

Full Length Research Paper

***In vitro* evaluation of antimicrobial activity of methanolic extract from selected species of Cephalopods on clinical isolates**

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Bioactive substances from marine biota have been found useful as special tools in pharmacological and biomedical research. In the present study, the *in vitro* antimicrobial activity of crude methanolic extracts of six species of cephalopods (*Sepia kobeensis*, *Sepiella inermis*, *Sepioteuthis lessoniana*, *Octopus aegina*, *Octopus aerolatus*, *Octopus dollfus*) from Cuddalore (Southeast coast of India) was studied. The antimicrobial activity was screened against 10 species of clinically isolated human pathogenic bacteria namely *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 equisetii* and *Candida albicans*. Different concentrations such as 25, 50, 75 and 100% were prepared and tested against the microbial strains for their inhibitory activities, using the disc diffusion method. The minimum inhibitory concentration (MIC) of methanolic extract of cephalopods ranged from 60 to 100 mg/ml. The results were discussed in the light of positive and negative control apart from the concentrations tested.

Key words: Antimicrobial activity, cephalopods, minimum inhibitory concentration (MIC), pathogenic microorganisms

INTRODUCTION

The Class: Cephalopoda includes nautilus, cuttlefishes, squids and octopods which are exclusively marine, varying in their form, size and nature (Voss and Williamson, 1971; Voss, 1973, 1977; Worms, 1983). They occupy littoral and benthic to pelagic environments of oceans. Cephalopods are important as a food resource as well as animal models in scientific investigations (Ngoile, 1987) and they are the store-house of many biologically important substances. There is an everlasting 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 re-emerging infectious diseases. A further big concern is the development of resistance to the antibiotics in current clinical use (Ilhan et al., 2007).

The first systematic search for antibiotics resulted in the discovery of actinomycin from *Actinomycete* bacteria. In United States and Japan between 1953 and 1970 approximately 85% of the antibiotics were produced by *Actinomycetes*, 11% by fungi and 4% by bacteria (Reiner, 1982). Although antibiotics are life saving drugs but now-a-days, due to careless and promiscuous use of antibiotics, various pathogenic microbes are gaining resistance. Among marine invertebrates, cephalopods belong to a molluscan group comprising of 700 species in which bacterial associations have been known for a long time (Pierantoni, 1917; Bloodgood, 1977) which include the reproductive organs (accessory nidamental glands) of

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myopsids, sepiolids and sepiids (Kaufman et al., 1998; Grigioni et al., 2000; Pichon et al., 2005) and the light organ of sepiolids (McFall-Ngai and Ruby, 1991; Nishiguchi, 2002).

Marine invertebrates offer a good source of potential antimicrobial drugs (Bansemir et al., 2006; Mayer et al., 2007; Jayaraj et al., 2008). Discovered bioactive compounds in molluscs were identified essentially as peptide, depsipeptide, sterols, sesquiterpene, terpenes, polypropionates, nitrogenous compounds, macrolides, prostaglandins and fatty acid derivatives, miscellaneous compounds and alkaloids which presented specific types of activities (Maktoob and Ronald, 1997; Balcázar et al., 2006; Blunt et al., 2006). Studies on antimicrobial activity that lead to valuable information for new antibiotic discoveries and give new insights into bioactive compounds in aquacultured molluscs. In most of the publications concerning antimicrobial activity in molluscs, either single body compartments alone, like haemolymph and egg masses, or extracts of whole body have been tested for activity. Recently crustaceans (Haug et al., 2002a) and echinoderms (Haug et al., 2002b) are reported to possess antibacterial factors in different tissues. The antimicrobial activity of polysaccharides extracted from cephalopods such as *Sepia aculeata* and *Sepia brevimana* and heparin and heparin – like glycosaminoglycans (GAGs) from the cephalopod *Euprymna berryi* was reported against the human pathogenic microorganism (Shanmugam et al., 2008a; Shanmugam et al., 2008b). In the present investigation, an attempt has been made to screen the antibacterial and antifungal activity of the crude methanolic extract of selected cephalopods on some important human pathogens.

