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Full Length Research Paper

Crude extract of *Nigella sativa* inhibits proliferation and induces apoptosis in human cervical carcinoma HeLa cells

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Cervical cancer is a major cause of morbidity in women worldwide and chemotherapy for this cancer seems unsatisfactory, which demands exploring new therapeutic options. The seeds and oil from *Nigella sativa* have been reputed to have many curative properties in traditional medicine. Utilizing different techniques (4',6-diamidino-2-phenylindole (DAPI) staining, deoxyribonucleic acid (DNA) laddering, Comet assay and reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analyses), this study was carried out to evaluate the impact of *N. sativa* on the growth of human cervical carcinoma HeLa cells. The ethanol extract from *N. sativa* (EENS) significantly inhibited proliferation and colony formation and induced apoptosis in HeLa cells. The apoptotic induction was associated with the release of mitochondrial cytochrome *c*, increase of Bax/Bcl-2 ratio, activation of caspases-3, -9 and -8 and cleavage of poly (ADP-ribose) polymerase (PARP). EENS decreased expression of oncoproteins such as c-Myc, human telomerase reverse transcriptase (hTERT), cyclin D1 and cyclin-dependent kinase-4 (CDK-4), but increased expression of tumor-suppressor proteins including p53 and p21. These findings demonstrate that EENS inhibits proliferation and induces apoptosis in HeLa cells and suggest this extract may be a promising agent for the prevention/treatment of human cervical cancer.

Key words: *Nigella sativa*, cervical cancers, apoptosis, caspases, cell cycle.

INTRODUCTION

Cervical cancer is the second leading cause of cancer deaths in women worldwide and is the most prevalent female malignancy in many developing countries (Parkin et al., 2005). Recent advances have led to a better understanding of the pathogenesis of cervical cancer.

The human papilloma virus (HPV) has been consistently identified as the major causal factor of this disease (Soliman et al., 2004). In addition, other specific genetic abnormalities may also play an important role in carcinogenesis and the aggressiveness of cervical tumors. The inactivation of tumor-suppressor proteins, Rb and p53, together with improper activities of the *c-myc* oncogene, the *ras* gene family (K-ras, H-ras and N-ras), the cyclins, cyclin-dependent kinases (CDKs), the CDK-inhibitory proteins (CDKIs), p16, p21 and p27, may also have a role in the pathogenesis of cervical cancer. Each of these genes has been described to have deregulated expression in cervical cancer and several have been associated with poor prognosis (Soliman et al., 2004). Therefore, better and more effective chemotherapeutics are apparently needed for cervical carcinogenesis. Currently, natural products, including plants (vegetables, herbs and spices) used in folk and traditional medicine, have been

Abbreviations: EENS, Ethanol extract from *Nigella sativa*; **AENS,** aqueous extract from *Nigella sativa*; **cyt *c*,** cytochrome *c*; **PARP,** poly(ADP-ribose) polymerase; **RT-PCR,** reverse transcription-polymerase chain reaction; **CDK,** cyclin-dependent kinase; **hTERT,** human telomerase reverse transcriptase; **TQ,** thymoquinone; **HR-HPV,** high-risk human papilloma viruses; **PCR,** polymerase chain reaction.

found to be a potential source of novel anticancer drugs over the decades and have much contributed to cancer chemotherapy (Gupta et al., 2010; Aggarwal et al., 2009). Among natural products, the seeds and oil from *N. sativa* have attracted the interest of medical scientists. *N. sativa* is an annual herbaceous plant with black seeds, commonly known as black seeds, black cumin, black caraway seed, and *Habbatul* barakat, belongs to Ranunculaceae family and grows in countries bordering the Mediterranean Sea, Pakistan and India (Ali and Blunden, 2003).

Its beneficial effects are related to their antioxidant, antidiabetic, antihistaminic, antiepileptogenic, anti-infective, antitumor and antiperoxidative properties (Ali and Blunden, 2003; Kaleem et al., 2006; Kanter et al., 2006; Mabrouk et al., 2002). Furthermore, numerous studies have shown that seeds and oil from this plant are characterized by a very low degree of toxicity (Ali and Blunden, 2003). The major biologically active compound of *N. sativa* is thymoquinone (TO), which is notorious for its antitumor and antiinflammatory effects (Gali-Muhtasib et al., 2005; El Gazzar et al., 2006). Several researches have shown that the growth inhibitory effects of TQ are specific to cancer cells and that the beneficial effects of the use of the seeds and TQ might be related to their cytoprotective and antioxidant actions, and to their effect on inflammation mediators (Gali-Muhtasib et al., 2005).

Based on the aforementioned reported scientific data and considering the fact that in some cases herbal extracts are showing more potency than the purified components (Seeram et al., 2004, 2005), the present study was undertaken to investigate the anti-proliferative potentiality of the crude extract of *N. sativa* on the human cervical cancer cell line, HeLa, and further to elucidate the molecular mechanism underlying this action. The data obtained demonstrate that the crude extract from *N. sativa* strongly suppressed growth of HeLa cells by induction of apoptotic cell death.

MATERIALS AND METHODS

Preparation of herbal extracts

Fresh black seeds (*N. sativa*) were purchased from the local market of Alexandria, Egypt. They were properly washed and used to prepare ethanol and aqueous extracts. Ethanol extract was obtained from the dried seeds that were mechanically powdered and extracted by cold percolation with 70% ethanol for 24 h. The extract was recovered and 70% ethanol was further added to the ground seeds and the extraction continued. The process was repeated three times. The three extracts were pooled together and the combined extract was then concentrated using a rotary evaporator in water bath set at 70°C.

For final use, the ethanolic residue was dissolved in dimethyl sulfoxide (DMSO) and further diluted in tissue culture media to give the final concentrations (25, 50, 75 and 100 µg/ml) and termed as ethanol extract of *N. sativa* (EENS). To prepare aqueous extract the same procedure was carried out with one modification, extraction processes were carried out using distilled water, rather than 70% alcohol. Before use, the aqueous residue was further diluted in tissue culture media to give the final concentrations (25, 50, 75 and 100 µg/ml) and termed as aqueous extract of *N. sativa* (AENS).

Cell culture

The human cervical cancer cell lines, HeLa, HEp-2 and the non-malignant human foreskin fibroblasts HF-5 were obtained from King Fahd Center for Medical Research, King Abdulaziz University, Kingdom of Saudi Arabia. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin antibiotics in tissue culture flasks under a humidifying atmosphere containing 5% CO_2 and 95% air at 37°C. The cells were sub-cultured at 3 to 4 days interval.

