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Screening of antimicrobial potential of polysaccharide from cuttlebone and methanolic extract from body tissue of *Sepia prashadi* Winkworth, 1936

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ABSTRACT

Objective: To evaluate the antimicrobial activity of polysaccharide from cuttlebone and methanolic extract from body tissue of *Sepia prashadi*, against ten human pathogenic bacteria and five fungi. **Methods:** The activity of polysaccharide and methanolic extract was investigated against *Vibrio cholerae, Pseudomonas aeruginosa, Klebsiella pneumoniae, Vibrio alginolyticus, Staphylococcus aureus, Vibrio parahaemolyticus, Streptococcus* sp., *Streptococcus pneumoniae, Salmonella* sp. and *Escherichia coli*, and five fungal strains such as *Alternaria alternata, Candida tropicalis, Penicillium italicum, Fusarium equiseti* and *Candida albican* using disc diffusion method and minimum inhibitory concentration (MIC) were also calculated. **Results:** Both polysaccharide and methanolic extract was active against gram positive than that of gram negative pathogenic bacteria but inactive against fungi. The MIC of both the extract ranging from 60 to 100 mg/mL. **Conclusions:** These results suggest that cephalopod polysaccharide and methanolic extract possess relatively good antibacterial activity.

1. Introduction

The cephalopod which includes the Nautilus, cuttlefishes, squids and octopods, is the most advanced class of phylum: Mollusca adapted to a swimming existence. They are exclusively marine, diverse in form, size and nature[1] and occupy littoral and benthic to pelagic environments of all world oceans. Cephalopods are considerably important as a food resource as well as in scientific investigations. There is an ever continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action due to the alarming increase that has been witnessed in the incidence of both new and reemerging infectious diseases. A further big concern is the development of resistance to the antibiotics in current clinical use[2].

Although antibiotics are life saving drugs but nowadays, as a result of careless and promiscuous use of antibiotics, various pathogenic microbes are gaining resistances. Among marine invertebrates, cephalopods belong to a molluscan group comprising 700 species in which bacterial associations have been known for a long time and can include the reproductive organs (accessory nidamental glands) of myopsids, sepiolids and sepiids and the light organ of sepiolids[3-5].

In recent years, human pathogenic microorganisms have developed resistance in response to the indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases. This situation, the undesirable side effect of certain antibiotics, and the emergence of previously uncommon infections, has forced scientists to look for new antimicrobial substitutions from various sources such as from plant origin and animal origin[6]. Marine invertebrates offer a source of potential antimicrobial drugs[7-9]. Discovered bioactive compounds in molluscs were identified essentially as peptide, depsipeptide, sterols, sesquiterpene, terpenes, polypropionate, nitrogenous compounds, macrolides, prostaglandins and fatty acid derivatives, sterols, miscellaneous compounds and alkaloids; they all presented specific types of activities[10-12]. Defer *et al*[13] reported the antibacterial and antiviral activities in three bivalve and two gastropod marine molluscs (*Cerastoderma edule*, *Ruditapes philippinarum*, *Ostrea edulis*, and *Buccinum undatum*).

Studies of antimicrobial activity provide valuable

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information for new antibiotic discoveries and give new insights into extraction of bioactive compounds from aquacultured molluscs. Romanenko *et al*[14] investigated the isolation, phylogenetic analysis and screening of marine mollusc-associated bacteria for antimicrobial, hemolytic and surface activities. In most of the publications concerning antimicrobial activity in molluscs, either single body component alone, like haemolymph and egg masses, or extracts of whole body tissues have been tested for activity.

Typhoid fever is a more classical systemic infection caused by the typhoid bacillus, *Salmonella enterica* serovar Typhi, the most common cause of enteric fever, which also includes paratyphoid fever caused by *Salmonella paratyphi*. With an estimated 16-33 million cases resulting in 500 000 to 600 000 deaths annually in endemic areas, the WHO identifies typhoid as a serious public health problem[15]. Due to the permanent resistance of the microorganisms to available drugs, continuous search for new antimicrobials is a scientific challenge. In our continuous search of antimicrobial agents from natural sources, this study was designed to assess the antimicrobial activity of the polysaccharides extracted from cuttlebone and methanolic extract from body tissue of *Sepia prashadi* (*S. prashadi*).