MATERIALS AND METHODS

Sampling and identification

Cephalopods such as, *S. kobeensis*, *S. inermis* (Cuttlefishes), *S. lessoniana* (Squid), *Octopus aegina*, *O. aerolatus* and *O. dollfusi* (Octopods), used in this study were obtained from Cuddalore landing centre (Latitude 10° 42'N; Longitude 79° 46'E) which is situated in the Southeast coast of India. The studies carried out by Voss (1973), Voss and Williamson (1971), Roper et al. (1984), Jothinayagam (1987) and Shanmugam et al. (2002) have been of considerable help on developing the identification keys and description which in most cases have also been corroborated with examination of actual specimen.

Preparation of extracts

Cephalopods 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 for 24 - 48 h (Ely et al., 2004). Then the methanolic extract was centrifuged to collect the supernatant and concentrated under vacuum in a rotary evaporator (LARK, Model: VC-100A) at low temperature. The crude methanolic extract was assayed for antibacterial and antifungal activities using standard disc diffusion method.

Microbial cultures

Ten species of bacteria and five species of fungi were used as test organisms. (Bacterial strains- Gram- positive: *Staphylococcus aureus*, *Streptococcus sp.*, *Streptococcus pneumoniae*; Gram-negative: *Escherichia coli*, *Vibrio cholerae*, *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Salmonella sp.*; Fungal strains- *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, and Annamalai Nagar, India.

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°C for 24 h. Mueller Hinton Agar (MHA, Himedia) was prepared, sterilized in an autoclave at 15lbs pressure for 15 min and poured into sterile petridishes and incubated at 37°C for 24 h. The 24 h old bacterial broth cultures were inoculated in the petridishes by using a sterile cotton swab.

Inoculum preparation for fungi

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

Disc diffusion method

Antibacterial and antifungal activity was determined following the method of (El-Masry et al., 2000). Briefly, a suspension of each tested microorganism was carefully mixed in the tube containing bacterial and fungal inoculums and media for bacterial and fungal were plated separately, respective strains were cotton swabbed on petridishes. Sterile antimicrobial disc (Hi-media) was impregnated with 50 µl of crude methanolic extract of the four concentrations tested. Positive control discs containing 50 µl of tetracycline (1 mg/ml) and negative control, 50 µl of methanol were used. The stocks for methanolic extracts were prepared in the concentration of 100 mg/ml. These impregnated discs were allowed to dry at laminar air flow chamber for 3 h, and were placed at the respective bacterial and fungal plates and incubated at 37°C for 24 h for bacteria and 72 h for fungi. The diameter (mm) of the growth inhibition halos produced by the methanolic extracts of cephalopods was examined. Result was calculated by measuring the zone of inhibition in millimeters. All the tests were performed in triplicates.

Determination of the minimum inhibitory concentration (MIC)

The methanolic extract of cephalopods which showed significant antimicrobial activity was selected for the determination of MIC followed by the method of Rajendran and Ramakrishnan (2009). A stock solution of 100 mg/ml was prepared and was serially diluted to obtain various ranges of concentrations between 20 and 100 mg/ml. 0.5 ml of each of the dilutions of different concentrations was transferred into sterile test tube containing 2.0 ml of nutrient broth. To the test tubes, 0.5 ml of test organism previously adjusted to a concentration of 10⁵ cells/ml was then introduced. A set of test

Table 1. Antibacterial activity of methanolic extract of cephalopods.

Bacterial strains	<i>S. inermis</i> (%)				<i>S. kobiensis</i> (%)				<i>S. lessoniana</i> (%)				<i>O. aegina</i> (%)				<i>O. dollfusi</i> (%)				<i>O. aerolatus</i> (%)			
	25	50	75	100	25	50	75	100	25	50	75	100	25	50	75	100	25	50	75	100	25	50	75	100
<i>Vibrio cholerae</i>	-	+	+	++	-	-	-	-	-	++	++	++	-	+	+	++	-	-	+	+	-	+	+	++
<i>P. aeruginosa</i>	+	+	+	+	+	+	+	++	-	+	+	++	-	-	-	-	-	-	-	-	-	-	-	-
<i>Klebsiella pneumoniae</i>	+	+	++	++	-	+	+	++	-	-	+	++	-	-	+	++	-	-	-	-	-	-	-	-
<i>V. alginolyticus</i>	-	-	-	-	-	-	-	++	+	+	+	++	+	+	+	++	+	+	++	++	-	-	-	++
<i>Staphylococcus aureus</i>	+	+	++	++	-	-	-	-	-	+	++	++	-	-	-	+	-	+	++	++	+	+	+	++
<i>V. parahaemolyticus</i>	-	+	+	+	-	-	-	-	+	+	+	++	+	+	++	++	-	+	+	+	-	-	-	++
<i>Streptococcus sp.</i>	+	+	+	++	-	+	+	++	+	+	++	++	-	-	-	-	+	++	++	++	-	-	-	+
<i>S. pneumoniae</i>	-	+	+	+	-	-	-	++	-	-	-	-	-	+	+	++	-	-	+	+	-	-	-	-
<i>Salmonella sp.</i>	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	+	++	++	+++	-	-	+	++
<i>E. coli</i>	-	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	++	++	+++	-	-	-	-

