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Antifungal activity of some marine organisms from India, against food spoilage *Aspergillus* strains

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Abstract

Crude aqueous methanol extracts obtained from 31 species of various marine organisms (including floral and faunal), were screened for their antifungal activity against food poisoning strains of *Aspergillus.* Seventeen species exhibited mild (+ = zone of inhibition 1–2 mm) to significant (+++ = zone of inhibition 3–5 mm) activity against one or the other strain under experiment. However, extracts of 12 species were active against all the three strains. Organisms like *Salicornia brachiata* (obligate halophyte), *Sinularia leptocladus* (Soft coral), *Elysia grandifolia* (Mollusks), Gorgonian sp. 2 and *Haliclona* sp. exhibited significant (inhibition zone of 3–5 mm) antifungal activity against one or the other strains. However, extracts of *A. ilicifolius*, *Amphiroa* sp., *Poryphyra* sp., Unidentified sponge, *Suberites vestigium*, *Sinularia compressa*, *Sinularia* sp., *Sinularia maxima*, *Subergorgia suberosa*, *Echinogorgia pseudorassopo* and *Sabellaria cementifera* were mild (inhibition zone of 1–2 mm) to moderate (inhibition zone of 2–3 mm) active against the respective strains. The growth of *A. japonicus* was significantly inhibited by the extracts of *S. leptocladus* ($r = 0.992$, $p < 0.0001$) and *E. grandifolia* ($r = 0.989$, $p < 0.0001$).

Key words: marine organisms, bioactivity, antifungal, *Aspergillus*, bioactive molecules

Introduction

The marine biota of various kinds, are of immense ecological and of great socioeconomic importance. Tropical oceans are rich in marine biodiversity. Chemical ecological investigations on marine organisms (Bakus et al., 1986; Wylie and Paul, 1989), have inspired the search for bioactive molecules of medicinal importance. Secondary metabolites produced in marine organisms could be the source of bioactive substance and useful in modeling compounds for drugs (Bhakuni and Silva , 1974; Baslow, 1977; Rinehart and Shield, 1978; Baker and Marphy, 1976; Bakus et al., 1986). Marine organisms have received great attention during recent years for natural product chemistry, a promising new area of study. Lately, a large number of marine organisms have been reported to exhibit various kinds of bioactivities (Caccamese, et al., 1980; Thompson et al., 1985; Bakus et al., 1986; Bernord and Pesondo, 1989; Ballesteros et al., 1992; Schmitz, 1993; Padmakumar and Vethanayagam, 1995).

Larger number of marine plants have been observed to exhibit antibacterial properties, but a few of them have been reported to have antifungal activity (Bernard and Pesando, 1989; Ballestros et al., 1992). In view of this, the present investigation was undertaken to explore the potential of marine organisms for antifungal activity.

The commonly occurring and easily accessible marine organisms including fauna and flora have been studied for their antifungal properties, against species of *Aspergillus.* These *Aspergillus* spp. have been commonly observed to spoil the food in the tropics on a variety of substrates (Onion et al., 1981; Jay, 1978). They contaminate foodstuffs by producing mycotoxins (aflatoxins) which are harmful to mankind and animals (Williams and Blany, 1992; Hussain et al., 1989). These species also have been reported to be potential opportunistic pathogens to a number of commercially important marine organisms (Bhattacharya, 1995, Anand et al., 1996). Therefore, the present investigation was undertaken to identify bioactive potential marine organisms, against *Aspergillus* spp. The

data generated in the present investigation would be of importance in follow up studies in the field of marine natural products chemistry, particularly in identifying potential bioactive molecules from marine living resources.

Materials and methods

I. *Collection and preparation of extracts*

Commonly occurring marine organisms (Table 1) from various localities along the Indian coast (Fig. 1) were collected during the year 1996–98. The collections were made during the period of March to October when the weather and sea conditions remains favorable, and the biodiversity (Qualitatively and quantitative) in general remains greater. The samples were hand-picked from the intertidal and subtidal shallow and deeper (up to 20 m) regions. Subtidal collections, were made by SCUBA diving. Samples were collected in bulk depending upon the abundance of individual organisms , washed with freshwater to remove adhering debris and associated biota, air dried in the shade, and extracted in 90% aqueous methanol. These extracts were further concentrated as described earlier (Wahidulla et al., 1987). The concentrated methanol extracts were stored in a refrigerator and used for antifungal activities. Specimens used for the present study were preserved in 4% formalin and are deposited in the taxonomy reference center, of National Institute of Oceanography (CSIR), Goa, India, for their taxonomic identification and reference. The taxonomic identifications of various types of organisms were done by respective experts in the field, using standard literature and taxonomic keys.

