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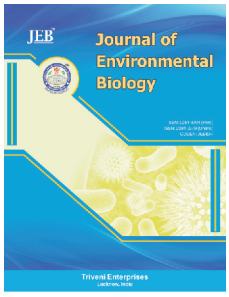

Antibacterial activity of garlic extracts on fish pathogenic bacteria

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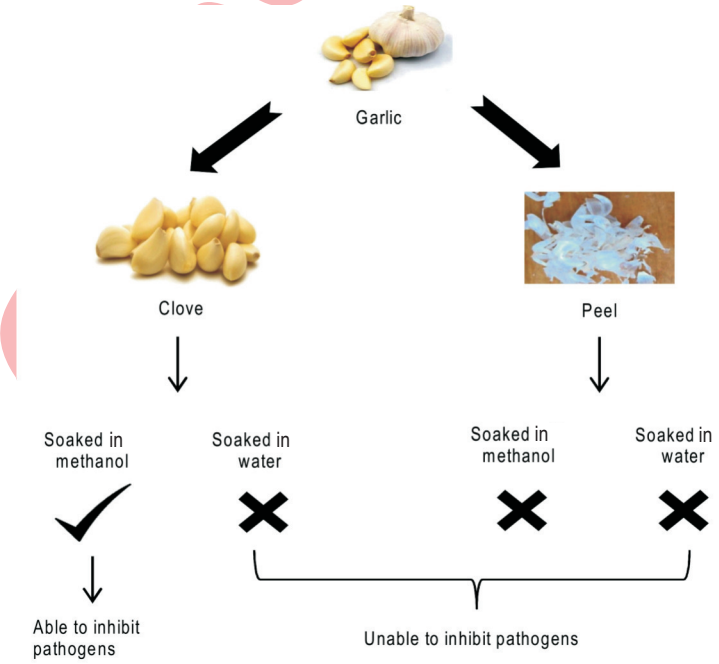
Abstract

Aim : Aqueous and methanol extracts of *Allium sativum* (clove and peel) were investigated for their antibacterial properties in *in-vitro* assay against four marine pathogens, *Aeromonas hydrophila, Vibrio anguillarum, Vibrio alginolyticus* and *Vibrio harveyi*.

Methodology : Different concentrations of methanol and aqueous garlic extracts were used in different methods including the disc-diffusion, minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) assay.

Results : The aqueous extract of *A. sativum* (clove and peel) had no antibacterial effect against the pathogenic bacteria tested, while the clove extract of methanol showed a strong inhibition activity against pathogens tested. The largest inhibition zone was observed against *A. hydrophila* (15.3±0.3) and the least were detected against *V. anguillarum* (11.0±0.6) and *V. harveyi* (11.0±0.6). The MIC and MBC values revealed that *A. hydrophila* was inhibited by a supernatant extract of clove at the lowest concentration of 0.6 mg ml^{-1}, while other pathogens were inhibited at the concentration of 0.8 and 1.0 mg ml^{-1}.

Interpretation : This study suggests that clove extract of *A. sativum* has the potential to be used as a phytobiotics in controlling the growth of marine pathogens.



Introduction

Infectious diseases are one of the main factors contributing to high losses in the aquaculture industry as the demand in this sector increases (Bondad-Reantaso *et al.,* 2005). The awareness of safe food demand has lead to the search of natural products as growth promoters and/or prophylactics in aquatic feeds (Kareem *et al.,* 2016; Sheikhlar *et al.,* 2017).

Phytobiotic is one of the alternatives used in the aquaculture sector. Phytobiotic is defined as natural compounds derived from plants, which can enhance animal productivity once included in the diets. Many plant products such as Aloe vera, onion, ginger, garlic, thyme, neem, peppermint, medlar and rosemary have been used as immunity stimulation and growth promoter in aquaculture (Kolkovski *et al*., 2011; Hoseinifar *et al.,* 2017). Moreover, they contain beneficial proteins, amino acids, vitamins, lipid, trace elements, and some unknown growth promoting factors that can strengthen metabolism of aquatic animals, enhance the composition of protein and enzymes, and expedite the growth of animals (Yin *et al.,* 2006). Cristea *et al.* (2012) demonstrated various properties of phytobiotics including enhancing appetites, promoting growth, acting as antioxidants, stimulating the digestive system, being anticarcinogenic or having antiparasitic, antimicrobial or insecticidal activities.

