CHAPTER 4   PHASE I STUDY

CHEMOSENSITIZING POTENTIAL OF FERULIC ACID IN CERVICAL CANCER CELL LINES

4.1. INTRODUCTION

Carcinoma of the uterine cervix is a common kind of cancer in women in which cancerous cells develop in the tissues of the cervix. It is one of the most common malignancies in women worldwide and prognosis for advanced stage of the disease is poor. On the global scale, cervical cancer represents the second most frequent cancer in women and the fifth most common cancer in humans with approximately 5,30,000 new cases registered each year and >2,74,000 deaths worldwide (Lopez et al. 2012; Thulaseedharan et al. 2013).

Cervical cancer development includes three major steps initiation, promotion and progression in which oxidative stress is involved. Oxidative stress is defined as an imbalance between the level of prooxidant and antioxidant defense system (Pajovic et al. 2003). When produced in excess reactive oxygen species (ROS) can seriously alter the structure of biomolecules such as proteins, lipids, lipoproteins and DNA (Breimer 1990). Cellular fatty acids are rapidly oxidized by ROS to produce lipid peroxyl radicals and lipid hydroperoxide (Rice-Evans et al. 1993). The cervical cancer progresses with lipid peroxidation and increased oxidative stress was observed in cervical cancer cells (Srivastava et al. 2009). Cervical cell membrane is one of the major targets for lipid peroxidation. Carbonyl group and malondialdehyde (MDA) interact with amino group of proteins and phospholipids forming Schiff base conjugate such cross linking mechanism might be initiated in lipid bilayer of cervical cell membrane. Increase in lipid
peroxides, oxidize sulphhydryl group of cross linking proteins leading to oxidative deterioration and peroxidation in cervical cell membrane (Sun 1999; Manoharan et al. 2002). Elevated lipid peroxidation and altered antioxidant status were reported for cervical cancer patients relative to health subjects (Manju et al. 2002).

![Figure 4.1. Cervical neoplasia](image)

Chemotherapy has played an important role in the treatment of cervical cancer. Cisplatin is considered as one of the most effective anticancer drugs against cervical carcinoma (Alberts et al. 1991; Benedetti et al. 2002; Neoadjuvant Chemotherapy for Locally Advanced Cervical Cancer Metaanalysis Collaboration 2003). It is a DNA-interactive agent that induces lesions in DNA by forming mono adducts and intra and interstrand cross-links.
(Chen et al. 2007). Cisplatin treatment induces DNA damaging stress, oxidative stress and endoplasmic reticulum stress (Mandic et al. 2003). Apart from the advantages, chemoresistance of cancer cells is the main disadvantage of cisplatin therapy against cervical and other cancers (Torigoe et al. 2005).

It is necessary therefore, to develop a medicine which has lower toxicity and potent therapeutic effect to substitute cisplatin or to combine with it. Many agents have been developed to improve the cytotoxicity of chemotherapy. Phytochemicals are one among those which are widely employed in cancer research. The presence of phytochemicals amplifies the effects of chemotherapy by inducing toxic reactions of free radicals. Free radicals are toxic entities known to cause cellular damage (Yamamoto et al. 2003). Recent epidemiological data revealed that polyphenols can help conventional chemotherapy by sensitising cancer cells to drugs (Garg et al. 2005).

Plant polyphenols are generally recognized as physiological antioxidants but they also exhibit prooxidant properties in vitro (Bhat et al. 2007). There appears to be a differential effect of polyphenols on normal and malignant cells (Raffoul et al. 2007). The effect of polyphenols largely depends on the environment of the cell. Tumor tissues contain microenvironments that are both hypoxic and acidic. As cancer cells possess centrally acidic region, phytochemicals could not able to act as antioxidants and instead they act as prooxidants due to the presence of high levels of peroxidases which act on phenolics and produce phenoxy radicals (Lee and Lee 2006). Some phenolic compounds such as catechin, quercetin and curcumin have been reported to exert prooxidant actions and DNA damaging ability (Azam et al. 2004; Tan et al. 2009). The prooxidant action of phenolic phytochemicals has been predicted to be an important mechanism for the chemosensitization property in cancer cells (Garg et al. 2005).
Mitochondria are the major site of reactive oxygen species (ROS) production in eukaryotic cells. Tumor cells have higher levels of ROS than their normal counterparts and are therefore more sensitive to the additional oxidative stress generated by anticancer agents. As a result, these polyphenols further induce DNA damage and apoptosis of the cancer cells (Trachootham et al. 2009). Phenolic phytochemicals have been shown to induce apoptosis by the generation of ROS (Fan et al. 2009). Elevated ROS levels inhibit cancer progression through the stimulation of pro-apoptotic-signals, leading to the death of cancer cells (Park et al. 2008).

Cancer cells are known to be the consequence of resistance to apoptosis, it is logical to search for agents that can trigger and modulate the oxidative stress in the cells (Bauer and Bauer 1999). Due to the prooxidant nature of polyphenols, the ROS levels were increased in cells. The accumulation of ROS damages the DNA of cancer cells by the formation of single and double stranded breaks (Igor Afanas’ev 2011). The stress-induced damage may be manifested in clonogenic cell death or may be reflected in altered signaling cascades resulting in activation of responsive genes inducing apoptosis (Nomiya 2013). Various forms of cellular stress can cause mitochondrial alterations which results in mitochondrial membrane depolarization (Mouria et al. 2002). Research evidences showed that polyphenols can induce stresses in cancer cells and thereby can cause changes in mitochondrial membrane potential. Recent study reports by Prasad et al. (2011) showed that Caffeic acid, a plant polyphenol enhanced mitochondrial membrane depolarization which finally resulted in the apoptosis of fibrosarcoma cell lines.

Apoptosis requires the coordinated action of a family of proteases called caspases that are divided into initiator caspases (caspase 8 and 9) and executioner caspases (caspase 3, 6 and 7) (Strasser et al. 1995; Thornberry and
Lazebnik 1998). In addition to caspases, members of the bcl-2 family also play an important role in the regulation of cell survival/apoptosis by either serving as anti-apoptotic or pro-apoptotic proteins.

p53 is a tumor suppressor protein that in humans is encoded by the TP53 gene. p53 is crucial in multicellular organisms, where it regulates the cell cycle and, thus, functions as a tumor suppressor that is involved in preventing cancer. As such, p53 has been described as "the guardian of the genome" because of its role in conserving stability by preventing genome mutation. The tumor suppressor protein p53 has a critical role in regulation of the Bcl-2 family of proteins, however the exact mechanisms have not yet been completely elucidated. This tumour suppressor gene is mutated in more than half of all tumours. Most chemotherapeutic drugs cause DNA damage, which is sensed by p53; the cell can then try to repair the damage or induce cell suicide. If the p53 machinery is defective, effective chemotherapy is made more difficult (Susan 2007).

Based on these reports, the Phase I study was designed to verify the chemosensitizing effect of FA in cisplatin chemotherapy in cervical cancer cell lines HeLa and SiHa.
4.2. MATERIALS AND METHODS

4.2.1. Chemicals

Ferulic acid (FA), cisplatin, thiobarbituric acid (TBA), trichloroacetic acid (TCA), phenazine methosulphate (PMS), nitroblue tetrazolium (NBT), 5, 5-dithiobis 2-nitrobenzoic acid (DTNB), 3-(4, 5-dimethyl-2-thiaozolyl)-2, 5-diphenyl-2H tetrazolium bromide (MTT), 2-7-diacetyl dichlorofluorescein (DCFH-DH), ethidium bromide (EBr), acridine orange (AO), dithiothreitol, propidium iodide (PI), Rhodamine 123, N,N,N',N' tetramethylethylenediamine (TEMED), N,N'-bisacrylamide, sodium dodecylsulfate (SDS), sodium orthovanadate, reduced glutathione (GSH) and cell culture chemicals such as heat inactivated fetal bovine serum (FBS), Dulbecco's modified Eagle's culture medium (DMEM), glutamine, penicillin - streptomycin, trypsin were purchased from Sigma Chemicals Co., St. Louis, USA. Low melting agarose, normal melting agarose, bovine serum albumin (BSA), ethylene diamine tetra acetic acid (EDTA), dimethyl sulphoxide (DMSO), bromophenol blue, ammonium persulphate (APS), β-mercaptoethanol, ponceau, phenyl methyl sulfonyl fluoride, phosphate buffered saline (PBS) and sodium hydroxide were purchased from (Himedia, Mumbai). All other chemicals and solvents of analytical grade were obtained from S.D. fine chemicals, Mumbai, Fisher Inorganic and Aromatic Limited, Chennai and Central Drug House (P) Ltd, New Delhi, India.

4.2.2. Antibodies

Antibodies against caspase 3 and caspase 9 (rabbit polyclonal), p53 (mouse monoclonal), Bcl-2 (rabbit Polyclonal), and β-Actin (rabbit polyclonal) were purchased from Santa Cruz Biotechnology, CA, USA. Corresponding horse radish peroxidase-linked secondary antibodies against
rabbit and mouse IgG were also purchased from Santa Cruz Biotechnology CA, USA.

4.2.3. Cell lines

The phase I studies of the present work were carried out in two human cervical cancer cell lines (HeLa and SiHa). These cell lines were obtained from National Centre for Cell Sciences (NCCS), Pune, India.

