CHAPTER-3

ELUCIDATING THE ANTI-CANCER POTENTIAL OF FICUS RELIGIOSA IN SiHa AND HeLa CELLS

The research work given in the chapter has been published in Biotechnol. Bioinf. Bioeng (2011) and Plos One (2013)
Abstract:
In the present study, we have explored the anti-oxidant and anti-neoplastic potential of *F. religiosa* bark. The aqueous (FR$_{aq}$) and ethanolic (FR$_{et}$) extracts of the bark exhibited significant ‘total antioxidant capacity’ as determined by Oxygen Radical Absorbance Capacity (ORAC) method, however FR$_{aq}$ showed higher ORAC than FR$_{et}$. Both FR$_{aq}$ and FR$_{et}$ exhibited significant increase in anti-lipid peroxidative (ALP) activity with IC$_{50}$ values of 29.06 and 34.39µg/ml, respectively. The total phenol content present in one milligram of FR$_{aq}$ and FR$_{et}$ was found to be around 497.77 and 375.23µg, respectively, equivalent to gallic acid control. Interestingly, both FR$_{aq}$ and FR$_{et}$ showed significant cytotoxicity in cervical cancer cell lines SiHa (HPV 16+) and HeLa (HPV18+).

Based on the antioxidant potential, polyphenol content and ALP activity, FR$_{aq}$ was selected for further experiments. Its anti-cancer activity was further delineated with underlying molecular mechanisms in human cervical cancer cell lines, SiHa and HeLa. FR$_{aq}$ altered the growth kinetics of SiHa and HeLa cells in a dose-dependent manner. It blocked the cell cycle progression at G$_{1}$/S phase in SiHa that was characterized by an increase in the expression of p53, p21 and pRb proteins with a simultaneous decrease in the expression of phospho Rb (ppRb) protein. On the other hand, in HeLa, FR$_{aq}$ induced apoptosis through an increase in intracellular Ca$^{2+}$ leading to loss of mitochondrial membrane potential, release of cytochrome-c and increase in the expression of caspase-3. Moreover, FR$_{aq}$ reduced the migration as well as invasion capability of both the cervical cancer cell lines accompanied with downregulation of MMP-2 and HER-2 expression. Interestingly, FR$_{aq}$ reduced the expression of viral oncoproteins E6 and E7 in both the cervical cancer cell lines. All these data suggest that *F. religiosa* could be explored for its chemopreventive potential in cervical cancer.
3.1 Introduction

*F. religiosa* L. family Lauraceae, the most popular member of the genus Ficus, is native of the sub-Himalayan tract, Bengal and central India. It has been extensively distributed worldwide through cultivation (McFarland et al., 1944; Galil et al., 1984). It is found in the areas up to 1500 m elevation having annual rainfall varying from 50 to 500 cm during the monsoon season and tolerates a wide variation in temperature (below 0°C and above 40°C) (Pullaiah et al., 2006) Chemically *F. religiosa* had been found to contain phytosterols, amino acids, furanocoumarins, phenolics components, hydrocarbons, aliphatic alcohols, volatile components and a few other classes of secondary metabolites (Singh et al., 2011). The bark of *F. religiosa* shows the presence of bergapten, bergaptol, lanosterol, β-sitosterol, stigmasterol, lupen-3-one, β-sitosterol-d-glucoside (phytosterolin) and vitamin K1 (Singh et al., 2011). The bark also contains tannin, wax, saponin, β-sitosterol, lupeol, ceryl behenate, lupeol acetate, α-amyrin acetate, leucoanthocyanin, leucoanthocyanin, leucocyanidin-3-0-β-D-glucopyranoside, leucopelargonidin-3-0-β-D-glucopyranoside and leucopelargonidin-3-0-α-L-rhamnopyranoside, (Husain et al., 1992).