Cell viability and colony formation assays

The dose-dependent effects of the EENS on the viability of the HeLa, HEp-2 and HF-5 cells were determined by trypan blue dye exclusion assay. Early log phase cells were trypsinized and re-grown in 24-well cell culture plates at the concentration of 50×10^3 cells/ml in 1 ml of complete culture medium. 24 h post-treatment, the medium was removed and replaced with fresh medium containing increasing concentrations of the EENS (25, 50, 75 and 100 µg/ml) or AENS (25, 50, 75 and 100 µg/ml) for the indicated time intervals. At the end of these treatment interval, both floating and adherent cells were collected (taking care that none of the floating cells were lost during washes), and pelleted by centrifugation at 700 g for 5 min. The cells were re-suspended in 50 µl phosphate-buffered saline (PBS), mixed with 50 µl of 0.4% trypan blue solution and counted using a hemocytometer under an inverted microscope. The effect of EENS/AENS on growth inhibition was assessed as percent cell viability, where control (DMSO) treated cells were taken as 100% viable. For these studies, all experiments were repeated three or more times in triplicate.

Colony forming assays were performed as described earlier (Baeshen et al., 2012). Briefly, log growth phase HeLa cells were trypsinized and plated onto 6-well plates at initial cell concentrations of 1×10^3 cells/ml. 24 h later, the medium was removed and fresh medium was added with increasing concentrations (25, 50, 75 and 100 µg/ml) of the EENS for ten days to allow cells to form colonies. The resulting colonies were stained with 0.4% trypan blue solution for 10 min and counted. Colonies with >50 cells were counted under a dissection microscope. Colony formation was calculated as a percentage of untreated control cultures. Each treatment was repeated in at least duplicate.

Apoptotic assay

The nuclear morphological changes associated with apoptosis was analyzed using 4',6-diamidino-2-phenylindole (DAPI) staining as described previously (Baeshen et al., 2012). Briefly, HeLa cells (3×10^4) were placed on coverslips, allowed to attach overnight, and exposed to vehicle (DMSO) or increasing concentrations (25, 50, 75 and 100 µg/ml) of EENS for 24 h. The cells were washed with PBS and fixed with 3.7% paraformaldehyde (Sigma-Aldrich, USA) in PBS for 10 min at room temperature. Fixed cells were washed with PBS, and stained with 4,6- diamidino-2-phenylindole (DAPI; Sigma-Aldrich) solution for 10 min at room temperature. The cells were washed twice more with PBS and analyzed; the cells with condensed and fragmented deoxyribonucleic acid (DNA) (apoptotic cells) were scored under a fluorescence microscope (Carl Zeiss, Germany) at x40 objective lens magnification.

DNA fragmentation assay

DNA gel electrophoresis was used to determine the presence of internucleosomal DNA cleavage as described previously (El-Kady et al., 2011). Briefly, HeLa cells ($3x10^6$ cells/100 mm dish) treated with increasing concentrations (25, 50, 75 and 100 µg/ml) of EENS for 24 h were collected, washed in PBS and purified using a DNA purification kit, DNeasy Blood and Tissue Kit (QIAGEN) according to the manufacturer's recommendations. The DNA was resolved by electrophoresis on 1% agarose gel. After electrophoresis at 80 to 100 V, the gel was stained with 0.5 µg/ml ethidium bromide, and DNA was visualized by a UV trans-illuminator (BIO-RAD).

Single-cell gel electrophoresis (Comet assay)

EENS-induced DNA damage was determined using the comet assay. Cells were treated with 25 and 50 µg/ml EENS for 24 h in complete medium, and the comet assay was done as described earlier (Singh et al., 1998). Briefly, after treatment with EENS, the cells were harvested and resuspended in ice-cold PBS. Approximately, 1×10^4 cells in a volume of 75 µl of 0.5% (w/v) low-melting-point agarose were pipetted onto a frosted glass slide coated with a thin layer of 1.0% (w/v) agarose, covered with a coverslip, and allowed to set on ice for 10 min. Following removal of the coverslip, the slides were immersed in ice cold lysis buffer containing 2.5 mol/L NaCl, 10 mmol/L Tris, 100 mmol/L Na2-EDTA, and 1% (w/v) N-lauroylsarcosine, adjusted to pH 10.0, and 1.0% Triton X-100 was added immediately before use.

After 2 h at 4°C, the slides were placed into a horizontal electrophoresis tank filled with buffer [0.3 mol/L NaOH, 1 mmol/L EDTA (pH 13)] and subjected to electrophoresis for 30 min at 300 mA. Slides were transferred to neutralization buffer (0.4 mol/L Tris-HCl) for 3 to 5 min washes and stained with ethidium bromide for 5 min. After a final wash in double-distilled water, the gels were covered with glass coverslips. To prevent additional DNA damage from visible light, all the steps described above were conducted under a dimmed light. Slides were viewed and nuclei images were visualized and captured at 400x magnifications with an Axioplan 2 fluorescence microscope (Zeiss) equipped with a CCD camera (Optronics). Hundreds of cells were scored to calculate the overall percentage of comet tail-positive cells.

RNA extraction and reverse transcriptase-PCR

The HeLa cells were plated as aforementioned. 24 h later, the medium was removed and replaced with fresh medium containing indicated concentrations of EENS for 24 h. Both floating and adherent cells were collected (taking care that none of the floating cells were lost during washes), and pelleted by centrifugation at 700 g for 5 min. total ribonucleic acid (RNA) was extracted, reverse transcribed and amplified by polymerase chain reaction (PCR) using QIAamp® RNA Blood Mini Kits (QIAGEN) following manufacturer's instructions. Expression of tested genes was examined by PCR method using gene-specific primers. The PCR was run as reported previously (El-Kady et al., 2011). The PCR products were analyzed on 1.0% agarose gel and stained with 0.5 µg/ml ethidium bromide.

Bands on the gel were scanned as digitalized images, and the areas under the peaks will be calculated by densitometric analysis using a computerized digital imaging system. The areas are reported in arbitrary units. The relative expression level was calculated by comparing the expression level of the sample with that of the internal standard (HPRT1). At least a 2-fold decrease in the relative expression level was considered to be reduced, which was verified by repeat real time (RT)-PCR assays. Primer sequences of the Bcl-2 and HPRT1 were described earlier (El-Kady et al., 2011), while the primer sequeces of the Bax were described in a current study (Baeshen et al., 2012).

