ABSTRACT

Objective: Mechanism of cell death inducing pathway of chloroform fraction of methanolic extract of Solanum nigrum were studied in a panel of cervical cancer cell line.

Methods: Cells were treated with IC50 doses (previously determined by MTT assay), and various experiments were performed. For cellular and nuclear morphology, cells were stained with Hoechst 33258 and observed under phase and fluorescence microscopy. Cell cycle shift and apoptotic assay (Annexin V-FITC/PI) were studied by flow cytometry. Expressions of relevant genes were studied at the transcriptional and translational levels. ROS induction and loss of MMP and presence of γH2AX were studied by fluorescence microscopy. Constituents of the fraction were separated by column chromatography and bioassayed by MTT assay. Bioactive sub-fractions were identified by GC-MS.

Results: Typical apoptotic morphological features were found in the treated cells. Most cells were found in late apoptotic stages. In HeLa and C33A cell lines, cells were blocked at G1/S, in SiHa, subG0 population increased. Expression of proapoptotic Bax and p21 decreased and that of proapoptotic Bcl-2 decreased and that of anti-apoptotic Bcl-2 decreased. ROS mediated DNA damage was detected and induced apoptosis. Different fatty acids along with other compounds were detected in the chloroform fraction, all of which are reported to have anticancer properties.

Conclusion: From the study it can be concluded that fatty acid rich chloroform fraction of S. nigrum is capable of inducing apoptosis in cervical cancer cells, through ROS mediated DNA damage.

Keywords: Apoptosis, DNA damage, ROS, p53, Solanum nigrum, HeLa, SiHa, C33A

INTRODUCTION

Solanum nigrum commonly known as black nightshade is an erect annual herb. Though the plant is native to Africa, it is found widely throughout the Indian peninsula. In Ayurveda medicine, the plant is considered as an important medicinal herb, having antiseptic and anti-inflammatory properties. The plant is used as diuretic, diaphoretic, anodyne and expectorant. The whole plant is used to treat and cure several ailments such as fever, dropsy, asthma, ulcer, heart burn etc. in recent times the plant is reported to have diaphoretic, anodyne and expectorant. The whole plant is used to treat and cure several ailments such as fever, dropsy, asthma, ulcer, heart burn etc. in recent times the plant is reported to have antidiabetic, antinociceptive and antinflammatory. From the study it can be concluded that fatty acid rich chloroform fraction of S. nigrum is capable of inducing apoptosis in cervical cancer cells, through ROS mediated DNA damage. The plant is used to treat and cure several ailments such as fever, dropsy, asthma, ulcer, heart burn etc. in recent times the plant is reported to have antidiabetic, antinociceptive and antinflammatory. From the study it can be concluded that fatty acid rich chloroform fraction of S. nigrum is capable of inducing apoptosis in cervical cancer cells, through ROS mediated DNA damage. The plant is used to treat and cure several ailments such as fever, dropsy, asthma, ulcer, heart burn etc. in recent times the plant is reported to have antidiabetic, antinociceptive and antinflammatory. From the study it can be concluded that fatty acid rich chloroform fraction of S. nigrum is capable of inducing apoptosis in cervical cancer cells, through ROS mediated DNA damage.
stained with Annexin V-FITC antibody and PI for 15 min at dark and subjected to flow cytometry (BD FACs Verse).

Cell cycle shift
Due to induction of cell death, some changes occurred in the cell cycle pattern. So the cell cycle patterns of control and treated sets were evaluated by measuring the total DNA content using flow cytometric technique. After 24 hours treatment with the IC50 doses, cells were harvested, washed with PBS, fixed with 70% ethanol, treated with RNase A at 37 °C for 2 h, and then stained with PI, incubated at dark for 15 min and subjected to flow cytometry.

Gene expression study by semi Q RT-PCR
After 24 h treatment, cells were harvested and washed with PBS. Total RNA was isolated from the cells using Tri reagent (Sigma) according to manufacturer's protocol. RNA was quantified by UV spectrophotometry and integrity of the RNA samples was checked by running in Agarose gels containing sodium hypochlorite [15]. Using first strand cDNA synthesis kit, cDNA was prepared taking 1µg RNA as starting material following manufacturer's protocol 1 µl cDNA was taken to do PCRs for several apoptotic genes. GAPDH served as internal control for normalization of the cDNAs. Primers for the PCR reactions (Sigma) were designed by Primer 3 software using FASTA sequences of target genes from NCBI. The primer sequences and PCR profiles were listed in table 1.

