RESEARCH ARTICLE

Induction of Mitochondrial-Mediated Apoptosis by *Morinda Citrifolia* (Noni) in Human Cervical Cancer Cells

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Abstract

Cervical cancer is the second most common cause of cancer in women and has a high mortality rate. Cisplatin, an antitumor agent, is generally used for its treatment. However, the administration of cisplatin is associated with side effects and intrinsic resistance. *Morinda citrifolia* (Noni), a natural plant product, has been shown to have anti-cancer properties. In this study, we used Noni, cisplatin, and the two in combination to study their cytotoxic and apoptosis-inducing effects in cervical cancer HeLa and SiHa cell lines. We demonstrate here, that Noni/Cisplatin by themselves and their combination were able to induce apoptosis in both these cell lines. Cisplatin showed slightly higher cell killing as compared to Noni and their combination showed additive effects. The observed apoptosis appeared to be mediated particularly through the up-regulation of p53 and pro-apoptotic Bax proteins, as well as down- regulation of the anti-apoptotic Bcl-2, Bcl-X_L proteins and survivin. Augmentation in the activity of caspase-9 and -3 was also observed, suggesting the involvement of the intrinsic mitochondrial pathway of apoptosis for both Noni and Cisplatin in HeLa and SiHa cell lines.

Keywords: *Morinda citrifolia* (Noni) - Cisplatin - HeLa - SiHa - apoptosis

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Introduction

Cervical cancer is a major health problem worldwide and is the most frequent cause of cancer in women in India (Jemal et al., 2011; Pathak et al., 2012). In majority of the cases, it is associated with the presence of human papilloma virus (HPV) infection, with high-risk HPV types 16 and 18 being responsible for more than 70% of cases of cervical cancer (Boulet et al., 2008; Franco et al., 2001). Apoptosis is controlled by a balance of pro-apoptotic and anti-apoptotic genes and is the most convenient manner for tumor cell elimination. Many chemotherapeutic drugs have been found to exert their cytotoxicity through this mechanism (Kamesaki et al., 1998).

Morinda citrifolia, also known as Great Morinda, Indian Mulberry, or Noni, is a plant belonging to the family Rubiaceae. A number of major chemical compounds have been identified in the leaves, roots, and fruits of Noni plant (Pawlus et al., 2005; Akihisa et al., 2007; Alitheen et al., 2010). Several studies have demonstrated that Noni shows cytotoxic and apoptosis-inducing affect by itself and enhances the efficacy of anticancer drugs like Cisplatin and doxorubicin (Hiramatsu et al., 1993; Furusawa et al., 2003; Taskin et al., 2009).

The antitumor drug, Cisplatin (CP), is employed as a first-line chemotherapeutic agent in the treatment of epithelial malignancies, including cancer of cervix, lung, ovarian, testicular, and others. It induces tumor killing by interacting with the DNA and activating mitogen-activated protein kinase (MAPK) signaling pathway, which controls a wide spectrum of cellular processes including growth, differentiation, and apoptosis (Hernandez-Flores et al., 2011). However, the administration of Cisplatin is associated with serious side effects, including nephrotoxicity, hepatotoxicity, and neurotoxicity. Furthermore, in advanced stages of cervical cancer, intrinsic resistance to Cisplatin is developed due to several factors (Kartalou et al., 2001). Therefore, new agents or new regimens in combination with Cisplatin are being sought in order to increase anticancer activity and decrease adverse effects (Jurado et al., 2009).

One such approach could be the use of a combination of the herbal Noni with Cisplatin. Even though some studies have examined the effect of Noni on cancer and have elucidated some of the mechanisms involved, there is still very little information available on the usefulness of Noni in the field of cancer, especially in terms of its effect in cervical cancer. The aim of this study was to study the cytotoxic and apoptosis-inducing effects of Noni by itself, Cisplatin by itself, and combination of both, in human cervical cancer cell lines.

