ELUCIDATING THE ROLE OF SAPONIN IN TREATING OVARIAN CANCER CELLS USING TWO – DIMENSIONAL ELECTROPHORESIS COUPLED WITH MASS SPECTROMETRY PROTEOMIC APPROACH

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ABSTRACT

Ovarian cancer remains a devastating disease worldwide. It affects young individuals and after that their whole lifestyle is changed. Though many advancements are developed in the treatment strategies for cancer, still the success rate is very low. To increase the survival rate of the affected cancer patients, novel therapeutic strategies should be implemented. Such a novel compound is Saponin. Saponin possess many important bioactivity functions which are used in different areas to benefit mankind. The aim of the study to understand and evaluate the significance of Saponin in treating ovarian cancer cells. After treating Saponin to the ovarian cancer cells, MTT analysis was performed followed by apoptotic staining. Further to understand the protein profile changes, the protein samples were analyzed by two-dimensional electrophoresis coupled with mass spectrometry. Interestingly identified two key candidate proteins which are involved in apoptotic pathway namely BID and BCL2. All the analysis showed significant apoptotic induction in the saponin treated PA1 ovarian cancer cells than untreated cells. The data clearly depicts the significance of Saponin in treating ovarian cancer cells. This preliminary data evidently proves the impact of Saponin for better therapeutic strategies in treating PA1 ovarian cancer cells.

Keywords: *PA1 cells, 2D electrophoresis, Mass spectrometry, Bioactive function, Apoptosis, Cytotoxicity.*

INTRODUCTION

In recent days, treating cancer becoming a difficult scenario and have many challenges. Researchers are screening for nature-based derivatives for cancer treatment management. Well known nature-based compounds include Taxol and vincristine, and these are practically applied in many types of cancer treatment strategies. Saponins are well known plant-based derivative and these are known to possess glycosides which contain aglycones of triterpene sapogenins or steroidal sapogenins. Saponins possess different kind of bioactivities such as pharmacological effects, including cardiovascular protective activity [1], anti-inflammatory [2], antiviral [3], and immunoregulatory effects [4]. In addition, current reports states that Saponin possess efficient anti-tumor activity by inhibiting the proliferation [5], metastasis [6], angiogenesis [7] and even helps in reverting the effects of multi-drug resistance (MDR) [8]. All these are achieved by inducing the apoptosis and eventually promoting the cell differentiation. Additional reports suggest that it even helps in minimizing the side effects radiotherapy and chemotherapy [9].

Carcinogenesis is defined as a crucial biological process which is comprised of different levels of interaction analysis. Hence, identifying and enrichment of antineoplastic molecules which aims multiple factors is the need of the hour. In coherence to this scenario, saponin was studied and found to be showing efficient anticancer property in different kinds of cancer cell lines by arresting the cell growth and by apoptosis induction. Few saponins known to possess anti-metastasis [10, anti-angiogenesis [11], and anti-inflammatory [12] properties and on the whole, everything insists the importance of this compound with very vast bioactivities in cancer treatment. Few other reports suggest the importance of this saponin in reversing the MDR and this helps in increasing the efficiency of chemo treatment [13], all these additional properties of Saponin highlights the significance in the cancer treatment strategies. Hence, in the current study, we analyzed the effect of saponin in treating ovarian cancer cells.

MATERIALS AND METHODS

Cell culture

The PA1 cells were obtained from NCCS, Pune, India. Standard DMEM (HIMEDIA) media was adapted to grow the cells along with 10 % FBS (HIMEDIA) and penstrep antibiotic solution (HIMEDIA). The cells were kept at 37°C with 5% CO_2.

MTT assay

The cytotoxicity of Saponin on PA-1 cells was determined by the method of Mosmann [14]. Briefly, the cells were taken and count was measured to reach a final concentration of 1×10^4 cells/ml and the same was added to 96 well plates. Cells were incubated for 24 h for complete cell settling in the plate. Then saponin (10 – 50 µg/ml) were added to the corresponding designated wells and incubated for 24 hrs at appropriate condition as described earlier in the methods. After specified incubation hours, the wells were rinsed with fresh media and the MTT (5 mg/ml in PBS) dye was mixed with each well, further incubated for 4 h at 37°C. Further DMSO (HIMEDIA) was added and reading was taken at 540 nm. The IC 50 was calculated for saponin based on the triplicates analysed.

