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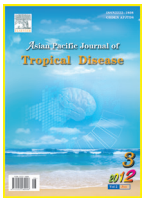

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Cytotoxic and antioxidant activity of selected marine sponges

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ABSTRACT

Objective: To evaluate the anticancer activity of the crude extracts of *Rhabdastrella globostellata* (*R. globostellata*) and *Spirastrella inconstans* (*S. inconstans*) var. moeandrina Dendy. **Methods:** Soxhlet extraction method was used to extract the secondary metabolites and various assays antioxidant, anticancer and various assays were carried out. The extract were tested anticancer activity against a HeLa, Raw 264.7 and Hek-293. **Results:** The sponge extracts tested exhibited from median to high toxicity in at least one of the toxicity bioassays performed. The antioxidant activity of the isolated metabolite in ethylacetate solution was assessed by SOD and GTH assays and compared with that of other known natural antioxidants. **Conclusions:** Potent antioxidants have been detected among both phenolic metabolites and alkaloids. Antioxidant effects of tested compounds have been attributed to their action as chain-breaking antioxidants and/or as scavengers of radicals

1. Introduction

Natural products and their analogs or molecules derived there comprise approximately 50% of the drugs presently used for clinical purposes. Regarding anticancer drugs, 63% of them fall into this category[1,2]. Despite the high number of available drugs, there is a growing need to develop more specific agents to treat cancers, particularly chemo-resistant tumors[3]. Biodiscovery is the extraction and testing of molecules for biological activity, identification of compounds with promise for further development, and research on the molecular basis for the biological activity[4].

Sponges are known to produce bioactive metabolites as part of their defensive system[5]. They occasionally develop symbiotic (mutually beneficial) relationship with both algae and microorganisms, and symbionts are to an extent the true source of secondary metabolites found in sponges[6].

Seasonal changes influence various abiotic factors such as temperature, pH, salinity *etc* as well as biotic factors like morphology and epifaunal diversity ultimately responsible for the biosynthesis of secondary metabolites[7,8].

The semi-purified fractions were screened for anticancer, antibacterial and antioxidative effects. The methods used in the anticancer screening are based on the methods utilized by the National Cancer Institute (NCI, United States) in their search for anticancer drugs. In the anticancer screening the cancer cells were exposed to the semipurified fractions and the viability was checked with cell lines from human carcinomas. A normal cell line was used as a control for toxicity. The antibacterial screening was carried out by exposing a panel of Gram-positive and Gram-negative bacteria to the semipurified fractions, and the inhibition of bacterial growth was determined. Bacteria strains in this study were selected because they cause serious infections and frequently lead to problems for human health. The antioxidant screening was carried out by testing the ferric reducing ability of plasma in order to measure the antioxidant capacity of the extracts.

Most chemotherapeutic drugs are directed against actively dividing cells, and so the malign cells will be influenced to the larger extent than normal cells. These drugs will effect normal cells in the dividing stage and therefore patients experience adverse side effects. Targeted cancer therapy uses drugs that specifically should attack cancer cells, and hence may have fewer side effects[9]. In this project cancer cell lines and one normal cell line were used for the anticancer screening.

An imbalance between the production of ROS and antioxidant defense can cause oxidative stress and lead to oxidative damage[10]. Proteins, lipids and nucleic acids are

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prone to oxidative damage which in a worst case scenario may lead to a number of serious conditions such as cancer, cardiovascular diseases and Alzheimer's disease[11].

Various phytochemicals present in sponges help in providing protection against cancer, cardiovascular diseases, dementia, cataract, macular degeneration, ageing and various other disorders associated with increased oxidative stress. These phytochemicals act as antioxidants which intercept free radicals and protect the cells from the oxidative damage[12]. Marine sponge extracts from unused by-products such as head and viscera of salmon have been investigated as cheap sources of natural antioxidants[13]. Besides, the accumulation of antioxidant molecules in fish gonads has also been studied mainly exploring the protection of embryos during the first stages of development[14].