After PCR, products were run in Agarose gels and photographed in Gel documentation system (UVP MultiDoc-It). Denatometric analysis was done by NIH-Image software following NIH guidelines. Relative densities of the gene expression with respect to the non-treated sets were calculated and histograms were produced (Microsoft Excel).

Table 1: Primer sequences and PCR profiles of the genes analyzed in RT-PCR

| Gene | Primer sequence (5’→3’) | Forward | Reverse |
|------|-------------------------|---------|---------|
| p53  | ATGGCCTACTTACAGACGAG    | AGATCTAAAGGCAAGACGGAGAGAGA | CTGTCTCAGAGATAGCCAGCTG|
| p21  | ATGAAATTCACCCCTTCTCC    | CCGTATCAGGTGGTTTGAGA | CATGAGGAATCTTGCAG|
| BAX  | GTGGCCGCTGAGATTTTTC    | AAAAGAAATGCTTTTCCATG | CGATGAGACACATG|
| Bcl-2 | GTGACGAATCAACGGAGAT         | GTGAGCCAGCTTTCACACAC | GTGAGGACATG|
| Bcl-xL| TCTCTTCAGCTGCTCCCTCCTC | CAGAACTCCACCTGCTGCT | GGCCAGCTG|
| HPV16 E6 | TGGTTTCTTCGGGATTATAGC | TGGTGGTGTCGGTTGTT | GGCCAGCTG|
| HPV18 E6 | TGAAAGAACGATGTCCTCAGCAG | TTGTTTTCGGGATTATAGC | GGCCAGCTG|
| GAPDH | CAGAAGTCATCCTAGACACTTTG | GTCCACCCACCTGGTTCAG | GTCCACCCACCTG|

PCR profile

| Initial denaturation (°C-min) | Denaturation (°C) | Annealing (°C-s) | Extension (°C-s) | Number of cycles | Final extension (°C-min) | Product length (bp) |
|------------------------------|------------------|------------------|-----------------|------------------|---------------------|-----------------------|
| p53                          | 94-4             | 94-30            | 58-30           | 72-45            | 35                  | 72-7                  | 210                  |
| p21                          |                  |                  | 57-30           | 72-30            | 174                 |                       |
| BAX                          |                  |                  | 58-30           | 72-45            | 151                 |                       |
| Bcl-2                        |                  |                  | 58-30           | 72-45            | 395                 |                       |
| Bcl-xL                       |                  |                  | 57-30           | 72-45            | 242                 |                       |
| HPV16 E6                     | 52-30            | 72-30            | 106             |                  |                     |                       |
| HPV18 E6                     | 53-30            | 72-30            | 285             |                  |                     |                       |
| GAPDH                        | 58-30            | 72-45            | 496             |                  |                     |                       |

Protein expression study by immunoblotting
After 24 h treatment, cells were harvested and washed in PBS. Then protein was extracted by lysing cells with 1X sample loading buffer. Total protein in the cell lysates were quantified by Lowry method and 50 µg of each sample was run in 10/12% polyacrylamide gels according to need. ß-tubulin served as the loading control. After gel run, proteins were blotted in nitrocellulose membrane (Pall-0.22 µm) and checked with Ponceu S stain. Membranes were blocked with 5% BSA for 3 h at room temperature and subsequently incubated with Primary antibody for overnight at 4 °C with gentle shaking. Then washed with TBST (Tris buffered saline with 0.05% Tween 20 buffer) and incubated with AP-conjugated secondary antibody for 3 h at room temperature. Then washed well with TBST and TBS and incubated with the substrate NBT-BCIP until bands appear. After the appearance of bands, reaction was stopped with water and photographed in gel documentation system (UVP-multipdoc It). Densitometric analysis was done as described previously.

ROS generation
After 3 h treatment, cells were briefly washed with PBS and stained with H2DCFDA for 30 min at 37 °C and then re-washed with PBS and visualized under epi-fluorescent microscope (Leica).

