 100 µM of H_2O_2 induced 64.4 ± 4.6% of DNA damage in L929 cells compared with the untreated cells ($p < 0.05$). Interestingly, the scale of DNA damage induced by the H_2O_2 was reduced to 46.3 ± 12.7% when treated with 0.4 mg/mL of *Nannochloropsis* sp. methanolic extract ($p < 0.01$). The DNA damage was further reduced to 7.3 ± 2.9% when treated with 20 µM of caffeic acid ($p < 0.01$), a compound found in the microalgae extract. In conclusion, *Nannochloropsis* sp. methanolic extract which contained phenolic compounds, was able to protect L929 normal cells from oxidative DNA damage and therefore may confer protection from genetic mutation and cancer formation.

Keywords: Caffeic acid, DNA damage, microalgae, *Nannochloropsis*

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INTRODUCTION

Nannochloropsis sp. is a unicellular green microalga, which is normally found in marine and freshwater habitats. *Nannochloropsis* sp. produces important antioxidants such as omega-3 fatty acid and polyphenolic compounds (Azim et al., 2018). Antioxidant compounds found in microalgae, play a major role in preventing the formation of reactive oxygen species (ROS) and oxidative stress, which are involved in DNA damage (Goiris et al., 2012). Antioxidants such as polyphenolic compounds exert an effective antioxidant properties by quenching and neutralizing free radical chain reactions in biological systems (Zhao et al., 2014). This is due to their ability to donate electron and thus protect the cells from damaging effects (Zhao et al., 2014). Most species of unicellular green microalgae have ability in producing valuable secondary metabolites for different needs such as antioxidants, several different carotenoids, polyunsaturated fatty acids vitamins, anticancer and antiviral drugs (Skjånes et al., 2013). Most of the compounds produced (cyanovirin, oleic acid, linolenic acid, palmitoleic acid, vitamin E, B12, β-carotene, phycocyanin, lutein, and zeaxanthin) have antimicrobial antioxidant, and anti-inflammatory capacities, with the potential for the reduction and prevention of diseases (Markou & Nerantzis, 2013). In many cases, these secondary metabolites were produced when the algae are exposed to stress conditions linked to nutrient deprivation, light intensity, temperature, salinity and pH (Skjånes et al., 2013).

ROS form as products under normal physiological conditions due to the partial reduction of molecular oxygen. ROS, that is, superoxide anion (O_2^-), hydroxyl radical ($OH^•$), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$), arise in many ways, as a product of the respiratory chain in mitochondria, in photochemical and enzymatic reactions (Nita & Grzybowski, 2016). ROS are generated in large amount by excessive physical activities or environmental chemicals which creating oxidative damage to genomic DNA resulting in single and double strand breaks (Nimse & Pal, 2015). These damages can lead to changes in genetic sequence, which might transform cultured normal cells into rapidly proliferating, cancer-type of cells. In plants, ROS are always formed by the inevitable leakage of electrons onto O_2 from the electron transport activities of chloroplasts, mitochondria, and plasma membranes or as a by-product of various metabolic pathways localized in different cellular compartments (Sharma et al., 2012).

Previously, we had shown that *Nannochloropsis* sp. methanolic extract contained high total phenolic and flavonoid contents with strong anti-oxidant activities (Zainoddin et al., 2018). We postulate that methanolic extract of *Nannochloropsis* sp., contains phenolic compounds, which may exhibit preventive effects against hydrogen peroxide-induced DNA damage in the normal cells.

MATERIALS AND METHODS

Microalgae Sample

Nannochloropsis sp. was obtained from Fisheries Research Institute (FRI) Pulau Sayak, Kedah, Malaysia. The microalgae obtained were cultured in 1000 mL flask with three replicates using the sea saline water (10 ppt) with Walne (1970) medium according to protocols described in Zainoddin et al. (2018). Media for stock culture were replaced every two weeks and the cells were maintained under 25 ± 1°C under continuous lighting. The cells were cultured under continuous exposure of white fluorescent lamps (50.05 µmol photons m^{-2} s^{-1}) with aeration of normal gas. The cells were harvested for the extraction at the exponential phase of growth i.e. 1.5 × 10^6 cells/mL.

