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Original Article

COMPARATIVE EVALUATION OF ANTI-PROLIFERATIVE ACTIVITY OF *SOLANUM NIGRUM* METHANOLIC AND AQUEOUS EXTRACT ON HELA, SIHA AND C33A CELLS

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ABSTRACT

Objective: Comparative evaluation of anti-proliferative activities of aqueous (SNA) and methanolic extract (SNM) of *Solanum nigrum* was studied on HeLa, SiHa (HPV-infected) and C33A (non-HPV) cell lines.

Methods: Cellular and nuclear morphology of treated cells were studied by phase contrast and fluorescence microscopy respectively. Cell cycle analysis was carried out by flow cytometry. DNA fragmentation assay and Annexin-V assay were conducted to study apoptosis induction. Expression of apoptotic proteins – Caspase and Bax were studied at translational level.

Results: SNM treated cells showed chromatin condensation, nuclear distortion and fragmentation as well as DNA laddering, whereas extensive cytoplasmic vacuolation was observed on treatment with SNA. In case of treatment with SNM, higher percentage of cells in sub G0 phase was observed in HeLa (40.83%) and C33A (44.72%) as compared to SiHa (11.95%). Flowcytometric assays showed higher percentage of late and early apoptotic cells in HeLa (58.5%) as compared to SiHa (31.3%) and C33A (41.5%) cell lines. Down regulation of Caspase and Bax was observed in all three cell lines treated with SNM and SNA. Cleaved Caspase was absent in case of all the three cell lines.

Conclusion: Absence of cleaved Caspase suggests that cell death by SNM might be taking place by Caspase –independent apoptotic pathway, while occurrence of cytoplasmic vacuoles in SNA treated cells suggest that cell death might be taking place by autophagic pathway.

Keywords: Cervical cancer cell line, *Solanum nigrum*, DNA laddering, Caspase-independent apoptosis, Annexin V, Autophagy.

INTRODUCTION

Solanum nigrum Linn. (Solanaceae), commonly known as "Black Nightshade" has been extensively used in traditional medicine in different parts of the world to cure liver disorders, chronic skin (psoriasis and ring worm) inflammatory conditions, fever, diarrhea, eye diseases, hydrophobia, edema, mastitis, seizures, etc. [1, 2]. *S. nigrum* has been reported to have several compounds, among them the major bioactive compounds are glycoalkaloids, glycoprotein, polyphenols and polysaccharide. Due to its various uses in traditional system of medicine the plant is recently been assayed to cure various kinds of cancers and neoplasias [3]. Cytotoxicity of the hydro-alcoholic extracts of *S. nigrum* (SNE) was studied on CHO, HepG2, CT26 and 3T3 cell lines. Methanolic extracts of *S. nigrum* showed inhibitory effects on HeLa and Vero cell lines [4]. Extract of *S. nigrum* was found to be significantly inhibitory on U266 cells [5]. Organic and aqueous extracts obtained from *S. nigrum* has been shown to have antiproliferative activities on Jurkat and HL-60 cell lines in a dose dependent manner [6]. Aqueous extract of *S. nigrum* (SNA) was reported to induce autophagy in DLD1 and HT29 (colorectal carcinoma cells) showing enhanced accumulation of LC3A/BII (an autophagic marker) [7].

