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Effect of neem extract against the bacteria isolated from marine fish

N.B. Dhayanithi*, T.T. Ajith Kumar and K. Kathiresan

Centre of Advanced Study in Marine Biology, Annamalai University, Parangipettai - 608 502, India

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Abstract: *Marine ornamental fishes are exceedingly valuable due to their high demand in domestic and international markets. There is a growing global interest to rear the fishes in captivity. But problem due to bacteria and fungi are the major hitch in captive condition. Since, the use of antibiotics is banned, an attempt was made to ascertain in vitro assay of the neem leaves extract against the bacterial pathogens isolated from infected fishes. Bacterial strains isolated from infected regions of the clown fishes Amphiprion sebae and A. ocellaris were identified as Aeromonas hydrophila, Enterobacter sp., E. coli, Pseudomonas aeruginosa, Proteus sp., Streptococcus sp., Vibrio cholerae, V. alginolyticus, V. parahaemolyticus and Yersinia enterocolitica. Ethanol and methanol extracts were highly inhibitory to the bacterial isolates when compared to other solvents. Ethanol extracts exhibited low minimum inhibitory concentration (75 - 250 µg ml^{-1}) as compared to other extracts. The present finding revealed that the neem leaf extract significantly reduces the bacterial pathogens and their infection in marine ornamental fishes.*

Key words: *Bacteria, Crude extract, Marine ornamental fishes, Minimum inhibitory concentration, Zone of inhibition*
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Introduction

The marine ornamental fishes have tremendous economic potential with a trade of US $ 43.8 million in 2006 (Choudhury *et al*., 2005). Intensive management of the marine ornamental fishes and their captive breeding could not make much headway till date in India. Bacterial diseases are responsible for heavy mortality in fishes. The problems in the culture systems are usually tackled by preventing disease outbreaks or by treating the disease with antibiotics or chemicals (Choudhury *et al*., 2005).

Antibiotics used in medicines have been tried experimentally to treat bacterial infections of fish. Problems including solubility, palatability, toxicity, cost, delivery and governmental restrictions have limited the available antibiotics to a select few, especially in ornamental fish culture (Smith *et al*., 1994). Increasing failures in antibiotic resistance exhibited by microbial pathogens has led to screening of several medicinal plants for their potential antimicrobial activity (Ritchkro *et al.,* 1996; Colombo *et al.,* 1996; Martins *et al*., 2001; Scazzocchio *et al.,* 2001). Neem (*Azadirachta indica*) of family Meliaceae is evergreen tree of potential medicinal value found in most tropical countries (Wafaa *et al*., 2007). The medicinal and industrial uses of neem tree have been reviewed (Schmutter, 1995). The antibacterial activity of neem has been known from ancient times (Chaurasia and Jain, 1978; Chawla *et al*., 1994). Neem has been considered to have various activities such as astringent, antiseptic, insecticidal and antiulcer in human (Chattopadhyay, 1999). Other than this, the leaf extract of neem showed superior antiviral and antihyperglycemic activity (Parida *et al*., 2002). However, application of neem extract in controlling bacterial pathogens of marine ornamental fish is yet to be studied. Therefore *In vitro* assay of the neem leaf extract was studied against bacterial pathogens isolated from infected marine ornamental fish.

* **Corresponding author:** dhaya_micro@yahoo.co.in

Materials and Methods

Fresh leaves of *Azadirachta indica* Juss (Meliaceae) were collected from coastal region of Parangipettai, Cuddalore District, Tamil Nadu. After shade drying, the leaves were powdered by using mechanical grinder (Premanathan *et al*., 2000). Five grams of the dry powder were extracted in 50 ml of ethanol, methanol, chloroform and acetone, using a rotary shaker for 30 min at 100 rpm and the extract was separated by centrifugation at 10,000 rpm for 30 min. After centrifugation, the solvent portion was collected and evaporated.

Bacteria were isolated from moribund stages of *Amphiprion sebae* and A. *ocellaris*. Infected parts of the fishes were serially diluted and plated on Zobell marine agar and nutrient agar with 50% seawater, incubated at 37°C for 24 hr. After incubation colonies were pure cultured and subcultured on Zobell marine agar slants. The morphological and biochemical tests were performed according to Bergey's manual of systematic bacteriology (Elizabeth, 2005).