(-) No activity, (+) Weak activity (7-10 mm dia.), (++) Good activity (11-15 mm dia.), (+++) Very good activity (above 16mm dia.) *The statistical significance: P values ≤ 0.05 (DMRT).

tubes containing broth alone was used as control. All the test tubes and control were then incubated at 37°C for 24 h. After the period of incubation, the tube containing the least concentration of extract showing no visible sign of growth was taken as the minimum inhibitory concentration.

Statistical analysis

Data on the inhibitory effect of methanolic extracts of cephalopods was analyzed by one-way analysis of variance (ANOVA) using SPSS-16 version software followed by Duncan's multiple range test (DMRT). P values ≤ 0.05 were considered as significant.

RESULTS

The methanolic extract of cephalopods showed antibacterial and antifungal activity against all pathogenic strains which were concentration-dependent i.e., the activity was higher in 100% concentration and lower in 25% concentration but activity was absent in negative control (Table 1).

In 100% concentration, the highest inhibition

zone of 17 mm was observed against *E. coli* in *O. dollfusi* extract, 15 mm against *S. aureus* in *S. inermis* extract, 15 mm against *V. parahaemolyticus* in *S. lessoniana* extract, 15 mm against *V. parahaemolyticus* in *O. aegina* extract, 14 mm against *K. pneumoniae* in *S. kobiensis* extract and 13 mm against *S. aureus* in *O. aerolatus* extract. The lowest inhibition zone of 8 mm was observed against *Salmonella sp.* in *S. inermis* extract, 9 mm against *S. pneumoniae*, *E. coli*, *S. aureus* and *Streptococcus sp.* in *O. dollfusi*, *S. lessoniana*, *O. aegina* and *O. aerolatus* extract respectively and 11 mm against *S. pneumoniae* in *S. kobiensis* extract.

In 75% concentration, methanolic extract showed the maximum activity of 14 mm against *E. coli* in *O. dollfusi* extract, 13 mm against *V. parahaemolyticus* in *O. aegina* extract, 13 mm against *V. cholerae* in *S. lessoniana* extract, 12 mm against *K. pneumoniae* in *S. inermis* extract, 10 mm against *K. pneumoniae* in *S. kobiensis* extract and 10 mm was exhibited against *Salmonella sp.* in *O. aerolatus* extract. The

minimum activity of 7 mm against *K. pneumoniae* in *O. aegina* extract, 8 mm against the *K. pneumoniae* in both *S. kobiensis* and *S. lessoniana* extract, 8 mm against *V. cholerae* and *Salmonella sp.* in *O. dollfusi* and *O. aerolatus* extract respectively.

In 50% concentration, the maximum activity of 12 mm was noticed against *E. coli* in *O. dollfusi* extract, 11 mm against *V. cholerae* in *S. lessoniana* extract, 10 mm against *V. parahaemolyticus* in *O. aegina* extract, 10 mm against *K. pneumoniae* in *S. inermis* extract, 8 mm against *K. pneumoniae* in *S. kobiensis* extract and 8 mm against *V. cholerae* in *O. aerolatus* extract. The minimum activity of 7 mm was recorded against *E. coli*, *P. aeruginosa*, *V. alginolyticus*, *V. cholerae* in *S. inermis*, *S. kobiensis*, *S. lessoniana* and *O. aegina* extract respectively and 7 mm was recorded against *S. aureus* in both *O. dollfusi* and *O. aerolatus* extracts.

