



## IN VITRO ANTI-CERVICAL CANCER EFFECT OF PROTEOGLYCANS ISOLATED FROM DEEP SEA SHARK CARTILAGE BY INDUCING APOPTOSIS AND CELL CYCLE ARREST

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### ABSTRACT

The present study was aimed at studying *in vitro* anti-cervical cancer effect of proteoglycans (PGs) isolated from deep sea shark. Anti-proliferative activity and apoptotic involvement were confirmed by following assays viz, the cell viability assay by MTT assay, morphological analysis and colony formation assay. The sample showed a significant cytotoxic activity for HeLa cells and results showed that the PGs at a concentration of 250µg/mL inhibit the colony formation capacity. Apoptosis induction was confirmed by the chromatin condensation assay by Hoechst 33342 nuclear staining. A number of apoptotic HeLa cells displayed a round and shrunken cell body and chromatin condensation inside the nucleus suggesting that PGs-induced apoptosis caused cell death. Apoptosis of HeLa cells was further verified by annexin V-FITC (fluorescein isothiocyanate) and PI (propidium iodide) staining by flow cytometry. PGs (250 µg/ml) were found to induce apoptosis and the ratio of apoptotic cells were 30.5%. Cell cycle arrest at G1/M and G2/M phase confirmed the cell death due to cell cycle arrest. Our study determined that proteoglycans effectively induced apoptosis and caused cell cycle arrest establishing their anti-proliferative effect against cervical cancer.

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## INTRODUCTION

Cervical cancer is the fifth most common cancer in humans and the second most common cancer in women (Manos *et al.*, 1999) living in less developed regions with an estimate of 84% of the new cases worldwide. As per the statistics of WHO in 2012, approximately 270 000 women died from cervical cancer, more than 85% of these deaths occurring in low- and middle-income countries like India. This high level of incidence makes it a serious concern over this disease especially in Indian women population and there is urgent need for studies that can offer solutions. Reports says that India is likely to have over 17.3 lakh new cases of cancer and over 8.8 lakh deaths due to the disease with cancers of cervix, breast and lung topping the list.

Indian Council of Medical Research stated said in 2016, the total number of new cases of cancer is expected to be around 14.5 lakh and figure is likely to reach nearly 17.3 lakh by around 2020. In vitro cell line studies are an excellent option to study the anti-cervical cancer effect of PGs to evaluate the effect of different drugs and its possible mechanism of action. HeLa cell line is the most widely used cervical cancer cell line to study the role of drugs against cervical cancer (Liu *et al.*, 2010). More than 100 chemotherapeutic drugs are in use today either alone or in combination with other drugs or treatments. Chemotherapy drugs have different side effects such as hair loss and thinning, nausea and vomiting, mouth and dental problems, fertility, fatigue etc. High dose of doxorubicin, a chemotherapy drug causes cardio toxicity in humans (Vishnu *et al.*, 2017). It has an IC<sub>50</sub> value of 1000 nM on HeLa cells (Larasati, 2010) which shows that it is not too sensitive with the treatment of doxorubicin (Maruthi *et al.*, 2015). Hence, it is needed to develop a new approach of treatment with a little or

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no side effects. For over 40 years, natural products have severed as well in combination cancer. Researchers have isolated approximately 7000 marine natural products especially from marine invertebrates to fight against different diseases including cancer. However, vertebrates like shark especially shark cartilage proteins are seldom being exploited. Proteoglycans (PGs) are important macromolecules of extracellular matrix found in vertebrate and invertebrate tissues. It is involved in various cellular functions including cell growth, adhesion, and differentiation (Kjellen and Lindahl, 1991; R.V. Iozzo *et al.*, 1996). They occur in virtually almost all mammalian tissues and are especially prominent in cartilage. The predominant proteoglycans present in cartilage is the large chondroitin sulfate proteoglycan 'aggrecan'. The other important types of proteoglycan present in cartilage are decorin, biglycan, epiphygan, versican etc. Studies reported that a special type of proteoglycan, Proteoglycan (P1) isolated from *Phellinus linteus* mushroom was found to exhibit anti-cancer activities (Li *et al.*, 2011). Apart from this small leucine rich proteoglycans (SLRP) like lumican and decorin are also found to prevent different types of cancer (Vuillermoz *et al.*, 2004; Hamid *et al.*, 2013). Till date, there is no report regarding the anti-cervical cancer activity exerted by proteoglycans of shark origin. Hence current work is designed to investigate the anti-cervical cancer effect of proteoglycans isolated from deep sea shark cartilage *Echinorhinus brucus*.

