ANTIMICROBIAL ACTIVITY OF *STACHYTARPHETA JAMAICENSIS* AGAINST FISH PATHOGENIC BACTERIA AND ITS TOXICITY ON BRINE SHRIMP

MUHAMMAD HAZIQ BIN ISMAIL

(D17A0021)

A RESEARCH PAPER SUBMITTED TO
THE FACULTY OF VETERINARY MEDICINE
UNIVERSITI MALAYSIA KELANTAN
IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF
DOCTOR OF VETERINARY MEDICINE

JUNE 2022
UNIVERSITI MALAYSIA KELANTAN

CERTIFICATION

It is hereby certified that we have read this proposal entitled "**Antimicrobial Activity of *Stachytarpheta jamaicensis* Against Fish Pathogenic Bacteria and Its Toxicity on Brine Shrimp**" by Muhammad Haziq bin Ismail and in our opinion, it is satisfactory in term of literature review, problem to be addressed, research question or hypotheses, objectives, materials and methods and results.

Dr. Ruhil Hayati binti Hamdan
BSc Conservation and Biodiversity Management (UMT), MSc Biotechnology: Aquatic Animal Health (UMT), PhD Aquatic Animal Health (UPM)
Senior Lecturer
Faculty of Veterinary Medicine
University Malaysia Kelantan
(Supervisor)

Dr Tan Li Peng
BSc of Forestry (UPM), PhD of Entomology (UPM)
Senior Lecturer,
Faculty of Veterinary Medicine
Universiti Malaysia Kelantan
(Co-supervisor)

ACKNOWLEDGEMENT

Special thanks for those who have given their support, guidance, advice, and aid for the completion of this project paper:

Dr. Ruhil Hayati binti Hamdan

Dr. Tan Li Peng

Lab assistants of FPV UMK

Family

DVM 5 class of 2017/2022

Thank You

DEDICATION

I dedicate this dissertation to my family and friends. My deepest gratitude to both of my parents, Ismail bin Baharom and Halijah binti Wahab, for their endless love and care for me as well as my siblings and close friends for their encouragement and prayers.

I also dedicate my dissertation work to my lecturers who have assisted and guided me throughout the process. Special appreciation goes to my Supervisor, Dr. Ruhil Hayati binti Hamdan for her supervision, guidance and valuable knowledge during the research period and thesis writing. Huge thanks to Dr. Tan Li Peng as well as my co-supervisor for her help and support during the research.

I dedicate this work to my fellow seniors, Aiman Shafiq and Rasul Arif for always helping me with my study and for their useful advices throughout the entire program of Doctor Veterinary Medicine. I would like to thank Ms. C.W. Salma binti C.W. Zalati, Madam Norsyamimi Farhana binti Mat Kamir, Ms. Nani Izreen binti Mohd Sani and Madam Nora Faten Afifah binti Mohamad for assisting and guiding me in the laboratory works.

Table of Contents

LIST OF TABLES

ABBREVIATIONS

DMSO	Dimethyl Sulfoxide
IZ	Inhibition Zone
LC_{50}	Lethal Concentration 50
MAS	Motile *Aeromonas* septicemia
MHA	Mueller-Hinton Agar
RS	Rimler-Shotts Agar
TCBS	Thiosulphate Citrate Bile Salts Sucrose Agar
TSA	Trypticase Soy Agar

ABSTRACT

An abstract of the research paper presented to the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, in partial requirement on the course DVT 5436 – Research Project

Stachytarpheta jamaicensis has been used traditionally in humans to treat digestive problems, allergies, respiratory conditions, hepatitis, wounds and ulcers in various countries. This valuable plant is known to have some pharmacological effects like antimicrobials due to its active phytochemicals. The objectives of this study were (i) to determine the antimicrobial activity of *S. jamaicensis* ethanolic extract against *Vibrio parahaemolyticus, Streptococcus agalactiae* and *Aeromonas hydrophila* and (ii) to investigate its toxicity effect on brine shrimp. *S. jamaicensis* was collected at Tanah Merah, Kelantan and the ethanolic extract of the plant was prepared by using the whole plant including the stems and leaves. Disc diffusion assay was done to assess the antibacterial properties of *S. jamaicensis* ethanolic extract. Then, brine shrimp lethality assay was employed to determine the possible toxicity of ethanolic extract of *S. jamaicensis.* LC_{50} was calculated by using Probit analysis. Results revealed that the ethanolic extract of *S. jamaicensis* has the most effective antimicrobial properties towards *A. hydrophila* but less effect on *V. parahaemolyticus* and *S. agalactiae*. The extract is also safe to use as the LC_{50} calculated was 26915.35 µg/ml which is considered non-toxic based on Meyer's toxicity index. Current study showed ethanolic extract of *S. jamaicensis* possessed antibacterial properties against *A. hydrophila* and safe to be used as therapeutic application in aquaculture.

Keywords: *Antimicrobial, Brine shrimp, Disc diffusion assay, Ethanolic extract, Stachytarpheta jamaicensis, Toxicity*

ABSTRAK

Abstrak daripada kertas penyelidikan dikemukakan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan untuk memenuhi sebahagian daripada keperluan kursus DVT 5436 – Projek Penyelidikan

Stachytarpheta jamaicensis telah digunakan secara tradisional dalam manusia untuk merawat masalah penghadaman, alahan, masalah pernafasan, hepatitis, luka dan ulser di kebanyakkan negara. Tumbuhan berharga ini mempunyai beberapa kesan farmakologi seperti antimikrobial kerana fitokimia aktifnya. Objektif kajian ini adalah (i) untuk menentukan aktiviti antimikrob ekstrak etanol *S. jamaicensis* terhadap *Vibrio parahaemolyticus*, *Streptococcus agalactiae* dan *Aeromonas hydrophila* dan (ii) untuk menyiasat kesan ketoksikannya terhadap udang air masin. *Stachytarpheta jamaicensis* dikutip di Tanah Merah, Kelantan dan ekstrak etanol tumbuhan tersebut disediakan dengan menggunakan seluruh pokok itu termasuk batang dan daunnya. Ujian resapan cakera dilakukan untuk menilai sifat antibakteria ekstrak etanolik *S. jamaicensis*. Kemudian, ujian kematian udang air garam digunakan untuk menilai kemungkinan ketoksikan ekstrak etanolik *S. jamaicensis*. LC_{50} dikira dengan menggunakan analisis Probit. Keputusan menunjukkan bahawa ekstrak etanol *S. jamaicensis* mempunyai sifat antimikrob yang paling berkesan terhadap *A. hydrophila* tetapi kurang berkesan terhadap *V. parahaemolyticus* dan *S. agalactiae*. Ekstrak ini juga selamat digunakan kerana LC_{50} adalah 26915.35µg/ml yang dianggap tidak toksik berdasarkan indeks ketoksikan Meyer. Kajian ini menunjukkan ekstrak etanol *S. jamaicensis* mempunyai sifat antibakteria terhadap *Aeromonas hydrophila* dan selamat digunakan sebagai aplikasi terapeutik dalam akuakultur.

