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Biopotential of secondary metabolites isolated from marine sponge *Dendrilla nigra*

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ABSTRACT

Objective: To evaluate the biopotential activity of secondary metabolites from marine sponge *Dendrilla nigra* (*D. nigra*) collected from the Gulf of Mannar. **Objective:** Soxhlet extraction method was used to extract the secondary metabolites and various assays were carried out. **Results:** *D. nigra* showed potent antibacterial, antioxidant, anti-inflammatory and anticancer activities and it was also subjected for brine shrimp lethality and cytotoxicity assays. The secondary metabolites were characterized by gas chromatography-mass spectrometry (GC-MS) analysis. **Conclusions:** Based on the present study, it can be inferred that the bioassay guided fractionation and purification of *D. nigra* may come up with potent bioactive drug.

1. Introduction

Sponges are the most primitive multicellular invertebrates, considered as a gold mine during the past 50 years, have fascinated scientists for isolation of promising bioactive compounds for human welfare[1]. The work on sponge natural products was systematically started and three nucleosides from the Caribbean sponge *Cryptothethya cypta* Laubenfels[2] were isolated. These entities are the sources of new leads for treatment of many diseases such as cancer, AIDS, inflammatory conditions and a large variety of viral, bacterial and fungicidal diseases[3]. Although many bioactives have been discovered in sponges only a few of these compounds have been commercialized[4]. Therefore, exploration of chemical ecology of secondary metabolites is a promising value[5] and some of these secondary metabolites offer avenues for developing potent drugs[6]. In light of this, the present study was initiated to find out the biopotential of marine sponge *Dendrilla nigra* (*D. nigra*) collected from Gulf of Mannar. However, this coast is rich in sponges[7], the willful collection of sponges is now banned by the Government of India due to species depletion and endangerment. Non destructive collection was made in the present study.

2. Materials and methods

2.1. Collection of sample

Sponge samples were collected as entangled specimens from a bottom trawl fish net operated off Manoli and Hare Islands of Mandapam group of Islands, Gulf of Mannar. The samples were placed inside sterile ethyl polythene bags under water and transferred to the lab aseptically in ice boxes.

2.2. Bioactive compound extraction

Prior to the extraction, samples were washed with water, cleaned, air dried, lyophilized and powdered. They were stored for further use. For the extraction of crude bioactives, 100 g of powdered material was exhaustively extracted with 200 mL of ethyl acetate using Soxhlet apparatus and concentrated in a rotary evaporator at reduced pressure[8,9]. The extract was stored at 4 ℃ in air-tight plastic vials for further studies.

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2.3. Antibacterial activity

In vitro antibacterial assay was done using disc diffusion method[10] against human pathogens *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella* sp., *Proteus* and *Pseudomonas*. Nutrient agar plates were prepared and wells were made using gel puncture. Test culture was swabbed aseptically and inoculated on the surface of the nutrient agar so as to make a lawn. This was allowed for 5 min for the agar surface to dry before making the wells. The extracts and respective solvent were added to the wells and the plates were incubated for 16 to 18 h at 37 ℃. The zone of inhibition was observed around the well.

2.4. Antioxidant activity

The ability of the extract to scavenge 2, 2-diphenyl-1-picrylhydazyl (DPPH) radicals was determined by the method of Gyamfi *et al* with minor modifications. A volume of 20 μL of test extract at different concentrations in methanol was mixed with 0.5 mL of 100 mM methanolic solution of DPPH. After 30 min of incubation in darkness and at ambient temperature, the resultant absorbance was recorded at 517 nm and the percentage inhibition was calculated using the following formula: Percentage inhibition = (Absorbance control − Absorbance sample)×100/ Absorbance control.

