Apoptosis Regulating Efficacy of Chosen Indian South Peninsular Coast Marine Sponge Extracts

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Abstract : Apoptosis, the programmed cell death regulates various functions of an organisms. If it plunge their regulation the cell become tumor/ Cancer. Apoptosis induction is one of the new methods of cancer treat. Lot of commercial apoptotic regulating drug are available in the market and they are used very little amount because of their deprived effects and cost. 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 sponge extracts collected from south peninsular coast were screened with standard protocols. Among that the methanolic extracts of sponge *Sigmodoicia* proven their significant activity over the other two tested sponge extracts through its cytotoxicity, Hydrogen peroxide scavenging, DPPH scavenging assays. More over that the same extract produced effective DNA degradation than other two tested sponge extracts.

Key words: Apoptosis, DPPH assay, MTT assay, Hydrogen peroxide scavenging assay.

I. INTRODUCTION

Apoptosis is an essential and fundamental phenomenon occurring in the cells during various biological process including growth, differentiation and remodeling and immunological development (Bowen, 1993).The nuclear DNA of apoptotic cells shows a characteristic laddering pattern of oligonucleosomal fragments. This results from inter –nuceosomal chromatin cleavage by endogenous endonucleus in multiples of 180 base pairs (Telford *et al.,* 1991). This fragmentation is regarded as a hallmark of apoptosis. Furthermore, apoptotic cell death is the consequence of a series of precisely regulated events that are frequently altered in tumor cells. The development of tumors arises as a consequence of disregulated proliferation and a suppression of apoptosis, and each of these primary defects provides an obvious opportunity for clinical intervention. Many of the current chemotherapeutics designed to perturb proliferation do so in such a crude manner that resulting damage to normal cells limits their clinical efficacy. Bharat, (2006) reported that tumerigenesis is due to the expression of apoptosis inhibiting protein (IAP) genes (bcl-2, bcl-XL). This provides the opportunity for selective clinical intervention to bring about the death of the tumor cell without damage to normal cells. So apoptosis is the most desirable target mechanism for induction of cell death in tumor cells.

A wide variety of biological activities from natural products have recently been reported in addition to their traditional medicinal effects. In particular it is well established that ethanomedicinal plants are useful sources of clinically relevant antitumor compounds (Cragg *et al.,* 1997) Plants and plant products both as extracts and derived compounds are known to be effective and versatile and chemo preventive agents against various types of cancers (Moongkurndi *et al*., 2004). Traditional background of Indian medicine shows extensive use of plant products in cancer (Husain *et al.,* 1997).

Based on this row, the present study was intended to screen apoptotic regulating potential of three potential antimicrobial marine sponges such as *Sigmadocia sp*., *Clathria gorgonoides* and *Callyspongia sp*., collected from south peninsular coast of India.

II. MATERIALS AND METHODS

Cell lines and its preparation for experiment

Human leukemia cell lines (ATCC CCL-2) were obtained from American Type Culture Collection (Manassas, VA), 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. These cell lines were maintained in the Animal tissue lab, Biogeno laboratory, Hydrabad.

MTT assay

MTT assay is a standard colorimetric assay (an assay which measures changes in colour) for measuring cellular proliferation (Cell growth). It is used to determine cytotoxicity of potential medicinal agents and other toxic materials. Yellow MTT (3-4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple Formosan in the mitochondria of living cells. 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. The media was removed and cells were resuspended 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. On the second day, cells were incubated overnight with three extracts of *Sigmadocia sp*., *Clathria gorgonoides* and *Callyspongia sp*. with the dose of 10 mg/ml each. 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 plates were incubated for 0, 12, 24, 36, 48, 60, 72 hours at 37°C 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. Sponge 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. The percentage of hydrogen peroxide scavenging by the extracts and standard compounds was calculated as follows:

% Scavenged (H2O2) = [(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 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.

DNA Fragmentation assay(Triton X100 lysis method)

4X10^6 Human leukimia cells (ATCC CCL-2) 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 with 0.5 ml PBS. 55 microliter of Triton X100 lysis buffer was added in the cell mixture at 4^0C. 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 tubes were agitated gently for 10 minutes and centrifuged again at 3000 rpm and kept in ice conditions. 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).

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. 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 initially run at 5V for 5min before increasing to 100V. 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.

III. RESULT

3.1MTT assay

The result of MTT assay after the administration of chosen sponge methanolic extracts of *Sigmadocia sp*., *Clathria gorgonoides* and *Callyspongia sp*. has a killing effect. Maximum cytotoxicity was observed in *Sigmadocia sp* extracts over human leukmia Cell lines (78%) at 48 hrs incubation. Among the concentrations, 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: 1. The cytotoxicity potential of choosen sponge extract over leukmia Cell lines at different concentration.