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Materials and Methods

Cell lines

Two cell lines, i.e. HeLa (HPV18+) and SiHa (HPV16+) cervical cancer cell lines were used in this study. These cell lines were cultured in DMEM (Sigma), supplemented with 10% fetal bovine serum (FBS), 100 U/ml Penicillin, and 100 μg/ml Streptomycin. Cultures were maintained at 37^{0}C in 5% CO_2 and 95% humidified atmosphere.

Cell culture and treatment

Noni juice was obtained from Health India Laboratories (a unit of Noni BioTech Pvt. Ltd., Chennai, India). Cisplatin was purchased from Sigma, Aldrich, St. Louis, MO, USA. The cells were treated with different concentrations of CP and Noni and incubated at 37^{0}C for 24 hrs.

MTT assay

Cells were seeded in a 96-well plate at 1×10^4 cells per well and cultured for 24 hrs. Cells were then incubated with different concentrations of Noni (5, 7.5 or 10%, v/v), Cisplatin (CP) (5, 7.5, 10 μg/ml), and their various combinations for 24 hrs. After cell treatment, the media was removed and 100 μl of MTT stock (5 mg/ml) was added, followed by incubation for 4 hrs at 37^{0}C. The media was aspirated and 150 μL DMSO was added to each well to dissolve the formazan crystals. Cell cytotoxicity was determined spectrophotometrically by measuring the absorbance at 570 nm using a micro-titer plate reader (Kuhar et al., 2006). Finally, a dose of 10% for Noni and a dose of 10 μg/ml Cisplatin were chosen for the various experiments as these caused apoptosis without toxicity.

Detection of apoptosis by flow cytometry

Apoptosis was identified by the presence of fragmented DNA in cells of the sub-G1 phase. After treatment of both HeLa and SiHa cells (2×10^6 cells) with Noni (10%, v/v), Cisplatin (10 μg/ml), and their combination for 24 hrs, the cells were pelleted and washed twice with phosphate buffered saline (PBS), fixed with ice-cold 80% methanol, and stored at –20^{0}C for 24 hrs. Subsequently, the cells were pelleted, washed with PBS, re-suspended in 400 μl of citrate buffer, and incubated with 0.2 mg/ml RNaseA for 1 hr at room temperature (RT). Propidium iodide was added to a final concentration of 10 µg/ml and cells were incubated for at least 1-2 hrs in the dark at 4^{0}C. Post incubation, the cells were analyzed in the Flow Cytometer (Becton Dickenson, USA) using Diva Software (Reddy et al., 2001).

Western blot analysis

Cells were lysed in RIPA Lysis buffer containing 1 X protease inhibitor cocktail (Sigma, Aldrich, St. Louis, MO, USA) and the lysate was centrifuged at 10,000 x g for 10 min at 4^{0}C to remove the debris. The supernatants were transferred to fresh micro-centrifuge tubes and total amount of protein was estimated by Bradford method. Lysate containing equal amount of total protein (40-80 μg) were resolved on either 15% or 12% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane. After transfer, the membrane was incubated in blocking buffer for 4 hrs at RT. This was followed by incubation with primary antibody diluted (1:350-1:2000) in Tris buffer saline - Tween-20 (TBST) containing 1% bovine serum albumin (BSA), for 4 hrs at RT with gentle shaking. Post incubation, the membrane was washed twice with Tris buffer saline (TBS), followed by once washing with TBST for 10 min. The Alkaline phosphatase conjugated secondary antibody was then diluted (1:2000) in TBST containing 1% BSA. The membrane was then incubated in secondary antibody for 2 hrs at RT, followed by washing with TBS and TBST. The membrane was then stained with pre-mixed BCIP/NBT solution for 1-5 min at RT (Khanna et al., 2000).

Assay of caspase-9, -3, and -8 activities

Both untreated and treated HeLa and SiHa cells were washed with PBS and lysed in Lysis buffer. Aliquots of cell lysate (50-100 µl) were then added to reaction buffer along with 50 µM fluorogenic substrate and reactions were incubated for 1 hr at 37^{0}C. Amounts of fluorogenic AMC/AFC moiety released were measured using a spectrofluorimeter (ex. 400nm, em. 490-520 nm). The results are expressed as Arbitrary Fluorescence Units/mg protein (Sen et al., 2005).