Microalgae Extracts

Cells of *Nannochloropsis* sp. were harvested according to the method as described in Pereira et al. (2015) with some modifications. Briefly, the cultured microalgae cells were dried using freeze drier for 72 hours. The dried microalgae were then subjected to sonication to disrupt the cell wall and extracted using 100% methanol at the ratio of 1 part of algae to 40 parts methanol (w/v). The extraction was performed overnight at room temperature (20°C) under continuous stirring. The extracted biomass was centrifuged at 10,000 × *g* for 10 minutes and supernatant was collected, filtered and dried by using rotary evaporator at 45°C under vacuum condition. Dried extracts were then resuspended in methanol to a final concentration of 20 mg/mL and stored at -20°C until further use.

High Performance Liquid Chromatography (HPLC) Analysis of *Nannochloropsis* sp.

Preparation of Standard Solutions. A total of 7 standards which include caffeic acid, *p*-coumaric acid, quercetin, naringenin, hesperetin, kaempferol and baicalin were prepared as a stock solution (15 ppm) in methanol and diluted with a final concentration of 5 ppm as a working solution. All the working solutions were stored in -20°C until further analysis.

Preparation of Mobile Phase. The analysis of HPLC involved 2 mobile phases as carrier solvent. In mobile phase A, a mixture of HPLC-grade methanol: acetonitrile: deionized water (40: 5: 55) and addition of 0.1% formic acid was prepared. For mobile phase B, same solvent with different ratio was mixed which includes HPLC-grade methanol: acetonitrile: deionized water (80: 5: 15) with addition of 0.1% formic acid. All the solvents were prepared according to the ratio and filtered with 0.22 µm nylon filter (Pall Gelman, Sigma Aldrich, USA), followed by degassing of the solvent (10 minutes sonication).

Chromatographic Conditions. The HPLC analysis was carried out using Varian Prostar HPLC, Germany. The separation method in this analysis was carried out using Eclipse Plus C18, 5 µm particle size and 4.6 mm ×

150 mm diameter (Agilent, Germany). Ten microliters of standards and samples were injected for analysis. The gradient mobile phase was run at a flow rate of 1.2 mL/min (Table 1).

Table 1
Gradient method for HPLC analysis

Time (min)	Flow-rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
Pre-run	1.2	100	0
2	1.2	92	8
4	1.2	91	9
12	1.2	90	10
18	1.2	81	19
20	1.2	0	100
20	1.2	100	0
30	1.2	100	0

Cell Culture and Treatments

L929 cell line which was derived from mouse fibroblast adipose tissue was used in this study. Cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Thermo Fisher Scientific, USA) and 0.1% (v/v) penicillin-streptomycin (PenStrep) (Thermo Fisher Scientific, USA). A stock concentration of *Nannochloropsis* sp. methanol extract (10, 000 ppm) was prepared and working concentrations were diluted to 0.16 – 5.0 mg/mL prior to use.

Cell Viability Assay

Cell viability was measured using the [3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyltetra zolium bromide (MTT) (Sigma Aldrich, USA) assay. L929 cells were seeded at 5000-10000 cells for each well in 96-well plate, cultured for 48 hours until it reached 80-90% confluency. The cells were then treated with *Nannochloropsis* sp. methanol extract concentrations ranging from 0.16 mg/mL to 5.0 mg/mL and incubated for 24 hours. Ten (10) microliters of MTT solution (5 mg/mL) were added to each well and the plate was incubated for 4 hours at 37°C incubator. The solution in each well was then aspirated and the formazan product was thoroughly dissolved using 100 µL of DMSO. The optical density (O. D.) of each well was measured at 570 nm wavelength using microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA). The experiment was carried out independently three times.

Cell proliferation was determined using the following formula:

$$\frac{O.D.\ treatment - O.D.blank}{O.D.untreated\ cell - O.D.\ blank} \times 100\% \quad [1]$$