Cell lines	Plant extracts	Concentration of crude extract (µ/ml)	Percentage Cytotoxicity %	
			24hrs	48hrs
Humen leukmia Cell lines	*Sigmadocia sp.,*	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

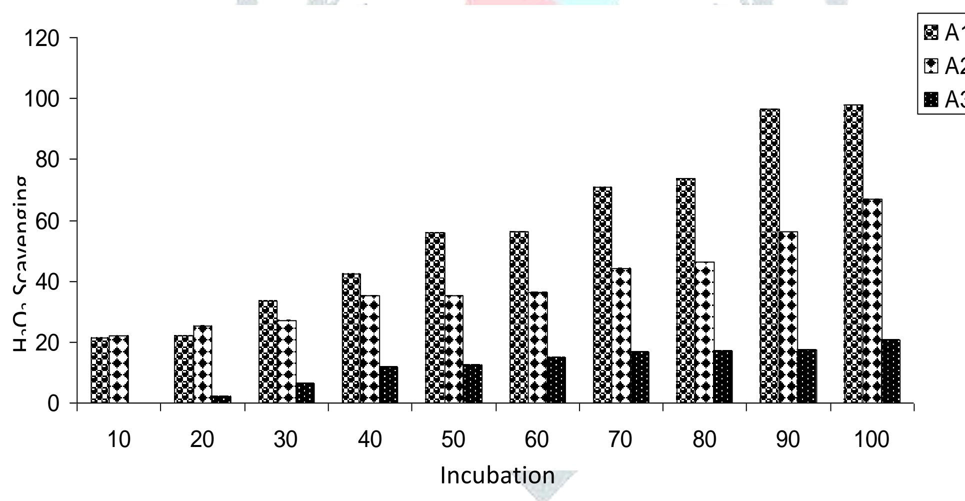
3.2Hydrogen peroxide scavenging assay

The results of H2O2 scavenging activity is shown in fig 1. The results clearly displayed that the A1 (*Sigmadocia*) produced high percentage of Hydrogen peroxide scavenging activity. (Values) It was followed by A2 and A3 .It was also noted that that the concentration of extracts play an important role in Hydrogen peroxide scavenging activity. Here Hydrogen peroxide scavenging activity is directly proportional to the concentration.

Table: 2 : Hydrogen Peroxide Scavenging Activity of choosen sponge extract over leukmia Cell lines

Incubation time	*Sigmadocia* **(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

Fig 1. Hydrogen Peroxide Scavenging Activity



3.3 DPPH radical Scavenging activity

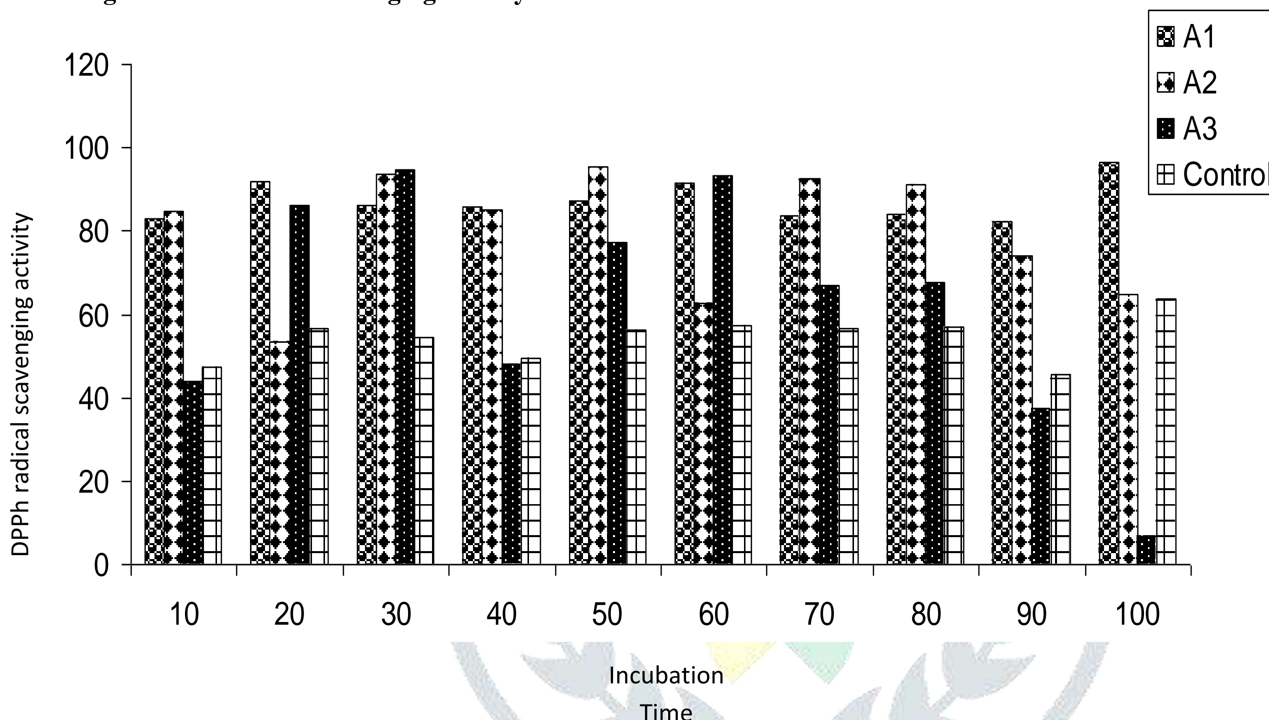
The results of DPPH radical Scavenging activity are depicted in fig 2. and table 12. The results clearly indicated that the plant extract of *Sigmadocia sp* induced more DPPH scavenging profile than the other groups in all concentrations.Interesingly in highr concentration (90 and 100 µg) The *Callyspongia (*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 choosen sponge extract over leukmia Cell lines