From all the previously reported studies, it was observed that *S. nigrum* extract (methanolic/aqueous) has potential cytotoxicity against a wide array of cell lines, suggesting their antiproliferative role in cervical cancer, breast cancer, leukemia etc. Among all these types of cancers, cervical cancer is the third most common cancer in women. The prevalence and burden is much higher among women of low socioeconomic status as well among rural women in India [8, 9]. The main risk factor for the development of cervical cancer is Human Papilloma Virus (HPV) infection, although there are several strains of HPV infection, HPV 16 & 18 accounts for 82.5% of invasive cervical cancer cases. Frequency of cervical cancer associated with HPV 16 is higher (67.6% in India) than HPV 18 (14.9% in India [10]. As the conventional treatments (radiotherapy, surgery and chemotherapy) are highly expensive and comes with unwanted side effects, our target is to develop drugs from natural herbs with low side effects and less toxicity. Therefore a comparative study was undertaken on HeLa (HPV18), SiHa (HPV16) and C33A (HPV negative) cell lines to evaluate the antiproliferative activity of *S. nigrum* extract. Induction of apoptosis was studied by Annexin FITC assay, and DNA fragmentation assay. The expression of Caspase and pro apoptotic gene Bax was studied at translational levels to investigate the pathway(s) of cell death.

MATERIALS AND METHODS

Preparation of plant extracts

Sequential extraction steps with hexane, methanol and water were carried out. Dried plants had cut into small pieces and defatted in hexane for 72 h. The pieces were then kept in methanol and incubated in dark for 72 h. These pieces were then further crushed and the filtrate obtained was reserved as methanolic fraction. The plant residue was dried and water was added to that. Then the mixture was boiled to obtain a concentrated aqueous fraction and subsequently lyophilized. The residues obtained with methanolic [SNM] and aqueous extracts [SNA] were used to treat the cells at a concentration of 1 mg/ml.

Cell culture

Human cervical cancer cell lines – HeLa (HPV18), SiHa (HPV16) and C33A (HPV-negative) were procured from NCCS, Pune. Cells were cultured in DMEM media having L-glutamine and non essential amino acids supplemented with 0.35% Sodium bicarbonate, fetal bovine serum and antibiotic antimycotic solution (100 unit/ml penicillin, 100 µg/ml Streptomycin and 0.25 µg/ml Amphotericin B). Cultures were maintained in humidified, 5% CO_2 incubator at 37 °C. Cells were grown in T25 flasks, 60 mm petri plates and Poly-L-Lysine coated cover slips – as per experimental requirements. Cell splitting and sub-culturing had carried out using 0.25% Trypsin with 0.02% EDTA (TED).

Each experiment was carried out using four sets–control (non-treated), vehicle control (media replaced by appropriate amount of methanol), Me-SN set (media replaced with methanolic extract of *S. nigrum* to get a final concentration 1 mg/ml) and Ae-SN set (media replaced with aqueous extract of *S. nigrum* to get a final concentration 1 mg/ml).

Morphological study of the effect of *Solanum nigrum* extracts

Cell coated cover slips from treatment and control sets were washed with PBS solution and fixed in fixing solution (denatured alcohol: formaldehyde: glacial acetic acid = 30:15:1) for 30 min at 4 °C. The cells were then washed and stained with 0.5 ml Hoechst33258 (sigma) for 5 min at room temperature. The cover slips were then washed and mounted on clean, grease free slides using 9:1 solution of 0.1M n-propylgalate in glycerol: PBS and were observed under a fluorescent microscope (Olympus).

Cell cycle study

Cells of control and treatment sets were harvested and washed in PBS. Cells were then collected by centrifugation and fixed in chilled methanol for 2 min at-20 °C. Then the pellets were suspended in 500 µl PBS and 3 µl of RNaseA (10 mg/ml) was added and incubated for 2 h at 37 °C. The mixture was incubated for 2 h at 37 °C and 2.5 µl of Ethidium Bromide (10 mg/ml) was added to it. Following incubation in the dark for another 15 min at room temperature, cell cycle was studied by measuring the total DNA content of the cells by flow cytometry (BD FACS verse).

Study of apoptosis

Assay of apoptosis by flowcytometry

Cells from control and treated sets were washed with PBS and collected as the pellet by centrifugation. The pellets of cells were suspended in 500 µl of 1X Binding buffer [10X binding buffer composition being: 0.1 M Hepes, 1.4 M NaCl, 25 mM $CaCl_2$]. 100 µl of the solution from each set was transferred to fresh tubes and 5 µl of AnnexinV FITC and 5 µl PI were added to it. The mixture was vortexed and incubated in the dark for 15 min at room temperature. 400 µl of binding buffer was added to each tube and the cells were analyzed by measuring the Annexin V and PI positive cells by BD FACS verse.