Statistical analysis

Results of each experiment represent the mean±standard deviation (SD) of three independent experiments carried out in triplicate. All the data were analysed using Student's t-test. Statistical analysis showing a value of $P<0.05$ was considered significant.

Results

Noni, cisplatin, and their combination induce apoptosis in human cervical carcinoma HeLa and SiHa cells

Cell cytotoxicity was measured by MTT assay and apoptosis by Flow Cytometry. Cell cytotoxicity of different concentrations of Noni (N), Cisplatin (CP), and their combinations (N+CP) was assessed 24 hrs post treatment by MTT assay (Figure 1). Treatment with Noni (10%, v/v), CP (10 μg/ml), and their combination (Noni 10%, v/v + Cisplatin 10 μg/ml) decreased cell survival by 22.3%, 29.7% and 52.9% respectively in HeLa cells; and by 21.3%, 31% and 50.1% respectively in SiHa cells, as compared to their respective controls (Figure 1).

Flow Cytometry data was found to be in agreement with the MTT data. Noni, CP, and their combination (N+CP) at the above-mentioned doses resulted in 22.8%, 26.4%, and 51.2% apoptosis respectively in HeLa cells, whereas its control showed 4.2% apoptosis; and 22.2%, 32%, and 53.1% apoptosis respectively in SiHa cells, whereas its control showed 3.3% apoptosis (Figure 2). Thus, the cell death induced by CP and Noni appears to be apoptosis. CP showed slightly higher cell killing as compared to Noni and their combination was additive.

Up-regulation of pro-apoptotic proteins and down-regulation of anti-apoptotic proteins by both Noni and Cisplatin

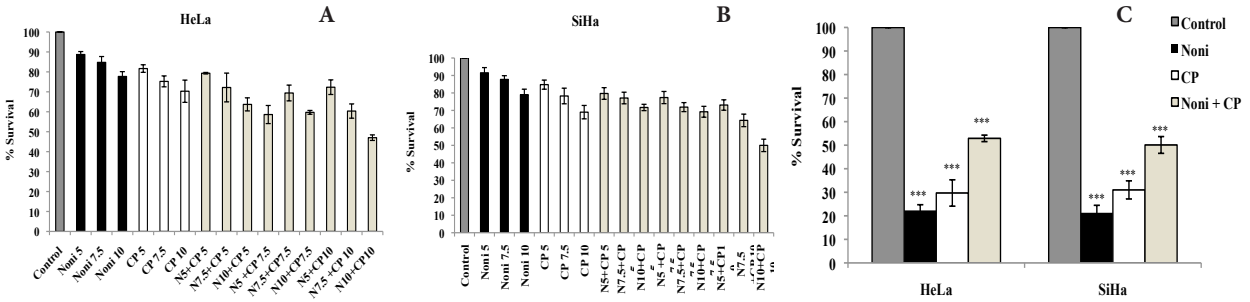

Figure 1. Cell Cytotoxicity by MTT Assay after Treatment with Noni (N) (5-10%, v/v), Cisplatin (CP) (5-10 μg/ml), and Their Various Combinations (N+CP) for 24 hrs. (A) HeLa cells (B) SiHa cells and (C) The percentage survival shown in the bar diagrams is mean±SD of three individual experiments done in triplicates

The expression of pro-apoptotic (Bax) and anti-apoptotic (Bcl-2 and Bcl-X_L) proteins was studied in untreated and treated HeLa and SiHa cells. Noni, CP, and their combination (N+CP) caused increase in Bax protein expression, with maximum increase being observed when used in combination. This was accompanied by a simultaneous decrease in anti-apoptotic proteins, Bcl-2 and Bcl-X_L. The increase in Bax expression by Noni was 16%, by CP 57%, and by their combination was 66% respectively in HeLa cells. It was 25%, 67% and 82% respectively in SiHa cells. This was found to be accompanied by decrease in Bcl-2 expression by 22%, 27% and 41% respectively in HeLa cells; and by 43%, 51% and 55% respectively in SiHa cells; and decrease in Bcl- X_L expression by 21%, 29% and 48% respectively in HeLa cells; and by 28%, 46% and 63% in SiHa cells respectively, as compared to their respective controls (Figure 3).