Allium sativum, commonly known as garlic, has been known for centuries for its importance in the dietary role and medicinal properties. Block (1985) revealed that the typical odor and flavor of garlic were attributed to water-soluble organosulfur compounds and some specific oils that had therapeutic and prophylactic effects. This was later supported by Ariga and Seki (2006) who suggested that the extensive variety of dietary and medicinal properties of garlic is due to the compounds of sulfur found in the garlic. These sulphur compounds are also responsible for the characteristic flavour of fresh garlic (Li *et al.,* 2007).

Currently, antibiotics are sometimes applied in aquaculture to control bacterial infections, but their application has led to increased development of drug-resistant bacteria. Moreover, the antibiotic accumulation in aquatic animals and environment are generally unsafe to consumers and the environment (Alderman and Hastings, 1998). A study conducted by Durairaj *et al.* (2009) showed that foods and consumers can be protected from the risk of pathogenic bacteria by using garlic. Thus, natural products have been considered as an alternative to control bacterial infections in aquaculture. Moreover, Corzo-Martinez *et al*. (2007) stated that garlic helps in controlling the pathogens, especially bacteria and fungi, and increases the well being of aquatic animals. Unfortunately, large quantities of garlic are consumed but the outer layer is treated as waste. Thanikachalam *et al*. (2010) reported that the outer layer of garlic has bioactive substances that are capable of acting as immunostimulant in fish. Thus, all the parts of garlic (peels and cloves) have their own active compounds. In this study, *A. sativum* (garlic) was investigated for its antibacterial activity against four major marine pathogens; *Aeromonas hydrophila*, *Vibrio harveyi*, *Vibrio anguillarum* and *Vibrio alginolyticus*. Different extractants (water and methanol) were used to extract garlic peel and clove, and were used to test their effectiveness against different pathogenic bacteria in fishes.

Materials and Methods

Preparation of garlic extracts : Garlic was purchased from grocery store in Serdang, Selangor. Two types of extracts were prepared; methanol and aqueous extracts of peels and cloves. The garlic cloves and peels were cleaned, peeled, sliced and oven dried at 70°C for 24 hrs. Each of the dried samples was grounded into powder using a blender. A 10 g powder of garlic cloves and garlic peels were soaked in different extracts including 100 ml of distilled water and 10%, 20% and 40% of methanol, respectively. The flasks were then shaken at 120 rpm and incubated for 72 hrs at room temperature.

The supernatant from each extract (water, 10%, 20% and 40% of methanol) were collected and labeled as supernatant extract of clove and supernatant extract of peel, respectively. Each of the dried crude extracts was dissolved in 100 mg ml^{-1} of distilled water as final concentrations. In order to remove the excess residues, the samples were further centrifuged at 10,000 rpm. The previous steps were repeated at 25ºC for 10 min. The extract solutions were stored at -20°C for further use. These extracts were labeled as crude extract of clove and crude extract of peel. For control, methanol and distilled water were prepared by following the same methods ascribed above and were tested for their antimicrobial properties (Fig. 1).

Pathogenic bacteria strains : *Vibrio alginolyticus, V. harveyi, V. anguillarum and A. hydrophila* were obtained from the Labarotory of Fish Health in the Aquaculture Department, Universiti Putra Malaysia. All the isolates were cultured in Tryptic Soy Agar (TSA) and Tryptic Soy Broth (TSB) at 30°C for overnight.

Stagnant Method: Disc-diffusion assay : A 6 mm diameter paper discs were sterilized and different concentrations of garlic extracts were prepared (1.0 mg ml^{-1}, 2.0 mg ml^{-1}, 4.0 mg ml^{-1}, 6.0 mg ml^{-1}, 8.0 mg ml^{-1}, 10.0 mg ml^{-1}). The discs were introduced with 10 µl of each garlic extract concentration. The discs were left for few minutes to allow the absorption of the solution.

