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Proteoglycans Isolated from Bramble Shark Cartilage (*Echinorhinus brucus*) Inhibits Proliferation of MCF-7 Human Breast Cancer Cells by Inducing Apoptosis

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Abstract

Proteoglycans (PGs) were isolated from deep sea shark *Echinorhinus brucus* (bramble shark) cartilage and their anti-proliferative activity against MCF-7 cell lines was evaluated. To establish apoptosis involvement for cytotoxicity, the following assays were performed i.e., caspases 3 and 9 assay, double fluorescent staining and DNA laddering assay. PGs were isolated by DEAE-sephacel ion exchange chromatography and identification and purity confirmed by chemical composition such as total carbohydrate, total protein, hexosamine, uronic aicd, sulfated GAG, wavelength scan and modified AGE. To confirm apoptosis pathway expression of genes BAX, Bcl-2 and p53 were carried out. PGs (100 µg/mL) induced a significant rise in activity of caspase 3 and 9, major apoptotic markers. A significant rise in the expression was also observed for Bcl-2 and p53. Whereas, low BAX expression was observed for PGs (100 µg/mL) treated sample. Significant anti-proliferative effect was observed and the mechanism behind cell death was concluded to be apoptosis.

Keywords: Proteoglycans; Apoptosis; Cytotoxicity; Caspases

Introduction

Breast cancer is the most common cancer form in women; with an estimated 1.4 million new cases worldwide each year, breast cancer constitutes about 25% of all cancer cases in women and is the second most common cancer form overall [1]. As lifestyles change in India, there is a steep rise in the occurrence of breast cancer especially among the younger women. From a current figures of 1,55,000 new emerging cases, it is predicted to go to 200,000 by the year 2030. During the last decade focus has been on prevention, early detection and introduction of new drugs and treatment modalities. The largest survival improvements were seen in cases where early detection was combined with the introduction of effective drug therapies. Chemotherapy is one of the commonly used strategies in breast cancer treatment. This therapy is usually associated with adverse side effects, ranging from nausea to bone marrow failure, digestive problems, leucopenia, hair loss and development of Multidrug Resistance (MDR) [2].

Because of the heterogeneous nature of breast cancer both histopathologically and genetically, the etiology remains uncertain. Use of *in vitro* cell lines has proven to be an excellent option for evaluating the effects of various drugs and also for determining possible mechanisms of action. MCF-7 cell line is one of the most widely used breast cancer cell line in the world, which was first introduced in 1973 at the Michigan Cancer Foundation and became the ideal model for studying the breast cancer *in vitro* [3].

Apoptosis, also called programmed cell death, plays a key role in protecting against uncontrolled proliferation of cells enabling the body to eradicate unwanted cells thereby maintaining proper homeostasis [4]. Cancer cells respond to treatment with chemotherapeutic drugs because the latter trigger apoptosis pathway. Activation of apoptosis is characterized by the triggering of the caspase cascade of enzymes belonging to aspartate proteases family (e.g. caspase 3, caspase 8, caspase 9 etc.) [5]. The enzymes are responsible for various mechanisms involved in apoptosis such as DNA laddering, cytochrome *c* activation, breakdown of different proteins and finally cell death [6].

p53 gene is considered as one of the major regulators of cell division and is a gene associated with mutations that eventually lead up to cancer development. It is also a potential inducer of positive and negative mediators of apoptosis such as BAX, BCl2 etc. [7]. *p53* gene is a subject of extensive investigations in relation to its role in apoptosis-induced cell death. The possible ways in which these mediators control apoptosis is by either regulating mitochondrial permeability or by controlling cytochrome *c* release [8,9]. Therefore a study of expression of these proteins could indicate the involvement of apoptosis during investigations into anti-cancer effect of biomolecules.

