CHAPTER-4

ANALYZING THE ANTI-NEOPLASTIC POTENTIAL OF A NOVEL HERBAL FORMULATION (FC)

The manuscript on the research work given in this chapter is communicated and a patent has been filed (2039/MUM/2010)
**Abstract:**

A novel herbal formulation (FC) was developed from the bark materials of *Ficus religiosa* and *Cinnamomum zeylanicum*. FC was standardized biochemically and its anticancer properties were delineated through in vitro and *in vivo* approaches. FC induced apoptosis in SiHa and HeLa cells through early generation of reactive nitrogen and oxygen species (RNOS) and loss of mitochondrial membrane potential (MMP). In C57BL/6 mouse melanoma model, the oral administration of FC inhibited tumor growth significantly. Interestingly, FC treatment led to an appreciable increase in the serum levels of Th1 cytokines (IFN-γ and IL-2) and subsequent decrease in the levels of Th2 cytokine (IL-4). These data suggest that FC formulation could be explored further for its chemopreventive potential in cervical cancer.
4.1. Introduction

Cancer is the leading cause of death worldwide, accounting for about 8.2 million deaths in 2012 (Globocan., 2012). Survival and progression of cancer cell strongly depends upon its ability to overcome apoptosis as well as host immune response (Brandacher et al., 2006).

In the recent years, there is a renewed interest in the use of herbal medicines in the management of wide range of human diseases, including cancer. The herbal formulations from Ayurveda, traditional Chinese medicines, Japanese (Kampo) and others are composed of mixture of different herbs, which may work synergistically to produce maximum therapeutic efficacy with minimum or no side effects (Ahmed et al., 2013). Moreover, extensive research has revealed that these formulations can induce apoptosis in cancer cells and may also stimulate the immune system (Liu et al., 2012a). For example, many polyherbal formulations such as PHY906 (Liu et al., 2012b), PC-SPES (Wang et al., 2013) and YWKLF (Li et al., 2008) have revealed their efficacy against cancer and some of them are even in phase II clinical trials. Likewise, herbal formulation of Withania somnifera (WSF) has been reported to have both anticancer and immune modulatory activities (Malik et al., 2008).

In previous chapter, we have elucidated the anticancer mechanism of aqueous extract of Cinnamomum cassia (Chapter-2) and Ficus religiosa (Chapter-3) in cervical cancer cells. Moreover, earlier work from our group had shown that Cinnamomum zeylanicum exhibited cytotoxic activity in a panel of cancerous cell lines (Singh et al., 2009), including cervical cancer cell line. We wanted to analyze whether combination of Ficus and Cinnamon could reduce the drug dose through the synergistic activity of both the plant materials. Thus, we prepared a herbal formulation (FC) of C. zeylanicum with
We did not take C. cassia since it is known to contain high amounts of coumarins that have been reported to exhibit toxicity in humans (Wang et al., 2013).

Our data suggests that FC inhibited the growth of SiHa and HeLa by inducing apoptosis. Interestingly, FC retarded tumor growth significantly and modulated the immune system in C57BL/6 melanoma tumor model through regulation of Th1 and Th2 cytokines.

4.2. Materials and Methods

4.2.1. Plant material and extract preparation

The bark of Cinnamomum zeylanicum (CZ) was purchased from Green Pharmacy Pune Maharashtra, India. The bark of Ficus religiosa (FR) was collected from Pune District, Maharashtra, India. Barks of both CZ and FR were chopped into small pieces, shade dried at ambient temperature and ground into coarse powder in a grinder. The aqueous extract was prepared as per the standard Indian Pharmacopoeia by combining the two bark materials (FR: CZ) in different ratios of 1:1,1:2 and 2:1. The extract obtained was centrifuged at 13000 rpm for 15 min and the supernatant was filtered through swiney filter (pore size, 0.45 µm). The extract was stored at -80ºC until use. (Note: FR:CZ composition at ratio of 2:1, is termed as FC formulation)

