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# Antibacterial Activity of Methanolic Extract of Whole Body Tissue and Ethylene Diamine Tetra Acetate Extract of Cuttlebone of *Sepiella inermis* (Orbigny, 1848)

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## ABSTRACT

Marine animals have great potential for providing novel drug leads with novel mechanism of action. The aim of the present study was to evaluate the antimicrobial potency of methanolic extract of whole body tissue of *Sepiella inermis* and Ethylene Diamine Tetra Acetate (EDTA) extract of cuttlebone (polysaccharides) against ten human pathogens. *S. inermis* tissue was extracted with 100% methanol for about 48 h, centrifuged and supernatant was concentrated under vacuum in desiccator. The polysaccharide extract was obtained from the internal shell using 10 mM hot EDTA. The final product was used for assaying the antibacterial activity by disc diffusion method in different concentrations. In 100% concentration, the highest inhibition zone of 12 mm was observed against *Klebsiella pneumoniae* and *Staphylococcus aureus* in methanolic extract of whole body tissue and *Staphylococcus aureus* alone in EDTA extract from cuttlebone. In 75% concentration, methanolic extract showed highest activity of 9 mm against *K. pneumoniae, Staphylococcus aureus* and *Staphylococcus pneumoniae* whereas the EDTA extract showed highest activity 8 mm against *Vibrio cholerae, Klebsiella pneumoniae* and *Vibrio alginolyticus*. In 50% concentration, the maximum activity of 9 mm was recorded against *Klebsiella pneumoniae* and *Staphylococcus aureus* in methanolic extract; whereas maximum activity (11 mm) was recorded against *E. coli* in EDTA extract. In 25% concentration, the both maximum and minimum activity 7 mm was recorded against *Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus* and *Streptococcus aureus* in methanolic extract; *Vibrio alginolyticus* in EDTA extract. This study reveals that both the methanolic extract of the whole body tissue and EDTA extract of cuttlebone have good antimicrobial activity depending on the concentration.

**Key words:** Antimicrobial potency, methanolic extract, human pathogens, cephalopods

## INTRODUCTION

The marine organisms are quite interesting in having several new compounds that show many important biological activities which are under investigation by researchers all over the world and provide many leads and basic principles in the development of new pharmaceuticals (Faulkner, 2000a, b; Da Rocha *et al*., 2001). Molluscs are the second largest animal group represented by 100,000 species so far recorded which includes monoplacophorans to more specialized group, cephalopods that inhabit almost all the environments and habitats. The class: Cephalopoda

includes the nautilus, cuttlefishes, squids and octopods which are exclusively in marine and vary in their form, size and nature (Voss, 1973). Cephalopods are commonly available throughout the world. The cephalopod diversity in India is represented by 80 species and in Parangipettai 17 species are reported (Silas *et al*., 1985; Shanmugam *et al*., 2002). These are important as a food resource as well as in scientific research (Ngoile, 1987). Since there is an alarming increase in the resistance obtained by the microbes against majority of the antibiotics under clinical use, it becomes very important to look for novel natural products showing newer mechanism of action against those microbes (Bansemir *et al*., 2006; Ilhan *et al*., 2007). Among the invertebrates, the discovered bioactive compounds in mollusks were identified essentially as peptides, depsipeptides, sterols, sesquiterpenes, terpenes, polypropionates, nitrogenous compounds, macrolides, prostaglandins and fatty acid derivatives and alkaloids which presented specific types of activities (Balcazar *et al*., 2006). The crude products extracted from plants and animals are proving themselves as good source of many drugs or acting as a source of basic active principles (Kamboj, 1999). Though there are many studies made on mollusks to report their biomedical importance (particularly antimicrobial), they are from individual body parts of the mollusks (Rajaganapathi, 2001) like mucus from *Achatina fulica* (Kubota *et al*., 1985); egg mass and purple fluid from *Aplysia kurodai* (Yamazaki, 1993); body wall of *Dolabella auricularia* (Iijima *et al*., 2003). In this series of study, an antibacterial and antifungal peptide was isolated by Charlet *et al*. (1996) from the blood of immune-challenged and untreated *Mytilus edulis*.