Proteoglycans (PGs) are specialized biomolecules that are conjugated forms of protein and Glycosaminoglycans (GAGs). The GAG portions have negative charge, due to the presence of acidic sugar moieties and/or derivatization by sulphate groups [10]. Major GAGs are present in association with PGs are Dermatan Sulfate (DS), Keratan Sulfate (KS) Heparin Sulfate (HS), heparin Chondroitinsulfate (CS), and Hyaluronic Acid (HA). GAGs either present on the cell membrane surface in connection with core proteins covalently, form proteoglycans, or as part of the extracellular matrix (ECM). GAGs are known to control several physiological functions such as tissue regeneration, cell differentiation, cell signaling etc. [11-13]. Cartilage is a rich source of different mixture of proteoglycans namely, aggrecan, decorin, biglycan, fibromodulin and lumican. They play a vital role in

many metabolic functions because of the presence of sulfated glycosaminoglycan chains and protein core [14].

Some proteoglycans have been found to be effective against the growth of melanoma cancer [15] and proteoglycans extracted from some species of mushroom have been observed to control colorectal carcinoma proliferation [16]. But there are a few or no reports of anti-cancer studies with proteoglycans of marine origin. The shark is a rich source of proteoglycans and is being considered as a better source of PGs over the mammalian sources [17]. Moreover, no evidence is available in the literature to establish the involvement of apoptosis in anti-proliferative activity against MCF-7 cell lines by proteoglycans. Therefore the objective of this study is to understand the anti-proliferative activity of proteoglycans from the cartilage of deep sea shark (bramble shark) *E. brucus* and to delineate the mechanisms involved.

Materials and Methods

Sample preparation

A deep sea shark *Echinorhinus Brucus* (bramble shark) sample was brought to the laboratory in a chilled condition. PGs were extracted from the separated cartilage with help of chaeotropic agent 4 M guanidine hydrochloride, 0.05 M EDTA, 0.1 M sodium acetate buffer and protein inhibitor iodoacetamide and 0.05 M benzamidine hydrochloride at 4°C for 12 h. After extraction, isolation was carried out by the help of DEAE- sephacel anion exchange chromatography with 7 M urea and 0.1, 0.2 and 0.4 M NaCl gradient as eluent. The collected eluent is dialysed with 12000 MWCO (molecular weight cut off) against water for 42 h with continuous stirring at 4°C to remove urea, NaCl and remaining traces of contaminants. After dialysis the proteoglycans in eluent was precipitated with ethanol in cold condition and centrifuged. The pellet obtained contains pure proteoglycans and is used for characterization and anti-cancer studies.

Chemical composition analysis

Proteoglycan purified by chromatography method were analysed for carbohydrate [18], protein [19], uronic acid [20], Hexosamine [21] and sulphated GAG content through UV/Visible Spectroscopy. Measurement of Total Sulfated GAGs was done by the kit DMMB method as per manufacturer's protocol (Blyscan Kit).

Agarose/polyacrylamide minislab gel electrophoresis of intact cartilage proteoglycans

A modified method [22] was used to electrophoretically separate PGs with molecular masses greater than 2.5 million Da was performed. The bands were visualized by toluidine blue staining. Standard aggrecan purchased from Sigma Aldrich was used to track the PGs presence.

Wavelength scan for GAGs in PGs

Purity of isolated PGs was done by wavelength scan based on a peak maximum of 656 nm. Blyscan Dye reagent (contains DMMB reagent) and dissociation Reagent) were used as per manufacturer's protocol.

MTT assay (Cell viability and cell toxicity)

The viability of cells was assessed by MTT assay [23] using MCF-7 cell lines. PGs used in a dose dependent manner, i.e., 5, 10, 25, 50 and 100 µg/mL to assess the cytotoxicity.

Caspase- activation assay

Caspase-3 is an intracellular cysteine-requiring aspartate protease that exists as a proenzyme, which is activated during the cascade of events associated with apoptosis. Activities of caspases were determined by chromogenic assays using caspase-3 activation kits according to the manufacturer's protocol (Calbiochem, Merck) [24] with PGs in a dose dependent manner.

DNA laddering assay

The experiment is characterized by the activation of endogenous endonucleases with subsequent cleavage of chromatin DNA into inter nucleosomal fragments of roughly 50 base pairs (bp) and multiples thereof (100, 150 etc.). This effect can be used to detect apoptosis via the DNA laddering assay. PGs in a dose dependent manner used for this assay.

