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ANTIBACTERIAL ACTIVITY OF METHANOLIC EXTRACT OF THE INK OF CUTTLEFISH, *SEPIA PHARAONIS* AGAINST PATHOGENIC BACTERIAL STRAINS

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ABSTRACT: The aim of the present study is an attempt to evaluate the antimicrobial activity of the methanolic extract of the ink of cuttlefish, *Sepia pharaonis* on bacterial pathogens. The methanolic extract of the ink of cuttlefish, *Sepia pharaonis* were prepared and the antibacterial activity was determined by agar well diffusion method on different bacterial strains (*Bacillus sp., Pseudomonas sp., E coli., Staphylococcus sp. and Klebsiella sp.*). Isolation and the purification of the antibacterial principle were carried out by using Thin Layer Chromatography (TLC). The thermal stability of the extract was determined by incubating the extract at different temperatures. The methanolic extract of the ink of cuttlefish showed antibacterial activity on different bacterial strains. Of the five different species used, the bacterial species *Bacillus* were found to be more sensitive than all the other species. The bacterial strains *Pseudomonas sp., Staphylococcus sp*. and *Klebsiella sp.* were found to be moderately sensitive to the methanolic extract of the ink of cuttlefish. The species *E coli* is found to be least sensitive than all the other species. So the results revealed that the bacterial species *Bacillus* were found to be more sensitive than all the other species. The thin layer chromatography revealed that the active principle in the ink extract may be a peptide compound. Study also revealed that the active principle in the extract is a thermally stable compound. In the present study, the ink extract of cuttlefish, *Sepia pharaonis* have been showed promising antibacterial activity against human pathogens, which reveals the therapeutic potential of the compound.

INTRODUCTION: Marine invertebrates are potential source of natural antimicrobial compounds [1]. Several natural bioactive compounds like peptides, depsipeptides, sterols, sesquiterpenes, terpenes, polypropionates, nitrogenous compounds, macrolids, prostaglandins, fatty acid derivatives and alkaloids were reported from molluscs which were identified as essential with specific types of activities [2]. Natural bioactive substances have the least quantum of side effects when compared to synthetic products.

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Although most antibiotics have been derived from the terrestrial products it is the marine world that provide the pharmaceutical industry the next generation of medicines [3]. The class Cephalopoda comes under the phylum Mollusca, which involves squid, cuttlefish, octopuses and nautilus[4]. Cephalopods occur in all marine habitats and are famous for their defenses, from their fast getting escape movements to changes in colouration that can be cryptic, disruptive or startling, to arm autotomy, to toxin venom and to inking [5, 6].

Squid and cuttlefish eject ink from their ink sac to escape from their predators[7]. Cuttlefish and squid form a major marine fishery resource of India and they are exploited mainly for export. A number of squid and cuttlefish processing plants have come to operate along the coastal areas of Kerala in

particular and all along the Indian coast in general. The ink sacs form the waste material in squid and cuttlefish processing industry which leads to serious environmental pollution. The utilization of the ink will reduce the environmental pollution. Hence proper technology has to be developed for the utilization of these waste materials for the production of materials which are beneficial to human kind. Cephalopod ink has shown potential antiretroviral activity [8]. The peptidoglycan and acetone delipidated ink of squid (*Illex argentinus*) showed antitumour activity [9,10].

The ink and tissue extracts of cuttlefish and squid showed antibacterial effect [11, 12, 13, 14, 15]. Attempts are currently being made to isolate bioactive substances from these waste materials for biomedical research. The present study was designed to investigate the antibacterial activity of the methanolic extract of the ink of cuttlefish, *Sepia pharaonis* on human pathogens.

MATERIALS AND METHODS:

Collection of ink from cuttlefish:

The ink sacs of cuttlefish, *Sepia pharaonis* were collected from the fishing harbor, Neendakara, Kerala, India. Before collecting the ink, the species of cuttlefish were identified with the help of a fishery biologist. The collected ink sacs were brought to the laboratory under ice cold condition and they were kept in freezer (-20^0 C) till use.

Preparation of methanolic extract of ink:

About 5 gm of ink were collected aseptically from the ink sac of cuttlefish and homogenized with 50 ml of 80% ethanol in a glass pestle homogenizer. The homogenized samples were heated under a reflex condenser in a boiling water bath for 15 min. The homogenate is then centrifuged for 15 minutes at 4^{o}C. The residue were washed two times with a small volume of 80% ethanol and centrifuged to collect the supernatant. The supernatants are combined and concentrated to a fixed volume using a rotary vacuum flash evaporator. This ethanol extract were kept in a refrigerator till use in sealed tubes.