Preparation of cytosolic and mitochondrial lysates

After EENS treatment as described above, cells were harvested by scraping, washed twice in ice-cold PBS, and resuspended in 500 µl of cytosol extraction buffer (20 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1mM EGTA) containing protease inhibitors (1 mM PMSF, 1% aprotinin, 1 mM leupeptin and 1 µg/ml pepstatin A). After 30 min of incubation on ice, cells were homogenized in the same buffer using a dounce homogenizer (30 strokes). Then, Cell homogenates were spun at 1,000 × g to remove unbroken cells, nuclei, and heavy membranes. The supernatant was collected and spun again at 14,000 × g for 30 min to collect the mitochondria-rich (pellet) and cytosolic (supernatant) fractions. The supernatant was used as cytosolic lysate, while the pellet was suspended in lysis buffer (137 mM NaCl, 20 mM Tris, pH 7.9, 10 mM NaF, 5 mM EDTA, 1 mM EGTA, 10% (v/v) glycerol, 1% Triton X-100) and protease inhibitor cocktail, before being spun to obtain the mitochondrial lysate. Protein concentration was determined by BCA assay kit (Pierce), before being subjected to Western blot analysis as described below.

Western blot analysis

The total cell lysates and Western blot were carried out as reported by El-Kady et al. (2011). Briefly, after treatment, the cells were collected, washed with cold PBS and lysed. Then, aliquots of the total cell lysates containing the same quantity of proteins were boiled for 5 min in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 5% β-mercaptoethanol, electrophoresed on 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. After transfer, the membranes were incubated with primary antibody against tested proteins (aprox.1:2000), followed by incubation with a secondary horseradish peroxidase–conjugated antibody (aprox.1:3000). The first and second antibodies were purchased from Spring Bioscience. The signals were visualized with enhanced chemiluminescent (ECL) substrates (Pierce, Rockford, IL, USA) on X-ray film (ISC BioExpress, Kaysville, UT, USA). Equal loading of proteins was confirmed by stripping the blots and reprobing with β-actin (Sigma).

Statistical analysis

All experiments were performed in triplicates and repeated at least five times and the data were presented as mean ± SD. Statistical analyses were evaluated by Student's *t*-test. Probability values $P < 0.05$ were considered statistically significant.

RESULTS

EENS and AENS inhibit proliferation of human cervical carcinoma cells

Initially, the effect of *N. sativa* extract on the cell survival was determined using a well-characterized human cervical carcinoma cell line, HeLa, as a model. The cells were incubated with increasing concentrations of either EENS or AENS for 24, 48 and 72 h, before being harvested and assayed for cell viability by trypan blue

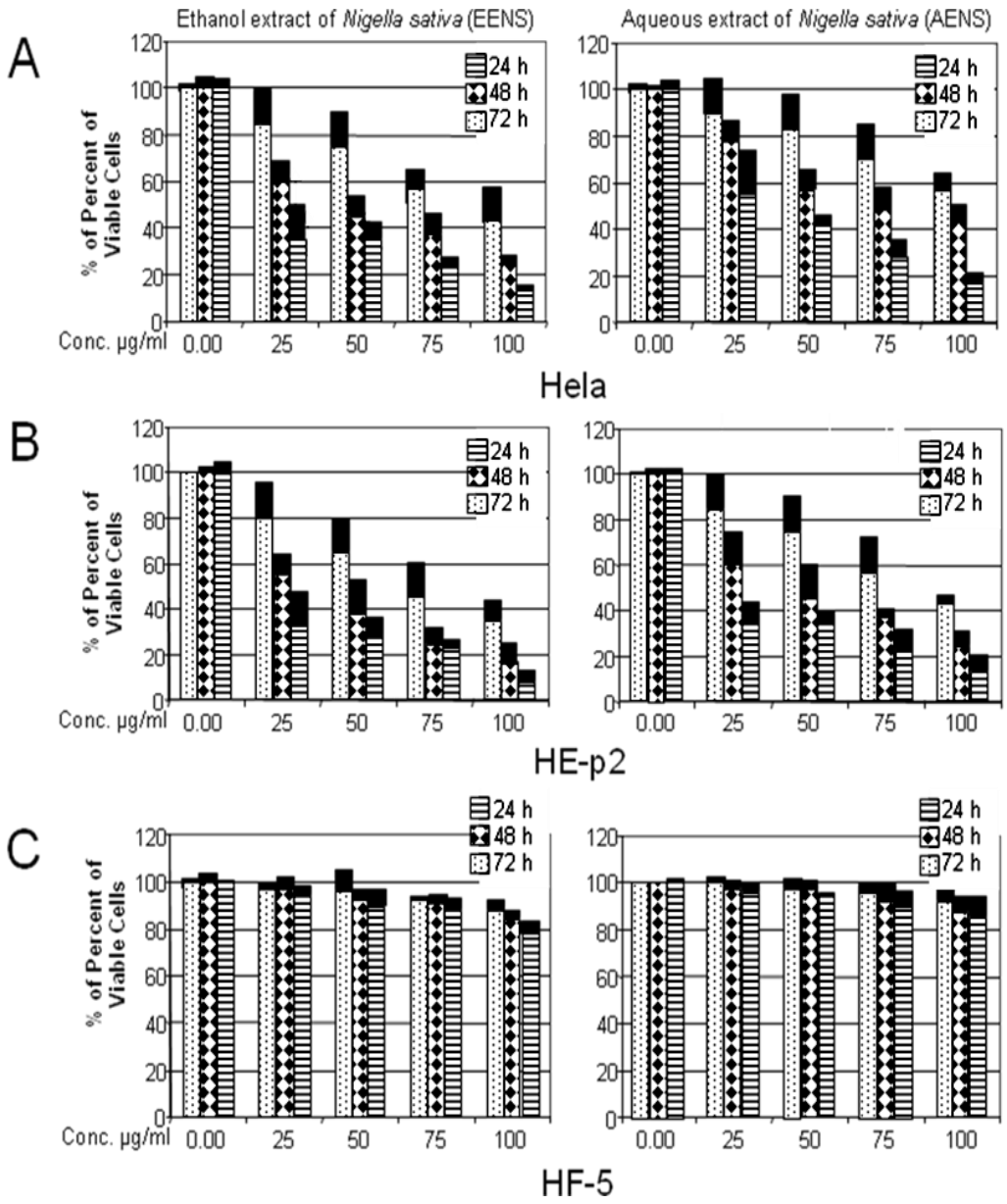

Figure 1. Dose- and time-dependent anti-proliferative activity of EENS and AENS on cellular growth of HeLa (A) HEp-2 (B) and HF-5 (C) cells. Each cell line was incubated with indicated concentrations of EENS/AENS for indicated time intervals. The cell viability was measured by trypan blue dye exclusion assay and expressed in terms of percent of control cells not treated with EENS/AENS. Results are presented as means ± SD of independent experiments performed in triplicate.