2. Materials and methods

2.1. Sampling and identification

The specimen (*S. prashadi*) were collected from Cuddalore landing centre (Lattitude 10°42′ N; Longitude 79°46′ E), southeast coast of India. The Publication of Roper *et al*, Jothinayagam and Shanmugam *et al* were used in identification[16-18].

2.2. Preparation of polysaccharide extract from cuttlebone

The polysaccharide extact was prepared using the method described by Okutani K, *et al*[19]. The air dried cuttlebones were pulverized and washed with acetone. The powder was extracted with hot 10 mM EDTA solution and filtered using Whatman No. 1 filter paper with hyflo super-cel. Then saturated barium hydroxide solution was added to the filtrate. The precipitate obtained after standing overnight was collected on Whatman No. 1 paper with hyflo super-cel and washed with water. The precipitate was dissolved in 10 mM EDTA solution and was dialyzed against deionized water. The dialysate was freeze-dried which was then used in the present investigation.

2.3. Preparation of methanolic extracts from the body tissues

The methanolic extracts of the body tissues were prepared by following the method of Ely R, et al[20]. *S. prashadi* were brought to laboratory; body tissues were removed, cut into small pieces and homogenized (REMI, RQ-127 A) and extracted with MeOH at room temperature fore 24-48 h. Then the methanolic extract was centrifuged to collect the supernatant and concentrated under vacuum in a rotary evaporator at low temperature.

2.4. Microbial cultures

Ten species of bacteria and five species of fungi were used as test organisms. The bacterial strains contains Gram-positive strains: *Streptococcus* sp., *Streptococcus pneumoniae* (*S. pneumoniae*), *Staphylococcus aureus* (*S. aureus*); Gram-negative strains: *Escherichia coli* (*E. coli*), *Vibrio cholerae* (*V. cholerae*), *Vibrio alginolyticus* (*V. alginolyticus*), *Vibrio parahaemolyticus* (*V. parahaemolyticus*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Klebsiella pneumoniae* (*K. pneumoniae*) and *Salmonella* sp.. The fungal strains contains *Alternaria alternata*, *Candida tropicalis*, *Penicillium italicum*, *Fusarium equiseti* and *Candida albicans*. All the bacterial and fungal strains were clinical isolates, obtained from the Raja Muthaiah Medical College Hospital, Annamalai University, Annamalai Nagar, India.

2.5. Inoculum preparation for bacteria

Nutrient broth was prepared and sterilized in an autoclave at 15 lbs pressure for 15 min. All the ten bacterial strains were individually inoculated in the sterilized nutrient broth and incubated at 37 ℃ for 24 h. Mueller Hinton agar (MHA, Himedia) was prepared, sterilized in an autoclave at 15 lbs pressure for 15 minutes and poured into sterile petridishes and incubated at 37 ℃ for 24 h. The 24 hour-old bacterial broth cultures were inoculated in the petridishes by using a sterile cotton swab.

2.6. Inoculum preparation for fungi

Czapek dox (Hi-media) broth was prepared and sterilized in an autoclave at 15 lbs pressure for 15min. Five fungal strains were inoculated in the broth and incubated at 37℃ for 72 h. The sterilized Czapek dox agar was poured into sterile petridishes and incubated at 37℃ for 3 days. The 72 hour -old fungal broth cultures were inoculated in the petridishes using a sterile cotton swab.

2.7. Disc diffusion method

Antibacterial and antifungal activity was determined following the method of El-Masry[21]. The stock solution of polysaccharide and methanolic extracts was prepared at a concentration of 100 mg/mL. Sterile antimicrobial disc (Hi-media) was impregnated with 50 μL of polysaccharide and methanolic extract of the four concentrations tested. Positive control disc containing 50 μL of tetracycline (1 mg/mL) and as negative control, 50 μL of 10 mM EDTA and methanol were used. These impregnated discs were allowed to dry at laminar air flow chamber for 3 hours, and were placed at the respective bacterial and fungal plates and incubated at 37℃ for 24 h for bacteria and 72 hours for fungi. The diameter (mm) of the growth inhibition halos produced by the polysaccharide and methanolic extract of cephalopods was examined. Result was calculated by measuring the zone of inhibition in millimeters. All the tests were performed in triplicate.

