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Full Length Research Paper

Studies on the bioactivity of *Holothuria atra* extracts collected from the South east coast of India

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Many bioactive compounds have been extracted from various marine animals like tunicates, sponges, soft corals, echinoderms, sea hares, nudibranchs, bryozoans, sea slugs, etc. The aim of this study is to identify the methanolic extracts of *Holothuria atra* with various *in vitro* experiments such as antibacterial, larvicidal and immunomodulatory activities *of H. atra* commonly called lolly fish. Antibacterial activity was evaluated using the agar well diffusion method. The results showed that *H. atra* extracts were active against *Klebsiella pneumoniae, Serratia liquefaciens,* and *Staphylococcus aureus*. Specifically, *H. atra* inhibited the growth of *Aedes aegypti.* The LC_{50} value detected was 0.58%. Strong hemolytic activity of *H. atra* was identified. Similarly, significant immunostimulant activity was analyzed using NBT assay. Therefore it could be suggested that *H. atra* has the ability to induce biological activities due to the availability of bioactive compounds. These promising results have led this study to consider purification of the active compounds and for the development of drugs.

Key words: *Holothuria atra*, antibacterial activity, larvicidal, hemolytic, immunostimulant.

INTRODUCTION

The “marine world” has a much richer biodiversity than the terrestrial one. Approximately 22,000 natural products of marine origin have been discovered (Blunt et al., 2011). Rajendran (2000) stated that the Gulf of Mannar and Palk Bay are rich in echinoderms like sea cucumber, star fishes and sea urchins. Sea cucumbers, *Holothuria atra, Holothuria scabra* and *Holothuria pinifera*, contain saponins (glycosidic steroids) and holothurin compounds are ichthyotoxic in nature. This property was studied on fish fingerlings, mice and erythrocytes (haemolytic activity). Ogushi et al. (2005) stated that *Bech-de-mer,* a processed form of sea cucumber, has the curative power for ailments like high blood pressure and muscular disorders with pharmacological effects.

Sea cucumbers are a delicacy for the Chinese, the Japanese and the Koreans. They are consumed fresh, chilled, frozen, dried and in the processed forms. The processed product commands a high price of US $ 400 per kg in the international markets at Hong Kong, Singapore, Taiwan, etc. They find an important place in the traditional Chinese medicines (Zhang et al., 2012). The fucosylated chondroitin sulphate from sea cucumber, *Stichopus japonicas* and *Ludwigothurea grisea*, has been found to exhibit various biological activities, such as antiviral, antitumour and antithrombotic properties. Furthermore, the kind of novel fucosylated chondroitin sulphate has shown a heparin-like anticoagulant activity, together with an undesirable effect of platelet aggregation (Zhao et al., 2009).

The antimicrobial peptides found in sea cucumbers like *Cucumaria frondosa* such as steroidal glycosides and polyhydroxylated sterols indicate remarkable activity against microbes. Therapeutic properties and medicinal benefits of sea cucumbers can be linked to the presence of a wide array of bioactives especially triterpene glycosides (saponins), chondroitin sulfates, glycosamino-glycans (Beauregard et al., 2001). Secondary metabolites

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obtained from using polar extracts of *H. scabra* are the sulfated triterpene glycosides, scabraside A and B and they have effective cytotoxicity against human tumor cell lines (Zou et al., 2003). New antifungal active triterpene glycosides of sea cucumber *H. scabra* were identified as scabraside A, echinoside A and holothurin A1 (Han et al., 2009). Zhang et al. (2010) reported that the sulfated polysaccharide, a metabolite from the body wall of the sea cucumber *Stichopus japonicus*, has the ability to regulate the cell proliferation rate in neurodegenerative disorders.

Hillaside C, a triterpene derived from sea cucumber *Holothuria hilla* inhibited the growth of human leukaemia, breast and colon cancer cells *in vitro* in a dose and time-dependent manner by a mechanism that required induction of apoptosis and the concomitant reduction of the apoptosis-suppressing protein Bcl-effect (Wu et al., 2006).

Coelomocytes, the immune mediator cells seen in the echinoderm *Holothuria tubulosa*, act as the source of novel antimicrobial peptides. They are antimicrobial and antibiofilm agents, and are found to be active against *staphylococcal* and *Pseudomonas aeruginosa* strains (Schillaci et al., 2013). The present investigations were carried out to evaluate the bioactivity potentials.