dye exclusion assay. The results are summarized in Figure 1. Generally, both extracts consistently exhibited a dose- and time-dependent anti-proliferative effect on the cell viability. In addition, the anti-proliferative potentiality of the EENS was a stronger than that of AENS, since in all experiments the IC_{50} values (the concentration of test compound that inhibits 50% of the cell growth) of the EENS were lower than those of AENS. For effect of EENS on HeLa cells, the IC_{50} values for 25, 50, 75 and 100 µg/ml were observed after 72, 48, 24 and 24 h of treatment, respectively.

Meanwhile in case of AENS, the IC_{50} values for 25, 50, 75 and 100 µg/ml were noticed after 72, 72, 48 and 48 h of treatment, respectively. To find out whether the anti-proliferative potentiality of EENS is restricted to HeLa cells, the above experiments were repeated using other human cervical carcinoma cell line, HEp2, as a model. The findings displayed in Figure 1B demonstrate both EENS and AENS recapitulated their growth-inhibitory potentialities in the presence of HEp-2 cells, in a dose-

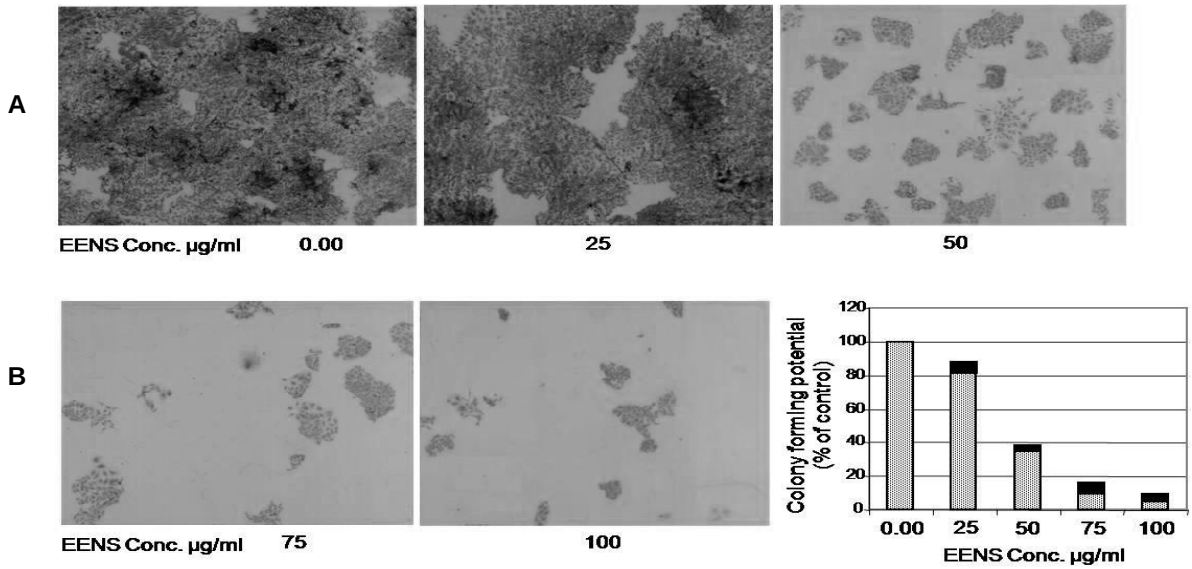

Figure 2. Effects of EENS on clonogenicity of HeLa cells. Cells were seeded onto a 6-well plate at 1000/well and treated with indicated concentrations of the EENS for 10 days. Colonies were counted under a dissection microscope. A survival of 100% corresponds to the number of colonies obtained with cells not treated with EENS. The experiment was repeated three times, and the colony forming potential of the cells at each dose of EENS is expressed in terms of percent of control and is reported as the mean ± SD.

and time-dependent manner. Next, a question was raised, whether EENS- and AENS-dependent growth inhibition was selective toward cancer cells, which is a highly desirable feature of potential cancer preventive and therapeutic agents.

This question was addressed by determining the effect of EENS and AENS treatments on viability of non-malignant human foreskin fibroblasts, HF-5 cells. As can be seen in Figure 1C, the HF-5 cell line was significantly more resistant to growth inhibition by the EENS or AENS. The strongest effect was seen at the highest dose of the EENS, where survival of HF-5 cells was inhibited by only aprox.15% on a 72 h exposure. Collectively, these results indicated that the EENS and AENS markedly inhibited the human cervical carcinoma cell growth, and that the growth-suppressive potentiality of the EENS is the stronger. The cytotoxic potentiality of the EENS or AENS is limited in the context of the normal human fibroblast cell line. Since the EENS had a stronger anti-proliferative potentiality than AENS, the EENS was selected for further mechanistic studies.

EENS suppresses colony formation in HeLa cells

Next, the anti-proliferative and cytotoxic effects of the EENS on HeLa cells were further determined and verified by using anchorage-dependent colony formation assay (also referred to as clonogenicity). As shown in Figure 2A, the EENS suppressed colony formation in a dose-dependent manner. Figure 2B demonstrates that 25, 50, 75 and 100 µg/ml of EENS reduced colony formation in HeLa cells down to 20, 65, 85 and 90%, respectively, of its original value.

EENS induces apoptosis in HeLa cells

A growing body of evidences support that the anti-proliferative effect of many naturally occurring cancer chemopreventive agents is tightly linked to their ability to induce apoptosis (Khan et al., 2007). To determine whether the proliferation-inhibitory effect of EENS is related to the induction of apoptosis, cells were treated with escalated doses of EENS for 24 h and incidents of apoptotic cell death was assessed by several approaches. First, alterations in HeLa cell morphology were observed under the inverted light microscope. As shown in Figure 3A, the untreated HeLa cells grew well forming confluent monolayer. On the other hand, the EENS-treated cells exhibited marked morphological changes including cell shrinkage, cellular detachment and lost of the originally confluent monolayer.