DNA Damage Analysis

Analysis of DNA damage activity was determined by using comet assay kit following the manufacturer's instructions (Trevigen, USA). L929 cells were seeded in T75 cm^2 flask in DMEM complete growth medium overnight to allow for cell attachment. The growth medium was then replaced with medium prior treatment. For this assay, L929 cells were treated with 100 µM of pre-chilled H_2O_2 and incubated for 20 minutes. After incubation with H_2O_2, cells were treated with the extract (0.4 mg/

mL) and caffeic acid (20 µM) for 24 hours. Cells were treated with H_2O_2 and serum-free medium for positive and negative controls, respectively. After 24 hours of incubation, cells were harvested from T75 cm^2 flask by trypsinization and washed with PBS. Thirty microliters of cells (1×10^5 cells/mL) were counted and mixed with 300 µL (ratio of 1: 10, v/v) of molten Comet LMAgarose and incubated at 37°C. Thirty microliters of mixture were pipetted and layered onto an area of the CometSlide™. The slide was incubated at 4°C for 10 minutes in the dark to accelerate gelling of the agarose disc. Then, the slides were transferred and immersed into pre-chilled lysis solution for 30 minutes at 4°C. The excess buffer from the slides was drained and the slides were concomitantly immersed in freshly prepared alkaline unwinding solution (0.2 M NaOH, 1 mM EDTA) at room temperature for 20 minutes, in dark condition. The slides were then transferred into pre-chilled alkaline electrophoresis solution for 10 minutes prior electrophoresis. Electrophoresis was run for 30 minutes. Once completed, the excess electrophoresis solution was drained from the slides. The slides were gently immersed twice in distilled water of 5 minutes each before being immersed in 70% ethanol for 5 minutes and air dried. All the cells were observed in a single plane after completely dried and stained with SYBR safe staining solution before being observed by fluorescence microscopy with a 490 nm filter. Scoring of Comet assay was carried out using Image J (NIH, USA).

Statistical Analysis

All results were presented as mean ± standard deviation. One-way ANOVA was used to compare the differences between the treatment groups. All analyses were conducted using SPSS software version 22.

RESULTS

High Performance Liquid Chromatography (HPLC) Analysis

The constituents present in *Nannochloropsis* sp. methanol extract were characterized using HPLC and each retention time was compared with the standard compounds. The major compounds that matched the retention time of the standard compounds (with the accepted deviation of less than + 0.1 min) found in *Nannochloropsis* sp. methanol extract were caffeic acid, *p*-coumaric acid, naringenin and hesperitin (Table 2).

Table 2
Retention times of the standards and the compounds found in the extract

Peak number	Name of compound	Retention time (min) (standards)	Retention time (min) (extract)	Area (mAU.min)
1	caffeic acid	1.984	2.002	159.4
3	*p*-coumaric acid	2.747	2.774	375.8
5	naringenin	8.172	8.236	9.7
6	hesperetin	9.529	9.594	17.2

Analysis of DNA Damage Activity

Treatment concentration for *Nannochloropsis* sp. methanol extract was determined using cell viability assay. L929 cells were treated with *Nannochloropsis* sp. methanol extract at concentration ranging from 0.16 mg/mL to 5.0 mg/mL) and the IC_{20} (inhibitory concentration which inhibits 20% of cell population) was extrapolated based on the proliferation curve of the MTT assay on L929 cells (Figure 1). We had chosen the IC_{20} as at this concentration, the cells were least toxic. As shown in Figure 1, the IC_{20} of *Nannochloropsis* sp. methanol was determined at 0.4 mg/mL.

The effect of treatments (*Nannochloropsis* sp. extract and caffeic acid) on H_2O_2-induced DNA damage on L929 cells was shown in Figure 2. The untreated cells (without any treatment) were

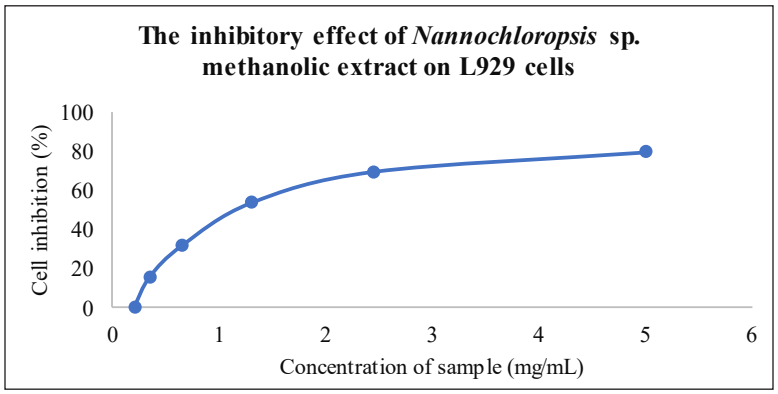

Figure 1. The effect of *Nannochloropsis* sp. methanol extract on the viability of L929 cell line and the IC_{20} value was determined after extrapolation from the graph