MATERIALS AND METHODS

Collection of sea cucumber (*Holothuria atra*)

H. atra specimens with a size range of 10 to 30 cm in length and 30 to 180 g weight were collected from fishing nets operated off Kanyakumari (8° 03' and 8° 35' of the north Latitudes and 77° 15' and 77° 36' of the east longitudes).

Extract preparation from sea cucumbers

Immediately upon collection, *H. atra* specimens were dissected to remove the internal organs and packed using ice after which they were kept at -80°C for extraction. The skin portion was peeled off and stored in methanol in separate containers. The biologically active compounds were extracted based on their polarity using methanol, an organic solvent, as per the method given by Rashid et al. (2001) with appropriate modifications. About 200 g of frozen samples were homogenized with deionized water and methanol. The mixture was continuously stirred in the dark at 4°C for 24 h. Then it was centrifuged at 5000 rpm for 15 min. The supernatant was collected and filtered. The extracts thus collected were freeze-dried and kept at -80°C, while the insoluble solid materials were re-extracted with methanol (100%). The organic extracts were combined and the solvents were removed by rotary evaporation at 40°C under low pressure to avoid degradation of compounds.

Antibacterial activity

The antibacterial activities of the methanol extracts of sea cucumber *H. atra* were determined by the standard agar well diffusion assay using Muller Hinton agar (Hi Media). The bacterial cultures were obtained from the microbial type culture collection and gene bank (MTCC), Institute for Microbial Technology, Chandigarh, India. The Gram positive bacterial strains included: *Bacillus thuringiensis* MTCC 4714, *Enterococci faecalis* MTCC 439, *Listeria monocytogenes* MTCC 1143, *Staphylococcus aureus* MTCC 737 and *Proteus vulgaris* MTCC 426. The Gram negative strains such as the *Escherichia coli* MTCC 443, *Klebsiella pneumonia* MTCC 109, *Pseudomonas putida* MTCC 1688 and *Serratia liquefaciens* MTCC 3039 were used in the test. Wells of 6.0 mm diameter were punched using a sterilized cork borer. Cultures of each microbial pathogenic strain were swabbed with sterile cotton on the surface of the medium. *H. atra* extracts were tested with different aliquots (50, 100 and 150 µl) in each well. The plates were incubated for 24 h at 37°C and solvent control was performed in each case. Areas of inhibited microbial growth were observed as clear zone around the well after 24 h in bacteria (Li et al., 2005).

Larvicidal activity

The larvicidal activity of methanolic extracts of sea cucumber species was evaluated against the third instar stage of *A. aegypti*. The egg cards of *A. aegypti* were obtained from the Centre for Research in Medical Entomology, Indian Council of Medical Research, Madurai. The susceptibility or resistance of the mosquito larvae to the selected concentrations of the extracts was carried out by adopting standard bioassay protocol given by WHO (1981). The extract was tested to determine the larval bio-efficacy by diluting the original extract to 1.0 - 5.5% levels. The bioassays were performed at a room temperature of 27 ± 1°C by exposing 20 larvae in a final volume of 250 ml water in 500 ml glass beaker with minimum of four replicates for each concentration. Simultaneously, control groups were also maintained in beakers without addition of extract but with the solvent alone. Observations were made after 12, 24, 36, 48, and 60 h of treatment for larvicidal activity. Based on the percentage of mortality, the LC_{50} values of the extracts were determined using Probit Scale Analysis (Newman and Crag, 2004).