4.2.2. HPLC analysis

The HPLC fingerprint of FC formulation was determined by Shimadzu High Performance Liquid Chromatographic System LC 2010 CHT with UV detector in combination with Class LC solution software. Chromatographic separation was performed with a Kromasil C18 reversed-phase column (4.6 mm id x 250 mm) with a 5 µm particle size (Sigma-Aldrich, USA). Column temperature was set at 40ºC. Gradient
flows for the two solvent systems (solvent A, 0.14% anhydrous potassium dihydrogen orthophosphate (KH$_2$PO$_4$) in water; solvent B, acetonitrile) were as follows: 0 min, 5% B; 12 min, 15% B; 35 min, 70% B; 40 min, 5% B; and hold at 5% B for 5 min. The flow rate of the mobile phase was maintained at 1.5 ml/min. The injection volume was 20 ul and the chromatogram was monitored at wavelengths of 254 nm throughout the experiment. The reference standards protocatechuic acid, catechin, cinnamic acid, cinnamyl alcohol and cinnamaldehyde, were used to confirm their presence in FC formulation.

4.2.3. Cell culture

Human cervical carcinoma cell lines SiHa (HPV-16) and HeLa (HPV-18) were obtained from National Center for Cell Science (NCCS), Pune, Maharashtra, India. PBMCs were isolated from a healthy donor and used as primary cells. The cells were grown in DMEM with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and antibiotics (100 units/ml penicillin and streptomycin). Cells were maintained at 37°C in 5% CO$_2$/air atmosphere.

4.2.4. Cytotoxic activity

Cytotoxic potential of each component (FR and CZ) and three various combinations (1:1, 1:2, 2:1) of FR:CZ were determine in SiHa and HeLa, by MTT dye uptake (Choudhari et al., 2011). Briefly, SiHa, and HeLa, were seeded at 1 x 10$^5$ ml density in a 96-well plate. Next day, the cells were incubated with various concentrations (0-640 µg/ml) of FR and CZ (used alone or in combination) in triplicates for 24 h in 5% CO$_2$ incubator at 37°C. An untreated group was kept as a control in all the cell lines used. The MTT solution (5 mg/ml) was added to each well and the cells were cultured for another 4 h at 37°C in 5% CO$_2$ incubator. The formazan crystals formed were dissolved by addition of 90 µl of
SDS-DMF (20% SDS in 50% DMF). After 15 min, the amount of colored formazan derivative was determined by measuring optical density (O.D) with the ELISA microplate reader (BioRad, Hercules, CA) at OD 570-630 nm. The percentage viability was calculated as:

\[
\% \text{ Viability} = \left( \frac{\text{OD of treated cells}}{\text{OD of control cells}} \right) \times 100
\]

4.2.5. Isolation of peripheral blood mononuclear cells from human blood and cytotoxicity assay

Peripheral blood mononuclear cells (PBMCs) were separated from whole heparinized blood by Ficoll-Hypaque density gradient centrifugation method (Fuss et al., 2009). Briefly, 6 ml of Ficoll–Hypaque (Sigma, St. Louis, MO) was stratified under 10 ml of peripheral blood and centrifugation was performed at 400 × g for 30 min at room temperature. Recovered PBMCs were washed three times with 1X PBS and the number of living cells was counted by trypan blue (Sigma, St. Louis, MO) staining. PBMCs were seeded at 1 x 10^5 cells/ml density in a 96-well plate. Next day, the cells were incubated with various concentrations (0-640 µg/ml) of FC. The experiment was repeated thrice and for each experiment freshly isolated PBMCs were used.

4.2.6. Assessment of apoptosis by Annexin FITC/PI staining

Apoptosis was determined by Annexin FITC/PI staining (Malo et al., 2010). Briefly, HeLa and SiHa cells were plated at a seeding density of 1 x 10^5 cells/ml in a 96 well black culture plate and allowed to adhere overnight at 37°C in a CO2 incubator. Next day, the cells were treated with FC (0-40 µg/ml) for 24 h at 37°C in 5% CO2. Following incubation, cells were stained with Annexin V-FITC according to the
manufacturer's instructions (Annexin V-FITC apoptosis kit #3, Invitrogen, Grand Island, NY). The plate was centrifuged at 400 g for 5 min at 37°C and the supernatant was aspirated and discarded. Hundred microliters of assay binding buffer was added to each well and the fluorescence intensity was measured using the Fluostar Omega microplate reader (BMG Labtech). Dead cells were stained by propidium iodide which displays strong fluorescence intensity with excitation and emission at 560 nm and 595 nm, respectively. Early stage apoptotic cells, stained by Annexin V FITC, were detected at the excitation and emission wavelength of 485 nm and 535 nm, respectively.

