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In vitro antimicrobial activity of betel, *Piper betle* leaf extract against *Vibrio alginolyticus* isolated from Asian sea bass, *Lates calcarifer*

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ABSTRACT

Betel plant, *Piper betle* has excellent potential to be used as an alternative antimicrobial agent to replace the use of commercial antibiotic in aquaculture. The present study evaluates the *in vitro* antimicrobial activity of betel leaf extract against *Vibrio alginolyticus* isolated from Asian sea bass, *Lates calcarifer*. Disc diffusion method was used to evaluate the antimicrobial activity of different concentrations of betel leaf extract and to compare the antimicrobial activity with commercial antibiotics. Broth dilution method was used to determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the extract against *V. alginolyticus*. It was found that 100 mg/mL of the extract produced significantly ($P < 0.05$) largest inhibition zone (19 mm) compared to other concentrations. Concentrations between 10 and 80 mg/mL of the extract produced inhibition zone similar ($P > 0.05$) as the commercial antibiotics. The MIC and MBC for the extract at 100 mg/mL were 0.2 mg/mL and 0.39 mg/mL, respectively. The results show that ethanolic crude extract of betel leaves at 100 mg/mL is a potential alternative to antibiotic against *V. alginolyticus* infection.

1. INTRODUCTION

Aquaculture is an important industry as it helps to overcome the rising demand for food fish. Since commercial fishing industry involving wild stock of fish has reached the production limit, aquaculture is an alternative initiative to overcome this problem [1]. Asian sea bass, *Lates calcarifer* is one of the important fish species that is being cultured in Southeast Asia [2]. It is a euryhaline species with the ability to tolerate a wide range of salinity thus a popular fish for culture [3].

In intensive aquaculture environment, increasing stress factors such as poor water quality and high stocking density expose fish to diseases [4]. Vibriosis is one of the main bacterial diseases that affect cultured marine fish, inflicting high losses, and *Vibrio alginolyticus* has being identified to be one of the bacteria associated with vibriosis in aquaculture around the world [5,6]. The symptoms are quite similar with other bacterial infections, which include drowsiness, loss of appetite, discoloration, reddened and ulcerative skin, disoriented swimming pattern, exophthalmia, and bloating of the abdomen [7].

The most common treatment against bacterial infections in aquaculture is antibiotic since it is effective against a wide range of bacteria and cost effective. The most frequently used antibiotics in aquaculture are tetracycline, quinolones, and phenols while the route of administration is either oral, injection, or bath [8].

Plants have been known to have various medicinal properties [9]. One of the plants that have been identified to contain antimicrobial compounds is betel plant, *Piper betle*. Betel plant is a native of Malaysia but is more popular and being used widely in other places such as India [10]. Betel is a vein plant with yellowish–green to bright green, heart-shaped leaves that belong to the family Piperaceae (Pepper family). There are about 100 varieties of betel plants around the world with different chemotypes in each region [11].

Betel leaves contain various compounds such as water, carbohydrates, proteins, fat, minerals, vitamins, tannin, fiber, alkaloid, steroidal compounds, and essential oil [12]. However, the main active compounds that are responsible for the antibacterial effect are the hydroxychavicol, sterol, and tannin [13,14]. This study determines the *in vitro* efficacy of ethanolic betel leaf extract against *V. alginolyticus* isolated from diseased Asia sea bass.

2. MATERIALS AND METHODS

One kg of fresh local betel leaves was collected and thoroughly washed with distilled water. The leaves were air dried for 72 h and grounded into powder form before 100 g of the betel leaves powder were immersed into 3 L of absolute ethanol for 48 h. This was followed by filtration and concentrated using rotary evaporator.

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V. alginolyticus was isolated earlier from an outbreak of vibriosis among Asian sea bass in Besut, Terengganu. The isolate was identified as *V. alginolyticus* using the API identification system (Biomerieux) and polymerase chain reaction. It was subcultured onto tryptone soy agar (TSA, Merck, Germany) containing 1.5% sodium chloride for 24 h at 30°C.

Five colonies of *V. alginolyticus* grown on TSA containing 1.5% sodium chloride were cultured in 250 mL tryptone soy broth containing 1.5% sodium chloride for 24 h at 30°C with shaking at 150 rpm. Then, 1 mL of the suspension was collected and serially diluted for 10 times in 9 mL phosphate-buffered saline (PBS, Merck, Germany) before 0.1 mL of each serial dilution was plated onto plate count agar containing 1.5% sodium chloride. The colony-forming unit/mL was determined after 24 h of incubation at 30°C.

Thirty µl of 1.25 mg/mL, 2.5 mg/mL, 5 mg/mL, 10 mg/mL, 20 mg/mL, 40 mg/mL, 80 mg/mL, and 100 mg/mL dilutions of the extract was dispensed onto 6 mm antibiotic assay discs, respectively. Absolute ethanol (99.98%) was used as a control. Each concentration was prepared in triplicate before the discs were allowed to dry at room temperature. Stock culture of *V. alginolyticus* was inoculated into the TSA containing 1.5% sodium chloride and incubated for 24 h before being further incubated in Mueller-Hinton broth containing 1.5% sodium chloride for 24 h. PBS was used to adjust the inoculum to 0.5 McFarland turbidity standard. The bacterial suspension was swapped onto the Mueller-Hinton agar using a sterile cotton swab before the discs with the diluted extract were put onto the agar. The commercial oxytetracycline disc (30 µg), oxolinic acid (2 µg), and chloramphenicol (30 µg) were used for comparison. The agar plates were then incubated for 24 h at 30°C. The experiment was carried out in triplicate, and the inhibition zone was measured and recorded. The results were compared with BBL zone interpretative chart to determine the sensitivity of the isolate to the antibiotics.