Hemolytic activity

The hemolytic activity of the methanolic extracts of sea cucumber species *H. atra* on human red blood cells

(RBC) was tested by a micro-hemolytic method. Human "B" positive blood was obtained from the Vivek Institute of Laboratory Medicine, Nagercoil, Kanyakumari District, Tamilnadu in EDTA solution (2.7 g in 100 ml of distilled water) as an anticoagulant at 5% of the blood volume and brought to the laboratory. Then 1% erythrocyte suspension was prepared by adding 99 ml of PBS, pH 7.4, to 1 ml of packed RBC. The micro-hemolytic test was performed on 96-well U-bottom micro titer plates. Serial two fold dilutions of the extracts were carried out in 100 µl of PBS, pH 7.4. For the control set, 100 µl of distilled water was added to 3% RBC suspension. This served as the positive control and an equal amount of PBS, pH 7.4, acted as the negative control. The plate was gently shaken and allowed to stand for two hours at room temperature. The percentage of the hemolytic cells was calculated using the formula:

$$\% \text{ Hemolysis} = \frac{\text{Absorbance of sample} - \text{Absorbance of blank}}{\text{Highest absorbance for positive control}} \times 100$$

Nitro Blue Tetrazoliun assay (NBT assay)

The *in vitro* immunomodulation study was done on the methanolic extracts of *H. atra.* Phagocytic index (PI) in percentage was assessed by NBT reduction assay as per the method described by Park et al. (1968). In five test tubes, the reaction mixture consisted of leucocytes suspension (0.4 ml), endotoxin activated plasma as standard (0.1 ml), and PBS solution used as control at 0.1 ml. To each test tube, 0.1 ml of different concentrations of test samples (25, 50, 100, 200 µg/ml) were added and 5%s NBT solution (0.8 ml) was added and incubated at 37°C in water bath with shaker for 30 min, after which the reaction was stopped with cold PBS (pH 7.2). A drop of this reaction mixture was spread on a clean glass slide, dried and fixed in methanol for 2 min, after which it was stained with 0.8% aqueous safranin for 2 min. The smear was washed, dried and mounted. However, NBT positive cells were represented by dark blue colour.

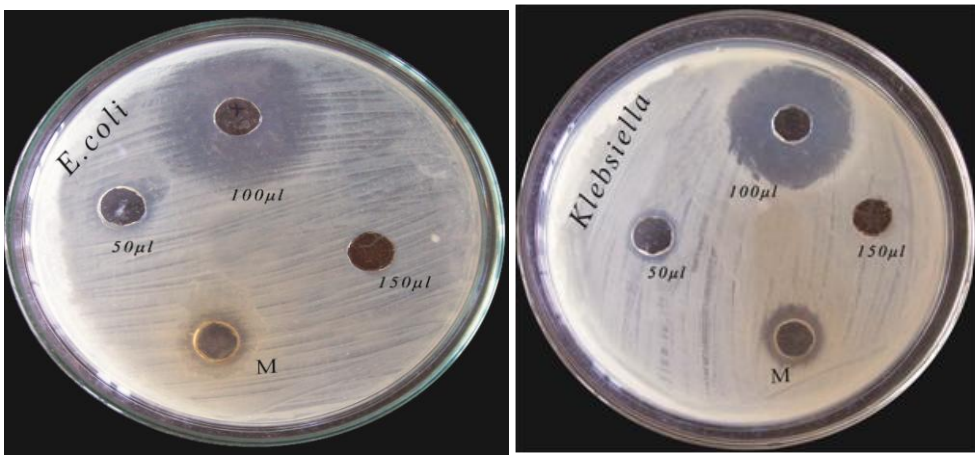
RESULTS

Figure 1 represents the effect of antibacterial activity of the methanolic extracts of the sea cucumber *H. atra.* An effective inhibitory action against the Gram negative organisms such as *K. pneumoniae*, *S. liquefaciens, E. coli* and *S. aureus* and Gram positive organisms such as *L. monocytogenes*, was shown. The remaining organisms were found to be less active. In *L. monocytogenes*, the zone of inhibition formed at 150 µl was 14 mm. In *E. coli*, the zone formed at 50 µl was 2 mm in diameter, and that formed at 100 µl was 16 mm in diameter. *K. pneumoniae* formed the zone of 3 mm and 14 mm diameter size at the concentrations of 50 and 100 µl. *S. liquefaciens* showed the zone of inhibition as 12 mm and 4 mm diameter size in 100 and 150 µl concentrations. Finally in *S. aureus*, the zone of inhibition found at 100 and 150 µl was 5 mm and 16 mm diameter size.