![Figure 3.1: A typical Ficus religiosa tree in its natural habitat](image)
Ficus religiosa L. (Figure 3.1), has been extensively used in traditional medicine to treat various disorders (Panda et al., 2005; Kirtikar et al., 1993). The bark forms the part of many Ayurvedic formulations such as “Pancha Valkala Kashaya” (decoction containing F. religiosa, F. benghalensis, F. glomerata, F. infectoria and Azadirachta indica) and “Pancha Valkaladi Tailum” (oil containing F. religiosa, Ficus benghalensis L., Ficus glomerata Roxb., Ficus infectoria Willd., A indica, Curcuma longa L. and Hemidesmus indicus R. Br.) (Panda et al., 2005; Singh et al., 2005). It has been shown to exhibit diverse pharmacological activities (Haneef et al., 2012) including wound healing (Choudhary et al., 2006), anti-bacterial (Nair et al., 2007), anti-convulsant (Singh et al., 2009), anti-diabetic (Deshmukh et al., 2007; Kirana et al., 2009), anti-inflammatory (Sreekshmi et al., 2007), acetyl cholinesterase inhibitory activity (Vinutha et al., 2007), and anti-anxiety activity (Ratnasooriya et al., 1998). The acetone extract of F. religiosa leaves has been shown to induce apoptosis in breast cancer cell lines (Haneef et al., 2012).

In the present study, we have investigated the antioxidant potential of aqueous (FR\textsubscript{aq}) and ethanolic (FR\textsubscript{et}) extracts of F. religiosa bark. Based on the antioxidant potential, polyphenol content and Anti Lipid Peroxidation (ALP) activity, FR\textsubscript{aq} was selected for further experiments. The putative molecular mechanism underlying the antineoplastic potential of (FR\textsubscript{aq}) was elucidated in cervical cancer cell lines, SiHa and HeLa. Our data suggests that Ficus inhibits the growth of SiHa and HeLa by inducing cell cycle arrest and apoptosis, respectively. Interestingly, FR\textsubscript{aq} significantly reduces the expression of viral oncoproteins E6 and E7, thereby suggesting the therapeutic potential of F. religiosa in cervical cancer.
3.2. Materials and Methods

3.2.1. Chemicals and Reagents

Tissue culture plasticware was purchased from BD Biosciences (CA, USA) and Axygen Scientific Inc (CA, USA). Dulbecco’s Modified Eagles Medium (DMEM) powder, penicillin and streptomycin were obtained from Invitrogen/ Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylthiazolium bromide (MTT), FCCP, Ionomycin and JC-1 were purchased from Sigma-Aldrich (St. Louis, MO). Primary antibody against p53 (DO-1), p21 (187), caspase-3 (H-277), cyto-c (7H8), HER-2 (F-11), pRb (C-15), ppRb (SER 807/811), HPV16 E6/18 E6 (C1P5), HPV16 E7 (ED17), HPV18 E7 (N-19) or tubulin (B-7) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Annexin V-FITC apoptosis kit #3 was purchased from Invitrogen (CA, USA). All other common reagents were procured from Qualigens Fine Chemicals (Mumbai, India).

3.2.2. Preparation of aqueous and ethanolic extract of Ficus religiosa

Bark of Ficus religiosa L. was collected from Pune District, Maharashtra, India. Botanical identification of plant material was carried out with the help of standard flora (18) and a voucher specimen (MPCC 2417) of authentic plant species have been deposited at the herbarium of Medicinal plants Conservation Center (MPCC), Pune, Maharashtra, India. Bark was chopped into small pieces, shade dried at ambient temperature and ground into coarse powder in a grinder. Aqueous and ethanolic extracts were prepared as per standard Indian Pharmacopoeia and soxhlet method, respectively. The extract obtained, was centrifuged at 13000 rpm for 15 min and the supernatant was filtered through Swiney filter (pore size, 0.45 µm) and the extract was stored at -80°C until further use.
3.2.3. Estimation of total phenolic content by Folin-Ciocalteu method

The total phenolic content of FR_	ext{aq} and FR_	ext{et} extracts was determined spectrophotometrically by Folin-Ciocalteu method (Pourmorad et al., 2006). The extract (100 µg mL\(^{-1}\)) was mixed with 5 ml of Folin-Ciocalteu reagent, previously diluted in distilled water (1:10), and 4 mL of sodium carbonate (1M in H\(_2\)O). The mixture was incubated at 37ºC for 15 min for the color development. The absorbance was measured at 765 nm using a Perkin Elmer spectrophotometer (lambda EZ201). Samples of the extracts were evaluated at a final concentration of 1 mg mL\(^{-1}\). Total phenolic content was expressed as mg g\(^{-1}\) gallic acid equivalent using the equation obtained from the standard calibration curve: \(y = 0.004x + 0.0201\), \(R^2 = 0.9953\).