Kata kunci: *Antimikrobial, Ekstrak etanol, Ketoksikan, Stachytarpheta jamaicensis, Udang air masin, Ujian resapan cakera.*

1.0 INTRODUCTION

Stachytarpheta jamaicensis (L.) Vahl, also referred to as Brazilian tea, blue porter weed, rooter comb, or verbena cimarrona, belongs to the Verbenaceae family (Idu *et al*., 2007; Okwu *et al*., 2010). This plant is usually found in America's tropical regions, as well as Asia, Oceania and Africa's subtropical forests. *S. jamaicensis* has long been regarded as a valuable medicinal plant with a wide range of therapeutic characteristics in conventional and folk medicine. This plant has traditionally been used by the elderly to treat allergies, respiratory disorders, cough, cold, fever, constipation, digestive issues, dysentery, and stimulate menstruation (Sivaranjani *et al*., 2014).

The extant literature reflects the interest in fish bacteria, which reflects their importance as pathogenic agents. Bacteria is abundant in the aquatic ecosystem, and nearly all fish pathogenic bacteria can survive exterior of the fish (Roque *et al*., 2009). The most serious issue in aquaculture industry is diseases caused by bacteria that infect the fish, resulting in outbreaks and financial damage (Huzmi *et al*., 2019). Common cases of disease outbreaks are rising in both manifestation and severity in numerous marine taxa internationally. Large-scale production via intensive culture with high density condition has led to the outbreaks of diseases, which usually caused by bacteria (Huzmi *et al*., 2019).

Antibiotics are the foundation treatment against bacterial infections. However, bacteria's enormous genetic diversity allows them to evolve antibiotic resistance quickly, allowing them to resist antibiotic action (Ruma *et al*., 2015). Bacterial infections in fish are generally treated by applying antibiotics to water, and this has led to its spread in water sources and development of resistance in aquatic bacteria (Akmal *et al*., 2020). To overcome this problem, investigations towards new, more effective and alternative antibiotics has continued. Studies for plant components with possible antibacterial activity has expanded due to their cost-effectiveness, safety, rising failure of chemotherapy, and antibiotic resistance displayed by pathogenic microbial agents (Ruma *et al.*, 2015).

Even though many plants have beneficial characteristics, toxicological features are also known to exist in some of them. Recent research shows that many plants are consumed as food for nutrients, but some of them might have genotoxic or mutagenic potential (Çelik & Aslantürk, 2007). The toxicity and pharmacology of medicinal herbs applied by humans have recently been the subject of numerous studies. This is critical to ensure the safety of any treatment involving plant materials (Parra *et al.,* 2001). The toxicity effect of natural plants can be caused by various contaminants or by chemical compounds found in plants. Research into the possible toxicity of herbal extracts is conducted using a variety of methods, including in vivo tests on laboratory animals. A variety of other biological assays, such as those including the *Artemia salina, Artemia urmiana and Artemia franciscana,* have been utilized in recent studies. These toxicity trials are known as useful tool for preliminary investigation of toxicity (Hamidi *et al.*, 2014).

2.0 RESEARCH PROBLEM

Fish disease prevalence is regarded as one of the primary causes of bad production in fish farming around the world. In addition, many others in various countries have reported income reductions due to disease problems. Such losses impact the livelihoods of aquaculture workers and the communities in which they occur due to decreased food availability, loss of income and employment, and other social consequences (Faruk *et al*., 2004).

Major concerns contribute to the application of antimicrobial drugs in aquaculture include emergence of antimicrobial-resistant pathogens in fish and the aquatic environment. At the moment, due to the proliferation of resistant bacteria, certain antibiotic drugs widely used in aquaculture are only partially effective against some fish pathogenic bacteria. Besides, a recent study showed an increase in antimicrobial resistance of *Aeromonas* strains against tetracycline and quinolones groups, however least study was conducted on *Vibrio* strains (Miller & Harbottle, 2018). There are possible alternative sources of natural antimicrobials from herbs with various mode of actions, some of these plants are applied in traditional medicine for a long time and was found to have competitive effects compared to a few commercial antibiotic medicine (Abdallah, 2011).

Although there are several reports claiming antimicrobial activities of *S. jamaicensis* plant extract, but there is no study of its effect on aquatic bacteria. Besides, the toxicity profiling of this plant is still lacking and further investigation is needed to confirm its safety for the therapeutic cure of diseases. Hence, this study is aimed at determining the antimicrobial activities of *S. jamaicensis* against the aquatic bacteria (*V. parahaemolyticus*, *S. agalactiae* and *A. hydrophila)* and also its toxicity effect to brine shrimp.

3.0 RESEARCH QUESTION

1. Does ethanolic extract of *Stachytarpheta jamaicensis* has antimicrobial activities against *Vibrio parahaemolyticus, Streptococcus agalactiae* and *Aeromonas hydrophila*?
2. Is *Stachytarpheta jamaicensis* ethanolic extract toxic to brine shrimp?

4.0 RESEARCH HYPOTHESIS

1. Ethanolic extract of *Stachytarpheta jamaicensis* has antimicrobial activities against *Vibrio parahaemolyticus, Streptococcus agalactiae* and *Aeromonas hydrophila.*
2. *Stachytarpheta jamaicensis* ethanolic extract is not toxic to brine shrimp.

5.0 RESEARCH OBJECTIVES

1. To determine the antimicrobial activity of *Stachytarpheta jamaicensis* ethanolic extract against *Vibrio parahaemolyticus, Streptococcus agalactiae* and *Aeromonas hydrophila.*
2. To investigate the toxicity effect of *S. jamaicensis* ethanolic extract on brine shrimp.

6.0 LITERATURE REVIEW

6.1 *Stachytarpheta jamaicensis* Botanical Aspect

Stachytarpheta jamaicensis, also referred to as Bastard vervain or Brazillian tea, is a member of the Verbanaceae family. It is a weedy herbaceous plant that completes its life cycle in one year, occasionally perennial, that grows to a height of 60 to 120 cm and reproduces via seeds. The plant's stem is smooth and dark green in colour, although it becomes woody near the base (Idu *et al.*, 2007). *S. jamaicensis* usually produces flowers that have colour of bluish, pinkish or mix of both. The leaves are opposite, greyish green in colour, smooth on the surface, round at the apex, and have distinguishable petioles. The herb is usually found in tropical and subtropical woods in America, as well as Europe, Russia and Nigeria. It is common in acclimatised tropics too like Malaysia and Indonesia. In these countries, it is known as 'Selasih Dandi' or sometimes 'Jolok Cacing' (Sulaiman *et al*., 2009).