2.5. Brine shrimp lethality assay

This technique is an *in vivo* lethality test in a tiny crustacean, the brine shrimp (*Artemia salina*). It has been previously utilized in various bioassay systems including the analysis of pesticide residues, mycotoxins, stream pollutants, anesthetics, dinoflagellate toxins, morphine-like compounds, toxicity of oil dispersants, cocarcinogenicity of phorbol esters and toxicants in marine environments. This test takes into account the basic premise that pharmacology is simply toxicology at a lower dose, and that toxic substances might indeed elicit, at a lower non-toxic dose, interesting pharmacologic effects.

The method described by Mongelli *et al*[11], was adopted to study the cytotoxicity activity of the compound. Water life brand brine shrimp (*Artemia salina*) eggs were purchased and the eggs were hatched in a flask containing 300 mL of sea water. The flask was well aerated with the aid of an air pump, and kept in a water bath at (29-30 ℃). A bright light was left on. The nauplii hatched within 48 h. The extracts and pure compounds were dissolved in normal saline. Different concentrations were obtained by serial dilution. Solution of each concentration was added. A check count was performed, and the number alive was noted after 24 h. The mortality end point of the bioassay was determined as the absence of controlled forward motion during 30 sec of observation.

2.6. Cytotoxicity assay

Cytotoxicity of extracts at various concentrations (15-1 000 μg/mL) was assessed for Hep2 and MCF7 using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) but with minor modification, following 72 h of incubation. Assay plates were read using a spectrophotometer at 520 nm. Data generated were used to plot a dose-response curve of which the concentration of extract required to kill 50% of cell population (IC_{50}) was determined.

Cell viability (%) = Mean OD/ Control OD × 100

Where, OD means optical density.

2.7. Anti-inflammatory activity

Blood was collected from healthy volunteer and mixed with equal volumes of Alsever solution. The blood was centrifuged at 3 000 rpm and the packed cells were washed with isosaline and 10% v/v was made with isosaline. A volume of 0.5 mL of this was taken in a tube and to this was added 1 mL of PBS, 2 mL of hyposaline and 0.5 mL of the extract. Indomethacin was used as a reference drug[12]. Instead of hyposaline 2 mL of distilled water was used in the control. All the assay mixture was incubated and centrifuged. The haemoglobin content in the supernatant was read at 540 nm. The percentage of haemolysis was calculated by assuming the haemolysis produced in the presence of distilled water ad 100%. The percentage of protection was calculated using the formula:

% Protection=100-OD of drug treated sample×100/OD of control

Where, OD means optical density.

2.8. Gas chromatography-mass spectrometry (GC-MS) analysis

The crude extract was quantified using gas chromatograph (Shimadzu QP2010) equipped with a VF-5 ms column (diameter 0.25 mm, length 30.0 m, film thickness 0.25 μm) mass spectrometer (ion source 2000C; EI-70 eV), programmed at temperature 40-650 ℃ with a rate of 4 ℃/min. Injector flow rate was 200 ℃; carrier gas was He 99.9995% purity, column flow rate 1.51 mL/min, injection mode-split.

3. Results

Drug from marine resources is an area which has offer an unprecedented opportunity hence has received great attention during recent years[13]. Emergence of antibiotic resistance among pathogenic microorganisms limits treatment options[14]. The crude extracts were subjected to antimicrobial assay using the agar well diffusion method[15]. Based on the preliminary screening, ethyl acetate was found to have potent antimicrobial activity for sponge *D. nigra*. Minimum inhibitory concentrations (MICs) of the extracts were determined[16]. Ethyl acetate was determined to be the best solvent for isolation of bioactive secondary metabolites. The sensitivity of ethyl acetate to all of the mangroves extracts could be attributed due to the presence of common bioactive compounds that had inhibitory effects on the microorganisms[17,18]. Antimicrobial activity of extracts was examined and found to exhibit good antibacterial activity for crude extract at 100, 125 μL against most of the gram

Table 1
Antibacterial activity of *D. nigra* at different concentrations.

