5.1. INTRODUCTION

The results of phase I study showed the chemosensitizing effect of ferulic acid in a cell culture model of cervical cancer. It was difficult to explore and confirm the efficacy of any phytochemical by testing it against a single type of cancer cell line alone. On the whole, any property of a drug cannot be ascertained only by the results obtained against a single type of cancer. So, the purpose of the Phase II study was to find out the sensitizing potential of ferulic acid in a cell culture model of lymphoma (Dalton’s lymphoma) to prove that ferulic acid not only sensitizes cervical cancer cells but also other cancer cells (ex: lymphoma).

Lymphoma is a type of blood cancer that occurs when B or T lymphocytes that form a part of the immune system and protect the body from infection and disease, divide faster than normal cells or live longer than they are supposed to. Lymphoma may develop in the lymph nodes, spleen, bone marrow, blood or other organs and eventually they form a tumor (Medical News today: Lymphoma research foundation).

Lymphoma is a solid tumor of lymphoid cells and treatment might involve chemotherapy and in some cases radiotherapy and/or bone marrow transplantation. Lymphomas can be curable depending on the histology, type, and stage of the disease. These malignant cells often originate in lymph nodes, presenting as an enlargement of the node (a tumor). It can also affect other
organs in which case it is referred to as extranodal lymphoma. Extranodal sites include the skin, brain, bowels and bone. Lymphomas are closely related to lymphoid leukemias, which also originate in lymphocytes but typically involve only circulating blood and the bone marrow and do not usually form static tumors. There are many types of lymphomas, and in turn, lymphomas are a part of the broad group of diseases called hematological neoplasms (Parham and Peter 2005). Cisplatin can be used in the treatment of lymphomas (Velasquez 1988; Crump et al. 2004).

Dalton’s lymphoma (DLA) is a spontaneous and highly invasive T cell lymphoma that develops an ascitic tumor in murines. DLA cell lines were non-adherent cell lines and like other cancer cell lines it can be grown in DMEM or RPMI medium. Research reports showed that DLA cell lines were used in cancer research to study the cytotoxic effects of various compounds including plant extracts and phytochemicals. Adhvaryu et al. (2008) studied the antitumor activity of ayurvedic herbs using DLA cells. In vitro cytotoxic potential of Emilia sonchifolia extracts were also studied using DLA cells (Shylesh and Padikkala 2000).

Variety of phytochemicals is found to enhance the cytotoxicity and alter the antioxidant and lipid peroxidation status in cisplatin treatment. Recently Periasamy et al. (2013) showed that tea polyphenols enhanced the cytotoxicity and modulated the antioxidant redox system during cisplatin treatment. Enhancement of cytotoxicity and alteration of antioxidant and lipid peroxidation status during chemotherapy is considered to be an important requirement in chemosensitization.

The phase II study was designed to explore the chemosensitizing effects of FA based on cytotoxicity and redox status in cisplatin treated Dalton’s lymphoma cell lines in vitro, a cell line other than cervical cancer cell line.
5.2. MATERIALS AND METHODS

5.2.1. Chemicals

Ferulic acid (FA), cisplatin, 3-(4, 5-dimethyl-2-thiaozolyl)-2, 5-diphenyl-2H tetrazolium bromide (MTT), trypan blue, thiobarbituric acid (TBA), trichloroacetic acid (TCA), phenazine methosulphate (PMS), nitroblue tetrazolium (NBT), 5, 5-dithiobis 2-nitrobenzoic acid (DTNB), 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 and trypsin were purchased from Sigma Chemicals Co., St. Louis, USA. 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.

5.2.2. Cell lines

Dalton's lymphoma ascites (DLA) cells (arose as a spontaneous carcinoma of thymus) were initially obtained from Cancer Institute, Chennai, South India. The cell lines were maintained in the laboratory by transplanting in the peritoneal cavity of mice. 5x10^6 DLA cells were suspended in Dulbecco’s Minimum Essential Medium (DMEM) supplemented with 10% FBS in culture flasks and maintained at 37° C in 5% CO₂ atmosphere.

