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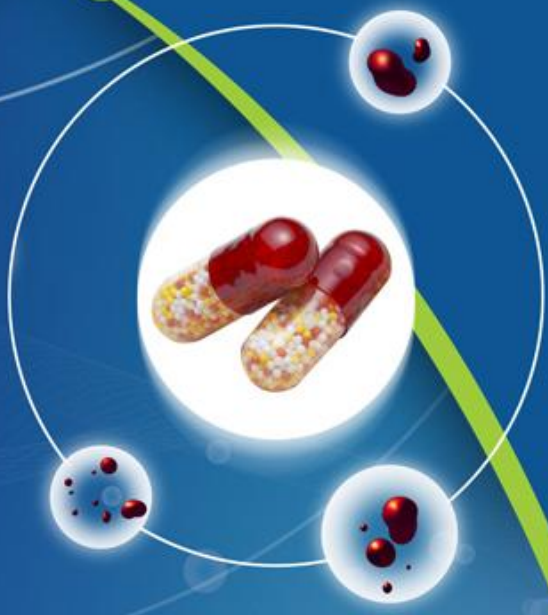

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Research Article

DETERMINATION OFAPOPTOSIS INDUCING ACTIVE PRINCIPLES IN CHOSEN MARINE SPONGES

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ABSTRACT

Secondary or primary metabolites produced by marine organisms may be potential bioactive compounds of interest in the pharmaceutical industry. Marine organisms exhibit a wide range of biological activities like anti-fertility, antiviral, antibiotic, antifungal and antimicrobial activities. In this study an attempt has been made to purify and partially characterize the active metabolites of the metabolic extract of the marine sponges, Sigmadocia carnosa and Clathria gorgonoides, which showed more potency in the pharmacological studies. The sponge Sigmadocia carnosa has showed rich source for several bis-1-oxaquinolizidine alkaloids, exhibiting diverse biological properties such as cytotoxic, antifungal, antimalarial, anti-tuberculosis and anti-rat brain nitric oxide synthase activities (Venkateswarlu et al., 2004; Rao et al., 2004). It was also evident that the efficacy of methanolic sponge extracts has been reduced in the lower temperature. The GC MS analysis of Sigmadocia carnosa showed that it contains around 10 compounds. The two major peaks at retention time 5.85 and 7.26 are identified as Iodophenyl (19.28 %) and Ephedrine (51.85 %). In conclusion, Sigmadocia carnosa offers a valuable candidate lead compound to counter growing drug resistance in cancers.

Key words: *Apoptosis, Ani-cancer activity, Biotoxicity, Leukemia cell lines*

INTRODUCTION

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.

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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. Sponges with their chemical defense mechanisms are one of the most studied organisms for the isolation of Natural Product Analogues (NPAs) [5]. Marine sponges are the excellent source of bioactive compounds such as carotenoids, dietary fibre, protein, essential fatty acids, vitamins and minerals [3], [4]. 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], [7]. 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 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 &Apoptosis regulating efficacy of chosen sponge extracts using standard protocols
- To determine the Apoptosis Inducing Active Principles from the selected Marine Sponge extracts

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.

Screening the bio-toxicity

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) LD50 values were determined.

Evaluation of Apoptosis regulating efficacy of chosen marine sponge extracts

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% CO2.

MTT Assay

Day 1: 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. The media was removed and cells were re-suspended to 1.0 ml with complete media. The cells per ml were counted. The cells diluted to 75,000 cells per ml with complete media. 100 µl of cells were added (7500 total cells) into each well and incubated overnight.

Day 2: The cells were incubated overnight with three extracts of *Sigmadocia carnosa*., *Clathriagorgonoides* and *Callyspongia sp*. with the dose of 10 mg/ml each, in a different experimental setups.

Day 3: About 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 plates were incubated for 0, 12, 24, 36, 48, 60, 72 hours at 37oC in culture hood. 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. Plant Extracts (1 mg ml-1) in distilled water were added to a hydrogen peroxide solution (0.6 ml, 40 mM). The absorbance of hydrogen peroxide at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without hydrogen peroxide.

