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Dendrilla nigra, a marine sponge, as potential source of antibacterial substances for managing shrimp diseases

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Abstract

Secondary metabolites of marine sponge *Dendrilla nigra* were tested for determining the efficacy of controlling shrimp bacterial pathogens. Based on the exploratory experiments, the chosen dose of *D. nigra* (500 mg/kg of shrimp) was used for pilot experiment. The percent relative protection (PRP) of shrimps treated with *Dendrilla* feed and challenged with various concentrations of bacterial pathogen was evaluated. *Dendrilla* feed elicited complete protection (100% survival) against the most common shrimp pathogens such as luminescent *Vibrio harveyi* and *Vibrio alginolyticus*. Results of combined bacterial challenge indicated that *Dendrilla* was a broad spectrum vibriostatic agent. Invariably, the survival of treated shrimp against the bacterial infection was significant at $p<0.01$ level. Based on the present findings, it could be inferred that the secondary metabolites of *D. nigra* form an excellent source for developing potent antibacterial agents to combat bacterial diseases of shrimp and replace the conventional antibiotics.

Keywords: *Penaeus monodon*; Bacterial-diseases; *Vibrio*; Disease management; *Dendrilla nigra*; Marine sponge

1. Introduction

Shrimp farming is a lucrative industry in several countries of Asia particularly Indonesia, Taiwan, China and India. However, diseases are recognized as a major constraint as well as

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a limiting factor for sustainable shrimp farming. It is well known that aquatic organisms come in direct contact with the ambient microbes continuously, which may act as opportunistic pathogens (Raa et al., 1992). Therefore, it is very difficult to prevent diseases caused by opportunistic or secondary pathogens during the entire culture period. Medication of aquatic organisms cannot be restricted to the diseased individuals and as a result, resistant microbial strains may develop, which change the normal microbial composition leading to massive outbreaks of the disease. Initial management of such outbreaks by reducing the stocking density or carrying capacities is considered as uneconomical due to increased production costs. The total sterility of rearing water in the entire culture period by pretreatment is practically impossible (Alabi et al., 1999). The pre-infection therapy such as vaccine and immunostimulants are still under investigation for the field use. Although post-infection therapy using medicated feeds incorporated with antibiotics is possible to a certain extent (de la Pena et al., 1992), the impact of antibiotics in the environment and consumer health is highly risky (Selvin and Lipton, 2003a). Therefore, cost-effective treatment technologies need to be developed for controlling/preventing such outbreaks especially due to opportunistic and secondary pathogenic invaders.

Sponges are filter feeders, which live in areas with strong currents or wave action. Most carnivorous animals avoid sponges because of the splinter-like spicules and toxic chemicals produced/sequestered by the sponge. Sponges were reported as potential source of antibacterial secondary metabolites (Nakamura et al., 1991; Mokashe et al., 1994; Pettit et al., 1996; Shen et al., 1998; Matsunaga et al., 2000, 2001). However, the overexploitation of sponges may pose imminent threat to their natural bed (Thomas, 1998). Therefore, in the present study, due emphasis was given for the ecofriendly utilization of bycatch sponges as source material for the development of novel shrimp therapeutants.

2. Materials and methods

Forty days old farm reared juveniles of 200 black tiger shrimp, *Penaeus monodon* were collected from an extensive farm located in Kanyakumari coast (southeast coast of India). They were conditioned in 1000 l capacity fibre reinforced plastic tanks at optimum hydrological conditions such as salinity (35 ppt), temperature (30±2 °C), pH (7.8) and constant aeration with 50% water exchange daily. They were fed with pellet feed at a rate of 3.2% of total biomass in three equal installments. The feeding rate and schedule were adjusted based on the unutilized feed remains. At the onset of experiments, shrimp density was adjusted to 1 individual per 10 l of water in 100 l glass aquaria. Prior to the challenge experiments, random sampling of shrimp was made for the bacterial isolation to ensure the shrimp were pathogen free.

The sponge, *Dendrilla nigra* collected as bycatch in fishing nets, were used for the isolation of secondary metabolites using methanol–dichloromethane (1:1) (Selvin and Lipton, 2004). In brief, the sponge was cut into small pieces and homogenized in a tissue homogenizer (Omni) using methanol as solvent. They were extracted thrice with methanol–dichloromethane (1:1) and the combined extract was concentrated in a rotary vacuum evaporator (Buchi) at room temperature. Commercial pelleted shrimp grower feed No. 1 (C.P. feeds, Cochin) was used for the preparation of sprayed medicated feed. The