3.2.4. Oxygen radical absorbance capacity (ORAC) assay

The total antioxidant capacity of the extract was determined using ORAC method (Huang et al., 2002). A freshly prepared fluorescein (150 µl of a 5 nM solution) was mixed with 25µl of various concentrations of the extract (0-200 µg/ml) in the flat-bottom black 96-well plate and incubated for 30 min at 37ºC. After incubation, fluorescence measurements (excitation, 485 nm; emission, 520 nm) were taken every 100 seconds to determine the background signal. After 3 cycles, 25 µl (250 mM) of AAPH (2,2'-azobis-2-methyl-propanimidamide) was added manually with a multi-channel-pipette. The test was resumed and fluorescent measurements were taken upto 150 min using FLUOstar omega multiplate reader, BMG labtech (Offenburg, Germany). The net area under the curve (AUC) of the standards and samples was calculated. The standard curve was obtained by plotting Trolox concentrations against the average net AUC of the three measurements for each concentration. The AUC was calculated as \(\text{AUC} = 1 + f1/f0 + ... + f149/f0 + (f150/f0)\), where, \(f0 = \) initial fluorescence reading at 0 min and \(fi = \)
fluorescence reading at time \( t \). The data were analyzed in Microsoft Excel to calculate the AUC. The net AUC was obtained by subtracting the AUC of the blank from that of the sample.

### 3.2.5. Anti-lipid peroxidation activity by TBARS method

Inhibition of lipid peroxidation activity by both aqueous and ethanolic extracts was determined using goat liver homogenate and thiobarbituric acid-malondialdehyde (TBA-MDA) (Wade et al., 1985). Animal tissue (goat liver) was perfused with KCl (0.15M in H\(_2\)O) and the perfused tissue (10\% w/v) was homogenized in ice cold KCl (0.15M) using mortar and pestle. The reaction mixture was set up using 0.5 ml of homogenate, 0.5 ml of different concentrations (0-200\( \mu \)g/ml) of FR\(_{aq}\) and FR\(_{et}\) extracts and 1 ml KCl (0.15 M). Lipid peroxidation was induced by adding 100\( \mu \)l FeCl\(_3\) (1mM in H\(_2\)O) and the reaction mixtures were incubated at 37\(^\circ\)C for 30 min. Trichloroacetic acid (15\% in 0.25N HCl), thiobarbituric acid (0.38\%) and 200\( \mu \)l butylated hydroxyl toluene (0.05\%) were added to stop the reaction. The reaction mixtures were heated at 80\(^\circ\)C for 60 min, cooled at room temperature and centrifuged at 6000 rpm for 15 min. Supernatant was collected and measured at 532 nm. The percent inhibition of lipid peroxidation was calculated by using the formula:

\[
\% \text{ inhibition of lipid peroxidation} = \left[ \frac{OD \text{ of induced sample} - OD \text{ of test sample}}{OD \text{ of induced sample}} \right] \times 100
\]

### 3.2.6. Cell Culture

The human cervical carcinoma cell lines, SiHa (HPV-16), HeLa (HPV-18) and C33A (HPV-negative) were obtained from National Center for Cell Science (NCCS), Pune, Maharashtra, India. The cells were grown in DMEM supplemented with 10\% FBS, 2mM
L-glutamine, and antibiotics (100 units/ml penicillin and streptomycin). The cells were incubated in a humidified 5% CO$_2$ incubator at 37ºC.

3.2.7. Cell viability
The anti proliferative activity of FR$_{aq}$ and FR$_{et}$ was determined by MTT dye uptake method on SiHa, HeLa as describe in chapter-2 section 2.2.4 (Koppikar et al., 2010). The anti proliferative activity of FR$_{aq}$ was further evaluated on C33A (HPV negative cell line).