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 electrophoresis)

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. The microscopic slides were diped in NMA up to one-third the frosted area. To the agarose coated slide, 75 µL of LMPA (0.5%; 37ºC) priorly mixed with ~10,000 lymphocytes was added. The agarose on the slides were allowed to solidify in the room temperature for 20 minutes. Third agarose layer (80 µL LMPA) was added to the slide and coverslip was placed. Then the slides were let to incubate in the alkaline buffer for 20 minutes to allow for unwinding of the DNA and the expression of alkali-labile damage. 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 micropippet 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

The 4X106 cell lines were incubated with 2 mg of dried sponge extract from each sample and 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 cell pellet was then suspended with 0.5 ml PBS. 55 microliter of Triton X100 lysis buffer was added in the cell mixture at 40C. The tubes were centrifuged at 40C 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 tubes were agitated gently for 10 minutes and centrifuged again at 3000 rpm. The above step was repeated for 3 times and finally centrifuged at 5000 rpm. The pellet was re-suspended in 30 micro liter of deionized water with RNAse solution (0.4ml water + 5µl of RNase).

Determination of Apoptosis Inducing Active Principles from Chosen Marine Sponge extracts

Separation and identification the compounds

HPLC Purification

- Crude extracts which showed comparatively high apoptotic induction against human leukemia cell lines such as *Sigmadocia carnosa* and *C. gorgonoides* extracts were dissolved in ethyl acetate and filtered through 0.8-µm nitrocellulose membranes.
- The ethyl acetate extracts were rotary evaporated at 32°C, and the dried extracts were re-suspended in 50% (v/v) acetonitrile: water in 1 µL/ mL of ethyl acetate.

- This solution was fractionated on a reverse-phase C18 analytical HPLC column (5 ì m, 250-4, Waters, USA) mounted with a guard column by injecting 200- µL samples, eluting for 5 min with water, then for 40 min with a linear gradient of increasing acetonitrile to 100% (v/v), and maintaining 100% (v/v) acetonitrile elution for an additional 15 min.
- The elutions were recorded and graphs were collected using an automated fraction collector for further structural determination.

Structural Analysis using NMR spectroscopy

Hydrogen NMR (H1) and Carbon NMR (C13)

- Solvent: Chloroform (CDCl3) (deuterated solvent)
- Few drops of a internal standard (Tetramethylsilane (TMS) added with solvent
- Residual water in the solvent is removed by the addition of activated 4 armstrong sieves
- The solvent is then neutralized with anhydrous granular K2CO3.
- The purified compound as the result of HPLC elucidation is dried to remove any solvent residues.
- The dry NMR tube is placed with kimwipe pipet filter.
- Approximately 10 mg of sample is collected from the pipet is dipped inside the NMR tube with deuterated solvent.
- The NMR tube is caped tightly and placed in Varian Y-3 NMR) Spectrometer. In case of Carbon NMR, the NMR tube is caped tightly and placed in Varian XL-300 Spectrometer
- The chemical shifts were recorded and tabulated at 400 Hz frequency.

Fourier Transform Infra-red Spectroscopic analysis of compounds

- For FTIR spectroscopy, the sample is ground using an agate mortar and pestle to give a very fine powder.
- A small amount is then mixed with nujol to give a paste and several drops of this paste are then applied between two sodium chloride plates (these do not absorb infrared in the region of interest).
- The plates are then placed in the instrument sample holder ready for scanning (Nicolet 6700 FT-IR Spectrometer, Thermo Scientfic).

GC MS Analysis

- The crude marine sponge extract *Sigmadocia carnosa* was subjected to centrifugation at about 10,000 rpm for about 30minutes to remove the particulates.
- The clear supernatant was aspirated using a pipette and transferred into a clean vial and labeled. Then the supernatants were subjected to gas chromatography analysis using a Varian Cp 3,800 model gas chromatography equipped with two flame ionization detectors and connected with Cp-ware (Polyethylene glycol)(60m x0.25nm) and Cp-5 (100% dimethyl polysiloxane) capillary column (50 mts x0.25 nm),(film thickness0.2µm).
- The peak area calculations were done by star work station and peak identification by comparison with authentic, wherever available calculations of Kovats Retention index was done.
- Mass spectrometry analysis was performed on a Shimadzu GC 17 A QP 5,000 MS coupled with a mass detector , fitted non-polar DB-5 (Diphenyldiphenylsiloxane) capillary column of length 25 m × 0.25 mm id. GC MS operation conditions at initial temperature 60 0 c – 300 0 c. The injection volume was 0.1µl with helium gas as carrier at the flow rate of 0.6ml per minute.

