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**Antimicrobial Activity of Polysaccharide Isolated from the Cuttlebone of
Sepia aculeata (Orbingy, 1848) and *Sepia brevimana* (Steenstrup, 1875):
An Approach to Selected Antimicrobial Activity for
Human Pathogenic Microorganisms**

A. Sharmugam, T.S. Mahalakshmi and A. Barwin Vino
Centre of Advanced Study in Marine Biology, Annamalai University,
Parangipettai-608502, Tamil Nadu, India

Abstract: Polysaccharide isolated from the cuttlebone of *Sepia aculeata* and *Sepia brevimana* using 10 mM EDTA were studied for their antibacterial and antifungal activity against nine bacterial (*Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Shigella* sp.) and four fungal (*Candida* sp., *Rhizopus* sp., *Aspergillus flavus* and *Aspergillus fumigatus*) pathogens at different concentrations such as 25, 50, 75 and 100% against control. The activities were found to be increasing with the increasing concentration of the extracts. No antibacterial activity was recorded against *V. cholerae* in all concentrations of *S. brevimana*. In *S. aculeata*, maximum (8 mm inhibition zone in 100%) and minimum (2 mm inhibition zone in 25%) activity was recorded against *E. coli*, but in *S. brevimana* the highest and lowest activity was recorded as 17 mm (100%) and 2 mm (25%) (inhibition zone) against *P. aeruginosa* and *E. coli*, respectively. In the antifungal activity study, the highest and lowest inhibition zones of 12 mm (100%) and 3 mm (25%) were noted against *A. flavus*, *Candida* sp. and *A. fumigatus* and *Rhizopus* sp. respectively, but in *S. brevimana*, maximum and minimum activity of 9 mm (100%) and 2 mm (25%) (inhibition zone) were observed against *A. flavus* and *A. fumigatus*, respectively. In both species the cuttlebone polysaccharide showed no activity against *Candida* sp.

Key words: Cuttlefish, cuttlebone, polysaccharide, antibacterial and antifungal activity

INTRODUCTION

The organisms from marine environment have been found possessing a vast array of new pharmaceutical compounds with novel activities that will provide new drug leads to combat microbial pathogens currently developing resistance to conventional antibiotic therapies. In recent years, the microbial metabolites have gained significant importance in drug research. The study of natural products that exhibit biological activity, derived from plants and animals has long been showing significant biomedical value and crude products isolated from marine organisms have served as source of many drugs (Kamboj, 1999). In most of the publications concerning antimicrobial activity in molluscs, either single body compartments alone, like haemolymph and egg masses, or extracts of whole bodies have been tested for activity (Haug *et al.*, 2003). Further the cuttlebone also reported antibacterial and antifungal activities against some of the human pathogenic microorganisms (Rajaganapathi, 2001). The ocean served just not only as the source of antibiotics but it is indeed reservoir of other bioactive compounds too. The presence of antimicrobial activity in Mollusca has been reported from the mucus of the giant snail *Achatina fulica* (Kubota *et al.*, 1985; Iguchi, 1982),

Corresponding Author: A. Sharmugam, Centre of Advanced Study in Marine Biology, Annamalai University, Parangipettai-608502, Tamil Nadu, India Tel: 04144-243223*212 Fax: 914144243555

from the egg mass and purple fluid of the sea hare *Aplysia kurodai* (Yamazaki, 1993; Kamiya *et al.*, 1984) and from the body wall of the sea hare *Dolabella auricularia* (Iijima *et al.*, 2003). Charlet *et al.* (1996) isolated from the blood of immune-challenged and untreated *Mytilus edulis* antibacterial and antifungal peptides. The mytilin isoforms C, D and G1 were isolated from *Mytilus galloprovincialis* and exhibited complementary antimicrobial properties (Mitta *et al.*, 2000a). In addition, a novel antifungal peptide that delays the growth of *Neurospora crassa* and *Fusarium culmorum*, mytimycin, has been isolated and partially characterized in conjunction with the defensins and mytilins from *Mytilus edulis* (Charlet *et al.*, 1996). Further these compounds are being extracted not only from the whole animal but also from the different body parts including the skeleton (internal shell in the case of cuttlefishes), which showed many pharmacological properties and hence medicinal value. Keeping the importance of cephalopod, in terms of bioactive compounds with antibacterial and antifungal properties etc., the present study has been attempted to focus the attention on to the study antibacterial and antifungal activity shown by the polysaccharides extracted from the cuttlebone of two cuttlefish species such as *S. aculeata* and *S. brevimana*.

MATERIALS AND METHODS

Collection and Preparation of Cuttlebone Powder

Cuttlebone of the two species such as *S. aculeata* and *S. brevimana*, were freshly collected from Annankoil landing centre of Parangipettai coast (Lat. 11° 46'E; Long. 79° 29'N), Tamil Nadu, India during September 2005 to August 2006. Cuttlebones were washed several times with tap water and dried.