6.2 Traditional Usage and Pharmacological Activities of the Plant

Stachytarpheta jamaicensis is well-known for its important role in traditional medicine systems practiced by humans in many countries. This natural herb has been said to have pharmacological effects because it has a lot of bioactive phytochemicals. Medicinally, *S. jamaicensis* plant was proven to have analgesic and antacid properties, as well as hypotensive and anthelminthic properties. It also has laxative, lactagogue, vermifuge, sedative, spasmogenic, vasodilator, diuretic and purgative properties. (Liew & Yong, 2016). *S. jamaicensis* has been used frequently by the elderly as a stomach tonic to relieve nausea and vomiting. Women in Southern Nigeria use *S. jamaicensis* to treat menstruation abnormalities and other feminine issues, and it is considered traditional medicine. The herb is also used as topical application for treating wounds, lesions, cuts, and ulcers on the skin (Okwu *et al.*, 2010). Some of the pharmacological activities of *S. jamaicensis* include antimicrobial, antifungal, antioxidant, anti-inflammatory, antinociceptive, antidiarrheal, antihypertensive, and hepatoprotective properties (Liew & Yong, 2016).

6.3 Phytochemical of The Plant Substances of *Stachytarpheta jamaicensis*

Stachytarpheta jamaicensis contains a high concentration of secondary metabolites, also described as bioactive compounds. Nowadays, it has been established that these bioactive chemicals are responsible for the manifestation of their therapeutic effects. There are numerous essential classes of secondary metabolites found in the herbage which are alkaloids, terpenoids, phenolic compounds, steroids and flavonoids, all present in significant amounts. These bioactive metabolites are abundant throughout the plant's entire structure (Liew & Yong, 2016). Researchers are particularly interested in the phytochemicals found in *S. jamaicensis* phenolic components, including coumarins, flavonoids, tannins (Putera *et al*., 2010), and saponins (Idu *et al*, 2007), due to their medicinal potential. These chemicals eventually produce a variety of therapeutic characteristics. The active phytochemicals found in *S. jamaicensis* like saponins, tannins, and flavonoids have been shown to have antibacterial property against microbes such *as Escherichia coli*, *Proteus vulgaris, Staphylococcus aureus*, and *Candida albicans* (Idu *et al.*, 2007).

6.4 *Aeromonas hydrophila, Vibrio parahaemolyticus* and *Streptococcus agalactiae* as Causative Agent in Fish Diseases

Aeromonas spp. are ubiquitous bacteria found in freshwater, and these microorganisms are most of the times related to severe infections in cultured fish. Motile *Aeromonas* septicemia (MAS), sometimes known as tail and fin rot, is caused by *Aeromonas hydrophila* which is a gram-negative and rod-shaped bacteria. This bacterium causes significant illnesses in a variety of freshwater fishes, including the *Misgurnus anguillicaudatus*, *Ictalurus punctatus*, and *Cyprinus carpio*, as well as a lesser extent in some marine fish species. Ulcers, stomach distension, fluid accumulation, anemia, and bleeding are some of the symptoms of these illnesses, which cause huge mortality in fish all over the world (Akmal *et al.*, 2020).

Vibriosis is thought to be the one of most common bacterial diseases in fish and other cultured species. Vibriosis outbreaks are most common when organisms are exposed to high temperatures, densely populated environments, poor water quality, and other stressors. Dark coloured skin, pale gills, bleeding at the base of the fins, exophthalmia, ulcers of the skin, corneal opacity, enlarged spleen, and enteritis are all symptoms of Vibrio infection in fish (Marudhupandi *et al*., 2017). *Vibrio* spp. infections can lead to high mortality in both brackish and salty water fish. Vibriosis is caused by *Vibrio harveyi*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, *Vibrio anguillarum*, and *Vibrio vulnificus*. Among others *V. harveyi* produces serious infection, which kills a lot of grouper fish and causes financial losses (Huzmi *et al*., 2019).

Streptococcal infections occur in tropical water with warm temperature where fish farms are established, and they sometimes caused death in wild fish. Several *Streptococcus* spp. cause diseases in fish, but the most common and harmful are *Streptococcus iniae* and *Streptococcus agalactiae*. In experimental models, these two pathogens caused rapid onset of generalised septicaemia, meningitis, exophthalmia of both eyes, and death with mortality rates frequently more than 70% within a few days of infection (Barnes & Silayeva, 2016).

6.5 Brine Shrimp Lethality Assay

Brine shrimp is an invertebrate that has been generally utilized for studies of ecotoxicology, common toxicology of chemicals (Cleuvers, 2003) and natural substances (Caldwell *et al*., 2003). Brine shrimp cysts are easily commercially obtainable and cheap. Therefore, this assay is really useful in situations where fast and cheap cost are needed, makes it practical to experiment with large number of samples for preliminary toxicity assessment. Many researchers have used the hatched nauplii of brine shrimp for the investigation of toxicity effect of natural products (Favilla *et al*., 2006; Manilal *et al*., 2009 and Syahmi *et al*., 2010). Few assays also were performed by observing the suppression of hatching of the brine shrimp cysts (Caldwell *et al*., 2003).

Toxicity study of plant extracts using *Artemia salina* in comparison with the mice bioassay was conducted by Parra *et al.*, (2001). The result of relative toxicity of each plant extracts assessed using brine shrimp assay was nearly the same as that of the mice bioassay. The researchers concluded that the results of the brine shrimp bioassay coincide well with those of the mouse bioassay as there was a good correlation between both tested methods. This experiment suggested brine shrimp bioassay as a useful alternative model to the mice bioassay as it is a simple, rapid, cheap and practical method.

According to Svensson *et al.*, (2005), living things in saltwater has a higher tolerance to chloride ions. Since most leachate waters from urban areas have high content of chloride ions, a salt-tolerant life form like brine shrimp is fit for analyzing toxicity caused by other sources.

7.0 METHODOLOGY

7.1 Samples Collection

Stachytarpheta jamaicensis was collected at Taman Sri Jelatok, Tanah Merah, Kelantan. The whole plant including the stems and leaves were harvested and washed with distilled water. The plant was dried in an oven at 40°C for 3 days.

7.2 Extraction of *Stachytarpheta jamaicensis*

The dried plants were pulverized using an electric blender. Then, 50 g of grinded plant sample was put into separate beakers containing 1 L of ethanol. The mixture was mixed well and soaked overnight. Then, the mixture was filtered using Whatman No. 1 filter paper. Another 1 L of ethanol was added into particular beakers and the steps were repeated for three days in order to get more extracts. All the extracts were concentrated by using the rotary evaporator and stored in a sterile container. Then, the extracts were stored in freezer at -20°C until further use.

7.3 Bacterial culture

Aeromonas hydrophila, *Vibrio parahaemolyticus* and *Streptococcus agalactiae* were obtained from glycerol stock which were clinically isolated and stored at -80°C freezer in Zoonotic Diseases Laboratory, Faculty of Veterinary Medicine. The bacterial stocks were streaked on Trypticase Soy agar, TSA (Oxoid, UK). The plates were incubated at 30°C for 24 h. The bacterial isolates were further confirmed by colony morphology on specific agar (Rimler-Shotts and TCBS agar), Gram staining and oxidase test. Rimler-Shotts (RS) agar is a selective agar for *A. hydrophila*, while Thiosulfate Citrate Bile Salt (TCBS) agar is a selective agar for *Vibrio* spp.