4.2.7. Estimation of cellular Reactive Oxygen Species (ROS)

Cellular ROS production was determined by dihydrodichlorofluorescein diacetate probe (DCF-DA) (Zhou et al., 2010). Briefly, SiHa and HeLa cells were seeded at a density of $1 \times 10^5$ cells/ml in a 96 well black culture plate and allowed to adhere overnight at 37°C in a CO$_2$ incubator. Next day, the medium was removed and the cells were incubated with fresh culture media containing DCF-DA (10 µM) dye (Molecular Probes) for 30 min in the dark followed by washing twice with 1X PBS. The cells were then treated with various concentrations of FC (10-40 µg/ml). The fluorescence intensity generated from intracellular ROS was detected using a Fluostar Omega microplate reader (BMG Labtech) with excitation and emission wavelengths of 480 nm and 520 nm, respectively. ROS was determined by comparing the changes in fluorescence intensity at a give point (F) with that of the baseline (control) fluorescence intensity ($F_0$).

4.2.8. Analysis of intracellular Nitric Oxide (NO)

Nitric oxide production in cervical cancer cells was measured using 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) (Vardi et al., 2006). Briefly, SiHa and
HeLa cells were seeded at a density of $1 \times 10^5$ cells/ml in a 96 well black culture plate and allowed to adhere overnight at 37°C in a CO$_2$ incubator. Next day, the medium was removed and the cells were incubated with fresh culture medium containing DAF-FM (2.5 µM) dye (Molecular Probes) for 30 min in the dark, followed by washing twice with 1X PBS. The cells were then treated with various concentrations of FC (10-40 µg/ml). The fluorescence intensity generated from NO was detected by using a FluoStar Omega microplate reader (BMG Labtech) with excitation and emission wavelengths of 480 nm and 520 nm, respectively. NO was determined by comparing the changes in fluorescence intensity at a given point (F) with that of the baseline (control) fluorescence intensity (F0).

**4.2.9. Analysis of Mitochondrial Membrane Potential ($\Delta \psi_m$)**

Cervical cancer cell lines were seeded at a density of $1 \times 10^5$ cells/ml in a 96 well black culture plate and allowed to adhere overnight at 37°C in a CO$_2$ incubator. Next day, the cells were treated with different concentrations of FC (10-40 µg/ml). After 24 h of incubation, the medium was removed and the cells were washed twice with 1X PBS followed by incubation with fresh culture medium containing JC-1 (2.5 µg/ml) dye (Sigma-Aldrich, St. Louis, MO) for 30 min in the dark. Fluorescence intensity was measured using the FluoStar Omega microplate reader (BMG Labtech) at 520 nm for JC-1 monomers and at 590 nm for JC-1 aggregates.

**4.2.10. Tumor retardation study**

The *in vivo* anti-cancer study was performed at Advanced Centre for Treatment Research and Education in Cancer (ACTREC), Navi-Mumbai. Briefly, B16F10 melanoma ($1 \times 10^6$ cells/mouse) cells were inoculated subcutaneously (s.c) into the right flanks of six weeks old C57BL/6 mice. Eight days after the injection, animals were
randomized into five groups, each group having six mice. Group I was kept as a negative control and received normal saline; Group II received Doxorubicin (4 mg/kg body weight (bw)) intravenously on 1st, 5th and 9th day; Group III and IV were given oral gavage of FC at concentrations of 100 and 200 mg/kg bw, respectively, daily for 15 days. During the treatment period, the tumor size was measured with vernier calipers after every 2 days, and the average tumor volume was calculated using the formula: $1/2(\text{length} \times \text{width}^2)$.

The percent tumor growth inhibition in treated groups was calculated as follows:

$$\text{% Tumor inhibition} = \left[\frac{\text{Av. tumor volume of control group} - \text{Av. tumor volume of test group}}{\text{Av. tumor volume of control group}}\right] \times 100$$

After 15 days of treatment, animals were scarified and blood samples were collected for cytokine analysis.