Incubation time	**A1**	**A2**	**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

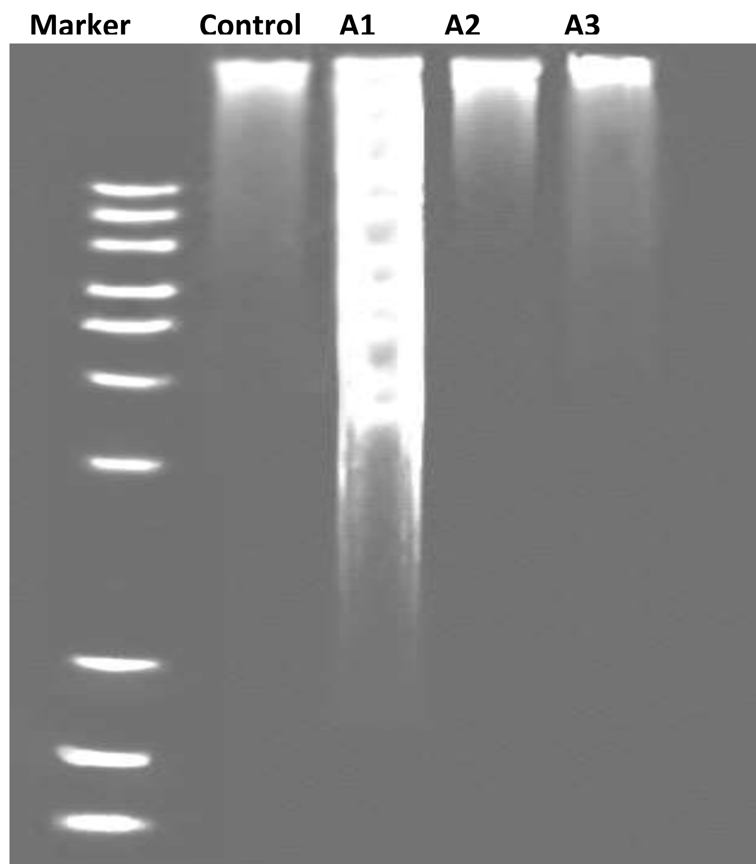
Fig 2: DPPh radical scavenging activity



3.4 DNA fragmentation assay

The result of sponge extracts induced DNA fragmentation was shown in plate. The results clearly displayed that the A1 *(Sigmadocia*) 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.

Plate 3 DNA Fragmentation assay



IV. DISCUSSION

Defective apoptosis represents a major causative factor in the development and progression of cancer. The ability of tumor cells to evade engagement of apoptosis can play a significant –role in their resistance to conventional therapeutic regimens. Our understanding of the complexities of apoptosis and the mechanisms evolved by tumor cells to resist engagement of cell death has focused research effort in to the development of strategies designed to selectively induce apoptosis in cancer cells. Programmed cell death is a highly conserved mechanism of self defense, also found to occur in plants. Hence it is natural to assume that chemicals must exist in them to regulate programmed cell death in them. Thus plants are likely to prove to be important sources of agents that will modulate programmed cell death. The use of herbal medicines and their market potentiality are growing widely patient switch over to use of complimentary or alternative medicine is increasing rapidly. Traditional Chinese and Korean oriental therapy involves the use of multiple herbs and their extracts which are found to be attractive, alternative therapy.