![HeLa](image1.png) ![SiHa](image2.png)

Figure 4.2. Morphology of cervical cancer cell lines

4.2.4. Culturing cells

HeLa (HPV 18+) and SiHa (HPV 16+) cervical cancer cell lines were maintained in vitro and propagated in Dulbecco's modified Eagle's culture medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS), 1% L-glutamine (2 mM final concentration) and antibiotics (100 U/mL penicillin/streptomycin) and incubated at 37°C in humidified atmosphere containing 95% air and 5% CO₂. Stock cultures were maintained in 25 cm² culture flasks. After cell numbers are counted, cells were seeded at 5 x 10⁴ cells per well in 24-well plates. After reaching 80 - 90% confluency, the cells were harvested by trypsinization, subcultured and used for further experiments.
Table 4.1. Characteristics of cell lines used in the present study

<table>
<thead>
<tr>
<th>Designation</th>
<th>HeLa</th>
<th>SiHa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue</td>
<td>Uterine cervical cancer cell line</td>
<td>Uterine cervical cancer cell line</td>
</tr>
<tr>
<td>Gene transfer vehicle</td>
<td>T25-Flask</td>
<td>T25-Flask</td>
</tr>
<tr>
<td>Bioluminescence <em>in vitro</em></td>
<td>860 photons/cell</td>
<td>860 photons/cell</td>
</tr>
<tr>
<td>Recommended media</td>
<td>DMEM with 10% FBS</td>
<td>DMEM with 10% FBS</td>
</tr>
<tr>
<td>Cell doubling time</td>
<td>17 - 21 hours</td>
<td>34 - 37 hours</td>
</tr>
<tr>
<td>Other conditions</td>
<td>Cells are ready to expand next day</td>
<td>Cells are ready to expand next day</td>
</tr>
</tbody>
</table>

4.2.5. Preparation of drug and dose fixation study

A stock solution of FA (1 mg/mL) and cisplatin (1 mg/mL) was prepared in 0.5% dimethyl sulphoxide (DMSO) (v/v) and stored at 4° C. Further dilutions were made in culture media to obtain the desired concentrations. The final concentrations of DMSO in the culture medium were not more than 0.01% (v/v). 0.01% DMSO was used as a sham control. Cells were treated with different concentration of FA (5, 10, 15, 20, 25, 30, 35, 40, 45, 50 µg/mL) or cisplatin (5, 10, 15, 20, 25, 30, 35, 40, 45, 50 µg/mL) and the cytotoxicity was observed by MTT assay. IC 50 (half maximal inhibitory
concentration) values for FA and cisplatin were calculated by MTT assay and the optimum concentration was employed for the study.

4.2.6. Study groups

Cervical cancer cells (HeLa and SiHa) were divided into four groups. Group 1 (control) consists of untreated HeLa and SiHa cells; group 2 cells were treated with ferulic acid (IC 50 concentration) alone and group 3 cells were treated with cisplatin (IC 50 concentration) alone. In group 4, the cells were pretreated with ferulic acid for a period of 1 hour (Karthikeyan et al. 2011) prior to the treatment of cisplatin.

<table>
<thead>
<tr>
<th>Groups</th>
<th>HeLa</th>
<th>SiHa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Untreated HeLa cells (Control)</td>
<td>Untreated SiHa cells (Control)</td>
</tr>
<tr>
<td>Group II</td>
<td>HeLa + FA (10 µg/mL)</td>
<td>SiHa + FA (10 µg/mL)</td>
</tr>
<tr>
<td>Group III</td>
<td>HeLa + Cisplatin (5µg/mL)</td>
<td>SiHa + Cisplatin (5µg/mL)</td>
</tr>
<tr>
<td>Group IV</td>
<td>HeLa + FA pretreatment (10 µg/mL) for 1 hour followed by Cisplatin (5µg/mL)</td>
<td>SiHa + FA pretreatment (10 µg/mL) for 1 hour followed by Cisplatin (5µg/mL)</td>
</tr>
</tbody>
</table>

Table 4.2. Experimental design for phase I studies on cervical cancer cells
4.2.7. Measurement of cell proliferation (MTT assay)

The proliferation activity of cell populations both untreated and treated with ferulic acid and cisplatin was determined by MTT assay based on the detection of mitochondrial dehydrogenase activity in living cells (Mosmann 1983).

Principle

In MTT (3-(4, 5 - dimethylthiazol-2-yl) - 2, 5-diphenyl-tetrazolium bromide) assay, the yellow tetrazolium salt is metabolized by NAD-dependent dehydrogenase present in the mitochondria of living cells to form a dark blue formazan product which is read colorimetrically at 490 nm. From the concentration of the purple formazan cell viability or cytotoxicity can be calculated.

Reagents

1. 0.5 g/L MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)

2. Dimethyl sulfoxide (DMSO)

Procedure

Yellow colour MTT is converted to purple formazan product only by metabolically active mitochondria. The absorbance of purple formazan is directly proportional to the number of viable cells. MTT solution (0.5 g/L) was added to each culture well 24 hours after treatment with FA or cisplatin or FA plus cisplatin and the colour was allowed to develop for additional 4 hours incubation. An equal volume of DMSO was added to stop the reaction and to solubilize the purple crystals. Samples were transferred into culture plates and the absorbance was measured at 490 nm in microplate reader.
4.2.8. Clonogenic cell survival assay

Colony formation capacity of cells was estimated by the method of Kamer et al. (2009).

Principle

In this method, the survival and reproducible rate of the cells after drug treatment was measured by counting the number of colonies (≤ 50 cells) formed in the culture plate.

Materials Required

1. Growth medium
2. Trypsin
3. Petri dishes
4. Methanol
5. Crystal violet

Procedure

Cervical cancer cells of about $2 \times 10^5$ cells/mL were seeded in 25 cm$^2$ tissue culture plates and allowed to grow for 24 hours at 37° C in a CO$_2$ incubator. Cells were treated with FA, cisplatin and FA plus cisplatin (FA pretreatment). The drugs were added directly to the growth media and kept for incubation. After incubation, the cells were trypsinized, diluted, counted and seeded into 100 mm plates at densities of 100–20,000 cells/dish. Colonies were allowed to grow in a humidified environment (5% CO$_2$, 37° C) for 8 days. After incubation, they were stained with crystal violet solution (10 g/1,000 mL MeOH) and colonies containing more than or equal to 50 cells (≤ 50 cells) were scored. Clonogenic survival curves were plotted as percentage of colonies versus the concentration of the drug.
4.2.9. Measurement of intracellular reactive oxygen species (ROS) in cells

Intracellular reactive oxygen species (ROS) was measured spectrofluorimetrically by DCFH-DA staining according to the method of Jesudason et al. (2008).

**Principle**

ROS was measured by using a non-fluorescent probe, 2, 7 - diacetyl dichlorofluorescein diacetate (DCFH-DA) that can penetrate into the intracellular matrix of cells. The non-fluorescent DCFH-DA is oxidized by intracellular ROS and forms the highly fluorescent (dichloro fluorescein) DCF which is measured spectrofluorimetrically at emission filters set at 485 ± 10 nm and 530 ± 12.5 nm respectively.

**Reagents**

1. Phosphate buffered saline (PBS)
2. 2, 7 - diacetyl dichlorofluorescein (DCFH-DA)

**Procedure**

The percentage ROS was estimated in the cancer cells treated with FA, cisplatin and ferulic acid (pretreatment) plus cisplatin. Briefly, an aliquot of the isolated cells (8 x 10^6 cells / mL) were made up to a final volume of 2 mL in normal phosphate buffered saline (pH 7.4). From this 1 mL was taken, to which 100 µl DCFH-DA (10 µM) was added and incubated at 37° C for 30 minutes. Fluorescent measurements were made with excitation and emission filters set at 485 ± 10 nm and 530 ± 12.5 nm respectively. All initial fluorescent values (time 0) were found to differ from each other by less than 5%. Results were expressed as percentage increase in fluorescence calculated using the formula \([\frac{(F_{t30} - F_{t0})}{F_{t0} x 100}]\); Ft0 and Ft30 are the fluorescent intensities at 0 and 30 minutes.
4.2.10. Biochemical Determinations

The cancer cells were harvested by trypsinization and washed with phosphate buffered saline (PBS). The cells were suspended in 130 mM KCl, 50 mM PBS containing 10 µM dithiothreitol and centrifuged at 20,000 x g for 15 minutes (4° C). The supernatant was collected and used for biochemical estimations.

4.2.10.1. Lipid peroxidation

4.2.10.1.1. Estimation of thiobarbituric acid reactive substances (TBARS)

The concentration of TBARS in the cancer cells was estimated by the method of Niehaus and Samuelson (1968).

Principle

In this method, malondialdehyde and other thiobarbituric acid reactive substances (TBARS) react with thiobarbituric acid in an acidic condition to generate a pink colour chromophore which was read at 535 nm.

Reagents

1. Trichloroacetic acid (TCA) : 15%
2. Hydrochloric acid: 0.25 N
3. Thiobarbituric acid (TBA) : 0.375% in hot distilled water
4. TBA-TCA-HCl reagent: Solution 1 to 3 was mixed in the ratio of 1:1:1 freshly prepared prior to use.
5. Stock standard: 4.8 molar solution of stock was prepared from 1, 1', 3, 3’ tetramethoxypropane purchased commercially.
6. Working standard: Stock solution was diluted to get a concentration of 48 nM/mL.

