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Antibacterial property of neem nanoemulsion against *Vibrio anguillarium* infection in Asian sea bass (*Lates calcarifer*)

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Fish vibriosis is among the most common diseases that is caused by a bacteria belonging to the genus Vibrio (Vibrio anguillarium). It causes considerable economic loss in the commercial cultivation of Asian sea bass (Lates calcarifer). The resistance of microorganisms to antibiotics has resulted in a growing need for developing a new antibacterial therapy that is effective in aquaculture. The aim of this study is to develop neem nanoemulsion with antibacterial activity against V. anguillarium to identify a possible alternative to the commonly used antibiotics in aquaculture. Neem nanoemulsion was prepared and the effectiveness was studied both in vitro and in vivo (agar well diffusion assay and artificial infection). Injection and immersion challenge of neem nanoemulsion formulated the fish less susceptible to V. anguillarium infection. The results confirmed the potential use of neem nanoemulsion as a source of antibacterial compounds or as a health-promoting medicine for fish culture.

[**Keywords**: Vibrio anguillarium; Lates calcarifer; Antibacterial therapy; Neem nanoemulsion; Asian sea bass]

Introduction

Asian sea bass (Lates calcarifer) is an economically important marine fish, but is susceptible to disease during larval and nursery rearing stages in aquaculture farms. Susceptibility of the fish to various diseases during these stages has been well documented, mainly for bacterial diseases caused by Vibrio alginolyticus, V. parahaemolyticus, V. cholera, V. vulnificus, V. anguillarum, V. ordalii, V. damsela, V. carchariae, and V. salmonicida[1,3]. Lymphocystis disease has also been found in the Asian sea bass. The first report of nodavirus infection in sea bass was made by Glazebrook[4], who described a picorna-like virus associated with mortalities in larval stage. This disease was also investigated by Munday[5] in Asian sea bass. *Vibrio anguillarum* is a Gram-negative, facultative anaerobic, and rod-shaped bacteria, which is the main causative agent of vibriosis in fish. Previously, the treatment of diseases was focused on chemicals, antibiotics, and vaccines. The treatment of affected fish with antibiotics is effective, but gives rise to problems such as accumulated resistance in the bacteria, which renders the antibiotic useless. Current vaccination research is concerned with alternate conventional vaccines with more effective and safer approaches, such as DNA vaccines. Immunization with antigen-encoding plasmid DNA can cause very strong and long-lasting humoral and cellular immune responses.

The vaccination approach also suggests economic, environmental, and safety advantages, which are particularly attractive for the aquaculture industry[5,6]. Recently, nanoemulsions have been identified as promising delivery systems. Neem (*Azadirachta indica*) is a traditional plant in India which has excellent medicinal and insecticidal properties due the presence of Azadirachtin[7,9]. However, all these strategies are not efficient to control the spread of this very serious disease. In the present study, we describe the formulation of nanoemulsion using neem oil and its potential therapeutic usage in Asian seabass (L. calcarifer), against *V. anguillarium* by experimental infection.

Materials and Methods

Fish

Juveniles of Asian seabass (L. calcarifer) (10 g in weight) were collected from grow-out ponds of Rajiv

Gandhi Centre for Aquaculture, Sirkali, Tamil Nadu, India and transported to the laboratory. They were maintained under laboratory condition in 1000 l fiberglass tanks with continuous aeration in UV-treated seawater at 24 °C (salinity 33 ppm). The collected fish were confirmed that they were free from *V. anguillarum* infection. The fish were fed twice a day with boiled fish meat during acclimatization and experimental periods.

Bacterial strains

V. anguillarum, *B. cereus*, and *M. luteus* were purchased from MTCC (Microbial Type Culture Collection) and confirmed according to the biochemical reactions scheme provided by Bergey's Manual of Systemic bacteriology.

Preparation of neem nanoemulsion

Neem (*Azadirachta indica*) nanoemulsion was prepared following the method described by Jerobin (2015)[10]. Fresh neem oil, Tween 20 and Milli-Q water were used in the preparation of nanoemulsion. The emulsions were formulated by sonication for 1–3 h using high-energy sonicator (Rivotex Ultrasonicatore, 230 W, and 50 kHz) in different ratios of 1:1, 1:2, and 1:3 (v/v) and used for the treatment study. These ratios were confirmed by advance stereo microscope and transmission electron microscopy (TEM).

