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Evaluation of Apoptosis Regulating Efficacy of Chosen Marine Sponge Extracts

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Abstract

Apoptosis is the most desirable target mechanism for induction of cell death in tumor cells. In this study three potent sponges such as *Sigmadocia carnosa, Clathria gorgonoides* and *Callyspongia sp.* were evaluated for their apoptosis regulating efficacy. These sponges were collected, extracted with methanol for bio-toxicity screening. The apoptotic inducing ability of sponge extracts have been evaluated using different bioassays. Various activities such as antimicrobial, biotoxicity and cytotoxicity were studied. *Sigmadocia carnosa* extract strongly inhibited Human leukemia cell lines (ATCC CCL-2), and was non-toxic to normal cells. It was cleared by all the results of tested bioassays such as MTT cytotoxicity, Hydrogen Peroxide Scavenging assay, DPPH Radical scavenging assay, COMET assay (Single cell electrophoresis), Trypan blue exclusion cell viability assay and DNA Fragmentation assay. In conclusion methanolic extract of *Sigmadocia carnosa* offers a valuable candidate lead compound to counter growing drug resistance in cancers.

Keywords: Apoptosis, Ani-cancer activity, Biotoxicity, Leukemia cell lines.

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INTRODUCTION

Cancer is one of the most dangerous diseases reported in the world of medical science. The world cancer report, issued by International Agency for Research on cancer tells that cancer prevalence are set to increase at a global alarm to aware of this epizootics [1]. The report also reveals that cancer has emerged as a major public health problem in developing countries. This shows the harmful growth of this killer disease, so the management of cancer disease is very important. Recently, National Cancer Institute screened around 114,000 extracts from an estimated 35,000 samples showed anticancer activity. Among that the significant antioxidant activity and apoptotic induction of marine group has generated interest as potential sources of chemotherapeutic compounds [2]. In this perspective, the present study intended to develop novel, potent, safe anticancer drugs especially against leukemia from chosen marine sponges of Indian peninsula.

Marine sponges are the excellent source of bioactive compounds such as carotenoids, dietary fibre, protein, essential fatty acids, vitamins and minerals [3], [4]. Sponges with their chemical defense mechanisms are one of the most studied organisms for the isolation of Natural Product Analogues (NPAs) [5]. The constant threat from competitors, by way of over growth, poisoning, infection or predation has armed sponges with a store house of potent chemical defense agents [6]. Sponges produce a wide array of secondary metabolites ranging from derivatives of amino acids and nucleosides to macrolides, porphyries, terpenoids, aliphatic cyclic peroxides and sterols [8].

Many of the published reviews showed the importance of sponges as potential source of pharmaceutical leads [9]. In the present study an attempt has been made to purify and also partially characterize the active metabolites of the metabolic extract of the marine sponge, Sigmadocia carnosa, which shows more potency in the pharmacological studies.

Objectives

To collect and extract chosen sponges from south peninsular coast of India

To screen the bio-toxicity of chosen sponge extracts using standard protocols

To determine the apoptosis regulating efficacy of chosen sponge extracts using leukemia cell lines

MATERIALS AND METHODS

Collection of sponges and crude extract

A diverse variety of sponges (*Sigmadocia carnosa*, *Clathria gorgonoides* and *Callyspongia sp*.) were collected off the peninsular coast of India. Immediately, they were immersed in methanol for extraction and filtered through a Whatman no.1 filter paper fitted with a Buchner funnel using suction. They were extracted thrice and the concentrated crude extract was collected in airtight plastic containers and kept in the refrigerator.

In vitro antibacterial and Biotoxicity screening of extracts

Antibacterial studies were carried out using ten bacterial type cultures obtained from Microbial Type Culture Collections (MTCC), Chandigarh. Likewise, Brine shrimp cytotoxicity (*Artemiasalina*), larvicidal activity (*Culex* sp.) and Ichthyotoxicity assay (*Oreochromismossambicus*) were carried out using standard protocols. From the mortality percentage (probit scale) LD_{50} values were determined.