**Procedure**

The sample was diluted to 0.5 mL with double distilled water, mixed well and then 2 mL of TBA-TCA-HCl reagent was added. This mixture was kept in a boiling water bath for 15 minutes. After cooling, the tubes were centrifuged at 1000 x g for 10 minutes and the supernatant was estimated for TBARS. A series of standard solution in the concentration of 2 - 10 nM was treated in a similar manner. The absorbance of the chromophore was read at 535 nm against reagent blank.

The values were expressed as nM/mg of protein for cells.

**4.2.10.1.2. Estimation of conjugated dienes (CD)**

Conjugated dienes were assayed by the method of Recknagel and Rao (1968).

**Principle**

Lipid peroxidation is associated with rearrangement of the double bonds in the polyunsaturated fatty acids leading to the formation of conjugated dienes. The measurement of the formation of conjugated dienes reflects the extent of lipid peroxidation taking place.

**Reagents**

1. Chloroform
2. Methanol
3. Cyclohexane
Procedure

One mL of sample was treated with 5 mL of chloroform - methanol mixture, vortexed and the contents were filtered. Aliquots of lipid extracts were evaporated to dryness. The lipid residue was resuspended in 5 mL of cyclohexane and the absorbance at 240 nm and 214 nm were measured against a solvent blank.

Conjugated diene was expressed as ratio of OD at 240 and 214 nm.

4.2.10.1.3. Estimation of lipid hydroperoxides (LOOH)

Lipid hydroperoxide in the cancer cell lines were estimated by the method of Jiang et al. (1992).

Principle

Oxidation of ferrous ion (Fe$^{2+}$) under acidic conditions in the presence of xylenol orange leads to the formation of a chromophore with an absorbance maximum at 560 nm.

Reagents

1. Fox reagent: 88 mg butylated hydroxytoluene (BHT), 7.6 mg xylenol orange and 9.8 mg ammonium iron (II) sulphate were added to 90 mL methanol and 10 mL H$_2$SO$_4$ (250 mM) mixture.

Procedure

Fox reagent (0.9 mL) was mixed with 0.1 mL of sample, incubated for 30 minutes at room temperature and the absorbance was read in a spectrophotometer at 560 nm.

Lipid hydroperoxides were expressed as µM/mg of protein.
4.2.10.2. Enzymic and nonenzymic antioxidants

4.2.10.2.1. Assay of superoxide dismutase (SOD, EC 1.15.1.1)

Superoxide dismutase in the cancer cell lines was assayed by the method of Kakkar et al. (1984).

Principle

The assay is based on the inhibition of the formation of NADH–phenazine methosulphate, nitroblue tetrizolium formazon. The reaction was initiated by the addition of NADH. After incubation for 90 seconds, addition of glacial acetic acid stops the reaction. The colour developed at the end of the reaction was extracted into n-butanol layer and measured in a spectrophotometer at 520 nm.

Reagents

1. Sodium pyrophosphate buffer: 0.052 M, pH 8.3
2. Absolute ethanol
3. Chloroform
4. n-butanol
5. Phenazine methosulphate (PMS): 186 µM
6. Nitroblue tetrizolium (NBT): 300 µM
7. Reduced nicotinamide adenine dinucleotide (NADH): 780 µM

Procedure

The supernatant (0.5 mL) was diluted to 1.0 mL with distilled water followed by addition of 2.5 mL of ethanol and 1.5 mL of chloroform (chilled reagents were added). This mixture was shaken for 90 seconds at 4°C and then centrifuged. The enzyme activity in the supernatant was determined as follows. The assay mixture contained 1.2 mL of sodium pyrophosphate buffer,
0.1 mL of PMS, 0.3 mL of NBT and appropriately diluted enzyme preparation in a total volume of 3 mL. The reaction was started by the addition of 0.2 mL NADH solution. After incubation at 30° C for 90 seconds, the reaction was stopped by the addition of 1 mL glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4 mL n-butanol. The mixture was allowed to stand for 10 minutes, centrifuged and n-butanol layer was separated. The colour intensity of the chromogen in n-butanol was measured at 520 nm. A system devoid of enzyme served as control. The enzyme concentration required to inhibit the chromogen produced by 50% in one minute under standard conditions was taken as one unit.

The specific activity of the enzyme was expressed as Unit/minute/mg of protein.

4.2.10.2.2. Estimation of catalase (CAT, EC 1.11.1.6)

The activity of catalase in the cancer cells was determined by the method of Sinha (1972).

Principle

Dichromate in acetic acid was converted to perchromic acid and then to chromic acetate, when heated in the presence of H₂O₂. The chromic acetate formed was measured at 620 nm. The catalase preparation was allowed to split H₂O₂ for various periods of time. The reaction was stopped at different time intervals by the addition of dichromate - acetic acid mixture and the remaining H₂O₂ as H₂O₂ - chromic acetate was determined colorimetrically at 590 nm.

Reagents

1. Phosphate buffer : 0.01 M, pH 7.0
2. Hydrogen peroxide : 0.2 M
3. Potassium dichromate: 5% (w/v)

4. Dichromate - acetic acid reagent: 5% potassium dichromate and glacial acetic acid was mixed in the ratio of 1:3. From this, 1 mL was diluted again with 4 mL of acetic acid

5. Standard hydrogen peroxide (H$_2$O$_2$): 0.2 mM

**Procedure**

To 0.9 mL of phosphate buffer, 0.1 mL of supernatant and 0.4 mL of H$_2$O$_2$ were added. The reaction was arrested after 15, 30, 45 and 60 seconds by adding 2.0 mL of dichromate - acetic acid mixture. The tubes were kept in a boiling water bath for 10 minutes, cooled and the colour developed was read at 590 nm.

The specific activity was expressed as $\mu$M of H$_2$O$_2$ consumed/minute/mg of protein.

**4.2.10.2.3. Estimation of glutathione peroxidise (GPx, EC 1.11.1.19)**

The activity of glutathione peroxidise (GPx) in the cancer cells was measured by the method of Rotruck *et al.* (1973).

**Principle**

A known amount of enzyme preparation was allowed to react with H$_2$O$_2$ in the presence of GSH for a specified time period. Then the remaining GSH content was measured at 340 nm.

**Reagents**

1. Tris buffer: 0.4 M, pH 7.0
2. Sodium azide solution: 10 mM
3. Trichloro acetic acid (TCA): 10%
4. Ethylene diamine tetra acetic acid (EDTA) : 0.4 mM
5. H$_2$O$_2$ solution : 0.2 mM
6. Glutathione (GSH) solution : 2 mM

**Procedure**

To 0.2 mL of tris buffer, 0.2 mL of EDTA, 0.1 mL of sodium azide and 0.5 mL of sample were added. To this mixture, 0.2 mL of GSH followed by 0.1 mL of H$_2$O$_2$ was added. The contents were mixed well and incubated at 37° C for 10 minutes, along with a control containing all reagents except the sample. After 10 minutes, the reaction was arrested by the addition of 0.5 mL of 10% TCA. The tubes were centrifuged and the supernatant was assayed for GSH colorimetrically at 340 nm.

The activity was expressed as μg of GSH consumed/minute/mg of protein.

**4.2.10.2.4. Estimation of reduced glutathione (GSH)**

Reduced glutathione in the cancer cells was estimated by the method of Ellman (1959).

**Principle**

This method was based on the formation of 2-nitro-5-thiobenzoic acid (a yellow colour compound) when 5, 5’-dithio-bis (2-nitrobenzoic acid) (DTNB) was added to compounds containing sulphhydryl groups. The yellow color was read at 412 nm.

**Reagents**

1. Phosphate buffer : 0.1 M, pH 8.0
2. TCA: 5%
3. Ellman’s reagent: 34 mg of DTNB in 10 mL of 0.1% sodium citrate
4. Disodium hydrogen phosphate: 0.3 M
5. Standard glutathione solution: 10 mg/100 mL

Procedure

About 0.5 mL of sample was pipetted out and precipitated with 2 mL of 5% TCA. 2 mL of supernatant was taken after centrifugation. To this 1 mL of Ellman’s reagent and 4 mL of 0.3 M disodium hydrogen phosphate were added. The yellow colour developed was read in a spectrophotometer at 412 nm with a blank containing 1 mL of buffer.

The amount of glutathione was expressed as nM/mg protein.

4.2.11. Estimation of protein

Protein in the cancer cells was determined after trichloroacetic acid precipitation by the method of Lowry et al. (1951).

Principle

The phenolic group of amino acids tyrosine and tryptophan in protein reacts with the sodium tungstate molybdate and phosphate of Folin - Ciocalteau reagent to give a blue purple colored complex which is read colorimetrically at 620 nm. The intensity of the colour developed is proportional to the amount of protein in the sample.

Reagents

1. Trichloroacetic acid (TCA): 10%
2. Sodium hydroxide (NaOH): 0.1 N
3. Alkaline copper reagent:
   Reagent A: 2% sodium carbonate in 0.1 N NaOH
Reagent B: 0.5% copper sulphate in 1% sodium potassium tartarate
Reagent C: 50 mL of reagent A was mixed with 0.5 mL of reagent B just before use

4. Folin’s phenol reagent: The commercial reagent was diluted in the ratio of 1:2 with distilled water
5. Stock standard: 100 mg of bovine serum albumin / 100 mL of water
6. Working standard: 10 mL of the stock standard was diluted to 100 mL to get a working standard containing 0.1 mg / mL.