Measurement of apoptotic induction using acridine orange/ethidium bromide (AO/EB) dual staining method

The fluorescence microscopic analysis of apoptotic cell death is done as performed earlier [15]. PA-1 cells were added at 5 x 10^4 cells/well in a 6 well dish and incubated for 24 hours. After treatment with sample 15 µg/ml for 24 h, the cells were detached, washed with cold PBS and then stained with a mixture of AO (100µg ml−1)/ EB (100µg ml−1) ratio (1:1) at room temperature for 5 min. The stained cells were observed by a fluorescence microscope at 40x magnifications. At the end of treatment, the cells were collected and washed three times with PBS. The plates were stained with acridine orange/ethidium bromide (AO/EB 1:1 ratio; 100 µg/ml) for 5 minutes and examined immediately under fluorescent microscope 40x magnification. The number of cells showing feature of apoptosis was counted as a function of the total number of cells present in the field.

Two-dimensional Gel Electrophoresis

For proteomic analysis, the amount of protein subjected to 2DGE was 100 µg. Coomassie Brilliant Blue (CBB)-G250 dye was used to stain the gels. The protein was added with 350 µL rehydration buffer (7 M urea, 0.5% ampholytes, 4% CHAPS, 2 M thiourea, 50 mM dithiothreitol, and 0.004% of bromophenol blue) with slight modifications [16]. The Immobilized pH Gradient (IPG) strips were rehydrated passively and kept for 14 h. Once this process was done, the strips were placed in an isoelectric- focusing machine, and a voltage of 82 kV was applied at 20°C with the following conditions: 500V step-n-hold for 1h, 1 kV gradient for 1 h, 8kV gradient for 3 h, and 8 kV step-n-hold for 8h. Equilibration of the IPG strips was done using buffers consisting of 2% dithiothreitol and 2.5% iodoacetamide [17]. Until the 2DGE was done, the focused strips were stored at -70 °C. To start the second-dimension separation of the proteins using sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE), the focused strips were incubated with an optimized buffer for 30 min. These incubation steps include both the reduction and alkylation process of the focused proteins [18]. On completing these processes, the second-dimension analysis was executed in SE600 Vertical Unit (Cytiva – GE HEALTHCARE, Uppsala, Sweden) using polyacrylamide gels containing 12.5% polyacrylamide, at 1 W/gel for 1 h and 13 W/gel for 5 h in dim-light. To minimize the experimental variation, both GC and control samples were simultaneously examined.

Staining

After completing the SDS-PAGE run of 2DGE, fixing the segregated proteins was performed by treating the gels with a fixative containing 40% methanol, 10% glacial acetic acid, and distilled water for at least 1 h. The gels were then rinsed thrice with water for 10 min, followed by staining with CBB-G250 staining solution for 1 min. The gel was then rinsed with water to remove the stain [18].

In-gel Tryptic Digestion

In-gel trypsin digestion was performed as detailed earlier [19]. Initially, the selected spots were rinsed twice with water for 10 min. Destaining of gel pieces was carried out using 25 mM Ammonium Bicarbonate (AmBic) in 50% Acetonitrile (ACN) thrice or till the gel gets completely decolorized. After dehydration, the gel pieces were incubated with 100% ACN for 15 min, followed by vacuum drying for 30 min. After drying, the gel pieces were incubated with a solution of 500 ng trypsin dissolved in 5 µL of 100 mM AmBic (in 10% ACN) for 30 min in ice. Furthermore, total 30 µL of 40 mM AmBic (in 10% ACN) was added to the gel pieces and incubated at room temperature for 12-14 h. The samples were then spun shortly for 10 s, and the supernatant was preserved. For further elution process, 35 µL of 0.1% trifluoroacetic acid (TFA; in 50% ACN) was added to the leftover pieces and incubated for 10 min. Both supernatants were mixed, and vacuum dried. The dried peptides were suspended in 8 µL of 0.1% TFA (in 5% ACN).