4.2.11. **Th1/Th2-like cytokine determination in tumor bearing mice sera**

Blood samples were collected from FC treated and un-treated tumor-bearing mice on day 15 of FC treatment. The concentrations of serum cytokines (IFN-γ, IL-2 and IL-4) were measured by using mouse Th1/Th2 enzyme-linked immunosorbent assay (ELISA) Ready SET Go Kit (BD Bioscience, San Diego, CA, USA). The detection procedures were performed according to the manufacturer's instructions. The sensitivity of assay for each cytokine was as follows: 4 ρg/ml for IL-2 and IFN-γ; and 2 ρg/ml for IL-4.
4.3 Results:

4.3.1 Effect of individual extracts and its combination on cell viability

The cervical cancer cell lines, SiHa and HeLa were treated with Ficus religiosa (FR), Cinnamon zyelanicum (CZ) and three different combinations (1:1, 1:2, 2:1) of Ficus religiosa:Cinnamomum zyelanicum (FR:CZ) at various concentrations (0-640 µg/ml) for 24 h. The cytotoxicity of FR, CZ and FR:CZ (1:1, 1:2, 2:1) in SiHa and HeLa has been shown in Table 4.1 and 4.2, respectively.

In SiHa, FR exhibited significant cytotoxicity at 320 µg/ml concentration whereas CZ showed cytotoxicity at 640 µg/ml concentration (Table 4.1). However, when the cells were treated with different ratios (1:1, 2:1 and 1:2) of FR:CZ, all the different compositions exhibited significant cytotoxicity at relatively lower doses compared to either FR or CZ.

Table 4.1: Cytotoxicity of FR, CZ and FR:CZ (1:1, 1:2, 2:1) in SiHa cells

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>C. zyelanicum (CZ) % viability</th>
<th>F. religiosa (FR) % viability</th>
<th>FR:CZ (1:1) composition % viability</th>
<th>FR:CZ (1:2) composition % viability</th>
<th>FR:CZ (2:1) composition % viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100.00± 0.00</td>
<td>100.00 ± 0.00</td>
<td>100.00 ± 0.00</td>
<td>100.00 ± 0.00</td>
<td>100.00 ± 0.00</td>
</tr>
<tr>
<td>20</td>
<td>106.20± 2.43</td>
<td>112.86 ± 3.4</td>
<td>113.85 ± 5.13</td>
<td>112.26 ± 4.23</td>
<td>103.94 ± 5.28</td>
</tr>
<tr>
<td>40</td>
<td>106.76± 1.03</td>
<td>116.02 ± 3.14</td>
<td>120.80± 8.21</td>
<td>122.22± 7.45</td>
<td>100.01± 1.77</td>
</tr>
<tr>
<td>80</td>
<td>107.14± 4.6</td>
<td>115.37 ± 5.31</td>
<td>122.97± 8.52</td>
<td>141.43 ± 3.43</td>
<td>86.86 ± 5.02*</td>
</tr>
<tr>
<td>160</td>
<td>108.60± 6.22</td>
<td>113.35± 6.79</td>
<td>87.25± 3.64*</td>
<td>85.47± 3.43*</td>
<td>57.75± 2.17*</td>
</tr>
<tr>
<td>320</td>
<td>101.27± 3.64</td>
<td>69.88 ± 5.10*</td>
<td>78.62± 0.96*</td>
<td>66.36± 7.06*</td>
<td>43.25± 2.14*</td>
</tr>
<tr>
<td>640</td>
<td>83.66± 1.16*</td>
<td>22.32± 2.43*</td>
<td>29.78± 1.66*</td>
<td>32.84± 4.70*</td>
<td>32.31± 1.08*</td>
</tr>
</tbody>
</table>

Similarly, in HeLa (Table 4.2), FR induced significant cytotoxicity at 320 µg/ml, whereas, CZ showed cytotoxicity at 640 µg/ml concentration. However, when the cells
were treated with different ratios (1:1, 2:1 and 1:2) of FR:CZ, the different compositions exhibited significant cytotoxicity at relatively lower doses compared to either FR or CZ.