**Procedure**

About 0.5 mL of the cell supernatant was mixed with 0.5 mL of 10% TCA and centrifuged for 10 minutes. The precipitate was dissolved in 1 mL of 0.1 N NaOH. From this, an aliquot was taken and 4.5 mL of alkaline copper reagent was added. This was allowed to stand at room temperature for 10 minutes. 0.5 mL of Folin’s phenol reagent was added and the blue colour developed was read after 20 minutes at 620 nm. A standard curve was obtained with standard bovine serum albumin and was used to assay the protein level for enzyme activity.

Values are expressed as mg / dL.

**4.2.12. Detection of apoptotic morphological changes**

Morphological changes during apoptosis in cancer cells were measured by dual stains (acridine orange and ethidium bromide) according to the method of Lakshmi et al. (2008).

**Principle**

Acridine orange (AO) and ethidium bromide (EBr) were allowed to stain with DNA for the visualization of chromatin of dead apoptotic cells. Apoptotic nuclei exhibiting typical changes such as nuclear condensation and
segmentation were stained by AO/EBr. Ferulic acid and cisplatin treated cells (2 x 10^4/well) were seeded into 6-well plate and incubated in CO_2 incubator for 24 hours and then the apoptotic morphological changes were observed using a fluorescence microscope under blue filter.

**Reagents**

1. Methanol
2. Glacial acetic acid
3. Phosphate buffered saline (PBS)
4. Acridine orange (AO)
5. Ethidium Bromide (EBr)

**Procedure**

The control and FA and / or treated HeLa and SiHa cells were seeded in 6-well plates (3 x 10^4/well) and incubated in CO_2 incubator for 24 hours. The cells were fixed in methanol : glacial acetic acid (3:1) for 30 minutes at room temperature. The cells were washed in PBS and stained with AO/EBr in the ratio of 1:1. Stained cells were immediately washed again with PBS and viewed under a fluorescence microscope with a magnification of 40 X. The number of cells showing features of apoptosis was counted as a function of the total number of cells present in the field.

**4.2.13. Measurement of mitochondrial transmembrane potential changes**

Mitochondrial transmembrane potential changes in cancer cells was determined by Rhodamine 123 staining according to the method of Bhosle _et al._ (2005).
**Principle**

Alteration in mitochondrial membrane potential (depolarization) is an indication of early stages of apoptosis. Rhodamine 123 (Rh 123) is a lipophilic cationic dye highly specific for mitochondria. Upon staining, polarized mitochondria are marked by orange-red fluorescence and depolarized mitochondria are marked by green fluorescence.

**Reagents Required**

1. PBS: 1%
2. Fluorescent probe: Rhodamine 123
3. Stock standard: 1mg/mL of 1% PBS
4. Working standard: 10 µL from stock was made up to 1mL with PBS

**Procedure**

The changes in mitochondrial transmembrane potential were estimated in the control, ferulic acid alone treated, cisplatin alone treated and ferulic acid plus cisplatin treated cancer cells. Briefly, an aliquot of the above mentioned isolated cells (8 x 10^6 cells/mL) was made up to a final volume of 2 mL in normal PBS (pH 7.4). One mL aliquot of cells was taken to which fluorescent dye Rhodamine 123 (10 µg/mL) was added and incubated for 30 minutes in CO₂ incubator. The cells were washed with warm PBS solution and the images were captured under fluorescence microscope using blue barrier filter. Fluorescent measurements were made with excitation and emission filters set at 485 ± 10 nm and 530 ± 12.5 nm respectively. All initial fluorescent values (time 0) were found to differ from each other by less than 5%. Results were expressed as percentage increase in fluorescence calculated using the formula [(Ft30 - Ft0)/ [Ft0 x 100]]. Ft0 and Ft30 are the fluorescence intensities at 0 and 30 minutes.
4.2.14. Estimation of DNA damage by single cell gel electrophoresis (Comet assay)

DNA damage was estimated by alkaline single cell gel electrophoresis (Comet assay) according to the method of Singh et al. (1988).

Principle

In this method, the cells were first lysed to form nucleoids. During electrophoresis, DNA fragments (from damaged DNA) streamed towards anode, while the undamaged DNA trapped within the nucleus. When they are stained with propidium iodide (PI), damaged DNA would give the appearance of a comet tail and undamaged DNA would give a spherical appearance.

Reagents

1. Normal melting point agarose: 1%
2. Low melting point agarose: 0.8%
3. Lysis solution: 7.3 g of sodium chloride, 1.8 g of EDTA and 0.06 g of tris was mixed with 35 mL of distilled water. To this, 0.06 g of sodium hydroxide, 1 mL of triton X-100 was added and the solution was made up to 50 mL with distilled water
4. Electrophoresis buffer: 6 g of sodium hydroxide and 186 mg of EDTA was dissolved in 500 mL of distilled water. The solution was stored in the refrigerator 1 hour before electrophoresis
5. Neutralising buffer: 0.4 M tris-HCl, pH 7.4
6. Staining solution: Ethidium bromide (20 µg/mL)

Procedure

The slides were prepared by pouring 3–5 mL of 1% normal agarose over frosted glass slides (Gold Coin Microslides, Blue Label Scientifics). It
was allowed to dry at room temperature and placed in hot-air oven at 70 – 80\(^\circ\) C for 30 minutes.

Freshly suspended cancer cells from all groups (groups 1 - 4) were mixed with 200 \(\mu\)L of 0.8% low-melting point agarose (LMPA) (1:3 ratio). This mixture was cast on to frosted microscopic slides, immediately covered with cover slip and kept for 10 minutes in a refrigerator to solidify. Then, the cover slip was removed and a top layer of 100 \(\mu\)L of LMPA was added and the slides were again cooled for 10 minutes. The cells were then lysed by immersing the slides in the lysis solution for 1 hour at 4\(^\circ\) C. After lysis, slides were placed in a horizontal electrophoresis tank. The unit was filled with electrophoresis buffer to a level of 0.25 cm above the slides. The cells were exposed to the alkaline electrophoresis solution for 20 minutes to allow DNA unwinding. Electrophoresis was conducted in cold condition for 20 minutes at 25 V and 300 mA. After electrophoresis, the slides were placed horizontally and neutralised with tris-HCl buffer. Finally, 50 \(\mu\)L of ethidium bromide was added to each slide and analysed using a fluorescence microscope. To prevent additional DNA damage, all steps were conducted under dim light or in the dark.

Twenty five images were randomly selected from each sample and were examined at 40 X magnification in a fluorescence microscope connected to a personal computer-based image analysis system. Images were captured with a digital camera with networking capability and analyzed by image analysis software, CASP, developed by Konca et al. (2003). The relative amount of DNA appearing in the tail of the comet (percent tail DNA), tail length and tail moment (% tail DNA x tail length) were linearly related to DNA break frequency.
4.2.15. Determination of nuclear condensation by DAPI Staining

The nuclear condensation during apoptotic cell death in cancer cells was detected by DAPI staining adopted from Rashmi et al. (2003).

Principle

4', 6 - diamidino-2-phenylindole (DAPI) is a fluorescent stain that binds strongly to A-T rich regions of DNA. DAPI can pass through an intact cell membrane; therefore, it can be used to stain both live and fixed cells. It passes through the membrane less efficiently in live cells so, the effectiveness of the stain is lower when bound to double-stranded DNA. Its absorption maximum is at 358 nm and its emission maximum is at 461 nm.

Materials Required

1. Cultured cells
2. Growth medium
3. Trypsin
4. Microslides
5. DAPI stain (4',6 - diamidino-2-phenylindole )

Procedure

Cancer cells grown in complete DMEM medium were treated with FA or cisplatin alone or FA (pretreatment) and cisplatin for specific time periods as mentioned in the table 4.2. After incubation the cells were trypsinized and the harvested cells were washed once with PBS, then resuspended in PBS containing 0.1 % Triton X (to induce holes in the cell membrane which would increase permeability) and incubated for 10 minutes in ice. Then, the cells were spun down and resuspended at a concentration of 5000 cells/µl in 4% PBS buffered paraformaldehyde solution containing 10 µg/mL of 4'6-
diamidino-2-phenylindole (DAPI, Sigma). 10 µL of this suspension were placed on a glass slide and covered with a cover slip ready for viewing. The morphology of the cells’ nuclei was observed using a fluorescence microscope (Olympus BH Series) at the excitation wavelength of 350 nm. Nuclei are considered to have the normal phenotype when glowing bright and homogenously. Apoptotic nuclei can be identified by the condensed chromatin gathering at the periphery of the nuclear membrane or a total fragmented morphology of nuclear bodies.

4.2.16. Determination of molecular markers in apoptosis

The apoptotic and non-apoptotic proteins were determined by western blotting according to the method of Laemmli (1970).

The protein expressions of p53, Bcl-2, caspase 3 and caspase 9 in treated and untreated cervical cancer cells was carried out by immunoblot analysis. The results were normalized to β-actin gene expression.