Bacterial pathogens	Zone of inhibition (mm)				
	Standard	Concentrations of crude extract (μL)			
		25	50	75	100
Proteus	9	10	15	17	18
S. aureus	9	11	13	15	17
Pseudomonas aeroginosa	14	12	13	16	18
E. coli	13	10	15	17	18
Klebsiella	12	12	15	17	18

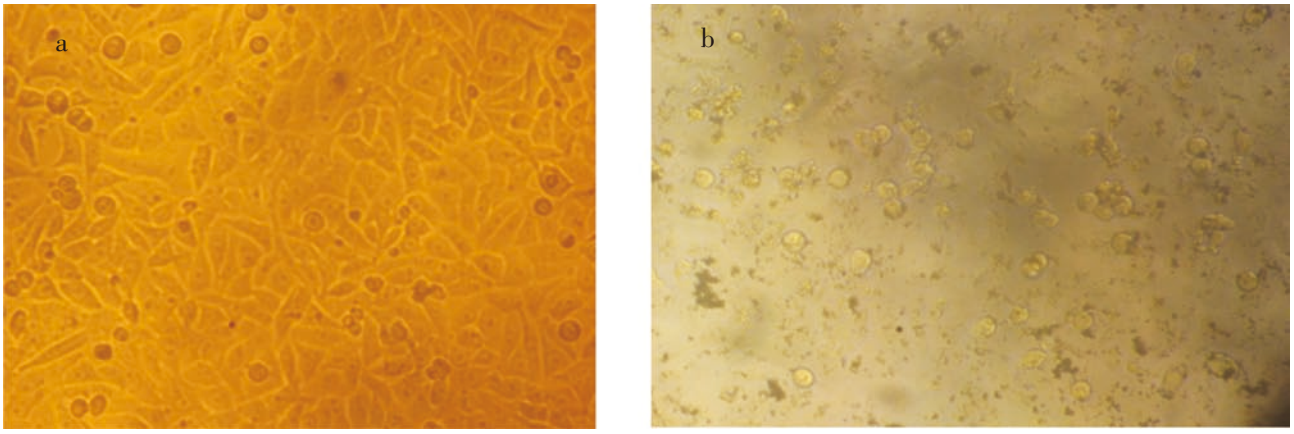

Figure 1. Anticancer activity of *D. nigra* against Hep2 cell line.
a: Control; b: Hep2 cell line treated with the drug.