Loss of mitochondrial membrane potential
After 4 h treatment, cells were briefly washed with PBS and stained with JC1 for 15 min at 37 °C and re-washed with PBS and visualized under epi-fluorescent microscope (Leica).

Incorporation of γH2AX
After 6 h treatment, cells were briefly washed with PBS and fixed with 4% PFA at room temperature for 15 min, washed, incubated overnight with anti-γH2AX antibody at 4 °C in a humidified container. Then washed with PBS and incubated with FITC conjugated antibody for 3 h at room temperature, re-washed with PBS, mounted in mounting medium (PBS: Glycerol=1:9) containing propyl gallate and visualized under epi-fluorescent microscope (Leica).

Column chromatography
Chloroform fraction was concentrated and mixed with silica gel (60-120) and packed in a column with silica gel (60-120). Then the column was eluted with hexane, ethyl acetate and methanol with increasing polarity. Fractions were collected, checked with TLC and pooled if necessary. Fractions were checked for their cytotoxicity by MTT assay. Positive samples were subjected to GC-MS study.

GC-MS
Samples were TMS derivatized with BSTFA (Sigma) following manufacturer's protocol and run in HP5-MS capillary column (30m X 0.25 mm X 0.25 µm) and run through a temperature gradient of 70-260 °C with a ramping rate of 5 °C per min. Fragmentation pattern of the peaks was matched with NIST library.

RESULTS
Cellular and nuclear morphology
Drastic changes in both cellular and nuclear morphology were observed in all the treated cell lines. Morphology of apoptotic features, such as cell shrinkage associated with extensive cytoplasmic blebbing was observed. Along with that, nuclear condensation and fragmentation were also visible.

Apoptotic assay (AnnexinV FITC/PI assay)
For 24 h treatment, most of the treated cells were in late apoptotic phase (fig. 2). In HeLa cells, 22.86% cells were found to be apoptotic, whereas in SiHa and C33A cells, higher percentages of cells (34.27% and 57.9% respectively) were apoptotic.
Fig. 1: Representative phase and fluorescence pictures of control and treated sets in HeLa, SiHa and C33A cells. Arrows show apoptotic features. Scale represents 50 µm.

Fig. 2: Representative dot plots along with Bar graph showing cell distribution in different sets of HeLa, SiHa and C33A cells after AnnexinV-FITC/PI staining; columns represent cell populations while bars represent standard deviations. * denotes significant difference between control and treated sets (P<0.05) [One way ANOVA+Dunnet’s post hoc test].

Fig. 3: Representative histograms along with Bar graph showing cell distribution in different phases of cell cycle in different sets of HeLa, SiHa and C33A cells after ethidium bromide staining; columns represent cell populations while bars represent standard deviations. * denotes significant difference between control and treated sets (P<0.05) [One way ANOVA+Dunnet’s post hoc test].
Cell cycle shift

Two types of responses were observed in the cell cycle pattern of treated sets. With SNC treatment, cell cycle arrest at G1/S was observed in HeLa and C33A, whereas in SiHa, SubG0 population was found to be increased indicating greater cell death.

In HeLa cells, 4.28% increase in G0/G1 population was observed along with 4.52% decrease in S phase population, with more or less no change in G2/M and subG0 populations. In SNC treated SiHa cells, 9.23% decrease in G0G1 and 5.25% increase in sub G0 population was observed whereas, S and G2/M populations were increased slightly. In C33A cells, only 0.21% increase in sub G0 population was observed, along with 2.13% increase in G0/G1 population, 4.45% decrease in S and 2.79% increase in G2/M phase population.

Gene expression study

In all the cell lines, the treated sets had showed elevated levels of expression of pro-apoptotic genes and reduced level of anti-apoptotic genes. Expression of viral HPV 16/18 E6 gene was found to be decreased in SiHa and HeLa cells along with the decrease in expression of Endothelin-1 (ET-1).

In HeLa cells, Bcl-2 expression was decreased by 0.86 fold, while BAX expression was elevated, 0.06 fold in treated set. p53 expression was enhanced, 0.36fold while p21 expression was increased slightly (0.008fold). ET-1 expression was reduced moderately (0.11fold) and significantly in HPV E6 (0.65fold).