Isolation of Polysaccharide

The polysaccharides were obtained from the cuttlebone of *S. aculeata* and *S. brevimana* by following method of Okutani and Morikawa (1978). The air-dried shell powder was pulverized and washed with acetone. The powder was extracted with hot 10 mM EDTA solution and filtered (Whatman No. 1) with hyflosuper cel. Then saturated barium hydroxide solution was added to the filtrate and allowed to stand over night. Then the precipitate was placed on a filter paper (Whatman No. 1) with hyflosuper cel and washed with distilled water. The precipitate was dissolved in 10 mM EDTA solution and was dialyzed against deionised water. The dialyzate solution present in the dialysis membrane was then freeze-dried and a pure white colored powder was obtained. This lyophilized powder was used for the antibacterial and antifungal activity.

Bacterial and Fungal Strains

Nine bacterial (*Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Shigella* sp.) and four fungal (*Candida* sp., *Rhizopus* sp., *Aspergillus flavus* and *Aspergillus fumigatus*) strains were used for studying the antibacterial and antifungal activities of the cuttlebone polysaccharide. These strains isolated from the HIV patients, were obtained from Raja Muthiah Medical College Hospital, Annamalai University, Tamil Nadu, India.

Antibacterial and Antifungal Assay

In vitro antibacterial and antifungal activity was determined by disc diffusion technique of Gunthorpe and Cameron (1987). 60 mg of lyophilized powder was dissolved in 0.6 mL of solvent (10 mM EDTA). From this 0.24, 0.18, 0.12 and 0.06 mL of sample were taken and each was made up to 0.24 mL with respective solvent. The control with respective solvent was also prepared. These different concentrations of the cuttle bone (0.24, 0.18, 0.12 and 0.06 mL were known as 100, 75, 50

and 25%, respectively) and 0.6 mL of 10 mM EDTA solvent (control) were applied to 6 mm sterile disc; allowed to dry at room temperature and placed on agar plate seeded with microorganisms (nutrient medium for bacterial strains and potato dextrose medium for fungal strains). These agar plates were incubated at room temperature for 24 h in the inverted position aerobically. The zones of growth inhibition, if any were measured after the incubation period. Each extract was tested thrice for confirming their activity.

RESULTS

Antibacterial Activity

The results of antibacterial activity (Table 1). In the case of control, no activity was recorded against any bacterial strain with respect to the two species of cuttle fish studied. In 100% concentration, the highest activity was recorded through the 8 mm inhibition zone against *E. coli*, whereas a minimum of 4 and 5 mm inhibition zones were observed against *K. pneumoniae* and *V. cholerae* and *V. parahaemolyticus*, respectively. In the same concentration, *S. brevimana* showed the differential activity of 17, 11 and 10 mm (inhibition zone) against *P. aeruginosa*, *S. typhii* and *Shigella* sp. while the minimum value of 5 mm was observed against *B. subtilis*.

At 75%, the extract of *S. aculeata* showed maximum activity against *E. coli* in which the inhibition zone was found to be 6 mm. The minimum of 3 mm inhibition zone was recorded against *K. pneumoniae*. In the same concentration, *S. brevimana* was found to record the maximum inhibition zone of 10 and 13 mm against *S. typhii* and *P. aeruginosa*. The minimum inhibition zone of 4 and 5 mm were recorded against *B. subtilis* and *E. coli*, respectively.

At 50% of concentration, *S. aculeata* showed more or less similar activity in all the strains with the inhibition zone of 3 to 5 mm. In the same concentration, *S. brevimana* extract was found to show the maximum inhibition zone of 11 and 7 mm against *P. aeruginosa* and *S. typhii*, respectively. The minimum of 3 to 6 mm inhibition zone was recorded against *B. subtilis*, *Shigella* sp., *V. parahaemolyticus*, *S. aureus*, *K. pneumoniae* and *E. coli*.

At 25% of concentration, *S. aculeata* showed 3 mm inhibition zone in all bacterial strains tested except in *P. aeruginosa* and *S. aureus* in which 2 and 4 mm of the inhibition zones were noted, respectively. At the same time the minimum inhibition zone of 1 mm was noticed against *K. pneumoniae*. In the same concentration, *S. brevimana* showed no activity at all against *V. cholerae*. However, the same concentration showed different activity in all other strains. The maximum of 8 and 6 mm inhibition zones were observed against *P. aeruginosa* and *S. typhii*, respectively. Whereas the minimum of 2 and 3 mm inhibition zones were recorded against *E. coli* and *B. subtilis*, respectively.