Matrix-Assisted Laser Desorption/Ionization – Time of Flight (MALDI-ToF) Spectrometry of Tryptic Digests

Extracted tryptic peptides were subjected to MS analysis as explained earlier [17]. The α-cyano-4-hydroxycinnamic acid (CHCA) matrix (2 mg/mL, prepared in 70% ACN and 0.03% TFA) was used in this study. Peptide samples were applied on a stainless steel MALDI target plate using the sandwich method. Total 0.5 µL of the CHCA matrix was layered first on the plate and allowed to dry. The same volume of sample was then layered over the matrix, followed by the layering of 0.5 µL of the matrix on the sample. In MS analysis, reflector mode was adapted to acquire the peptide mass spectrum. The parameter used in the instrument was an acceleration voltage of 20 kV fixed to pulsed extraction. Bradykinin (757.39 Da), P14R (1533.85 Da), angiotensin II (1046.54 Da), and adrenocorticotropic hormone fragment (2465.19 Da) were used as calibration standards. Search parameters in the database were set as explained earlier [20]. Databases adapted for the search were NCBInr, MSDB, and SWISS-PROT. The MASCOT score with $p<0.05$ was only considered significant.

RESULTS AND DISCUSSION

To understand the efficacy of the Saponin on ovarian cancer cells, PA1 cells were treated with different doses such as for known standard compound Saponin (10, 20, 30, 40 and 50 ug) as shown in (Table 1). The cells were analysed after 24 h treatment, notable morphological changes were observed. Treated cells were showing drastic morphological changes such as rounding up of cells, connection and contact were lost with adjacent cells and observed to be easily detachable from the dish.

Table 1: Data points for the Effect of saponin in inhibiting the viability of ovarian cancer cell lines in a dose dependent manner

Control	5 ug	10 ug	20 ug	30 ug	40 ug	50 ug

0.02	0.64	19.57	34.51	49.7	62.11	68.44
0.00	-0.53	17.39	29.24	44.29	59.68	66.23
-0.01	1.56	20.2	26.42	41.16	56.48	64.2

The cells showed cytoplasmic condensation, shrinkage, tendency to float in the medium, and reduction in size compared to the untreated cells shown in Fig. 1.

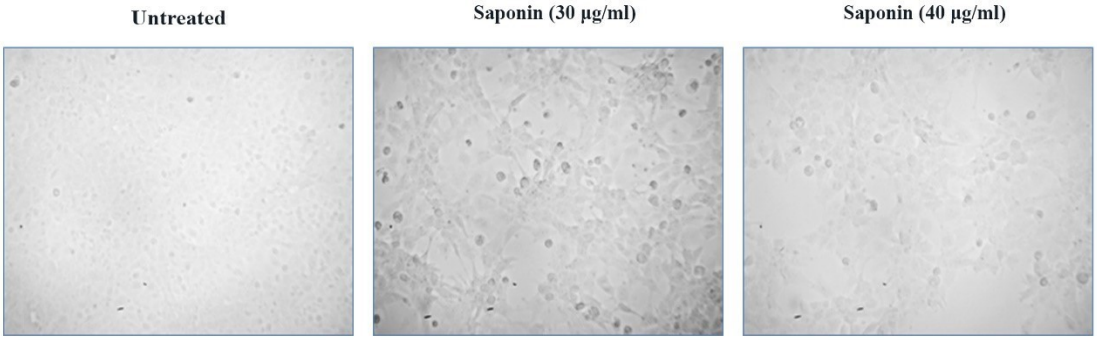

Fig. 1: Cell morphology of the untreated and treated ovarian cancer cells

To investigate the viability of the cells upon treatment with Saponin, MTT experiment was done is presented in Fig. 2. The results showed that saponin inhibits the viability of ovarian cancer cell lines in a dose dependent manner is shown in Fig. 2. Taken together Saponin triggers the morphological changes in ovarian cancer cells.

