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In vitro cytotoxicity and antimicrobial activity of methanolic extracts of *Holothuria* (*Semperothuria*) *cinerascens* (Brandt, 1835) and *Stomopneustes variolaris* (Lamarck, 1816) from the southwest coast of India

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Original Article

Abstract

Marine organisms are prolific sources of novel biologically active organic molecules with diverse structures. The antimicrobial and *in vitro* cytotoxic activity of methanolic extracts of *Holothuria cinerascens* and *Stomopneustes variolaris* were examined. The antimicrobial activity against various pathogens such as *Aeromonas hydrophila, Edwardsiella tarda, Enterobacter ludwigii, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella typhi, Vibrio cholerae*, and *V. parahaemolyticus* was also evaluated. The *in vitro* cytotoxicity was tested in Ehrlich Ascites Carcinoma cells. Results of the study highlighted that the methanolic extract of *S. variolaris* had high antimicrobial and cytotoxic activities compared to that of *H. cinerascens. E. coli* and *K. pneumoniae* were highly sensitive to these extracts.

Keywords: *Cytotoxicity, antibacterial activity, trypan blue dye exclusion assay, broth microdilution assay, Holothuria cinerascens, Stomopneustes variolaris*

Introduction

The discovery of antibiotics revolutionized pharmacology and medicine in the 20th century. As a result of the property of antibiotics to kill or inhibit the growth of bacteria, they are generally used to treat bacterial infections (ECDC, 2014; NHS, 2014). The easy access and effectiveness of antibiotics led to overuse and inappropriate treatments, which led to the emergence of multidrug-resistant bacteria (Larson, 2007). This circumstance prompted the researchers to search for novel and potent natural antimicrobial compounds. Nowadays, interest in drugs from natural products is gaining preference as they are safer than synthetic drugs. Marine organisms are prolific sources of novel biologically active organic molecules with diverse structures (Baker and Murphy, 1976; Scheuer, 1978; Nandi and Sharma, 2019). Sponges, molluscs, bryozoans, ascidians, and echinoderms contributed many biologically active secondary metabolites to the pharmaceutical field (Liu *et al.,* 2019). These secondary metabolites are normally produced by the host to ward off competition and predation in the natural environment where they inhabit. Several studies and reports were published on the diversity and chemical characteristics of secondary metabolites produced by marine organisms (Faulkner, 2002; Proksch *et al.,* 2002; Haefner, 2003; Jha and Zirong, 2004; Jimeno *et al.,* 2004; Carvalhal *et al.,* 2018).

Echinoderms are ecologically important, they recycle the nutrients trapped in reef sediments and lagoons, thus called keystone species in oligotrophic coral reef environments (Uthicke, 1999; Uthicke *et al.,* 2003). Sea cucumbers have been fished for food and traditional medicine (Conand and Byrne, 1993; Pangestuti and Arifin, 2018). Sea urchins are algal grazers and inhabit algal colonies and coral reefs. They maintain the balance of their ecosystem, controlling the overgrowth of algae and herbivores. During the last decade, scientists showed interest in the medicinal properties of echinoderms because of the unique biologically active organic molecules produced by them (Haug *et al.,* 2002; Dang *et al.,* 2020). Bioactive compounds from echinoderms are reported to show a broad spectrum of biological

activities such as anti-HIV, antiviral, antitumor, antibacterial, antifungal, anticoagulant, antiprotozoal, anti-tuberculosis, and anti-inflammatory (Laurienzo, 2010; Samuel *et al.,* 2011; Uzair *et al.,* 2011; Mayer *et al.,* 2013; Claereboudt *et al.,* 2019). However, most of these studies are reported from elsewhere in the world, and virtually no detailed reports are available on the bioactive compounds from sea cucumber *Holothuria cinerascens* and sea urchin *Stomopneustes variolaris*, which are two commonly available echinoderm species on the southwest coasts of India (James, 2007, Vijay Kumar *et al*., 2017). Hence the present study explored the antimicrobial and cytotoxic effects of methanolic extracts of *H. cinerascens* and *S. variolaris* from the southwest coast of India.

Material and methods

Sample collection and extraction

The specimens of sea cucumber and sea urchin were collected from rocky intertidal and subtidal regions of Vizhinjam and Kovalam, Thiruvananthapuram District, Kerala, southwest coast of India. Two common species *H. cinerascens* (Fig. 1) and *S. variolaris* (Fig. 2) were selected for the study (Clark and Rowe, 1971). The samples collected were immediately brought to the laboratory in containers filled with seawater.