The mytilin isoforms C, D and G1 were isolated from *Mytilus galloprovincialis* and exhibited complementary antimicrobial peptides (Mitta *et al*., 2000). In addition, a novel antifungal peptide that delays the growth of *Neurospora crassa* and *Fusarium culmorum*, mytomycin has been isolated and partially characterized in conjugation with defensins and mytilins from *M. edulis* (Charlet *et al*., 1996). The increasing resistance of antibiotics by the pathogenic microorganisms develops the demand for the isolation of novel alternative antimicrobial substances (Obeidat *et al*., 2012). Further these compounds are being extracted not only from the whole animal but also from different body parts including skeleton (internal shell in the case of cuttlefishes) which showed many pharmacological properties and hence medicinal value. Keeping the importance of mollusks as a potential source of many bioactive compounds, the cuttlefish *S. inermis* was taken to study the antimicrobial activity of the methanolic extract of whole body tissue and the polysaccharides extracted from the cuttlebone.

## MATERIALS AND METHODS

**Chemicals and reagents:** Muller Hinton Agar, Czapek Dox broth, Czapek Dox agar and sterile antimicrobial disc were purchased from Hi-media. Ethylene Diamine Tetra Acetate (EDTA) and Barium hydroxide were obtained from Loba Chem. Company. Acetone and Methanol were obtained from Merck. All other chemicals used were of analytical grade.

**Collection of animals:** The cuttlefish *S. inermis* was collected from Thondi landing centre, situated at Lat 9°44'N; Long 079°02'E, South East coast of India.

**Tissue extracts:** The extraction process was completed at a few steps. The cuttlebone and ink sac were removed from *S. inermis*; remaining tissues were cut into small pieces and extracted with 100% methanol for 24-48 h by incubating at room temperature. The extract was centrifuged to collect the supernatant and concentrated under vacuum in desiccators. The crude methanolic extract of whole body tissue was assayed for antibacterial activity using standard disc diffusion method.

**Isolation of polysaccharides:** The polysaccharide extract was obtained from the internal shell of *S. inermis* by following the 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 Hyflo Super Cel. Then saturated Barium hydroxide solution was added to the filtrate and allowed to stand overnight. Then the precipitate was collected on a filter paper (Whatman No.1) with Hyflo Super Cel and washed with distilled water. The dialyzate solution present in the dialysis membrane was then freeze-dried and white colour powder was obtained. The lyophilized powder was used for assaying the antibacterial activity.

**Microbial cultures:** The ten strains of bacteria used in the present study included Gram-positive: *Staphylococcus aureus*, *Staphylococcus pneumoniae* and *Streptococcus aureus*; Gram-negative: *Vibrio cholerae*, *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella* sp. and *E. coli*. All the bacterial strains were clinical isolates, obtained from Raja Muthayiah Medical College, Annamalai University.

**Extraction and preparation of stock solution:** Nutrient broth was prepared and sterilized at 15 lbs pressure for 15 min. Ten bacterial strains were inoculated in the sterilized Nutrient broth and incubated at 37° for 24 h. Mueller Hinton Agar (MHA) were prepared, sterilized in an autoclave at 15 lbs for 15 min pressure and poured into sterile petri dishes to incubate at 37° for 24 h. The 24 h bacterial broth cultures were inoculated in the petri dishes by using a sterile cotton swab. Then tissue extracts were impregnated in the sterile disc (5 mm dia) and after evaporation the discs were placed in respective swabbed plates. Both positive (tetracycline) and negative (methanol) control discs were also placed in all the plates.

The lyophilized powder of crude extract (stock solution) was prepared at a concentration of 1 mg 0.1 $mL^{-1}$ in 10 mM EDTA. From this 0.24, 0.18, 0.12 and 0.06 mL of sample was taken and each was made up to 0.24 mL with respective solvent to represent the corresponding concentration of 100, 75, 50 and 25%, respectively.

**Antibacterial assay:** Antibacterial activity of the whole body tissue extracts and polysaccharides from cuttlebone was determined by the agar disc diffusion method (Barry, 1980). Briefly, a suspension of each tested microorganisms was carefully mixed in a tube with 18 mL of MHA and then poured on petri plates. Sterile filter-paper discs (Whatman No.1; 5 mm in diameter) were impregnated with 50 μL of the extracts. Positive control discs containing 50 μL of Tetracycline and negative control containing 50 μL of methanol and 10 mM EDTA each were used. These plates were allowed to dry at room temperature for 3 h and after their impregnation in each petri plates, the petri plates were incubated at 37° for 48 h. The diameters of the inhibition zones were measured in millimeters. Each extract was tested thrice for the confirmation of their activity.