Activation of p53 by Noni and Cisplatin

p53 plays a central role in apoptosis. As a tumor suppressor, p53 is essential for preventing inappropriate cell proliferation and maintaining genome integrity following genotoxic stress, such as DNA damage by chemotherapeutic agents, by infectious virus, and by oncogene over expression. An increasing trend in p53 expression was seen. With Noni alone, CP alone, and their combination, increase in expression of p53 was seen by 9%, 18% and 43% respectively, in HeLa cells; and by 26%, 41% and 54% respectively, in SiHa cells, as compared to their controls (Figure 3).

Modulation of survivin by Noni and Cisplatin

The expression of Survivin, one of the members of the Inhibitor of Apoptosis (IAP) family, is regulated in a cell cycle-dependent manner with maximum levels occurring during the G2/M phase. There was a decrease in the expression of Survivin on treatment with Noni, Cisplatin, and their combination, as compared to their controls. HeLa cells showed 17%, 27% and 20% decrease respectively in survivin expression with Noni, CP, and combination, whereas in SiHa cells, decreases of 46%, 33% and 32% respectively, were observed (Figure 3).

Activation of Caspase-9, -8, and -3 in cervical cancer cell lines

On treatment with Noni, CP, and their combination, the activities of both Caspase-9 and -3 were found to be

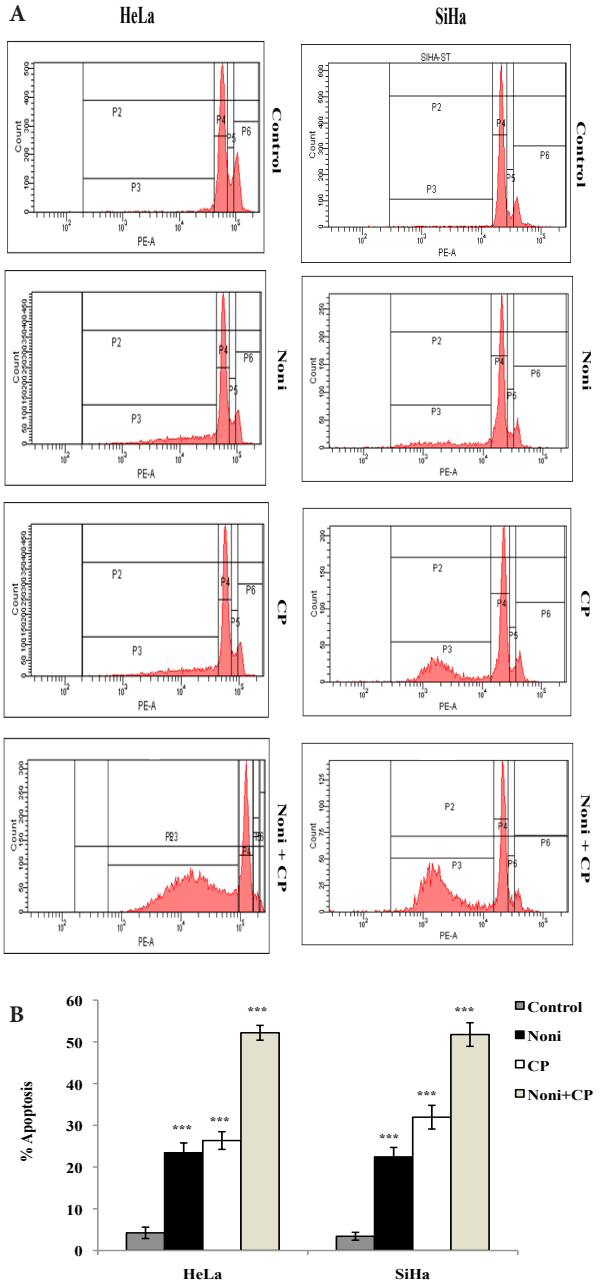

Figure 2. (A) Representation of Flow Cytometric analysis of apoptosis in both HeLa and SiHa cells on treatment with Noni (N) (10%), Cisplatin (CP) (10 μg/ml), and their combination (Noni 10% + CP 10μg/ml) for 24 hrs. (B) The bar diagram represents percentage apoptosis as mean±SD of three individual experiments done in triplicates

