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Antibacterial Activity of Neem (*Azadirachta indica*) Leaves on *Vibrio* spp. Isolated from Cultured Shrimp

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ABSTRACT

The use of antibiotics in aquaculture to treat infections has resulted in the development of resistant strains which have rendered antibiotic treatment ineffective. Therefore, alternative antibacterial materials must be found. Extracts of neem tree (*Azadirachta indica*) leaves were tested against *Vibrio parahaemolyticus* and *Vibrio alginolyticus* isolated from cultured shrimp. Aqueous extract of neem leaves did not produce any inhibitory zone while the neem juice produced inhibitory zone that showed linear relationship to the concentration of neem juice on both bacteria. The Minimum Inhibitory Concentration (MIC) for *V. parahaemolyticus* and *V. alginolyticus* was 3.13 and 6.25%, respectively. The Minimum Bactericidal Concentration (MBC) for *V. parahaemolyticus* and *V. alginolyticus* was 12.50 and 25.00%, respectively. It is concluded that neem juice is an antibacterial agent and is useful for inhibition of vibrios in shrimp.

Key words: *Azadirachta indica*, neem, *Vibrio* spp., antimicrobial sensitivity test, minimum inhibitory concentration, minimum bactericidal concentration

INTRODUCTION

Aquaculture is the fastest growing industry and is an important economic activity contributing to the world protein supply. To meet the growing demand for fish and seafood throughout the world, traditional farming systems have given way to intensive aquaculture. Intensive culture and adverse environmental conditions are often attributed to disease due to immune suppression or physiological stress. High mortality and serious economic losses have been reported due to vibriosis, a major disease problem in shrimp and prawn (Balasundaram *et al*., 2012) aquaculture.

According to Ramalingam and Shyamala (2006), *Vibrio* spp. are often considered opportunistic pathogens in shrimp, but primary disease can also be caused by highly virulent strains. The major species causing vibriosis in shrimp are *Vibrio alginolyticus*, *V. anguillarum*, *V. harveyi* and *V. parahaemolyticus* based on the phenotypic data (Goarant *et al*., 1999).

The use of antibiotics is a common practice for the treatment of diseases, but excessive use of antibiotics has encouraged the evolution of antibiotic-resistant bacteria (Cabello, 2006). The impact of the intensive use of antimicrobial agents worldwide for prophylactic and therapeutic purposes

has been associated with the increase of bacterial resistance in the exposed microbial environment. Currently, multiple antibiotic resistances have been reported in a wide range of human pathogenic bacteria and also in fish pathogens. Reservoirs of antibiotic resistance can interact between different ecological systems and potential transfer of resistant bacteria or resistant genes from animals to human may occur through the food chain (Sarter *et al*., 2007). It has been reported that luminous strains of *Vibrio* isolated from shrimp larvae are resistant to antibiotics such as erythromycin, kanamycin, penicillin and streptomycin (Baticados *et al*., 1990). The indiscriminate use of oxytetracycline and chloramphenicol has led to an increase in the incidence of bacterial resistance in shrimp farms and hatcheries. According to Immanuel *et al*. (2004), Philippines have isolated *V. harveyi* strains from diseased shrimp that are resistant to most of the chemotherapeutic agents used in aquaculture systems.

It might be clear from the above that global efforts are needed to promote more judicious use of antibiotics in aquaculture and that new strategies to control pathogenic bacteria are needed to make the industry more sustainable. The limited number of treatment options for aquatic species in certain countries makes widespread resistance to even one antibiotic class a concern (Uhland and Higgins, 2006). Therefore, new strategies to control infections are urgently needed.

In general, a good therapeutic antimicrobial agent should have wide spectrum of activity, must not trigger any adverse reactions or be resistant to their therapeutic effects. A number of natural products, specifically some herbal plant, could possess some of these ideal characteristics. The search for antimicrobial agents has continued to be concentrated on lower plants, fungi and bacteria. Less research has focused on higher plants although identified plant compounds such as berberine, emetine, quinine and sanguinarine still find specialized uses.

Neem or the Margosa tree (*Azadirachta indica*) is abundantly prevalent in the tropical countries of the world and is well known for its insecticidal and various types of biomedical properties. Almost every part of neem tree has been known to possess a wide range of pharmacological properties (Farah *et al*., 2006). The medicinal and insecticidal properties of different parts of neem tree have been well documented. Neem leaves are traditionally being used as curative against certain fungal and bacterial diseases (Parida *et al*., 2002). The present study investigates the inhibitory activity of the extracts of *Azadirachta indica* leaves against two most common shrimp pathogens *Vibrio parahaemolyticus* and *Vibrio alginolyticus* under *in vitro* conditions.