7.4 Disc diffusion assay

Disc diffusion method was used for screening of antimicrobial activity of the extract against *A. hydrophila, V. parahaemolyticus* and *S. agalactiae.* Overnight bacterial isolates were inoculated into sterile normal saline. The turbidity of the bacterial suspension was adjusted equivalent to 0.5 McFarland standard. A sterile swab was dipped into the suspension and the dipped swab was rolled firmly against the side of the tube several times to remove excess inoculum from the swab. The entire surface of MHA plate was streaked by the swab, where the plate was rotated and streaked repeatedly up to 8 times to ensure to form a bacterial lawn on the agar surface on the next day. A stock solution of extract was prepared by dissolving 0.2 g of extract with 1 mL of solvent (5% DMSO) to produce a final concentration of 200 mg/ml. The stock solution was then diluted using 5% DMSO to concentrations of 40, 60, 80, and 100 mg/ml of extract. Then, 20 μl of each dilution was impregnated into sterile blank discs. 20 μl of extract was spotted alternately on both sides of the discs and allowed to dry before the next 20 μl was spotted to ensure precise impregnation. DMSO loaded discs was used as negative controls for the disc diffusion assay. All discs were fully dried before the application on bacterial lawn. Oxytetracycline disc (30µg/ml) (Sensi-Disc, UK) was used as a positive control. Oxytetracycline is a broad-spectrum tetracycline antibiotic and it was chosen in this study as it is one of the most used antibiotics in aquaculture to treat bacterial infections. The discs were placed by a sterile forceps and each disc were gently pressed to ensure that the discs adhere to the surface of the agar. The plates were incubated at 30°C for 18 h. Antibacterial activity was evaluated by measuring the diameter of the inhibition zone (IZ) around the discs. Antibacterial activity is expressed as the mean zone of inhibition diameters (mm) produced by the plant extract. Zone of inhibition range interpretation for the extract was as follows: (+) as present and (-) Absent; < 9 mm as weak (+), 9-11 mm as moderate (++) and > 11 mm as strong (+++) (Turker *et al*., 2009).

7.5 Brine shrimp hatching procedure

NaCl solution was made by mixing 38 g of NaCl in 1 L distilled water. The optimum hatching temperature was 25 to 28°C. Then, 1 g of the brine shrimp cysts were added in 1 L of saltwater. Strong aeration and light were provided to keep cyst in suspension. Cysts were hatched in 24 h. After the brine shrimp hatched, aeration and light were turned off and waited for few minutes for shell and nauplii to separate. Newly hatched nauplii swam towards the source of light and the shells were floated on water surface. The nauplii were siphoned with pipette once separated.

7.6 Two-fold dilution *of S. jamaicensis* extract preparation

The prepared concentrated extract was diluted using NaCl solution to produce a two-fold dilution series of concentration. Six different concentrations which included 1000 µg/mL, 2000 µg/mL, 4000 µg/mL, 8000 µg/mL, 16000 µg/mL and 32000 µg/mL were prepared. Then, the solutions (0.25 ml each) will be mixed with NaCl solution (19.75 ml). NaCl solution was used as the negative control.

7.7 Brine shrimp lethality assay

Ten nauplii were pipetted into each of petri dishes containing 20 mL of the prepared two-fold concentrations of the leaf extract mixtures. The set up was allowed to stand for 24 h under continual light. Then, the petri dishes were examined and the number of alive nauplii that exhibited movement during few seconds of observation was counted after 24 h. The experiment was replicated three times. The percentage of mortality was calculated as:

$$\text{Mortality (\%)} = \frac{\text{Total nauplii} - \text{Alive nauplii}}{\text{Total nauplii}} \times 100$$

7.8 Statistical analysis

The percentage of the brine shrimp mortality was corrected according to the Abbott formula by using mortality in the control group as a correcting factor (Abbott, 1925).

$$\frac{(\%\text{ mortality in treatment} - \%\text{ mortality in control})}{100 - \%\text{ mortality in control}} \text{ x } 100$$

The statistical analysis was conducted using Microsoft Excel 2016 software. The data obtained was used to decide the LC_{50} value by performing Probit analysis. The LC_{50} was taken as the concentration of plant extract required to produce fifty percent mortality of the nauplii of brine shrimp. According to Meyer *et al.*, (1982) and Clarkson *et al*., (2004), extracts derived from natural products which have LC_{50} more than 1000 µg/ml are considered as non-toxic.

8.0 Results

8.1 Disc diffusion assay

The results of the antimicrobial activity of the ethanolic extract of *S. jamaicensis* against *A. hydrophila, V. parahaemolyticus* and *S. agalactiae* are presented in table 1. From the result, the extract shows highest antimicrobial activity against *A. hydrophila* with the inhibition zone ranged from 10 to 12 mm. There is only weak activity against *V. parahaemolyticus* and *S. agalactiae* with their highest inhibition zone at 8 and 7 mm, respectively. Besides, the result also shows that the inhibition zone showed by the bacteria increased as the concentration of the extract increased. The higher the concentration of the extract, the bigger the inhibition zone showed by the bacteria was observed. Meanwhile the positive control which was oxytetracycline shows high activity against all the organisms where the inhibition zone ranged from 20 to 24 mm. Inhibition zone were absent in all of the negative control (DMSO).

Table 1: Inhibition of *Aeromonas hydrophila, Vibrio parahaemolyticus* and *Streptococcus agalactiae* by ethanolic extract of *Stachytarpheta jamaicensis.*

Extract Concentration (mg/ml)	Average diameter of inhibition zone (mm)		
	Aeromonas hydrophila	*Vibrio parahaemolyticus*	*Streptococcus agalactiae*
40	10 (++)	0 (-)	0 (-)
60	11 (++)	7 (+)	0 (-)
80	12 (+++)	7 (+)	7 (+)
100	12 (+++)	8 (+)	7 (+)
Negative control (DMSO)	0 (-)	0 (-)	0 (-)
Positive control (oxytetracycline)	24 (+++)	20 (+++)	20 (+++)

Interpretation: (+) as present and (-) Absent; < 9 mm as weak (+), 9-11 mm as moderate (++) and > 11 mm as strong (+++).