Principle

Following the protein estimation, the samples were separated using SDS- polyacrylamide gel electrophoresis (PAGE) and the separated molecules are blotted onto a polyvinylidene fluoride (PVDF) membrane. After blocking, the primary antibody was added and allowed to bind to the protein followed by washing (which removes non-specifically bound antibody); then an enzyme-labeled secondary antibody was added, to detect the primary antibody. The location of the secondary antibody was determined by adding an appropriate substrate for the enzyme conjugated to the secondary antibody.
Reagents

1. Acrylamide stock: 30% acrylamide, 0.8% N, N'-methylene bisacrylamide
2. Separating gel buffer: 1.5 M Tris, pH 8.8
3. Sample buffer: 0.5 M Tris, pH 6.8
4. Sodium dodecylsulfate (SDS): 10%
5. Ammonium per sulfate (APS): (10%)
6. N, N, N', N'-tetramethylethylenediamine (TEMED)
7. Separating gel overlaying solution: Water-saturated isobutanol
8. Sample buffer
   a. Tris (0.5M, pH6.8) : 2.5 mL
   b. SDS (10%) : 4.0 mL
   c. Glycerol (100%) : 2.0 mL
   d. β – Mercaptoethanol :0.8 mL (or 1 M DDT-0.5 mL)
   e. Bromophenol blue (0.1%) : 300 µl
   f. Distilled water : 10.0 mL
9. Running gel buffer
   a. Tris : 6.05 g
   b. Glycine : 28.80 g
   c. 10% SDS : 10.0 mL or (1.0 g)
   d. Distilled water : 1000 mL
10. Staining solution
    a. Coomassie brilliant blue R250: 300 g
    b. Methanol : 80 mL
    c. Acetic acid : 20 mL
    d. Distilled water : 100 mL
11. Destaining solution
   
a. Acetic acid: 100 mL  
b. Methanol: 300 mL  
c. Distilled water: 1000 mL  

Procedure

Preparation of whole cell lysate

FA and/or cisplatin treated cells were washed thrice with ice-cold phosphate buffered saline (PBS) and lysed in ice-cold lysis buffer (50 mM Tris HCl, pH 7.5, with 120 mM NaCl, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1% NP-40 (Tergitol-type NP-40, a nonyl phenoxypolyethoxylethanol) and protease inhibitor cocktail. Cellular-lysates were clarified by centrifugation at 15,000 rpm for 30 minutes. Supernatant was used as whole cell protein sample. Protein concentration was quantified using protein assay reagent (Pierce).

Transfer of proteins to membrane

Samples containing 50 μg of total cellular proteins were loaded and separated using 10% SDS polyacrylamide gel electrophoresis. Following electrophoresis, the proteins were transferred from the gel to a membrane by using semi-dry blotting system. Before assembling the transfer system, soaked (polyvinylidene difluoride) PVDF membrane in methanol for 10 minutes and blotting papers in cold transfer buffer. Sandwich of blotting paper, membrane, gel and blotting paper was prepared and placed in the transfer apparatus. Few drops of transfer buffer was added and subjected to an electric current 20 V for 1 hour under cold condition. After the transfer, the sandwich was removed.
from the transfer system. Membrane was stained with 0.5% ponceau in 1% acetic acid to confirm equal loading and then washed with distilled water.

The PVDF membrane was blocked with 5% blocking solution (containing 5% BSA in 0.5 M Tris-buffered saline, pH 7.5) for 2 hours to reduce the non-specific protein binding sites and then incubated with β-actin (rabbit polyclonal; 1:500), Bcl-2 (rabbit polyclonal; 1:750), caspase 3 and 9 (rabbit polyclonal), in blocking solution with gentle shaking overnight at 4º C. After this, the membranes were washed with TBST (Tris-buffered saline and 0.05% Tween-20) thrice for 10 minutes interval and then incubated with horseradish peroxidase conjugated secondary antibody (diluted 1:1000; anti-rabbit, anti-goat IgG and anti-mouse conjugated to horseradish peroxidase) in blocking solution for 2 hours at room temperature. Then the membranes were washed with TBST thrice for 10 minutes interval.

Protein bands were visualized by an enhanced chemiluminescence method using ECL-kit (GenScript ECL kit, USA) and scanned using a scanner.

4.2.17. Statistical analysis

All values were expressed as means ± SD. The data were statically analyzed using one-way analysis of variance (ANOVA) and the significant difference among treatment groups were evaluated by Duncan’s Multiple Range Test (DMRT). The results were considered statistically significant at P<0.05. All statistical analysis were made using SPSS 17.0 (Statistical package for social sciences) software package (SPSS, Tokyo, Japan).
4.3. **RESULTS**

Resistance of cancer cells to anticancer drugs such as cisplatin is a major obstacle to cancer chemotherapy. In the present study, the sensitizing effects of ferulic acid on cisplatin chemotherapy by its potential to alter cytotoxicity, clonogenic cell survival, intracellular reactive oxygen species (ROS), cellular lipid peroxidation and antioxidant status, morphological changes in apoptosis, mitochondrial membrane potential, DNA damage, nuclear condensation and levels of apoptotic and anti-apoptotic signaling proteins such as p53, caspase 3, caspase 9 and Bcl-2 in human cervical cancer cell lines (HeLa and SiHa) were investigated.

4.3.1. **Drug sensitivity and dose fixation studies of FA and cisplatin in cervical cancer cells**

The cell sensitivity of HeLa and SiHa cells to *in vitro* treatment with FA and cisplatin alone was first evaluated. Figure 4.3 and figure 4.4 showed the percentage of cytotoxicity induced by FA and cisplatin on HeLa and SiHa cell lines. A significant increase (p<0.05) in the cytotoxicity of both cell lines were observed with an increase in the concentration of FA and cisplatin in a dose dependant manner. A 100% cell death of both cell lines was attained at 20 µg for FA and at 10 µg for cisplatin. Hence, the IC 50 value for FA and cisplatin for HeLa and SiHa cell lines was calculated to be 10 µg/ml and 5 µg/ml respectively, which were used further in the present study. These results suggested that each drug individually decreased the viability of cancer cells but in combination the effects may be increased.
Data are presented as the means ± SD; n=6

FA (5-50 µg) on percentage cytotoxicity in HeLa and SiHa cells as measured by the reduction of MTT dye (MTT assay).

IC 50 value for FA was found to be 10 µg for both HeLa and SiHa cells.

**Figure 4.3. Cytotoxicity of FA on HeLa and SiHa cells**
Data are presented as the means ± SD; n=6
Cisplatin (5-50 µg) on percentage cytotoxicity in HeLa and SiHa cells as measured by the reduction of MTT dye (MTT assay)
IC 50 value for cisplatin is found to be 5 µg for both HeLa and SiHa cells

**Figure 4.4. Cytotoxicity of cisplatin on HeLa and SiHa cells**
Data are presented as the means ± SD; n=6
p<0.05, the values not sharing a common superscript letter (a–d) are significantly different
Cells were pretreated with FA (10 µg/ml) 1 hour before cisplatin (5 µg/ml) treatment

**Figure 4.5. Synergistic effect of FA pretreatment on the cytotoxicity of cisplatin treatment** HeLa and SiHa cells
Data are presented as the means ± SD; n=6
FA (10 µg/ml) pretreated HeLa and SiHa cells were exposed to different concentrations of cisplatin (1-11 µg/ml). The cell viability of cisplatin was measured by MTT assay.
The IC 50 value of cisplatin was reduced from 5 µg/ml to 3 µg/ml in the presence of FA.

Figure 4.6. Effect of FA pretreatment on the IC50 value of cisplatin
4.3.2. Synergistic effect of FA pretreatment on cytotoxicity of HeLa and SiHa cells treated with cisplatin

According to the optimum dosage of FA indicated by the above assays, 10µg/ml of FA alone or in combination with 5µg/ml of cisplatin was applied. As shown in the figure 4.5, the cell viability in FA primed-cisplatin treated cells was approximately 17.12% for HeLa and 20.47% for SiHa. But in FA alone treated cells, the viability was observed to be about 60.43% for HeLa and 65.31% for SiHa and in cisplatin alone treated cells, the viability was found to be 55.27% for HeLa and 52.34% for SiHa. Hence, the cell viability indices of FA pretreated-cisplatin treated cells were significantly (p<0.05) decreased in both HeLa and SiHa cell lines when compared to cisplatin alone treated cells. Treatment of cervical cancer cells with a combination of cisplatin and FA markedly reduced tumor cell viability, in contrast to the results for cisplatin alone. FA also induced cell death in a dose-dependent manner (Figure 4.3). When FA and cisplatin were combined, the cytotoxicity was greatly induced.

4.3.3. Effect of FA pretreatment on the IC 50 value of cisplatin

In the presence of FA (10µg/ml), the IC 50 value of cisplatin was significantly (p<0.05) decreased from 5 µg/ml to 3 µg/ml (Figure 4.6). Moreover, while keeping the concentration of FA constant (10µg/ml) and varying the concentrations of cisplatin (1-11µg/ml), the cytotoxicity of cisplatin was increased. These results suggested that FA may synergistically combine with cisplatin in blocking the cell growth of human cervical cancer cells. The therapeutic synergy of FA and cisplatin was confirmed further in other assays.
4.3.4. Effect of FA and/or cisplatin on clonogenic cell survival of HeLa and SiHa cells

Treatment of HeLa and SiHa cell lines with FA and/or cisplatin resulted in the decline of cell survival (Figure 4.7). FA pretreatment showed significant (p<0.05) decrease in the surviving fractions of both cell lines when compared to FA or cisplatin alone treated cells. Also it was found that treatment with FA alone reduced the number of cell colonies.