Next, 10 ml of TSB was used as a medium to inoculate each of the pathogenic bacteria and incubated overnight at 30ºC. The culture was swabbed on the TSA agar using a sterilized cotton swab. Later, the discs that were prepared earlier with different concentrations of each supernatant extracts of clove, supernatant extract of peels, crude extracts of clove and crude extract of peel, were placed on the inoculated TSA. After half an hour of discs application, the plates were incubated at 30°C for a period of 24 hrs. Meanwhile, the disc that contained only sterile

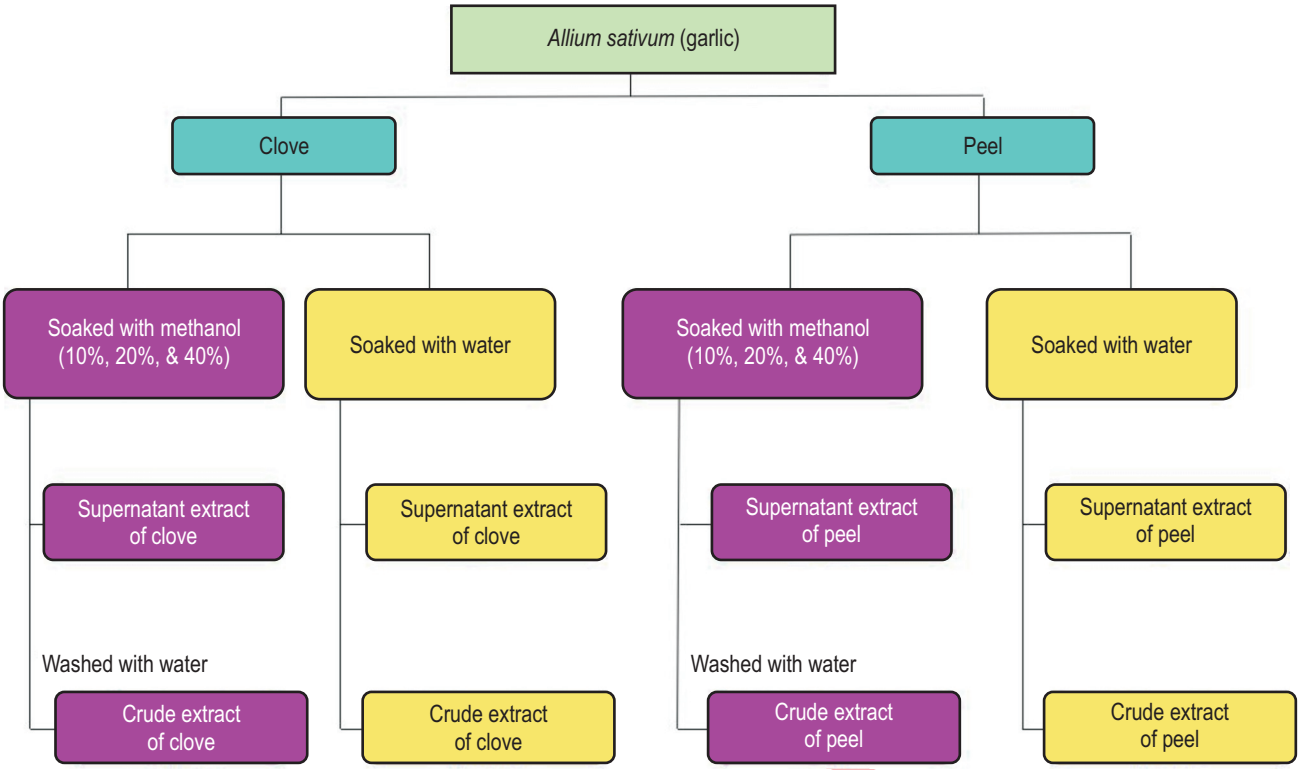


Fig. 1 : Extraction of garlic

distilled water and methanol was used as control. Inhibition zone formed was observed on the plate on the next day. The diameter of the inhibition zone was measured and recorded accordingly (Durairaj *et al.,* 2009).