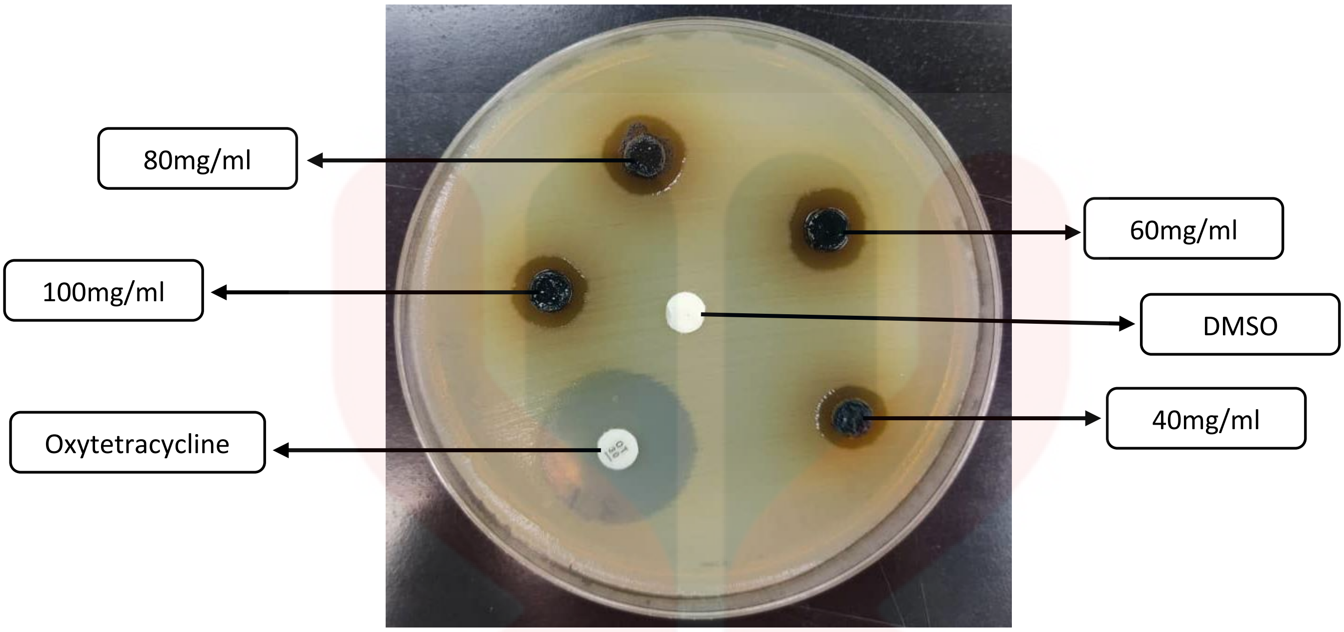


Figure 1: Disc diffusion assay of ethanolic extract of *Stachytarpheta jamaicensis* against *Aeromonas hydrophila*.

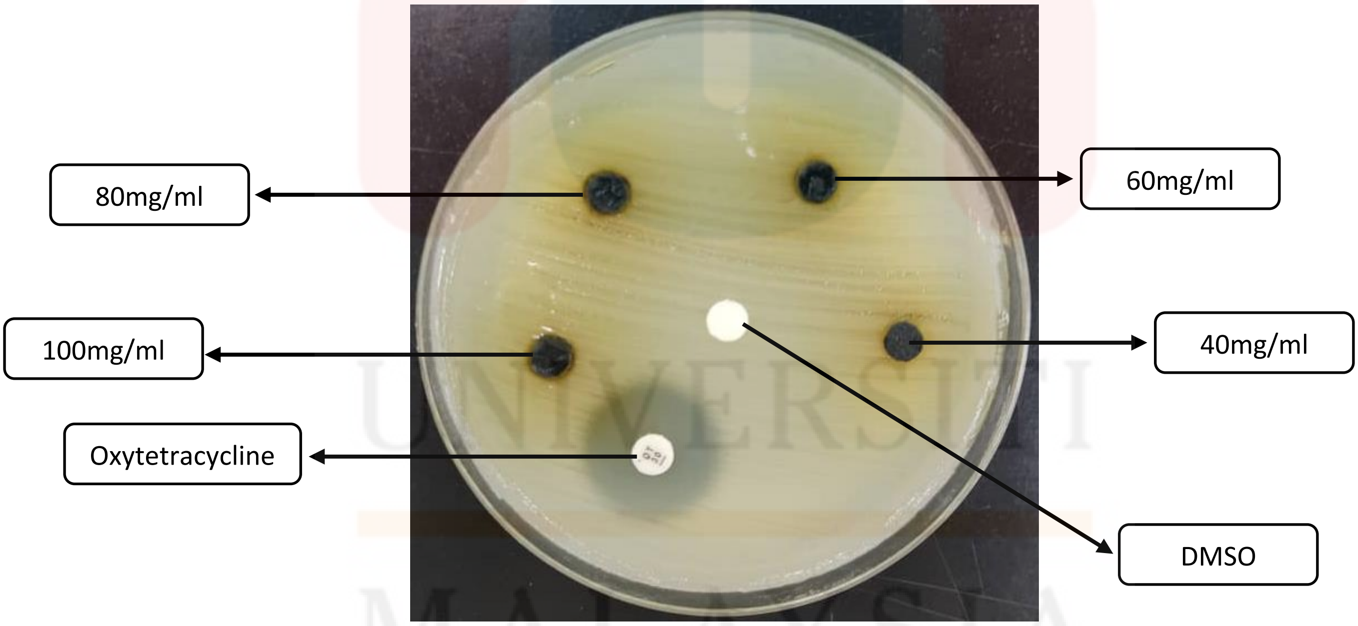


Figure 2: Disc diffusion assay of ethanolic extract of *Stachytarpheta jamaicensis* against *Vibrio parahaemolyticus*.

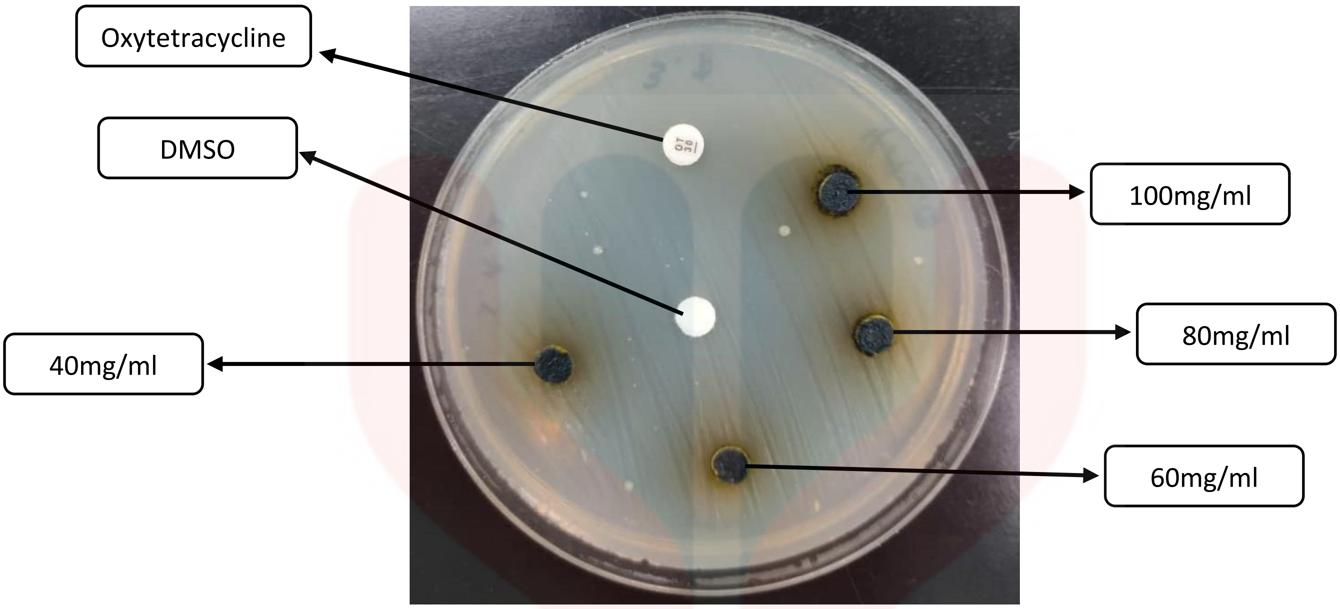


Figure 3: Disc diffusion assay of ethanolic extract of *Stachytarpheta jamaicensis* against *Streptococcus agalactiae*.