DNA Laddering assay of apoptosis

The treated and the control cell samples were collected in microcentrifuge tubes, washed with PBS and suspended in Tris-EDTA (TE) buffer. They were then incubated overnight at room temperature with lysis buffer (pH 8.0) containing 20 mM EDTA, 50 mM Tris, 1% SDS, 1% NP40. Cells were then incubated at 37 ºC for 1h with RNase A. Phenol-Chloroform-IAA extraction was done to purify DNA from protein contamination and then the DNA was precipitated from aqueous solution with the help of chilled isopropanol and ammonium acetate. The DNA was precipitated by centrifugation at 17000 G, washed with 70 % alcohol and fully dissolved in appropriate amount of water. The DNA samples were then electrophoresed through 1.5% agarose gel (containing 0.5 µg/ml EtBr) immersed in TAE buffer and was visualized under UV.

Western Blotting technique

Cells were harvested, washed in PBS and suspended in suspension buffer. The 2X Sample loading buffer was added to the cell suspension and the mixture was boiled in water bath for 5 min. It was then cooled to room temperature and centrifuged at 14000 G. The supernatant was collected and total protein from each set was estimated by Micro Lowry Method. Protein separation was carried out in 12 % SDS gel. Protein was transferred to nitrocellulose paper by the semi-dry method at 0.8 mA/cm^2 for 40 min. It was then incubated in blocking buffer for 2 h at room temperature followed by washing in TBST buffer and then incubated with primary antibody for overnight at 4 ºC. The NC paper was then washed with TBST buffer and incubated with alkaline phosphatase conjugated secondary antibody for 2 h at room temperature. The paper was then washed with TBST buffer followed by washing in TBS buffer and the western blot pattern was developed using BCIP/NBT-Blue liquid substrate from Sigma.

RESULTS

Cellular and nuclear morphology study

Cells from control sets did not display any significant morphological or nuclear changes, whereas treatment with the methanolic extract (SNM) displayed shrinkage in cell size accompanied by chromatin condensation and nuclear fragmentation in case of all the three cell lines. Treatment with aqueous extract (SNA) showed extensive cytoplasmic vacuolation in case of HeLa and C33A cell line and moderate vacuolation in case of SiHa cell line as seen in fig. 1. Slight to moderate nuclear distortion was observed in case of HeLa, SiHa and C33A cell lines treated with SNA respectively.