Antibacterial activity of neem extracts was assessed by using disc diffusion method. About 500 µg of crude extract was impregnated on 5 mm sterile Whatman No.1 filter paper disc placed on Muller Hinton agar plates swabbed with 18 hr bacterial culture, incubated at 37°C for 24 hr and zone of inhibition was measured. Oxytetracycline and unloaded disc were used as a positive control and negative control respectively (Lenette *et al*., 1985).

Minimum inhibitory concentration (MIC) of ethanol, methanol, chloroform and acetone extracts of neem was determined by broth dilution assay method (NCCLS, 1993). Stock concentration of the

plant extract was prepared by using DMSO and methanol in the ratio of 1:1 which in turn was diluted with equal volume of phosphate buffer saline (pH 7.0). Muller Hinton agar was prepared, sterilized and kept ready in molten condition. Twenty ml of the molten Muller Hinton agar was mixed with known concentrations of plant extracts swirled and poured on to the plate. After solidification, the test bacteria were inoculated and incubated at 37°C for 24 hr. MIC was recorded based on the growth of the bacteria incubated at a range of concentrations 25, 50, 75, 100, 125, 150, 175, 200, 250, 300, 400 and 500 µg ml^{-1}. The statistical method used was one way ANOVA by SPSS software package version 13.

Results and Discussion

The present study isolated ten predominant bacterial strains from infected regions of *Amphiprion sebae* and *A. ocellaris* through plating methods and they were identified as *Aeromonas hydrophila, Enterobacter* sp., *E. coli, Pseudomonas aeruginosa, Proteus* sp., *Streptococcus* sp., *Vibrio cholerae, V. alginolyticus, V. parahaemolyticus and Yersinia enterocolitica* (Table 1). Seul ki park *et al*. (2009) pointed out *Pseudomonas aeruginosa* is a common environmental bacterium and this is an opportunistic pathogen under certain conditions. Yanong and Floyd (1995) reported that *Streptococcus* sp. was isolated from five families of fish groups from Florida tropical fish farms. However all the bacteria are present in the culturing system in low count and cause infection under favourable environmental conditions and it may be tedious to maintain marine ornamental fishes in captivity. Therapy may be useful tool to maintain their survival during these circumstances. Antibiotic treatment of bacterial diseases in fish culture has been applied for many years. The occurrence of antibiotic resistant bacteria associated with fish diseases is a worldwide problem in aquaculture, which has received considerable attention in the last years and this issue continues to increase due to the absence of a more effective and safer use of antibiotics (Smith *et al*., 1994). Resistance to antibiotics and chemical treatments subject to increasing restriction because of their potential harmful impact on the environment and eradication of an infected fish group because by the time disease is diagnosed, most of the fish are infected. Neem leaves extracted in different organic solvents were tested for antibacterial activity. Ethanol and methanol extracts were highly inhibitory to bacterial isolates when compared to other solvents (Fig. 1). Gram positive bacteria *Streptococcus* sp. was highly susceptible to the extracts and *Aeromonas hydrophila* was resistant when compared with other test bacteria. *Enterobacter* sp. and *E. coli* showed 15 and 14 mm zone of inhibition for ethanol and methanol extracts respectively. Wafaa *et al*. (2007) confirm that native extracts of neem leaves with concentration of 20 µg $disc^{-1}$ are inhibitory to *Staphylococcus aureus, E. coli, Candida albicans, Aspergillus niger* and *Penicilium citrinum. Azadirachta indica* is well known in India and its neighbouring countries for more than 2000 years as one of the most versatile medicinal plants having a wide spectrum of biological activity (Chopra *et al*., 1956). Aqueous extract of the neem is reportedly possessing anti-inflammatory, antimicrobial and immunomodulatory activities (Van Der Nat *et al*., 1987). Dialysis of acidic, aqueous and alkaline plant extracts allows removing the low molecular weight molecules and the separation of macromolecules such as proteins and polysaccharides. Wafaa *et al*. (2007) reported the antibacterial and antifungal activities of the native and chemically modified extracts from neem seeds, seed-hulls and leaves.