In 25% concentration, maximum activity of 10 mm was recorded against *Salmonella sp.* in *O.*

Table 2. MIC of methanolic extracts of cephalopods against clinically isolated human pathogens.

Bacterial strains	<i>S. inermis</i> (mg/ml)					<i>S. lessoniana</i> (mg/ml)					<i>O. dollfusi</i> (mg/ml)				
	100	80	60	40	20	100	80	60	40	20	100	80	60	40	20
<i>Vibrio cholerae</i>	*	+	++	+++	+++	-	*	+	++	+++	*	+	++	+++	+++
<i>P. aeruginosa</i>	+	++	+++	+++	+++	*	+	++	++	+++	++	++	+++	+++	+++
<i>Klebsiella pneumoniae</i>	-	*	+	++	+++	*	+	++	+++	+++	++	++	+++	+++	+++
<i>V. alginolyticus</i>	++	+++	+++	+++	+++	*	+	++	++	+++	*	+	++	++	+++
<i>Staphylococcus aureus</i>	-	*	+	++	+++	-	*	+	++	+++	*	+	++	+++	+++
<i>V. parahaemolyticus</i>	+	+	++	+++	+++	*	+	++	+++	+++	+	++	+++	+++	+++
<i>Streptococcus</i> sp.	-	*	+	++	+++	-	*	+	++	+++	-	*	+	++	+++
<i>S. pneumoniae</i>	*	+	++	+++	+++	++	+++	+++	+++	+++	*	++	+++	+++	+++
<i>Salmonella</i> sp.	+	++	+++	+++	+++	+	++	+++	+++	+++	-	*	+	++	+++
<i>E. coli</i>	*	+	++	+++	+++	+	++	+++	+++	+++	-	-	*	+	+++

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

dollfusi extract, 8mm against *Streptococcus* sp. in *S. lessoniana* extract, against *V. parahaemolyticus* in *O. aegina* extract and against *K. pneumoniae* in *S. inermis* extract. The lowest activity of 7 mm against *V. alginolyticus* in both *O. aegina* and *O. dollfusi* extract, against *P. aeruginosa* in *S. inermis* extract and the same level of inhibition found *V. parahaemolyticus* in *S. lessoniana* extract. But at the same time, no activity was recorded against all the fungal strains studied.

MIC of the active extract against the test organisms

The MIC results are given in Table 2. MIC values of *S. inermis* against bacterial strains such as *V. cholerae*, *K. pneumoniae*, *S. aureus*, *Streptococcus* sp., *S. pneumoniae* and *E. coli* showed 100, 80, 80, 80, 100 and 100 mg/ml respectively. In *S. lessoniana* the MIC for *V. cholerae*, *P. aeruginosa*, *K. pneumoniae*, *V. alginolyticus*, *S. aureus*, *V. parahaemolyticus* and *Streptococcus* sp.

was recorded as 80, 100, 100, 100, 80, 100 and 80 mg/ml respectively. Whereas in *O. dollfusi* the MIC for *V. cholerae*, *V. alginolyticus*, *S. aureus*, *Streptococcus* sp., *S. pneumoniae*, *Salmonella* sp. and *E. coli* was recorded as 100, 100, 100, 80, 100, 80 and 60 mg/ml respectively.

DISCUSSION

The overall objective of the current study is to compare the ability of antibacterial and antifungal activity of crude methanolic extract of six species of cephalopods collected from the same habitat. In the present study the results clearly showed (Tables 1 and 2) that majority of extracts exhibited appreciable antimicrobial activity against most of the clinically isolated human pathogens. The effects of extracts were different for different bacterial strains. Interestingly the present study reported no antifungal activity for all methanolic extracts from the cephalopods studied.

In recent years, great attention has been paid to

study the bioactivity of natural products due to their potential pharmacological utilization. The rationale of searching for drugs from marine environment stems from the fact that marine plants and animals have adapted to all sorts of habitats in the marine environment and these creatures are constantly under tremendous selection pressure including competition for space, predation, surface fouling and reproduction. Many of these organisms are showing antimicrobial properties. Although most of the antibacterial agents isolated from marine sources have not been active enough to compete with classical anti-microbials obtained from microorganisms (Rinehart et al., 1981). However, majority of marine organisms are yet to be screened for discovering useful antibiotics.