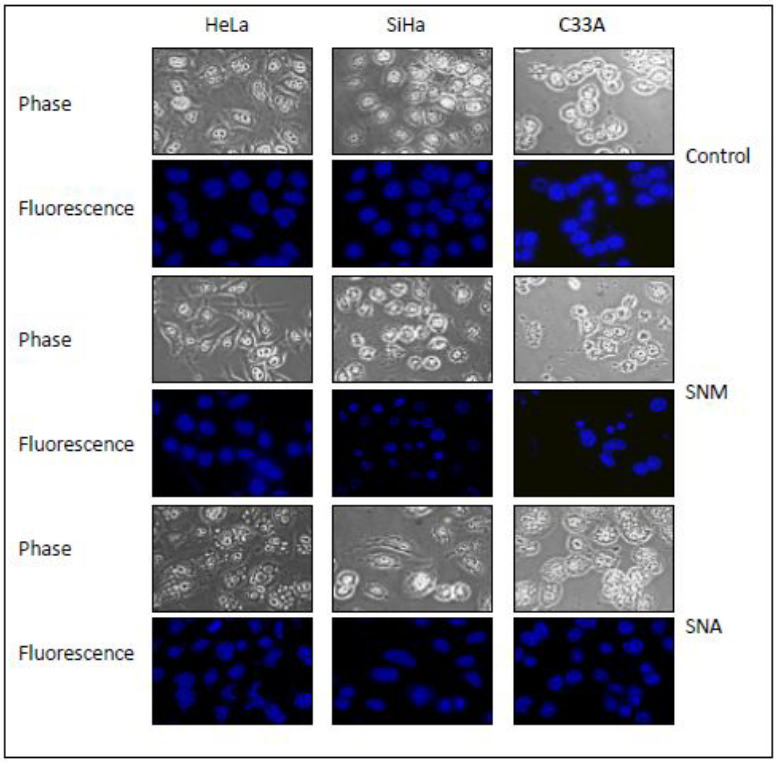

Fig. 1: Cellular and nuclear morphology as studied under fluorescence and phase contrast microscope

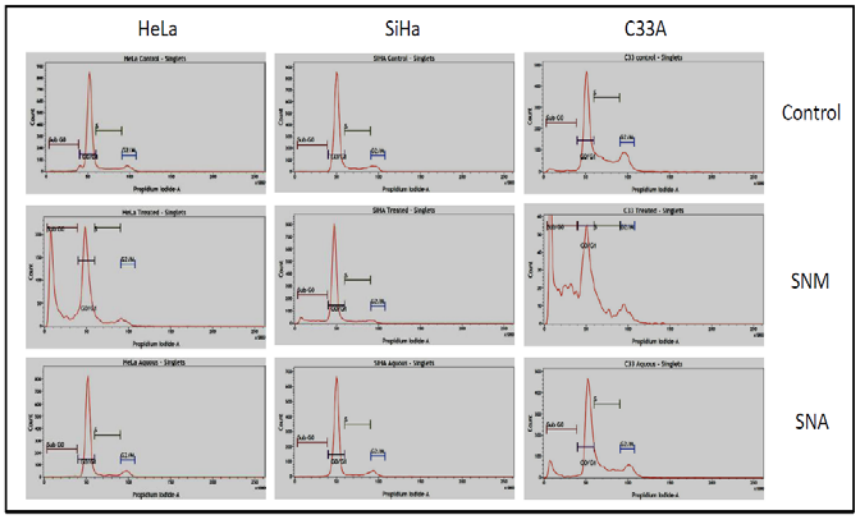

Fig. 2: Cell cycle analysis of SNA and SNM treated HeLa, SiHa and C33A cells analyzed by Flowcytometery

Cell cycle analysis

Percentage of subG0 phase cells in SNM treated cells were 40.83%, 11.95% and 44.72% in HeLa, SiHa and C33A cell lines respectively suggesting this fraction's cytotoxicity (fig. 2), while that of SNA treated cells were quite low, indicating its less cytotoxic effects on cervical cancer cells.

Annexin V-FITC/PI double staining apoptosis assay

Control sets of all the 3 cell lines have showed high percentages of live cells. In SNM treated sets, the highest amount of early apoptotic cells (53.4%) was found in HeLa cells. Whereas that in SiHa and C33A cell lines the number of apoptotic cells was 13.1% and 40.8% respectively. For SNA treated cells, only 5.1%, 18.2% and 0.7% early apoptotic cells were found in HeLa, SiHa and C33A cells (fig. 3). From the study it was observed that HeLa cells showed maximum sensitivity towards SNM fraction, as the total percentage of early and late apoptotic cells combines to 73.5% whereas, SiHa cells responded least.

DNA laddering assay

A single prominent band of DNA was observed in Control sets of all the 3 cell lines. Clear DNA laddering shown in fig. 4 was observed in the SNM treated cells but absent in SNA treated cells, indicating that SNM fraction can induce apoptotic cell death in the cervical cancer cells.