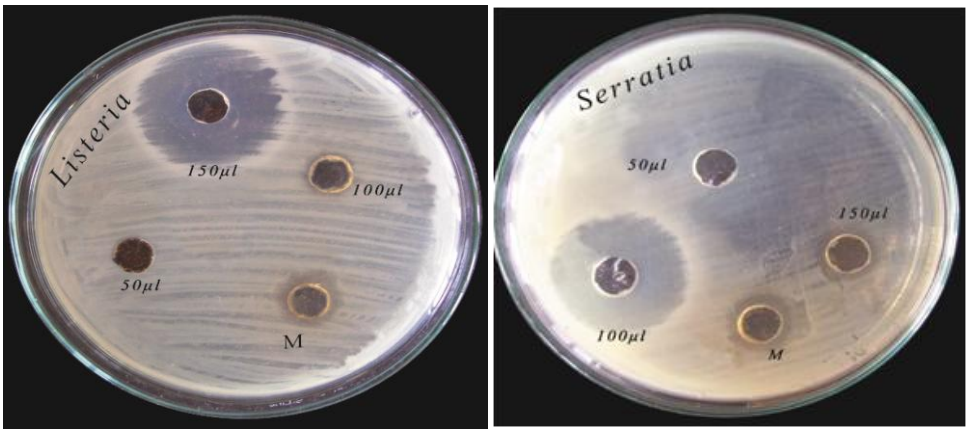
The larvicidal effect of the extracts of sea cucumber *H. atra* based on the mortality of the third instar stage of *A. aegypti* is given in Table 1. The mortality rate was calculated at different intervals of time from 12, 24, 36, 48 and 60 h. The extracts produced 100% mortality at 24 h interval with 5.0% concentration of the extracts, 100% mortality at 48 h interval with 4.5% concentration of extracts and 100% mortality at 60 h interval with 4.0% concentration of the extracts. LC_{50} values calculated based on the potency of the extracts at various concentrations form 1.0 to 5.5% and at different intervals of time such as 12, 24, 36, 48 and 60 h using the mortality rate against the third instar stage of *A. aegypti.* It was also observed that the extracts of the sea cucumber *H. atra* had good larvicidal activity with less toxic effects amid LC_{50} value of 0.58% in 24 h. Strong hemolytic activity of *H. atra* in methanolic extract is described in Table 2. The optical density value of the sample was 0.550 at the highest concentration of extract of 1 mg/ml. The positive hemolysis was measured by uniform red colour suspension and a button formation at the bottom of wells showed negative hemolysis. The calculated hemolytic activity was 100%. Table 3 shows the *in vitro* model of Qualitative NBT test. Significant immunostimulant activity of the *H. atra* was found at a dose of 50%. Although the effect of *H. atra* was found to be moderate, in this study it showed less effect at higher concentrations.

DISCUSSION

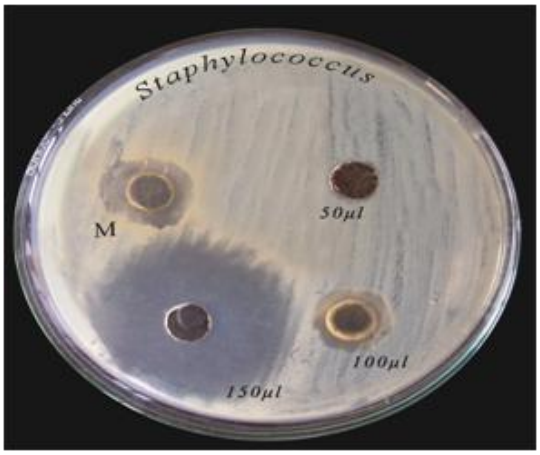
The alcoholic extracts of *H. atra* inhibited human pathogens such as *K. pneumonia, E. coli, L. monocytogenes* and *S. aureus*. This could be due to the availability of compounds such as triterpene glycosides and aglycones. Similarly, sea cucumber (*H. scabra*) showed strong broad spectrum antibacterial activity against both Gram positive and Gram negative bacteria such as the *E. coli, Staphylococcus sp.,* and *Proteus* (Nagaraj et al., 2008). The body wall extracts of *H. atra* showed antibacterial activity which could be due to the availability of various natural products. Assay of antibacterial activity with purified lectin and cell free coelomic fluid of sea cucumber, revealed that it exhibited strong antibacterial activity against both Gram positive and Gram negative bacteria. It inhibited the growth of *Staphylococcus* sp., and also inhibited effectively the growth of other Gram negative *Serratia sp., Proteus sp., Shigella sp., and E. coli* (Tunkijjanukij and Olafsen, 1998).



a. *Escherichia coli* b. *Klebsiella pneumoniae*



c. Listeria monocytogenes *d. Serratia liquefaciens*



e. Staphylococcus aureus

Figure 1 (a-e). Antibacterial activity of the methanolic extracts of the Sea cucumber *Holothuria atra* in well diffusion.