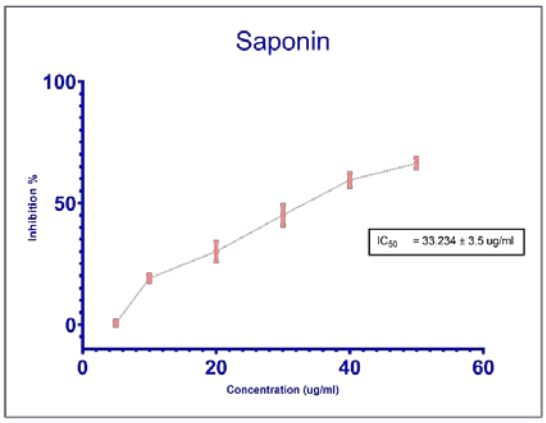

Fig. 2: Effect of saponin in inhibits the viability of ovarian cancer cell lines in a dose dependent manner

Hoechst 33342 staining was performed to detect the nuclear morphological changes in PA1 cells after exposure to the Saponin (30, 40, 50 and 100 µg/ml) at 24 h, and then analysed by fluorescence microscopy is shown in Fig. 3. Living cells were appeared as normal green nucleus, early apoptotic condensed or fragmented form of yellow colour nucleus with chromatin, late apoptotic chromatin condensation or fragmentation orange-stained nuclei and necrotic cells (uniformly red-stained cell nuclei).

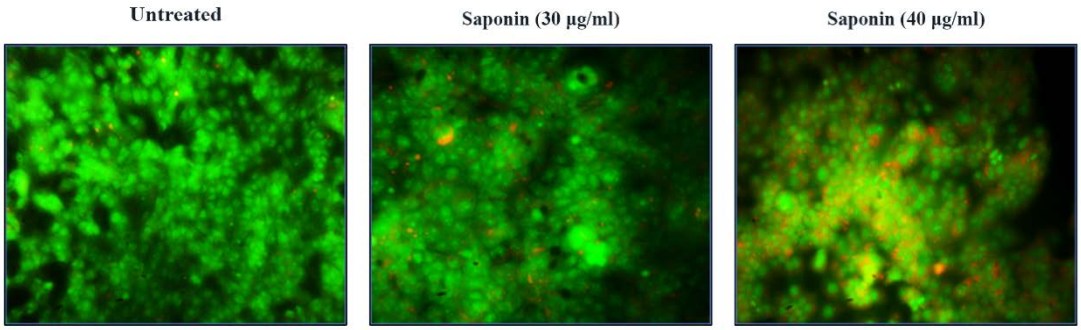

Fig. 3: Fluorescence microscopy analysis of Hoechst 33342 staining to detect the nuclear morphological changes in PA1 cells after exposure to the Saponin (30 & 40 µg/ml) at 24 h.

Saponins are described as a nature-based glycosides group, and it exhibits vast application with pharmacological activity with cytotoxic property. Saponins are described as secondary metabolites of glycosidic in nature and these are broadly present in many plants and sometime exist in animals also it will be mostly in marine invertebrates. Though it possesses huge diversity in structure, these saponins have specific bio functional properties such as the property to lyse erythrocytes or to foam [21, 22].

Many different studies described about the property of cytotoxicity and chemo preventive property of saponins which were elaborated and reported [23]. Reports suggested that triterpenoids specifically with ginseng, soy saponins possess the property of chemo prevention and tumor control of steroidal sapogenin – diosgenin [24].

Fig. 4 describes the 2D electrophoresis gel analysis for the untreated control cells and saponin treated PA1 ovarian cancer cells. The differentially regulated proteins spots were identified by mass spectrometry analysis and represented in the 2D gel along with their regulation status. Bcl2 found to be downregulated and BID found to be upregulated in the saponin treated PA1 ovarian cancer cells.