MATERIALS AND METHODS

Extract preparation: Leaves of neem were collected from trees growing in the Universiti Putra Malaysia, Malaysia, campus. The collected neem leaves were thoroughly washed with water to remove dirt. Twenty grams of neem leaves was then shade dried and ground well by using mixer grinder. The powder was then put into a filter bag and distilled water was added. Aqueous extract was collected by squeezing the bag manually. Aqueous extract was prepared in 10, 25, 50, 100 and 200 mg mL^{-1} concentration. Another portion of the fresh neem leaves was blended to get the juice of the leaves. The juice was mixed with distilled water to make into different concentration: 10, 25, 50 and 75%. Hundred percent concentration was also prepared without mixing with distilled water.

Bacteria: *Vibrio parahaemolyticus* and *Vibrio alginolyticus* were isolated from cultured shrimp and maintained at the Aquatic Animal Health Unit, Universiti Putra Malaysia and used in this experiment. All cultures subsequently grown from stored stocks were streaked to obtain single colony prior to use. The bacteria were cultured on tryptic soy agar (TSA; Merck, Germany) plates and incubated at 37°C.

Agar plate preparation for sensitivity test: Thirty-eight grams of Mueller-Hinton agar powder (Merck, Germany) was suspended in 1 L of distilled water together with 30 g of sodium chloride (3% NaCl) and autoclaved at 121°C for 15 min. The agar was allowed to cool to 62°C in a water bath before pouring into petri dish (Ruangpan and Tendencia, 2004).

Agar plate for minimum bactericidal concentration (MBC): Forty grams of TSA powder was suspended in 1 L of distilled water together with 30 g of sodium chloride (3% NaCl) and autoclaved at 121°C for 15 min. The agar was allowed to cool to 62°C in a water bath before pouring into petri dish (Ruangpan and Tendencia, 2004).

Broth preparation for minimum inhibitory concentration (MIC): Fifteen grams of Tryptic Soy Broth (TSB; Merck, Germany) was suspended in 500 mL of distilled water together with 15 g of NaCl and was autoclaved at 121°C for 15 min. The broth was allowed to cool to 62°C in a water bath before pouring into bijou bottles (Ruangpan and Tendencia, 2004).

Disc diffusion method: Disc diffusion test was done according to the method of Austin *et al.* (1995). A single colony from each of the bacteria species was selected and suspended in sterile saline until turbidity comparable or adjusted to 0.5 McFarland standard was obtained. A sterilized cotton swab was dipped into the suspension. The inoculum was then swabbed over the entire agar surface. Five pieces of 6 mm disc previously impregnated with different concentrations of herbal extracts were placed onto the agar surface with sterile forceps. The plates were then incubated at 37°C for 24 h. The antimicrobial activity of the test materials was observed through zone of inhibition by measuring the diameter in millimeters (mm) inclusive of the disc. Commercial antibiotic disc of sulfamethoxazole-trimethoprim (10 μg), erythromycin (15 μg), doxycycline (30 μg), ampicillin (10 μg), tetracycline (30 μg) and oxolinic acid (2 μg) was placed on agar plate for comparison of the zone of inhibition.

Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values: MIC of neem juice was assessed using the broth microdilution method. An inoculum of the bacteria was prepared and suspension was adjusted with a turbidity equivalent to 0.5 McFarland standards. Dilutions of neem juice by two-fold dilution were prepared using sterile TSB so as to get different range of concentrations. One milliliter of cultured suspension was added into each tube. Control tubes contained no neem juice. After 24 h of incubation at 37°C the test tubes were examined for possible growth and MIC was determined as the lowest concentration that ended with no growth. Tubes without bacterial growth in the MIC test were streaked onto TSA plates to achieve MBC against tested bacteria. Bacterial growth was observed after incubation. The minimum concentration of neem juice that prevents bacterial growth is reported as the MBC value (Ruangpan and Tendencia, 2004).

RESULTS

Antimicrobial sensitivity test: Aqueous extract of neem leaves did not produce any zone of inhibition on the culture of *V. parahaemolyticus* and *V. alginolyticus*. Mean diameter of inhibition zone produced by neem juice of different concentration tested against *V. parahaemolyticus* and

Table 1: Antibacterial activity of *Azadirachta indica* leaves juice on *Vibrio parahaemolyticus* and *Vibrio alginolyticus* at different concentrations

	Zone of inhibition (mm)				
	Concentration of neem juice (%)				
Bacteria	10	25	50	75	100
Vibrio parahaemolyticus	10.8±1.10	12.8±1.10	13.6±0.89	16.4±0.89	19.6±0.89
Vibrio alginolyticus	8.0±0.00	8.8±1.10	10.4±0.75	12.8±1.10	14.8±1.10

Values are Mean±SE, n = 3

Table 2: Antibacterial activity of commercial antibiotic disc on *Vibrio parahaemolyticus* and *Vibrio alginolyticus*

	Zone of inhibition (mm)					
	Commercial antibiotic disc					
Bacteria	DO-30	SXT-25	E-15	AMP-10	TE-30	OA-2
Vibrio parahaemolyticus	25.3±2.3	25.3±0.9	21.3±0.9	34.0±2.0	19.3±0.9	14.7±0.9
Vibrio alginolyticus	21.3±0.9	20.0±2.0	20.7±0.98	26.7±0.9	19.3±0.9	14.0±2.0

Values are Mean±SE, n = 3, DO-30: Doxycycline, SXT-25: Sulfamethoxazole-trimethoprim, E-15: Erythromycin, AMP-10: Ampicillin, TE-30: Tetracycline, OA-2: Oxoliuic acid

V. alginolyticus are shown in Table 1. Hundred percent neem juice produced 19.6 mm inhibition zone on *V. parahaemolyticus* and 14.8 mm on *V. alginolyticus*. Compared with their sensitivity towards commercial antibiotic disc, the inhibition zone produced was only larger than the zone produced by oxolinic acid (2 μg).