## MATERIALS AND METHODS

BD FACS, multimode reader, confocal microscope, phase-contrast microscope, refrigerated centrifuge, incubator, Annexin V – FITC apoptosis kit (cayman), propidium iodide, Rnase, Hoechst 33342 stain, crystal violet, DMEM, Trpsin, foetal bovine serum, phosphate buffer Saline, DMSO, MTT[3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide], hydrogen peroxide, antibiotic-antimycotic were purchased from Gibco Invitrogen (Carlsbad, CA, USA). All the chemicals used were of high quality analytical grade chemicals.

### Isolation and characterization of Proteoglycans (PGs)

Isolation and characterization of PGs were described in previous study (Ajeeshkumar *et al.*, 2017).

### Cell culture and treatment

HeLa cells (human epithelial cervix carcinoma) were obtained from NCCS, Pune and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (GibcoBRL) and 2% antibiotic-antimycotic (GibcoBRL) mixture were added at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells were seeded at appropriate density on plate for each analysis. After attachment, cells were incubated in 37°C with different concentrations of PGs sample. Initial study started with MTT to confirm anti-proliferative activity. Untreated cells served as negative control. The positive control of apoptosis was induced with 500µM H<sub>2</sub>O<sub>2</sub> (Sigma) in the medium for 10 min.

### Cell viability Assay and cell morphology

Cell proliferation was determined by MTT assay (Mosmann *et al.*, 1989). HeLa Cells were trypsinized and seeded on 96 well plate. After 80% confluency, different concentrations of sample (10 µg/ml, 50 µg/ml, 100 µg/ml, 250 µg/ml, 500 µg/ml

PGs) were incubated at 37°C for 24 hrs. Sample-free media was used as negative control. Cell morphology was observed after 24 hr incubation with sample using phase-contrast microscope. 100µl MTT reagent (50µg/well) was added and incubated for 4hrs in dark. After incubation, the reagent was removed and 200µl of DMSO was added to all wells, covered with aluminium foil and cells were agitated on shaker for 45 min. After shaking absorbance was read at 570nm using multimode reader. Each assay was carried out three times, and the results were expressed as mean.

Percentage viability = (OD of test /OD of control)\*100

### Colony formation assay

Colony formation assay serves as a useful tool to test whether a compound can reduce the clonogenic survival of tumor cells. A colony is defined as a cluster of at least 50 cells that can often only be determined microscopically. It is the method of choice to determine the effectiveness of cytotoxic agents. HeLa cells were seeded onto 6-well plates with a density of  $2 \times 10^5$  cells per well. After 24 h incubation, three different concentrations (10, 100 and 250 µg/ml) of PGs were added to the cells and incubated for 24 hrs. After incubation, the cells were stained with 0.5% crystal violet solution and further incubated at RT for 30 minutes. The stained cells were observed using phase contrast microscope.