To further examine the morphological changes in responding to EENS treatment, both control and EENS treated cells were stained with the fluorescent dye DAPI and visualized by a fluorescent microscope. As depicted in Figure 3B, the control cells appeared normal with round and homogenous nuclei. By contrast, the EENS-treated cells exhibited the typical characteristics of apoptotic death, such as nuclear condensation and fragmentation. Scoring numbers of apoptotic cells with

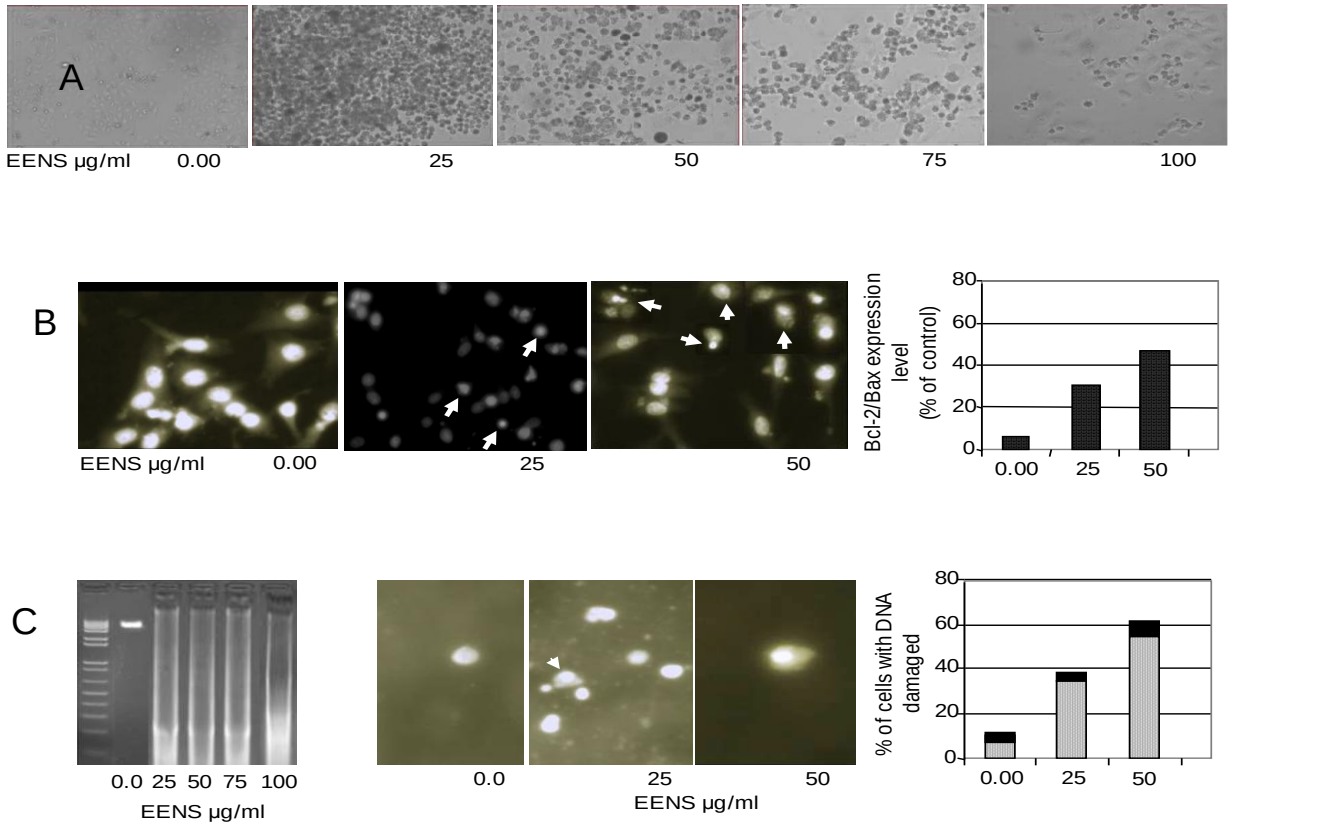

Figure 3. EENS induces apoptotic cell death in HeLa cells. HeLa cells were treated with indicated concentrations of EENS for 24 h and assayed for existence of apoptotic cell death. Depicted results are representative for independent experiments with almost identical observations. (A) Microphotographs showing EENS affected morphology of HeLa cells. The photographs were taken directly from culture plates using a phase microscope. (B) DAPI staining showing EENS induced chromatin condensation and nuclei fragmentation (arrow heads) in HeLa cells. The histogram demonstrates percentages of cells with condensed and fragmented nuclei. (C) Agarose gel showing EENS induced DNA fragmentation in HeLa cells. (D) Comet assay showing formation of DNA tail in EENS-treated Hela cells. The histogram shows percentage of cells with damaged DNA.

condensed and fragmented DNA from control and EENS-treated cultures revealed that the percentage of apoptotic cells was increased by aprox. 30 and 47-fold upon a 25 and 50 µg/ml EENS treatment, respectively.

DNA fragmentation is a hallmark of apoptosis and the detection of DNA ladder in agarose gel electrophoresis is commonly used as a biochemical marker for the measurement of apoptosis (Nagata et al., 2000). Consistent with this, exposure of HeLa cells to the EENS resulted in marked DNA fragmentation (Figure 3C). Next, DNA fragmentation by EENS was further confirmed using comet assay (or single-cell electrophoresis), a specific assay for detection of apoptosis ((Tice et al., 2000). As shown in Figure 3D, treatment of HeLa cells with 25 and 50 µg/ml of EENS for 24 h resulted in significant DNA damage compared with cells that were not treated with EENS. The percentage of DNA released to the comet tail was used as the most sensitive parameter to quantify the DNA damage (Figure 3D, histogram). Taken together, these independent methods of measuring apoptosis provided similar results, suggesting that the anti-proliferative potentiality of EENS is linked to its ability to induce apoptosis in HeLa cells.