5.2.3. Study design

Dalton’s lymphoma (DLA) cells were divided into 4 groups. Control group (group1) comprised of untreated DLA cells, groups 2 DLA cells were treated with FA alone and group 3 cells were treated with cisplatin alone. The cells in group 4 were primed with FA for 1 hour and then exposed to cisplatin.
Groups | Treatments
--- | ---
Control | Untreated DLA cells
DLA + FA | DLA cells treated with FA (10 µg/mL) alone
DLA + Cisplatin | DLA cells treated with cisplatin (5 µg/mL) alone
DLA+FA+Cisplatin | DLA cells pretreated with FA (10 µg/mL) (1 hour) and thereafter treated with cisplatin (5 µg/mL)

Table 5.1. Experimental design for Phase II studies on Dalton’s lymphoma cells

5.2.4. Short term *in vitro* cytotoxicity assay

Short term *in vitro* cytotoxicity studies for FA and cisplatin in Dalton’s lymphoma (DLA) cells were performed by trypan blue dye exclusion method (Gupta 2002).

**Principle**

Trypan blue is a cationic chromosomal dye that intercalates with DNA. The reactivity of trypan blue is based on the fact that the chromopore is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, all the cells which exclude the dye are viable. By calculating the number of viable cells, the cell viability and cytotoxicity can be calculated.

**Materials required**

1. Trypan blue dye : 0.4%
2. Light microscope
3. Haemocytometer and cover slip
**Procedure**

Different concentrations of ferulic acid (FA) or cisplatin were added in four sets of 1x10⁶ isolated DLA cells in a final volume of 1 mL with PBS and incubated at 37° C for 30 minutes and 3 hours respectively. The cells were dispersed by gentle tapping and 0.1 mL of 0.4% trypan blue was added to all the sets of cells incubated with drugs (30 minutes and 3 hours respectively). After 5 minutes, the cell viability was determined using a hemocytometer under a microscope.

The percentage of viable cells was calculated using the formula,

\[
\text{% Cell viability} = \frac{\text{Total number of viable cells}}{\text{Total number of viable and non-viable cells}} \times 100
\]

**5.2.5. Long term \textit{in vitro} cytotoxicity studies**

Long term \textit{in vitro} cytotoxicity studies for Dalton’s lymphoma (DLA) cells were performed by trypan blue dye exclusion method (Gupta 2002).

**Principle**

Trypan blue is a cationic chromosomal dye that intercalates with DNA. Loss of viability results in the nuclear uptake of the dye, giving characteristic blue colour to the nucleus. In a viable cell, uptake of the dye is prevented by the presence of the intact plasma membrane.

**Materials required**

1. Trypan blue dye (0.4%)
2. Light microscope
3. Haemocytometer and cover slip
Procedure

Long-term cytotoxicity of FA or cisplatin to DLA cells was determined by seeding $1 \times 10^6$ isolated DLA cells in 24-well-plate containing 1 mL DMEM medium supplemented with 10% FBS, streptomycin (100 µg/mL) and penicillin (100 units/mL). The drugs were added separately in the culture medium and cells were grown at 37°C in a humidified environment of 5% CO$_2$ and 96% air for 20 hours. To the trypsinized cells 0.1 mL of 0.4% trypan blue was added and the cell viability was determined using a haemocytometer under a microscope.

The percentage of viable cells was calculated using the formula,

$$\text{% Cell viability} = \frac{\text{Total number of viable cells}}{\text{Total number of viable and non-viable cells}} \times 100$$

5.2.6. Measurement of cell proliferation (MTT assay)

The proliferation activity of cell populations 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 active mitochondria, to form a dark blue formazan product which was measured at 490 nm.

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 the purple formazan product only by metabolically active mitochondria and the absorbance is directly proportional to the number of viable cells. MTT solution (0.5 g/L) was added to each culture well after treatment with ferulic acid and/or cisplatin for 24 hours 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.

5.2.7. Biochemical determinations

The cancer cells were harvested by trypsinization and washed with phosphate buffered saline (PBS). The cells were suspended in 130 mM KCl and 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 further biochemical estimations.

5.2.7.1. Lipid Peroxidation

5.2.7.1.1. Estimation of thiobarbituric acid reactive substances (TBARS)

The concentration of TBARS in the Daltons lymphoma 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 : 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

About 0.2 mL sample was diluted to 0.5 mL with double distilled water, mixed well and then 2 mL of TBA-TCA-HCl reagent was added. The 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 used for estimation of 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 cell lines.
5.2.7.2. Enzymic and non enzymic antioxidants

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

Superoxide dismutase in the Dalton’s lymphoma 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 tetrazolium formazan. 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 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 tetrazolium (NBT): 300 μM
7. Reduced nicotinamide adenine dinucleotide (NADH): 780 μM

Procedure

About 0.5 mL of supernatant was diluted to 1 mL with distilled water followed by 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 PMT, 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. 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 the 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 produce 50% inhibition of chromogen formation in one minute under standard conditions was taken as one unit.