RESULTS

MTT Assay

The results of MTT assay were shown in plate 1. a,b,c,d. It was noted that methanolic extracts of *Sigmadocia carnosa*., *Clathriagorgonoides* and *Callyspongia* sp. has cytotoxic effect. Maximum cytotoxicity was observed in *Sigmadocia carnosa* extracts over human leukemia Cell lines (78%) at 48 hrs

incubation. The different concentrations of MSEs 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 *Clathriagorgonoides* (58 and 48%) and *Callyspongia* (28.48 and 30. 64%) extracts administered Cell lines. The cytotoxic variation of different sponge extracts in different cell lines are depicted in Table 1.

Table 1: *Artemia* cytotoxicity profile of Marine sponge extracts (MSE) at 30 and 20^{0}C

MSE	Concentration	Mortality (%)	
		30 ±2^{0}C	20 ±2^{0}C
Clathriagorgonoides	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
Callyspongiasp	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
Sigmadocia carnosa	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

Mean ±SD n-10 experiments

Hydrogen Peroxide scavenging assay

The results of H_2O_2 scavenging activity is shown in Table 2. 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.

Table 2: Hydrogen Peroxide Scavenging Activity of chosen sponge extract over leukemia Cell lines (%)

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

DPPH Radical scavenging assay

The results of DPPH radical Scavenging activity (Table 3) 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.

Table 3: DPPH radical 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

COMET assay (Single cell electrophoresis)

The results of COMET assay is 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. In other marine sponge extracts, administered groups also produced some DNA damage (less than the control)

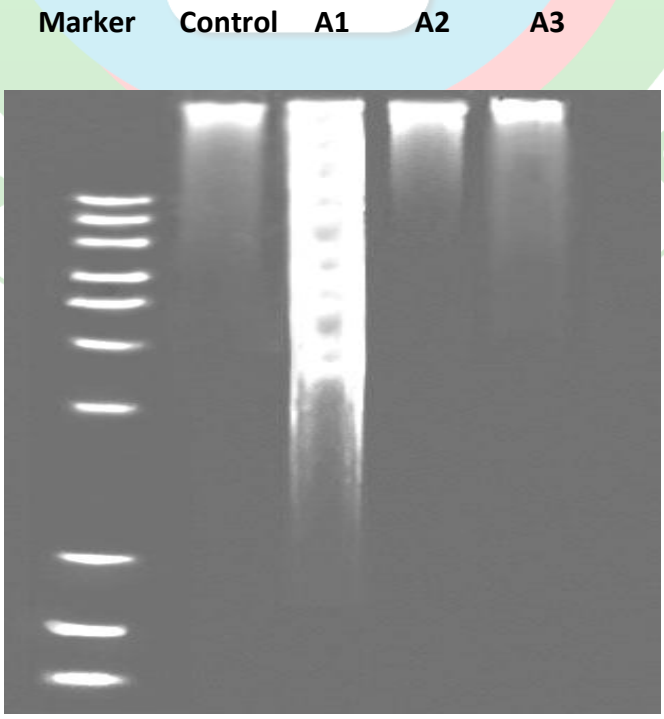


Figure 1: DNA Fragmentation assay

Trypan blue exclusion cell viability assay

The result (Table 4) indicates that the methanolic extracts of *Sigmadocia carnosa* (A1) shows decreasing trend in terms of viability. However in the other extracts showed the major variation than the control in 24 hours, 48 hours and 72 hours of incubation. It indirectly indicated that over trypane blue inclusion in A1 administered group produced more cell death (Cancer cell death).