EENS treatment induces release of cyt *c*

The mitochondrial apoptotic pathway has been described as an important signaling of apoptotic cell death for mammalian cells (Wong, 2011). Release of cyt *c* from the mitochondria into the cytosol is a key event in apoptosis (Wong, 2011). To examine whether the EENS-induced apoptosis is mediated by the release of mitochondrial cyt *c*, a cytosolic fraction was prepared from EENS treated cells under conditions that maintain the integrity of mitochondria and accumulation of cyt c in the cytosol was monitored by Western analysis. As seen in Figure 4A, the amount of cyt *c* released from mitochondria into the

cytosol was markedly increased, with a concomitant decrease in the level of cyt *c* in the mitochondrial compartment, in a dose-dependent manner. These findings suggest that EENS-induced apoptosis in HeLa cells is mediated, at least in part, by the mitochondrial signaling pathway.

EENS treatment alters Bax/Bcl-2 ratio in favor of apoptosis

The release of cyt *c* from mitochondria is tightly regulated by Bcl-2 family proteins, where the anti-apoptotic members (such as Bcl-2) prevent the release of cyto *c* from mitochondria and pro-apoptotic members (such as Bax) increase the release of cyto *c* from mitochondria (Burlacu, 2003). To obtain further evidence confirming role of EENS in release of mitochondrial cyt *c*, the expression levels of Bcl-2 and Bax in EENS-treated HeLa cells have been assessed. As shown in Figure 4B, exposure of HeLa cells to increasing concentrations of EENS for 24 h markedly decreased the protein expression of anti-apoptotic Bcl-2, which was associated with a concomitant increase in the expression of pro-apoptotic Bax protein, in a dose-dependent manner. To substantiate these data, the expression of Bcl-2 and Bax mRNAs was examined. As seen in Figure 4B, the EENS treatment decreased the expression level of Bcl-2 mRNA, but increased that of Bax, resulting in an increase in Bax/Bcl-2 ratio. These findings indicate that the EENS mediating up-regulation of Bax and down-regulation of Bcl-2 might tip the balance toward release of mitochondrial cyt *c*.

EENS treatment mediates activation of casp-9, -8 and -3, and poly (ADP-ribose) polymerase (PARP) cleavage

A key step in apoptotic signaling pathways is activation of caspase-3, which proteolytically cleaves and activates other down-stream caspases and other relevant target molecules in the cytoplasm or nucleus (Van Cruchten and Van Den Broeck, 2002). To address the apoptotic pathway in EENS-treated HeLa cells, the protein levels of the activated/cleaved caspase-9, -8, and -3 were examined by Western analysis following EENS treatment for 24 h. As shown in Figure 4C, the protein levels of the activated caspase-9, -8, and -3 were dose-dependently increased (note decreased expression of the procaspase isoforms). Once activated, caspase-3 proteolytically cleaves the 116 kDa PARP protein into an 85 kDa fragment, which is a biochemical characteristic of apoptosis (Cruchten and Broeck, 2002). As seen in Figure 4C, incubation of cells with EENS resulted in the formation of 85 kDa protein fragments. These results revealed that the activation of caspase-9, -8, and -3 had an important role in EENS-induced apoptosis in HeLa cells.

EESN alters the expression of cell cycle-associated proteins

In addition to induction of apoptosis, chemotherapeutic agents also kill cancer cells by inhibition of cell proliferation (Meerana and Katiyara, 2008). To obtain further evidence confirming proliferation-inhibitory potentiality of EENS, impact of EENS treatment on the expression of key proteins playing essential roles in cell proliferation and deregulated in cervical cancer were examined. To this end, HeLa cells were treated with increasing doses of EENS for 24 h and cell lysates were prepared and expression of levels of c-Myc, the human telomerase reverse transcriptase (hTERT), cyclin D1, cyclin-dependent kinase-4 (CDK-4), p21 and p53 were examined.

The data depicted in Figure 5, explain that EENS down-regulated, in a dose-dependent manner, expression of c-Myc hTERT, cyclin D1 and CDK-4proteins. On the other hand, expression levels of the tumor suppressor proteins, p53 and p21, were up-regulated. All these changes were not due to differences in the amounts of proteins loaded on the gels as the equivalent protein loading was confirmed by probing stripped blots for *β*-actin as shown. Taken together, these findings demonstrate that EENS treatment modulated expression levels of the cell cycle regulating proteins.

DISCUSSION

Cervical cancer is a major cause of morbidity in women worldwide (Parkin et al., 2002), whereas chemotherapy for this type of cancer still seems unsatisfactory (Rahaman et al., 2009). Therefore, it is necessary to develop a new anti-cancer agent for treatment of this cancer. With increasing use of plant-derived cancer, chemotherapeutic agents exploring the antiproliferative effects of phytochemicals has gained increasing momentum for cancer therapy (da Rocha et al., 2001). Among natural products, the seeds and oil of *N. sativa* are gaining attention as therapeutic agents for cancer (Ali and Blunden, 2001).

Several workers have investigated the possible anti-tumour activity of some crude and purified components of *N. sativa in vitro* and *in vivo* (Ali and Blunden, 2001; Gali-Muhtasib et al., 2005; Al-Johar et al., 2008; Banerjee et al., 2010). However, its role in cervical cancer remained to be elucidated. The present study aimed to investigate the ability of the crude extracts of *N. sativa* to suppress proliferation of cervical cancer cell line, HeLa, and to elucidate the molecular mechanisms underlying growth suppression. The study focused on crude extracts since