3.2.8. Preliminary phytochemical investigation of aqueous extract of Ficus religiosa (FR$_{aq}$)
The freshly prepared FR$_{aq}$ extract was qualitatively tested for the presence of flavonoids, phenols, saponins, tannins and carbohydrates using standard procedures of analysis (Khandelwal et al., 2005).

3.2.9. Cell growth analysis
The assay was performed on FR$_{aq}$ (0-80 μg/ml) treated and untreated SiHa and HeLa cells as describe in chapter-2 section 2.2.5 (Koppikar et al., 2010; Kaul et al., 2003).

3.2.10. Colony formation assay
The assay was performed on FR$_{aq}$ (0-80 μg/ml) treated and untreated SiHa and HeLa cells as describe in chapter-2 section 2.2.6 (Koppikar et al., 2010).

3.2.11. Soft agar assay
The assay was performed on FR$_{aq}$ (0-80 μg/ml) treated and untreated SiHa and HeLa cells as describe in chapter-2 section 2.2.7 (Koppikar et al., 2010; Kaul et al., 2003).
3.2.12. Wound healing assay

The assay was performed on FR$_{aq}$ (0-80 μg/ml) treated and untreated SiHa and HeLa cells as described in chapter-2 section 2.2.8

3.2.13. Matrigel transmembrane invasion assay

For invasion studies, 24-well BioCoat Matrigel Invasion Chambers (BD Bioscience, Bedford, MA) were used (Kim et al., 2012). SiHa and HeLa cells ($5 \times 10^4$) with or without FR$_{aq}$ treatment (0-80 μg/ml) were seeded in serum-free medium into the upper invasion chambers and allowed to invade across the Matrigel-coated membrane for 24 h. The medium containing 10% FBS was added to the lower chamber which served as a chemo attractant. After 24 h of incubation, non-invading cells were removed from the top of each membrane with wet cotton swabs; invading cells attached to the bottom of the membrane were fixed with 4% formalin and stained using 0.5% crystal violet. The cell numbers were counted in ten random high-power (20X) fields using Axiovert 200M microscope (Carl Zeiss, Germany) equipped with a Sony Cyber-shot 3.3 mega pixels camera.

3.2.14. Gelatin zymography

SiHa and HeLa cells were seeded at a density of $4 \times 10^5$ cells/ml in 6-well plates and allowed to adhere overnight at 37°C in 5% CO$_2$ incubator. Next day, the cells were treated with various concentrations of FR$_{aq}$ (0-80 μg/ml) prepared in serum-free medium and incubated for 24 h. The following day, the culture medium was collected and centrifuged at 14,000 rpm for 20 min at 4°C to remove the cellular debris. The activity of MMP-2 in the conditioned medium was determined by gelatin zymography as described in chapter-2 section 2.2.7 (Koppikar et al., 2010).
3.2.15. Immunoblotting

SiHa and HeLa cells were plated at a seeding density of $4 \times 10^5$ cells/ml in 6-well plates and allowed to adhere overnight at 37°C in CO₂ incubator. Next day, the cells were exposed to various concentrations of FR$_{aq}$ (0-80 µg/ml) and incubated for 24 h. Following incubation, the cells were harvested by trypsinization, washed with 1X PBS and protein was extracted as described in chapter-2 section 2.2.11 (Koppikar et al., 2010). For cytochrome-c release, cytosolic and mitochondrial fractions were prepared as described previously (Sánchez-Alcázar et al., 2010). The protein was estimated using Bradford reagent (Biorad Laboratories Inc, CA, USA). Equal amount of protein was loaded on to either 10% or 12% (for E6 protein) SDS-polyacrylamide gel and transferred electrophoretically to Amersham Hybond-P PVDF membrane (GE Healthcare, UK) in sodium phosphate buffer (pH 6.8). The membrane was blocked in 5% BSA in TST and incubated at 4°C overnight with primary antibody against p53, p21, caspase-3, cyto-c, HER-2, pRb, ppRb, HPV16 E6/18 E6, HPV16 E7, HPV18 E7 or tubulin (Santacruz, CA, USA) at a 1:500 dilution. The membrane was washed in TST and incubated with secondary IgG HRP conjugate at 1:5000 dilution. Proteins were visualized with a chemiluminescence kit (Amersham ECL Advance western blotting detection kit, GE Healthcare, UK) and densitometry analysis was performed on scanned immunoblot images using the Image J gel analysis tool.