Table 4: Trypan blue Cell Viability of chosen sponge extract over leukemia Cell lines (%)

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

DNA Fragmentation assay

The result of marine sponge extracts induced DNA fragmentation was shown in Figure 1. The results clearly displayed that the A1 (*Sigmadocia carnosa*) extract produced more DNA damage than the other extracts. It was clearly indicated that this extract has effective killing mechanism of cancer cells by DNA damage.

HPLC Purification

The HPLC analysis of *Sigmadocia carnosa* and *C. gorgonoides* are given in Fig.2. The results produced 4 peaks between 5 mins to 20 mins duration in *Sigmadocia carnosa*. But *C. gorgonoides* produced one major peak within 5min of retention time. It also produced three meager peaks within 20 min.

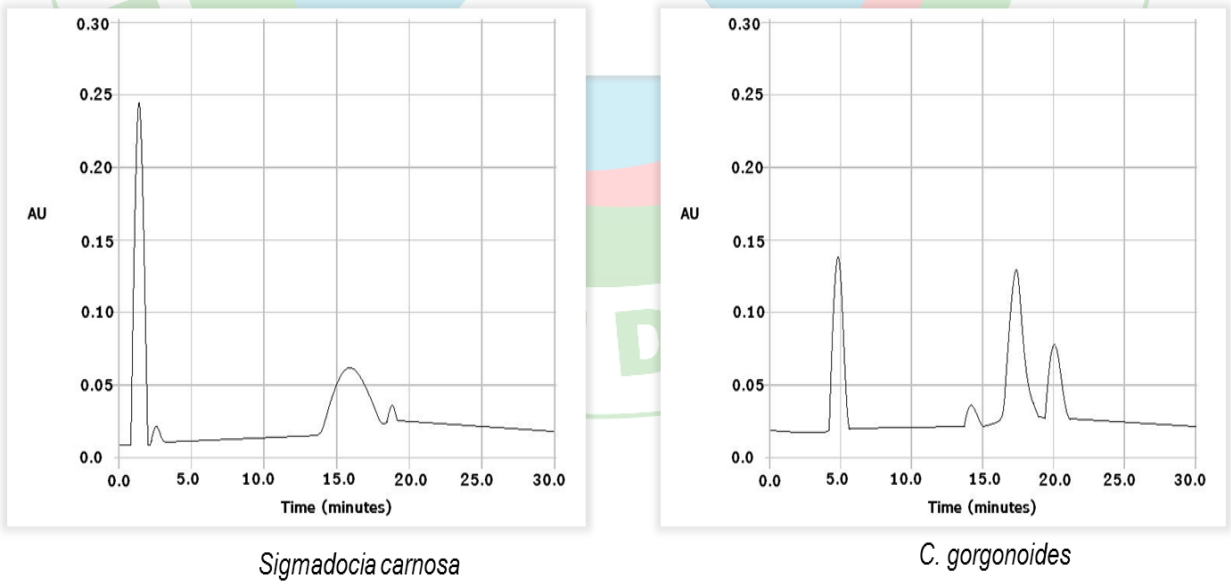


Fig. 2. HPLC Analysis of *Sigmadocia carnosa* and *C. gorgonoides*

Structural Analysis using NMR spectroscopy

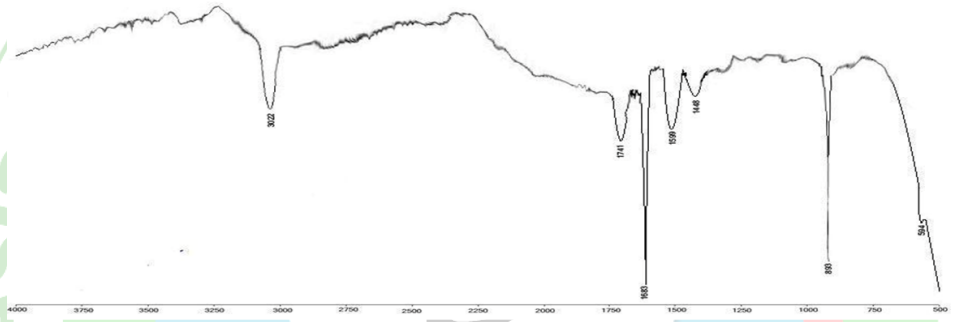
The results of C13NMR and H1 NMR indicate that the Peaks (Chemical shift) of *Sigmadocia carnosa* are nearly similar. Because both have same number of carbon atoms. Based on these studies it was concluded that the compound behind these activity might be 2- Bromo 3-heptinic acid.