Both bacteria tested were sensitive to doxycycline, sulfamethoxazole-trimethoprim, ampicillin and tetracycline and showed an intermediate susceptibility towards erythromycin and oxolinic acid (Table 2) (based on the NCCLS publication M31-A2-Performance Standards for Antimicrobial Disc and Dilution Susceptibility Test for Bacteria Isolated from Animals).

Minimal inhibitory concentration (MIC): The MIC for the neem juice against *V. parahaemolyticus* was 3.13% and against *V. alginolyticus* was 6.25%.

Minimal bactericidal concentration (MBC): The MBC for the neem juice against *V. parahaemolyticus* and *V. alginolyticus* were 12.50 and 25.00%, respectively.

DISCUSSION

According to Immanuel *et al.* (2004), treating microbial infections in fish and shrimp involves dissolving higher quantities of broad spectrum of chemotherapeutic agents in the culture medium. A disadvantage of this method is the requirement of large amount of expensive drugs which are used and discharged in the environment that poses risk to the animals and human health. An alternative is resorting to herbal compounds having antimicrobial characteristics instead of synthetic antibiotic drugs.

In the present investigation, two different preparations of neem leaves (aqueous extract and leaf juice) were used to determine their antimicrobial activity against *V. parahaemolyticus* and

V. alginolyticus that were isolated from cultured shrimp. This experiment revealed that aqueous extract that is prepared from powder of neem leaves did not show any antibacterial activity. This may be due to deactivating or denaturing of the antimicrobial properties in the neem leaves during the process of drying (Parida *et al*., 2002).

Zone of inhibition produced by neem juice show a linear relationship with the concentration of the juice. Higher concentration produced bigger zone of inhibition. One hundred percent concentration of neem juice produced the largest inhibition zone and the diameter of the zone decreased as the neem juice becomes diluted. This is sufficient to prove that fresh neem juice contains compound with antibacterial activities. By comparing the size of inhibition zone, *V. parahaemolyticus* showed a higher sensitivity to 100% neem juice.

Compared to the inhibitory zone produced by commercial antibiotics, the inhibitory zone of neem juice was smaller. This may be because the active compound in neem juice is not high or concentrated enough to produce the same antibacterial effect as compared to commercial antibiotics where their bacteriostatic and bactericidal effect has been determined by a lot of research. *Vibrio parahaemolyticus* and *V. alginolyticus* were moderately sensitive to neem juice at 100% concentration based on inhibition zone.

Although the result showed lower activities of this neem juice relative to previous findings (Nkuo-Akenji *et al*., 2001) which reported higher potency of this plant against bacteria, they however corroborate their reports. This variation may be due to the dose of extract in different studies, the age of the plant and the part of the plant used. The masking effect by other compounds in the extract may also account for the low activity demonstrated in this study. However, the weak activity demonstrated by these extracts *in vitro* to the bacteria does not necessarily imply that they would demonstrate weak activities *in vivo*. Oliver-Bever (1986) and Garcia *et al*. (2003) had demonstrated immuno-modulation of chemical compounds from medicinal plants, many of which had been proven to be inactive or weakly active *in vitro* against pathogens. Also, as with some drugs, some of these plant maybe more potent *in vivo* due to metabolic transformation of their components into highly active intermediates (Ngemenya *et al*., 2006).

According to Yamamoto (2003), an antimicrobial agent can be considered as bactericidal agent when the MBC value is no more than four times of the MIC value. Based on the MBC values, only 12.5% is needed to inhibit growth of *V. parahaemolyticus* and 25% to inhibit *V. alginolyticus*. Both of these values are less than four times of the MIC value for *V. parahaemolyticus* and *V. alginolyticus*. Based on this, 100% neem juice can be considered as a bactericidal agent.

CONCLUSION

Crude preparation of neem juice is an antibacterial agent and is useful for inhibition of vibrios in shrimp. It is possible that neem may take a role as an adjuvant to the use of antibiotics or as a replacement of current antibiotics. The present study showed the effectiveness of neem juice in inhibiting bacteria. It is recommended that identification of herbs and their medicinal value need further exploration. Further study also needs to be conducted to determine the active compound (s) that poses antimicrobial activities and their amount. Evaluation of neem extract against other important aquatic pathogens also needs to be conducted.

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