### Annexin-V apoptosis assay

HeLa cells were seeded onto 6-well plates with a density of  $2 \times 10^5$  cells per well. After 24 h incubation, three different concentrations (10, 100 and 250 µg/ml) of PGs sample were added to the cells and incubated for 24 h. Cells were then trypsinized, aspirated and re-suspended in 1000 µL assay binding buffer. This was mixed well and centrifuged at 400x g for 5 min. The supernatant was discarded and cells were further stained with FITC-Annexin-V (2 µL) and propidium iodide solution (2 µL) in binding buffer, from the Annexin-V apoptosis detection kit (Cayman) and then kept undisturbed for 10 min at room temperature in darkness. After 5 min, this was centrifuged at 400xg for 5 min and then re-suspended in 1 mL assay binding buffer. This was followed by flow cytometry analysis. Ratio of apoptotic cells was measured as described by manufacturer's instructions.

### Nuclear Staining with Hoechst 33342(Chromatin condensation)

The nuclear morphology of the cells was evaluated using the cell-permeable DNA dye, Hoechst 33342. Presence of chromatin condensation or fragmentation is an indication of apoptosis. The HeLa cells were seeded in 24-well plates at a concentration of  $1.0 \times 10^5$  cells/mL. After cells attachment, the cells were treated with various concentrations of the PGs sample and further incubated for 24hrs at 37 °C in a humidified atmosphere. Then Hoechst 33342, a DNA-specific fluorescent dye, was added at a final concentration of 10 µg/mL, followed by 10 min of incubation at 37°C. The stained cells were then observed under a confocal fluorescence microscope to examine the degree of nuclear condensation.

### Cell cycle analysis

Cell cycle distribution was detected using propidium iodide staining. HeLa cells were seeded onto 6-well plates for 24 h

and rinsed twice with PBS. Three different concentrations (10, 100 and 250  $\mu\text{g/ml}$ ) of PGs were added to the cells and incubated for 24 h. After 24 h, the cells were washed twice with PBS and then trypsinized and fixed using 70% absolute ethanol. This was vortexed gently and kept under ice for 30 minutes. The samples were then centrifuged at 2000 rpm for 5 minutes. Further, the cells were resuspended in 0.25ml PBS and 5 $\mu\text{l}$  RNase A and incubated at 37  $^{\circ}\text{C}$  for 30 minutes. After incubation 10 $\mu\text{l}$  propidium iodide was added and kept in dark at 4 $^{\circ}\text{C}$ . The cells were further washed with PBS to remove the unbound PI and then centrifuged for 5 min at 1500 rpm. The supernatant was discarded and the cell pellet was further resuspended in 1 mL PBS.

## RESULTS

### Cell viability and morphological analysis

The results of anti-proliferation study done with MTT assay are shown in Fig 1. It was observed that the proteoglycans extracted from shark cartilage showed cytotoxic effect in a dose-dependent manner. High rate of cell cytotoxicity observed indicates the effectiveness of extracted proteoglycans against proliferation of HeLa cell lines. Cell cytotoxic effect of PGs against HeLa cell lines was further confirmed by cell morphology analysis (Fig.2). The morphology of the HeLa cells was examined using a phase contrast microscope. PGs treated cells showed a round morphology of cells with shrinkage. A proportion of the cells revealed swelling, cell membrane lysis and disintegration of organelles, suggesting PGs-induced toxicity in cells. Through phase-contrast microscopy, a decrease in the total number of cells and an accumulation of cells floating in the culture medium, indicating cell death was observed. In the control group, the cells exhibited an intact morphology of nucleus and cytoplasm. The cell morphology became more round and floated compared to the untreated healthy cells, showing a dissimilar cytoskeleton. Based on the MTT assay three different concentrations viz., 10, 100 and 250 $\mu\text{g}$  PGs were used for further analysis.