4.3.5. Effect of FA and/or cisplatin on intracellular ROS levels in HeLa and SiHa cells

The levels of intracellular ROS were significantly (p<0.05) increased in FA or cisplatin alone treated groups when compared to control group. In cells pretreated with FA before cisplatin treatment, a significant (p<0.05) increase in the percentage of intracellular ROS was observed when compared to untreated and FA or cisplatin alone treated cells (Figure 4.9). The photomicrograph (Figure 4.8) captured in different treatment regimens of HeLa and SiHa cell lines showed a gradation in the fluorescent intensity from low to high depicting increased intracellular ROS generation due to treatment. In particular, cells pretreated with FA before cisplatin treatment showed significantly increased fluorescence when compared to other groups.
Data are presented as the means ± SD; n=6
a–d p<0.05, the values not sharing a common superscript letter are significantly different
*Cells were pretreated with FA (10 µg/ml) 1 hour before cisplatin (5 µg/ml) treatment

**Figure 4.7. Effect of FA and/or cisplatin on clonogenic cell survival of HeLa and SiHa cells**
Figure 4.8. Photomicrograph of intracellular ROS in FA and/or cisplatin treated HeLa and SiHa cells (40x)
Data are presented as the means ± SD; n=6
a–d p<0.05, the values not sharing a common superscript letter are significantly different

**Figure 4.9. Effect of FA and/or cisplatin on intracellular reactive oxygen species (ROS) levels in HeLa and SiHa cells**
4.3.6. Effect of FA and/or cisplatin on cellular lipid peroxidation and antioxidant status in HeLa and SiHa cells

Cellular lipid peroxidation and antioxidant levels play an important role in the treatment of cancer. So in the present study, the levels of lipid peroxidation and antioxidants were measured in FA and/or cisplatin treated cells. Cells treated with FA or cisplatin alone increased the levels of lipid peroxidation indices such as thiobarbituric acid reactive substances (TBARS), conjugated dienes (CD) and lipid hydroperoxides (LOOH). Whereas, cells primed with FA prior to cisplatin treatment significantly (p<0.05) enhanced the levels of lipid peroxidation indices when compared to FA or cisplatin alone treated cells and untreated cells (Figure 4.10).

Table 4.3 shows the alteration in the levels of antioxidants such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) due to FA and/or cisplatin treatment. FA and/or cisplatin treatment significantly (p<0.05) decreased the levels of these antioxidants when compared to control cells. A more pronounced effect lipid peroxidation decline was found in the cisplatin treated cells primed with FA when compared to FA or cisplatin alone treated HeLa and SiHa cells.
Figure 4.10. Effect of FA and/or cisplatin on lipid peroxidation markers in untreated and treated cervical cancer cells

Lipid peroxidation markers Vs nM/mg protein; Values are given as mean ± S.D.; n=6. Values not sharing a common superscript (a, b, c & d) differ significantly at p<0.05 vs. control (DMRT).
<table>
<thead>
<tr>
<th>Groups</th>
<th>Superoxide dismutase (SOD)</th>
<th>Catalase (CAT)</th>
<th>Glutathione peroxidase (GPx)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HeLa</td>
<td>SiHa</td>
<td>HeLa</td>
</tr>
<tr>
<td>Control</td>
<td>17.38 ± 1.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.55 ± 2.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.45 ± 0.53&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>FA</td>
<td>12.60 ± 1.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.85 ± 1.81&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.98 ± 0.30&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>10.87 ± 1.10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15.05 ± 1.06&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.76 ± 0.21&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>FA + Cisplatin</td>
<td>5.08 ± 0.42&lt;sup&gt;d&lt;/sup&gt;</td>
<td>12.63 ± 1.10&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.82 ± 0.09&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>e</sup> Enzyme concentration required for 50% inhibition of nitroblue tetrazolium reduction in one minute/mg protein

<sup>a</sup> µM of H₂O₂ consumed / minute/mg protein

<sup>¥</sup> µg of glutathione consumed / minute/mg protein

Values are given as means ± S.D., n=6.

Values not sharing a common marking (a, b, c & d) differ significantly at p< 0.05 vs. control (DMRT).

**Table 4.3.** Effect of FA and/or cisplatin on superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in HeLa and SiHa cells
### Table 4.4. Effect of FA and/or cisplatin on reduced glutathione (GSH) in HeLa and SiHa cells

<table>
<thead>
<tr>
<th>Groups</th>
<th>Reduced glutathione (GSH)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HeLa</td>
</tr>
<tr>
<td>Control</td>
<td>3.07 ± 0.27(^a)</td>
</tr>
<tr>
<td>FA</td>
<td>2.34 ± 0.22(^b)</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>1.66 ± 0.11(^c)</td>
</tr>
<tr>
<td>FA + Cisplatin</td>
<td>0.76 ± 0.06(^d)</td>
</tr>
</tbody>
</table>

\(^a\) nM/mg protein
Values are given as means ± S.D.; n=6
Values not sharing a common marking (a, b, c & d) differ significantly at p < 0.05 vs. control (DMRT)

4.3.7. Effect of FA and/or cisplatin on reduced glutathione (GSH) levels in HeLa and SiHa cells

The tripeptide glutathione (GSH) is a detoxifying antioxidant which plays a vital role in chemotherapy and chemoresistance. In table 4.4, FA or cisplatin treatment caused a decrease in GSH levels when compared to untreated HeLa and SiHa cells. Further, FA pretreated cells significantly (p<0.05) reduced the levels of GSH when compared to other treatment regimens.
4.3.8. Effect of FA and/or cisplatin on morphological changes of apoptosis in HeLa and SiHa cells

The photomicrographs on morphological changes of apoptosis in HeLa and SiHa cells treated with FA and/or cisplatin was shown in figure 4.11. Cells with characteristic apoptotic features such as condensed or fragmented chromatin were observed in FA and/or cisplatin treated cells. The signs of apoptotic changes where more in cisplatin treated cells primed with FA. Non-apoptotic chromatin showing green fluorescence alone was seen in untreated control cells.

Effect of FA and/or cisplatin in percentage of apoptosis of HeLa and SiHa cells was shown in table 4.5. The percentage of apoptotic cells was found to be increased in cells treated with FA or cisplatin alone when compared to untreated control cells. In cancer cells pretreated with FA and then treated with cisplatin, the number of apoptotic cells was found to be increased significantly (p<0.05) when compared to other treatment groups.
<table>
<thead>
<tr>
<th>Groups</th>
<th>% of Apoptotic cells</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HeLa</td>
<td>SiHa</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5.62 ± 0.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.32 ± 1.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>FA</td>
<td>24.75 ± 1.53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.66 ± 1.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td>67.80 ± 5.44&lt;sup&gt;c&lt;/sup&gt;</td>
<td>70.23 ± 7.56&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>FA + Cisplatin</td>
<td>88.48 ± 7.27&lt;sup&gt;d&lt;/sup&gt;</td>
<td>93.01 ± 9.56&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

Values are given as means ± S.D.; n=6
Values not sharing a common marking (a, b, c & d) differ significantly at p < 0.05 vs. control (DMRT)

Table 4.5. Effect of FA and/or cisplatin on percentage of apoptotic cells in treated and untreated HeLa and SiHa cells by Dual staining method

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mitochondrial membrane potential (MMP)*</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HeLa</td>
<td>SiHa</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>525.64 ± 48.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>367.88 ± 35.93&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>FA</td>
<td>467.98 ± 43.54&lt;sup&gt;b&lt;/sup&gt;</td>
<td>313.26 ± 31.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td>403.71 ± 40.71&lt;sup&gt;b&lt;/sup&gt;</td>
<td>296.17 ± 25.31&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>FA + Cisplatin</td>
<td>374.22 ± 32.77&lt;sup&gt;c&lt;/sup&gt;</td>
<td>234.91 ± 22.62&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

* Fluorescence Intensity; Values are given as means ± S.D.; n=6
Values not sharing a common marking (a, b, c & d) differ significantly at p < 0.05 vs. control (DMRT)

Table 4.6. Effect of FA and/or cisplatin on mitochondrial membrane potential (MMP) in cervical cancer cells HeLa and SiHa
Arrow marks indicate the presence of apoptotic cells with characteristic morphological changes in FA and/or cisplatin treated HeLa and SiHa cells.

Figure 4.11. Effect of FA and/or cisplatin on apoptotic morphological changes in HeLa and SiHa cells by Dual staining (40x)
Photomicrograph shows changes in fluorescence from orange red to green indicating increased depolarization of mitochondrial membrane.

**Figure 4.12.** Effect of FA and/or cisplatin on mitochondrial membrane potential changes in HeLa and SiHa cells (40x)
4.3.9. Effect of FA and/or cisplatin on mitochondrial membrane potential changes in HeLa and SiHa cells

Figure 4.12 show the effect of FA and/or cisplatin on mitochondrial transmembrane potential changes in HeLa and SiHa cells. FA and/or cisplatin treatment significantly increased the mitochondrial depolarization in cancer cells when compared to the control cells. There is a significant (p<0.05) increase in the fluorescent intensity in FA and cisplatin treated cells when compared to HeLa and SiHa cells treated with FA or cisplatin alone as indicated in table 4.6. This showed that FA and cisplatin treatment significantly increased mitochondrial depolarization than FA or cisplatin treated cells.