II. *Isolation of* Aspergillus *strains*

Three strains of *Aspergillus* were isolated from wheat bread, with sterile needles, grown in mycological agar and subcultured for purification, following standard methods (Collins and Allsopp, 1967). Pure cultures were used for testing antifungal properties of various methanol extracts. Taxonomic identification of fungal strains were done by using various standard keys (Ainswort et al., 1973; Onions et al., 1981) and were further confirmed from Dr.(Mrs.) Alaka Pande, Agharkar Research Institute, Pune.

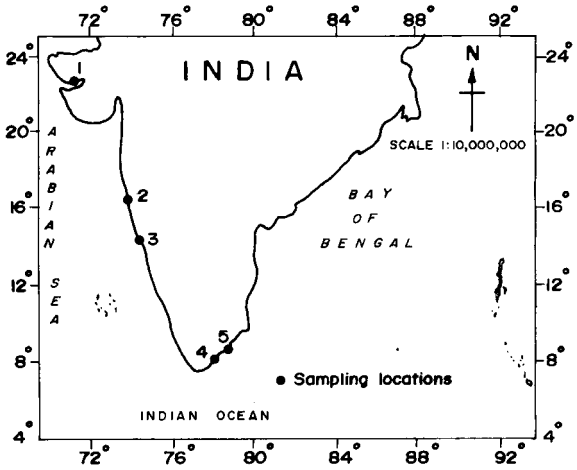


Figure 1. Map showing sampling locations: (1) Okha (Gulf of Kutchch,Gujarat); (2) Malvan (Maharashtra); (3) Anjuna (Goa); (4) Tuticorin (Tamilnadu); (5) Mandapam (Tamilnadu). Note: The names in parentheses' indicate the states in which sampling locations situated).

III. *Antifungal activity of crude organic extracts against* Aspergillus *strains*

Whatman (GF/C) filter paper disc of 6 mm diameter were prepared, sterilized for 15 minutes under 15 lb/m^2 pressure. The sample consisting of 0.5 to 1 mg of extract was loaded on to the paper disc at room temperature and allowed to dry completely, the process was continual load the extract drop by drop till the final concentration of 500 μg disc^{-1}was obtained. The disc were placed in mycological agar plates seeded with different strains of *Aspergillus* as described by (Pesando et al., 1979). The cultures were incubated for 24–198 hours at room temperature, to obtain maximum growth in the culture media. Due precautions were taken not to contaminate paper discs and glasswares by subjecting them to UV treatment. The zones of inhibition around the discs were measured and the activity for extract was scored using symbols and expressed as inactive (−), not - detectable (ND), mild (+), moderate (++) and significant (+++) and strong (++++). Standard discs of Amphotericin (100 u/disc) was used to check the sensitivity. Control tests with the solvents loaded on to discs were performed for every assay. Based on the preliminary results, the extracts of *Sinularia leptocladus* and *Elysia grandifolia*, which exhibited significant antifungal property, were further tested on the *Aspergillus* strains, using different concentrations ranging from 100–1000 μg disc $^{-1}$.