All the samples (300 g of wet weight from each species) were thoroughly washed with seawater to remove mud and other attached sand particles. The whole body of organisms were then frozen at -80 ºC and lyophilized (PENGUIN 4KG, Lark instruments, India). The dried samples were ground to a fine powder and soaked in methanol in the ratio of 1:3 (W/V) and maintained in a dark chamber for two weeks. After two weeks, the extract was filtered through Whatman®No. 1 filter paper. After the first filtration, the filtrate was stored at 4 ºC and the powder was again kept for extraction in methanol. The extraction procedures were repeated three times to collect maximum compounds from the organisms. The filtered methanolic extracts were pooled and dried under reduced pressure using a rotary evaporator (RE100- Pro, Lark instruments, India) at 37 ºC and the crude extracts were stored at 4 ºC for further analysis (Chellaram *et al.,* 2004). Cytotoxicity and antimicrobial activities of crude extracts were measured by trypan blue dye exclusion assay and broth microdilution assay, respectively.

In vitro cytotoxicity- Trypan blue dye exclusion assay

In vitro cytotoxicity of crude extracts of organisms was studied using Ehrlich Ascites Carcinoma (EAC) cells, following the standard protocol of Talwar (1974). The tumour cells aspirated from the peritoneal cavity of tumour bearing mice were washed thrice with Phosphate Buffered Saline (PBS). Test compounds were dissolved in dimethyl sulphoxide (Merck). Viable cell suspension ($1x10^6$ cells in 0.1 mL) was added to test tubes containing various concentrations of the test compounds and the volume was made up to 1.0 ml using PBS. The control tube contained only cell suspension. The assay mixture was incubated for 3 hours at 37 ºC, after which the cell suspension was mixed with 0.1 ml of 1% trypan blue and kept for three minutes. The treated mixture was then loaded on a haemocytometer and the numbers of stained and unstained cells were counted separately. While the viable cells exclude the dye, damaged cells take up the dye and become blue. The percentage of cytotoxicity of extracts was calculated using the following formula

Percentage of cytotoxicity = (Number of dead cells /Total number of cells) × 100

Antimicrobial assay -broth microdilution

The antimicrobial activity of extracts was studied by the broth

Fig. 1. *H. cinerascens*

Fig. 2. *S. variolaris*

microdilution method using Mueller-Hinton Broth. The activity was assessed against nine Gram-negative bacteria; *Aeromonas hydrophila* (MTCC 1739), *Edwardsiella tarda* (MTCC 2400), *Enterobacter ludwigii, Escherichia coli* (MTCC 1610), *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* (MTCC 741), *Salmonella typhi, Vibrio cholerae* (MTCC 3906) and *V. parahaemolyticus* (MTCC 451). All the organisms were inoculated in Mueller-Hinton Broth and incubated overnight at 30 ºC in a shaking incubator.

The stock solutions of crude extracts were prepared by dissolving 10 mg extracts in 1 ml dimethyl sulphoxide (DMSO). Five different concentrations (mg/ml) from each species, such as 10, 5, 2.5, 1.25, and 0.625 μg/mL were tested during the study. The assay was performed in 96 well microtiter plates. To each well, 50 μl bacterial inoculums and 50 μl test compounds were added, which is then made up to 200 μl by adding 100 μl of Mueller-Hinton Broth. The absorbance was measured at 630 nm for three hours at an interval of five minutes. While taking the reading, the temperature of the instrument was maintained at 37 ºC with shaking.

Results and discussion

Screening for antimicrobial activity and *in vitro* cytotoxicity was conducted using methanolic crude extracts of two echinoderm species. Chemotherapeutically, cytotoxicity is an indication of antitumor activity (Negin *et al.,* 2014; Stabili *et al.,* 2018). Saponins are one of the highly biologically active secondary metabolites; specifically found in phylum Echinodermata. Sea cucumbers use saponins for their chemical defense against predators. Saponins are more localized in the viscera than the body wall of sea cucumbers. Sea urchin exoskeleton contains unique compounds and dimethyl sulfoxide is the major constituent present in the exoskeleton (Bahrami *et al.,* 2014). From the results we obtained, some interesting observations can be inferred.