Significant literature on the chemical composition of these herbs and their effectiveness are scarcely available. This set the agenda for researchers around the world to elude a clear picture on the curative mechanisms implicated of these compounds. Understanding and unraveling the mechanisms of anti-cancer drugs-induced apoptosis is of prime importance not only for designing effective therapeutic interventions and development of novel cancer therapeutic strategies but also for monitoring cancer responses to chemotherapeutic drugs. This allows better understanding and rational usage of chemotherapeutic drugs specifically for certain tumor types. In the cellular response to genotoxic stress by various chemotherapeutic anti-cancer agents, cell cycle checkpoint and apoptosis are considered to be two of the major biological events in maintaining genomic stability (Sandeepsingh *et al* ., 2006). Lot of earlier reports demonstrated that sponges have anticancer activity (Parimala and Sachdanadam, 1993). The 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 H2O2 scavenging assay, DPPH scavenging, DNA fragmentation, Trypan blue staining assays were performed in the present investigation.

The micro culture assay based on metabolic reduction of MTT to evaluate the cytotoxic effect. Tetrazoleum salt is metabolically reduced by viable cells to yield a blue formasan product measurable in a multiwell scanning spectrophotometer. The intrinsic mitochondrial pathway is characterized by the rapid release of cytochrome c from the mitochondrial inter membrane space in to the cytosol. Most apoptosis-inducing conditions involve the disruption of the mitochondrial inner transmembrane potential as well as the so called permeability transition (PT), a sudden increase of the inner mitochondrial membrane permeability to solutes with a molecular mass below approximately 1.5 KilloDalton. Concomitantly, osmotic mitochondrial swelling has been observed by influx of water into the matrix with eventual rupture of the outer mitochondrial membrane, resulting in the release of pro apoptotic proteins from the mitochondrial inter membrane space into the cytoplasm released proteins include cytochrome c, which activates the apoptosome and therefore the caspase cascade, but also other factors such as

the apoptosis-inducing factor AIF [Susin *et al.,* 1999], the endonuclease endoG, Smac/Diablo and Htr/Omi. The release of cytochrome c requires permiabilization of the outer mitochondrial membrane. And is tightly regulated by the pro-and anti apoptotic members of the Bcl-2 family (Green and Reed 1988; Gross *et al*., 1999;Martinou *and* green,2001).In the present study Viability of cell was studied by MTT assay . MTT assay was done in human leukmia cell lines. *Sigmadocia* extract was found to be good cytotoxicity effect compared with other two sponge extracts (*C. gorgonoides* and *Callyspongia sp*.). High level cytotoxicity was observed after 48 hours at 800 µg/ml concentration.

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

H2O2 and DPPH are the tumor inducing compounds. In the present study the scavenging activity of H2O2 and DPPH were done .Various concentration of MSEs were tried. Among these *Sigmadocia* extract (100 µg) showed good scavenging activity against H2O2 and DPPH. It was followed by *C. gorgonoides* (45 % of scavenging activity at 100 µg levels). The results of DNA fragmentation assay demonstrated that MSEs have the apoptotic effect .To ascertain the mechanism of apoptosis DNA fragmentation is the hall mark event of apoptosis (Zhang and Huang, 2005). Apoptosis is charrecterized by a series of morphological changes ,such as chromatin condensation ,cell shrinkage ,membrane bebbling ,packing of orgenells ,the formation of apoptotic bodies ,inter nucleosomal DNA fragmentation (Fleisher ,1997; Zhang andXUM, 2000). The morphology of apoptotic cell shows a highly condensed chromatin in a fragmented nucleus (Wyllie *et al.,* 1998) .Same trend was noticed in the present study. DNA loss and decrease of DNA accessibility to the die were observed in the present investigation The same characters of apoptotic cells were reported by Zamai *et al*., 2000 . Among the MSEs, *Sigmadocia* (A1) were observed to be better apoptotic inducing capacity than the other two extracts. The phytochemical studies of *Sigmadocia* showed that the presence of oil and phytostrols, flavanoids, esters, tannins and steroids (Chandro *et al*., 1994 ; Deepak *et al*., 1995; Roy *et al* ., 2002; Sircar *et al*., 2007). In conclusion methanolic extract of *Sigmadocia* offers a valuable candidate lead compound to counter growing drug resistance in cancers.

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