Table 1: Antibacterial activity of various concentrations of the polysaccharides extracted from the cuttlebone of *Sepia aculeata* and *S. brevimana*

Strains	<i>Sepia aculeata</i>				<i>Sepia brevimana</i>			
	25	50	75	100	25	50	75	100
	Conc. (%)							
<i>B. subtilis</i>	3	4	4	6	3	4	4	5
<i>Shigella</i> sp.	3	4	5	6	4	5	8	10
<i>P. aeruginosa</i>	2	3	5	6	8	11	13	17
<i>S. typhii</i>	3	4	5	6	6	8	10	11
<i>V. parahaemolyticus</i>	3	4	4	5	5	5	6	8
<i>V. cholerae</i>	3	4	4	5	-	-	-	-
<i>S. aureus</i>	4	5	5	6	4	5	7	8
<i>K. pneumonia</i>	1	3	3	4	4	5	6	8
<i>E. coli</i>	3	4	6	8	3	3	5	8

Table 2: Antifungal activity of various concentrations of the polysaccharides extracted from the cuttlebone of *Sepia aculeata* and *S. brevimana*

Strains	<i>Sepia aculeata</i>				<i>Sepia brevimana</i>			
	25	50	75	100	25	50	75	100
	Conc. (%)							
<i>Candida</i> sp.	4	5	7	12	-	-	-	-
<i>Rhizopus</i> sp.	3	6	7	10	-	2	3	4
<i>A. fumigatus</i>	3	3	4	5	2	2	3	4
<i>A. flavus</i>	5	6	9	9	4	6	9	12

Antifungal Activity

All the results of antifungal activity are shown in Table 2. In control no activity was recorded for all the fungal strains in both *S. aculeata* and *S. brevimana*. The 100% concentration of *S. aculeata* exhibited the maximum activity against the two species i.e., 12 mm inhibition zone against *A. flavus* and *Candida* sp., whereas the minimum of 5 mm inhibition zone was recorded against *A. fumigatus*. At the same time *Rhizopus* sp. showed 10 mm inhibition zone. In *S. brevimana* showed the minimum value of 4 mm inhibition zone against *A. fumigatus* and *Rhizopus* sp; whereas the maximum activity of 9 mm (inhibition zone) against *A. flavus*.

At 75% concentration, *S. aculeata* showed the maximum and minimum activity of 9 and 4 mm inhibition zone against *A. flavus* and *A. fumigatus*, respectively. Whereas *Candida* sp. and *Rhizopus* sp. recorded the same value of inhibition zone. In *S. brevimana*, maximum (9 mm) against *A. flavus* and the minimum of 3 mm against *A. fumigatus* and *Rhizopus* sp.

At 50% concentration, *S. aculeata* showed the maximum activity against *A. flavus* and *Rhizopus* sp. in which the inhibition zone was found to be 6 mm, whereas *Candida* sp. and *A. fumigatus* recorded the zone of inhibition measuring 5 and 3 mm, respectively. The same concentration of *S. brevimana* showed maximum activity against *A. flavus* (6 mm) and the minimum (2 mm) activity was showed against *A. fumigatus* and *Rhizopus* sp.

At 25% concentration, *S. aculeata* showed maximum inhibition zone of 4 mm against *A. flavus* and *Candida* sp. and the minimum activity (3 mm inhibition zone) was recorded against *A. fumigatus* and *Rhizopus* sp. The maximum of 5 mm inhibition zone was recorded against *A. flavus* and the minimum zone of inhibition was found to be 2 mm against *A. fumigatus*.

DISCUSSION

For the first time attempts to study the antimicrobial activity in marine organisms were initiated around 1950s (Berkholder and Berkholder, 1958). Since that time, a large number of marine organisms from a broad range of phyla have been screened for their antimicrobial activity (Rhinhart *et al.*, 1981; Shaw *et al.*, 1976).

Potent antibacterial activity in haemocytes and haemolymph has been detected in various mollusks (Anderson and Beaven, 2001; Charlet *et al.*, 1996). Antimicrobial peptides have been isolated and characterized from the haemocytes of *Mytilus edulis* (Mitta *et al.*, 2000a; Charlet *et al.*, 1996) and *M. galloprovincialis* (Mitta *et al.*, 200a, b) and from the sea hare *Dolabella auricularia* (Iijima *et al.*, 2003).

A screening of antibacterial and antifungal activity in cuttlebone extracts of *S. aculeata* and *S. brevimana* was conducted. The results show that *in vitro* antibacterial and antifungal in cuttlebone extracts tested. Antibacterial activity has previously been described in a wide range of mollusk species (Anderson and Beaven, 2001; Benkendorff *et al.*, 2001; Prem Anand *et al.*, 1997; Gunthorpe and Cameron, 1987; Constantine *et al.*, 1975). In most of the species studied, the haemolymph, egg masses or the whole body have been tested for activity. The present study demonstrates the presence of

antibacterial and antifungal factors in cuttlebone extracts. The antimicrobial factors might therefore have an important function as a first line defense against pathogenic microorganisms. Whether the antimicrobial activity increases following microbial stimuli is not known.