The specific activity of the enzyme was expressed as enzyme required for 50% inhibition of NBT reduction/minute/mg of protein for cells.

5.2.7.2.2. Estimation of catalase (CAT, EC 1.11.1.6)

The activity of catalase in the Dalton’s lymphoma 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 CAT 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 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 chromate (w/v) : 5%
4. Dichromate–acetic acid reagent: 1:3 ratio of 5% potassium dichromate was mixed with glacial acetic acid. 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$_2$ was added. The reaction was arrested after 15, 30, 45 and 60 seconds by the addition of 2 mL of dichromate - acetic acid mixture. The tubes were kept in a boiling water bath for 10 minute, cooled and the colour developed was read at 620 nm.

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

**5.2.7.2.3. Estimation of glutathione peroxidase (EC 1.11.1.19)**

The activity of GPx in the Dalton's lymphoma 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. TCA : 10%
4. EDTA : 0.4 mM
5. H$_2$O$_2$ solution : 0.2 mM
6. Glutathione solution : 2 mM

Procedure

To 0.2 mL of tris buffer, 0.2 mL of EDTA and 0.1 mL of sodium azide, 0.5 mL of sample was added and mixed. To the 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 at 340 nm.

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

5.2.7.2.4. Estimation of reduced glutathione (GSH)

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

Principle

This method is 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 colour compound 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: 10.0 mg/100 mL

**Procedure**

About 0.5 mL of supernatant was pipetted out and precipitated with 2 mL of 5% TCA. 2 mL of supernatant was taken after centrifugation and 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.

**5.2.8. Estimation of protein**

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

**Principle**

Protein reacts with the Folin-Ciocalteau reagent to give a coloured complex. The colour so formed is due to the reaction of alkaline copper with protein and the reduction of phosphomolybdate by tyrosine and tryptophan present in the protein. The intensity of the colour depends on the amount of these aromatic amino acids present.

**Reagents**

1. 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

2. Folin’s phenol reagent: The commercial reagent was diluted in the ratio of 1:2 with distilled water just before use

3. Stock standard: 100 mg of bovine serum albumin/100 mL of water

4. Working standard: 10 mL of the stock standard was diluted to 100 mL to get a working standard containing 0.1 mg/mL.

5. 10% Trichloroacetic acid (TCA)

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 minute. Then, 0.5 mL of Folin’s phenol reagent was added and the blue colour developed was read after 20 minutes at 640 nm. A standard curve was obtained with standard bovine serum albumin and was used to assay the tissue protein level for enzyme activity.

Values are expressed as mg / dL.

5.2.9. 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).
5.3. RESULTS

The sensitizing effect of FA and/or cisplatin was tested in Dalton’s lymphoma (DLA) cell lines and the results were presented below.

The effect of FA and cisplatin on DLA cells on short term cytotoxicity was shown in table 5.2. It was found that, when DLA cells were incubated with varying concentrations of FA and cisplatin for 30 minutes and 3 hours, the percentage of cytotoxicity was increased with an increase in concentration of the drugs. In short term cytotoxicity, at 10 µg of FA and 5µg of cisplatin, the cytotoxicity in DLA cells was less than 50%.

<table>
<thead>
<tr>
<th>Concentration (µg)</th>
<th>FA (30 minutes)</th>
<th>FA (3 hours)</th>
<th>Cisplatin (30 minutes)</th>
<th>Cisplatin (3 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>5.27 ± 0.02a</td>
<td>10.94 ± 0.65a</td>
<td>15.69 ± 0.81a</td>
<td>25.37 ± 0.62a</td>
</tr>
<tr>
<td>5</td>
<td>11.86 ± 0.73b</td>
<td>32.85 ± 1.44b</td>
<td>26.34 ± 0.94b</td>
<td>44.86 ± 2.13b</td>
</tr>
<tr>
<td>10</td>
<td>24.96 ± 1.01c</td>
<td>41.70 ± 2.13d</td>
<td>47.96 ± 2.06d</td>
<td>87.96 ± 4.29c,d</td>
</tr>
<tr>
<td>15</td>
<td>27.84 ± 0.95d</td>
<td>64.69 ± 2.60c</td>
<td>63.74 ± 1.57c</td>
<td>91.27 ± 2.42d</td>
</tr>
<tr>
<td>20</td>
<td>29.94 ± 0.89d</td>
<td>79.36 ± 1.78f</td>
<td>69.08 ± 1.11f</td>
<td>93.50 ± 3.75d</td>
</tr>
<tr>
<td>25</td>
<td>37.66 ± 1.24e</td>
<td>84.89 ± 3.96f</td>
<td>74.36 ± 3.14f</td>
<td>94.39 ± 2.04d,e</td>
</tr>
</tbody>
</table>

