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Inhibitory effect of Indian Sponge extracts on bacterial strains and larval settlement of the barnacle, *Balanus amphitrite*

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Scientific relevance: Natural Product Antifoulants (NPAs) are one of the most promising alternatives to the toxic and non-biodegradable antifouling agents such as TBT. Considering the wealth of potential marine biota such as sponges and octocorals in Indian waters, there is wide scope for isolation of NPAs. Successful formulation of NPAs-based antifouling coatings will certainly help mitigate the load of toxic agents being released into the marine environment.

Abstract

The total ban imposed on TBT based antifouling paints, since 2008, has triggered the search for Natural Product Antifoulants (NPAs) as a promising alternative for the development of environmentally benign coatings. In view of this, thirty six species of sponges collected from the Gulf of Mannar, India, were screened for their inhibitory effect on fouling bacterial strains and cyprids of *Balanus amphitrite*. Of these, *Fasciospongia cavernosa* and *Petrosia nigricans* had high activity against the fouling bacteria and *Iotrochota baculifera* significantly inhibited larval settlement. *Oceanapia fistulosa*, *Haliclona exigua* and *H. cribricutis* exhibited considerable activity against both bacterial strains as well as barnacle larvae. These observations indicate high prospects for isolation of effective NPAs from Indian sponges.

Keywords: Gulf of Mannar; sponges; antifouling; fouling bacteria; *Balanus amphitrite*

1. Introduction

Biofouling, the attachment and growth of sessile marine organisms and its consequent adverse effects on the intended performance of submerged surfaces such as ship hulls and platforms, is a cause of serious concern to all maritime nations. Strategies for containing fouling have probably been initiated since man's first venture into the sea. However, the development of environmentally compatible antifouling procedure remains an unresolved problem till date (Raveendran and Limna Mol, 2009). The imposition of global ban on the most efficient antifouling agent, Tri butyl tin (TBT), due to its environmental toxicity, triggered the search for natural product antifoulants (NPAs) as one of the most viable alternatives, especially, during the last two decades. The soft-bodied marine organisms, well known for their inherent chemical defense mechanisms against epibiosis, are identified as the prospective source for NPAs (Wahl et al., 1994).

Intensive efforts on screening, isolation and characterisation of potential NPAs are currently progressing in various laboratories worldwide (Raveendran and Limna Mol, 2009). Of the 145 NPAs identified so far, more than 50% are from sponges. Yet, sponges remain as one of the most under-explored group of organisms (hardly less than 100 species out of more than 10,000 described species have been investigated) as far as NPAs research is concerned.

The Gulf of Mannar is located on the southeastern tip of India in the state of Tamil Nadu. It is the first marine biosphere reserve in all of South and Southeast Asia, extending from Rameswaram Island to Tuticorin in a NE-SW direction to a distance of 140 km with a width of 25 km. It comprises 21 islands running almost parallel to the coastline between latitude 8°47' N and 9°15'N and longitude 78°12'E and 79°14'E with three distinct marine ecosystems namely corals, seagrass and mangroves. Around 3,600 species of fauna and flora have been identified in the Gulf of Mannar area including approximately 108 species of sponges (Patterson et al., 2004). However, studies on antifouling properties of sponges from Indian waters are rather limited (Sarma et al., 1991; Mokashe et al., 1994; Prabha Devi et al., 1998; Wilsanand et al., 1999; Thakur and Anil, 2000; Bhosale et al 2002; Thakur and Muller 2004). Considering the vast virgin resources available in Indian waters, there is wide scope for isolation of novel NPAs.

As part of our continuing research on NPAs, we have carried out the screening of crude extracts of 36 species of sponges from the Gulf of Mannar for their antifouling activity against fouling bacterial strains and cyprids of the cosmopolitan barnacle, *Balanus amphitrite* (= *Amphibalanus amphitrite*, Clare and Hoeg, 2008; Carlton and Newman, 2009). Bacteria were selected as test organisms owing to their contribution in microfilm formation, while barnacles were included in the assay due to their significance as one of the major macrofoulers.