median lethal dose (MLD) of *D. nigra* was determined in separate experimental set-up. Exploratory (dose selecting) experiments were conducted with broad range of the test compounds. Chosen concentrations such as: 100, 1000 and 2000 mg/kg shrimp of *D. nigra* was prepared in normal saline (NS). Ten shrimps (abw=8.5 g) per group (in triplicates) were injected intramuscularly with 0.1 ml of appropriate dose. The immediate reflexes and mortality were observed every hour for the first 6 h and every 24 h for 7 days. Based on the exploratory experiments, narrow ranges were administered to the experimental shrimp to determine the LD_{50}. LD_{50} value of *D. nigra* was determined (420 mg/kg of shrimp) by probit analysis. The dosing regimens were determined on the basis of active ingredients (in terms of minimum inhibitory concentration=9 mg/ml) in *D. nigra* (Selvin and Lipton, 2004). Based on the exploratory experiments, the chosen dose of *D. nigra* (500 mg/kg of shrimp) was incorporated in the feed by spraying appropriate concentration of extract on the surface of the feed at a rate of 3.2% of the shrimp body weight daily (Selvin et al., 2004).

The shrimps (100 numbers) with an average body weight of 8 and 10.5 g range was treated with medicated feed at a rate of 3.2% of the body weight for a period of 15 days. On the 16th day, three groups each of healthy pretreated and control shrimps (10 nos/group) were challenged with MLD and lethal dose of appropriate bacterial pathogens. For the challenge experiment, one type culture *Vibrio fischeri* (ATCC 7744, American Type Culture Collection, Manassas, VA), two shrimp isolates *Vibrio harveyi* (obtained from marine biotechnology laboratory) and *Vibrio alginolyticus* (Selvin and Lipton, 2003) and one fish isolate *Aeromonas* sp. in addition to a potentiated pathogen (*V. harveyi,* 5×10^6+*V. alginolyticus,* 5×10^6=10^7 cfu/shrimp) were used. An 18 h shake culture of appropriate pathogens was centrifuged at $4800\times g$ for 15 min for the preparation of inoculum. Cell pellets were washed twice with NS, resuspended and serially diluted in NS and enumerated using a Petroff–Hausser chamber and by plating on nutrient agar (supplemented with 2% NaCl) plates to obtain counts of colony forming units (cfu). The appropriate MLD and lethal dose of the isolates were injected intramuscularly at the ventral side between the second and third segment in 0.1 ml NS using a 1 ml tuberculin syringe. They were observed for a period of 15 days for mortality and infections. The infected and moribund shrimps were examined for external signs of infection using standard methods (Austin and Austin, 1989). The mortality/infectivity percentages were estimated after Sung et al. (1994).

$$\%\,\text{Mortality} = \left(\frac{\text{No. of dead/infected shrimp} - A}{\text{Total no. of dead} - A}\right) \times 100$$

where, A is the number of dead shrimp in 1 day after infection, which is considered to die due to administration stress. Based on the mortality rate, the percent relative protection (PRP) of challenged shrimps was evaluated.

$$\text{PRP} = 1 - \left\{\frac{\%\text{ of morality (treated)}}{\%\text{ of mortality (control)}}\right\} \times 100$$

Tissue level accumulation of active principles of *D. nigra* in shrimp tissue was detected after 24 h of post-treatment. Randomly harvested shrimps were killed under ice and peeled

off the shell and head. The pooled tissue was minced in a tissue homogenizer (Omni) and extracted three times with methanol–dichloromethane (1:1). The extract was centrifuged at 10000 rpm at 4 °C for 15 min. The combined extract was adjusted to pH 6.4 and diluted with KH_2PO_4 buffer (pH 6.4) for microbial assay. *Micrococcus luteus* (ATCC 9341), which showed maximum sensitivity in the in vitro antibacterial activity, was used as test organism for the detection of residual antibacterial agents in shrimp tissue (Selvin and Lipton, 2003a) using modified cylinder plate method (USP, 1995).

3. Results and discussion

The survival rate was found to be significant ($p<0.01$) in the *Dendrilla* treated group (Table 1). The shrimps challenged with lethal dose and potentiated pathogen were produced more infection or mortality in the treated group than those caused by the MLD of individual pathogens. In the case of *V. fischeri*, the MLD did not cause mortality in the treated group while all the control shrimps died. At 10^8 cfu/shrimp, 60% mortality and 40% infection in the *Dendrilla* fed group was noted. The infected shrimps showed external signs such as shell necrosis and black spots on the shell, necrotised chelate legs, anorexia and folded tail. *Dendrilla* feed elicited complete protection (100% survival) against the most common shrimp pathogens such as luminescent *V. harveyi* and *V. alginolyticus*.