Table 4.2: Cytotoxicity of FR, CZ and FR:CZ (1:1, 1:2, 2:1) in HeLa cells

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>C. zyelanicum (CZ) % viability</th>
<th>F. religiosa (FR) % viability</th>
<th>FR:CZ (1:1) composition % viability</th>
<th>FR:CZ (1:2) composition % viability</th>
<th>FR:CZ (2:1) composition % viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100.0±0.00</td>
<td>100.0±0.00</td>
<td>100.0±0.00</td>
<td>100.0±0.00</td>
<td>100.0±0.00</td>
</tr>
<tr>
<td>20</td>
<td>110.65±1.44</td>
<td>104.43±1.47</td>
<td>102.77±10.78</td>
<td>112.39±2.44</td>
<td>97.06±3.91</td>
</tr>
<tr>
<td>40</td>
<td>111.79±2.09</td>
<td>100.93±2.43</td>
<td>103.15±11.15</td>
<td>108.35±1.65</td>
<td>91.37±2.05</td>
</tr>
<tr>
<td>80</td>
<td>109.82±2.03</td>
<td>102.49±0.78</td>
<td>104.91±6.91</td>
<td>111.93±3.12</td>
<td>70.46±3.21*</td>
</tr>
<tr>
<td>160</td>
<td>104.09±2.84</td>
<td>91.09±2.79</td>
<td>112.49±9.9</td>
<td>71.94±5.52*</td>
<td>38.75±3.69*</td>
</tr>
<tr>
<td>320</td>
<td>99.26±3.03</td>
<td>76.49±1.71*</td>
<td>44.46±11.57*</td>
<td>44.76±11.21*</td>
<td>35.58±4.54*</td>
</tr>
<tr>
<td>640</td>
<td>84.11±2.10*</td>
<td>57.97±1.38*</td>
<td>11.56±2.21*</td>
<td>9.80±1.41*</td>
<td>25.92±9.47*</td>
</tr>
</tbody>
</table>

Interestingly, at 2:1 ratio of FR:CZ significant cytotoxicity was observed, in both SiHa and HeLa at relatively lower concentration compared to other ratios. Thus, further studies were carried out with 2:1 ratio of FR and CZ. (Note: FR:CZ composition at ratio of 2:1, has been termed as FC formulation in further work)

4.3.2 FC selectively inhibited growth of cervical cancer cells

Since FC showed toxicity at relatively lesser concentration compared to the individual plant materials and other combinations, its cytotoxicity was investigated in normal human peripheral blood mononuclear cells (PBMCs). The cells were treated with various concentrations (0-640 µg/ml) of FC for 24 h and cytotoxicity was measured by MTT assay. The cytotoxicity of FC in SiHa, HeLa and PBMCs has been shown in Figure 4.1. Compared to IC₅₀ value of 203.16 and 119.70 µg/ml in SiHa and HeLa, respectively,
the IC$_{50}$ value of FC in PBMCs was > 640 µg/ml concentration. This suggests that FC was safe to the normal cells.

Figure 4.1: Cytotoxic effect of FC in cervical cancer lines and normal cells. SiHa, HeLa and PBMCs were treated with different concentrations (0-640 µg/ml) of FC for 24 h. The viability was measured by MTT assay.

4.3.3 Standardization of FC formulation

Before evaluating the therapeutic efficacy of FC, it was important to standardize it with respect to its marker compounds. Thus, chemo-profiling of FC was performed based upon five standard marker compounds (protocatechuic acid, catechin, cinnamic acid, cinnamyl alcohol and cinnamaldehyde) present in it employing HPLC. Comparisons of the retention times of sample peaks (Figure 4.2 A) were done with respect to the standard peaks (Figure 4.2 B-F). The retention times of protocatechuic acid, catechin, cinnamic acid, cinnamyl alcohol and cinnamaldehyde were 6.97, 17.15, 20.50, 21.26, 23.93 minutes, respectively (Figure 4.2 B-F). Catechin was the most abundant marker component (Average Content (AC) was around 140.67 mg/g), followed
by cinnamaldehyde (AC, 1.20 mg/g), protocatechuic acid (AC, 0.89 mg/g), cinnamic acid (AC, 0.80 mg/g) and cinnamyl alcohol (AC, 0.30 mg/g).

A) FC formulation

B) Protocatechuic acid

C) Catechin

D) Cinnamic acid
Figure 4.2: Representative chromatogram of (A) FC formulation and standard (B) protocatechuic acid (C) catechin (D) cinnamic acid, (E) cinnamyl alcohol, (F) cinnamaldehyde. HPLC was performed using potassium dihydrogen orthophosphate (KH₂PO₄): acetonitrile gradient. Gradient flows for the two solvent systems (solvent A, 0.14% anhydrous potassium dihydrogen orthophosphate (KH₂PO₄) in water; solvent B, acetonitrile) were as follows: 0 min, 5% B; 12 min, 15% B; 35 min, 70% B; 40 min, 5% B; and hold at 5% B for 5 min, flow rate 1.0 ml/min, detection wavelength UV 254 nm, injection volume 20 µL.