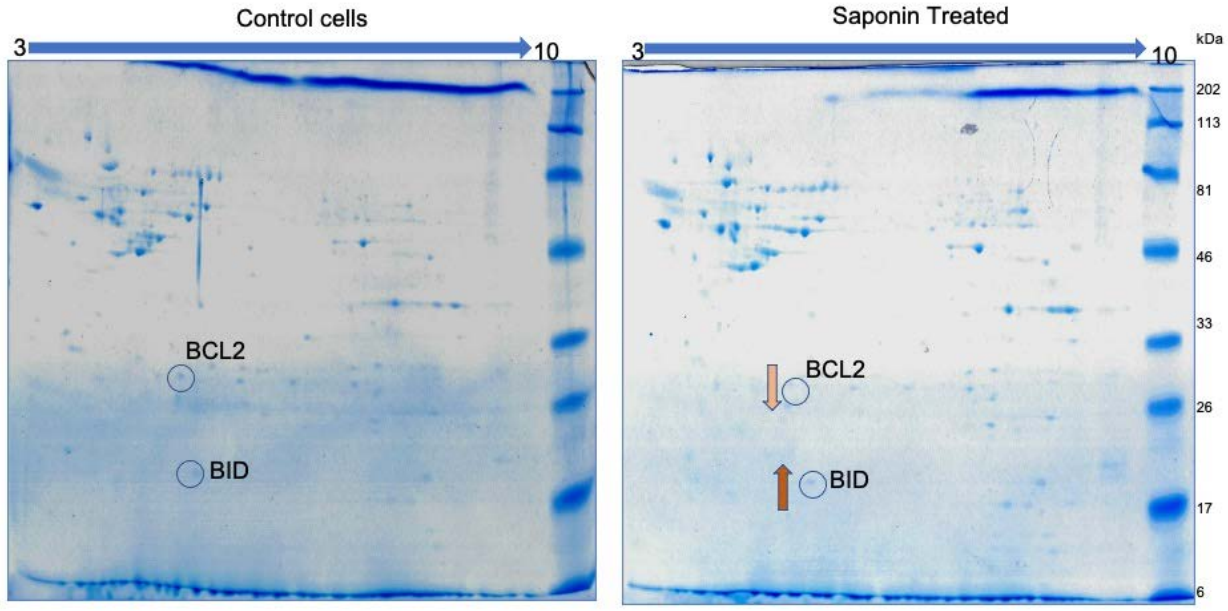

Fig. 4: 2D gel image representing the analysis for control cells and saponin treated PA1 ovarian cancer cells. Identified protein spots BCL2 and BID are marked with their regulation status.

Fig. 5 describes the gene ontology analysis for the identified candidate proteins BID & BCL2 to understand more about the impact of saponin in PA1 ovarian cancer cells. The bio-informatic analysis clearly depicts that these proteins actively involved in the apoptotic pathway, oxidative stress response and CCKR signalling map. In addition, it has various biological process including response to stimulus, signalling, cellular process, metabolic process and biological regulation of the cells. String interaction analysis describes that these proteins have strong interactions with other key players in apoptosis pathway.