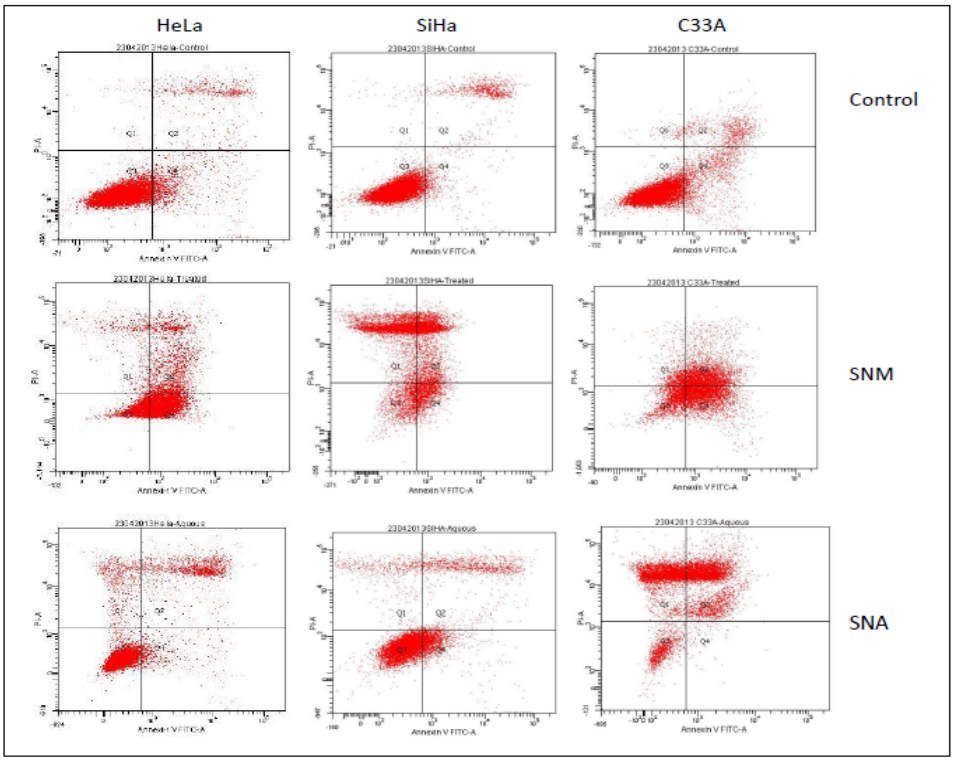

Fig. 3: Flow cytometric analysis of Annexin V-FITC assay of HeLa, SiHa and C33A cell lines treated with SNA and SNM for 24 hours. The 1st quadrant (Q1) represents necrotic cells, 2nd quadrant (Q2) represents late apoptotic, 4th quadrant (Q4) represents early apoptotic and 3rd quadrant (Q3) represents live cells

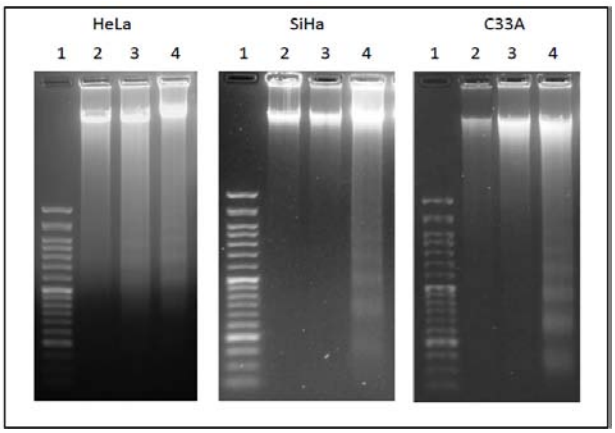

Fig. 4: Analysis of DNA in SNA and SNM treated HeLa, SiHa and C33A cells. DNA fragmentation assay showing laddering of DNA in SNM treated cells. (Lane 1: 100bp DNA marker; Lane 2: Control; Lane 3: SNA treated; Lane 4: SNM treated)

Protein expression study (Western Blotting)

Expression of beta-tubulin was normalized and expressions of other apoptotic genes were compared. In HeLa cells, pro-caspase3 expression was decreased in SNM treated set. In other cell lines, the expression was found to be same. Bax expression remained constant for SNM and SNA treated C33 and SiHa cells. However, both SNA and SNM treated HeLa cells, showed down regulation of Bax expression (fig. 5).