4.3.4 FC induced apoptosis in cervical cancer cells

To analyze the mechanism behind the FC mediated cell death in cervical cancer cells, we investigated apoptosis in SiHa and HeLa after treating the cells with different concentrations (0-40 µg/ml). On staining with Annexin V-FITC, both SiHa and HeLa showed a dose-dependent increase in both early as well as late apoptotic cell population (Figure 4.3). Interestingly, in SiHa, at 40 µg/ml FC concentration, there was ~1.69-fold (p=0.039) and ~2.31-fold (p≤0.001) increase in both early as well as late apoptotic cell
population, respectively, compared to the untreated control cells (Figure 4.3A). On the other hand, at 40 µg/ml concentration of FC, HeLa cells exhibited ~5.1-fold (p=0.47) and ~1.47-fold (p≤0.001) increase in early and late apoptotic cell population, respectively, compared to the untreated control cells (Figure 4.3B).

![Figure 4.3: FC induced apoptosis. (A) SiHa and (B) HeLa cells treated with different concentrations (0-40 µg/ml) of FC for 24 h. Apoptosis was measured by Annexin V-FITC/Propidium iodide staining. Dead cells were stained by propidium iodide which displays strong fluorescence intensity with excitation and emission at 560 nm and 595 nm, respectively. Early stage apoptotic cells stained by Annexin V FITC were detected at the excitation and emission wavelength of 485 nm and 535 nm, respectively.](image)

4.3.5 FC generated intracellular Reactive Oxygen Species (ROS) in cervical cancer cells

Elevated amounts of intracellular ROS are sufficient to trigger cell death, and it has been suggested that ROS are biochemical mediators of apoptosis (Brodská et al., 2011). Therefore, to determine whether ROS is involved in the regulation of apoptosis induced by FC, we performed the ROS kinetic assay using DCF-DA, a selective probe for ROS measurement. Cells were loaded with DCF-DA, followed by treatment with FC (0-40 µg/ml). ROS was measured every 10 min after FC treatment for 6 h and then at 24 h. Compared with the untreated control group, a dose dependent increase in the ROS
content was observed in FC-treated SiHa and HeLa cells. FC significantly increased endogenous ROS within 6 h of the treatment with a maximal at 40 μg/ml concentration in SiHa (~11.63±2.00 fold; p≤0.001) and HeLa (~13.39±1.41 fold; p≤0.001) (Figure 4.4A and B, respectively). However, the fold increases in ROS when measured after 24 h treatment with FC, at 40 μg/ml concentration, was ~7.06±1.46 (p≤0.05) and ~17.58±1.27 (p≤0.05), for SiHa and HeLa, respectively, compared to the untreated cells (Figure 4.4C and D, respectively).

Figure 4.4: FC treatment increased intracellular Reactive Oxygen Species. (A) SiHa and (B) HeLa cells were loaded with DCF-DA (2.5 μM) and exposed to FC, during which measurements (495/515 nm) were recorded every 1 min for 6 h. Representative graphs of intracellular ROS in (C) SiHa and (D) HeLa after 24 hours of FC treatment; (n = 4) are shown for three independent experiments.
4.3.6 FC causes increase in nitric oxide (NO)

The extent of NO generation in cells was analyzed by performing its kinetics using DAF-FM, a selective probe for NO measurement. Cells were loaded with DAF-FM, followed by treatment with FC (0-40 µg/ml) and measurement of NO was done after every 10 min for 6 h and then at 24 h. NO levels increased significantly in cells treated with FC compared to the untreated control cells (Figure 4.5).

Figure 4.5: FC treatment increased intracellular Nitric oxide. (A) SiHa and (B) HeLa cells were loaded with DAF-FM diacetate (2.5 μM) and exposed to FC, during which measurements (495/515 nm) were recorded every 1 min for 6 h. Representative graphs of intracellular NO in (C) SiHa and (D) HeLa after 24 hours of FC treatment; (n = 4) are shown for three independent experiments.

FC significantly increased intracellular NO within 6 h of the treatment with a maximal at the concentration of 40 µg/ml in both SiHa (~19.54±1.54 fold; p≤0.001) and HeLa
(~22.96±2.2 fold; p≤0.05) (Figure 4.5A and B, respectively). However, at 40 µg/ml of FC, the fold increase in NO at 24 h was ~3.28±0.20 (p≤0.05) and ~9.22±2.16 (p≤0.05), for SiHa (Figure 4.5C) and HeLa (Figure 4.5D), respectively, compared to the untreated control cells.