8.2 Brine shrimp lethality assay

The results of the brine shrimp lethality assay are presented in table 2. From the result, the mortality of the nauplii of brine shrimp can only be seen starting from the concentration of 16000 µg/ml. The mortality (%) of the nauplii of brine shrimp also increased as the concentration of the extract increased. The LC_{50} of the ethanolic extract of *Stachytarpheta jamaicensis* was calculated using Probit analysis and the result is 26915.35 µg/ml. By comparison to Meyer's or to Clarkson's toxicity index, it is non-toxic (LC_{50}>1 mg/ml) and considered as safe concentration.

Table 2: Percentage of mortality of brine shrimp nauplii.

Extract Concentration (µg/ml)	Total of alive nauplii (Average)	Mortality (%)
1000	10/10	0
2000	10/10	0
4000	10/10	0
8000	10/10	0
16000	8/10	20
32000	4/10	60
Negative control	10/10	0

9.0 Discussion

Stachytarpheta jamaicensis has the ability to sustain the microbial activity of a wide range of pathogenic microbes. Many studies have proven the antimicrobial potential of this plant extracts towards harmful microorganisms, including bacteria and fungi. Important phenolic compounds like tannins, saponins, flavonoids and coumarins can be found abundantly in all parts of the *S. jamaicensis* plant including its stem and leaves (Liew & Yong, 2016). These phytochemicals are responsible for the plant's therapeutic activity in treating many disease conditions. Usually, plants that possess these metabolites have greater antimicrobial activity than others (Idu *et al*., 2007). These phytochemicals are known to be the bioactive antimicrobial agent of the plant (Ruma & Zipagang, 2015).

The result of this study revealed *A. hydrophila* was the most susceptible organism against the ethanolic extract of *S. jamaicensis* plant among all tested bacteria. The extract had weaker antimicrobial activities against *V. parahaemolyticus* and *S. agalactiae*. At the highest concentration level of the extract, the growth of *A. hydrophila* was inhibited at strong level (>11 mm) while *V. parahaemolyticus* and *S. agalactiae* at weak level (<9 mm). The finding also means that *V. parahaemolyticus* and *S. agalactiae* are not sensitive or resistant to the ethanolic extract of the plant. Since occurrence of antibiotic resistant bacterial strains has been reported in aquaculture, the same mechanism involved in the antibiotic resistance could cause the inhibition of the antimicrobial action of plant extracts on the bacterial cells (Castro *et al*., 2008). There are three principle mechanisms of antibiotic resistance by bacteria which are prevention of antimicrobial accumulation by decreasing uptake or increasing efflux, inactivation of antibiotics by hydrolysis or modification and alteration of target sites that the antibiotic usually targets (Yoneyama & Katsumata, 2006).

The synergistic actions of active phytochemicals in the extract of *S. jamaicensis* are highly responsible for bactericidal activities of the plant against the bacterial growth (ZS *et al*., 2017). The plant extract can promote drug accumulation in bacterial cells which means the ethanolic extract of *S. jamaicensis* was able to cause damage to the bacteria especially to *A. hydrophila*. While, DMSO was unable to produce inhibition against those bacteria as it did not cause damage to the cell membrane of the bacteria as the protein and nucleic acid leakage from the bacterial cells did not change significantly when the cells were exposed to the solution (Swamy Thangiah, 2019). Besides, many researchers conducted studies to investigate the effectiveness of plant extracts and their active compounds as antibacterial agents to control fish bacterial infections. Some of them have proposed that antimicrobial components of the natural herb extracts including terpenoid, alkaloid and phenolic compounds can interact with enzymes and proteins of the bacterial cell membrane causing its interference to disperse a flux of protons towards cell exterior which induces cell death or may impede enzymes necessary for amino acids production (Burt, 2004, Gill & Holley, 2006). Other researchers described the inhibitory effect of these plant extracts to hydrophobicity characters of these plants extracts which allow them to react with protein of bacterial cell membrane and mitochondria disturbing their structures and changing their permeability (Friedman , 2004, Tiwari *et al*., 2009).

Bacterial infections are quite common in fish especially when they are reared in high density conditions. The infection may spread from one fish to another causing disease outbreaks in aquaculture farms. The outbreak is responsible for high mortality rate and decline in productivity efficiency, leading to high economic losses to the people involved with aquaculture industry (Castro *et al*., 2008). Antimicrobial resistance in fish is a major concern because of improper and continuous use of antimicrobials in large-scale settings of aquatic animal husbandry. To avoid the occurrence of antibiotic resistance in aquatic bacteria, excessive antibiotic usage needs to be reduced and alternative methods are highly suggested to treat fish bacterial diseases (Pandey *et al*., 2012). The use of plant extracts as an alternative to antibiotic drugs has been suggested as they have shown antimicrobial activity in several studies *in vitro* and *in vivo* in the marine lives. Besides, they are also overall safer, not expensive, easy to access, more environmentally friendly and easier to prepare than antibiotics. Not to forget that natural substance may present fewer side effects on the treated animals (Rangel-López *et al*., 2020). From this study, the ethanolic extract of *S. jamaicensis* showed strong inhibitory activity on the growth of *A. hydrophila* and mild inhibitory activity on *V. parahaemolyticus* and *S. agalactiae*. This means that the plant extract has the potential to be an alternative to commercial antibiotics in the treatment of bacterial infection in fish such as aeromoniasis.

Other than that, this study also revealed the ethanolic extract of *S. jamaicensis* is not toxic as the LC_{50} obtained from the brine shrimp lethality assay was more than 1 mg/ml based on the Meyer's toxicity index. Although many studies have shown that this plant has no toxicity effect, toxicology study of the plant is important as it is useful in determining the upper limits of administration of the plant for therapeutic use and for the registration of the product as well (Liew & Yong, 2016). There are few studies conducted regarding the toxicity of *S. jamaicensis* and the overall results showed the plant extracts did not show toxicity effect and considered safe for therapeutic cure of diseases. A study conducted by Idu *et al*., (2006) where rats were administered with powdered *S. jamaicensis* showed no significant difference in the normal serum biochemistry when compared with control. The ultrasound of organs of the tested rats also showed similar findings with the control. On the other hand, a study conducted by Indrayani *et al*., (2006) where the toxicity effect of several extracts of *S. jamaicensis* leaves were tested on brine shrimp and thin layer chromatography was performed. The results revealed that the hexane, chloroform and ethyl acetate extracts of the plant were toxic and they contained terpenoid compound.