Table 1. Antifungal Properties of some marine organisms form Indian coast

Sr. No.	Species name	*Aspergillus fresenii*	*Aspergillus japonicus*	*Aspergillus niger*
	PLANTS			
	Marine angiosperms			
1	*Acanthus ilicifolius*	−	−	++
2	*Avicennia marina*	−	−	−
3	*Salicornia brachiata*	+	+	+++
	Seaweeds			
4	*Amphiroa sp.*	+	++	+
5	Unidentified Algae	−	−	−
6	*Poryphyra sp.*	+	+	++
7	*Ulva fasiculata*	−	−	−
	ANIMALS			
	Sponges			
8	*Tadinia sp.*	−	−	−
9	*Haliclona sp.*	+++	++	+
10	*Tadinia anhelans*	−	−	−
11	Unidentified sponge	−	−	−
12	*Suberites vestigium*	+	+	−
13	*Spongia officinalis*	−	−	−
	Soft corals			
14	*Lobophyllum sp.*	−	−	−
15	*Sinularia leptocladus*	+++	+++	+++
16	*Sinularia compressa*	+	+	+
17	*Sinularia sp.*	+	−	−
18	*Sinularia maxima*	+	++	+
	Mollucsus			
19	*Elysia gradifolia*	+++	+++	+++
20	*Planexis sulcatus*	−	−	−
21	*Aplysia sp.*	−	−	−
22	*Littorina sp.*	−	−	−
	Ascidian			
23	*Ascidian sp.*	−	−	−
	Gorgonians			
24	*Subergorgia suberosa*	+	+	+
25	*Echinogorgia pseudorassopo*	+	+	+
26	Gorgonian unidentified sp.1	−	−	+
27	Gorgonian unidentified sp.2	+++	+	++
	Polycheates			
28	*Sabellaria cementifera*	+	−	−
	Star fish			
29	*Pentaceraster appinis*	−	−	+
	Jelly fish			
30	*Casiopela sp.*	−	−	−
	Sea anemone			
31	*Radianthus sp.*	−	−	−
	Standards			
A1	Amphotericin	++	++	++++

− No zone of inhibition (inactive); + 1–2 mm zone of inhibiton (mild active); ++ 2–3 mm zone of inhibition (moderately active); +++ 3–5 mm zone of inhibition (significantly active); ++++ 5–7 mm zone of inhibition (strongly active).

Table 2. The quantum of antifungal actvity by *S. leptocladus* and *E. grandifolia*

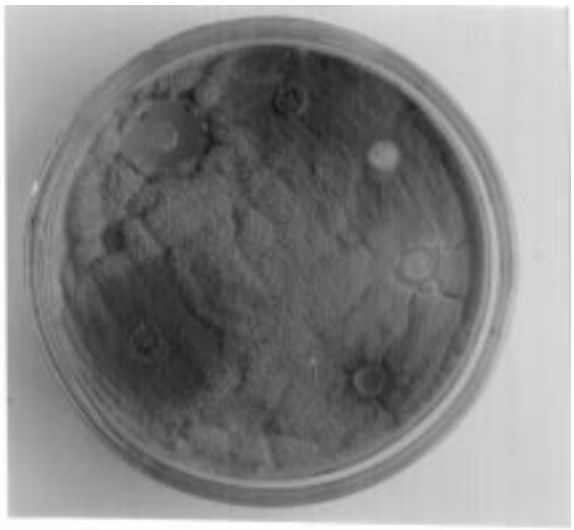
Marine bioextracts	Fungal strains	r	p
E. grandifolia	*A. niger*	0.982	***
	A. japonicus	0.989	***
	A. fresenii	0.972	***
S. leptocladus	*A. niger*	0.988	***
	A. japonicus	0.992	***
	A. fresenii	0.986	***

Level of significance (*** $p < 0.0001$).

Results and discussion

Out of the total 31 methanol extracts of marine organisms, seventeen (flora – 4 and animals – 13 nos.) exhibited antifungal activity against one or other strain of *Aspergillus*. However, out of the above, 12 extracts were active against all the three strain used (Table 1). The quantum of activity exhibited in the extract of individual species varied from mild (+ = zone of inhibition 1–2 mm) to significant (+++ = zone of inhibition 3–5 mm). Extracts of *Salicornia brachiata*, *Sinularia leptocladus*, *Elysia grandifolia*, *Haliclona* sp. and an unidentified Gorgonian sp. 2, showed activity against all the three strains. However, the intensity of activity varied from strain to strain. The extracts of *Sinularia leptocladus* and *E. grandifolia*, however, were observed to be significantly active ($r > 0.972$, $p < 0.0001$) against all the three strains and the antifungal activity increased at higher concentrations (Tables 2 and 3).

Though, mangroves species like *Acanthus ilicifolius* and *Avicennia marina* have been reported to possess medical properties such as analgesic and anti-inflammatory activities (Jongsuvat 1981; Kokpol et al., 1984; Padmakumar et al., 1993), only *A. ilicifolius* exhibited moderate antifungal properties against *A. niger. Avicinnia marina* was totally inactive against all the fungal strains. *Salicornia brachiata* was significantly active against *Aspergillus niger*, however, it exhibited mild activities against other two species of the *Aspergillus*. Marine algal species such as *Amphiroa* sp. (coralline alga) and *Porphyra vietnamensis* (red algae) exhibited moderate activity against *Aspergillus japonicus* and *A. niger*, respectively. Other species of *Amphiroa* have been reported to exhibit antitumor, oxytoxic, hypotensive and spasmogenic activities (Wahidulla et al., 1987; Mizokoshi et al., 1982).



