Antibacterial Activity and Toxicity Study of Selected *Piper* Leave Extracts Against the Fish Pathogen (*Aeromonas hydrophila*)

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ABSTRACT
The extensive use of antibiotics in aquaculture has resulted in the emergence of bacterial resistance strains. The medicinal importance of the herb such as *Piper betle*, *Piper sarmentosum*, and *Piper nigrum* evidently proved as one of the most promising commercial botanicals with earlier reported to possess a lot of therapeutic values. Thus, this present study was conducted to evaluate the antibacterial activity of crude aqueous extracts from these three local *Piper* extracted using the decoction method. The bioassays involved disc diffusion and resazurin microdilution assays against aquacultural pathogenic strain; *Aeromonas hydrophila* ATCC49140. Further minimum bactericidal concentration (MBC) also was determined. *Piper nigrum* crude aqueous extract showed the largest inhibition zone (12.71±2.14 mm), followed by *P. betle* crude aqueous extract (11.79±1.36 mm) and *P. sarmentosum* (7.33±0.81 mm). The lowest values for minimum inhibitory concentration (MIC) were obtained from *P. sarmentosum* and *P. nigrum* crude aqueous extract with the same concentration of 12.5 $mg{\cdot}mL^{-1}$ and the lowest MBC was obtained from *P. betle* crude aqueous extract (50 $mg{\cdot}mL^{-1}$). In the brine shrimp assay, all crude aqueous *Piper* extracts of the study demonstrated no toxicity levels. The findings suggested that these three crude aqueous *Piper* extracts prepared by decoction have the potential to prevent bacterial diseases, particularly in aquaculture. The extract preparation method is pursuing the green technic of plant extraction and is safer to be practiced.

Keywords: *Aeromonas hydrophila, Antibacterial, Bioassay, Brine shrimp, Piper herbs.*

1. INTRODUCTION

Aquaculture's fast growth and rising demand for fish resulted in a rise in agricultural production, exploitation of fish stressors, and rising disease risk. Chronic stress has a significant effect on the health of fish, reducing particular immunological responses and defense mechanisms, which results in pathogenic infections under favourable situations. *Aeromonas hydropila* is a gram-negative bacteria that is one of the common pathogens that cause disease in aquaculture. Besides, it can also be found in a diverse variety of habitats and it causes frequent disease of freshwater, brackish water, and ocean fish and shrimp. The presence of colonies may potentially spread to other animals and humans, resulting in death in the worst-case scenario [1, 2]. A virulent

epidemic *A. hydrophila* was first discovered in the United States, where it was originated in Asia [3]. The bacteria were extremely pathogenic in freshwater fish such as catfish, carp, and perch which caused visible clinical signs [4].

Chemotherapy has been the only method for preventing and treating aquaculture disease outbreaks until today. Previously, preventative or prophylactic methods used synthetic chemicals and antibiotics to treat fish diseases. In conjunction with this, chemical treatment is commonly used since it is more convenient to obtain pharmaceutical and medical supplies. However, excessive antibiotic usage results in the emergence of drug-resistant bacteria. Additionally, the biofilms generated by this bacterium inhibited antibiotics' antibacterial efficacy. Chemical drug use has a number of severe consequences for both the environment and human health. This issue of medication and antibiotic resistance has received increasing interest to seek novel antibacterial agents derived from natural sources to address fish diseases caused by a bacterial infection in the aquaculture sector [5, 6].

Plant extracts are particularly appealing due to their inexpensive cost and higher efficiency against certain bacteria as compared to antibiotics that may have negative impacts on the environment. The decoction method is a green extraction method that uses an aqueous extraction method. It minimizes or eliminates the use of solvents, reagents, preservatives, and other potentially dangerous compounds to human health or the environment, while also enabling quicker and more energy-efficient analysis without losing performance requirements. This approach is non-toxic, eco-friendly, and less expensive compared to using ethanol as a solvent [7, 8]. *Piper* species are scented plants that are frequently used as spices in food. These plants contain a high concentration of essential oils, which are present in their fruits, seeds, leaves, branches, roots, and stems. *Piper* species contain therapeutic and preventative potential that might be employed as natural antibacterial agents [9].

Thus, the goal of this study is to explore the antibacterial activity of *Piper* species extracted by decoction method against *A. hydrophila*, because *Piper* species can suppress pathogens. In addition, a brine shrimp lethality assay was performed to assess the extract's toxicity.