## RESULTS

The methanolic extraction of whole body tissue and the polysaccharides from cuttlebone of *S. inermis* showed activity against almost all pathogenic organisms. In general, the activity was higher in 100% concentration and lower in 25% concentration but activity was absent in negative control and positive control showed activity against all the bacterial strains (Fig. 1).

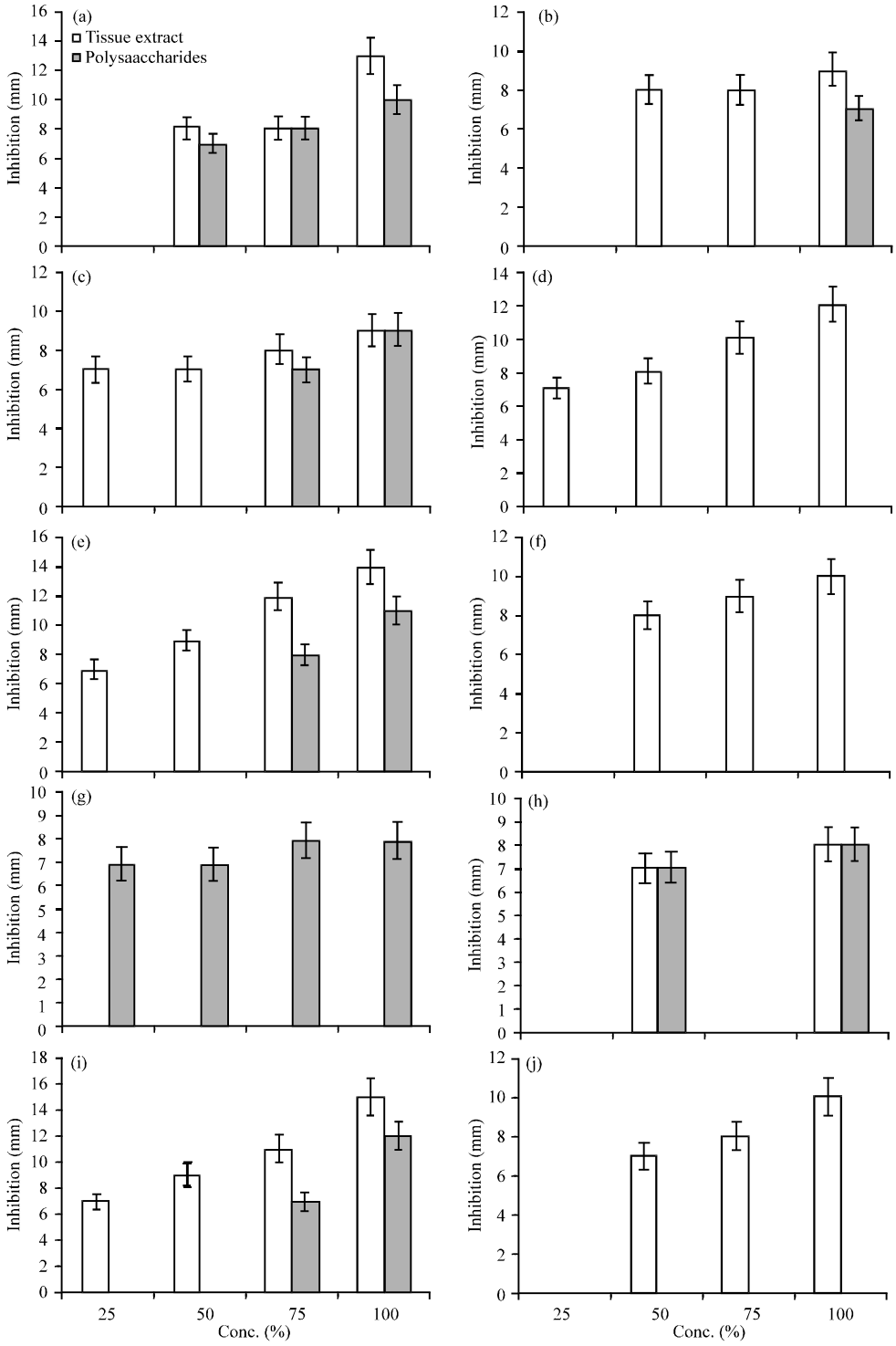


Fig. 1(a-j): The antibacterial activity of methanolic extract of whole body tissue and EDTA extract (polysaccharides) from cuttlebone of *S. inermis* against (a) *Vibrio cholerae*, (b) *Vibrio parahaemolyticus*, (c) *Pseudomonas aeruginosa*, (d) *Streptococcus aureus*, (e) *Klebsiella pneumoniae*, (f) *Staphylococcus pneumoniae*, (g) *Vibrio alginolyticus*, (h) *Salmonella* sp., (i) *Staphylococcus aureus* and (j) *E. coli*

In 100% concentration, the highest inhibition zone of 12 mm was observed against *K. pneumonia* and *S. aureus* in methanolic extract of whole body tissue and *S. aureus* alone in EDTA extract from cuttlebone. The lowest inhibition zone of 8 mm was observed against *V. parahaemolyticus* and *Salmonella* sp. in methanolic extract of whole body tissue and *V. parahaemolyticus* alone in EDTA extract from cuttlebone.

In 75% concentration, methanolic extract showed highest activity of 9 mm against *K. pneumonia*, *S. aureus* and *S. pneumoniae* whereas the EDTA extract showed highest activity of 8 mm against *V. cholerae*, *K. pneumoniae* and *V. alginolyticus*. The lowest activity of methanolic extract with 7 mm inhibition zone was observed against *P. aeruginosa*, *Salmonella* sp. and *E. coli*; whereas the EDTA extracts showed 7 mm inhibition zone observed against *P. aeruginosa* and *S. aureus*.

In 50% concentration, the maximum activity of 9 mm was recorded against *K. pneumoniae* and *S. aureus* in methanolic extract; whereas the maximum activity (11 mm) was recorded against *E. coli* in EDTA extract. The lowest activity of 7 mm was recorded against *P. aeruginosa*, *Salmonella* sp. and *E. coli* in methanolic extract and 7 mm against *V. cholerae*, *V. alginolyticus* and *Salmonella* sp. in EDTA extracts.