Fourier Transform Infra-red Spectroscopic analysis of compounds

The presence of sharp absorbance at 1800 cm-1 area of FTIR spectrum suggests the presence of halogen group in the purified compound (Figure 3 and 4). Molecular structures of these secondary metabolites were proposed on the basis of comprehensive analysis of the 1H NMR.

The 1H NMR recorded the presence of furnanone ring (d -525) and Bromine (d –545). The resonances of several methylene protons connected to the same carbon at different chemical shifts substantiate the presence of monocyclic in the molecule, as is evident from the 1H NMR spectrum.

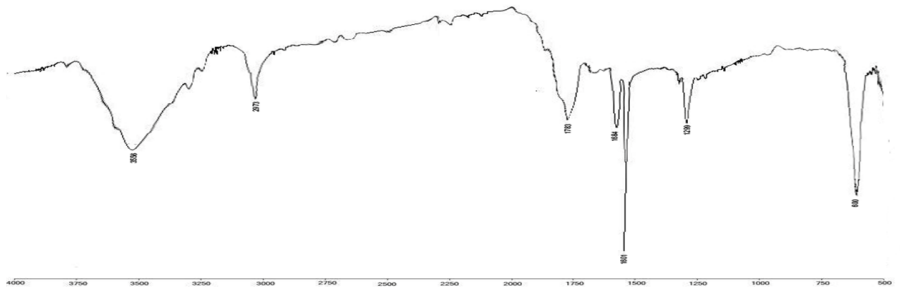
From spectroscopic data, it could be suggested that the active principle behind *Sigmadocia carnosa* compound is a brominated furanone.



Reference spectra interpretation with standard database.

C-H		900 cm^{-1}
C-H (alkyl)	METHYL	1380 cm^{-1}
C-Br	Medium	500–600 cm^{-1}
C=C (sp2)	Medium	1680 cm^{-1}
C-C	Strong	1600 cm^{-1}
C=C (aromatic)	Weak	1450 cm^{-1}

Fig. 3 A1 – *Sigmadocia carnosa*



Reference spectra interpretation with standard database.

C=O		1750 cm^{-1}
C≡C		1685 cm^{-1}
C-Br	Medium	500–600 cm^{-1}
O-H	Broad	3500–3560 cm^{-1}
C-O	Medium	1250–1300 cm^{-1}
C-C	Strong	1600 cm^{-1}
C-CH2	Medium	2975 cm^{-1}

Fig. 4 A 2 - *C. gorgonoides*

GC MS Analysis

The chemical compositions present in the apoptosis inducing plants were identified by using GC MS analysis. The data are interpreted in the figure and tabular column

The GC MS analysis showed *Sigmadocia carnosa* contains around 10 compounds. The two major peaks at retention time 5.85 and 7.26 are identified as Iodophenyl (19.28 %) and Ephedrine (51.85 %) (Table 5)

Three identified major peaks were 2, 3-Diflurophenyl hydrarine (17.71 %) at a retention time 17.91. 3.15% Dinitro phenyl hydraamino (11.10 RT) and Furan carboxaldehyde 2.97 % at 19.80 retention time (Fig.5).

Table. 5 The chemical Components of *Sigmadoiciacarnosa*

SI. No:	Name of the compound	Retention time	Percentage
1	Iodophenyl	3.70	19.28
2	Ephedrine	7.26	51.85
3	Carboxy methyl cyateine	9.32	0.96
4	Dinitro phenyl hydraamino	11.10	3.15
5	Dichlorodiathoxy	12.09	0.46
6	Methyllmidarole -4-carboxyaldehyde	14.98	0.63
7	Serine acetaldehyde	16.23	2.31
8	1,2- Dimethyl- cyclopent -2- ethyl	16.99	0.63
9	2,3-Diflurophenyl hydrarine	17.91	17.71
10	Furan carboxaldehyde	19.80	2.97