2. MATERIALS AND METHODS

2.1. Plant Extracts Preparation

Fresh leaves of *P. betle*, and *P. nigrum* was collected from farm at Banting (Selangor) and Ayer Hitam (Johor) respectively while *P. sarmentosum* was bought from Kuala Nerus (Terengganu). Leaves sample was transported to Food Biochemical Laboratory, FAST, UTHM. The collected leaves sample were first cleaned with tap water and air dried under the shaded area at room temperature for a week. Dried leave was ground into powder form. The extract was prepared by the decoction method. Ground powder of *P. betle*, *P. nigrum*, and P. *sarmentosum* (100 g) was added to 1.5L of distilled water and boiled for one hour. The extract was filtered using a muslin cloth and concentrated in an oven (50°C) until the moisture is less than 10%. By dividing the extract yield (g) by the weight of the leaves powder, the crude extract yield percent was calculated (100 g) [10].

2.2. Bacterial Strain and Culture

A virulent strain of the bacteria *Aeromonas hydrophila* (ATCC 49140) was bought from Universiti Malaysia Kelantan. The strain was sub-culture once on Mueller Hinton Agar (MHA) before use for pathogenicity study and maintain in MHA incubated at 30°C for 24 h to get young discrete colonies. After the optimum growth, the fresh cultures served as test pathogens for in vitro antibacterial activity assay.

2.3. Disc Diffusion Assay

The effects of the *P. betle*, *P. sarmentosum*, and *P. nigrum* crude extracts on bacteria *Aeromonas hydrophila* (ATCC 49140) strains were assayed using the disc diffusion method [11]. The extracts were diluted to a final concentration of 300 $mg \cdot mL^{-1}$ in sterile distilled water. The optical density of the pathogen in bacterial cultures was then determined using a UV spectrophotometer (PG Instrument, UK) set at 660 nm. The required pathogen concentration had an optical density of 10^8 CFU mL^{-1} which is equivalent to a MacFarland No. 0.5 standard solution. Dilution was used to adjust the pathogen's turbidity to match the optical density of the standards. On MHA plates, 100 L of bacterial suspension was spread using a sterile cotton swab. 20 µL of plant extracts were put onto sterile blank susceptibility discs (Thermo Scientific, United Kingdom) with a diameter of six mm. The disc was then air-dried for 2-3 minutes before being put on the agar plates. As positive controls, oxytetracycline (Thermo Scientific, United Kingdom) discs containing 30 µg of antibiotic were applied, while sterile distilled water was used as a negative control. Following that, all plates were incubated at 30°C for 24 hours. After 24 hours of incubation, each plate was observed and the diameter of the inhibitory zone was determined. Three replicates of the test were done. The diameter (mm) of the zone of inhibition (ZI) was determined as follows: 1-6 mm corresponds to low antimicrobial activity; 7-10 mm corresponds to moderate antimicrobial activity; 11-15 mm corresponds to moderate antimicrobial activity; 16-20 mm corresponds to extremely high antimicrobial activity, and 0 mm corresponds to no antimicrobial activity. [3, 11].

Table 1. Percentage of aqueous extract of plants yields used in the study.

Species	Plant Part	Solvent	Method	Extraction Yield (%)
Piper betle	Leaf	Distilled Water	Decoction	24.78
Piper nigrum	Leaf	Distilled Water	Decoction	15.48
Piper sarmentosum	Leaf	Distilled Water	Decoction	13.39

2.4. Resazurin Microdilution Assays and MBC Determination

The broth microdilution technique was used to determine the minimum inhibition concentrations (MIC) for all extracts. The assay was conducted by the Clinical and Laboratory Standards Institute's recommendations. 50 µL of each bacterial suspension (108 CFU mL-1) were loaded in each well of a 96-well plate followed by an aliquot of 100L serially diluted aqueous Piper extracts at diluted concentrations of 200, 100, 50, 25,12.5, 6.25, 3.125, 1.562, and 0.78 mg.mL-1 for each initial working concentration previously mentioned. As a control, wells without tested aqueous *Piper* extracts were used. Each test was performed in triplicate and incubated at 30°C under aerobic conditions for 24 hours. The minimum inhibitory concentration (MIC) of each sample was found to be the lowest concentration inhibiting the observable growth of *A. hydrophila* during incubation. Each well of the microtiter plate was diluted with 1% resazurin dye solution (Sigma Aldrichâ) and incubated at 37°C for 30 minutes. The wells that contained bacterial growth became pink, whereas the wells that did not contain bacteria maintained blue. The minimum inhibitory concentration (MIC) was determined as the extract concentration at which bacterial growth is completely inhibited. MBC values were calculated by re-inoculating 10L aliquots from wells where no growth was found on the MHA surface after 24 hours of incubation at 30°C under aerobic conditions. The MBC concentration was calculated using a sample that had no colony formation [12, 13].