Relatively few works were carried out to investigate antioxidant properties of marine natural products isolated from sponges[15,16]. The aim of this work was to study a distribution of sponges containing antioxidants in the phylum Porifera and to investigate marine natural products responsible for an antioxidant activity of ethylacetate extracts from sponges. Antioxidant activities of the majority of extracts have been caused by the presence of phenolic or heteroaromatic compounds.

Antioxidants are classified as preventative when they prevent formation of free radicals. Others are classified as radical scavengers, functioning to halt further propagation of chain reactions. The final class of antioxidants consists of repair molecules which ameliorate oxidative damage to a cell methods to quantify antioxidant activity have been an important component of biomedical research.

2. Material and methods

2.1. Sponge collection

Samples of *R. globostellata* and *S. inconstans* var. moeandrina Dendy were collected by scuba-divers at depths of 15 m (first collection) and 7 m (second collection), on Tuticorin gulf of Mannar, in the state of Tamilnadu. The sponge collection was made in March, 2010 and these samples were directly immersed in ethyl acetate. Samples from the second collection in May, 2011, were immediately frozen under dry ice (-20℃). Both collections were sent to the laboratories, Department of Zoology, Sri Paramakalyani College, and Alwarkurichi. All samples were maintained at -20 ℃, and subsequently identified by Dr. PA Thomas, National Institute of Marine Sciences and Technologies, New Delhi.

2.2. Extract preparation

The sponge was extracted in ethylacetate (which was reserved) and the remaining material was sequentially extracted four times by maceration in ethylacetate (0.3 g/mL), over 4 days. The ethylacetate solution was reserved each of these days. After the fourth day, the solutions were blended, filtered, concen-trated in a rotary evaporator and dried in a SpeedVaccum evaporator. A portion of the solution extracted from samples collected in 2011 was partitioned against hexane (1:1, v/v) (100%), the lipid portion was removed, and then the polar fraction was dried in a SpeedVaccum evaporator.

2.3. Cell culture and cytotoxicity evaluation

The HeLa (Human cervical cancer cell lines), Raw 264.7 (Mouse leukaemic monocyte macrophage cell line) and HEK-293 (Human kidney cell line) were obtained from American Type Culture Collection, Manassas, VA, and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 2mM glutamine and 100 μg/mL Penicillin-Streptomycin incubated at 37 ℃, humidified atmosphere with 5% CO_2. Cytotoxity of the crude extract II, III, A, B, C and D were measured using MTT[3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (Sigma, St. Louis, USA)]assay. For this, the cells were grown at a concentration of 5 x 103 cells/well in 96 well plates. After 24 h, cells were washed with fresh medium and were treated with different concentrations of crude extract (12.5, 25, 50 and 100 μg/mL). The cells without the addition of crude extract were taken as control. After 24 h incubation, 100 μL of MTT (1 mg mL-1) solution was added and further incubated for 4 h at 37 ℃. Finally 100 μL DMSO was added to solubilize the formazan salt formed and the amount of formazan salt was determined by measuring the OD at 540 nm using a GENios® microplate reader (Tecan Austria GmbH, Austria). The relative cell viability was determined by the amount of MTT converted into formazan salt. Viability of cells was quantified as a percentage compared to that of control. The experiment was carried out in triplicate and the data were expressed as mean from these three sets of experiments.

2.4. Antioxidant Assay

2.4.1. Animals used

Healthy and pure strain male albino rats (Rattus norvegicus), ranging from the body weight of 150-200 g were procured from the Department of Experimental Medicine, Central Animal House, Nagercoil and used for the experimental study. The animals were housed in polypropylene cages at (24 ± 2)℃ and were fed with proper food and water *ad libitum* throughout the experiment. The experiment got clearance from the Institutional Animal Ethical Committee.

2.4.2. Toxicities studies

Acute toxicity study for WAELI was performed by Miller and Tainter method 20 to determine lethal dose (LD_{50}). CMC (0.025%) was used as vehicle to suspend the extracts and the suspension was administered intraperitoneally. One tenth of the LD_{50} was used as a maximum dose of exacts tested for acute toxicity. The dose was selected for evaluation of heteroprotective activity *i.e.* 400 mg/kg.