Microbial cultures: Five species of bacteria were used as test organisms. The bacterial species include *Bacillus sp., Pseudomonas sp., E coli., Staphylococcus sp. and Klebsiella sp.*

Inoculum preparation for bacteria:

Nutrient broth was prepared and sterilized in an autoclave at 15 lbs pressure for 15 min. All the five bacterial species were individually inoculated in the sterilized nutrient broth and incubated at 37^{o}C overnight. The overnight bacterial cultures were inoculated in the petridishes by using a sterile cotton swab.

Culture Media:

Nutrient agar media purchased from Hi Media, Laboratories Pvt Ltd, Mumbai was prepared and sterilized in an autoclave at 15 lbs pressure for 15 min. and poured in to sterile petridishes and incubated at 37^{o}C for 24 h. Sterile plates were used for antibacterial studies.

Antibacterial assay-Agar well diffusion method:

The antibacterial activities of the ink extract of cuttlefish were evaluated by agar well diffusion method. The ink extracts of cuttlefish, *Sepia pharaonis* were tested for antibacterial activity against gram positive and gram negative bacterial strains, *Bacillus sp., Pseudomonas sp., E coli., Staphylococcus sp. and Klebsiella sp.* by agar well diffusion method. Autoclaved nutrient agar was plated and seeded with the 100 µl of inoculums concentration (10^6 CFU/ ml) and spreaded on the solid agar plates with sterile swab and 4 mm diameter was made on the agar media using sterile cork borer. 20 µl (0.5 mg) and 40 µl (1 mg) of the cuttlefish ink extract were filled in the respective wells.

The petri plates were then incubated at 37^{0}C overnight and the diameter of the inhibitory zone around the well was measured and compared. The standard antibiotic Gentamycin was used as a positive control and methanol was used as a negative control. The diameter of zone of inhibition was measured in mm [16].

Separation and isolation of Ninhydrin positive antibacterial principles using TLC:

To evaluate the ninhydrin positive (peptides) antibacterial principles in the extract, the methanolic extract of the ink of cuttlefish, *Sepia pharaonis* was subjected to thin layer chromatography (TLC). A known volume of the ink extract of cuttlefish were applied in silica gel G

TLC (Thin Layer Chromatography) plates and developed using a solvent system, n-butanol:acetic acid: water (80:20:20, v/v/v) after development, solvent front is marked and the plate is dried. The plate is then sprayed with 0.5% ninhydrin in acetone and heated to 105^0C in a hot air oven for 20–50 minutes. The number of purple spots obtained was noted and their distance from their origin was measured. The R_f values of the spots were calculated.

Separation and isolation of Iodine positive antibacterial principles using TLC:

To evaluate the iodine positive (lipids) antibacterial principles in the extract, the methanolic extract of the ink of cuttlefish, *Sepia pharaonis* was subjected to TLC (Thin Layer Chromatography). A known volume of the ink extract of cuttlefish were applied in silica gel G TLC plates and developed using a solvent system, hexane: ether: acetic acid (85:15:2, v/v/v) after development, solvent front is marked and the plate is dried. The plates were kept in iodine chamber to reveal iodine reactive lipid components.

Determination of the thermal stability of the antibacterial principle:

The thermal stability of the antibacterial principle (ink extract of cuttlefish) was tested under 40^0C-100^oC at 10^0C intervals. For this, the ink extract of cuttlefish were incubated for 30 minutes at respective temperature and the antibacterial activity was described against a standard culture.

Statistical analysis:

Diameter of zone of inhibition (excluding well diameter) were measured in mm. The antibacterial activity of the ink extract of cuttlefish was indicated by a clear zone of growth inhibition. The values obtained are mean inhibition zone (mm) ±standard deviation (SD) of three replicates of antibacterial activity [17].