2.5. Toxicity Testing

The toxicity screening in this study was carried out using brine shrimp lethality assay (BSLA) [14]. 34g Tropical Marine Sea Salt was dissolved in 1L of distilled water to create artificial saltwater. A sealed bottomless plastic bottle was inversely placed in another half bottle as the stand. The saltwater and 0.5 g brine shrimp (*Artemia salina*) eggs (Golden Dolphin) were added to the inverted plastic bottle. The saltwater was aerated with an air pump connected using an aeration tube. For 48 hours, brine shrimp eggs were incubated at 26°C. After incubation, the seawater was transferred to a shallow plastic container containing brine shrimp so that easier to collect the active swimming brine shrimp. A light source was placed at an angle over for approximately 30 minutes. As the brine shrimp is a phototactic organism, this was increased the sample size of the brine shrimp collection as the brine shrimp moved towards the light. 15 units of 2mL vials were prepared by adding 400 L seawater to each vial after 30 minutes. Each vial contained 25 live brine shrimp. Following that, a plant sample (400 µL of 1 mg.mL-1) was added to the vials. The positive control was potassium dichromate (1.6 mgmL-1), whereas the negative control was seawater (34 $mg{\cdot}mL^{-1}$). This experiment was conducted in triplicates. The dead brine shrimp were counted after 24 and 48 hours using a light microscope (Olympus) with a 40x magnification and a magnifying glass. After 48 hours of counting, a lethal dosage of acetic acid (Saarchem; 100 % (v/v); 50 ml) was applied to each vial. After 30 minutes, a final count was conducted, and the % mortality was calculated. A death rate of 50% or above was judged toxic. [15].

2.6. Statistical Analysis

The mean and standard deviation of the data was calculated and evaluated statistically using a one-way analysis of variance (ANOVA) in SPSS. Duncan's multiple tests were used to determine the level of significance between individual treatments ($p > 0.05$).

3. RESULTS

3.1. Yields of the Plants Extract Extraction

By using the decoction method, the highest yield of plant leaves extract was obtained from *P. betle* (24.78%) while the other yields were 15.48% and 13.39% for *P. nigrum* and *P. sarmentosum* respectively (Table 1). All

Table 2. Effect of different *Piper* extracts on inhibition of *Aeromonas hydrophila* using MHA.

Sample	Zone of Inhibition (mm)	Result of Assessment
Piper betle	11.79 ± 1.36^{b}	High Activity
Piper nigrum	12.71 ± 2.14^{b}	High Activity
Piper sarmentosum	7.33 ± 0.81^{c}	Moderate Activity
Sterile Distilled Water	0.00 ± 0.00^{d}	No Activity
Oxytetracycline	32.50 ± 2.85^{a}	Very High Activity

*Values are means of replicates groups±SD. Within the same column, means with the same letters are not significantly different ($p > 0.05$).

the extracts were kept in an airtight container in a refrigerator (5-7°C).

3.2. Disc Diffusion Assay

The effectiveness of all aqueous *Piper* extracts on inhibition of *A. hydrophila* is significant ($p < 0.05$) shown in Table 2. There is no significant difference ($p < 0.05$) found between aqueous *P. betle* and *P. nigrum* leaf extract against the inhibition zone of *A. hydrophila* ($p < 0.05$) while both extracts showed a significantly higher inhibition zone compared to aqueous *P. sarmentosum* extract.

3.3. Resazurin Microdilution Assays and MBC Determination

Table 3 shows the minimum inhibitory concentration (MIC) values of *P. betle*, *P. nigrum*, and *P. sarmentosum* extract against *A. hydrophila* (ATCC 49140). The lowest MIC values were obtained from *P. nigrum* and *P. sarmentosum* extracts with the same concentration value of 12.5 mg·mL^{-1} while the MBC was obtained from *P. betle* extract with the concentration value of 50 mg·mL^{-1}.