Cell lines and its preparation for experiment

Human leukemia cell lines (ATCC CCL-2) were obtained from American Type Culture Collection, maintained in continuous exponential growth by twice-a-week passage in RPMI 1640 medium supplemented with 8.25% fortified bovine calf serum (Hi Media) and penicillin (100 IU/ml)- streptomycin (100 g/ml), and incubated in the presence or absence of drugs at 37°C in a humidified atmosphere containing 5% CO_2.

MTT assay

On the first day of the experiment, one T-25 flask of Human leukemia cell lines was trypsinized and 5 ml of complete media was added to trypsinized cells. Further the cells were centrifuged in a sterile 15 ml falcon tube at 500 rpm in the swinging bucked rotor (~400 x g) for 5 min. On the 2nd day, cells were incubated overnight with three extracts of *Sigmadocia carnosa*, *Clathria gorgonoides* and *Callyspongia sp*. with the dose of 10 mg/ml each, in a different experimental setups. On the third day of the experiment, 20 µl of 5 mg/ml MTT was added to each well. One set of wells with MTT was incubated but no cells as the control group. The media was removed carefully and 150 µl MTT solvent was added. The cells were agitated on orbital shaker for 15 min and the absorbance at 590 nm was read with a reference filter of 620 nm.

Hydrogen Peroxide Scavenging assay

The ability of the extracts to scavenge hydrogen peroxide was determined. Hydrogen peroxide solution (40 mM) was prepared in phosphate buffer (pH 7.4). The concentration of hydrogen peroxide was determined by absorption at 230 nm using a spectrophotometer. Sponge Extracts (1 mg ml-1) in distilled water were added to a hydrogen peroxide solution (0.6 ml, 40 mM). The percentage of hydrogen peroxide scavenging by the extracts and standard compounds was calculated as follows:

% Scavenged (H_2O_2) = [(Ao -A1)/Ao] x 100

where Ao was the absorbance of the control and A1 was the absorbance in the presence of the sample of extract and standard.

DPPH Radical scavenging assay

10-100 µg of sponge extracts were added to 295 µl DPPH solution (4,5 mg DPPH (HIMEDIA) in 100 ml methanol) in each well of 96 well plates. The absorbance at 517 nm was then monitored at 15 seconds interval from 0 to 5 min. Methanol was used as the blank solution. Ascorbic acid as a positive control representing 100% radical scavenging activity in each experiment.

COMET assay (Single cell eletrophoresis)

1% (500 mg per 50ml Phosphate buffered Saline (PBS)) and 0.5% Low Melting Point Agarose (LMPA) (250 mg per 50 ml PBS) and 1.0% Normal Melting Agarose (NMA) (500 mg per 50 ml in Milli Q water) were prepared. The Agarose (LMPA and NMA) were boiled to get the agarose solidified. To the agarose coated slide, 75 µL of LMPA (0.5%; 37ºC) priorly mixed with ~10,000 lymphocytes was added. The slides were incubated in lysing solution at 4ºC for 2 hours. The slides were placed side by side on the horizontal gel box near one end, sliding them as close together as possible. The power supply was turned on to 24 volts (~0.74V/cm) with the current of 300 milliamperes.The slides were let for 15 minutes for electrophoresis and removed from the setup. For visualization of DNA damage, observations were made of EtBr-stained DNA using a 40x objective on a fluorescent microscope.

Trypan blue exclusion cell viability assay

The cells culture (treated with extract)was diluted to 100 cells/ml with 1x Phosphate buffered Saline (PBS) solution. 5 ml cell suspension was taken with equal volume of trypan blue solution. 50 microliter of cell trypan blue mixture was taken into micropipette and placed in the edge of Haemocytometer. The solution was allowed to run along the grooves with parallel gentle shaking of haemocytometer slide. The slide was placed under 10X objective lens in a Phase contrast microscope (NIKON) and cells were counted from each chamber.

DNA Fragmentation assay (Triton X100 lysis method)

$4X10^6$ cell lines were incubated with 2 mg of dried sponge extract from each sample was incubated separately in 1 ml eppendrof tube for 10 minutes. The incubated cells were further collected in 1.5 ml eppendorf tube and centrifuged at 3000 rpm to collect the cells. The supernatant was discarded. The cell pellet was then suspended and the tubes were centrifuged at 4^0C for 30 minutes and supernatant was transferred to 1.5 ml eppendorf tubes. 1:1 mixture of Phenol: Chloroform along with one tenth quantity of sodium acetate solution. The above step was repeated for 3 times and finally centrifuged at 5000 rpm. The pellet was resuspended in 30 micro liter of deionized water with RNAse solution (0.4ml water + 5µl of RNase).