I - a

I - b

Plate 1. Extracts of marine organisms showing significant antifungal activity against *A. fresenii* at concentration of 500 μg disc^{-1} (a: *Elysia grandifolia*, b: *Sinularia leptocladus*).

Table 3. Antifungal activity of significantly active extracts of *Sinularia lepticlades* (*) and *Elysia grandifolia* (♠) at different concentration

Conc./fungal strains	Control	100 μ g/disc	200 μ g/disc	300μ g/disc	400 μ g/disc	500 μ g/disc	600 μ g/disc	700 μ g/disc	800 μ g/disc	900 μ g/disc	1000 μ g/disc
Aspergillus fresenii	*ND	ND	+	+	+	+++	+++	+++	++++	++++	++++
	♠ND	ND	+	+	+++	+++	+++	+++	++++	++++	++++
Aspergillus niger	*ND	ND	+	+	+	+++	+++	+++	++++	++++	++++
	♠ND	ND	+	+	+++	+++	+++	+++	++++	++++	++++
Aspergillus japonicus	*ND	ND	+	+	+	+++	+++	+++	++++	++++	++++
	♠ND	ND	+	+	+++	+++	+++	+++	++++	++++	++++

ND – Not detectable.
Same as in Table 1.

Marine sponges possess various kinds of bioactivities (Amade et al., 1987; Mcclintoc and Ganthier 1992; Williams and Faulkener, 1996). In this study only *Haliclona* sp. Table 1 was found to be significantly active against *Aspergillus japonicus*. Earlier 19 species of sponges from India have been reported (Padmakumar and Vethanayagam, 1995) to exhibit antifungal properties against *Aspergillus niger*, *Candida* sp. And *Cryptococcus neoformans.* Several species of soft corals release chemicals toxic to other marine aquatic lives (Coll et al., 1982a, Coll and Sammarco, 1983). About 50% of the number of the existing soft coral species found in the world are ichthyotoxic (Coll et al., 1982b, La Barre et al., 1986). Extracts and secondary metabolites from soft corals, particularly from *Sinularia* spp. have been reported to be very effective against their predators (Wylie and Paul, 1989). Though all the four species of *Sinularia* exhibited antifungal properties in the present investigations,*S. leptocladus* was significantly active against *Aspergillus fresenii* (Plate I-a). Mollusks, such as *Aplysia* spp. produce toxic chemicals (terpene) or other protective compounds, dietarily derived from their algal food, as antipredator weapons (Norris and Fenchel 1982; Faulkner, 1984). *Elysia grandifolia* in the present studies exhibited strong antifungal activities against all three strains (Plate I-b). However, *Aplysia* sp. did not show antifungal property. Among other faunal groups Gorgonian sp. 2 exhibited strong antifungal property against *Aspergillus fresenii.* Most of the Gorgonian spp. have been reported to be ichthyotoxic (Gerhert, 1984) due to the presence of prostaglandin A2 in them.

Aspergillus spp. and many other moulds are often common contaminants of food, and affect the quality of the contaminated food by reducing starch and fat contents and lowering digestible energy level (Williams and Blaney, 1992). The genus *Aspergillus* is one of the most common and wide spread mould associated with food spoilage. Aspergilli are known to occur on cereals, treenuts, peanuts, cotton, fruits, meat, poultry and sea food products (Jay, 1978). *Aspergillus niger* has been reported to be the leading cause for the spoilage of various kinds of fruits, other two species, i.e., *A. fresenii* and *A. japonicus*, used in the present experiments were found to be commonly associated with the food products of wheat, in the coastal regions of India having warm and humid climate. Marine organisms exhibiting significant antifungal activities in the present investigation provide the basis for safe rational bioactive compounds from readily available marine organisms, which could be effective in controlling fungal (partial only *Aspergillus*) contamination in food and feed. Further testing of the efficiency of these compounds against toxigenic Aspergilli such as the aflotoxigenic *A. flavus* and *A. parasiticus* could enhance the utility if these compounds against the species of *Aspergillus*.

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