Broth dilution method - Minimum Inhibitory Concentration : The minimum inhibitory concentration (MIC) of the aqueous garlic extract was evaluated by using the broth dilution method with 96 well plates. Pathogenic strains were cultured in TSB. Each cell suspension was adjusted approximately to 10^4 CFU ml^{-1}. Different concentrations (1.0, 2.0 mg ml^{-1}, 4.0 mg ml^{-1}, 6.0 mg ml^{-1}, 8.0 mg ml^{-1}, 10.0 mg ml^{-1}) of garlic extracts were pre-incubated with 100 µl of pathogen suspension (10^4 CFU ml^{-1}) in the respective wells. Un-inoculated wells that contained TSB, garlic extracts only, and pathogens only were used as controls. The plate was incubated at 30°C for 24 and 48 hrs (Durairaj *et al.,* 2009). The minimum inhibitory concentration is defined as the minimum concentration of garlic extract that is able to inhibit the pathogenic bacteria significantly.

Minimum Bactericidal Concentration : Results were evaluated as outlines for MIC by pipetting the medium with a combination of all of the wells onto TSA and TCBS plates. The plates were incubated for 24 to 48 hrs at 30ºC. MBC indicates the concentration at which no microbial growth was formed on the plate.

Results and Discussion

This study assay demonstrated that supernatant extract of clove showed some antibacterial activities against the pathogenic bacteria tested. Meanwhile, the crude extract of clove, supernatant extract of peel and crude extract of peel showed no activity against the pathogenic bacteria tested (Table 1). Results also demonstrated that the supernatant extract of clove using 20% of methanol was active against all pathogens, higher when compared to 10% supernatant extract of clove. The largest inhibition zone was observed against *A. hydrophila* (15.3±0.3), while the smallest was against *V. harveyi* (11.0±0.6). Meanwhile, the 20% of methanol extract showed the largest inhibition zone against *A. hydrophila* when compared to the other pathogens. No inhibition zone was found in a control.

The MIC values for 10% and 20% of methanol clove extracts (supernatant) against the tested pathogens ranged between 0.6 and 0.8 mg ml^{-1}. The 10% methanol extract showed some activities at 0.8 mg ml^{-1} against *V. anguillarum*, *V. alginolyticus* and *V. harveyi* and the extract was active against *A. hydrophila* at 0.6 mg ml^{-1}. For the garlic extract on the MIC plate, there was no activity present against the pathogenic bacteria tested for concentrations above 1.0 mg ml^{-1}. Overall, the findings showed that the growth of most of the tested pathogens were found to inhibit 0.6 mg ml^{-1} to 0.8 mg ml^{-1} extract.

Table 1 : Antibacterial activity of supernatant clove extract of using methanol against marine pathogens by disc-diffusion method

Pathogens	Supernatant extract of clove (mm) 10% Methanol	20% Methanol	40% Methanol
Aeromonas hydrophila	15.3 ± 0.3	15.0 ± 0.6	-
Vibrio anguillarum	11.0 ± 0.6	12.6 ± 0.3	-
Vibrio alginolyticus	11.3 ± 0.3	13.0 ± 0.6	-
Vibrio harveyi	11.0 ± 0.6	12.0 ± 0.6	-

(-) = No inhibition

Table 2 : Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of supernatant extract of clove at 10% and 20% of methanol. Results are expressed in mg ml^{-1}

	Supernatant extract of clove (mm) 10% Methanol		20% Methanol	
Pathogen	**MIC**	**MBC**	**MIC**	**MBC**
Aeromonas hydrophila	0.6	0.8	0.6	1.0
Vibrio anguillarum	0.8	0.8	0.6	1.0
Vibrio alginolyticus	0.8	1.0	0.6	1.0
Vibrio harveyi	0.8	1.0	0.6	0.8