10.0 Conclusion

In conclusion, this study was able to demonstrate the antibacterial properties of the ethanolic extract of *S. jamaicensis* plant against fish pathogenic bacteria: *A. hydrophila, V. parahaemolyticus and S. agalactiae* without toxicity effect. The plant extract produced stronger antimicrobial activity against *A. hydrophila* compared to *V. parahaemolyticus* and *S. agalactiae*. Through brine shrimp lethality assay, the result showed the ethanolic extract of the plant is not toxic (LC_{50}>1mg/ml) hence it is considered safe for therapeutic use. The current study confirms the possible use of ethanolic extract of *S. jamaicensis* plant as an alternative to antibiotics and it has a great potential to combat antimicrobial resistance in fish pathogenic bacteria.

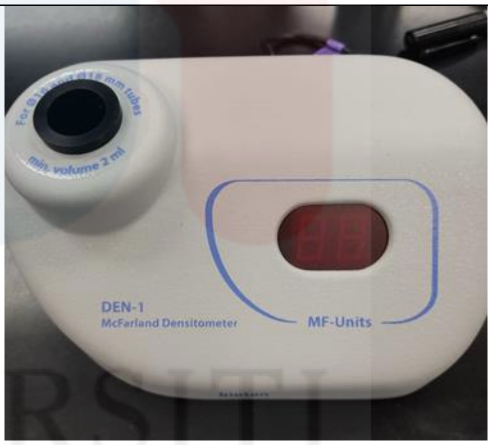
11.0 Recommendations.

In this study, only two replicates of disc diffusion assay were done due to time limitation. Three replicates of disc diffusion assay should be performed for better comparative variances for the extract tested and for more precise result of the study.

Besides, other different types of extract of *S. jamaicensis* using different solvents can be studied in the future for their antimicrobial activity and toxicity effect as well. Different kinds of extract of the plant may produce different results of the study. This might be due to different solubility of the plant phytochemicals in different solvents. According to Turker *et al*., (2009), the use of alcohol as solvent has a higher efficiency in generating antibacterial activities of plant extracts compared with aqueous extraction and some studies also proved that alcoholic extraction method produced higher antimicrobial activity than hexane, aqueous and ethyl acetate.

Other than that, it is preferrable to examine the number of alive nauplii which exhibited movement at different time interval such as every 12 h. So that conclusion can be made whether percentage of mortality is time dependent or not, whereby the longer the nauplii are exposed to the plant extract, the higher the mortality.

Appendices

<table>
<tr><td>
</td><td></td></tr>
<tr><td>Rotary evaporator machine</td><td>Concentrated crude extract stored in sterile container</td></tr>
<tr><td>
</td><td>
</td></tr>
<tr><td>Vortex mixer</td><td>Densitometer</td></tr>
</table>

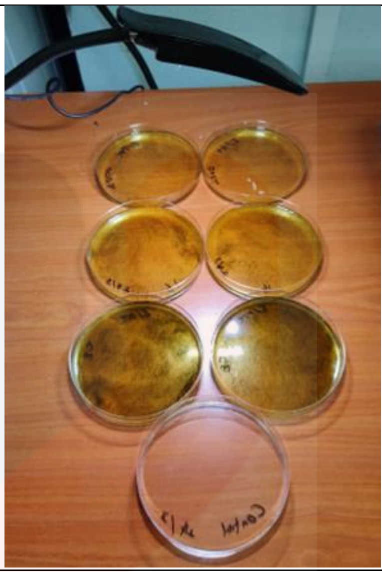
	
Brine shrimp hatching set-up	Brine shrimp lethality assay

Table 3: LC_{50} of ethanolic extract of *Stachytarpheta jamaicensis* mixed with NaCl

Concentration (µg/mL)	log10 (concentration)	% dead	Probit
8000	3.903089987	0	2.67
16000	4.204119983	20	4.16
32000	4.505149978	60	5.25
Intercept	-13.98919506		
X Variable 1	4.285287242		
y=ax+b			
y=4.29x+(-13.99)			
5=4.29x-13.99			
x=(5+13.99)/4.29			
x=4.43			
LC50= antilog x			
LC50= antilog 4.43			
LC50= 26915.35			

References

Abdallah, E. M. (2011). Plants: An alternative source for antimicrobials. *Journal of Applied Pharmaceutical Science, 1*(6), 16-20.

Akmal, M., Rahimi-Midani, A., Hafeez-ur-Rehman, M., Hussain, A., & Choi, T.-J. (2020). Isolation, characterization, and application of a bacteriophage infecting the fish pathogen *Aeromonas hydrophila*. *Pathogens, 9*(3), 215.

Barnes, A. C., & Silayeva, O. (2016). Vaccination against streptococcal infections in farmed fish. *Microbiology Australia, 37*(3), 118-121.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods—a review. *International journal of food microbiology, 94*(3), 223-253.

Caldwell, G. S., Bentley, M. G., & Olive, P. J. (2003). The use of a brine shrimp (*Artemia salina*) bioassay to assess the toxicity of diatom extracts and short chain aldehydes. *Toxicon, 42*(3), 301-306.

Castro, S., Leal, C., Freire, F., Carvalho, D., Oliveira, D., & Figueiredo, H. (2008). Antibacterial activity of plant extracts from Brazil against fish pathogenic bacteria. *Brazilian Journal of Microbiology, 39*(4), 756-760.

Çelik, T., & Aslantürk, Ö. (2007). Cytotoxic and genotoxic effects of *Lavandula stoecha*s aqueous extracts. *Biologia, 62*(3), 292-296.

Clarkson, C., Maharaj, V. J., Crouch, N. R., Grace, O. M., Pillay, P., Matsabisa, M. G., Folb, P. I. (2004). In vitro antiplasmodial activity of medicinal plants native to or naturalised in South Africa. *Journal of ethnopharmacology, 92*(2-3), 177-191.

Cleuvers, M. (2003). Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. *Toxicology letters, 142*(3), 185-194.

Facey, P. C., Pascoe, K. O., Porter, R. B., & Jones, A. D. (1999). Investigation of plants used in Jamaican folk medicine for anti-bacterial activity. *Journal of Pharmacy and Pharmacology, 51*(12), 1455-1460.

Faruk, M., Sarker, M., Alam, M., & Kabir, M. (2004). Economic loss from fish diseases on rural freshwater aquaculture of Bangladesh. *Pakistan Journal of Biological Sciences, 7*(12), 2086-2091.

Favilla, M., Macchia, L., Gallo, A., & Altomare, C. (2006). Toxicity assessment of metabolites of fungal biocontrol agents using two different (*Artemia salina and Daphnia magna*) invertebrate bioassays. *Food and Chemical Toxicology, 44*(11), 1922-1931.

Friedman, M. (2004). Applications of the ninhydrin reaction for analysis of amino acids, peptides, and proteins to agricultural and biomedical sciences. *Journal of agricultural and food chemistry, 52*(3), 385-406.

Gill, A., & Holley, R. (2006). Disruption of *Escherichia coli*, *Listeria monocytogenes* and *Lactobacillus sakei* cellular membranes by plant oil aromatics. *International journal of food microbiology, 108*(1), 1-9.

Hamidi, M., Jovanova, B., & Panovska, T. (2014). Toxicological evaluation of the plant products using Brine Shrimp (*Artemia salina* L.) model. *Maced pharm bull, 60*(1), 9-18.