4.3.7 FC decreased mitochondrial membrane potential

It is well known that loss of mitochondrial membrane potential (ΔΨm) is an important step in initiation and activation of apoptotic process in cells (Ahn et al., 2014). Since generation of reactive nitrogen and oxygen species (RNOS) is related with mitochondrial dysfunction (Ahn et al., 2014), the effect of FC on ΔΨm was examined in SiHa and HeLa. The cells were treated with various concentrations of FC (0-40 µg/ml) for 24 h and ΔΨm was measured by using JC-1 dye. FC caused concentration dependent decrease in ΔΨm within 24 h of the treatment with maximum decrease at 40 µg/ml concentration in both SiHa (~1.25-fold; p=0.010) and HeLa (~1.37-fold; p=0.008) (Figure 4.6).

**Figure 4.6:** FC decreased mitochondrial membrane potential. SiHa and HeLa cells treated with different concentrations (0-40 µg/ml) of FC for 24 h. The mitochondrial membrane potential was measured by JC-1 (2.5 µM). The data represents mean ± SD of three independent experiments.
4.3.8 FC inhibited tumor growth in mice melanoma model

For analyzing the anti-tumorigenic potential of FC, B16F10 mouse melanoma model was used. Subcutaneous tumors of B16F10 melanoma were induced in mice by the injection of $1 \times 10^6$ viable tumor cells. One week after development of subcutaneous tumors, mice were given oral gavage of FC at 100 and 200 mg/kg bw for 15 consecutive days. It was observed that the untreated control mice showed tumor volume of $4.74\pm0.58 \text{ cm}^3$ and the positive control Adriamycin (ADR) (2 mg/kg i.v) showed a tumor volume of $0.89 \pm0.28 \text{ cm}^3$ ($p<0.001$). On the contrary, mice treated with 100 and 200 mg/kg of FC showed a tumor volume of $2.97\pm0.30 \text{ cm}^3$ ($p=0.026$) and $1.58\pm0.25 \text{ cm}^3$ ($p<0.001$), respectively (Figure 4.7A). The percent tumor growth inhibition in 100 and 200 mg/kg of FC treated animals was found to be 37% and 66%, respectively. ADR treatment (2 mg/kg i.v) resulted into 81% tumor growth inhibition compared to the untreated tumor control. Treatment as well as control groups showed 100% survival by day 15, except for one mouse in FC (200 mg/kg) that died quite early during the study (day 5), due to unknown reasons (Figure 4.7 B). There was no significant difference between the body weight of the FC treated group and that of the untreated control group (Figure 4.7 C).
Figure 4.7: FC exhibited anti-tumor activity in the B16F10 mouse melanoma model. (A) Effect of FC on tumor growth. B16F10 melanoma (1× 10⁶ cells/mouse) was inoculated subcutaneously (s.c) into the flanks of C57BL/6 mice (6 weeks old male). Eight days after the injection, mice were divided into five groups and each group consisted of six mice. The mice were treated with FC (100 and 200mg/kg) for 15 consecutive days. Adriamycin (2mg/kg) was used as positive control which was administrated i.v at 1, 4 and 9th day. The tumor volume was measured every 3 days; ***p<0.001, *p<0.05 between FC and tumor control at same day (B) Animal survival. Kaplan-Meier survival analysis was compared using the log-rank test (C) Body weight. The mean ± SD body weight of mice in each group was calculated.

4.3.9 FC modulated Th1 and Th2 cytokine response in mice

To investigate the effect of FC on Th1 and Th2 cytokines, we measured the levels of IFN-γ, IL-2 and IL-4 cytokines in the serum samples of FC treated and untreated mice (Figure 4.8). With 200 mg/kg FC treatment, B16F10 tumor bearing mice showed a
significant increase in IL-2 (~5.60-fold; p<0.001) and IFN-γ (~5.66-fold; p<0.001) levels (Figure 4.8 A and B) with a significant decrease in IL-4 (~3.77-fold; p=0.006) level compared to the tumor control group (Figure 4.8 C). Interestingly, ADR did not alter the levels of Th1 cytokine but decreased the Th2 cytokine levels. These observations showed that FC not only retarded the tumor growth but also helped in the activation of Th1 cytokine response in mice.