Agarose Gel Electrophoresis

The lysed ingredients of DNA fragmentation assay were added in 250mL conical flask and stirred well. Then the conical flask was kept in microwave oven for one minute. 1µL of ethidium bromide (10mg/ml) was added with the solution and mixed thoroughly. The gel was poured at the gel setup without bubbles at 60 ^{0}C and the combs were inserted. The setup was kept undisturbed for 30 minutes to allow the gel to solidify.

Running the gel

0.5X of TBE buffer was poured into the gel tank until the gel is submerged for 2 mm depth. The Samples from Triton x100 lysis reaction was mixed with 5 µL of loading buffer and each sample was loaded into adjacent wells along with Hind III lambda DNA digest marker. The gel was allowed to run until the tracking dye reaches the end of the gel. Further the gel was taken from the setup and documented with BioRAD Gel documentation system.

RESULTS AND DISCUSSION

Collection and identification of sponges

The successful development of anticancer drugs from the sponges completely relies on the continuous availability of the source organism and the cost effective collection methods. The major distributed species were: *Callyspongia sp*. and *Sigmadocia carnosa* followed by *Clathria gorgonoides*. The identified sponges are presented in Table 1. These sponges are collected as bycatch in the fishing nets.

MSE	Concentration	Mortality (%)	
		30 ±2^{0}C	20 ±2^{0}C
Clathria gorgonoides	2%	20.0±4.14	0
	4%	66.4±0.89	20.0±3.4
	6%	90.0±3.94	60.0±5.6
	10%	100±0.0	80.0±7.3
Callyspongia sp	2%	20.2±3.6	0
	4%	60.0±7.0	0
	6%	90.6±3.2	20.0±1.26
	10%	100±0.0	40.0±2.19
Sigmadociacarnosa	2%	10.2±2.6	0
	4%	50.0±7.0	0
	6%	67.2±0.8	0
	10%	85.0±2.5	10.0±1.26

Table 1: *Artemia* cytotoxicity profile of Marine sponge extracts (MSE) at 30 and 20^{0}C
Mean +SD n-10 experiments

Screening for in vitro antibacterial activity

The extracts were initially used for primary antibacterial screening using different MTCC cultures.

The antibacterial activity was done by disc diffusion method. The result is presented in the Table 2.

MSEs	Concentration mg/ml	Mortality (%)	
		Second instar larvae	Fourth instar larvae
C. gorgonoides	10	100±0.0	20.2±1.72
	6	80.2±2.6	0
Callyspongia sp.	10	100±0.0	10.2±2.71
	6	78.2±3.0	0
Sigmadociacarnosa	10	100±0.0	0
	6	71.8±1.32	0

Table 2: Larvicidal profile of MSE on second and fourth instar larvae of *Culex sp.*

Brineshrimp cytotoxicity assay

The results of *Artemia* cytotoxicity bioassay are depicted in Table 1. The secondary metabolites of chosen sponge extract especially *Clathria gorgonoides* exhibited high toxicity against *Artemianauplii* followed by *Callyspongia sp*. and *Sigmadocia carnosa.* The medium lethal dose extrapolated from the graph indicated that *Clathria gorgonoides* was least toxic and it produced 50% mortality at 5.5% level. The LC_{50} values of *Clathria gorgonoides*, *Callyspongia sp*. and *Sigmadocia carnosa* are accounted for 0.20%, 0.28% and 0.32% respectively.

Larvicidal effect

The results of the mortality profile of second instar larvae by sponge extracts are showed in the table 2. The results indicated that the second instar larvae were most susceptible than the fourth instar larvae. The secondary metabolites of *Clathria gorgonoides* had more potent larvicidal activity followed by *Callyspongia sp*. and *Sigmadocia carnosa* effectively killed all the second instar larvae at 3.2% level. Larvicidal potential of *Callyspongia sp*. and *Sigmadocia carnosa* were more or less same kind of activity profile and they produced 100% mortality at 10% level.