DUAL staining for apoptosis

In this study, we used acridine orange/ethidium bromide (AO/EB) double staining assay [25,26]. MCF-7 cells were seeded in a 24-well plate (50,000 cells per well). After 24 h of cells incubation, the medium was replaced with 100 µL medium containing various doses of PGs samples at different concentrations (25, 50 and 100 µg/mL) range of different time intervals 1st h, 12th h and 24th h. Untreated cells served as the control. After 12th h, the media was aspirated and treated with prepared dye and observed under the fluorescent microscope in emission and excitation wavelength of 460-490 nm.

Gene expression-RNA isolation and RT-PCR of cell samples

This assay was done to study the apoptotic gene expression [27-30]. Total RNA was isolated using TRIZOL-(Sigma, India) according to the manufacturer's instructions. RT-PCR was performed in triplicate using SuperScript™ two Step RT-PCR with platinum® Taq kit according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA, USA).

For cDNA synthesis, complementary DNA was synthesized from 1 µg total RNA from each sample in 20 µL of reaction buffer (contained 50 mM Tris-HCl, pH 8.3, 75 mM KCl and 3 mM $MgCl_2$) using SuperScript II reverse transcriptase enzyme (Genetech, RT-PCR mix- Germany) in a 20 µL volume reaction containing 10 mM dithiothreitol, 10 U RNase inhibitor (Promega, Madison, WI, USA), 1 mMd NTPs and 2.5 µM random hexamers. Each sample was incubated for 45 min at 45°C, followed by 10 min at 72°C in a Agilent amplicon system (AGILENT Biosystems), the prepared cDNA was stored in -20°C for further use. The cDNA (1 µL) was then amplified in 20 µL of reaction buffer for 40 cycles of denaturation (96°C for 30 s), annealing (56°C for 30 s), and extension (72°C for 30 s) using primers.

Bcl-2: Fwd: 5'-CATGCTGGGGCCGTACAG-3';

Rev: 5'-GAACCGGCACCTGCACAC-3';

β-actin Fwd : 5'-TTCTACAATGAGCTGCGTGTG-3';

Rev: 5'-GGGGTGTTGAAGGTCTCAAA-3';

p53: FW, 5'-AGGGTTAGTTTACAATCAGC-3';

RW, 5'-GGTAGGTGCAAATGCC-3'.

The PCR products were visualized by agarose gels. A 100 bp DNA ladder molecular weight marker (Life Technologies, Rockville, MD) was run on every gel to confirm expected molecular weight of the amplification product. Band intensity was expressed as relative absorbance units. The ratio between the sample RNA to be determined and β actin was calculated to normalize for initial variations in sample concentration and as a control for reaction efficiency. Mean and standard deviation of all experiments performed were calculated after normalization to β-actin.

Results and Discussion

Chemical composition analysis

Isolated PGs were used for chemical composition analysis and the assays performed were total protein, total carbohydrate, uronic acid, Hexosamine and sulfated GAGs. The values observed to be 0.468, 0.530, 0.098, 0.20 and 0.06 mg/mg respectively (Figure 1). GAGs contents observed in our study has accordance with the GAGs composition analysis of squid [31]. In our study significant amount of sulfated GAG (Chondrotin-6-sulfate, Dermatan sulfate and Heparan sulfate) and non-sulfated GAG (hyaluronic acid) was observed. Which indicates the significant presence of GAG content in our sample? Since GAG has great therapeutic and cosmetic value shark cartilage can be a better alternative source of it [32,33].