RESULTS AND DISCUSSION:

Antibacterial Assay:

The antibacterial activity of the methanolic extract of the ink of cuttlefish, *Sepia pharaonis* was screened by agar well diffusion method. The methanolic extract of the ink of cuttlefish, *Sepia pharaonis* showed antibacterial activity towards five different bacterial strains *Bacillus sp., Pseudomonas sp., E coli., Staphylococcus sp. and Klebsiella sp.* with two different concentrations of methanolic extract of the ink of cuttlefish 20 µl (0.5 mg) and 40 µl (1 mg). The results are shown in **Table 1** Of the five different species used, the bacterial species *Bacillus* were found to be more sensitive than all the other species and this species exhibited maximum antibacterial susceptibility with zone of inhibition recorded was 10±0.31 mm and 11±0.34 mm respectively with concentrations of 20 µl and 40 µl.

It is clear that at concentration of 40 µl the species *Bacillus sp.* exhibited maximum antibacterial activity with zone of inhibition of 11±0.34 mm. With concentrations 20 µl and 40 µl the bacterial strain *Klebsiella sp* recorded the zone of inhibition 8±0.27 mm and 9±0.26 mm respectively. With the concentrations 20 µl and 40 µl the bacterial strains *Pseudomonas sp.* recorded the zone of inhibition 7±0.28 mm and 8±0.23 mm respectively. At concentrations 20 µl and 40 µl the bacterial strains *Staphylococcus sp.* recorded the zone of inhibition 6±0.21 mm and 8±0.30 mm respectively. Among the tested bacterial strains, the lowest inhibitory zone was observed in *E coli.* at concentrations 20 µl and 40 µl with zone of inhibition 5±0.20 mm and 6±0.25 mm.

Among all the tested microorganisms, *Bacillus sp*. recorded highest antibacterial activity at a concentration 40 µl with a zone of inhibition 11±0.34 mm. Gentamycin was used as a positive control and exhibited strong antibacterial activity with a zone of inhibition ranging from 12 ±0.22 mm to 20 ±0.23 mm against all tested microorganisms. Methanol was used as a negative control and it did not inhibit the growth of any of the tested strain.

Antibacterial principle in the methanolic extract of the cuttlefish ink:

To see whether the antibacterial principle in the methanolic extract of ink of cuttlefish, *Sepia pharaonis* is a peptide or lipid derivatives, the extract were separated by using thin layer chromatography and spots were developed by using Ninhydrin or iodine chamber. Purple spots were developed with two distinct bands 1& 2. The Rf

values of the bands were recorded as 0.20 (band 1) and 0.13 (band 2). The results revealed that peptide content may be present in the active principle of the cuttlefish ink extract. No bands were observed for the plates developed in iodine chamber and which indicated the absence of lipid derivatives. The findings suggested that the active principle in the ink extract may be rich in peptide molecules (**Figure 1**).

TABLE: 1. ANTIBACTERIAL ACTIVITY (ZONE OF INHIBITION) OF METHANOLIC EXTRACTS OF INK OF CUTTLEFISH, *SEPIA PHARAONIS* TOWARDS BACTERIAL STRAINS.

Bacterial Strains	Ink 20 µl	Ink 40µl	Positive control	Negative control
E coli	5±0.20	6±0.25	12 ±0.22	-
Pseudomonas sp.	7 ±0.28	8±0.23	20 ±0.23	-
Klebsiella sp.	8 ±0.27	9±0.26	20±0.30	-
Bacillus sp.	10 ±0.31	11±0.34	19 ±0.40	-
Staphylococcus sp	6 ±0.21	8 ±0.30	12±0.27	-

Values are mean inhibition zone (mm)±SD of three replicates

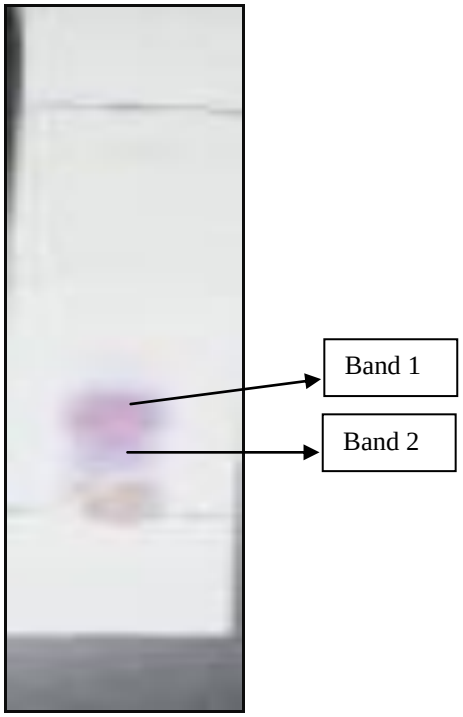

FIGURE 1: THIN LAYER CHROMATOGRAPHY-METHANOLIC EXTRACT OF INK OF *SEPIA PHARAONIS* SHOWED PURPLE SPOTS ON SILICA GEL PLATE