Table 3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of the *Piper* extract towards *Aeromonas hydrophila* (ATCC49140) strains.

Sample	MIC (mg·mL^{-1})	MBC (mg·mL^{-1})
Piper betle	25	50
Piper nigrum	12.5	100
Piper sarmentosum	12.5	100
Tetracycline	< 0.098	nill

3.4. Toxicity Testing

All of the plant extracts used in this study were shown to be safe to use at a concentration of 1 mgmL-1 using the brine shrimp lethality assay. The brine shrimp average percentage that died resulting from exposure to each plant extract (n = 3) is shown in Table 4 . All reported percentage fatality rates were less than 50%, showing that all plant extracts analyzed were non-toxic as previously stated. [15].

Table 4. Mortality percentage of brine shrimp after exposure to *Piper* extracts.

Sample	Percentage of Mortality (n=3)	
	24h	48h
**Piper betle*	13.33	32.00
**Piper nigrum*	6.67	10.67
**Piper sarmentosum*	9.33	33.33
**Potassium dichromate	100.00	100.00
Distilled water	33.33	45.33

*All the *Piper* extract used in the concentration of 1 mg·mL-

4. DISCUSSION

Extraction techniques of plant materials should be greener and more sustainable. In other words, it should use no harmful chemicals, practical and environmentally friendly [7]. As practiced in this study, an amount of 24.78% yield extract obtained from *Piper* betle leave is higher compared to leaves sample macerated with another solvent in the previous study. This *Piper betle* yield extract in this study is higher compared to a previous study using another solvent such as methanol, ethanol, n-hexane, and ethyl acetate. Aqueous extraction of *P. nigrum* and *P. sarmentosum* also shows a better yield percentage compared to another study. However, the quality of plant extract is depending on the purpose of their usage. The solvent used affect the solubility of several active compounds obtained since some of them react for certain biological function [8, 11, 16].

Surprisingly, this aqueous extract of *Piper* leaves has significant and moderate antibacterial efficacy against the microorganisms tested. In comparison, several researchers have used water as an extracting solvent, and the majority of their findings show that water extracts have little or no inhibition effect. This indicates that the decoction procedures commonly used in the manufacture of traditional medicines aid in revealing the antibacterial properties of the *Piper* leaves. This antibacterial activity is depending on the miscibility of the chemicals in water, as some will show antimicrobial activity [17].

Minimum inhibitory concentration and MBC were conducted to assess the bacteriostatic and bactericidal concentration of the aqueous *Piper* extracts against the tested pathogens. The lower the MIC and MBC values indicate the higher antibacterial potential of the plant extract. The variation of antibacterial activity of the extracts might be due to the different responses of antibacterial substances from *Pipers* towards *A. hydrophilla* [12].

The results of these studies showed the brine shrimp lethality tests suggest that all of the aqueous *Piper* extracts tested were confirmed to be safe to apply at a dose of 1 mgmL-1. Because all documented percentage death rates were less than 50%, all plant extracts investigated were judged non-toxic. The brine shrimp nauplii bioassay can be used to assess the toxicity of a wide variety of hazardous substances. It can detect a wide variety of bioactivities in crude extracts. Individual plant extracts have different degrees of toxicity, even when they come from the same family [3, 15].

In conclusion, the outcomes of this investigation showed the efficacy of crude aqueous *Piper* extracts as

antibacterial agents. Additionally, when tested on brine shrimp, the crude extracts demonstrated promising effectiveness against fish bacterial infections and had the least toxicity. Thus, the extracts of these chosen *Pipers* have potential and maybe further investigated for their use in aquaculture therapeutic practices rather than employing more expensive treatments and chemicals that would hurt the ecosystem and threaten other aquatic life species. Additional research is required to determine the efficacy of these extracts and to discover the phytochemicals responsible for their biological activity in the treatment of fish diseases.

AUTHORS' CONTRIBUTIONS

SFS, HMD, and NZO each contributed to the study's concept and workflow. NA was involved in the laboratory and experimental studies, as well as the preparation of the initial manuscript, evaluation, and editing. SFS was involved in the review and editing process. Supervision was assisted by SFS, HMD, and NZO.

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