Table - 1: Morphological and biochemical characteristics of bacterial strains isolated from infected marine fish

Characteristics	Bacterial strains									
	I	II	III	IV	V	VI	VII	VIII	IX	X
Gram staining	-	-	-	-	-	+	-	-	-	-
Shape	Rod	Rod	Rod	Rod	Rod	Cocci	Rod	Rod	Rod	Rod
Motility	M	M	M	M	M	NP	M	M	M	M
Indole test	+	-	+	-	+	NP	+	+	+	+
Methyl red test	+	-	+	-	+	NP	+	+	+	+
Voges proskeur test	-	+	-	-	-	NP	+	+	+	-
Citrate utilization test	-	+	-	+	-	NP	+	-	-	-
Urease test	-	+	-	+	+	NP	-	-	-	-
TSI test	K/A	A/A	A/A	K/K	A/A	NP	K/A	K/A	K/A	K/A
H_2S	-	-	-	-	+	NP	-	+	-	-
Gas	-	+	+	-	+	NP	-	+	-	-
Nitrate reduction test	+	+	+	+	+	NP	+	+	+	+
Catalase tests	+	+	+	-	+	NP	+	+	+	+
Oxidase test	+	-	-	+	+	NP	+	+	+	-
Carbohydrate test										
Glucose	+	+	+	+	+	NP	+	+	+	+
Maltose	+	+	+	+	+	NP	+	+	+	+
Sucrose	-	+	+	-	-	NP	+	+	+	+

- = Negative, + = Positive, M = motile, NP = not performed, A/A = Acid slant acid butt, K/A = Alkaline slant acid butt, I = *Aeromonas hydrophila,* II = *Enterobacter* sp., III = *E. coli*, IV = *Pseudomonas aeruginosa,* V = *Proteus* sp., VI = *Streptococcus* sp., VII = *Vibrio cholerae*, VIII = *V. alginolyticus,* IX = *V. parahaemolyticus,* X = *Yersinia enterocolitica*

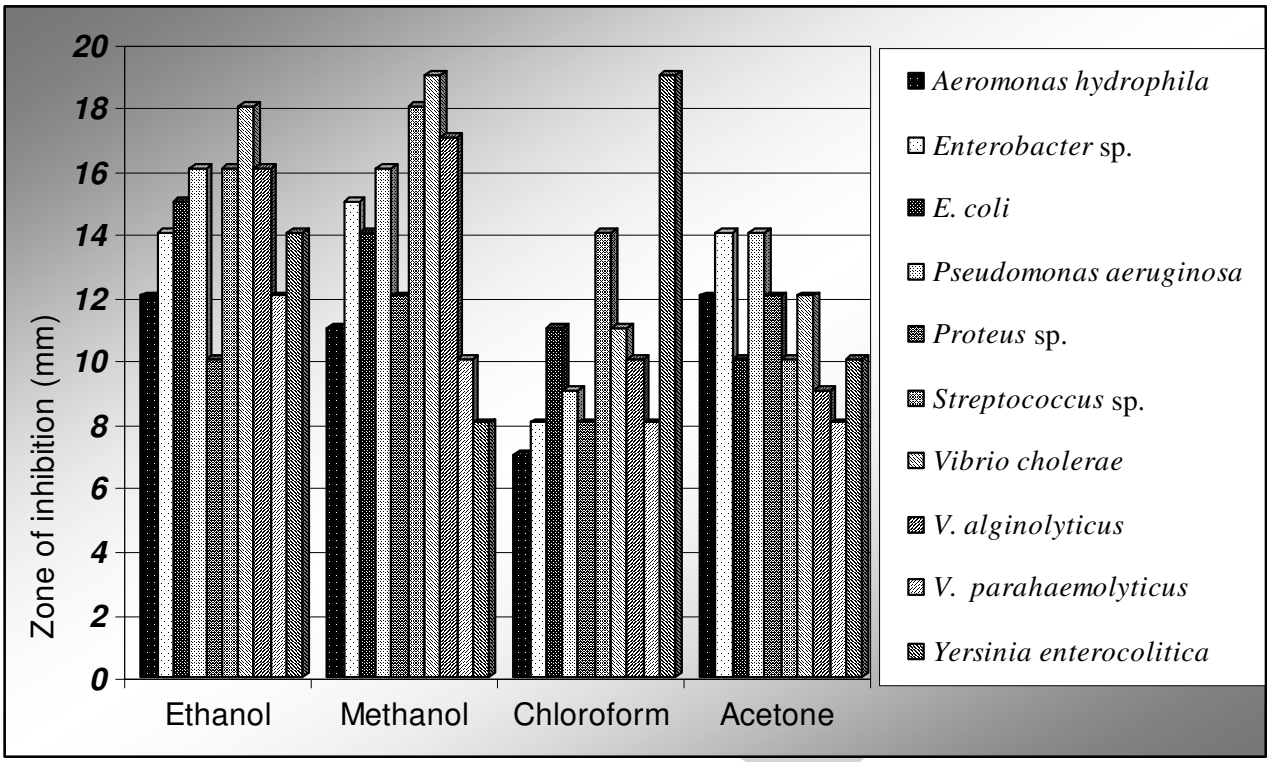

Fig. 1: Antibacterial activity of leaf extract of neem against bacterial strains isolated from infected marine fish (Values between extracts significant $p<0.05$, but not significant between test bacteria)