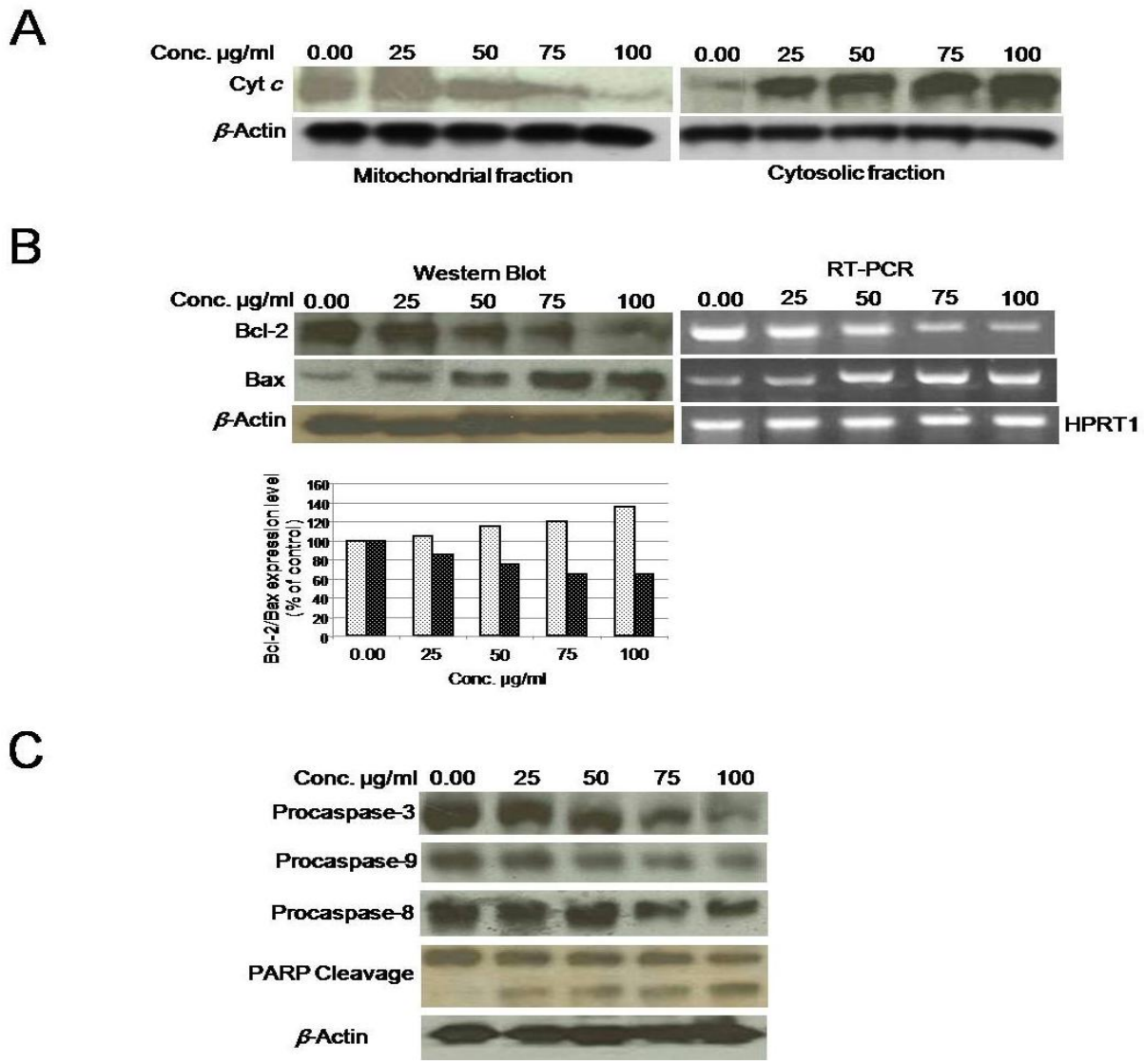

Figure 4. EENS induces mitochondrial cyc c release, increases Bax/Bcl-2 ratio and activates caspases. The HeLa ells were incubated with the indicated concentrations of the EENS for 24 h, then protein and total RNA lysates were prepared and subjected for Western and RT-PCR analyses. (A) EENS treatment induces release of mitochondrial cyt *c* into cytosol. (B) EENS treatment modulates expression of Bax/Bcl-2 ratio in favor of apoptosis. (C) EENS treatment mediates activation of casp-9, -8 and -3, and PARP cleavage. The experiments were repeated several times and typical results from independent experiments are shown.

in some cases herbal extracts exhibited more potency than the purified components (Seeram et al., 2004, 2005).

In this study, the EENS and AENS have been found to suppress growth of human cervical cancer cell lines, HeLa and HEp-2 cells, in both dose- and time-dependent manner. On the other hand, they exerted marginal effect on the non-malignant human fibroblasts HF-5, which suggests that the EENS and AENS may selectively target cervical cancer cells but spare normal cell line. These findings are significant because selectivity toward cancer cells is a highly desirable feature of potential cancer chemopreventive and therapeutic agents. These findings are reminiscent too to previous studies demonstrating a crude methanol extract of the *N. sativa* which exhibited a strong cytotoxic action on Erlich ascites carcinoma, Dalton's ascites lymphoma and sarcoma 180 cells, while exerting minimal cytotoxicity to normal lymphocytes (Salomi et al., 1992).

However, the assays herein cannot exclude the possibility that there are tissue-specific differences between HF-5 fibroblasts and adenocarcinoma HeLa and HEp-2 cells. A further proof for the anti-proliferative activity of EENS was concluded from colony formation assay, which demonstrated statistically significant reduction in the number and size of EENS-treated

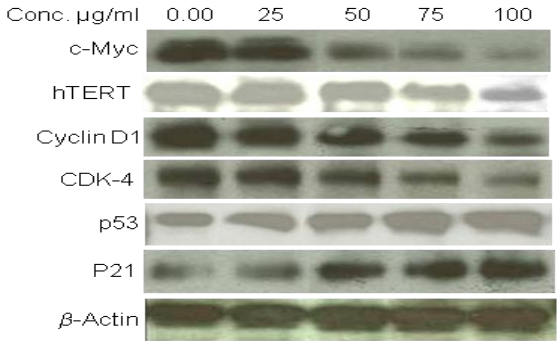

Figure 5. EESN altered expression of cell cycle-associated proteins. The HeLa cells were incubated with the indicated concentrations of the EENS for 24 h. Cell lysates were examined for the displayed proteins by Western blot. β-actin was used as the loading control. A representative blots from three independent experiments are shown.

colonies, compared to the control colonies. Since this assay measures the ability of tumor cells to grow and form foci in a manner unrestricted by growth contact inhibition as is characteristically found in normal, untransformed cells, thus, EENS could be proposed to be a promising candidate for restricting the growth of cervical cancer cells.

It is documented that the antitumor drugs kill the cancer cells by stimulating the apoptotic pathways (Khan et al., 2007). Likewise, the findings in the present study indicated that the proliferation inhibitory activity of EENS was related to the induction of apoptosis. This is because the EENS-treated HeLa cells exhibited typically morphological features of apoptosis, such as a loss of cell viability, cell shrinkage, irregularity in cellular shape, and cellular detachment (Protrka et al., 2010). In addition, DAPI staining assay revealed occurrence of nuclear condensation, DNA fragmentation, and perinuclear apoptotic bodies in EENS-treated HeLa cultures. Furthermore, DNA laddering and comet assay, which are widely used as biochemical markers of apoptosis (Nagata, 2000; Tice et al., 2000), demonstrated fragmentation of DNA isolated from EENS-treated cells. Collectively, these findings demonstrate that the cytotoxic effect observed in response to the EENS treatment is associated with the induction of apoptosis in the HeLa cells.