2.8. Determination of the minimum inhibitory concentration

The polysaccharide and methanolic extracts were selected for the determination of MIC following the method of

Rajendran and Ramakrishnan[22].

2.9. Statistical analysis

Data on the inhibitory effects of polysaccharide and methanolic extracts of cephalopods was analyzed by one-way analysis of variance (ANOVA) using SPSS-16 version software followed by Duncun's multiple range test (DMRT) and Mean ± SEM. P values at <0.05 were considered for describing the significant levels.

3. Results

The polysaccharide and methanolic extracts of *S. prashadi* showed antimicrobial activity against all pathogenic bacteria; but at the same time they didn't show any activity against fungi. The activity was highest in 100% concentration, lowest in 25% concentration and there was no activity in negative control (Table 1).

In 100% concentration, the highest inhibition zone of (13 ±1.53) mm was noticed against *V. parahaemolyticus* in polysaccharide extract, and (12±1.25) mm inhibition zone was recorded against *S. aureus* in methanolic extract. The lowest inhibition zone of (8±0.82) mm was observed against *S. pneumoniae* in polysaccharide extract and (7± 0.58) mm against *Salmonella* sp. in methanolic extract. In 75% concentration, polysaccharide extract showed highest activity of (11±1.00) mm against *S. aureus* and (10±1.53) mm for methanolic extract. The lowest activity with (7± 0.58) mm inhibition zone was observed against *Salmonella* sp. in polysaccharide extract and (7±0.82) mm against *K. pneumoniae* in methanolic extract.

In 50% concentration, the maximum inhibition zone of (11 ±0.82) mm was recorded against *Streptococcus* sp., in polysaccharide extract, and (9±0.82) mm against *S. aureus* in methanolic extract. The lowest activity of (7±0.58) mm against *V. alginolyticus* in polysaccharide extract and same level of inhibition against *P. aeruginosa*, *V. alginolyticus* and *Streptococcus sp*. was noticed for methanolic extract. In 25% concentration, maximum activity of (8±0.58) mm was recorded against *S. aureus* in polysaccharide extract and (7 ±0.58) mm against *S. aureus*, *V. parahaemolyticus* and *S. pnemoniae* in methanolic extract. But at the same time, no activity was recorded against all the fungal strains studied.

3.1. MIC of the active extract against the test organisms

The MIC results are given in Table 2. MIC values of polysaccharide against bacterial strains such as *V. cholerae*, *K. pneumoniae*, *V. alginolyticus*, *S. aureus*, *V. parahaemolyticus* and *Streptococcus* sp., were reported as 80, 100, 80, 80, 60 and 80 mg/mL respectively. Whereas for methanolic extract, the MIC for *V. cholerae*, *S. aureus*, *V. parahaemolyticus*, *S. pneumoniae* and *E. coli* was recorded as 100, 80, 100, 100 and 100 mg/mL respectively.

Table 1
Antibacterial activity of *S. prashadi*.

Bacterial strains	Inhibition zone in polysaccharide extract from cuttlebone (mm)				Inhibition zone in methanolic extract form body tissues (mm)				Positive control (Tetracycline) (mm)	Negative control (Methanol & EDTA) (mm)
	25% conc	50 % conc	75% conc	100% conc	25% conc	50 conc	75% conc	100 % conc		
V. cholerae	7± 0.58	9±0.58	10±1.00	11±0.58	–	8±0.58	9±0.58	10±0.58	23±1.53	–
P. aeruginosa	–	–	9±0.58	10±0.82	–	7±0.58	8±0.58	9±1.25	24±1.53	–
K. pneumoniae	–	–	9±0.82	12±1.53	–	–	7±0.82	8±0.58	27±1.25	–
V. alginolyticus	–	7±0.58	10±0.82	11±1.25	–	7±0.58	8±0.58	9±0.82	28±1.53	–
S. aureus	8±0.58	9±0.82	11±1.00	12±1.25	7±0.58	9±0.82	10±1.53	12±1.25	26±1.25	–
V. parahaemolyticus	7±0.58	9±0.58	10±1.25	13±1.53	7±0.58	8±0.58	9±0.82	10±0.82	24±1.53	–
Streptococcus sp.	–	11±0.82	10±0.82	12±1.25	–	–	–	–	22±1.25	–
S. pneumoniae	–	–	–	8±0.82	7±0.58	8±0.58	9±0.58	10±0.82	18±0.82	–
Salmonella sp.	–	–	7±0.58	10±0.82	–	–	–	7±0.58	29±1.53	–
E. coli	–	–	–	–	–	7±0.58	9±0.58	11±0.82	11±0.58	–