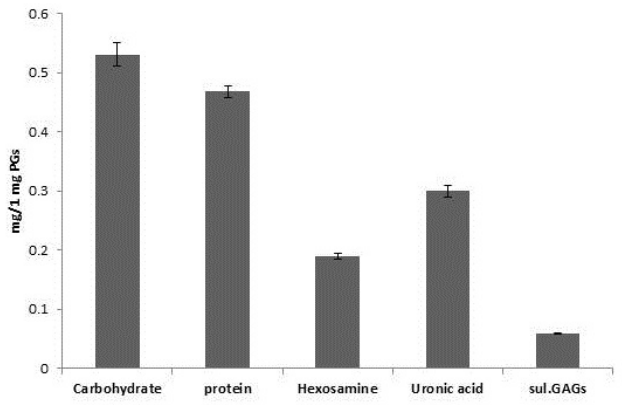

Figure 1: Chemical composition of PGs expressed as mg/1 mg PGs.

AGE of PGs

In electrophoresis proteoglycans stretched bands are commonly seen due to negatively charged GAG polymer and macromolecular structure (Figure 2) [22]. According to the results it was clear that both high molecular and low molecular weight PG is present in the isolated sample. Bands seen with respect to aggrecan standard band could be the high molecular weight PG such as aggrecan of cartilage origin. Whereas band formed well below the aggrecan band could be low molecular weight PGs namely, decorin, biglycan etc.

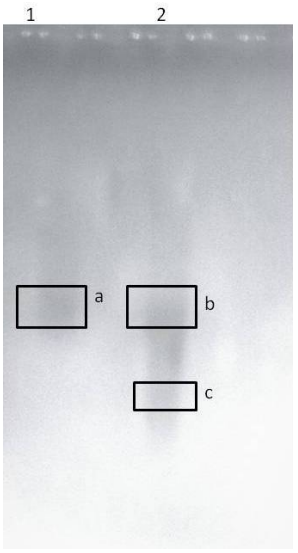

Figure 2: AGE pattern of PGs. Lane 1 (a) denotes the larger molecular weight cartilage proteoglycan aggrecan and similar pattern observed in lane 2 of (b) PGs sample. Band formed at bottom (c) of lane 2 shall be indicating smaller molecular weight PGs such as decorin, fibromodulin, byglycans, lumican etc.

Wavelength scan

Sulphated GAGs presence was determined according to the absorbance range of Blyscan reagent containing 1,9-dimethyl methylene blue (DMMB) (Figure 3). Chondroitin sulphate standards and sample were subjected to DMMB complexation and decomplexation and the absorbance was measured at 656 nm. Absorption curve of 1,9-dimethyl-methylene blue dye in Blyscan Dissociation Reagent has a scan range from 400 to 700 nm. Compared the Sulfated GAG reference Standard curve and the GAG Sample Curve obtained, with the curve formed by DMMB in Blyscan dissociation reagent. The significant similarity of standard chondroitin sulfate and sample observed for the wavelength scan assay. Reports suggest that absorption maximum at 656 nm indicates the presence and purity of GAGs [34] and which has good accordance with our work.

Cell cytotoxicity assays

After confirming the purity the PGs were used for anti-cancer studies Figure 4. It was observed that the PGs isolated from shark cartilage showed cytotoxic effect in a dose-dependent manner. IC50 was found to be 25 µg/mL and it indicates the effectiveness of isolated proteoglycans against proliferation of MCF-7 cell lines. Significant reduction of cell viability, i.e., cell cytotoxicity was observed in 50 and 100 µg/mL samples, the latter exhibiting 73% cell cytotoxicity. The above results are in good accordance with the previous work [35]. It was reported that PGs from mushrooms exhibited similar cytotoxic effect on colorectal carcinoma cells and it suggests the usefulness of proteoglycans in controlling tumour growth [16].

DUAL staining for apoptosis

The double fluorescence assay showed the presence of significant numbers of orange-red fluorescent cells in the MCF-7 cell line treated with 100 µg of PGs indicating active cell death (Figure 5). Double fluorescent staining with AO and EB is very helpful to assess the morphological changes during cell death. AO stains the viable cells and produces green fluorescence whereas EB is taken up by the dead cells that have lost their cytoplasmic membrane integrity and show uniform orange fluorescence [25,26,36,37].