4.3.10. Effect of FA and/or cisplatin on DNA damage in HeLa and SiHa cells

Figure 4.13. shows photomicrograph of DNA damage (as comet) in HeLa and SiHa cells treated with FA and/or cisplatin. The untreated cells showed largely non-fragmented intact nucleoid with no or less comet tail, whereas FA and/or cisplatin treated cells showed the presence of tail DNA, which appeared as comet during single cell gel electrophoresis. FA pretreated cells prior to cisplatin treatment resulted in a further increase in DNA damage.

The extent of DNA damage as measured by % of tail DNA, tail length, tail moment and olive tail moment in different treatment groups were shown in table 4.7. (a-d). In the present study, FA or cisplatin alone treatment increased DNA damage in HeLa and SiHa cell lines when compared to untreated cancer cells. FA pretreatment prior to cisplatin treatment caused significantly (p<0.05) increased % tail DNA, tail length, tail moment and olive tail moment when compared to other groups.
Arrow marks show the presence of tail DNA indicating DNA damage in cervical cancer cells.

**Figure 4.13. Photomicrograph showing DNA damage in Hela and SiHa cells treated with FA and/or cisplatin (40x)**
Table 4.7a. Effect of FA and/or cisplatin on % tail DNA (DNA damage) in HeLa and SiHa cells by Comet assay

<table>
<thead>
<tr>
<th>Groups</th>
<th>% of Tail DNA</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HeLa</td>
<td>SiHa</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.63 ± 0.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.03 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>FA</td>
<td>5.00 ± 0.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.41 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td>19.26 ± 1.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.62 ± 0.76&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>FA + Cisplatin</td>
<td>21.11 ± 1.81&lt;sup&gt;c&lt;/sup&gt;</td>
<td>13.72 ± 1.19&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

Values are given as means ± S.D.; n=6
Values not sharing a common marking (a, b, c & d) differ significantly at p < 0.05 vs. control (DMRT)

Table 4.7b. Effect of FA and/or cisplatin on % tail length (DNA damage) in HeLa and SiHa cells by Comet assay

<table>
<thead>
<tr>
<th>Groups</th>
<th>% of Tail length</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HeLa</td>
<td>SiHa</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>11.20 ± 1.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.00 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>FA</td>
<td>16.83 ± 1.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.36 ± 0.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td>45.06 ± 3.69&lt;sup&gt;c&lt;/sup&gt;</td>
<td>13.63 ± 1.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>FA + Cisplatin</td>
<td>58.65 ± 4.62&lt;sup&gt;d&lt;/sup&gt;</td>
<td>27.91 ± 2.45&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

Values are given as means ± S.D.; n=6
Values not sharing a common marking (a, b, c & d) differ significantly at p < 0.05 vs. control (DMRT)
### Table 4.7c. Effect of FA and/or cisplatin on tail moment (DNA damage) in HeLa and SiHa cells by Comet assay

<table>
<thead>
<tr>
<th>Groups</th>
<th>Tail Moment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HeLa</td>
</tr>
<tr>
<td>Control</td>
<td>2.43 ± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>FA</td>
<td>3.17 ± 0.30&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>12.78 ± 1.09&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>FA + Cisplatin</td>
<td>17.11 ± 1.17&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are given as means ± S.D.; n=6
Values not sharing a common marking (a, b, c & d) differ significantly at p < 0.05 vs. control (DMRT)

### Table 4.7d. Effect of FA and/or cisplatin on olive tail moment (DNA damage) in HeLa and SiHa cells by Comet assay

<table>
<thead>
<tr>
<th>Groups</th>
<th>Olive Tail Moment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HeLa</td>
</tr>
<tr>
<td>Control</td>
<td>3.30 ± 0.35&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>FA</td>
<td>4.70 ± 0.36&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>10.33 ± 1.08&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>FA + Cisplatin</td>
<td>18.81 ± 1.12&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are given as means ± S.D.; n=6
Values not sharing a common marking (a, b, c & d) differ significantly at p < 0.05 vs. control (DMRT)
Figure 4.14. Effect of FA and/or cisplatin on nuclear condensation in HeLa and SiHa cells (40x)

Arrow marks indicate the presence of apoptotic nuclei in HeLa and SiHa cells treated with FA and/or cisplatin.
4.3.11. Effect of FA and/or cisplatin on nuclear condensation of HeLa and SiHa cells

The effects of FA and/or cisplatin on nuclear condensation of HeLa and SiHa cell lines were depicted in the figure 4.14. Untreated cells showed intact nuclei which were seen clearly in the photomicrograph stained with DAPI. Whereas in the cells treated with FA or cisplatin, the nuclear material was condensed with signs of fragmentation. In FA and cisplatin treated cells, complete fragmentation was observed.

4.3.12. Effect of FA and/or cisplatin on the expression of apoptotic and non-apoptotic proteins in HeLa and SiHa cells

The effects of FA and/or cisplatin on the expression of the proteins Bcl2, caspase 3, caspase 9 and p53 in HeLa and SiHa cells were shown in figure 4.15a and 4.15b. In both HeLa and SiHa cell lines, pretreatment of FA prior to cisplatin treatment increased the levels of pro-apoptotic and apoptotic proteins such as caspase 3, caspase 9 and p53. Also it was found that, FA primed cisplatin treatment decreased the levels of anti-apoptotic protein Bcl2. This showed that the treatment of FA and cisplatin induced apoptosis in HeLa and SiHa cells.
Representative immunoblots of Bcl-2, caspase 3, caspase 9 and p53. β-actin was used as an internal control.

Protein samples of HeLa cells treated with FA and/or cisplatin were resolved on SDS-PAGE and probed with corresponding antibodies.

**Figure 4.15a. Effect of FA and/or cisplatin on the expression of the proteins Bcl2, caspase 3, caspase 9 and p53 in HeLa cells**
<table>
<thead>
<tr>
<th>CONTROL</th>
<th>FA</th>
<th>CISPLATIN</th>
<th>FA+ CISPLATIN</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Bcl-2" /></td>
<td><img src="image2" alt="Caspase-3" /></td>
<td><img src="image3" alt="Caspase-9" /></td>
<td><img src="image4" alt="p53" /></td>
</tr>
</tbody>
</table>

Representative immunoblots of Bcl-2, caspase 3, caspase 9 and p53. β-actin was used as an internal control.

Protein samples of SiHa cells treated with FA and/or cisplatin were resolved on SDS-PAGE and probed with corresponding antibodies.

**Figure 4.15b. Effect of FA and/or cisplatin on the expression of the proteins BCl2, caspase 3, caspase 9 and p53 in SiHa cells**
4.4. DISCUSSION

Cervical cancer is the leading cause of cancer death of women in the world, besides surgical resection. Cisplatin chemotherapy is the most commonly used method in cervical cancer treatment, especially for the patients with advanced, persistent or recurrent cervical cancer.

Inherent or acquired drug resistance remains a major obstacle to successful chemotherapy. Tumors may be intrinsically drug-resistant or acquire resistance during treatment. Therefore, exploring the alternative therapeutic modalities is necessary to overcome drug resistance in cancer treatment.

Various strategies have been explored to overcome tumor drug resistance, among which the combination of chemotherapy with plant polyphenols as a chemosensitizer has emerged as a promising one. Polyphenols are a family of natural compounds that are widely distributed in plant foods. Due to its tumor suppressive potential, the use of polyphenols as chemosensitizers provides an important milestone in cancer chemotherapy (Garg et al. 2005).

In the present study, the chemosensitizing potential of Ferulic acid (FA) in human cervical cancer cell lines (HeLa and SiHa) subjected to cisplatin chemotherapy was evaluated. And to the best of our knowledge the chemosensitizing effect of FA was reported for the first time.

Cisplatin is a known cytotoxic agent which can bring cytotoxicity of cancer cells (Basu and Krishnamurthy 2010). But an enhanced cytotoxic effect was seen in cisplatin treated cancer cells primed with FA compared to cisplatin alone treated cells. It was observed that FA (10µg/ml) when combined (as pretreatment) with cisplatin (5µg/ml), could greatly inhibit the
cell growth which may be due to the prooxidant activity of ferulic acid on cancer cells (Lee and Lee. 2006). This prooxidant property might disrupt mitochondrial dehydrogenase activity which might be the one of the reasons for increased cytotoxicity. In fact, FA itself was found to be cytotoxic to cervical cancer cells (figure 4.3). Previous studies by Karthikeyan et al. (2011) and Bandugula and Prasad (2012) have also shown that FA induced cytotoxicity in cervical cancer and lung cancer cells. This indicated that FA pretreatment sensitized cancer cells to cisplatin treatment.

The important criterion in chemosensitization is the decrease in the optimum dosage of chemotherapeutic drugs. In cells treated with a combination of FA and cisplatin (by keeping the doses of FA constant and varying the concentration of cisplatin), the IC 50 value (5µg) of cisplatin was decreased to 2-3µg. As a result of this, the cytotoxic effect of cisplatin was enhanced further. The enhanced cytotoxicity may be due to increased DNA cross linking by cisplatin or increased cellular accumulation of platinum or decreased repair of drug induced DNA damage (Sarna et al. 2007). A therapeutic synergy between FA and cisplatin in combination was observed in the present study.