Transmission electron microscopy (TEM)

The morphological observation of the nanoemulsion was determined by TEM. For TEM, a drop of nanoemulsion was prepared on a copper grid and allowed to dry in vacuum and examined in Tecnai G-10 instrument as described in the method by Anjali (2012a)[11]. The nanoemulsion was analysed by ultra high-resolution pole piece with a point-to-point resolution of 1.9 Å

Physical characterization of nanoemulsions

The pH, temperature stability, and centrifuge of nanoemulsion were determined following the method described by Anjali (2012b)[12].

Agar well diffusion method

The agar well diffusion method[13] was determined by antibacterial effects in nanoemulsion. Two wells of 8 mm diameter were punched into the agar on each plate using a sterile well cutter. About 40 µl of neem nanoemulsion was added into each well. Saline was added into negative control well. The solutions were allowed to diffuse for 2 h and the plates were incubated at 30 °C for 24–48 h. The antibacterial activity was evaluated by measuring the zone of inhibition around the wells. About 40 µl of the prepared emulsion were added to the sterile disc (Hi media) and dried under sterile condition at room temperature. The prepared discs were used in the study.

Kinetics of killing

Neem oil nanoemulsion formulation with oil-to-surfactant (v/v) ratio of 1:3 was preferred for analyzing the antibacterial efficacy owing to its lowest droplet diameter. The kinetics of killing experiment was performed by the method described by Al-Adham (2000)[14]. The overnight culture of *Vibrio anguillarum* was centrifuged at 4000 rpm for 15 min. It was then washed twice in phosphate buffered saline (PBS, pH 7.4). The washed culture with known inoculum size (6×10^5 CFU/ml) was prepared. About 1% v/v of this culture was then challenged with undiluted nanoemulsion formulations. For viable counts, about 0.1 ml of the sample was taken from each tube and spread onto TCBS (Thiosulfate Citrate Bile Salts Sucrose) agar plates. The colony count was done after incubation at 37 °C for 24 h.

Experimental pathogenicity

Healthy juveniles of Asian sea bass (10 fish per tank) were reared in glass tanks (100 l) containing UV-treated seawater with continuous aeration. The air stones and air tubes were sterilized by immersing them in 2.6% sodium hypochlorite. Aseptic techniques were used throughout the experiment. The Asian sea bass were fed with the commercial fish feed. The pathogenicity of *V. anguillarum* was studied in healthy seabass. For the experimentally induced infection, the fish were exposed to different concentrations of bacterial cells (10^3,10^4, 10^5, 10^6, and 10^7 CFU ml^{-1}). The control consisted of fish that were exposed only to seawater. This experiment is to determine the killing dosage of *V. anguillarumin* fish. Selected dosage was used in bioassay study.

Bioassay

The Asian seabass (10 per tank) were maintained in fiberglass tanks (100 l) containing seawater at 28 °C. *V. anguillarum* was inoculated into healthy fish near the dorsal fin by intramuscular injection at doses of 10^5 CFU/ml/animal. The antibacterial activity of neem nanoemulsion was tested by immersion challenge in fish for 10 days. The animals were fed twice a day