In 25% concentration, only 7 mm of inhibition zone was recorded against: *P. aeruginosa*, *K. pneumoniae*, *S. aureus* and *S. aureus* in methanolic extract; *V. alginolyticus* in EDTA extracts. There is no activity in all the concentrations of methanolic extract of *S. inermis* against *V. alginolyticus* and EDTA extract from cuttlebone of *S. inermis* against *S. aureus* and *S. pneumoniae*.

## 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 (Ray and Mukherjee, 1979). Several molecules extracted from marine invertebrates, including bivalves, reported broad-spectrum antimicrobial activities, affecting the growth of bacteria, fungi and yeasts (Mitta *et al*., 2000). The world's oceans, covering more than 70% of the earth surface represent an enormous resource for the discovery of potential chemotherapeutic agents. Because of the diversity of marine organisms and habitats, marine natural products encompass a wide variety of chemical class including terpenes, shikimate, polyketides, acetogenins and peptides, alkaloids of varying structures and multitude of compounds of mixed synthesis.

Antibacterial activity has previously been described in a wide range of molluscan species such as oyster (*C. virginica*), mussel (*M. edulis* and *Geukensia demissa*), muricid mollusks (*Dicathais orbita*) and sea hare (*Dolabella auricularia*) (Benkendorff *et al*., 2001). In most of the species studied, the haemolymph, egg masses or the whole body have been tested for their activity. However, by far the majority of marine organisms are yet to be screened and the potential for discovering a useful antibiotic is sufficient to warrant further research. This study dealt the antibacterial activity of methanolic extract of whole body tissue and EDTA extract (polysaccharides) from cuttlebone of *S. inermis*. The activity was recorded in almost all the concentration with variation.

The activity of methanolic extract of whole body tissue and EDTA extracts (polysaccharides) from cuttlebone was found to be high in 100% concentration than the three other concentrations. In general, the activity was found to be concentration dependent (Fig. 1).