In the present study, high antibacterial activity was found in *P. aeruginosa* and antifungal activity was found in *A. flavus*. The activity of the cuttlebone extracts was found to be high in 100% concentration than the other concentrations. In general, the concentration was increased activity also increased. *S. aculeata* showed against all the nine bacterial strains in all the concentrations tested with the maximum activity of 8 mm in 100% against *E. coli* and the minimum activity of 1.0 mm in 25% concentration against *K. pneumoniae*. For antifungal activity, the highest activity was recorded 12 mm against *A. flavus* and *Candida* sp. in 100% concentration.

S. brevimana also recorded the activity against all the strains tested except *V. cholerae* with the activity ranged from 2 mm in 25% against *E. coli* to 17 mm against *P. aeruginosa* and antifungal activity 5 mm was recorded against *A. fumigatus*. The difference in response may be due to species specific characteristics. The highest antibacterial activity was found in *S. brevimana* than the *S. aculeata* and the highest antifungal activity was *S. aculeata* than *S. brevimana*. In general, they were more antibacterial activity than antifungal activity.

In this study a wide spectral antibacterial and antifungal activity has been recorded in almost all the extracts which is the significant finding of this study. Kagoo and Ayyakkannu (1992) reported that the hypobranchial gland of *Chicoreus ramosus* exhibited a broad spectral activity against 10 bacterial strains. In this study also similar type of result was found in cuttlebone of *S. aculeata* and *S. brevimana*.

Prem Anand and Patterson Edward (2002) reported moderate antibacterial and antifungal activity from the extracts of various bivalve mollusks. Patterson and Murugan (2000) reported broad spectrum of antibacterial activity for aqueous ink extract of the cephalopods *L. duvauceli* and *S. pharaonis* against nine human pathogens. In the present study also promising activity was noticed for *S. aculeata* and *S. brevimana* against all bacterial strains, except *S. brevimana* against *V. cholerae*.

Only very few studies have been carried out on the antimicrobial activity of the internal shell of cuttlefishes. But many such studies are available for the extracts from the whole body tissue whose results could be compared with that of the present study. Jayaseeli *et al.* (2001) when studied the antibacterial activity of *Donax faba*, *D. modesta*, *Circe scripta* and *G. pectinatum* against nine pathogenic bacteria such as *S. aureus*, *K. pneumoniae*, *S. typhi*, *E. coli*, *B. subtilis*, *P. vulgaris*, *P. mirabilis*, *V. cholerae* and *S. flexneri* reported broad spectrum antibacterial activity for the water and heptane extracts.

Okuda and Sheuer (1985) isolated Latrunculin-A from *Chromodoris elisbenthina* that showed inhibition activity against *C. albicans*. But there are some reports on the isolation of antifungal compounds from marine animals in general: puuphenone from 87 species of marine sponges (Amede and Chevolot, 1982) and Dolastatin -10 from tunicate and spongistatin from sponges (Pettit *et al.*, 1998a, b).

The presence of antimicrobial activity in Mollusca has been reported from the mucus of the giant snail *Achatina fulica* (Iguchi *et al.*, 1982), from the egg mass and purple fluid of the sea hare *Aplysia kurodai* (Yamazaki, 1993; Kamiya *et al.*, 1984) and from the body wall of the sea hare *Dolabella auricularia* (Iijima *et al.*, 2003). Charlet *et al.* (1996) isolated from the blood of immune-challenged and untreated *Mytilus edulis* antibacterial and antifungal peptides. Relatively cuttlebone of polysaccharide extract of *S. aculeata* and *S. brevimana* showed highest antibacterial and antifungal activity.

The fact that some of the polysaccharide extracts showed high antibacterial and antifungal activity against human pathogens and indicate that polysaccharide is at least not solely responsible for the antimicrobial activity detected.

The result of the present study further supports the above i.e., the cuttlebone extracts of both *S. aculeata* and *S. brevimana* showed antibacterial activity against the nine important human pathogenic bacteria with an exception of *S. brevimana* extract against *V. cholerae* evincing the presence of some active principles of medicinal importance particularly with the bactericidal effect.

CONCLUSION

In the present study, the polysaccharides have been extracted from the cuttlebone of *S. aculeata* and *S. brevimana* which showed promising antibacterial and antifungal activity against the human pathogenic strains. We found potent antibacterial and antifungal activity against human pathogenic strains. This finding is very significant and may pave way for the discovery of new potent drugs against these dangerous pathogens. Further research on purification and characterization of these extracts should be carried out.

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