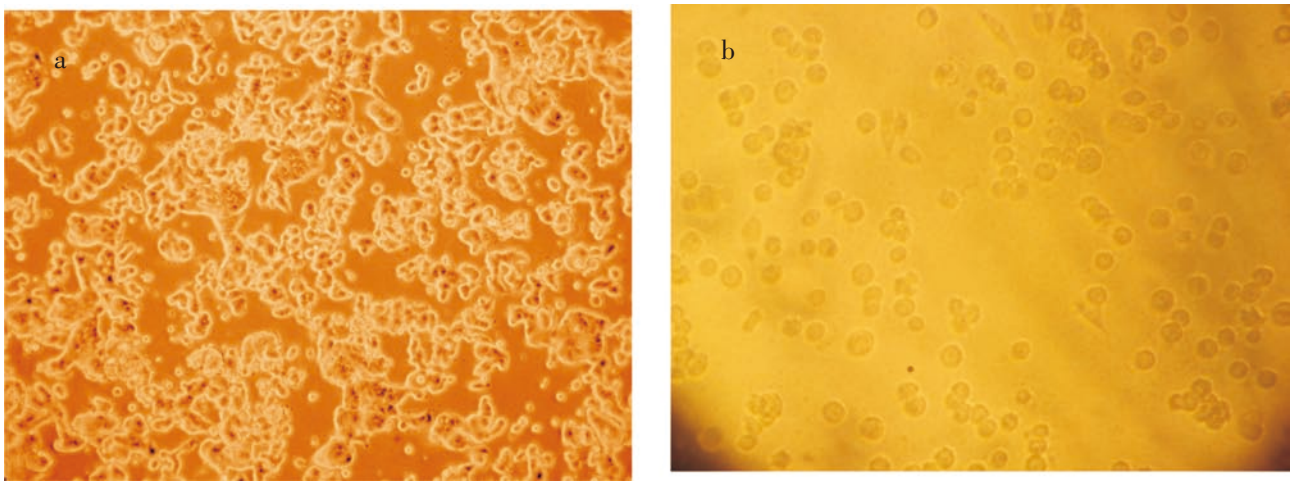

Figure 2. Anticancer activity of *D. nigra* against MCF 7 cell line.
a: Control; b: MCF7 cell line treated with the drug.

positive and gram negative organisms (Table 1).

The secondary metabolites of sponge exhibited high toxicity against artemia nauplii. The *in vitro* anticancer activity of the sponge extract was screened using MTT assay. Two cell lines Hep2 (Figure 1) and MCF7 (Figure 2) representing human larynx and breast malignancy were cultured with crude extracts to be tested at a concentration of 1% (v/v) for 48 h and cell viability was determined. The IC_{50} values for MCF7 and Hep2 were observed as 59.52 and 58.82 μg/mL, respectively (Figure 3 and 4).

The percentage of antioxidant activity of content was determined by DPPH assay. A dark colored crystalline powder containing the free radicals was used in this experiment which will be removed by the antioxidants. Sponge *D. nigra* showed activity of 50.83% when compared to the standard BHT which showed activity of 100.00%. The absorbance for *D. nigra* was 0.354 and blank was 0.720.

The lysosomal enzymes released during inflammation produce a variety of disorders. The extracellular activity of these enzymes is said to be related to acute or chronic inflammation. The non steroidal drugs function by inhibiting these lysosomal membranes[19]. Since human red blood cell (HRBC) membrane is similar to lysosomal membrane components, the prevention of hypotonicity induced HRBC membrane lysis is taken as a measure on anti-inflammatory activity of drugs[20]. The results were reported in Table 2. The crude extract showed significant anti-inflammatory activity at the concentration of 100 μL/mL which is comparable

to the standard drug indomethacin. A variety of natural products from marine sponges have been found to exhibit remarkable antitumour and anti-inflammatory activities[21].

Table 2
Anti-inflammatory activity of the extracts.

Concentration (100 μL)		% Protection	Concentration (200 μL)		% Protection
Control	Test		Control	Test	
1.09	1.26	15	1.29	1.21	7
Standard		72	Standard		58

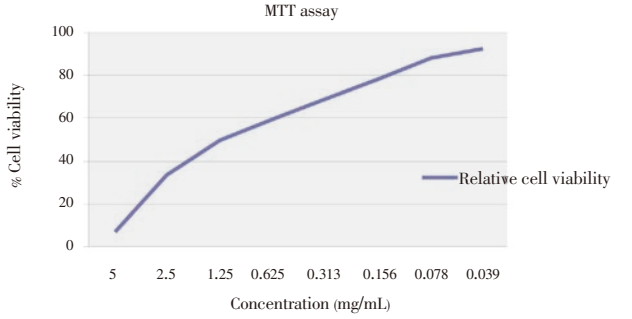

Figure 3. Cytotoxicity of *D. nigra* for breast cancer cell line.

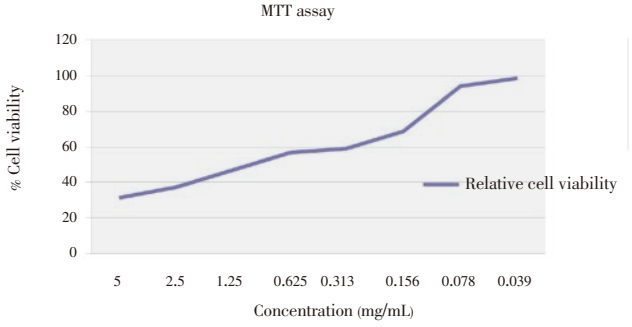

Figure 4. Cytotoxicity of *D. nigra* for Hep2 cancer cell.