conc: Conncentration; * The statistical significance: P values <0.05 (DMRT).

Table 2
MIC of polysaccharide and methanolic extracts of *S. prashadi* against tested microorganisms.

Bacterial strains	Polysaccharide extracts (mg/mL)					Methanolic extracts (mg/mL)				
	100	80	60	40	20	100	80	60	40	20
V. cholerae	–	*	++	+++	+++	*	+	++	+++	+++
P. aeruginosa	+	++	+++	+++	+++	+	++	+++	+++	+++
K. pneumoniae	*	+	++	++	+++	+	++	+++	+++	+++
V. alginolyticus	–	*	+	++	+++	+	++	+++	+++	+++
S. aureus	–	*	+	++	+++	–	*	+	++	+++
V. parahaemolyticus	–	–	*	+	++	*	+	++	+++	+++
Streptococcus sp.	–	*	+	++	+++	++	+++	+++	+++	+++
S. pneumoniae	+	++	+++	+++	+++	*	+	++	+++	+++
Salmonella sp.	+	++	+++	+++	+++	+	++	++	+++	+++
E. coli	++	++	+++	+++	+++	*	+	++	+++	+++

* MIC concentration; – No growth; + Cloudy solution (slight growth); ++ Turbid solution (strong growth); +++ Highly turbid solution (dense growth).

4. Discussion

In recent years, great attention has been paid to the bioactivity of natural products because of their potential pharmacological utilization. Most homeopathic medicines are either of plant or animal origin. Several molecules extracted from marine invertebrates, including bivalves, possess broad spectrum antimicrobial activities, affecting the growth of bacteria, fungi and yeasts[23]. The overall objective of the current study was to compare the ability of antibacterial and antifungal activity of methanolic extract of the body tissue and polysaccharide extract from the cuttlebone of *S. prashadi*. The results of the present study clearly showed that the 75% and 100 % concentrations exhibited appreciable antibacterial activity against human pathogens (Tables 1 & 2).

Antibacterial activity has previously been described in a wide range of molluscan species such as oyster (*Crassostrea virginica*), mussel (*Mytilus edulis* and *Geukensia demissa*), muricid mollusc (*Dicathais orbita*) and sea hare (*Dolabella auricularia*)[24-26]. Prem Anand and Patterson Edward reported moderate antibacterial and antifungal activity from the extracts of five species of *Cypraea* namely *C. errones*, *C. arabica*, *C. onyx*, *C. tigris* and *C. vitellas*[27]. Patterson and Murugan reported the broad spectrum of antibacterial activity for aqueous extract of ink of the cephalopods *Loligo duvaucelii* (*L. duvaucelii*) and *Sepia pharaonis* against nine human pathogens[28]. However the majority of marine organisms are yet to be screened for discovering useful antibiotics.

There are only a few studies carried out hitherto on the antibacterial activity of the internal bone of cephalopods. Barwin Vino[29], for the EDTA extract (polysaccharides) of *Doryteuthis sibogae* (*D. sibogae*) gladius reported 10 mm inhibition zone against *E. coli* and *K. pneumoniae*, 9 mm inhibition zone against *S. aureus* and 7 mm against *Salmonella typhi* (*S. typhii*) (excluding the disc size of 5 mm dia). Whereas the EDTA extract of *L. duvauceli* gladius showed only low activity *i.e.*, 5 mm against *P. aeruginosa*, 4 mm against *S. typhi* and *E. coli* (excluding the disc size of 5mm dia). At the same time, the gladius extract of both the species showed no activity against *V. cholerae*. The polysaccharide extract from the gladius of *D. sibogae* recorded potent antibacterial activity against all the bacterial strains mentioned above and at the same time the polysaccharide of the *L. duvaucelii* gladius extract recorded only low activity. The polysaccharide extract from the gladius of *L. duvauceli* showed activity against the fungi such as *Aspergillus fumigatus* (*A. fumigatus*), *Aspergillus flavus* (*A. flavus*) and *Rhizopus* sp.; whereas gladius extract of *D. sibogae* reported activity against *A. fumigatus* and *Rhizopus* sp. only. But at the same time both the species showed no activity against *Candida* sp. at all the concentrations tested.