Albeit *Dendrilla* gave 100% survival against *Vibrio* sp., the inoculation of lethal dose (10^7 cfu/shrimp) of *Aeromonas* sp. caused 40% mortality and 20% infection in the treated group. This might be due to the broad spectrum vibriostatic activity of *Dendrilla* (Selvin and Lipton, 2004). Invariably, maximum PRP was obtained in the treated shrimps challenged with MLD of appropriate pathogen. *Dendrilla* exhibited 75% survival in the *P. monodon* challenged with potentiated pathogen. It is well known that the residual accumulation of antibiotics in the shrimp tissue will pose severe environmental and public health problems (Selvin and Lipton, 2003a). The active ingredients of *Dendrilla* were not accumulated in the shrimp tissue after 24 h of post dosing. The mechanism of action of

Table 1
Efficacy of *Dendrilla* feed (500 mg/kg) and PRP of *P. monodon* towards shrimp pathogens

Bacterial pathogens	Dose (cfu/shrimp)	Cumulative mortality within 15 days (%)		PRP	Percentage infection in the treated shrimp (n=20)
		Control (n=10)	Treated[a] (n=20)		
V. fischeri	10^8	100	60	60	40
	10^7	100	0	100	0
V. harveyi	10^6	100	0	100	0
V. alginolyticus	10^6	100	0	100	25
Aeromonas sp.	10^7	100	40	60	20
	10^6	80	0	100	0
Potentiated pathogen	10^7	100	25	75	25
V. harveyi	5×10^6				
V. alginolyticus	5×10^6				

[a] Based on the cumulative mortality, the calculated survival rate was significant at $p<0.01$.

secondary metabolites of *Dendrilla* was not exactly known. However, it was found that the *Dendrilla* diet did not influence the host defense system of shrimp (Selvin et al., 2004). Therefore, the protection elicited by *Dendrilla* could be due to its antibiotic effect rather than its influence on the host defense system. Therefore, *Dendrilla* medication may form a suitable alternative to replace conventional antibiotics.

In the culture system, shrimps are constantly exposed to a myriad of bacterial pathogens (Raa et al., 1992; Lightner, 1988). It may be difficult to ascribe mortality to any single pathogen. It was reported that secondary infection among shrimp and red disease syndrome arose due to multiple bacterial pathogens (Karunasagar et al., 1997; Tendencia and Dureza, 1997). Results of combined bacterial challenge indicated that *Dendrilla* was a broad spectrum vibriostatic agent. Literature evidenced that biological potency of *D. nigra* is virtually an untapped resource. In vitro studies in our laboratory indicated that *D. nigra* contained biologically active agents. It exhibited potent antibacterial, antifungal, brine shrimp cytotoxicity, microalgal lethality, insecticidal, anticoagulant, anti-fouling and anti-predation (ichthyotoxic) activities (Selvin and Lipton, 2004). The actual mechanism of such broad spectrum of bioactivity exhibited by a single species was not known. As crude extracts only were used, they may contain more than one compound or active principles. Manzamine-type alkaloids isolated from the Philippine marine sponge *Xestospongia ashmorica* showed insecticidal, antibacterial and cytotoxicity activities (Edrada et al., 1996). Cacospongionolide, B a new sesterpene, isolated from *Lascispongia covernosa* showed antimicrobial activity, brine shrimp cytotoxicity and ichythyotoxicity (de-Rosa et al., 1995). The crude extracts of Caribbean sponges *Ircinia campassa*, *Verongula rigida*, *Agelas conifera* and *Discus oxeata* were found to have ichthyotoxic, cytotoxic and antimicrobial activities (Zea et al., 1986).

Findings of the present study indicated that bycatch collection of *D. nigra* forms a reliable source for the development of shrimp therapeutants. However, no literature available on the aspects of bycatch landing of sponges and their biological potential. Therefore, it is inevitable to explore the chemical ecology of secondary metabolite synthesis. A number of bacteria and cyanobacteria associated among sponges were found to be the sources of antibiotics and other bioactive compounds in the marine environment. It was reported that the wider biosynthetic capabilities of sponges were associated with the symbiotic microorganism (Abrell, 1997). A purple-coloured bacterium isolated from the *Adocia* sp. produced antibiotic *O*-aminophenol (Oclarit et al., 1994). The marine bacterium, *Pseudomonas* isolated from its host sponge *Suberea creba* collected from the coral sea of New Caledonia produced strong antibiotic quinones (Debitus et al., 1998). One of the study on the chemical ecology of sponges in our laboratory indicated that the antibacterial secondary metabolites of *Dendrilla* might have synthesized by the associated bacterial species. Research is being underway on the aspects of chemical ecology of sponge-associated endosymbionts and their potential in shrimp disease management.

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