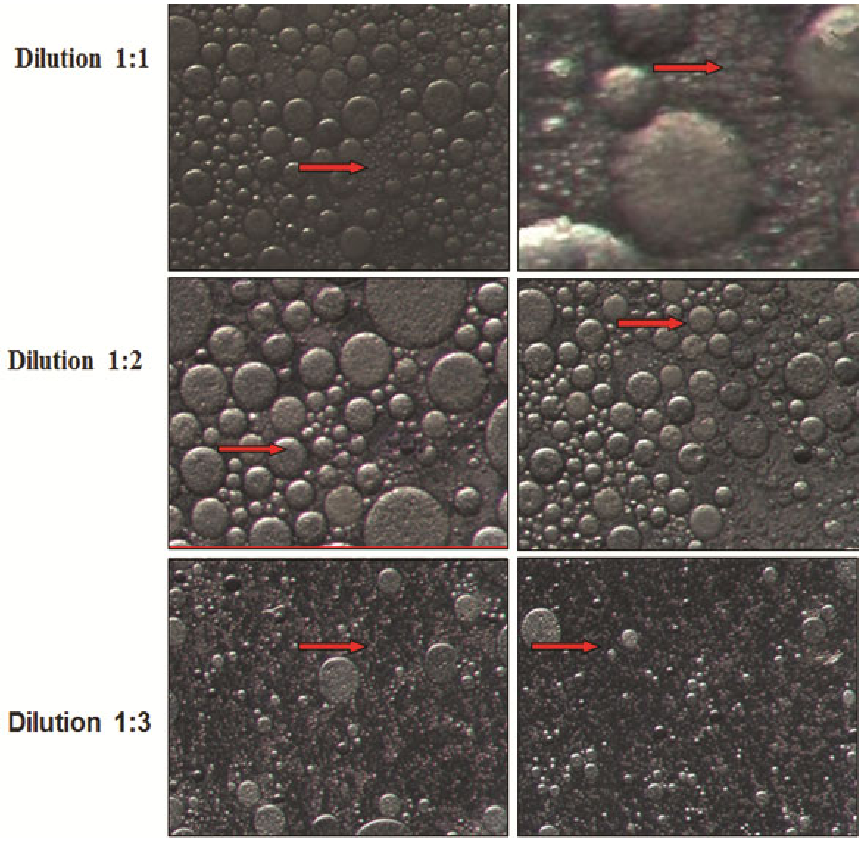

Fig. 1 — Droplet size of neem oil nanoemulsion by 1:1, 1:2 and 1:3 formulation of neem oil and non- ionic surfactant

with commercial fish feed. After 1 h of acclimatization, the emulsion was dissolved in water. The concentrations of emulsion used were 50, 100, 150, and 200 mg/l, and control, without emulsion. The control fish were injected only with sterile phosphate buffer saline (PBS). Fish mortality was observed and recorded daily for 10 days. The experiment was performed in triplicate.

Results

Droplet size and size distribution

Nanoemulsion was formulated using neem oil, tween 20, and water. The neem oil-based nanoemulsion formulations 1:1, 1:2, and 1:3 showed reduced droplet size. The droplet size is not clear under the advance stereo microscope (Fig. 1). With additional increase in oil-to-surfactant ratio, no significant decrease in droplet size was obtained. Hence, the 1:3 formulation was selected for further characterization and application studies. The TEM image illustrates the presence of well-distributed nanoemulsion (1:3) without any accumulation of air, indicating that the nanoemulsion was spherical in shape and was in the range of 30–50 nm (Fig. 2).

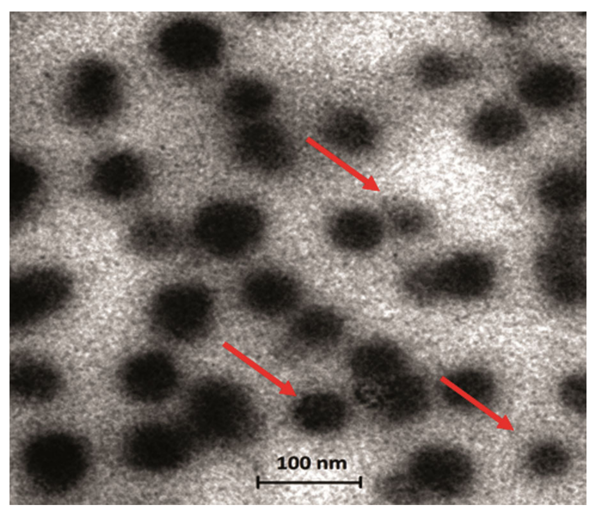

Fig. 2 — Transmission electron microscopic image of neem nanoemulsion

Physical characterization

By increasing the pH in the surfactant concentration, the pH of neem nanoemulsion surfactant formulation increased. The nanoemulsion formulation was found to be thermostable at room temperature. There was no noticeable phase formation in this experiment (Table 1).