Data are presented as the means ± SD; n=6
Values not sharing a common superscript (a-f) differ significantly at P<0.05 vs. control (DMRT)

Table 5.2. Effect of FA and cisplatin on short term cytotoxicity of DLA cells
The effect of FA and cisplatin on DLA cells on long term cytotoxicity was shown in table 5.3. In long term cytotoxicity, when the DLA cells were incubated with varying concentrations of FA and cisplatin for 20 hours, 50% cytotoxicity was attained at the concentration of 10 µg for FA and 5 µg for cisplatin (Table 5.3).

<table>
<thead>
<tr>
<th>Concentration (µg)</th>
<th>% cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FA</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>27.60 ± 0.17^a</td>
</tr>
<tr>
<td>5</td>
<td>36.40 ± 1.80^b</td>
</tr>
<tr>
<td>10</td>
<td>50.06 ± 2.31^d</td>
</tr>
<tr>
<td>15</td>
<td>77.92 ± 3.58^e</td>
</tr>
<tr>
<td>20</td>
<td>97.98 ± 2.09^f,g</td>
</tr>
<tr>
<td>25</td>
<td>100.00 ± 0.09^g</td>
</tr>
</tbody>
</table>

Data are presented as the means ± SD; n=6
Values not sharing a common superscript (a-f) differ significantly at P<0.05 vs. control (DMRT)

Table 5.3. Effect of FA and cisplatin on long term cytotoxicity of DLA cells
FA (5-50 µg) on percentage cytotoxicity of DLA cells as measured by the reduction of MTT dye (MTT assay). IC 50 value for FA was found to be 10 µg for DLA cells

**Figure 5.1. Effect of FA on cytotoxicity of DLA cells by MTT assay**
Cisplatin (5-50 µg) on percentage cytotoxicity of DLA cells as measured by the reduction of MTT dye (MTT assay). IC 50 value for cisplatin was found to be 5 µg for DLA cells

**Figure 5.2 Effect of cisplatin on cytotoxicity of DLA cells by MTT assay**

The effect of cytotoxicity on 24 hours treatment of FA and cisplatin on DLA cells by MTT assay was showed in figure 5.1 and 5.2. FA and cisplatin induced 50% cytotoxicity of DLA cells at the concentrations of 10 µg and 5 µg respectively. Hence, the IC 50 value of FA and cisplatin on DLA cells was found to 10 µg/ml and 5 µg/ml respectively.

Figure 5.3 showed the effect of FA pretreated cisplatin treatment on the cytotoxicity of DLA cells. As like in the cervical cancer cell lines, DLA cells
primed with FA 1 hour before cisplatin treatment, significantly increased the cytotoxicity of DLA cells when compared to FA or cisplatin alone treated cells.

Cells were pretreated with FA (10 µg/ml) 1 hour before cisplatin (5 µg/ml) treatment showed increased cytotoxicity than FA or cisplatin alone treated cells

**Figure 5.3. Effect of FA pretreatment on cytotoxicity of cisplatin treated Dalton’s lymphoma cell lines**

The effect of FA and/or cisplatin on antioxidant status of DLA cells were shown in tables 5.4 and 5.5. Treatment of FA and/or cisplatin significantly decreased the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and reduced glutathione (GSH) when compared to untreated DLA cells with a more pronounced effect found in the cells treated with both FA and cisplatin.
<table>
<thead>
<tr>
<th>GROUPS</th>
<th>Superoxide dismutase (SOD) $^x$</th>
<th>Catalase (CAT) $^y$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>13.67 ± 1.33$^a$</td>
<td>5.17 ± 0.28$^a$</td>
</tr>
<tr>
<td>FA</td>
<td>11.35 ± 1.03$^b$</td>
<td>4.73 ± 0.19$^b$</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>8.65 ± 0.08$^c$</td>
<td>2.88 ± 0.25$^c$</td>
</tr>
<tr>
<td>FA+Cisplatin</td>
<td>7.01 ± 0.04$^d$</td>
<td>2.00 ± 0.12$^d$</td>
</tr>
</tbody>
</table>