2. Materials and Methods

2.1. Collection and Extraction of Sponges

Sponges were collected from the Gulf of Mannar (Fig.1), Southeast coast of India (Lat 9°5' N; Long 79°5' E) by skin/SCUBA diving. After washing thoroughly with freshwater, the samples were cut into pieces and soaked in methanol/ acetone for three to four days. The extracts so obtained were concentrated using a rotary vacuum evaporator and subjected to antifouling assays. The samples were simultaneously identified and voucher specimens have been deposited at the National Institute of Oceanography, RC, Kochi.

2.2. Antibacterial assay

Seven different strains of fouling bacteria (*Bacillus cereus*, *B. pumilus*, *B. megaterium*, *Pseudoalteromonas haloplanktis*, *Pseudomonas chlororaphis*, *P. putida* and *P. aeruginosa*) were isolated from fouling panels immersed at a depth of 1m at Fore Shore Road boat jetty, Kochi (Lat 9°9'N; Long 76°2' E). The isolates were identified using taxonomic, physiological and biochemical methods based on Bergey's manual of systematic bacteriology (Bergey and Holt, 1994). Identification was further authenticated using gas chromatographic analysis of whole cell fatty acid methyl ester (FAME). Fatty acids of the bacteria were extracted according to the protocol of MIS-operating manual by MIDI Corporation, USA and compared with the library of Sherlock MIS System (Agilent GC-6950). The antibacterial activity was tested by the disc diffusion method (Bhosale et al., 2002; Murugan and Ramasamy, 2003). 18-hour-old cultures of the selected fouling bacterial strains were spread onto Zobell marine agar. Sterile 6-mm Whatman (GF/C) filter paper discs were impregnated with 500 µg of the crude extract dissolved in methanol, evaporated to dryness and placed onto the bacterial lawn. Streptomycin (10 µg/disc each) discs were used as

positive control and discs loaded with 25 μ l of absolute methanol served as negative control. The plates were incubated for 24 hours at $28\pm 1^\circ\text{C}$ and the resulting clear zones of bacterial growth inhibition were measured. The experiment was conducted in duplicate for each of the strains.

2.3. Larval Settlement Inhibition Assay

The adult barnacles were collected from their natural habitat at Vypeen beach, Kochi. They were maintained in the laboratory in 35 ppt aerated seawater on a diet of *Chaetoceros calcitrans* and artemia nauplii. Water and feed was changed every day, and the individuals showing symptoms of stress (weak operculum) were immediately removed from the mass. The barnacles were subjected to regular exposure periods to stimulate the release of nauplii (Rittschof et al., 1984; Hellio et al., 2004). The nauplii were reared in the laboratory at $28\pm 1^\circ\text{C}$ through the six different instars on a regular diet of *C. calcitrans* on a 12:12 hour Light: Dark cycle (Anil et al., 2001; Limna Mol et al., 2009). Cyprids were collected on the fifth and sixth day and stored at 5°C for use in the settlement inhibition assay.

The experiments were conducted in sterile 24-well polystyrene multiwell plates (Axigen) (Rittschof et al., 1992, Marechal et al., 2004, Limna Mol et al., 2009). The crude extract was dissolved in methanol and added to autoclaved $0.45\ \mu$ filtered sea water (FSW) to obtain a concentration of 100 $\mu\text{g/ml}$. 10 competent 3-day old cyprids were added to each of the 3 replicates of 2ml of the test solution. CuSO_4 solution (100 $\mu\text{g/ml}$) was used as positive control and wells containing only FSW with MeOH served as negative control. The plates were incubated for a 24 hour period under similar conditions at which the nauplii were reared. After the incubation period, the numbers of settled and metamorphosed cyprids were counted under a stereomicroscope and expressed as a proportion of the total number of larvae in the well. The experiment was repeated twice with different batches of larvae.

Statistical Analysis

The cyprid settlement inhibition data was subjected to statistical analysis using Two Factor ANOVA for comparison between batches of cyprids and between the sponge crude extracts (Conover and Iman, 1981).

3. Results

3.1. Antibacterial assay

Out of the 36 extracts tested for antibacterial activity, nine extracts showed growth inhibition of the fouling bacterial strains (Table 1). The highest zone of inhibition was exhibited by *Fasciospongia cavernosa* and *Oceanapia fistulosa* against two bacterial strains each. *Petrosia nigricans* and *Haliclona exigua* showed relatively high activity against two bacterial strains each. Also, *P. nigricans*, *H. exigua*, *H. cribricutis*, *Spongia officinalis* and *Dysidea* sp. showed moderate activity against some of the tested bacterial strains (Table 1).