Supernatant clove extract of 10% methanol had the MBC value of 1.0 mg ml^{-1} for *V. alginolyticus* and *V. harveyi*. At this concentration, no bacterial growth was observed. Meanwhile, the MBC values for *A. hydrophila* and *V. anguillarum* were 0.8 mg ml^{-1}. This research was further tested with MBC value of supernatant clove extract that was extracted with 20% of methanol. Results showed that *A. hydrophila*, *V. anguillarum* and *V. alginolyticus* were bacteriocidal at 1.0 mg ml^{-1} concentration while *V. harveyi* was bacteriocidal at of 0.8 mg ml^{-1} concentration, respectively. The results revealed that *V. harveyi* had a lower MBC value and bacteriocidal at a lower concentration of supernatant clove extract when compared to the other marine pathogens tested (Table 2).

This study was conducted to examine the ability of garlic clove and peel to inhibit the growth of marine pathogens as a natural alternative and as an antibacterial substance. This is with the purpose to substitute the use of antibiotics to treat pathogenic bacterial infections in farmed fishes. In this study, two types of solvents (methanol and water) were used for garlic extraction. Two concentrations of methanol extracts, 10% and 20% (supernatant extract of clove), showed the highest antimicrobial activities towards *A. hydrophila, V. anguillarum, V. alginolyticus* and *V. harveyi* when compared to the water extracts of clove and peel. In fact, the water extract of garlic showed no inhibitory effect on the pathogenic bacteria tested, both in the disc-diffusion assay and MIC assay. This is in agreement with the previous studies that reported methanol extracts of plant products had a stronger inhibition towards bacterial pathogens *in vitro* than aqueous ones (Parekh *et al.,* 2005; Sheikhlar *et al.,* 2017). It has also been reported that methanol was more effective than other solvents including hexane or ethanol (Eloff, 1998; Karaman *et al.,* 2003). This could be due to methanol being more effective at extracting bioactive compounds with antimicrobial activity.

Several studies have provided strong evidence that most of the biological function of garlic clove is caused by active substance known as allicin (Li *et al.,* 2007), which is the most important organosulphide present in garlic. Rattanachaikunsopon and Phumkhachorn (2007) reported that some herbal extracts produce antimicrobial effects on pathogenic bacteria by reducing the pathogenic bacteria's active compound. Another factor to the reduced efficacy of the aqueous extract may be due to the characteristic of allicin that decomposes faster (Ankri and Mirelman, 1999). Furthermore, allicin acts as an antibiotic, antidiabetic and antihypertensive (Shinkafi *et al.,* 2013).

On the other hand, 40% methanol supernatant clove extract was not able to inhibit the growth of all pathogens tested. This may be due to the use of methanol at high concentration which then was able to degrade the active compounds. Results clearly showed that 40% of methanol extract was incapable to interrupt the RNA production and lipid synthesis. If RNA is not hampered by allicin, protein synthesis will not be affected. Thus, it will result in the development of organism or bacteria as they get sufficient amount amino acids and proteins for their growth (Durairaj *et al.,* 2009).

The values obtained through MIC and MBC of the methanol extracts against *A. hydrophila, V. anguillarum, V. alginolyticus* and *V. harveyi* supported the results of the disc-diffusion assay. The MIC values obtained were lower when compared to the MBC values obtained in this study. This indicates that the supernatant extract of clove is bacteriocidal at a higher concentration and bacteriostatic at a lower level of concentration.

Based on the results of this study and findings from the previous reports, it can be concluded that *A. sativum* extract of clove is capable of fulfilling all criteria of antibacterial agents. It is cheap, easy to obtain and safe to be applied. Furthermore, the supernatant methanol extract of clove showed the greatest antimicrobial activities against the tested pathogenic bacteria. Thus, supernatant extract of clove should be tested as a potential phytobiotic candidate in aquaculture that may help reduce the reliance of antibiotics. The pure compound that is responsible for the antimicrobial activity should be isolated from the raw extract of garlic, for future research. In conclusion, the supernatant extract of *A. sativum* clove should provide several advantages to prevent and/or treat bacterial diseases as a natural alternative in aquaculture.

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