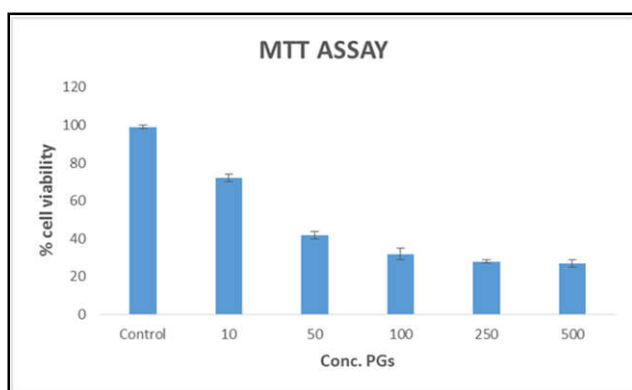


Fig. 1. Cell cytotoxicity of PGs cell lines on HeLa in dose dependent manner

### Colony formation assay

To explore the anti-proliferative properties of PGs, colony formation assay was performed. The results showed that the sample can inhibit the colony formation capacity, mainly at 250 $\mu\text{g/ml}$  concentration. PGs clearly reduce the colony to small scattered colonies as that of 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$  treated ie, positive standard groups. PGs showed a promising inhibitory

effect on colony formation of HeLa cells in a dose dependent manner. Colonies were photographed using phase-contrast microscope (Fig. 3)

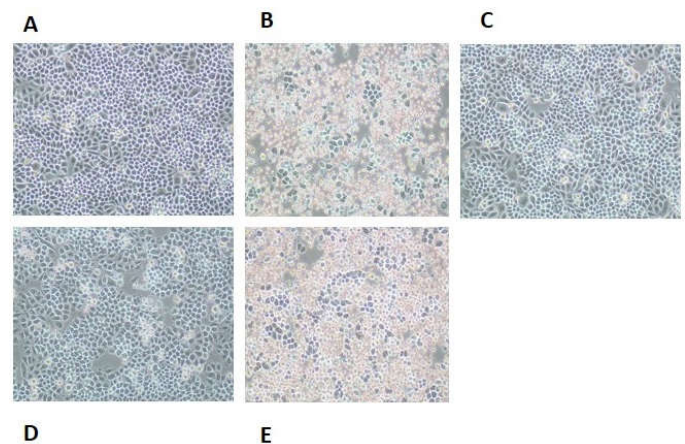


Fig. 2. Morphological analysis by light microscopy: morphological changes of HeLa cells treated with A) untreated as negative control B)  $\text{H}_2\text{O}_2$  treated as positive control C) 10  $\mu\text{g/ml}$  D) 100  $\mu\text{g/ml}$  and E) 250  $\mu\text{g/ml}$  PGs and cells were observed using phase-contrast microscopy. Original magnification, 40X