Ichthyotoxicity

Ichthyotoxicity profiles of MSEs are presented in Table 3. *Clathria gorgonoides* was extremely toxic and killed all the fingerlings (*O. mossambicus*) within a short exposure time of 40 min at 4% level, 1 h at 2% level and 2 h at 1% level. The medicinal *Callyspongia sp*. was toxic up to 4 mg/ml whereas 2 mg/ml was less toxic and did not influence mortality within 6 h. *Sigmadocia carnosa* was less toxic and as 20.0% mortality was recorded only at 2 mg/ml.

MTT assay

It was noted that methanolic extracts of *Sigmadocia carnosa*, *Clathria gorgonoides* and *Callyspongia sp*. has a killing effect. Maximum cytotoxicity was observed in *Sigmadocia carnosa* extracts over human leukemia Cell lines (78%) at 48 hrs incubation. The different concentrations of sponges were utilized in the present study. Among these 800 µl showed better response after 48 hours of incubation. The moderate cytotoxic responses were noted in the *Clathria gorgonoides* (58 and 48%) and *Callyspongia* (28.48 and 30. 64%) extracts administered Cell lines (Table 4)

Species	Concentration	Mortality (%)	Time of death (h)
C. gorgonoides	4%	100	2
	2%	60.4±3.0	6
	1%	10.0±3.34	-
Callyspongia sp.	4%	100	3
	2%	60.0±2.89	6
	1%	10.0±3.34	8
Sigmadociacarnosa	4%	80.4±3.0	1
	2%	40.6±5.6	6
	1%	0	-

Table 3: Ichthyotoxicity profile of MSE to *Oreochromismossambicus* fingerlings
Mean±SD n=10

Cell lines	Sponge extracts	Concentration of crude extract (µ/ml)	Percentage Cytotoxicity %	
			24hrs	48hrs
Human leukemia Cell lines	*Sigmadociacarnosa*	400	22	43
		800	49.16	78.40
	C. gorgonoides	400	Nil	23
		800	28.36	58.04
	Callyspongia sp.	400	Nil	Nil
		800	19.22	28.48

Table 4: Cytotoxicity potential of chosen sponge extract over leukemia Cell lines at different concentration

Hydrogen Peroxide scavenging assay

The results of H_2O_2 scavenging activity is shown in table 5. The results clearly displayed that the A1 (*Sigmadocia carnosa*) produced high percentage of Hydrogen peroxide scavenging activity. It was followed by A2 (*C.gorgonoides)* and A3 (*Callyspongia sp.)*.It was also noted that the concentration of extracts play an important role in Hydrogen peroxide scavenging activity.

DPPH radical Scavenging activity

The results of DPPH radical Scavenging activity are depicted in table 6. The results clearly indicated that the sponge extract of *Sigmadocia carnosa* (A1) induced more DPPH scavenging profile than the other groups in all concentrations. Interestingly in higher concentration (90 and 100 µg) The *Callyspongia (*A3) extract showed less activity than ascorbic acid (as

control) But the other extracts A2 (*C. gorgonoides*) showed consistent scavenging activity. It was also noted that the A2 produced high activity in low concentrations.

Incubation time (minutes)	*Sigmadocia carnosa* (A1)	*C. gorgonoides* (A2)	*Callyspongia* (A3)
10	21.4±1.14	22.0±0.70	0±0
20	22.0±1.58	25.2±0.83	2.0±0.70
30	33.4±2.4	27.2±0.83	6.4±1.40
40	42.2±1.92	35.2±1.30	11.6±0.89
50	56.0±1.58	35.2±1.30	12.6±1.14
60	56.2±1.78	36.2±1.30	14.8±0.83
70	71.0±1.58	44.0±1.0	16.8±0.83
80	73.8±1.48	46.4±1.67	17.2±0.83
90	96.4±1.14	56.4±1.40	17.6±0.54
100	97.8±1.30	66.8±0.85	20.6±1.14

Table 5: Hydrogen Peroxide Scavenging Activity of chosen sponge extract over leukemia Cell lines (%)