Huzmi, H., Ina-Salwany, M., Natrah, F., Syukri, F., & Karim, M. (2019). Strategies of Controlling Vibriosis in Fish. *Asian Journal of Applied Sciences, 7*(5).

Idu, M. (2006). Effect of *Stachytarpheta jamaicensis* L.(Vahl.) on Wistar Rats: Serum Biochemistry and Ultrasonography* M. Idu,"JE. Ataman," AO. Akhigbe,"EK I. Omogbai,"F. Amaechina and" EA Odia. *J. Med. Sci, 6*(4), 646-649.

Idu, M., Omogbai, E., Aghimien, G., Amaechina, F., Timothy, O., & Omonigho, S. (2007). Preliminary phytochemistry, antimicrobial properties and acute toxicity of *Stachytarpheta jamaicensis* (L.) Vahl. leaves. *Trends in Medical Research, 2*(4), 193-198.

Indrayani, L., Soetjipto, H., & Sihasale, L. (2006). Skrining fitokimia dan uji toksisitas ekstrak daun pecut kuda (*Stachytarpheta jamaicensis* L. Vahl) terhadap larva udang Artemia salina Leach. *Berkala Penelitian Hayati, 12*(1), 57-61.

Liew, P. M., & Yong, Y. K. (2016). Stachytarpheta jamaicensis (L.) Vahl: From Traditional Usage to Pharmacological Evidence. *Evid Based Complement Alternat Med, 2016*, 7842340. doi:10.1155/2016/7842340

Manilal, A., Sujith, S., Kiran, G. S., Selvin, J., & Shakir, C. (2009). Cytotoxic potentials of red alga, *Laurencia brandenii* collected from the Indian coast. *Global J Pharmacol, 3*(2), 90-94.

Marudhupandi, T., Kumar, T. T. A., Prakash, S., Balamurugan, J., & Dhayanithi, N. B. (2017). *Vibrio parahaemolyticus* a causative bacterium for tail rot disease in ornamental fish, *Amphiprion sebae*. *Aquaculture Reports, 8*, 39-44.

Meyer, B., Ferrigni, N., Putnam, J., Jacobsen, L., Nichols, D., & McLaughlin, J. L. (1982). Brine shrimp: a convenient general bioassay for active plant constituents. *Planta medica, 45*(05), 31-34.

Miller, R., & Harbottle, H. (2018). Antimicrobial drug resistance in fish pathogens. Microbiol Spectr 6. In.

Mostafa, A. A., Al-Askar, A. A., Almaary, K. S., Dawoud, T. M., Sholkamy, E. N., & Bakri, M. M. (2018). Antimicrobial activity of some plant extracts against bacterial strains causing food poisoning diseases. *Saudi journal of biological sciences, 25*(2), 361-366.

Okwu, D., & Ohenhen, O. (2009). Isolation, characterisation and antibacterial activity of lanostane triterpenoid from the leaves of *Stachyterpheta jamaicensis* Linn Vahl. *Der pharma chemica, 1*(2), 32-39.

Pandey, G., Madhuri, S., & Mandloi, A. (2012). Medicinal plants useful in fish diseases. *Plant Archives, 12*(1), 1-4.

Parra, A. L., Yhebra, R. S., Sardiñas, I. G., & Buela, L. I. (2001). Comparative study of the assay of *Artemia salina* L. and the estimate of the medium lethal dose (LD50 value) in mice, to determine oral acute toxicity of plant extracts. *Phytomedicine, 8*(5), 395-400.

Putera, K. A. S. I. (2010). *Antimicrobial Activity and Cytotoxic Effects of Stachytarpheta Jamaicensis (L), Vahl Crude Plant Extracts.* Universiti Teknologi Malaysia,

Rangel-López, L., Zaragoza-Bastida, A., Valladares-Carranza, B., Peláez-Acero, A., Sosa-Gutiérrez, C. G., Hetta, H. F., Rivero-Perez, N. (2020). In Vitro Antibacterial Potential of *Salix babylonica* Extract against Bacteria that Affect *Oncorhynchus mykiss* and *Oreochromis* spp. *Animals, 10*(8), 1340.

Roque, A., Soto-Rodríguez, S. A., & Gomez-Gil, B. (2009). Bacterial fish diseases and molecular tools for bacterial fish pathogens detection. *Aquaculture Microbiology and Biotechnology, 1*, 73-99.

Ruma, O. C., & Zipagang, T. B. (2015). Determination of secondary metabolites and antibacterial property of extract from the leaves of *Stachytarpheta jamaicensis* (L.) Vahl. *Journal of Medicinal Plants and Studies, 3*(4), 79-81.

Sivaranjani, R., Ramakrishnan, K., & Bhuvaneswari, G. (2014). Pharmacognostic studies on root of *Stachytarpheta jamaicensis*. *International Letters of Natural Sciences, 8*(2).

Sulaiman, M., Zakaria, Z., Chiong, H., Lai, S., Israf, D., & Shah, T. A. (2009). Antinociceptive and anti-inflammatory effects of *Stachytarpheta jamaicensis* (L.) Vahl (Verbenaceae) in experimental animal models. *Medical Principles and Practice, 18*(4), 272-279.

Svensson, B.-M., Mathiasson, L., Mårtensson, L., & Bergström, S. (2005). *Artemia salina* as test organism for assessment of acute toxicity of leachate water from landfills. *Environmental monitoring and assessment, 102*(1), 309-321.

Swamy Thangiah, A. (2019). Phytochemical Screening And Antimicrobial Evaluation Of Ethanolic-Aqua Extract Of *Stachytarpheta Jamaicensis* (L.) Vahl Leaves Against Some Selected Human Pathogenic Bacteria. *Rasayan Journal of Chemistry, 12*(1), 300-307. doi:10.31788/rjc.2019.1215042

Syahmi, A. R. M., Vijayarathna, S., Sasidharan, S., Latha, L. Y., Kwan, Y. P., Lau, Y. L., Chen, Y. (2010). Acute oral toxicity and brine shrimp lethality of *Elaeis guineensis* Jacq.,(oil palm leaf) methanol extract. *Molecules, 15*(11), 8111-8121.

Tiwari, B. K., Valdramidis, V. P., O'Donnell, C. P., Muthukumarappan, K., Bourke, P., & Cullen, P. (2009). Application of natural antimicrobials for food preservation. *Journal of agricultural and food chemistry, 57*(14), 5987-6000.

Turker, H., Yildirim, A. B., & Karakaş, F. P. (2009). Sensitivity of bacteria isolated from fish to some medicinal plants. *Turkish Journal of Fisheries and Aquatic Sciences, 9*(2).

Yoneyama, H., & Katsumata, R. (2006). Antibiotic resistance in bacteria and its future for novel antibiotic development. *Bioscience, biotechnology, and biochemistry*, *70*(5), 1060-1075.

ZS, O., Ogunmola, O., Kuyooro, S., & Abiona, O. (2017). *Stachytarpheta jamaicensis* leaf extract: Chemical composition, antioxidant, anti-arthritic, anti-inflammatory and bactericidal potentials. *Journal of Scientific and Innovative Research*, *6*(4), 119-125.