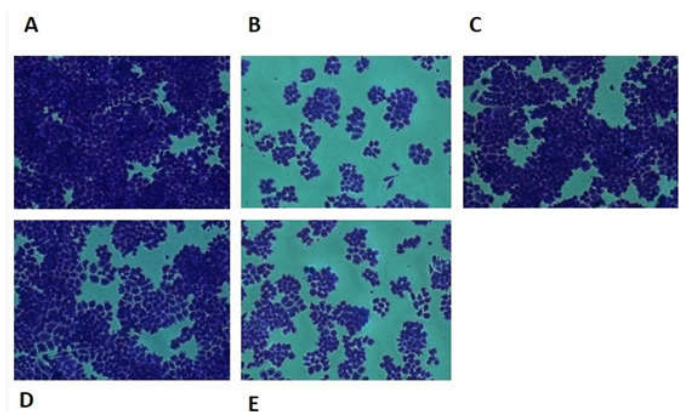


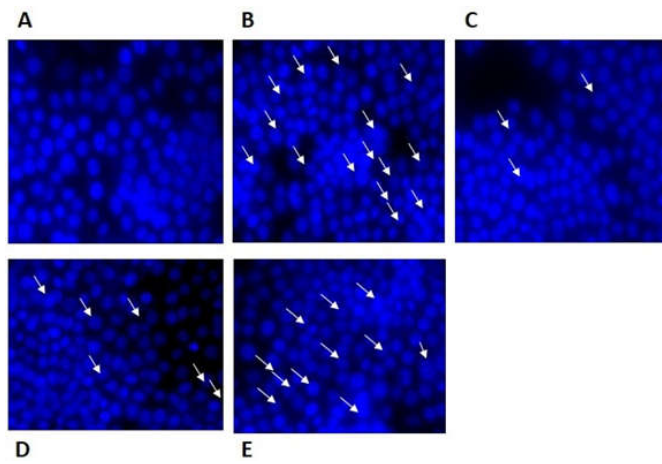
Fig. 3. Inhibitory effects of GAG on colony formation capacity of HeLa cells. A) untreated as negative control B)  $\text{H}_2\text{O}_2$  treated as positive control C) 10  $\mu\text{g/ml}$  D) 100  $\mu\text{g/ml}$  and E) 250  $\mu\text{g/ml}$  PGs

### Chromatin condensation

The Hoechst 33342 staining was used for the detection of chromatin condensation (DNA damage). Representative stained images are shown in Fig. 4. The number of apoptotic HeLa cells (white arrows) which displayed a round and shrunken cell body and chromatin condensation inside the nucleus suggesting the PGs-induced apoptosis. Control cells show an intact uniform nucleus and  $\text{H}_2\text{O}_2$  used as positive control with highly condensed chromatin.

### Effect of PGs on the degree of apoptotic cells by Annexin V-FITC staining

To further verify PGs-induced apoptosis in HeLa cells, cells were stained with annexin V-FITC and PI using Annexin V-FITC apoptosis kit (Cayman). Results of flow cytometry are presented in Fig. 5. The lower right quadrant (Q4) depicts the percentage of early apoptotic cells (annexin V-FITC-stained cells) and the upper right quadrant (Q2) represents the percentage of late apoptotic cells (annexin V-FITC and PI-stained cells). The fully apoptotic cells are those in the lower right and upper right quadrants.

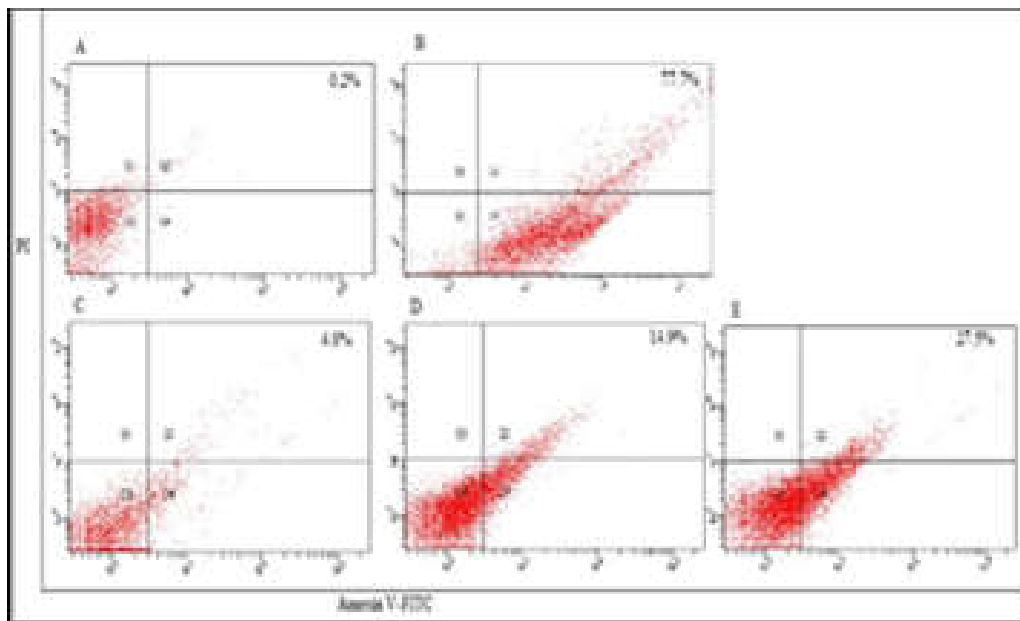


**Fig. 4. Cell apoptosis observed using Hoechst 33342 staining: HeLa cells were treated with A) untreated as negative control B) H<sub>2</sub>O<sub>2</sub> treated as positive control C) 10 µg/ml D) 100 µg/ml and E) 250 µg/ml PGs. Photographs were taken under a fluorescence microscope (200×, original magnification). Arrows represent apoptotic cells with chromatin condensation inside the nucleus**