The methanolic extract of whole body tissue of *S. inermis* showed activity against almost all the bacterial strains in the concentrations tested with the maximum activity of 12 mm in 100% against *K. pneumoniae* and *S. aureus* and the minimum activity of 7 mm in 25% concentration against *K. pneumoniae, S. aureus, P. aeruginosa* and *S. aureus*. The ethanolic extract of *Brillantaisia patula* was active against all the tested pathogens such as *S. aureus, Enterococcus faecalis, Proteus hauseri, P. aeruginosa* and *E. coli* whereas the methanolic extract inhibited all the bacterial growth except *S. aureus* (Faparusi *et al*., 2012). Hexane extract of *Senna didymobotrya* showed 16 mm of inhibition zone against *Microsporum gypseum* (Korir *et al*., 2012). Acetic acid extract of *Chlorophytum borivilianum* showed maximum antibacterial activity against *S. aureus* and least activity against *B. subtilis* (Sundaram *et al*., 2011).

The EDTA extract (polysaccharides) from cuttlebone showed antibacterial activity against all the bacterial strains except *S. aureus* and *S. pneumoniae* with the activity ranging from 7 mm inhibition zone in 25% concentration against *V. alginolyticus* to 12 mm in 100% concentration against *S. aureus*.

The results clearly showed that majority of the extracts exhibited appreciable antibacterial activity against human pathogens but reported varying activity against different bacterial strains. The level of activity measured by disc diffusion assay is dependent on both the rate of diffusion of extract into the agar and the potency of the extract. Extracts that contain highly active compounds (more potent) but have physical properties that generate a lower diffusion rate, may appear to have low activity in the assay (Kelman *et al*., 2006).

In the crude venom of hypobranchial gland of muricid gastropod *Rapana rapiformis*, the highest activity was recorded as 26 mm against *K. pneumoniae*, 22 mm against *V. cholerae* and 18 mm against *S. aureus* (Murugan *et al*., 1991). The hypobranchial gland extract of *Chicoreus ramosus*, inhibits the growth of ten bacterial strains; out of this, the broad inhibition zone was formed against *S. faecalis* and *S. aureus* (Kagoo and Ayyakkannu, 1992). The ethanol extracts of hypobranchial gland of *C. virgineus* showed 10 mm of inhibition zone against *S. typhi*, 7 mm against *Shigella flexneri*, 6 mm against *V. cholerae*, 5 mm against *K. pneumonia* and 4 mm against *B. subtilis* and *E. coli*; but methanol extract exhibited inhibition only against *S. pyogenes* (Rajaganapathi, 1996).

Anand and Edward (2001) recorded 10, 8 and 5 mm inhibition zone in the case of the ethanol extract of *T. delicatula, B. spirata, T. brunneus* and *L. arthritica* operculum, respectively against *B. subtilis*; but the activity was absent against *E. coli, K. pneumoniae, Proteus vulgaris, P. mirabilis, S. typhi, S. flexneri, S. aureus* and *V. cholerae*. Further the whole body tissue extracts of *Trochus radiatus* obtained with different solvents (acetone, ethyl acetate and dichloromethane) were screened for their antibacterial activity. All the extracts exhibited clear zones of inhibition for the seven of the nine human pathogens tested. The highest activity was formed against *Enterobacter aerogenes, S. aureus* and *E. coli* (MaryElizabeth *et al*., 2003).

In this context, the similar antibacterial activity was recorded by Rajaganapathi (2001), who has reported the broad-spectrum antibacterial activity for 13 species of molluscan extracts comprising seven gastropods, one bivalve and five cephalopods. The activity varied with extracts and bacterial species and the antibacterial activity was found to be greater in cephalopods than gastropods and bivalves. In study, the methanol and saline extracts of ink gland, salivary gland, body mucus and internal shell of cephalopods such as *Loligo duvauceli, Sepia pharaonis, Sepiella inermis, Octopus dollfusi* and *Cistopus indicus* recorded varying antibacterial activity against the different bacterial strains viz., *B. subtilis, E. coli, K. pneumoniae, P. vulgaris,*