The methanolic extracts of *H. atra* had larvicidal effect on *A. aegypti* with the LC_{50} of 0.58%. Antimicrobial substances obtained from the alcoholic extracts of *H. scabra*, and *Actinopyga echinites* such as steroidal

Table 1. LC_{50} values for 12, 24, 36, 48 and 60h with their 95% fiducial (lower and upper) limits, regression equation, Chi-square and P-levels of Sea cucumber *Holothuria atra* against the 3rd instar larvae of *Aedes aegypti*.

Holothuria atra	LC_{50} with fiducial limits		Regression equation	Chi-square	Median	P-level
	Upper	Lower	Log Y=(Y-bx) +bX	(df)	LC_{50} 1 mg/ml	
12 h	1.68	1.60	Y=6.53 +7.006X	0.60(3)	4.42	7.0
24 h	1.61	1.55	Y=12.95+11.313X	6.43(4)	3.86	11.31
36 h	1.59	1.52	Y=6.65+7.474X	1.71(4)	3.61	7.47
48 h	1.50	1.42	Y=5.40+7.106X	4.46(5)	2.90	7.10
60 h	1.42	1.32	Y=2.73+5.614X	5.76(5)	2.38	5.61

Table 2. Hemolytic activity of *Holothuria atra*

Holothuria atra	Absorbance at 541 nm
Blank	0.152
Sample (1 mg/ml)	0.550
Positive control	0.340
Negative control	0.242

Table 3. Effect of the methanolic extracts of *Holothuria atra* using qualitative nitroblue tetrazolium (NBT) test

Groups	Conc. of extract 1 mg/ml	% NBT positive cells
Control		23
Endotoxin activated plasma (standard)	10%	85
Holothuria atra	10%	26
	25%	34
	50%	46
	50%	52

sapogenins showed antibacterial and antifungal activities. It inhibited the organisms such as the *E. coli, K. pneumonia, P. aeruginosa and S. aureus* (Fuestani, 2004). Holothurians contained chondroiton and glucosamine bioactive substances as important cartilage building blocks with anti-inflammatory, anti-tumour activity and antifungal properties (Hamel, 1997). The strong mosquito larvicidal activity was displayed on the body wall methanolic extracts of *H. scabra and H. argus*, and it was attributed to the presence of steroids (Indap and Thakur, 1995).

The extracts of *H. atra* showed high hemolytic activity against the human erythrocytes. Sea cucumber triterpene glycosides have lanostane type aglycones. At milli- and micromolar concentrations, sea cucumber glycosides showed hemolytic, antifungal, cytotoxic and other kinds of biological activities caused by membranotropic action of these substances (Avilov et al., 2008). *H. atra* body wall extracts showed hemolytic activity against the erythrocytes. Marine invertebrates offer a source of potential antimicrobial drugs. Studies of antimicrobial mechanisms and compounds of marine invertebrates may provide valuable information for new antibiotic discoveries and give new insights into bioactive compounds (Mayer et al., 2007).

The *H. atra* extracts also showed immune modulatory activity which could be due to the presence of compounds such as sulponamides and diketones. Cucumarioside derived from the Sea cucumber *Cucumaria japonica* have potent immunomodulatory properties, and exhibited high efficacy against *E. coli*, *Proteus mirabilis*, *Neisseria meningitidis* BT-2, *Salmonella minnesota*, *Pertussis meningoencephalitis* and *Salmonella typhimurium* (Sedov et al., 1990). A variety of lectins have been isolated in the coelomic plasma and coelomocytes of marine invertebrates, specifically sea cucumbers. One of the roles of marine invertebrate lectins is to act as the humoral factors in the

defense mechanism, as the immunoglobulins in vertebrates (Hatakeyama et al., 1995).

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