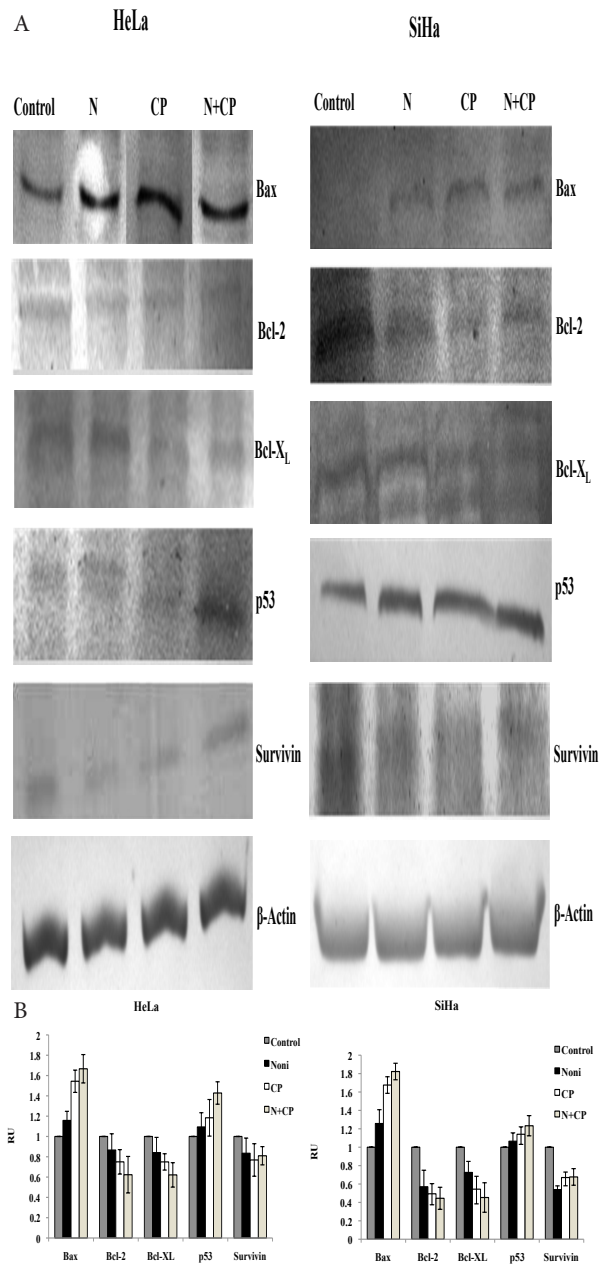

Figure 3. (A) Western Blot Analysis for Studying the Protein Expression of Bax, Bcl-2, Bcl-X$_L$, p53, and Survivin in HeLa and SiHa Cells, on Treatment with Noni (N) (10%), CP (10μg/ml) and their Combination (N+CP) for 24 hrs, (B) The Results Shown in the bar Diagram is mean±SD of Three Individual Experiments