Chemical characteristics of active fraction on the basis of spectral data by GC-MS were found to be a mixture of fatty acids which were observed with retention time as presented in Figure 5. The chromatogram of *D. nigra* active fraction found that the main phycoconstituents showed antibacterial, antioxidant, anti-inflammatory and anticancer activities. We have found a mixture of fatty acids on the basis of spectral data by GC-MS analysis.

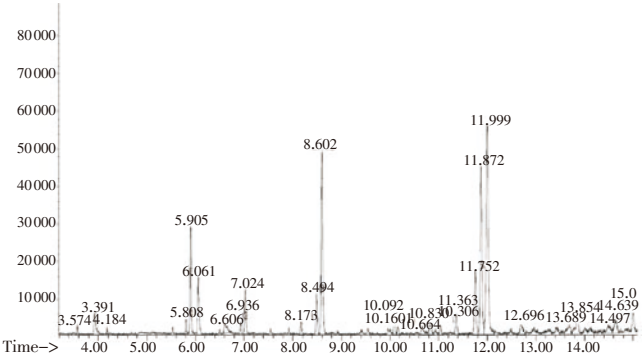

Figure 5. GC-MS spectrum of *D. nigra*.

4. Discussion

Marine sponges are a 'gold mine' with respect to the diversity of their secondary metabolites and can provide potential drugs against many major worldwide occurring diseases[22]. In our study, gram positive as well as gram negative bacteria were more or less equally represented in the producers encountered. Secondary metabolites of sponges exhibited significant antibacterial activity.

The secondary metabolite of sponges also exhibited significant bactericidal activity. The sponge *D. nigra* exhibited broad spectrum of antibacterial activity and it inhibited the growth of all the tested bacteria. Most of the available reports on antibacterial property of sponges revealed their activity on gram positive bacteria.

Antioxidants are capable of inhibiting the oxidation that produces free radicals. In turn, these radicals can start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radicals intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid or polyphones.

One of the well studied cytotoxic sponge *Axinella donnani* was reported with vast potential of antitumour activity[23]. The Philippine marine sponge, *Plakinastrella* sp. yielded peroxide containing metabolites and the crude methanolic extract was toxic to brine shrimp[24]. The crude extracts of marine sponges *Pachastrella* sp. and *Jaspis* sp. collected from the south sea of Korea, exhibited significant brine shrimp cytotoxicity. Guided by this bioassay, fractionation and purification gave pectenotoxin II and psammaplin A, which were cytotoxic to human cancer cell line[25]. In the present study, also the crude extracts of marine sponge *D. nigra* exhibited significant brine shrimp cytotoxicity. Therefore, bioassay guided purification of *D. nigra* may give potent cytotoxic drugs.

Cytotoxicity assays are a widely-used method in *in vitro* toxicology studies. It is not only rapid and standardized, but also a sensitive and inexpensive method to measure drug-induced alterations in metabolic pathways or structural integrity which may or may not be related directly to cell death[26-36]. In the present study, the crude extracts of sponge were made with only ethyl acetate, thus, the comparison between different extracts using different solvent systems cannot be made. The results revealed that the extracts showed different cytotoxic effects on MCF7 and Hep2 cell lines. The present study has planned to find out the newer compounds from the most unexplored sponge. These chemicals have been developed in the oceans for thousands of years[37] and remained largely untapped and thus there is a need to highlight the biomedical potential of the organisms[38]. The search for bioactive metabolites is a result of curiosity and interest as they prove to be new lead structures as generation next medicines by passing the age-old antibiotics.

Conflict of interest statement

We declare that we have no conflict of interest.

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