2.4.3. Selection of experimental animals

The animals were divided into 7 groups with 3 rats each.

Out of 7 groups, group I served as normal control and received water. Group II served as aspirin control received aspirin (200 mg/kg). The remaining 6 groups served as treated groups received ranitidine (50 mg/kg) – a standard drug and sponge extracts administered in different doses (2, 4, 6, 8 and 10 mg/kg). All the doses were administered orally once daily for 14 days. From the 6th day onwards, animals in groups III to VIII received aspirin (200 mg/kg) orally, one hour after the administration of the drugs.

2.4.4. Superoxide dismutase (SOD) activity

The inhibition of reduction of nitro blue tetrazolium (NBT) to blue colored formozan in presence of phenazine metha sulphate (PMS) and NADH was measured at 560 nm using n-butanol as blank. One unit (U) of enzyme activity was defined as the amount of enzyme that inhibits rate of reaction by 50% in 1 min under the defined assay conditions and the results had been expressed as U of SOD activity/mg protein.

2.4.5. Reduced glutathione activity

Reduced glutathione was estimated as total non-protein sulphydryl group by the method as described by Moron *et al* (1979). Homogenates were immediately precipitated with 0.1 mL of 25% trichloroacetic acid and the precipitate was removed after centrifugation. Free SH groups were assayed in a total 3 mL volume by adding 2 mL of 0.6 mM DTNB (5,5′-Dithio-bis 2-nitrobenzoic acid) prepared in 0.2 M sodium phosphate buffer (pH 8.0), to 0.1 mL of the supernatant and absorbance was read at 412 nm using a Shimadzu UV-160 spectrometer. GSH was used as a standard to calculate millimoles of SH content per gram of tissue.

2.4.6. Assay of catalase

Catalase activity was determined by the titrimetric method. To 1 mL sponge extract, 5 mL of 300 μM phosphate buffer (pH 6.8) containing 100 μM hydrogen peroxide (H_2O_2) was added and left at 25℃ for 1 min. The reaction was stopped by adding 10 mL of 2% sulphuric acid, and residual H_2O_2 was titrated with potassium permanganate (0.01N) till pink colour was obtained. Enzyme activity was estimated by calculating the decomposition of μM H_2O_2 per min per mg protein[17].

2.4.7. Assay of glutathione peroxidase

To 0.2 mL each of 0.8 mM EDTA, 10 mM sodium azide, 1mM GSH, 2.5 mM H_2O_2, 0.32 M phosphate buffer (pH 7.0) and sponge extract were mixed and incubated at 37 ℃ for 10 min. The reaction was arrested by the addition of 0.5 mL of 10% TCA and the tubes were centrifuged at 5 000 rpm for 5 min. To 0.5 mL of supernatant, 3.0 mL of 0.33 mM phosphate solution and 1.0 mL 0.6 mM DTNB reagent were added and the colour developed was read at 420 nm immediately. Graded amount of standards were also treated similarly. Glutathione peroxidase activity was expressed as μg of glutathione utilized per mg of protein[18].

3. Results

3.1. Cytotoxicity assay

These results were expressed as percent viability and as total number of viable cells (Figure 1-3). The tests started with ethylacetate extract. The extract (sample II, III, A, B, C, D) was cytotoxic only at concentrations of 12.5 g/mL and 100 g/mL. Viability decreased following a dose/time res-ponse curve. There was no significant difference between control viability and treated cells.