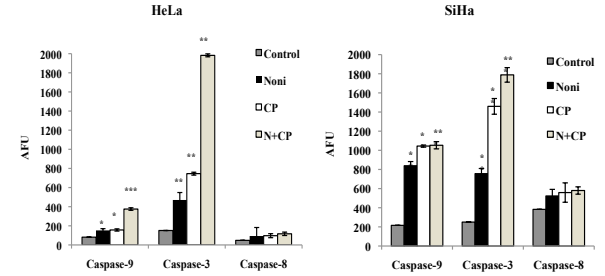

Figure 4. Caspase-3, -9, and -8 Activities in HeLa and SiHa Cells on Treatment with Noni (N) (10%), CP (10 ug/ml), and their Combination (N+CP) for 24 hrs. Data shown is mean±SD of three individual experiments

increased significantly as compared to control, in both HeLa and SiHa cell lines. In HeLa cells, the activity of Caspase-9 increased by 1.8 folds with Noni, 1.9 folds with CP, and by 4.6 folds with their combination; and Caspase-3 increased by 6.2 folds, 10.4 folds, 29.3 folds respectively, as compared to control. In SiHa cells, with the above treatments, Caspase-9 activity was found to be increased by 2.9 folds, 3.8 folds, and 3.9 folds respectively; and Caspase-3 activity increased by 11.5 folds, 23 folds, 28.5 folds respectively, as compared to control. However, Caspase-8 activity did not show any increase on these treatments in both the cell lines (Figure 4).

Discussion

Search for new chemopreventive and antitumor agents that are more effective but less toxic has kindled great interest in phytochemicals. Noni, fruit juice derived from the plant *Morinda citrifolia*, is one such compound which was used in this study. Noni is a herbal remedy with promising anticancer properties. It has shown to inhibit the growth of tumor cells in experimental model systems (Hiramatsu et al., 1993; Liu et al., 2001; Taskin et al., 2009), but little is known about its potential as an adjuvant chemotherapeutic agent. There is no data on the efficacy of Noni in modulating the cytotoxic effects of Cisplatin which is used to treat human cervical carcinoma. We studied the effect of Noni by itself, Cisplatin by itself, and their combination, on two cervical cancer cell lines (HeLa and SiHa). These cell lines were chosen for the study because they harbor the human papilloma virus (HPV) type 18 (HeLa) and type 16 (SiHa) genotypes. These HPVs have been shown in multi-institutional studies as etiological agents in the development of cervical cancer and these genotypes account for >70% of all HPV DNA positive invasive cervical cancers (Franco et al., 2001; Boulet et al., 2008).

Cisplatin is among the most effective and widely used chemotherapeutic agent employed in the treatment of solid tumors. It is a platinum-based compound that forms intra- and inter-strand adducts with DNA, thus it is a potent inducer of cell cycle arrest, and apoptosis in most cancer cell types (Schloffer et al., 2003). However, a major limitation of chemotherapy is that many tumors either are inherently resistant or acquire resistance to the drug after an initial response (Kartalou et al., 2001). Tremendous efforts are being made to improve the anticancer effect of Cisplatin. Naturally occurring compounds from medicinal plants are good candidates for increasing anticancer activity of Cisplatin.

Our aim of this study was to investigate whether or not Noni by itself and when combined with Cisplatin, could act additively on the cytotoxic effect of the latter, in the treatment of cervical cancer. The cytotoxicity of Noni/Cisplatin by themselves, and their combination, as detected by MTT assay, showed a decrease in cell survival of cervical cancer cells. Also, our in vitro results showed that treatment with the combination of Noni and Cisplatin induced additive cytotoxic effect, and this increase in cytotoxicity was found to be statistically significant when compared to either Noni alone ($p<0.001$)

or Cisplatin alone ($p<0.001$) in both HeLa and SiHa cells. The cytotoxic effect of Noni juice has been reported to be due to the presence of chemical compounds such as damnacanthal and alkaloids (Hiramatsu et al., 1993).