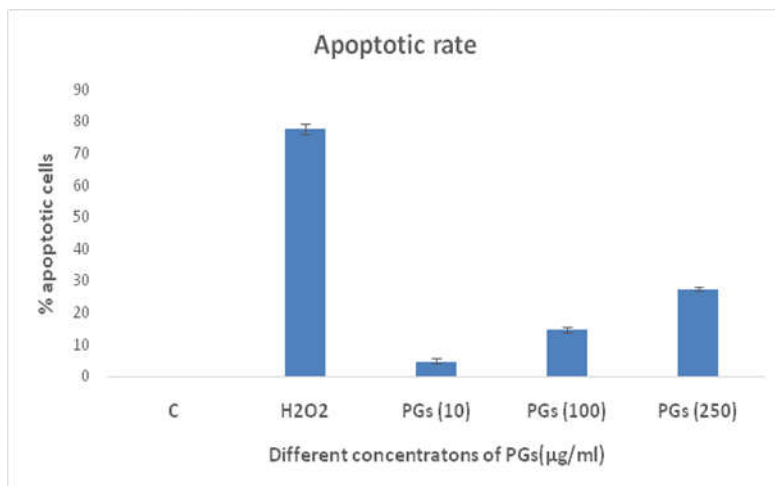
H<sub>2</sub>O<sub>2</sub>-treated cells were taken as positive control and untreated cells as negative control. As shown in Fig. 6, no apoptotic cells were detected in the control group. However, after treatment with PGs sample at different concentrations 10 µg/ml, 100 µg/ml, 250 µg/ml and H<sub>2</sub>O<sub>2</sub>, ratio of apoptotic cells were 4.8%, 14.9%, 27.5% and 77.7% respectively.

**Cell cycle distribution**

Fig 7 depicts PGs-induced cell cycle distribution in dose dependent manner. Cell cycle analysis was done by using propidium iodide staining. Cell cycle analysis revealed three distinct cell populations in HeLa cells, which were indicative of cells in the G<sub>0</sub>/G<sub>1</sub>, S and G<sub>2</sub>/M phases of the cell cycle. There were no significant differences in the cell population analysis between the negative control and the cells treated with PGs at different concentrations. Our result shown that the most effective concentration of 250µg/ml that induced the highest apoptosis in cells, also caused the accumulation of cells at G<sub>2</sub>/M phase of the cell cycle. G<sub>2</sub> phase in the cell cycle is where DNA repair might occur in cells, along with preparation for mitosis in M phase.

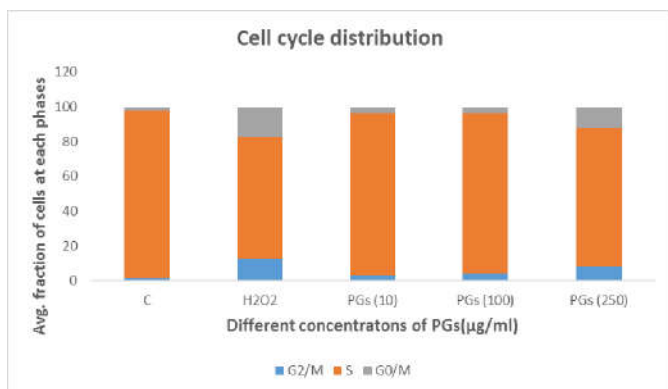


**Fig. 5. PGs-induced apoptosis in HeLa cells was determined by flow cytometry using annexin FITC-PI staining method. A) untreated as negative control B)H<sub>2</sub>O<sub>2</sub> treated as positive control C)10 µg/ml D)100 µg/ml and E)250 µg/ml PGs. The lower right quadrant (Q4) indicates the percentage of early apoptotic cells (FITC-stained cells) and the upper right quadrant (Q2) indicates the percentage of late apoptotic cells (FITC+PI-stained cells)**