Incubation time (Minutes)	Sigmadocia carnosa (A1)	C. gorgonoides (A2)	Callyspongia (A3)	Control
10	82.8±0.83	84.6±1.81	43.8±1.78	47.4±1.51
20	92±1.58	53.4±0.91	86.0±1.58	56.6±1.14
30	86±1.0	93.7±1.48	94.8±0.83	54.6±0.54
40	85.8±0.83	85.0±1.58	47.9±0.90	49.4±0.54
50	87.2±1.30	95.44±1.63	77.1±1.47	56.4±0.54
60	91.6±1.14	62.56±0.94	93.2±1.30	57.4±0.54
70	83.6±1.67	92.46±1.53	67.1±1.37	56.6±0.54
80	84.1±0.74	91.2±1.30	67.6±1.31	56.8±0.44
90	82.2±1.92	74.02±0.69	37.4±1.14	45.6±0.54
100	96.56±1.12	64.96±0.65	6.6±1.14	638±1.09

Table 6: DPPH radical scavenging activity of chosen sponge extract over leukemia Cell lines (%)

Trypan blue exclusion cell viability assay

Based on the result, it was cleared that the methanolic extracts of *Sigmadocia carnosa* (A1) shows decreasing trend in terms of viability. But in the other extarcts showed the major variation than the control in 24 hours, 48 hours and 72 hours of incubation (Table 7). It indirectly indicated that over trypane blue inclusion in A1 administered group produced more cell death (Cancer cell death).

Incubation time(hrs)	Sigmadocia carnosa (A1)	C. gorgonoides (A2)	Callyspongia (A3)	Control
0	100±0	100±0	100±0	100±0
24	81.3±0.93	97.2±0.83	100±0	100±0
48	57.0±1.58	93.8±0.83	94.0±1	97.4±0.54
72	41.46±1.45	83.2±1.09	93.6±1.14	96.8±0.83

Table 7: Trypan blue Cell Viability of chosen sponge extract over leukemia Cell lines (%)

COMET Assay for DNA fragmentation

The results of COMET assay displayed in figure 1. A1 (*Sigmadocia carnosa)* extract has showed perfect cell without any damage of DNA particle. But in control huge DNA degradation was noted. Like that in other sponge extracts administered groups also produced some DNA damage (less than the control)

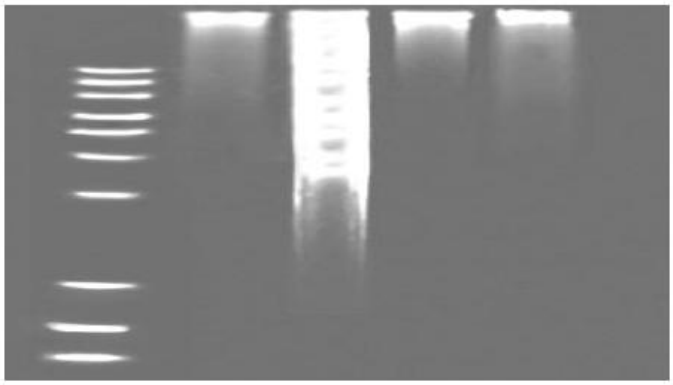

Figure 1: DNA Fragmentation assay

CONCLUSION

Apoptosis, the programmed cell death regulates various functions of organisms. If it plunge their regulation the cell become tumor/ Cancer. Apoptosis induction is one of the new methods of cancer treat. Now lot of plants and natural products were screened for their apoptotic regulating efficiency. In the present study, the apoptotic regulating efficiency of three marine sponges such as *Sigmadocia carnosa, Clathria gorgonoides* and *Callyspongia sp.* were collected and they were extracted with methanol. Various activities such as antimicrobial, biotoxicity and cytotoxicity were studied. *Sigmadocia carnosa* extract strongly inhibited Human leukemia cell lines (ATCC CCL-2), and was non-toxic to normal cells. It was cleared by all the results of tested bioassays such as MTT cytotoxicity, Hydrogen Peroxide Scavenging assay, DPPH Radical scavenging assay, COMET assay (Single cell electrophoresis), Trypan blue exclusion cell viability assay and DNA Fragmentation assay.

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