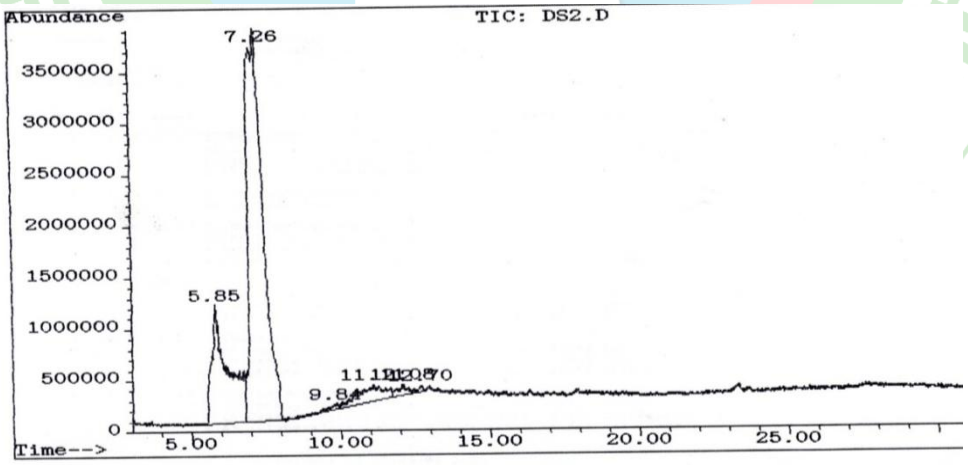


Fig. 5GC/MS analysis of *Sigmadoiciacarnosa*

CONCLUSION

Bioassay results revealed that *Sigmadocia carnosa* species (90%) exhibited antibiotic activity against most of the bacterial isolate followed by *Clathria (Clathria)gorgonoids.*

Methanolic extracts of *Sigmadocia carnosa*. *Clathriagorgonoides* and *Callyspongia sp*. has a killing effect. Maximum cytotoxicity was observed in *Sigmadocia carnosa* extracts over human leukmia Cell lines (78%) at 48 hrs incubation. Summary does not state anything

about bioactive principle screening of *Clathriagorgonoides* and *Sigmadocia*

A comparative study of these plants on the regulation of apoptosis in cancer cell line was studied in the present investigation. The anticancer studies like MTT assay H_2O_2 scavenging assay, DPPH scavenging, DNA fragmentation, Trypan blue staining assays were also conducted during the investigation.

The micro culture assay based on metabolic reduction of MTT to evaluate the cytotoxic effect. In the present study Viability of cell was studied by MTT assay in human leukemia cell lines. *Sigmadocia carnosa* extract was found to be good cytotoxicity effect compared with other two sponge extracts (*C. gorgonoides* and *Callyspongia* sp.).

In case of trypane blue exclusion cell viability, *Sigmadocia carnosa* extract (A1) showed less viability than the other two. So *Sigmadocia carnosa*induces apoptosis in cancer cells compared to the other two extracts. It was followed by *C. gorgonoides*. The extracts of *Sigmadocia carnosa* activate against the tumor cell within 24 hours of incubation. More than 60 % of the cells become apoptotic in *Sigmadocia carnosa* extract.

Among that the methanolic extracts of sponge *Sigmadocia carnosa* has proven their significant activity over the other two tested sponge extracts through its cytotoxicity, Hydrogen peroxide scavenging, DPPH scavenging assays. More over the same extract produced effective DNA degradation than other two tested sponge extracts.

The sponge *Sigmadocia carnosa*has been identified as a rich source for several bis-1-oxaquinolizidine alkaloids, Idophenyls, Ephedrine etc exhibiting diverse biological properties such as cytotoxic, antifungal, antimalarial, antituberculosis and anti-rat brain nitric oxide synthase activities

The results of this study produced a preliminary point that the crude methanolic extract of *Sigmadocia carnosa* has anticancer activity. However, further study of the activity associated with the different species, environmental conditions, and a detailed investigation including in-vivo studies of the *Sigmadocia carnosa* extract may provide useful comparative information in the future.

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