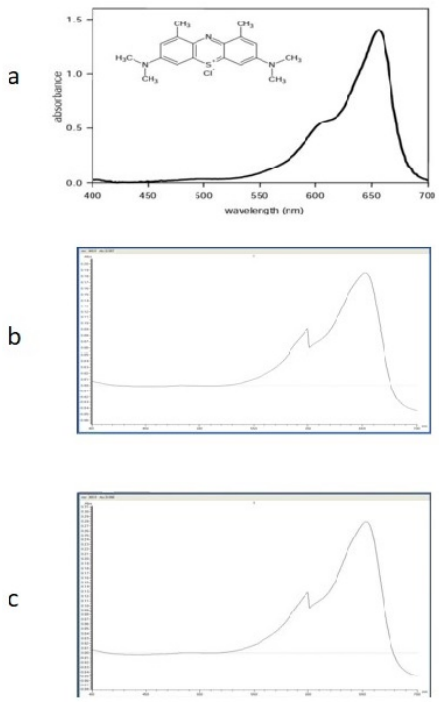

Figure 3: Wavelength scan of GAGs-DMMB complex showing peak maxima at 656 nm. (a) Denotes the corresponding GAGs-DMMB complex peak absorption pattern given by manufacture's protocol, (b) Denotes standard GAG absorption maxima at 656 nm, and (c) Absorption maxima for PGs sample.

Caspase- activation assay

Apoptosis or programmed cell death is responsible for removal of unwanted cells, stimulating the immune activity and controlling the growth of the cell [38]. Apoptosis could be the reason for cell cytotoxicity effects of proteoglycans of shark cartilage on MCF-7 cell line. Caspase 3 and 9 are the major apoptotic markers that are up regulated and whose expression is enhanced during activation of apoptosis. Caspase-3 is an intracellular cysteine protease that exists as a proenzyme, becoming activated during the cascade of events associated with apoptosis. Caspase 3 and 9 levels are summarized in Figure 6. A significant amount of caspases 3 and 9 i.e., 2.81 and 3.1 µg/mL respectively were observed in 100 µg of PGS-treated sample. Studies [39] have reported similar findings with respect to caspase activation during antiproliferation studies on MCF-7 cell lines. Production of caspase-3 is vital for the control of cell division during tumour growth and decreased production could lead to active tumour growth and resistance to chemotherapy [40]. This indicates the importance of caspase production during anti-proliferative action and the therapeutic agents that increase caspase production could be effective anti-proliferative agents. In our study, we observed increased caspase 3 expression which corroborates the anti-cancer effect of PGs from shark cartilage.

DNA laddering assay

DNA laddering assay is successfully used to establish the involvement of apoptosis and helps in ruling out the involvement of necrosis as the cause of cell death. The assay showed the formation of inter nucleosomal fragments of roughly 50 base pairs (bp) and multiples thereof (100, 150 etc.) in DNA isolated from cells treated with 100 µg of PGs (Figure 7). These nucleosomal fragments were generated as a result of activation of endogenous endonucleases with subsequent cleavage of chromatin DNA; a feature characteristically observed in apoptotic cell death. The DNA ladder pattern is often visible during early apoptotic stages and it has been accounted as an evidence of cell death due to apoptosis [41]. The similar DNA fragmentation pattern was reported in MCF-7 cell lines after sulforaphane treatment, an isothiocyanate present in cruciferous vegetables [42].

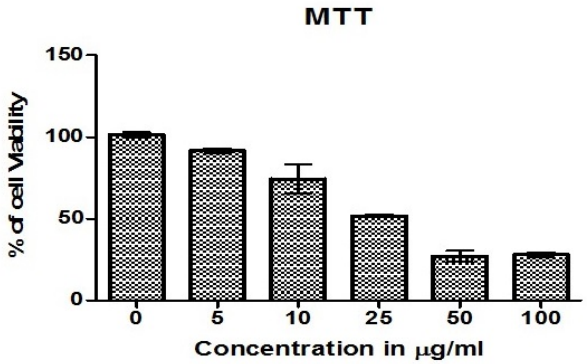

Figure 4: MTT assay showing the cell death at different concentration of PGs treatment i.e., 5, 10, 25, 50 and 100 µg/ml PGs after 24 h incubation.