Antibacterial principle in the methanolic extract of the cuttlefish ink is a thermally stable molecule:

Thermal stability of the ink extract was detected by testing the ink samples from 40^0C to 100^0C at 10^0C intervals for 30 minutes. The ink extract of cuttlefish, were tested for antibacterial activity towards the five different strains, *Bacillus sp., Pseudomonas sp., E coli., Staphylococcus sp. and Klebsiella sp*. and the results showed that the ink extract is a thermally stable.

DISCUSSION: Marine natural products have drawn the attention of researchers in recent years due to their pharmacological value. Extensive studies have been carried out with many marine plants and animals. As the marine plants and animals get adapted to different types of habitats in the marine environment they show several adaptations to overcome the difficulties they face in ecosystem. Marine invertebrates are potential source of natural antimicrobial compounds. The antibacterial activity of the methanolic extract from the body tissues of certain cephalopod species was studied. Antimicrobial activity of polysaccharide isolated from the cuttlebone of *Sepia aculeate* and *Sepia brevimana* and methanolic extract of body tissue of *Sepia parshadi* have been reported [18]. Studies have shown that methanolic extract of cephalopods showed maximum activity against human pathogens [19].

Only very few studies has been carried out on the antimicrobial activity of the ink of cuttlefish and squid. The present study was an attempt to understand the antibacterial activity of the ink extract of cuttlefish, *Sepia pharaonis* on bacterial pathogens. The results obtained from agar well diffusion assay indicated that both gram positive and gram negative bacteria were effectively inhibited by the ink extract of cuttlefish, *Sepia pharaonis.* The effect of the extract differs from strain to strain with respective to their concentration.

Among all the tested microorganisms, *Bacillus sp.* recorded highest antibacterial activity at a concentration 40 µl with a zone of inhibition

11±0.34 mm and the lowest inhibitory zone was observed in *E coli* at a concentration 20 µl with a zone of inhibition 5±0.20 mm. It was also observed that with an increase in concentration of ink extract, there was an increase in the antibacterial activity against tested pathogenic bacterial strains. It indicates that the antibacterial activity is concentration dependent [20]. A large number of marine bioactive peptides have been isolated from molluscs [21].

The present study also revealed that the antibacterial principle in the ink extract of *Sepia pharaonis* was a thermally stable peptide molecule. These findings are useful in designing of new strategies for the development of new therapeutic agents.

CONCLUSION: The results of this study clearly indicate that the methanolic extract of the ink of cuttlefish has potent antibacterial activity against common human pathogenic microbes. So the present study concluded that the ink extract of the cuttlefish, *Sepia pharaonis* showed very good antibacterial effect on five different bacterial strains, *Bacillus sp., Pseudomonas sp., E coli., Staphylococcus sp. and Klebsiella sp*. The chromatographic separation revealed that the antibacterial principle may be peptide molecules. Selection of cuttlefish ink is based on the isolation of novel biomolecules from a waste product that can be used from nature's source.

The present investigation also revealed that the antibacterial principle in cuttlefish ink extract is a thermally stable compound. Further analysis of elucidating the structure of bioactive molecule responsible for the antimicrobial activity is under progress.

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REFERENCES:

1. Mohanraju R, Dayanand Babi Marri P, Karthik, Sumanath Narayana K, Narayana Murthy and Ch. Ramesh: Antibacterial activity of methanolic extract of cephalopods from Andamans, India. Asia Pacific Journal of Tropical Biomedicine 2012; 1-4.
2. Blunt JW, Copp BR, Munro MHG, Northcote PT, Prinsep MR: Marine natural products. Natural product Reports 2006; 23: 26-27.
3. Smiline Girija AS, Vijayshree Priyadharshini J., Pandi Suba K, Hariprasad P and Reguraman R: Antibacterial effect of squid ink on ESBL producing strains of Escherichia coli and Klebsiella pneumonia. Indian Journal of Geo-Marine Sciences 2012. 41(4): 338-343
4. Voss GL: Cephalopod resources of the world. Food and Agricultural organization of the United Nations, USA 1973.
5. Hanlon RT and Messenger JB: Cephalopod behavior. Cambridge University Press, Cambridge1996. 232.
6. Norman MD: Cephalopods. A World Guide. Conch Books, Hackenheim, Germany 2000, 172
7. Ortonne JP, Voulot C, Khatchadourian C, Palumbo A and Prota G: A reexamination of melanogenesis in the ink gland of cephalopods. In: Pigment cell. Phenotypic Expression in Pigment cells (M.Seiji. Ed.) University Tokyo Press, Tokyo. 1981. 49-57.
8. Rajaganapathi J, Thyagarajan SP and Patterson Edward JK: Study on cephalopod's ink for antiretroviral activity. Indian Journal of Experimental Biology 2000. 36: 583-587.
9. Sasaki J, Ishita K, Takaya Y, Uchisawa H and Matsue H: Antitumour activity of squid ink. Journal of Nutritional Science and Vitaminology 1997. 43(4): 455-461.
10. Takaya Y, Uchisawa H, Matsue H, Okuzaki B, Narumi F, Sasaki J and Ishida KL: An investigation of the antitumour peptidoglycan fraction from squid ink. Biology Pharmacology Bulletin 1994. 17(6): 846-849.
11. Nithya M, Ambikapathy V and Panneerselvam A: Effect of pharaoh's cuttlefish ink against bacterial pathogens. Asian Journal of Plant Science Research 2011. 49:49-55.
12. Vennila R, Rajesh Kumar RK, Kanchana S, Arumugam M and Balasubramanian T: Investigation of antimicrobial and plasma coagulation property of some mulluscan ink extracts: Gastropods and cephalopods. African Journal of Biochemistry and Research.2011. 5(1): 14-21.
13. Zainab Dakhil Degiam and Ali Tahar Abas: Antimicrobial activity of some crude marine mollusca extracts against some human pathogenic bacteria. Thi-Qar Medical Journal (TQMJ) 2010. 4(3): 142-147
14. Annaian Shanmugam, Thangaraj Amalraj, Chendur Palpandi Gnanasekar Devanathan and Thangavel Balasubramanian: Antimicrobial activity of sulphated mucopolysaccharides (Heparin and heparine like Glycosaminoglycans (GAGs)) from cuttlefish, *Euprymna berryi* Sasaki, 1929.Trends in Applied Science Research 2008. 3: 97-102.
15. Shanmugam A, T.S Mahalekshmi and Barwin Vino A: Antimicrobial activity of Polysaccharide Isolated from the Cuttlebone of *Sepia aculeate* and *Sepia brevimana*. An Approach to selected antimicrobial activity for human

pathogenic microorganisms. Journal of fisheries and aquatic science 2008. 268-274.

16. Pranay Jain, Vibhor Aggarwal and Amrinder Singh: *In vitro* evaluation of antimicrobial properties of extracts of *Pterocarpus Santalinus* against oral pathogens and its synergistic effect with ciprofloxacin and fluconazole. International Journal of Pharmaceutical Science Review and Research 2013. 19 (1) 31-35.
17. Malik Tafazul Rashid, Yadav AS, Balaji A and Shabir AL: An assessment of antibacterial potency of aqueous leaf extract of *Clerodendrum Serratum* Linn. against pathogenic bacterial strains. Indo American Journal of Pharmaceutical Research 2013. 3 (2)
18. Ramaswamy P, Vino AB, Saravanan R, Subhapradha N, Shanamugam V and Shanmugam A: Screening of antimicrobial potential of polysaccharide from cuttlebone and methanolic extract from from body tissue of *Sepia prashadi* Winkworth, 1936. Asian Pacefic Journal of Tropical Biomedicine 2011: 244-248.
19. Ramaswamy P, Subhapradha N, Srinivasan A, Shanamugam V, Krishnamoothy J and Shanmugam A: *In vitro* evaluation of antimicrobial activity of methanolic extract from selected species of cephalopods on clinical isolates. African Journal of Microbiology research 2011.5:3884-3889.
20. Nair R and Chanda S: Activity of some medicinal plants against certain pathogenic bacterial strains. Indian Journal of Pharmacology 2006. 38; 142-144
21. Bhakuni DS and Rawat DS: Bioactive Marine Natural Products. Anamaya Publishers. First Edition 2005

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