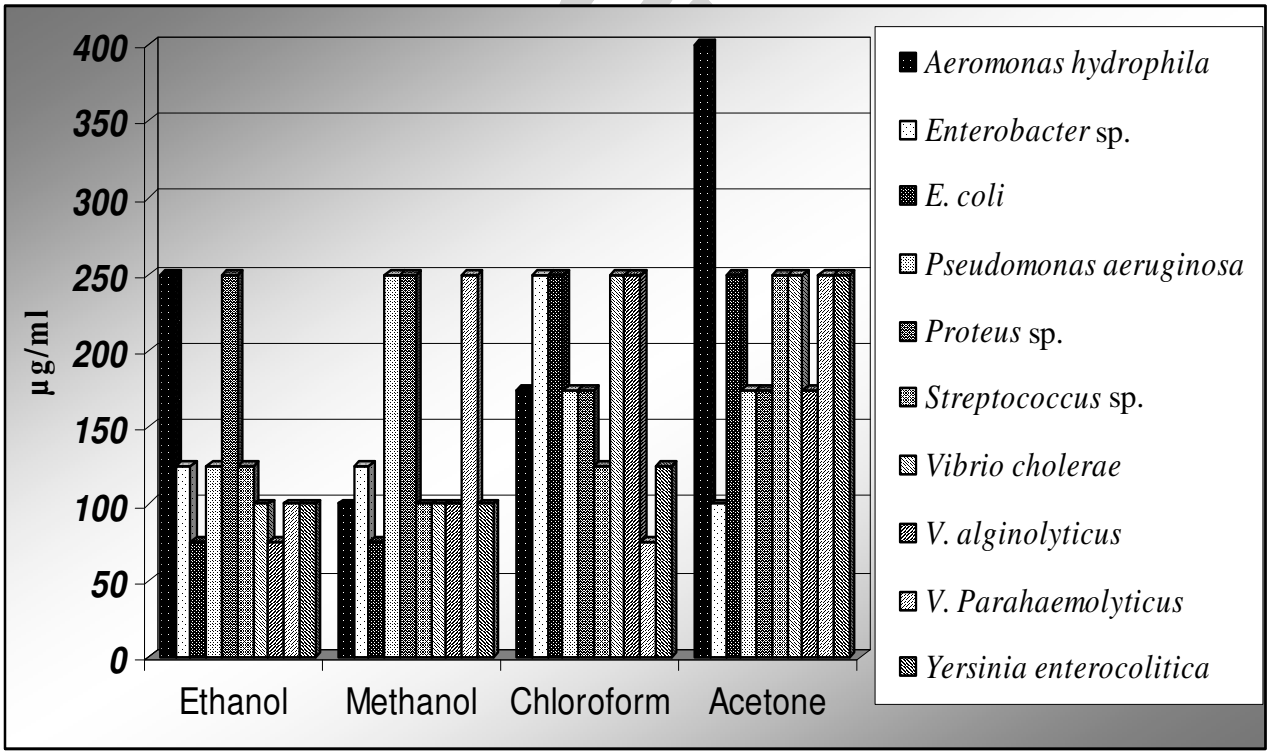

Fig. 2: Minimal inhibitory concentration of leaf extracts of neem against bacterial strains isolated from infected marine fish (Values between extracts significant $p<0.05$, but not between test bacteria)

Ethanol extracts showed good inhibitory activity with low MIC concentration (75-250 µg ml^{-1}) as compared to other extracts (Fig. 2). MIC results can indirectly predict the concentration of extract which should be used as minimum dosage. Ethanol has higher polarity when compared with other solvents and hence the antibacterial compounds are polar compounds which are under purification process. Neem is a one of the easily available tree by applying this concentration of neem extract will significantly reduce the bacterial population in fishes and reduce infection of fishes.

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