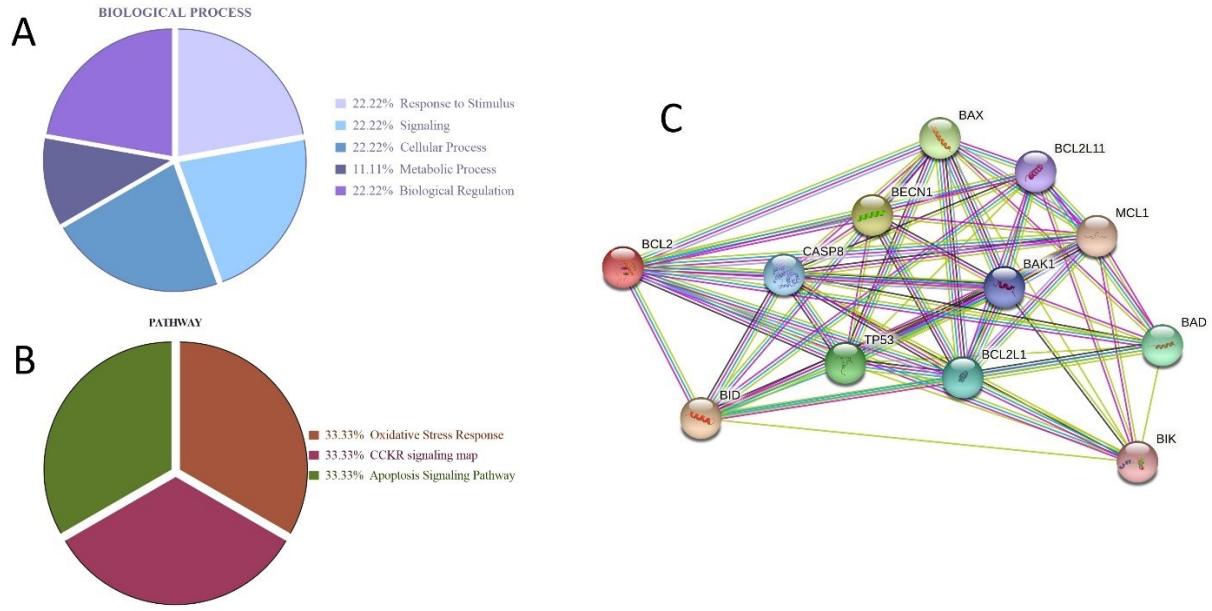

Fig. 5: Gene ontology analysis for the identified candidate proteins BID & BCL2

(A) Biological Process (B) Pathway analysis (C) String analysis for protein interaction

The unique and important property of dioscins's cytotoxic activity was detailed with steroid saponin [25]. Another report detailed that saponin exhibits an important activity in treating the tumours and these were performed by important compounds such as dioscins, saikosapo-nins, julibrosides, avicins, soy and ginseng saponins [26]. Major cytotoxic studies were analysed in specific range of cell lines, generally it ranges from 1-5 and maximum to 10. But in certain reports, triterpenoid saponins was analysed with 59 cancer cell line which were taken from 9 different human cancers including leukaemia, non-small cell lung, colon, CNS, melanoma, ovarian, renal, prostate and breast [27]. In another report, saponins obtained from Dios Corea was compared with different range of cancer cell lines and the efficiency of the cytotoxic activity was analysed [25]. Specifically, a saponin which is obtained from the plant rhizomes, exhibited the highest potential against different range of cancer cell lines such as leukaemia lines (CCRF-CEM, K562 and MOLT-4), colon cancer lines (HCT-15, KM12), CNS cancer line (SNB-75), melanoma line (M14), renal cancer line (CAKI-1), prostate cancer (DU-145) and breast cancer line (MDA-MB-435) [28].

Apoptosis was described as process of programmed cell death and it plays one of the most important functions in regulating the tissue generation and homeostasis. In recent years, most of the studies were focusing on finding the new potential candidate as anti-tumour agents which primarily have the potential

to target the pro – apoptotic properties. The whole mechanism possibly can be induced by one of the pathways (extrinsic or intrinsic) [26].

B-cell lymphoma-2 (Bcl-2) is the primary member of the Bcl-2 group of apoptotic regulatory proteins that assist oncogenesis, not by cell growth and proliferation but by enabling apoptotic resistance. In our study, Bcl-2 was expressed in control cells. When treated with the saponin, the Bcl-2 expression was reduced. This massive downregulation of the anti-apoptotic Bcl-2 by the test compounds, along with improved apoptotic rates in these samples, could explain the therapeutic value of these compounds for cancer therapy.

BH3-interacting-domain death agonist (BID) and Bcl-2 associated death promoter (Bad) are members of the pro-apoptotic Bcl-2 protein family characterized by the presence of only BH3 domains. Higher BID levels in saponin treated ovarian cancer cell lines could be correlated with increased apoptotic rates in these cells. Reports described that saponin derived from specific plants exhibit the extrinsic apoptotic pathway [29]. Saponin had the potential to induce the microtubule network disintegration which in turn eventually lead to sequence of either apoptotic or non-apoptotic cell death [30, 31].

CONCLUSION

Generally, process involved in cytotoxicity were reported for both triterpene and steroid forms of saponins. Most of the studies reported that the key process behind the saponin activity is to induce the intrinsic apoptotic pathway. In addition, non-apoptotic mechanism was also been initialized, including mechanism of arresting cell cycle, induction of autophagy related cell death, inhibition of metastasis and disintegration of cytoskeleton.

Moreover, saponin exhibits another important function to arrest the cancer cell angiogenesis process. In addition, saponin combined effect with other recombinant protein toxin was found to be significant in cancer treatment strategies. Based on the available literature, very few studies have explored the impact of Saponin in ovarian cancer cells. Hence, our study focused to evaluate the impact of saponin in ovarian cancer cells and we found an efficient impact of saponin in PA1 cancer cells both by MTT assay, apoptotic staining and proteomic analysis. It clearly states that saponin is a promising compound which can be further taken for validation studies and applied effectively for cancer treatment strategies.

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