**Fig. 6. PGs -induced apoptosis rate in dose dependent manner**





**Fig 7. Cell cycle distribution of different treatment A) untreated as negative control B) H<sub>2</sub>O<sub>2</sub>-treated as positive control C) HeLa cells treated with 10 µg/ml D) 100 µg/ml and E) 250 µg/ml PGs**

Increasing arrest of the cells in G2/M phase has been shown to be associated with enhanced apoptosis.

## DISCUSSION

Cell cytotoxicity analysis using MTT is the widely accepted method to determine cell cytotoxicity exerted by bioactive compounds. (Mosaman, 1983). Significant reduction of cell viability, i.e., cell cytotoxicity was observed for 250 µg/ml PGs-treated samples, exhibiting 73% cell cytotoxicity. The above results are in good accordance with the work done by Vuillermoz *et al.*, 2004. Proteoglycans extracted from mushrooms exhibited similar cytotoxic effect on colorectal carcinoma cells and it suggests the usefulness of proteoglycans in controlling tumour growth (Jambunathan *et al.*, 2014). The results obtained from the cytotoxicity assays indicate that inhibition of HeLa cells was gradually increased by the addition of PGs in MTT assay. It reveals that HeLa cell line is susceptible to PGs. Cell cytotoxicity was visually confirmed by reduction of HeLa cell count of PGs-treated samples by microscopic view. Confirmation of anti-proliferative activity of PGs was clearly observed in colony formation assay. Reduction of HeLa cell colonies indicated the role of PGs in the cytotoxicity of the HeLa cells by certain mechanism. Thus the overall study evaluates that PGs isolated from cartilage of *Echinorhinus brucus* has potential activity on HeLa cells. Apoptosis which is also called programmed cell death, is the protective pathway of the body to kill mutated or damaged cells to prevent the cells from becoming cancerous. So compounds that are able to trigger the apoptotic pathway always have an advantage to resist development and progression of cancer. Many drugs are developed by researchers which could possibly activate apoptosis and hence could have anti-cancer activities (Fulda and Debatin, 2006; Lin *et al.*, 2007).

There are assays like chromatin condensation and flow cytometry assisted identification of apoptosis activation of PGs by Annexin V-FITC staining. Chromatin condensation is a vital process that is specific to apoptosis mechanism. After the activation of apoptosis, cells reach the final stages of cell death by the action of both extrinsic and intrinsic pathways. Chromosomes of the cells become condensed and finally cell death occurs and this process is highly specific to apoptosis. These chromatin condensation changes of cells can be better visualized by staining with nuclear stain called Hoescht stain which imparts blue colour after staining and binds to chromatin of cells.

This enables the visualization of chromatin condensation after treating with apoptosis-activating anti-cancer compounds. Prominent chromatin condensation observed in PGs-treated sample indicated cell cytotoxicity activity of PGs against HeLa cell lines through the activation of apoptosis. Similar reports were observed by Zhao *et al.*, 2011 who have reported the anti-proliferative activity of compound called ergone, a plant steroid isolated from *Russula cyanoxantha*, towards HepG2 cells by the activation of chromatin condensation. In vitro and in vivo anti-colon cancer effects of *Garcinia mangostana xanthones* extract has been reported by the activation of apoptosis and involvement of chromatin condensation (Abdalahim *et al.*, 2012). Above studies are in accordance with our observation of involvement of chromatin condensation by PGs against HeLa cell lines. Hence, we assumed that cell cytotoxicity exerted by PGs could be due to the activation of apoptosis. Apoptotic activation of PGs in *in-vitro* anti-cervical cancer study was confirmed by Annexin V-FITC and PI staining with the aid of flow cytometry. Response for late and early apoptotic cells were obtained in annexin V-FITC and PI analysis.