Our next aim was to understand whether the observed cell cytotoxicity by Noni and CP was brought about by induction of apoptosis which is an important homeostatic mechanism that balances cell division and cell death and maintains the appropriate cell numbers. Many natural phytochemicals have been demonstrated to promote apoptosis in cancer cells, including phenolics, alkaloids, damnacanthal, and flavonoids (Alshatwi et al., 2011; Wong et al., 2012). In the present study, treatment with Noni/Cisplatin by themselves as well as their combination induced apoptosis in cervical cancer cells, as evident by Flow cytometry assay which showed accumulation of population of cells in hypodiploidy phase in both HeLa and SiHa cells. The results of this study indicate that treating the cells with combination of Noni and Cisplatin can enhance the apoptosis in vitro, as compared to either Noni alone ($p<0.001$) or Cisplatin alone ($p<0.001$). It seems that Noni can enhance the apoptotic efficacy of DNA-binding agents like Cisplatin. This also supports one of the study in which Noni-precipitate in combination with Cisplatin enhanced the antitumor effects; while in combination with DNA/RNA synthesis inhibitors and agents with immunosuppressive properties, were not beneficial on Sarcoma 180 ascites tumors in mice (Furusawa et al., 2003).

On elucidating the molecular mechanism of apoptosis, it was observed that Bcl-2 family of proteins is involved in the apoptotic signaling. There was an increase in Bax expression accompanied by decrease in Bcl-2 and Bcl-X_L expression, in both the cervical cancer cell lines studied. Bcl-2 tends to stabilize the barrier function of mitochondrial membranes; whereas pro-apoptotic Bax tends to antagonize such a function and permeabilizes the mitochondrial membrane (Guillemin et al., 2010). Though, both Noni and CP showed similar response but their combination was additive and the changes were more marked in SiHa cells as compared to HeLa cells.

The p53 tumor suppressor gene plays a critical role at the G1/S phase of cell cycle transition, where it can either block entry into S phase or activate apoptosis in response to DNA damage. Our study showed that, in both HeLa and SiHa cells, treatment with Noni, Cisplatin, and their combination led to p53-dependent apoptosis. Since pro-apoptotic Bax is known to be a p53 downstream target, our result of the higher expression of Bax protein also supports the involvement of p53 during this cell death mechanism. The increased expression of p53 and Bax; and decreased expression of Bcl-2 and Bcl-X_L perhaps confers higher sensitivity to apoptosis or favors the onset of apoptosis when treated with combination of Noni and Cisplatin as compared to that induced by either Noni or Cisplatin alone, in both HeLa and SiHa cells.

Survivin, a member of the IAP gene family, has been implicated in suppression of cell death, regulation of mitosis, surveillance checkpoints, and adaptation to unfavorable environments (Khan et al., 2011). Its aberrant, high protein expression in cancer cells and concomitantly low expression in most normal tissues makes survivin an important anticancer target. Our results showed a decrease in survivin following treatment with either Noni or CP, and the lowest expression was seen on their combination treatment, in both the cell lines. Since survivin expression is regulated in a cell cycle-dependent manner, with maximum expression occurring during the G2/M phase, the maximum Survivin expression in control (untreated) is understandable.

As there was an increase in protein expression of pro-apoptotic members and decrease in anti-apoptotic ones, we studied the effect of Noni, Cisplatin, and their combination on Caspase activity, as caspases are the initiator and effectors of apoptosis. Our results showed that there was significant increase in the activity of Caspase-9, and -3, by Noni, Cisplatin, and their combination. However, the activity of caspase-8 did not increase significantly on these treatments. This signifies that Noni, Cisplatin, and their combination primarily activated intrinsic mitochondrial mediated apoptotic pathway. The increase was more in SiHa cells as compared to HeLa cells, thereby showing slight variation from one cell type to another.

Taken together, this study showed that Noni/Cisplatin by themselves, and their combination were able to induce apoptosis through the mitochondrial pathway, in both HeLa and SiHa cells, particularly through the up-regulation of pro-apoptotic members and down-regulation of the anti-apoptotic members. This was accompanied by an increase in activity of caspases-9 and -3, thus primarily activating intrinsic pathway of apoptosis. Both Noni and Cisplatin appear to be inducing apoptosis through a similar mechanism in both the cervical cancer cells and their effects were additive when they were used in combination. However, the effects were more marked with Cisplatin treatment and were more in SiHa cells as compared to HeLa cells, thereby showing some variation from one cell type to another. Hence Noni offers potential to be used as a chemoadjuvant, especially for treatment of cervical cancer.

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