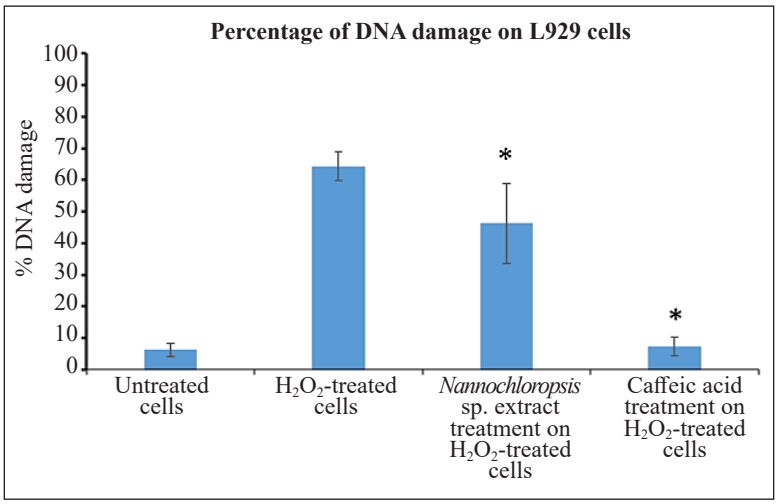

Figure 2. The effects of *Nannochloropsis* sp. methanol extract and caffeic acid treatments on hydrogen peroxide-induced DNA damage on L929 cells. * denotes significant difference when compared with H_2O_2-treated cells

used as a control. Treatment with 100 µM H_2O_2 had induced significant DNA damage (64.4 ± 4.6%, $p < 0.01$) in L929 cells when compared to the untreated cells, as measured by Comet assay. When H_2O_2 –treated cells were treated with the *Nannochloropsis* sp. methanol extract, the DNA damage induced by H_2O_2 was significantly reduced to about 46.3 ± 12.7% ($p < 0.01$). Treatment with a standard compound, caffeic acid at concentration of 20 µM on H_2O_2 –treated cells were able to further reduce the DNA damage to 7.3 ± 2.9% ($p < 0.01$).

DISCUSSION

Hydrogen peroxide induces oxidative stress and generates single and double strand breaks when the cells are exposed to it, causing oxidative DNA damage and decreased in cell viability (Driessens et al., 2009). DNA damage on the L929 cells was stimulated by the treatment with H_2O_2 at the concentration of 100 µM, which caused an average of 64.4% DNA damage when compared with the untreated cells. The concentration of H_2O_2 at 100 µM used in this study was shown to be sufficient in inducing the damage to the DNA in the normal cells, as previously shown in the other studies (Driessens et al., 2009; Luo et al., 2006; Miranda et al., 2008).

Our findings demonstrated that *Nannochloropsis* sp. methanolic extract at the concentration of 0.4 mg/mL was able to protect the DNA from H_2O_2-induced DNA damage for up to 21% in the L929 cells. We had postulated earlier that this preventive effect of *Nannochloropsis* sp. methanolic extract was attributed by the presence of phenolic antioxidant compounds. The high total phenolic content in the extract contributes to the high antioxidant capacity, measured by DPPH and ABTS assays in which we had published earlier (Zainoddin et al., 2018). Further analysis of the extract by HPLC showed that, based on the reference of retention time of the standards, there were 4 phenolic compounds identified such as caffeic acid, *p*-coumaric acid, naringenin and hesperitin (Table 2).

To demonstrate that these compounds, found in the extract might play an important role in the protection from H_2O_2-induced DNA damage, we had chosen one out of four compounds found, which was caffeic acid. Caffeic acid is commonly found in the microalgae species (Safafar et al., 2015) and it has been well-described to possess a strong antioxidant effect (Gülçin, 2006). Treatment with caffeic acid at concentration of 20 µM was able to markedly reduce the H_2O_2-induced DNA damage by 57.1%. The strong effect of caffeic acid in preventing the DNA damage in our study was not a surprise as caffeic acid has been shown previously to prevent DNA damage in human cell lines through the activation of ERK and lipoxygenase signalling pathways (Kang et al., 2006; Kim et al., 2016; Li et al., 2015). Activation of these pathways results in protective effects by decreasing in production of inflammatory cytokines and cytotoxicity (Kim et al., 2016), with an increase in cells differentiation and proliferation (Li et al., 2015).