The results of the clonogenic cell survival assay showed significantly decreased colony formation ability in the cells pretreated with FA before cisplatin treatment than the cells treated with FA or cisplatin alone. This suggested that the combination of FA and cisplatin not only induced death in tumor cells, but also reduced the number of cells with reproductive capacity after treatment. However, the biological mechanism responsible for increased therapeutic gain might deserve further investigation.

Cell death is often associated with a strong production of ROS. Cells pretreated with FA before cisplatin treatment caused a rapid increase of intracellular reactive oxygen species (ROS) by increase in DCF fluorescence
in HeLa and SiHa cell lines (figure 4.8) when compared to FA or cisplatin alone treated cells. The increased ROS levels during the FA pretreatment might be due to its prooxidant property. The prooxidant activity of FA was also confirmed in many similar studies (Karthikeyan et al. 2011; Bandugula and Prasad 2012). Increased intracellular ROS levels disrupt the biological membranes and cause cytotoxicity (Kovacic and Cooksy 2005). Moreover, the potent inducers of pro-apoptotic and apoptotic pathways are mediated through the production of ROS (Garg and Aggarwal 2002). Increase in ROS also increases the cells’ sensitivity to cisplatin treatment (Torigoe et al. 2005).

In the present study, a significant increase in lipid peroxidation levels in cervical cancer cells was observed. Increased ROS in turn are capable of initiating and promoting oxidative damage to the cells in the form of lipid peroxidation (LPO). The prooxidant activity of FA, a hydroxy cinnamic acid derivative might have played a significant role in elevating the levels of LPO. Prooxidant activity of hydroxy cinnamic acids on DNA damage and lipid peroxidation in the presence of Cu (II) ions and the structure-activity relationship have been elucidated (Zheng et al. 2008). In this study, significant decreased activities of antioxidant enzymes such as SOD, CAT and GPx was observed in FA primed cisplatin treated cancer cells than in FA or cisplatin alone treated cells. Many studies suggested that antioxidant enzymes are critical in protecting normal cells against tumor-promoting agents. But antioxidants in high amounts in cancer cells may contribute for their chemoresistance (Landriscina et al. 2009). Cisplatin also increases free radical production and decreases antioxidant production (Yilmaz et al. 2004).

Cellular glutathione (GSH) has been shown to be crucial for regulation of cell proliferation, cell cycle progression and apoptosis (Chen et al. 2005). A prominent decrease of GSH levels in cancer cells treated with FA was also noticed. Indap et al. (2006) reported that exposure of malignant cells to FA
leads to cell cycle arrest and cytotoxicity due to the depletion of GSH by FA. Depletion of GSH led to an increase in the sensitivity of cervical cancer cells to cisplatin (Indap et al. 2006). Previous studies have shown that phytochemicals depleted intracellular antioxidants, thereby induced cancer cell death (Bhosle et al. 2005). The role of GSH in DNA synthesis and its involvement in the repair of DNA damage has already been reported (Khynriam and Prasad 2003). Therefore reduction the levels of GSH in cancer cells may facilitate the DNA damage further.

Cells which die by apoptosis are characterized by morphological changes which include nuclear condensation, membrane blebbing and DNA fragmentation. Research evidences showed that FA has pro-apoptotic activity which leads particularly the cancer cells to apoptotic morphological changes and finally to suicidal death (Bandugula and Prasad 2012). In cells treated with FA and cisplatin, the apoptotic morphological changes and the percentage of apoptosis were significantly increased than FA or cisplatin alone treated cells. The cytotoxic activity, mitochondrial membrane potential changes and DNA damage potential by FA may be the reasons for this increased apoptotic changes.

Loss of mitochondrial potential ($\psi_m$) is an early stage of apoptosis. Mitochondrion is one of the most important organelles in regulating cell death as well as a marker of apoptosis (Metodiewa et al. 1999). During apoptosis, there would be changes in the membrane potential of mitochondria (Prasad et al. 2011). Depolarization of the membrane appears to be an important step in programmed cell death (Franco et al. 2006). In the present study, FA primed cisplatin treatment significantly increased mitochondrial depolarization in cancer cells when compared to FA or cisplatin alone treated cells. Growth inhibition due to the modulation of mitochondrial membrane potential of cancer cells by FA might be a reason for the increased cytotoxicity in cisplatin
treated cancer cells primed with FA (Hsu and Liu 2002). Reports of Galati et al. (2002) have showed that polyphenols alter the mitochondrial membrane potential and induce mitochondrial collapse. Caffeic acid, a hydroxy cinnamic acid derivative, also increased the depolarization of mitochondria (Prasad et al. 2011). Similar effects of FA on mitochondrial membrane potential were reported by Bandugula and Prasad (2012) on lung cancer cells.

DNA has been reported to be the critical site for cisplatin induced cytotoxicity. The mechanism of action of cisplatin is its ability to form covalent adducts with genomic DNA which leads to irreversible DNA damage as a result of DNA-platinum complexes (Sarna et al. 2007). In the present study, DNA damage was significantly increased in FA and cisplatin treated cells than FA or cisplatin alone treated cells. It is also suggested that FA enhanced the influx of cisplatin into tumor cells, leading to increased DNA damage. Reports revealed that FA could potentiate the activity of platinum complexes. Enhanced uptake of cisplatin by tumor cells might be due to modulation of permeability of the tumor cell membrane by altering the level of lipid peroxidation by FA (Indap et al. 2006).

A study by Zheng et al. (2008) showed that FA exhibited a remarkable DNA damaging activity than the other hydroxy cinnamic acid derivatives. In the presence of Cu, phenolic acids generate the •OH radical in a fenton-type reaction, and this hydroxyl radical has been proved to be very lethal to nucleic acids. The electron transfer between the hydroxy cinnamic acid FA and Cu (II) played a crucial role in DNA damage (Zheng et al. 2008). Another possible reason for DNA damage in cancer cells may be due to the increased intracellular accumulation of ROS by FA pretreatment. ROS and copper ions played an important role in FA induced DNA damage in cancer cells. Damage to DNA by ROS and RNS (Reactive Nitrogen Species) is reported to initiate signaling cascades and results in activation of transcription of specific groups
of genes which may lead to apoptosis (Valko et al. 2007). Genotoxic damage induces cell cycle arrest and/or apoptosis, an active physiological mode of death. Reed (2003) pointed out that a number of anticancer drugs are genotoxic and their damaging effects upon cells are mediated by this mechanism.

Hallmarks of the terminal stages of apoptosis are genomic DNA fragmentation and chromatin condensation. Chromatin condensation causes compact and smaller nuclei and/or the formation of apoptotic bodies. Through staining with fluorochrome DAPI, HeLa and SiHa cultures treated with a combination of FA and cisplatin showed signs of chromatin condensation with the formation of apoptotic bodies. Compact and smaller nuclei (indicating chromatin condensation) and apoptotic bodies was noticed in HeLa and SiHa cultures which suggested the treatment induced death by apoptosis in these cells.

Apoptosis has been found to be induced by transcriptional and non-transcriptional pathways which play through common effector events mediated by caspases 3 and 9 and regulated by members of the Bcl-2 family of genes. In the present study, FA and cisplatin treatment induced both initiator caspase 9 and executor caspase 3 much more effectively than either drug alone (Figure. 4.15a and 4.15b). The anti-apoptotic protein Bcl-2 is inappropriately over expressed in a variety of tumors including cervical cancer (Kaufmann and Vaux 2003). Increase in Bcl-2 increases cell survival and contributes to resistance to chemotherapy (Calvin et al. 2003). In the present study, when compared to cisplatin alone treated cells, FA primed cisplatin treated cancer cells down regulated the protein expression of Bcl-2 further. Several reports have shown that inhibiting Bcl-2 sensitizes tumor cells to chemotherapy. Kim et al. (2003) showed that inhibiting Bcl-2 increased the drug-sensitivity and apoptosis in thyroid carcinoma.
Cisplatin can induce two major distinct apoptotic pathways via various stress signalings: the first is p53-dependent mitochondrial apoptosis, which begins with translocation of the p53-induced Bax from the cytosol to the mitochondria, followed by cytochrome c release and activation of caspase 9 and 3 the second is the caspase induced apoptotic cascade (Torigoe et al. 2005).

Tumor suppressor protein p53 also plays a critical role in eliciting cellular responses to DNA damage (Tamm et al. 2001; Olson and Kornbluth, 2001). Many genes have been identified that affect cancer cells during programmed cell death following various genotoxic stresses. The activation of the typical tumor-suppressor protein p53 can result in cell-cycle arrest, DNA repair or apoptosis. Loss of p53 function confers resistance to cisplatin in various human cancer cell lines (Torigoe et al 2005). Furthermore, mutations in the p53 gene have been widely detected in various human cancer cells, indicating that p53 might be critical in determining drug sensitivity (Lowe et al. 1994). The p53 tumor-suppressor gene family proteins p53 and p73 are central to the cellular response to DNA damage. These proteins accumulate in nuclei after DNA damage and control cell proliferation. Cancer cells carrying loss of function mutants of p53 are also less sensitive to anticancer agents (Torigoe et al. 2005).

Taken together, the results suggested that FA and cisplatin treatment triggers cancer cell death by both activating caspases and regulating the expression of bcl-2 family proteins.

4.5. CONCLUSION

From the results of the Phase I studies it was clear that FA chemosensitizes cervical cancer cells HeLa and SiHa for cisplatin chemotherapy.