Most anticancer agents either directly induce DNA damage or indirectly induce secondary stress-responsive signaling pathways to trigger apoptosis by activation of the intrinsic (mitochondrial) apoptotic pathway, and some can simultaneously activate the extrinsic (death receptor) pathway (reviewed in Reed, 2000). Related to this, Shafi et al. (2009) have recently cited that the chloroform fraction of seed extracts of *N. sativa* induced apoptotsis in HeLa cells. The data here support the conclusion of this previous study and underscore that apoptotic signaling was mediated through the mitochondrial pathway, since HeLa cells treated with EENS exhibited marked increase in the cytosolic cyt *c*, which was associated with a concomitant decrease in the mitochondrial cyt *c*.

In addition, Western and RT-PCR analyses demonstrated up-regulation of Bcl-2 expression and down-regulation of Bax expression as well as enhanced cleavage of caspase-9, -8 and -3 and poly (ADP-ribose) polymerase (PARP). Therefore, it is tempting to speculate that EENS induced cyto *c* release and activation of caspase-9 and caspase-3 due to, at least in part, the disruption of a balance between Bax and Bcl-2. Subsequently, active caspase-3 mediated cleavage of PARP processes which account for DNA fragmentation as well as other morphological and biochemical changes during apoptosis (Saraste and Pulkki, 2000).

Since over expression of Bcl-2 has been found to play an important role in cervical carcinogenesis (Protrka et al., 2010), down-regulation of Bcl-2 expression by EENS draws attention to the potentiality of EENS as an ideal chemopreventive or therapeutic agent for cervical cancer. Other seminal finding, EENS treatment mediated activation of caspase-8, which raises a possibility that EENS implements the extrinsic (caspase-8-dependent) pathway to trigger apoptotic machinery. Thereby, EENS might induce apoptotic death of HeLa cells through intrinsic as well as extrinsic pathways.

A growing body of evidence document that natural products exert their anti-proliferative potentialities through modulating expression of cell cycle regulatory proteins (Meerana and Katiyara, 2008). Coherent to this notion, EENS modulated expression of cell cycle regulatory proteins, such as c-Myc, hTERT, cyclin D1, CDK-4, p53 and p21, which may provide a further mechanistic basis for the induction of apoptosis by EENS in HeLa cells. Over expression of c-Myc was frequently found in advanced stages of cervical cancers and was shown to be associated with tumor progression (Golijow et al., 2001; Brenna et al., 2002). More importantly, c-Myc activates expression of hTERT (Wu et al., 1999), thereby increases telomerase activity, which has been demonstrated to be associated with initiation and progression of cervical lesions (Iwasaka et al., 1998; Zheng et al., 1997) and an important marker for cervical cancer (Kailash et al., 2006). Therefore, at least in part, the decreased expression of c-Myc and hTERT (and hence telomerase activity) by EENS could be a candidate mechanism underlying the induction of apoptotic HeLa cell death, through a telomerase inhibitory pathway.

Furthermore, the high-risk human papilloma viruses (HR-HPV) have been identified as an essential prerequisite for the development of cervical cancer (Hausen, 2000) and HeLa cells harbor subtypes of HPV (Androphy et al., 1987; Seedorf et al., 1987). The two HR-HPV oncoproteins, E6 and E7, modulate cellular proteins that regulate the cell cycle (Mantovani and Banks, 2001; Münger et al., 2001). In particular, E6

induces transcription of hTERT (Veldman et al., 2001).

Therefore, down-regulation of hTERT expression by EENS brings to mind that EENS subverts mechanisms underlying activation of telomerase by E6, which leads to inhibition of cervical cancer development.

Deregulated expression of the cyclins and CDKs has been found to be a hallmark in various types of cancer including cervical cancer (Kim and Zhao, 2005). In particular, over-expression of CDK-4 has been proposed to play an important role in cervical carcinogenesis (Vermeulen et al., 2003), whilst cyclin D1 is a rate-limiting factor in progression of cells through the G1 phase of the cell cycle (Kim and Zhao, 2005). The experiments herein explained that EENS treatment drastically down-regulated expression of cyclin D1 and CDK-4. Therefore, these findings hint that EENS mediated negative regulation of these positive regulators of cell cycle progression would impair HeLa cell cycle progression through, at least in part, cyclinD1-CDK-4 complex-inhibition pathway.

A great amount of research data suggests that the inactivation of the tumor suppressor gene p53 is believed to play a major role in the carcinogenesis of the cervical carcinoma (Kim and Zhao, 2005). p53 can trigger apoptotic events implementing different mechanisms. Through transcription dependent pathways, it up-regulates proapoptotic genes, such as Bax, Noxa, Puma and Bid and/or down-regulates antiapoptotic genes, such as Bcl-2. Through transcription-independent pathways, p53 can directly bind to and inhibit the BclxL and Bcl2 proteins, leading to the release of cyt *c* and the initiation of caspase cascade (Mihara et al., 2003). In addition, p53 can induce cycle arrest at G1 phase provoking apoptotic events (El-Deiry et al., 1994). It exerts this activity through increasing expression of p21, which is a universal inhibitor of CDKs, involved in the regulation of cell cycle restriction points and has been shown to be up-regulated in G0/G1 as well as in G2/M arrests (Grana and Reddy, 1995).

However, it has been demonstrated that HeLa cells express low levels of p53, since the HPV18 E6 proteins bind effectively to the p53 protein, leading to p53 degradation (Werness et al., 1990). The results in this study showed that EENS dose-dependently enhanced expression of p53 and p21 suggesting that EENS was able to stabilize expression of p53 even in the presence of E6. Accordingly, enhanced level of p53-effector, p21, may, in turn, trigger a series of events resulting in cell cycle arrest-dependent apoptotic events in HeLa cells. Ongoing research will support this conclusion by cell cycle (flow cytometry) analysis.

Conclusion

In conclusion, EENS significantly inhibited proliferation and colony formation and induced apoptosis in HeLa cells. It exerted its apoptotic potentiality through increasing the Bax: Bcl-2 protein ratio leading to release of cyt *c* into cytosol and activation of downstream caspases (3, 9 and 8). In addition, it modulated expression levels of the cell cycle-related proteins including c-Myc, hTER, cyclin D1, CDK-4, p53 and p21. These findings suggest that EENS could potentially be a new therapeutic option in anticancer treatment for cervical cancer.

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