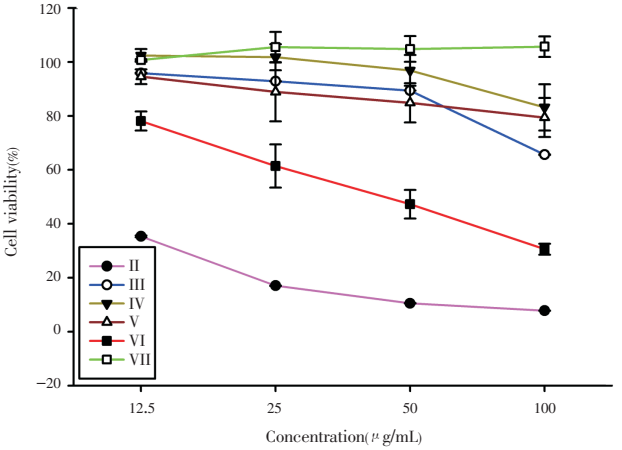

Figure 1. Anti Cancer Activity(Raw 264.7)

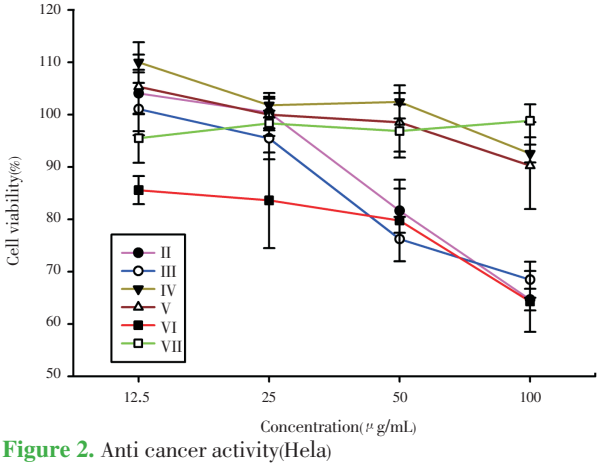

Figure 2. Anti cancer activity(Hela)

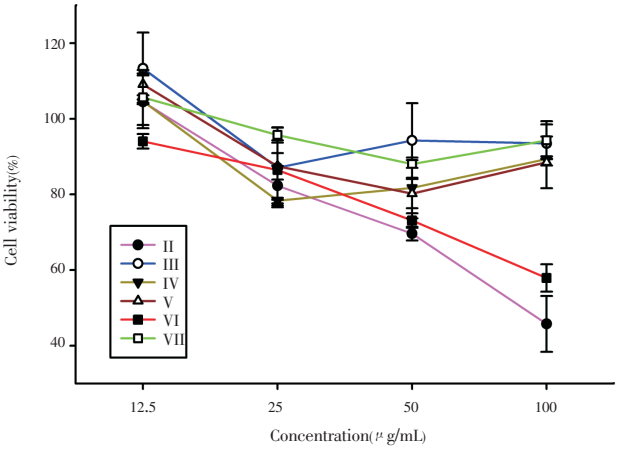

Figure 3. Cell viability normal cell line(HEK-293)

3.2. Antioxidant assay

The hepatic SOD activity was increased by 4.5±0.05 (P < 0.05) and 5.9± 0.06 (P < 0.05) in the extracts of *R. globostellata* and *S. inconstans* var. moeandrina Dendy compared with the control group. In immunosupressed animals, it showed a decrease in SOD activity (Table 1).

Table 1

Modulatory influence of *R. globostellata* and *S. inconstans* var moeandrina Dendy on antioxidant enzyme profiles in the extrahepatic organs of rats.

Group	SOD[a]	CAT[b]	GPX[c]	GR[d]	GSH[e]
I Control	4.5±0.005	29±0.10	20±1.86	33±1.80	0.97±0.13
II (Aspirin)	4.7± 0.05	44±0.06	33±2.55*	40±3.89*	1.60±1.19*
III (2 mg/100ml)	3.9± 0.05	96±5.20*	33±2.47*	41±2.77*	1.70±0.16*
IV (4 mg/100ml)	5.9± 0.06	98±5.20*	94±5.20*	44.±1.77	1.54±0.17*
V (6 mg/100 ml)	4.1±0.05	23±0.12	12±0.03	21±0.10	0.77±0.10
VI (8 mg/100ml)	4.4±0.04	38±0.16	25±0.16	40±0.07	1.53±0.03
VII (10 mg/100ml)	3.8±0.05	41±0.6	29±0.30	59±0.02	1.82±0.0

*: significant.