In SiHa cells, Bcl-2 expression was reduced by 0.43fold, but BAX, p53 and p21 expression were elevated (0.05, 0.33 and 0.12 fold respectively). ET-1 expression was reduced by 0.41fold, while E6 expression was slightly reduced, (0.12fold).

In C33A cells, surprisingly Bcl-2 expression was found to be increased slightly (0.02fold), p53 expression was increased 0.03fold, p21 was increased, 0.68fold . ET-1 expression was not detected as expected in this cell line, which didn’t have HPV viral load.

Protein expression study

In all the cell lines, the treated sets had showed elevated levels of effecter Caspase3, pro-apoptotic BAX, p53 and reduced levels of anti-apoptotic Bcl-2 proteins.

In HeLa cells, cleaved Caspase3 was significantly increased (8.87 fold). Bcl-2 was reduced, (0.54 fold). While BAX expression was elevated, 0.17 fold. p 53 expression was elevated 0.41fold intreated set.

For SiHa cells, cleaved Caspase3 expression was elevated 0.26 fold, Bcl-2 expression was decreased by 0.08foldwhile BAX and p53 expression was increased heavily, (0.28 fold and 0.83 fold) respectively, in treated set.

In C33A cells, cleaved Caspase 3 expression was increased 1.9 fold, Bcl-2 expression decreased by 0.16 fold, BAX expression increased heavily (0.61fold) and p53 expression remained same (increased by 0.01 fold) in treated cells.
Fig. 5: Representative western blots of proteins in control and treated sets in HeLa, SiHa and C33A cells [1: Control set, 2: SNC treated] along with densitometric analysis, columns represent the relative densities while bars represent standard deviations. * denotes significant difference between control and treated sets (P<0.05) [One way ANOVA+Dunnet’s post hoc test]

ROS generation

In all the treated cells, green fluorescence of DCF (formed by the oxidation of DCFDA with ROS) was detected. The effect was most pronounced in HeLa cells.

MMP assay

Loss of MMP was clearly visible. Only monomeric form of JC1 was visualized in treated sets of all cell lines.

Fig. 6: Representative phase and fluorescence pictures of control and treated HeLa, SiHa and C33A cells stained with DCFDA. Scale represents 50 µm

Fig. 7: Representative phase and fluorescence pictures of control and treated HeLa, SiHa and C33A cells stained with JC1. Scale represents 50 µm
Incorporation of γH2AX

For all three cell lines, treated sets showed the presence of γ-H2AX (detected by anti-γ-H2AX antibody) which is a marker of DNA damage. At 6 hours’ time point this incorporation of phospho variant of H2AX clearly indicated the presence of either single or double stranded DNA damage in the treated sets.

Column chromatography and GC-MS

Several subfractions were isolated from the chloroform fraction by column chromatography. Out of which, subfractions (SF) 17-18, 19-20, and 21 were found to be cytotoxic by MTT assay. Common constituents of the SFs were Stearic acid TMS ester (major constituent), Phenol 2, 4-Bis (1, 1-dimethyl-ethyl), Palmitic acid TMS ester, Butanedioic acid TMS ester.

SF17-18 had 2.42% Butanedioic acid TMS ester, 2.48% Phenol 2, 4-Bis (1, 1-dimethyl-ethyl), 16.10% Palmitic acid TMS ester and 31.54% Stearic acid TMS ester. SF19-20 had 2.48% Butanedioic acid TMS ester, 4.21% Phenol 2, 4-Bis (1, 1-dimethyl-ethyl), 23.12% Palmitic acid TMS ester and 31.48% Stearic acid TMS ester. SF21 had 5.96% Butanedioic acid TMS ester, 1.72% Phenol 2, 4-Bis (1, 1-dimethyl-ethyl), 20.86% Palmitic acid TMS ester and 28.25% Stearic acid TMS ester.