In vitro cytotoxicity study results are presented in Fig. 3 which explains that methanolic crude extract of *S. variolaris* showed high cytotoxic activity against Ehrlich Ascites Carcinoma (EAC) cells, followed by an extract of *H. cinerascens*. From the results obtained, it is evident that even the lowest concentration of methanolic extract of *S. variolaris* showed significant cytotoxic activity. Previous studies suggested that the high bioactivity of sea urchin extracts may be due to the presence of cytolytic peptides or proteins and a good amount of omega 3 fatty acids (Bragadeeswaran *et al.,* 2013). Methanolic crude extracts of the sea cucumber *H. cinerascens* showed moderate cytotoxic activity. Cytotoxic activity of sea cucumber extract against tumour cell line is correlated with the triterpene glycosides found in sea cucumbers for defense against predators (Drozdova *et al.,* 1997; Kalinin *et al.,* 1996; Kim and Himaya, 2012). The difference in

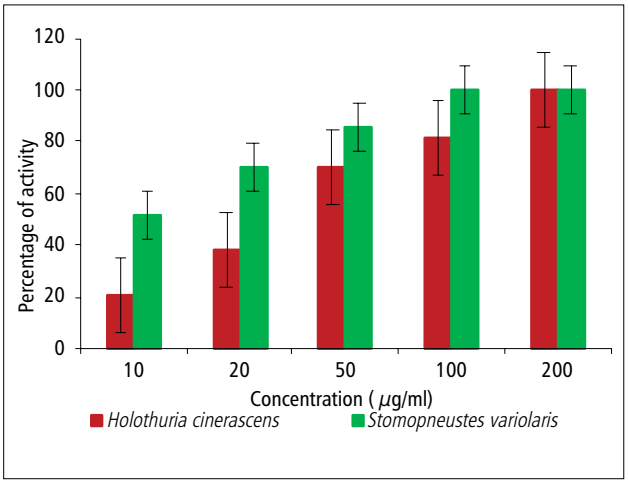

Fig. 3. *In vitro* cytotoxic activity of crude extracts of *H. cinerascens* and *S. variolaris*

expression of crude extracts of echinoderms may be because of the functionalization of different kinds of compounds present in certain organisms. Hence, it can be inferred that the two organisms showed promising cytotoxic activity against the cancer cell line, in which *S. variolaris* extract showed relatively superior activity.

The antimicrobial activity of two species of echinoderms was screened against nine selected Gram-negative microorganisms. The results showed significant variations in the first two hours. The results of the broth microdilution assay of *H. cinerascens* and *S. variolaris* extracts are graphically represented in Figs. 4 and 5, respectively. *E. ludwigii* exhibited high resistance against the crude extract of *S. variolaris,* whereas *E. coli* and *K. pneumoniae* were found to be highly sensitive against extracts. The extract of *S. variolaris* showed relatively high activity against all the test organisms. There are previous reports related to the antimicrobial activity of a methanolic crude extract of sea urchins which supports our observations (Uma and Parvathavarthini, 2010; Abubakar *et al.,* 2012). The coelomocytes of the coelomic fluid and the shells of sea urchins contain various polyhydroxylated naphthoquinone, pigments such as spinochromes and echinochrome A, which are reported to possess high antibacterial properties (Anderson *et al.,* 1969; Johnson, 1969; Service and Wardlaw, 1984; Gerardi *et al.,* 1990).

The methanolic crude extracts of *S. variolaris* showed high antimicrobial activity even in lower concentrations. This may be due to the nonspecific inhibition of signal pathways. Within the cell, the cell signalling functions as an integrated network. The termination or deactivation of signalling pathways are complicated and interacts through positive and negative feedback loops (Lemmon *et al.,* 2016). The crude extract will contain many biologically active compounds and some of them will inhibit the other compounds by binding to their specific targets. By diluting