3.2. Larval Settlement Inhibition Assay

The sponge crude extracts exhibited significant difference in their activity towards barnacle cyprids ($F_{1,36}=194.5$, $p<0.0001$, 2-factor ANOVA). However, no significant difference was observed among the two batches of larvae tested ($p<0.01$). Two of the extracts (*O. fistulosa* and *H. exigua*) having considerable activity against the fouling bacterial strains, exhibited high activity against the barnacle cyprids. At the same time, *F. cavernosa* and *P. nigricans*, having relatively high activity against the fouling bacterial strains, exhibited settlement promotion of the barnacle cyprids. On the contrary, *H. cribricutis* and *Iotrochota baculifera*, having moderate and no activity against the fouling bacterial strains respectively, exhibited high activity against the barnacle cyprids (Fig.2). Except for *Sigmatocia fibulata*, all the remaining sponges exhibited some degree of settlement inhibition activity (Table 2).

4. Discussion

Sponges are among the oldest inhabitants of the earth (Zhang et al., 2003). Till date, over 10,000 biologically active molecules have been isolated from them, making them the highest biosynthesizing organisms (Blunt et al., 2004; Thakur and Muller, 2004). Their sessile and unarmoured body propels the synthesis of secondary metabolites to defend the predators and competitors, as also to maintain a clean body surface (Becerro et al., 2003). Also, since sponges are voracious filter feeders, they take in along with regular food, the toxic chemicals excreted by other plants and animals, such as corals. They then modify and reuse these chemicals for their defense purposes. Thus, these sponge-produced and

sponge-modified metabolites form the chemical defense mechanism of sponges (Hooper, 2000).

In this study we have explored the ability of sponge extracts to inhibit the growth of fouling bacteria as also the settlement of *B. amphitrite* larvae. The results conform to the antifouling potential of sponges reported by earlier workers (Thompson et al., 1985; Willemsen, 1994). The concentration tested was one to two orders of magnitude lower than body tissue concentrations, thus mimicking the natural situation (Willemsen, 1994).

Among the 36 species of sponges screened for antifouling properties during the present study, six species (*F. cavernosa*, *O. fistulosa*, *H. exigua*, *H. cribricutis*, *P. nigricans* and *I. baculifera*) exhibited relatively high activity against fouling bacteria and/or barnacle cyprids. Majority of these species have been reported to have wide-ranging biological properties. For example, xestospongin and araguspongine from *H. exigua* is known to have cytotoxic, antifungal, antimalarial, antituberculosis and anti rat brain nitric oxide synthase activities (Venkateswarlu et al., 1994; Rao et al., 1998; Orabi et al., 2002; Liu et al., 2004); *Oceanapia* sp. and *Iotrochota* sp. are known for cytotoxicity (Venkateswarlu et al., 1999; Luter et al., 2007); *H. cribricutis* was found to have significant larvicidal and insecticidal activity (Rao et al., 2008) and polyacetylenes from *Petrosia* sp. was found to inhibit DNA replication at the level of initiation (Kim et al., 2002). However, no antifouling activity has so far been reported from any of these species, except for *H. exigua* (Limna Mol et al., 2009).

As evident from the present study, activity of an extract against one test species does not warranty activity against other fouling species. For eg. *I. baculifera* had high antisettlement activity, while it was totally inactive against the fouling bacterial strains. Another interesting case was that of *F. cavernosa* and *P. nigricans*, wherein both the species exhibited considerable growth inhibition of bacterial strains, but were found to promote settlement of barnacle cyprids. This observation ascertains the need for testing both anti-microfouling as well as anti-macrofouling activity of isolated extracts/compounds (Qian et al. 2010). In view of this, *O. fistulosa*, *H. exigua* and *H. cribricutis* exhibiting considerable activity against both, fouling bacteria and barnacle larvae, have better prospects as NPAs. We have already isolated bis-1-oxaquinolizidine alkaloids from *H. exigua* having significant antifouling activity within environmentally compatible limits (therapeutic ratio 2.7; Limna Mol et al., 2009).