CAT activity increased in both *R. globostellata* and *S. inconstans* var. moeandrina Dendy sponge extract treated animals by 29±0.10 (P < 0.05) and 98± 5.20 (P < 0.05) relative to control.

GRX activity: *R. globostellata* and *S. inconstans* var. moeandrina Dendy sponge extract augmented the GPX activity by 20±1.86 (P < 0.05) and 94±5.20 (P < 0.05) when compared to control group.

The level of GR, the non-enzymatic antioxidant protein was enhanced by 33±1.80 (P < 0.05) and 59±0.02 (P < 0.05) by the extracts of *R. globostellata* and *S. inconstans* var. moeandrina Dendy sponge (Table1).

4. Discussion

Antioxidant refers to any substance that hinders the reaction of a substance with dioxygen or any substance that inhibits free radical reaction[20-22]. Nowadays antioxidants have gained more importance on account of their positive effects, as health promoters in the treatment of cardiovascular problems, atherosclerosis, cancers, the ageing process, *etc*. Many antioxidant compounds which are naturally occurring in plant sources have been identified as free radical scavengers[23, 24]. In present study, *in vitro* antioxidant activity of the two marine extracts showed potential free radical scavenging activities expressed in IC_{50} value 29±0.10 (P < 0.05) and 98± 5.20 (P < 0.05), respectively.

Developing countries, which is induced by oxidative stress[25]. Hence antioxidants needs thorough search especially safer compounds from plant sources. Increased oxidative stress encountered in body due to either environmental hazard, or impairment in the body metabolism due to various disease conditions including drugs or having insufficient amount of dietary antioxidants, The oxidative stress has to be curbed by exogenous supply of antioxidants as a choice of therapy or preventive measure. Natural antioxidants that are present in herbs and spices are responsible for inhibiting or preventing the deleterious consequences of oxidative stress.

Currently there has been an increased interest worldwide to identify antioxidant compounds from plant sources which are pharmacologically potent and have small or no side effects for use in protective medicine and the food industry. Increasing acquaintance in antioxidant phytoconstituents and include them in daily uses and diet can give sufficient support to human body to fight those diseases. Phytochemical analysis revealed the presence of tannins, flavonoids, steroids and alkaloids. This study affirms the *in vitro* antioxidant potential of crude ethyl acetate extracts of the marine sponges, with results comparable to those of the standard compounds such as aspirin.

Cytotoxicity assays are widely used method *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 intergrity which may or may not be related directly to cell death[24]. The crude extracts of marine sponge *D. nigra* exhibited significant brine shrimp cytotoxicity[27]. In the present study, the crude extracts of sponge were made with only ethyl acetate, thus the comparision between different extracts using different solvent systems cannot be made. The results revealed that the extracts showed different cytotoxic effects on Raw 264.7 (Mouse leukaemic monocyte macrophage cell line) and HEK-293 (Human kidney cell line).

Reference to antioxidant enzyme status in liver, the specific activity of almost all the antioxidant related enzymes including glutathione peroxidase, glutathione reductase, catalase, and superoxide dismutase were found elevated above the control basal values in the *R. globostellata* extract treated rats. Superoxide dismutase plays an important role in catalyzing the dismutation of superoxide radicals. Increase in SOD activity accelerates the removal of the reactive oxygen species. Catalase activity was also augmented by the extracts of *S. inconstans* var. moeandrina Dendy sponge and *R. globostellata* at help to remove the hydrogen peroxide produced by the action of SOD. Induced SOD activity along with that of catalase explains the decrease in glutathione reductase, which is an indicator of oxidative stress that persists in the cell. In the phytochemical studies it was found that the sponge extracts have a high amount of flavonoid content.

The results of the present investigation showed potential bioactive properties of marine sponge extracts. To our knowledge, the results describes antioxidant and anti-inflammatory activity of marine compound and they are promising for the development of drug for anti-cancer.

Conflict of interest statement

We declare that we have no conflict of interest.

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