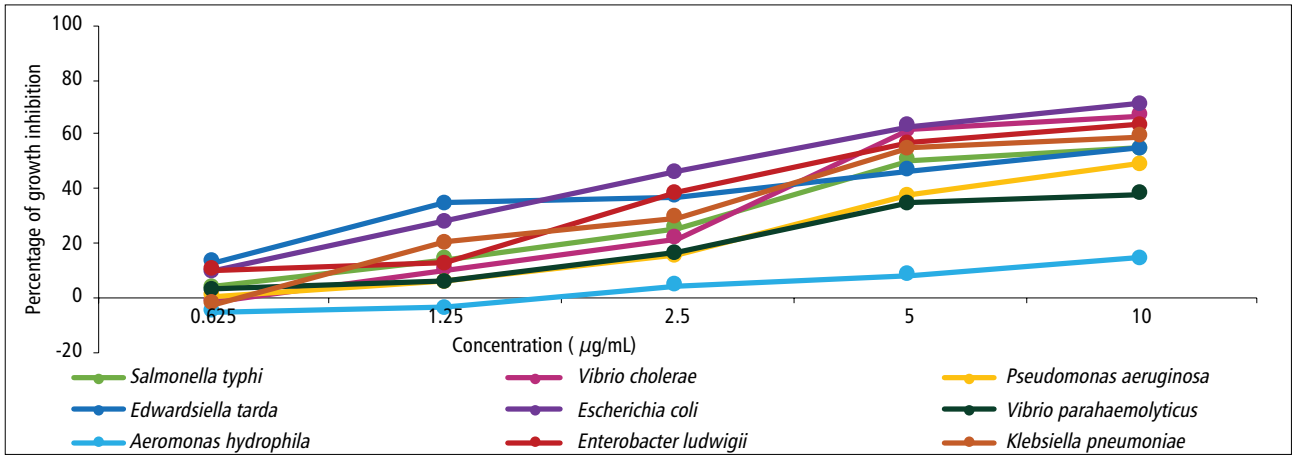

Fig. 4. Antimicrobial activity of crude extract of *H. cinerascens*

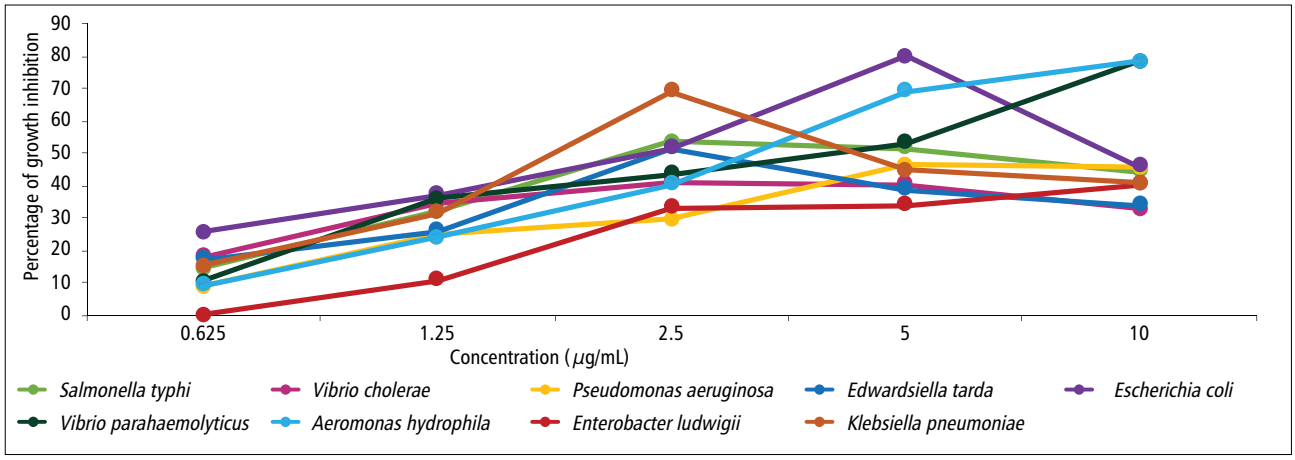

Fig. 5. Antimicrobial activity of crude extract of *S. variolaris*

the crude extract, the number of inhibiting molecules will get reduced. So that some of the targets of active compounds will be free and available for binding with the biologically active compounds. This may be the reason for the high activity of compounds in low concentrations.

The stocks of test materials were prepared in concentrated DMSO. DMSO functions as a drug carrier across membranes without causing cytotoxicity. The higher concentrations of DMSO inhibit the activity of certain compounds by affecting the partitioning properties. Different compounds will be inhibited in different ranges depending upon the partitioning of compounds between DMSO and an aqueous medium. These chemical characteristics of DMSO may affect the solubility of compounds in the broth (Jacob and Herschler, 1986; Kelava *et al.,* 2011; Popa-Burke and Russell, 2014). The extracts of *H. cinerascens* showed an increase in antimicrobial activity with increasing concentration. The antimicrobial properties of echinoderms may be mainly due to the presence of terpenoids, flavonoids, carbohydrates, steroids, tannins, and glycosidic compounds, specifically saponins or saponin like compounds (Ivanchina *et al.,* 2000; Avilov *et al.,* 2000; Maier *et al.,* 2001; Ceesay *et al.,* 2019).

The methanolic extracts of *H. cinerascens* and *S. variolaris* showed antimicrobial and cytotoxic activities. The extract of *S. variolaris* was highly active against Gram-negative bacteria and cancer cell lines. From the observations, these two echinoderms are potential sources of bioactive compounds, which may lead to the development of potential drugs in the pharmaceutical field. Fractionation, purification, and structural elucidation of compounds are necessary to evaluate the biologically active compounds.

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