$^x$ Enzyme concentration required for 50% inhibition of nitroblue tetrozolium reduction in one minute/mg protein

$^y$ µM of H$_2$O$_2$ 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 5.4. Effect of FA and/or Cisplatin on superoxide dismutase (SOD) and catalase (CAT) in Dalton’s lymphoma cell lines
<table>
<thead>
<tr>
<th>GROUPS</th>
<th>Glutathione peroxidase (GPx)(^p)</th>
<th>Reduced glutathione (GSH)(^q)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>4.23 ± 0.28(^a)</td>
<td>24.05 ± 1.47(^a)</td>
</tr>
<tr>
<td>FA</td>
<td>3.89 ± 0.25(^a)</td>
<td>19.37 ± 1.37(^b)</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>2.08 ± 0.12(^b)</td>
<td>15.43 ± 1.38(^c)</td>
</tr>
<tr>
<td>FA+Cisplatin</td>
<td>1.00 ± 0.09(^c)</td>
<td>10.34 ± 1.39(^d)</td>
</tr>
</tbody>
</table>

\(^p\)µg of glutathione consumed /minute/mg protein

\(^q\)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)

Table 5.5. Effect of FA and/or Cisplatin on glutathione peroxidase (GPx) and reduced glutathione (GSH) in Dalton’s lymphoma cell lines
Table 5.6. shows the effect of FA and/or cisplatin on the status of lipid peroxidation marker TBARS on DLA cells. It was found that cells treated with FA and/or cisplatin significantly increased the levels of TBARS when compared to untreated cells.

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>Thiobarbituric acid reactive substances (TBARS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>1.55 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>FA</td>
<td>4.19 ± 0.32&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>5.56 ± 0.43&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>FA+Cisplatin</td>
<td>6.04 ± 0.33&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* 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)

Table 5.6. Effect of FA and/or Cisplatin on Thiobarbituric acid reactive substances (TBARS) in Daltons lymphoma cell lines
5.4. DISCUSSION

The cytotoxicity of the combination of FA and Cisplatin was tested in Dalton’s lymphoma (DLA) tumour cell lines to prove that the sensitizing effect was influenced not only on cervical cancer cells but also on other cancer cell lines to chemotherapeutic drugs. In the short term cytotoxicity (for 30 minutes and 3 hours) by Trypan blue assay and percentage of cytotoxicity by MTT assay, both FA and Cisplatin showed cytotoxic action against Dalton’s lymphoma cell lines *in vitro*.

Cisplatin is a known cytotoxic agent (Prasad *et al.* 2010). Earlier studies showed that FA was cytotoxic over variety of cancer cell lines. Lee and Lee (2006) suggested that the cytotoxic action of FA may be because of its proxidant activity. In the phase I studies, it was found that FA showed cytotoxicity against cervical cancer cell lines HeLa and SiHa. Treatment of cisplatin (5 µg/ml) with FA (10 µg/ml) pretreated DLA cells, increased the percentage cytotoxicity or decreased the percentage survival in both Trypan blue assay and MTT assay compared to cisplatin alone treatment (Figure 4.3). The cytotoxic action of cisplatin may be enhanced in the presence of FA.

In a study by Sarna *et al.* (2007), vitamin E enhanced the cytotoxicity of cisplatin. They found that a lower concentration of cisplatin in combination with α-tocopherol (vitamin-E) was found to be more cytotoxic than the higher concentration of cisplatin alone. Similarly in the present study, FA enhanced the cytotoxicity of cisplatin. The significant enhancement of the cytotoxic index of cisplatin by FA may be the result of chemosensitizing effect of FA.

The pattern of changes in the levels of enzymic and non-enzymic antioxidants by the DLA cells treated with FA before the treatment of cisplatin follow similar pattern like that of cervical cancer cells lines (Phase I). When FA pretreated DLA cells were treated with cisplatin, the levels of lipid
peroxidation were significantly increased and the levels of antioxidants were significantly decreased respectively. The changes in the levels were higher in FA and cisplatin treatment than compared to the FA or cisplatin alone treated cells. This increase and decrease in the levels of lipid peroxidation and antioxidants respectively in the treated cells was due to the prooxidant activity of FA (Lee and Lee 2006).

5.5. CONCLUSION

Based on the above results, it was suggested that FA showed chemosensitizing effects on Dalton’s lymphoma (DLA) cells in vitro as like in cervical cancer cell lines. Thus the chemosensitizing efficacy of ferulic acid (FA) on cisplatin therapy further suggested based on its results on these two different cancer cell lines.