Other important antioxidant compounds including *p*-coumaric acid, naringenin and hesperitin found in this study had been showed to protect the DNA damage from oxidative stress. We believed that these compounds may act synergistically to protect and prevent the cells from oxidative DNA damage induced by chemical and physical factors in various cells models such as in the eyes (Larrosa et al., 2008), brain (Ekinci-Akdemir et al., 2017), prostate (Gao et al., 2006) and liver (Thangavel et al., 2012). Their mechanism of actions involved various signalling pathways which protect the DNA damage such as inhibiting the activation xanthine oxidase activity (Larrosa et al., 2008), increasing the SOD and GSH enzymes (Ekinci-Akdemir et al., 2017) and enhance base excision repair, where the mutagenic process can be prevented (Gao et al., 2006).

Our study has shown that the methanolic extract of *Nannochloropsis* sp. contains phenolic compounds, which have been reported to be able to protect DNA damage in a wide range of cells and animals' models. The molecular mechanism exerted by the *Nannochloropsis* sp. methanol extract in protecting the normal cells from DNA damage and oxidative stress needs to be carried out in future study.

CONCLUSION

Methanolic extract of *Nannochloropsis* sp. contains many beneficial phenolic compounds such as caffeic acid, *p*-coumaric acid, naringenin and hesperitin. The presence of these compounds in the extract may protect cell from oxidative DNA damage and thus prevent genetic mutation and cancer formation.

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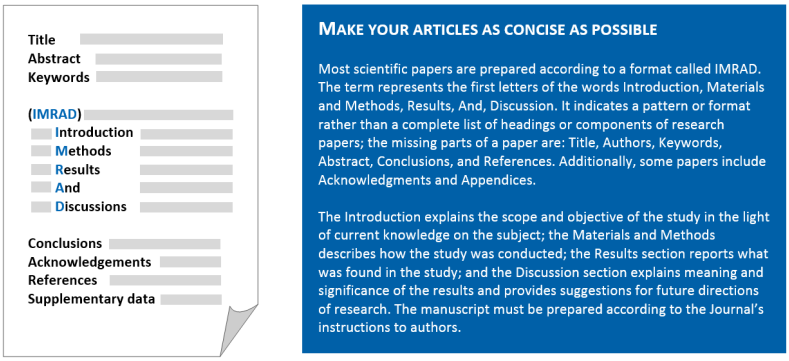

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Journal article with DOI	**Information prominent' (the author's name is within parentheses):** … (Shaw et al., 2005).. Or **'Author prominent' (the author's name is outside the parentheses):** Shaw et al. (2005) …	Shaw, K., O'Rourke, P., Del Mar, C., & Kenardy, J. (2005). Psychological interventions for overweight or obesity. *The Cochrane database of systematic reviews* (2). doi:10.1002/14651858.CD003818.pub2

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Newspapers	Insertion in Text	In Reference List
Newspaper article – with an author	… (Waterford, 2007)...	Waterford, J. (2007, May 30). Bill of rights gets it wrong. *The Canberra Times*, p. 11.
Newspaper article – without an author	… ("Internet pioneer", 2007) …	Internet pioneer to oversee network redesign. (2007, May 28). *The Canberra Times*, p. 15.
Article in an newsletter	... ("Australians and the Western Front", 2009) ...	Australians and the Western Front. (2009, November). *Ozculture newsletter*. Retrieved June 1, 2019, from http://www.cultureandrecreation.gov.au/ newsletter/

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Conference / Seminar Papers	Insertion in Text	In Reference List
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Online	… (Tester, 2008) … Or Tester (2008) …	Tester, J. W. (2008). The future of geothermal energy as a major global energy supplier. In H. Gurgenci & A. R. Budd (Eds.), *Proceedings of the Sir Mark Oliphant International Frontiers of Science and Technology Australian Geothermal Energy Conference*, Canberra, Australia: Geoscience Australia. Retrieved June 1, 2019, from http://www.ga.gov.au/image_cache/ GA11825.pdf

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Government Publications	Insertion in Text	In Reference List
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