Cells that underwent late and early apoptotic stages were clearly observed in PGs-treated samples which confirm the triggering of apoptosis by PGs to kill HeLa cells. Anticancer activity and apoptosis activation by quercetin in human lung cancer cell line A-549 has been already reported (Shi-Ying Zheng *et al.*, 2011). Our results also agree with the above studies and substantiated the evidence of activation of apoptosis by PGs against HeLa cell lines. In the present study, PGs isolated from deep sea shark *E. brucus* has shown apoptotic-triggered anti-cancer effect on in vitro HeLa cell lines. Hence PGs can be added to the list of natural compounds which could evoke the apoptotic pathway to fight against cancer. Importance of natural products to kill cancer cells by activating apoptosis is well established (Youn *et al.*, 2009). Cell cycle arrest enhances the activation of apoptosis and facilitates cell cytotoxicity against cancer cells. Reports suggested the involvement of apoptosis and cell cycle arrest in chitosan induced anti-cancer activity on oral cancer cell lines (Wimardhani *et al.*, 2014).

Activation of apoptosis in human cervical carcinoma HeLa cells was previously reported which highlight the role of apoptosis to kill cervical cancer cells (Liu *et al.*, 2010). Similar trend was observed in our study where PGs induced apoptosis-activated cell cytotoxicity on HeLa cervical cancer cell lines and clear cell cycle arrest at G1/S and G2/M phases were observed. Researchers suggest the role of cell cycle regulation at G1, S, G2 and M phases of the cell cycle to resist cancer progression (Tuteja and Tuteja, 2000). Compounds which arrest cell cycle phases especially at G2/M phases facilitate better protection from cancer occurrence and recovery. Role of cell cycle arrest in anti-proliferative action of mushroom extract against leukemia cells has been reported by Gu and Belury, 2005. Similarly anti-tumor activity exerted by the arrest in the G2/M phase and its apoptotic association has been reported in human anti-gastric adenocarcinoma (SGC-7901 cells) studies. They confirmed cell cycle arrest at G2/M phase and enhanced apoptotic events to prevent cancer progression in their study (Shi *et al.*, 2007; Cui *et al.*, 2007). Youn *et al.* (2009) evaluated the anti-proliferative activity of water extract of *I. obliquus* extract on murine melanoma (B16-F10) cells. They observed better apoptotic response followed by arrest of cell cycle at G0/G1 phase indicating the importance of cell

cycle arrest to block cancer cell proliferation and activation of apoptosis. All the findings confirm the better protection rendered by bioactive molecule against any cancer cells by arresting cell cycle and by enhanced activation of apoptosis. Similar response was exerted by PGs isolated from deep sea shark cartilage which emphasize the anti-cancer potential of PGs against HeLa cell lines. So we confirm that PGs used in our study exhibited anti-cancer activity on cervical cancer through combined action of apoptosis and cell cycle arrest.

## Conclusion

Cervical cancer is a big concern among women since numerous cases are increasingly being reported each day. At the same time current treatment with chemotherapeutic drugs are ridden with several side effects. There is a continual search for natural compounds with negligible side effects in the current scenario. Proteoglycans from marine sources especially from shark cartilage can be better utilized to fight against cervical cancer and other cancers too. Compounds having the capacity to activate apoptosis play vital role in fight against cancer. We observed higher dose of proteoglycans (250µg/ml) isolated from deep sea shark cartilage has a greater potential to fight against cancer through the activation of apoptosis and cell cycle arrest. However, detail investigations are further needed to elucidate the exact mechanisms of action of proteoglycans in apoptosis involved anti-cervical cancer effect.

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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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