Betel leaf extract at a concentration of 100 mg/mL was introduced into Muller-Hilton broth before serially diluted to obtain concentrations of 50 mg/mL, 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL, 3.13 mg/mL, 1.56 mg/mL, 0.78 mg/mL, 0.39 mg/mL, 0.20 mg/mL, and 0.09 mg/mL. Then, 1 mL of bacterial suspension was added into each diluted betel leaf extract. The broth without betel leaf extract was used as control. The mixtures were incubated at 30°C for 24 h before the turbidity was determined. The broth that did not show any turbidity was used as minimum inhibitory concentration (MIC) [15], while the lowest concentration that did not show any bacterial growth was recorded as minimum bactericidal concentration (MBC).

3. RESULTS

Crude extract of betel leaves at a concentration of 1.25 mg/mL produced an inhibition zone of 1 mm that was significantly ($P < 0.05$) increased to 3 mm at a concentration of 2.5 mg/mL and to 8 mm at 5 mg/mL. At higher concentrations between 10 and 80 mg/mL of betel leaf crude extract, there were no significant ($P > 0.05$) differences in the size of the inhibition zone. However, at 100 mg/mL, the inhibition zone was 19 mm, significantly ($P < 0.05$) larger than other tested concentrations [Figure 1].

When compared with oxytetracycline, betel leaf crude extract at 100 mg/mL showed significantly ($P < 0.05$) larger inhibition zone (19 mm) than the 14 mm oxytetracycline. However, chloramphenicol resulted in significantly ($P < 0.05$) largest inhibition with 23 mm [Figure 2].

Turbidity was absent at the concentrations between 0.2 mg/mL and 50 mg/mL of betel leaf crude extract. The turbidity first appeared at the concentration of 0.09 mg/mL of the extract.

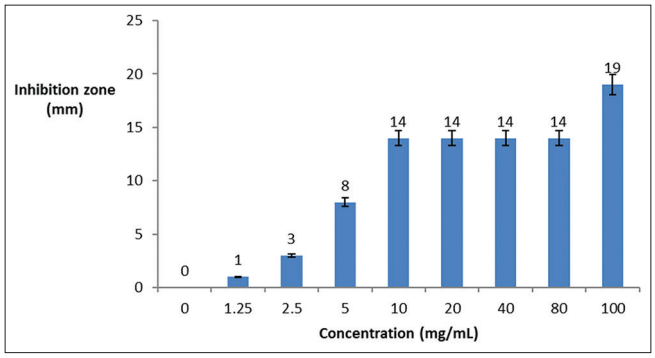

Figure 1: The inhibition zone obtained following disc diffusion method of different concentrations of betel leaf crude extract against *Vibrio alginolyticus.*

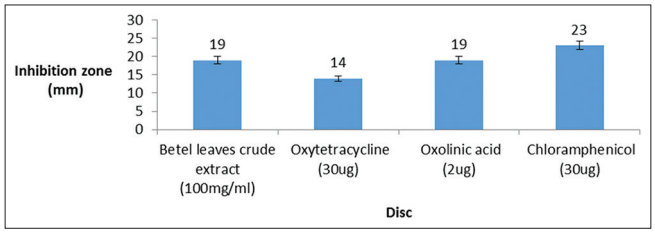

Figure 2: Comparative inhibition zone obtained following disc diffusion method between betel leaf crude extract (100 mg/mL), oxytetracycline (30 µg), oxolinic acid (2 µg), and chloramphenicol (30 µg) against *Vibrio alginolyticus.*

Bacterial growth was first detected at the concentration of 0.2 mg/mL of the extract. Higher concentrations of extract resulted in no bacterial growth.

4. DISCUSSION

Humans have used betel leaves for a long period of time as treatment for various diseases and ailments [16]. They contain various phytochemicals including sterol, an active compound that is an excellent antimicrobial [14]. Betel leaves also contain hydroxychavicol, another active compound that possesses powerful antimicrobial effect [13]. Combination of these active compounds resulted in excellent therapeutic effect [17].

Results of this study indicate that the antimicrobial activity of betel leaf crude extract against *V. alginolyticus* is maximum at a high concentration of 100 mg/mL while intermediate concentrations between 10 and 80 mg/mL resulted in activity similar to those of commercial antibiotics such as oxytetracycline. Although chloramphenicol showed the strongest antimicrobial activity, it has been banned for use in food animal industry [18]. Similarly, the use of oxolinic acid in aquaculture is harmful to the environment and other aquatic animals as it promotes the antimicrobial-resistant microorganism [19].

The MIC is the lowest concentration of compound that can inhibit bacterial growth while the MBC is the lowest concentration of the antimicrobial compound that can kill the bacterium. The low MIC and MBC of betel leaf extract indicate the potential of the extract for use as an alternative to antibiotic in controlling vibriosis in Asian sea bass.

5. CONCLUSION

This study revealed the potential use of betel leaf crude extract as antimicrobial agent against marine bacteria. The concentrations between 10 mg/L and 80 mg/L are comparable to the commercially

available antibiotics against *V. alginolyticus,* but the concentration of 100 mg/L is the best concentration to be recommended.

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