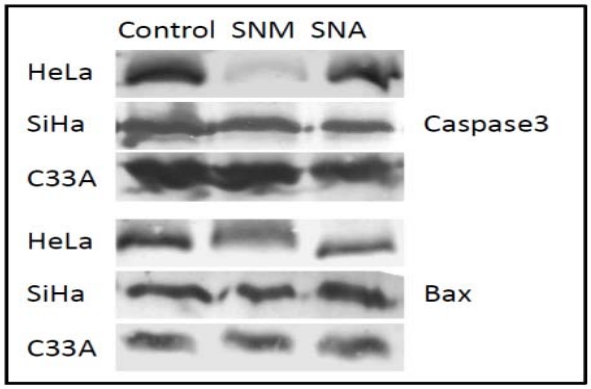

Fig. 5: Analysis protein expression in SNA and SNM treated HeLa, SiHa and C33A cells. Expression of caspase 3 and Bax in translational level as studied by western blot

DISCUSSION

Thus, it is observed that methanolic and aqueous extracts of *S. nigrum* acts differently on the three cell lines. Sensitivity of the three cell lines also differs from each other. Among the three cervical cancer cell lines HeLa showed maximum sensitivity to *S. nigrum* extracts while SiHa appears to be least sensitive. This differential sensitivity of HPV positive and HPV negative cell lines to SNM and SNA may be due to the presence of viral genome HPV 16 in SiHa, HPV 18 in HeLa and absence of viral genome in C33A cell lines. In particular, HPV positive cervical cancer cells differently expressed significant gene sets relative to HPV negative cervical cancer cells, including cell cycle regulatory genes and testis-specific genes with mechanistic, diagnostic, and therapeutic implications [11].

Induction of apoptotic cell death in the three cell lines by SNM is evident by the presence of DNA laddering and higher percentage of apoptotic cells in flow cytometric study, whereas SNA treated cell lines showed cell death other than apoptosis as is suggestive by absence of DNA laddering as well as an overall low percentage of apoptotic cells. In case of SNM treated cells, shrinkage of cells accompanied by pyknosis was observed, although up-regulation of apoptosis inducing genes such as Caspase3 and Bax could not be detected in transcriptional (data not shown here) or translational level in all the three cell lines. This is because cell death in these cell lines might be taking place by Caspase-independent apoptotic pathway involving Endonuclease G. In mammalian cell, it has been reported that Endonuclease G, like AIF (Apoptosis Inducing Factor) translocates from the mitochondria to the nucleus during apoptosis and is capable of inducing DNA fragmentation independent of caspases [12]. In these studies, Endo-G mediated DNA fragmentation was unaffected by zVAD (a cell-permeant pan Caspase inhibitor), suggesting that Endonuclease G can function in a caspase-independent manner. Studies examining AIF or Endo-G mediated DNA fragmentation has suggested that in nuclei from mammalian cells these factors can induce nuclear chromatin breakdown independent of one another [12, 13]. These studies are at par with the observations obtained from cells treated with SNM. The down regulation of Bax and Caspase in the three cell lines may be due to a low biological half life of these protein and mRNA. Since the entire study was carried out only by subjecting the cells to 24 h of treatment with SNM, further study involving lower time intervals of treatment is needed.