DISCUSSION

Earlier workers reported anti-proliferative activity of S. nigrum phytochemicals on cervical cancer cells. Methanolic extract of S. nigrum fruit showed cytotoxicity on HeLa cells with IC50 dose of 84.78 µg/ml [16] which is much higher than our observed IC50 dose of SNC (250±1.01µg/ml) for HeLa cells. For SiHa and C33A cells that was even less (87.5±0.82µg/ml and 100±0.03µg/ml). So chloroform fraction of methanolic extract of whole plant of S. nigrum worked better than the methanolic extract of S. nigrum fruits. GC-MS analysis of chloroform fraction of plant extracts (PCFs) were found to be enriched with fatty acids. Both short chained (C4-Butanedioic acid, C-7-Benzoiic acid) and long chained (C18-Steaiic acid, Olic acid, Linoleic acid) fatty acids were detected in the fractions. These natural fatty acids are useful for membrane integrity and played an important role in cell signaling in plants, whereas in animal cells these compounds exhibit an array of therapeutic activities. Along with anti-inflammatory and antioxidant activities, a number of unsaturated fatty acids are reported to have anticancer activities when tested on human cancer cell lines [17-20]. Saturated as well as unsaturated fatty acids were detected in the plant extracts. Phenol 2, 4-Bis (1, 1-dimethyl ethyl) was detected in SNC fraction. This compound was also detected in the bioactive (ethanolic acid) fraction of S. trilobatum inhibiting Erlich’s Ascites Carcinoma growth [21]. Stearic acid, a C18 long chain fatty acid was also present in both the fractions reported to have inhibitory activity against cervical cancer line HOG-1 [22]. Palmitic acid present in SNC, showed selective cytotoxicity to MOLT-4 (Human leukemic) cell line, probably by inhibiting DNA topoisomerase-1 [23].

With this fatty acid rich fraction, treated cells showed clear signs of late events of apoptosis. Morphological features, such as, membrane blebbing, shrinkage of cell size along with nuclear condensation and
fragmentation indicated apoptosis induction. Another late event of apoptosis was studied by AnnexinV-FITC/PI double staining, as AnnexinV protein binds to phosphatidylserine which externalizes, as a result of membrane flipping, after apoptosis induction. In HeLa and C33A cells, the increase in G0/G1 population and decrease in S phase population indicated G1/S cell cycle arrest. Crude polysaccharides isolated from S. nigrum showed significant growth inhibition on cervical cancer (U14) of tumor bearing mice showing increased expression of BAX and decreased expression of Bcl-2 [24]. It was reported that Endothelin-1 (ET-1) is responsible for neoplastic growth in HPV associated carcinoma cells [25]. At the transcriptional level, expression of ET-1 was found to decrease significantly in the treated HeLa cells and slightly in the SiHa cells, which may have help in attenuating the proliferation of these cells. SNC treatment also elevated the expression of BAX and down regulated Bcl-2 in all the cell lines. Along with that, expression of p53 was elevated in all the cells. Up regulation of p53 may occur in two ways. In one pathway, SNC mediated ROS production within the cells may results in the subsequent DNA damage which might leads to up regulation of p53. Presence of phospho loci in the treated cells also vindicated this. In the second pathway, down regulation of HPV 16/18 E6 by SNC may also help in stabilization of functional p53. p53 acted as the master regulator in this case. In one hand it increases the expression of BAX and down regulate Bcl-2, on the other hand up regulate p21 and cells were arrested at G1/S check point. With the BAX activation, subsequent loss of mitochondrial membrane potential and caspase activation took place. A probable mode of action of SNC on the cervical cancer cells is presented (fig. 9).

CONCLUSION
From the study it can be concluded that SNC is capable of inducing apoptosis in cervical cancer cells. Induction of ROS mediated DNA damage by this chloroform fraction might have induced cell death. Presence of damaged DNA was detected in the treated cells which might be the cause of p53 up regulation along with stabilization of p53 due to HPV E6 down regulation. With down regulation of anti-apoptotic Bcl-2 and up regulation of BAX, caspase dependent apoptosis was induced following intrinsic mitochondrial pathway. The SNC fraction was rich in different fatty acids, phenolics, alkaloids which might be responsible for the apoptosis induction singly or in a synergistic way.

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AUTHOR CONTRIBUTION
SP: Designed experiments, performed experiments, wrote manuscript.
RK: Hypothesized the study, designed experiments, supervised experiments, revised manuscript.

CONFLICT OF INTERESTS
Declared none

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