Gene expression assays

Gene expression studies have been widely used to confirm apoptosis activity. Pro-apototic and anti-apoptotic regulator genes such as BAX and Bcl-2 are actively expressed in order to regulate apoptosis [43]. The gene *p53* is well known for its ability to control cell division. Expressions of these genes are quite common during cell division and apoptosis. An increase or decrease in expression of these genes provides valuable information with respect to apoptosis and proliferation. Gene expression details are is illustrated in Figure 8. In the present study, significant increase in expression of BAX and decreased expression of Bcl-2 was observed in cells treated with 100 µg of PGs. These events indicate effective activation of apoptosis, which contribute to the prevention of MCF-7 cell proliferation. Also an increase in *p53* gene expression was observed in cells treated with 100 µg of PGS which shows the active involvement of *p53* gene product in bringing about effective cell division control. PGS may activate

apoptosis by the activation of the expression of Bcl-2 and BAX by *p53* mediated pathway. Apoptosis is characterized mainly by two pathways, extrinsic (cytoplasmic) pathway and/or the intrinsic (mitochondrial) pathway [44]. High BAX: Bcl-2 ratio could favor release of enzymes, namely cytochrome *c*, copies and eventually lead to active apoptosis [45]. A balanced apoptotic and pro-apoptotic ratio is vital for the control of cancer development. Effective chemotherapeutic agents should be capable of inducing these factors in ideal proportion, to elicit a good anti-proliferative effect. Our results showed up-regulation of BAX and subsequent down-regulation of Bcl-2 gene expression which could be one of the mechanisms by which PGs induced apoptosis activation against MCF-7 cell line proliferation [27].

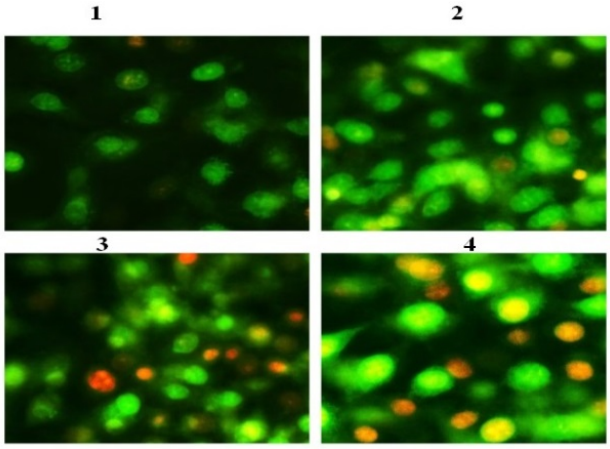

Figure 5: Double fluorescent images at different concentration of PGs treatment i.e., (1) 5 µg/ml, (2) 10 µg/ml, (3) 25 µg/ml, (4) 50 µg/ml, and (5) 100 µg/ml on MCF-7 cell line. Uniform orange fluorescent cells with condensed nucleus indicate the presence of apoptosis in treated sample compare to control.

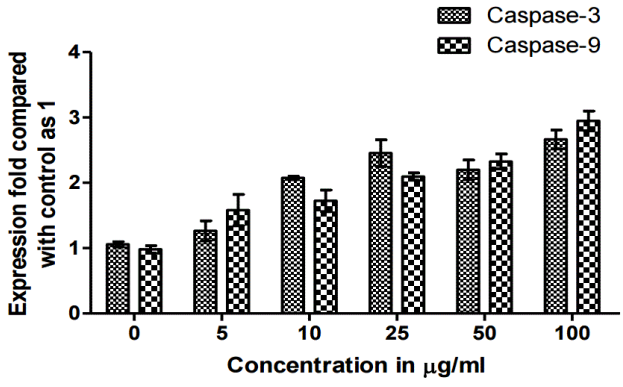

Figure 6: Level of Caspases 3 and 9 at different concentration of PGs treatment i.e., 5, 10, 25, 50 and 100 µg/ml on MCF-7 cell line.