Table 1 — Droplet pH and stability of nanoemulsion formulations

Ratio (Solution A: Solution B)	pH	Stability		
		30°C	20°C	4°C
1:1	4.47	Yes	No	No
1:2	4.52	Yes	No	No
1:3	4.61	Yes	No	No

Table 2 — Antibacterial screening of neem nanoemulsion against fish pathogens

Test organism	Zone of inhibition (mm)	
	Neem nanoemulsion (mm)	Saline
V. anguillarum	20	0
Bacillus cereus	18	0
Micrococcus luteus	18	0

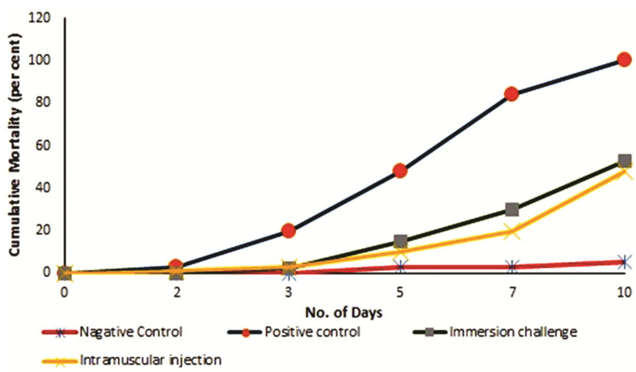

Fig. 3 — Development of cumulated mortality in neem nanoemulsion treated and control *Lates calcarifer* after challenge with *Vibrio anguillarum*

Antibacterial activity

The neem nanoemulsion showed significant antimicrobial activity against all the pathogenic bacteria. The nanoemulsion was found to be effective against pathogenic bacteria and had more than 70% inhibitory activity against *V. anguillarum* (20 mm), *Bacillus cereus* (18 mm), and *Micrococcus luteus* (18 mm) at the formulation of 1:3 (Table 2). The antibacterial activity was attributed to the presence of bioactive compounds.

Cumulative mortality rate

Clinical signs of lesions were observed in the healthy fishes after injection of V. *anguillaruminoculum*. The bacterial isolate from *V. anguillarum* caused mortality and thus produced vibriosis in the healthy fishes, whereas the clinical symptoms of *V. anguillarum* in tail and fin rot and dropsy. The mortality rate was calculated to be lower in fish treated with nanoemulsion when compared to control groups (Fig. 3). The neem nanoemulsions protected the fishes from *V. anguillarum* with 58% (intramuscular) and 56% (immersion). The positive groups showed 100% mortality within 10 days. There was no mortality reported in negative control groups.

Discussion

Neem, a very important tropical plant, is being used for the development of different medicinal products[14]. Nanoemulsions are thermodynamically stable liquid dispersions of oil, water, and surfactant with a droplet size ranging between 20 and 200 nm[15]. The formulation of nanodroplets in neem oil emulsion with a ratio of 1:3 of oil and surfactant occurs due to the presence of the surfactant that tends to help in the reduction of oil and water interface[16]. The bioefficacy of water and oil nanoemulsion formulated using the non-ionic surfactants having the smallest droplet size was found to be more effective. There was no phase separation observed in the nanoemulsion for a longer duration of time, which resulted in its prolonged stability in the nanoemulsion[11,17]. The nanoemulsion applied on seedlings and seeds also significantly controlled the wilt disease in tomato without showing any sign of phytotoxicity[18]. Nanoemulsions are used in agrochemicals in pesticide drug-delivery formulas[24].

The major conclusion of our experiment is that the nanoemulsions are round in shape with their droplet size ranging between 20 and 50 nm as revealed by TEM. Similar results were also obtained by the experiments conducted by Wen-Chien[20]. TEM studies of celecoxib nanoemulsion exhibited a size range between 19 and 78 nm[19]. Hence, the present results are in agreement with the above findings. This study demonstrates that *V. anguillarum* infection is a serious threat in the sea bass aquaculture and neem nanoemulsion is highly effective in controlling the vibriosis caused by *V. anguillarum*. Further research may focus on identifying the active molecules present in neem that are responsible for the antibacterial activity against *V. anguillarum.* There is also a need to study the antibacterial activity against other aquatic bacterial pathogens infecting the sea bass.

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