Figure 4.8: FC exhibited immunomodulatory activity. Levels of (A) IFN-γ, (B) IL-2 and (C) IL-4, in the sera of mice were determined by ELISA on day 15 of the treatment. The data represents mean ±SD of mice in each group.
4.4. Discussion:

A novel formulation, FC, was developed from the bark materials of *Ficus religiosa* and *Cinnamon zeylanicum* based on our previous experimental work related to their anti-cancer potential in cervical cancer. The standardized FC formulation strongly inhibited the cell viability of cervical cancer cells at relatively lower doses compared to either *Ficus religiosa* or *Cinnamon zeylanicum*. Interestingly, FC did not induce any cytotoxicity in normal cells which promoted us to further elucidate the molecular mechanism underlying the anti-cancer potential of FC in SiHa and HeLa cells.

Apoptosis or programmed cell death is an important mechanism to kill the tumor cells and is recognized as a strategy for identifying anti-cancer drugs (Ricci et al., 2006). It can be induced by generation of reactive nitrogen and oxygen species (RNOS) that result into loss of membrane potential ($\Delta \psi_m$), expansion of the matrix and rupture of the outer mitochondrial membrane (Ahn et al., 2014). Depolarization of mitochondrial membrane potential ($\Delta \psi_m$) is an important phenomenon responsible for the release of cytochrome c from mitochondria into the cytosol and subsequent activation of caspase (Ly et al., 2003). In cervical cancer cells, at an effective concentration of FC (40 µg/ml), we observed a significant proportion of cells undergoing apoptosis compared to the untreated control cells. Interestingly, at similar concentration, we observed increased intracellular RNOS and significant decrease in mitochondrial membrane potential ($\Delta \psi_m$).

Since FC induced apoptosis in cervical cancer cells, we examined its anti-cancer activity in C57BL/6 melanoma tumor model. This is a widely used model in cancer research to investigate the antitumor as well as immune response of anticancer drugs and their role in disease progression, as well as to test new therapies (Hannani et al., 2013). Daily oral administration of 100 and 200 mg/kg of FC significantly inhibited the growth
of B16F10 cells in mice. Adriamycin, was used as a positive control in the study. Interestingly, FC did not induce any adverse effect on the body weight of the treated mice. The treatment and the control groups did not show any major toxic effect till the end of the experiment, except for one mouse in FC (200 mg/kg) that died quite early during the study (day 5), due to unknown reasons.

Tumour reactive immune system is another checkpoint in cancer cell proliferation (Dobbelstein et al., 2014). The tumor inhibitory immune response is generally mediated by T-helper 1 (Th1) cells which express cytokines responsible for the destruction of tumor cells, whereas the expression of T-helper 2 (Th2) cytokines create a tolerogenic environment in which the tumor cells could grow (Smyth et al., 2001). In cancer patients or tumour-bearing animals, cell mediated immunity is weak with gradual shift from Th1 to Th2 cell phenotype leading to immune suppressive environment and tumor reactive immune dysfunction (Lauerova et al., 2001; Moretti et al., 2001). Plant extracts and herbal formulations have been shown to reduce cancer cell proliferation by modulating the immune system (Malik et al., 2009; Kamiyama et al., 2005). Consistent with this notion, our results showed significant up-regulation of Th1 (IFN-γ and IL-2) and down regulation of Th2 (IL-4) cytokine in FC treated groups. IFN-γ and IL-2 are important lymphokines that can activate Natural Killer (NK) cells, cytotoxic T lymphocytes, and tumoricidal macrophages, thereby, increasing the ability to kill tumor cells (Weigent et al., 1983). On other hand, IL-4, acts as the natural antagonist, and suppresses the function and production of IFN-γ (O'Garra et al., 2008). Thus, FC not only reduced the tumor size but also helped in the activation of Th1 immune system, whose down-regulation otherwise worsens the severity of the disease.

In conclusion, this study has demonstrated that standardized FC formulation caused NO and ROS production that could be responsible for the induction of apoptosis
through mitochondrial dependent pathway. Interestingly, FC retarded tumor growth in mice and simultaneously activated the immune system, favoring Th1 response. The immunomodulatory activity is a valuable addition to the therapeutic potential of FC. Our results clearly indicate that FC is a potent immuno-chemotherapeutic agent which may find usefulness in the management of cervical cancer either alone or as an adjunct to conventional radio or chemotherapy.