Antibacterial activity has previously been described in a wide range of molluscan species such as oyster (*C. virginica*), mussel (*Mytilus edulis* and *Geukensia demissa*), muricid mollusks (*Dicathais orbita*) and sea hare (*Dolabella auricularia*) (Constantine et al., 1975; Gunthorpe

and Cameron, 1987; Prem Anand et al., 1997; Anderson and Beaven, 2001; Benkendorff et al., 2001). In most of the species studied, the haemolymph, egg masses or the whole body have been tested for activity. Antimicrobial peptides have been isolated and characterized from the haemocytes of *Mytilus edulis* (Charlet et al., 1996; Mitta et al., 2000a) and *M. galloprovincialis* (Hubert et al., 1996; Mitta et al., 1999; Mitta et al., 2000b), and from the seahare *Dolabella auricularia* (Iijima et al., 2003). In the present investigation the crude methanolic extract from the whole body tissue of the cephalopods was used to study the antimicrobial activity against selected human pathogens.

Prem Anand and Patterson Edward (2002) reported moderate antibacterial and antifungal activity from the extracts of various bivalves molluscs. Patterson and Murugan (2000) reported broad spectrum of antibacterial activity for aqueous ink extract of the cephalopods *L. duvacei* and *S. pharaonis* against nine human pathogens.

EDTA extract (polysaccharides) of *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 *S. typhii*. Whereas the EDTA extract of *L. duvacei* showed only low activity i.e., 5 mm against *P. aeruginosa*, 4mm against *S. typhii* and *E. coli*. 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 extract of the *L. duvacei* gladius recorded only low activity. The polysaccharides extracted from the gladius of *L. duvacei* showed activity against the fungi such as *A. fumigatus*, *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 (Barwin vino, 2003). The cuttlebone extract (using EDTA) of *S. aculeata* and *S. brevimana* showed antibacterial activity against almost all the 9 pathogenic bacterial stains tested viz., *B. subtilis*, *E.coli*, *K. pneumoniae*, *S. aureus*, *V. parahaemolyticus*, *V. cholerae*, *S. typhii*, *P. aeruginosa* and *Shigella* sp. The activity was recorded in almost all the concentrations except in negative control. The antifungal activity of cuttlebone extracts of *S. aculeata* and *S. brevimana* against four fungal stains such as *A. fumigatus*, *A. flavus*, *Candida* sp. and *Rhizopus* sp. showed the maximum activity of 100% and 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* (Shanmugam et al., 2008a).

Shanmugam et al. (2008b) reported that the crude and purified sample of Glycosaminoglycans (GAGs) from *E. berryi* showed activity against five pathogenic bacteria and four fungal strains. The activity was higher in 100%

concentration and lower in 25% concentration; but activity was absent in negative control. The maximum antibacterial activity was shown in *Shigella* sp. (5 mm) for crude sample and *S. aureus* for purified sample. The minimum antibacterial activity showed (1.5 mm) against *E. coli* in crude sample. The maximum antifungal activity (5.5 mm) was observed against *C. albicans* and *A. fumigatus* in crude and purified sample, respectively. The minimum antifungal activity (0.5 mm) was recorded against *A. fumigatus* and *C. neoformans* in crude sample.

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 to control microbial diseases. In the present investigation highest inhibition was recorded in *Salmonella* sp. and *E. coli* against *O. dollfusi* extract. Kagoo and Ayyakkannu (1992) reported a broad spectral activity in the hypobranchial gland extract of *Chicoreus ramosus* against 10 bacterial strains. In the present study a wide spectrum of antibacterial activity has been recorded in almost all the extracts, (Table 1).

Conclusion

Cephalopods collected from Cuddalore landing centre, southeast coast of India showed potential antibacterial activity against human pathogenic bacterial strains. In the present evaluation good antimicrobial activity was seen in the extracts of *O. dollfusi*, *S. lessoniana* and *S. inermis* species, which indicates the presence of potent antimicrobial compounds in them. Further research on purification and characterization of these potentially active compounds, may pave the way for the development of potent antibacterial drugs.

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