Several studies have reported the triggering of apoptosis by natural compounds [46]. Our results also support the above observation as it was shown that PGs activated apoptosis mediated cell death against MCF-7 cell lines. In mammals each and every mutated/damaged or old cell has the capacity to be committed to apoptosis leading to its death. Whereas, due to some uncertain reasons cells escape from apoptosis mechanism and highly susceptible to become cancerous. And it was reported that eventual development of cells devoid of mechanisms for apoptosis activation could be the reason behind the induction of resistance to chemotherapy in tumor tissues. So uses of agents that can effectively activate the process of apoptosis have evolved to be the best way to Figureht against cancer cells [47].

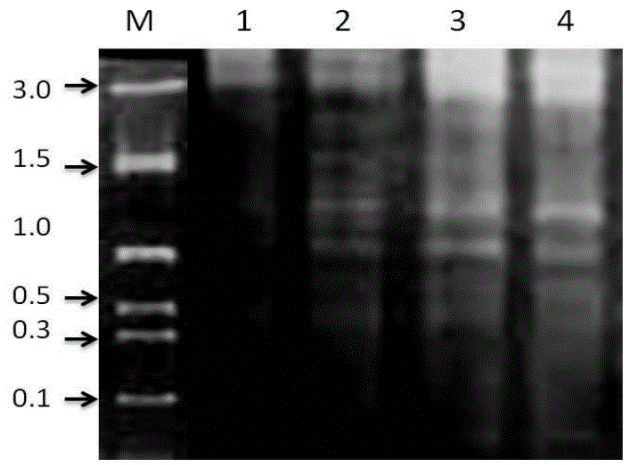

Figure 7: DNA laddering images at different concentration of PGs treatment i.e., lane (1) 10 µg/ml (2) 25 µg/ml (3) 50 µg/ml, and (4) 100 µg/ml on MCF-7 cell line. Fragments of roughly 50 base pairs (bp) and multiples thereof (100, 150 etc.) have seen maximum in lane 3 and 4 indicates apoptotic involvement.

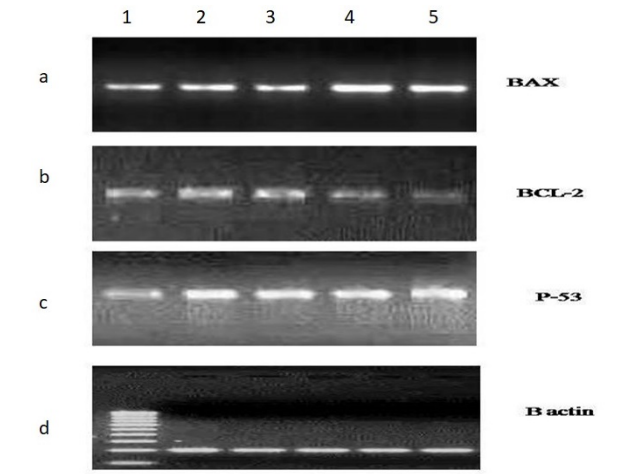

Figure 8: Apototic gene expression at different concentration of PGs treatment i.e., (1) 5, (2) 10, (3) 25, (4) 50 and (1) 100 µg/ml on MCF-7 cell line. a) BAX expression, b) Bcl-2 expression, c) *p53* gene expression, and d) β-actin.

Conclusion

Compounds which have the ability to evoke body's own anti-cancer mechanisms getting more attention due to its less side effects and

better accuracy in prevention of cancer. PGs can also be considered as a vital macromolecule into that list of compounds. As shark cartilage is a rich source of PGs, it could be a potential source of apoptotic triggering compounds since PGs has shown to activate apoptosis mediated anti-proliferative activity against MCF-7 cell lines. Individual PGs from shark cartilage need to be isolated and further studies have to be done in order to elucidate whether it is a combined effect of different PGs or individual effect. All these results point out the importance of PGs of marine sector as a promising source of anti-cancer and other bioactive agents. In order to strengthen the Figureht against cancer and other deadly diseases and for the well-being of human being PGs and other bioactive compounds of marine source can be better exploited.

Acknowledgements

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Conflict of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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