Thus, the present study provides baseline data for isolation of NPAs from sponges of Indian waters.

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Fig.1. Sampling locations in the Gulf of Mannar

Fig.2. Sponge crude extracts (100 $\mu\text{g/ml}$) exhibiting significant antifouling activity against *B. amphitrite* cyprids.

Table 1. Sponge species showing considerable activity against fouling bacterial strains at 500µg/disc concentration.

Sponge Species	Bc	Bp	Bm	Ph	Pc	Pp	Pa
Streptomycin (control)	1	4	3	4	11	5	4
<i>Fasciospongia cavernosa</i> Schmidt	14	--	--	--	19	--	--
<i>Oceanapia fistulosa</i> Bowerbank	15	8	--	--	--	--	--
<i>Petrosia nigricans</i> Lindgren	4	5	3	6	5	--	6
<i>Haliclona exigua</i> Kirkpatrick	6	--	--	--	6	--	5
<i>Haliclona cribricutis</i> Dendy	4	3	4	4	--	3	--
<i>Spongia officinalis</i> var. <i>ceylonensis</i> Dendy	4	3	--	--	3	--	--
<i>Myrmekioderma granulata</i> Esper	1	--	2	2	--	--	--
<i>Dysidea</i> sp.	3	--	4	--	--	3	4
<i>Ircinia fusca</i> Carter	2	1	--	--	2	--	1

Bc-*Bacillus cereus*; Bp-*B. pumilus*; Bm-*B. megaterium*; Ph-*Pseudoalteromonas haloplanktis*; Pc-*Pseudomonas chlororaphis*; Pp-*P. putida*; Pa-*P. aeruginosa*; 1-2 mm zone= low activity; 3-5 mm zone= Moderate activity; >5 mm zone= High activity

Table 2. Antifouling activity of sponge crude extracts (100 µg/ml) against cyprids of *Balanus amphitrite*.

Sponge species	Activity in test solution
<i>Gelliodes cellaria</i> Rao	*
<i>Thalysias reinwardti</i> Vosmaer	*
<i>Sigmatocia fibulata</i> Schmidt	--
<i>Oceanapia fistulosa</i> Bowerbank	***
<i>Cinachyrea cavernosa</i> Lamarck	**
<i>Callyspongia diffusa</i> Ridley	*
<i>Petrosia testudinaria</i> Lamarck	*
<i>Spongia officinalis</i> var. <i>ceylonensis</i> Dendy	*
<i>Callyspongia</i> sp.	*
<i>Psammaphysilla purpurea</i> Carter	*
<i>Sigmatocia</i> sp.	*
<i>Haliclona pigmentifera</i> Dendy	*
<i>Fasciospongia cavernosa</i> Schmidt	#
<i>Callyspongia fibrosa</i> Ridley & Dendy	*
<i>Aurora globostellata</i> Carter	*
<i>Haliclona exigua</i> Kirkpatrick	***
<i>Spirastrella inconstans</i> var. <i>digitata</i> Dendy	*
<i>Iotrochota baculifera</i> Ridley	***
<i>Petrosia nigricans</i> Lindgren	#
<i>Cliona</i> sp.	*
<i>Thalysias vulpine</i> Lamarck	**
<i>Thalysias vulpina</i> var. <i>dichela</i> Hentschel	**
<i>Axinella tenuidigitata</i> Dendy	*
<i>Agelas ceylonica</i> Dendy	*
<i>Myrmekioderma granulata</i> Esper	**
<i>Heteronema erecta</i> Keller	*
<i>Hyatella cribriformis</i> Hyatt	*
<i>Haliclona cribricutis</i> Dendy	***
<i>Sigmatocia carnosus</i> Dendy	**
<i>Phycopsis</i> sp.	**
<i>Axinella donnani</i> Bowerbank	*
<i>Axinella carteri</i> Dendy	*
<i>Endectyon fruticosus</i> Dendy	*
<i>Dysidea herbacea</i> Keller	*
<i>Dysidea</i> sp.	*
<i>Ircinia fusca</i> Carter	*

-- = No activity; * = Low activity (1- 30% inhibition); ** = Moderate activity (31- 80% inhibition); *** = High activity (81-100% inhibition); # = Promotion of settlement