*P. mirabilis*, *S. typhi*, *S. flexneri*, *S. faecalis* and *V. cholerae*. All the cephalopod extracts exhibited activity against at least three bacteria and the highest activity of 10.5 mm was recorded in the ink gland extract against *P. mirabilis*. Further, the methanol extracts of the cuttlebone of *S. pharaonis* showed activity against *S. flexneri* (5 mm), *S. faecalis* and *V. cholerae* (4.5 mm) and *S. typhi* (3.5 mm); whereas *S. inermis* extract of cuttlebone showed activity only against *K. pneumoniae* and *V. cholera* (3.5 mm). Such similar activities were found only in 50, 75 and 100% concentrations of *S. aculeata* but the *S. brevimana* extracts showed highest activity against all the strains at all concentrations. A wide spectral antibacterial activity has been recorded in almost all the concentrations of 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 (Ramasamy *et al.*, 2011). *Lannea velutina* showed potent bactericidal activity against *E. faecalis*, *B. subtilis*, *S. aureus*, *S. camorum*, *E. aerogenes*, *P. mirabilis* and *P. aeruginosa* (Ouattara *et al.*, 2011). Aqueous extract of *Valeriana wallichii* showed maximum activity against *S. aureus* whereas the methanolic extract showed highest activity against *B. subtilis* (Sati *et al.*, 2011).

The EDTA extract of *D. sibogae* gladius recorded 10 mm inhibition zone against *E. coli* and *K. pneumoniae*, 9 mm inhibition zone against *S. aureus* and 7 mm against *S. typhi*. Whereas the EDTA extract of *L. duvauceli* extract showed only low activity i.e., 5 mm against *P. aeruginosa*, 4 mm against *S. typhi* and *E. coli*. At the same time, the gladius extract of both the species showed no activity against *V. cholerae*. The EDTA extract from the gladius of *D. sibogae* recorded potent antibacterial activity against all the bacterial strain mentioned above and at the same time the polysaccharide of *L. duvauceli* gladius extract recorded only low activity. The EDTA extract from the gladius of *L. duvauceli* showed antifungal activity against *A. fumigatus*, *A. flavus* and *Rhizopus* sp. whereas gladius extract of *D. sibogae* recorded the antifungal activity against *A. fumigatus* and *Rhizopus* sp. only. There was no activity for both the species against *Candida* sp. (Barwin Vino, 2003). The antibacterial activity was predominant among cuttlebone extracts (using EDTA) of the cuttlefishes such as *S. aculeata* and *S. brevimana* against almost all the 9 pathogenic bacterial strains tested viz., *B. subtilis* and *E. coli*, *K. pneumoniae*, *S. aureus*, *P. aeruginosa* and *Streptococcus aureus*, *V. cholerae*, *V. parahaemolyticus*, *S. typhi* and *Shigella* sp. The activity was recorded in almost all the concentrations except in control. On comparison the activity was higher in the cuttlebone extract of *S. aculeata* than *S. brevimana* (Shanmugam *et al.*, 2008).

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 discovering new substances for drug development. In the present investigation highest inhibition zone of 12 mm was recorded against *K. pneumoniae* and *S. aureus* in *S. inermis* whole body tissue methanolic extract, highest inhibition zone of 12 mm was recorded against *Staphylococcus aureus* in cuttlebone EDTA extract (polysaccharide). There was no activity seen against *V. alginolyticus* with methanolic extract whereas no activity was found against *S. aureus* and *S. pneumoniae* with cuttlebone EDTA extract. Thus in the present study a wide spectral antibacterial activity has been recorded in almost all concentrations of the extracts which is the significant finding of the study. Further investigations intending to purify these active compound(s) should be considered to clarify their chemical nature.

## CONCLUSION

The methanolic extract of whole body tissue showed activity against 9 human pathogenic bacterial strains except *V. alginolyticus*. The polysaccharide from cuttlebone showed activity against eight human pathogenic bacterial strains except *Streptococcus aureus* and *S. pneumoniae*. The maximum activity of 12 mm in 100% against *K. pneumoniae* and *Staphylococcus aureus* in methanolic extract of whole body tissue and the highest activity were recorded 12 mm in 100% concentration against *Staphylococcus aureus*. Thus, the present study provides the baseline information to the future researchers in this field and also paves the way for the wise utilization of the cuttlebone and cuttlefish meat by the pharmaceutical technologist for the extraction of useful drugs in future.

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