Shanmugam *et al*[30] observed that the antibacterial activity was predominant among cuttlebone extracts (using EDTA) of the cuttlefishes such as *Sepia aculeata* (*S. aculeata*) and *Sepia brevimana* (*S. brevimana*) against almost all the 9 pathogenic bacterial strains tested *viz.*, *Bacillus subtilis*, *E. coli*, *K. pnemoniae*, *S. aureus*, *V. parahaemolyticus*, *V. cholerae*, *S. typhi*, *P. aeruginosa* and *Shigella* sp. The activity was recorded in almost all the concentrations except in negative control. The cuttlebone extract of *S. aculeata* and *S. brevimana* against four fungal stains such as *A. fumigatus*, *A. flavus*, *Candida* sp. and *Rhizopus* sp. showed the maximum antifungal activity in 100% concentration and the activity was found to be in an increasing order from the lower to higher concentration. On comparison the activity was higher in the cuttlebone extract of *S. aculeata* than *S. brevimana*. But in the present study at 100% concentration, the polysaccharide extract from the cuttlebone reported (13±1.53) mm of inhibition zone against *V. parahaemolyticus* and the methanolic extract from the body tissue showed (12±1.25) mm of inhibition zone against *S. aureus*. This highest activity of polysaccharides may be due to the destruction of cell wall of *V. parahaemolyticus* and *S. aureus*. This result was consistent with that of oleoyl-chitosan, which could destroy cell wall of *S. aureus* and lead to intracellular components being released[31]. Water-soluble intracellular protein of *S. aureus* treated with the polysaccharide increased rapidly, which indicated that cell wall and membrane of *S. aureus* might be disrupted by the polysaccharide [32].

When compared to the above mentioned studies, polysaccharides (EDTA) extract from *S. prashadi* cuttlebone exhibited better activity. In the present study the activity was also dose dependent. At the same time, the zone of inhibition was relatively higher than that of the previous above study. However there exists a difference in the activity shown by the compound(s) present in the extracts, in laboratory studies and natural environment while may be due to their varying concentration present in the extracts used in both the places[33].

Although different species and experimental procedures were used in the different studies, they indicated the high frequency of detectable antimicrobial activity in marine molluscs. These results enforce the idea that cephalopods are a source to be considered in the discovery of new substances for drug development. In the present investigation, there exists antibacterial against different bacterial strains. But at the same time no antifungal activity was recorded in both polysaccharide and methanolic extracts studied. Premanand and Patterson also reported no antifungal activity in the methanolic extracts from *Didemnum psammathodes*[27]. The known antimicrobial mechanisms associated to each group of chemical to which the isolated compounds belong may explain the antimicrobial potency of the crude extracts and compounds from *Vismia laurentii*. Membrane disruption could be suggested as one of the likely mechanisms of action of friedelin[34].

In the present study a wide spectral antibacterial activity has been recorded in almost all the concentrations. On comparison the maximum zone of inhibition was obtained for the methanolic extract than the polysaccharide extract from *S. prashadi* which explains and supports the presence of active principle in both the methanolic and polysaccharide extracts obtained from the body tissue and cuttlebone of *S. prashadi*. Further the dose as well as the concentration of the active principle in the extract shows either 'bactericidal' or 'bacteriostatic' action against the bacteria *i.e.* a low dose of a 'bactericidal' antibacterial agent may only inhibit bacterial growth, while a high dose of a 'bacteriostatic' antibacterial agent will be bactericidal[35]. Further investigation on the purification and chemical elucidation of the active principle present in both the extracts shall pave the way for the development of either the base or a new drug itself in future.

Conflict of interest statement

We declare that we have no conflict of interest.

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