Results indicate that treatment with SNA has significant cytotoxicity as is observed by a decrease in the percentage of live cells seen by flowcytometric analysis. Furthermore an overall reduction in expression level of apoptotic markers such as Bax and Caspase3 suggests that cell death may not be induced by the apoptotic pathway. It has also been found that autophagic cell death is a type of cell death occurring together with (but not necessarily by) autophagic vacuolation [14]. In an experiment involving the study of the effect of SNA on endometrial cancer cells, SNA was seen to induce autophagic cell death by the accumulation of LC3A/BII [7]. In this experiment presence of cytoplasmic vacuolation in all the cells treated with SNA is indicative of autophagic cell death, but this cannot be concluded for certain as further study involving the detection of the presence of LC3IIB (autophagic marker) is required.

CONCLUSION

The cell lines used in this study had behaved differently against the extracts. Presence and absence of viral factors may have played an important role in this regard. The presence of DNA laddering and an increase in Annexin-V positive cells in the treated sets indicated induction of apoptosis. Cytotoxicity caused by SNM may be due to induction of Caspase independent apoptotic pathway and SNM may be due to induction of autophagy.

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CONFLICT OF INTERESTS

Declared None

REFERENCES

1. Kritikar KR, Basu BD, Blatter E. Indian medicinal plants. 2nd Ed. (2), Allahabad, Lalit Mohan Basu Publications; 1935.
2. Jainu M, Devi CS. Antiulcerogenic and ulcer healing effects of *Solanum nigrum* (L.) on experimental ulcer models: Possible mechanism for the inhibition of acid formation. J Ethnopharmacol 2006;104:156-63.
3. Jain R, Sharma A, Gupta S, Sarethy IP, Gabrani R. *Solanum nigrum*: Current perspective on therapeutic properties. Altern Med Rev 2011;16:78-85.
4. Patel S, Gheewala N, Suthar A, Shah A. *In-vitro* cytotoxicity activity of *Solanum nigrum* extract against HeLa cell line and vero cell line. Int J Pharm Pharm Sci 2009;1(1):38-46.

5. Wang W, Lu DP. An *in vitro* study of cytotoxic and antineoplastic effect of *Solanum nigrum* extracts on U266. Beijing Da Xue Xue Bao 2005;37:240-4.
6. Gabrani R, Jain R, Sharma A, Sarethy IP, Dang S, Gupta S. Antiproliferative Effect of *Solanum nigrum* on Human Leukemic Cell Lines. Indian J Pharm Sci 2012;74(5):451-3.
7. Tai CJ, Wang CK, Tai CJ, Lin JF, Lin CS, Jian JJ, Chang JJ, *et al*. Aqueous Extract of Solanum nigrum Leaves Induces Autophagy and Enhances Cytotoxicity of Cisplatin, Doxorubicin, Docetaxel, and 5-Fluorouracil in Human Colorectal Carcinoma Cells. J Evid Based Complementary Altern Med 2013. Available from: http: //dx.doi.org/ID.1155/2013/514719.
8. Vallikad E. Cervical cancer: The Indian perspective. Int J Gynaecol Obstet 2006;95(1):S215-S233.
9. Kurkure AP, Yeole BB. Social inequalities in cancer with special reference to South Asian countries. Asian Pac J Cancer Prev 2006;7(1):36-40.
10. WHO/ICO Information Centre on HPV and Cervical Cancer (HPV Information Centre). Human papillomavirus and related cancers in India. Summary Report 2010. Available from: www.hpvcentre.net/statistics/reports/IND.pdf.
11. Dohun Pyeon, Michael A Newton, Paul F Lambert, Johan A den Boon, Srikumar Sengupta, Carmen J Marsit, *et al.* Fundamental differences in cell cycle deregulation in human papillomavirus–positive and human papillomavirus–negative head/neck and cervical cancers. Cancer Res 2007;67:4605-19.
12. Li LY, Luo X, Wang X. Endonuclease G is an apoptotic DNase when released from mitochondria. Nat 2001;412:95–9.
13. Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE. Molecular characterization of mitochondrial apoptosis-inducing factor. Nat 1999;397:441–6.
14. Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES. Classification of cell death: recommendations of the nomenclature committee on cell death 2009. Cell Death Differ 2009;16:3–11.