3.2.16. Assessment of cell cycle arrest

For cell cycle analysis, HeLa, SiHa and C33A cell lines were plated at a seeding density of $5 \times 10^5$ cells/well in 6-well plates and allowed to adhere for 24 h at 37°C in CO₂ incubator. Next day, the cells were treated with FR$_{aq}$ (0-80 µg/ml) for 24 h. The cells were harvested by trypsinization and fixed in ice-cold 70% ethanol at -20°C for 30 min.
Following washing with 1X PBS, the cells were treated with RNase A (100 mg/ml) at room temperature for 30 min and stained with Propidium Iodide (20 µg/ml). Stained cells were analyzed for DNA-PI fluorescence using a flowcytometer (FACS Calibur, BD). A minimum of 10,000 events were counted per sample; data were analyzed using FACS Calibur-cell quest software (Becton Dickinson) for the proportions of cells in G₀/G₁, S phase and G₂/M phases of the cell cycle.

3.2.17. Assessment of apoptosis
The assay was performed on FRₐq (0-80 µg/ml) treated and untreated SiHa and HeLa cells as describe in chapter-2 section 2.2.12.

3.2.18. Detection of Intracellular calcium using Fluo-3/AM
The assay was performed on FRₐq (0-80 µg/ml) treated and untreated HeLa cells as describe in chapter-2 section 2.2.13 (Koppikar et al., 2010).

3.2.19. Analysis of mitochondrial membrane potential (Δψm)
The assay was performed on FRₐq (0-80 µg/ml) treated and untreated HeLa cells as describe in chapter-2 section 2.2.14

3.2.20. Statistical analysis
All the experiments were performed in triplicates and repeated at least three times and the data has been presented as mean±SD. Statistical analysis was conducted with the SigmaStat 3.5 program (Systat Software, Inc.) using one-way ANOVA with α = 0.05.
3.3. Results

3.3.1 Comparison of total antioxidant capacity of FR$_{aq}$ and FR$_{et}$ by ORAC method

The antioxidant activity of *Ficus religiosa* has been reported by various groups (Panchawat et al., 2010; Rathee et al., 2010; Kumar et al., 2011). However, we have for the first time compared the ‘total antioxidant capacity’ of aqueous (FR$_{aq}$) and ethanolic (FR$_{et}$) extracts of bark of *F. religiosa* by ORAC method. It was found that both the extracts possessed significant free radical scavenging capabilities. However, FR$_{aq}$ inhibited fluorescein decay in a dose-dependent manner closer to that of the positive control, Trolox (Figure 3.2).

![Graph](attachment:image.png)

**Figure 3.2:** Oxygen Radical Absorbance Capacity (ORAC) assay. The graph represents the ORAC of the FR$_{aq}$ and FR$_{et}$ extracts which is expressed as Net Area Under Curve and compared with Trolox. \( n=3 \) experiments

3.3.2 Total phenolic content in FR$_{aq}$ and FR$_{et}$

The antioxidant activity exhibited by both aqueous and ethanolic *F. religiosa* bark extract may be due to phenolic compounds present in them. Folin-Ciocalteu method was
used to determine the total phenol content present in both the extracts and was compared with standard gallic acid (Figure 3.3). The results were expressed in terms of mg/g of extract. The total phenolic content present in FR\textsubscript{aq} and FR\textsubscript{et} was 497.77 and 375.23 mg/g equivalent of gallic acid, respectively.

![Graph showing standard gallic acid curve with equation: y = 0.004x + 0.020, R^2 = 0.996]

*Figure 3.3: Standard Gallic Acid Curve. The graph represents the absorbance of gallic acid at known increasing concentrations for Total phenol determination of FR\textsubscript{aq} and FR\textsubscript{et} extract. (n=3 experiments)*

### 3.3.3 FR\textsubscript{aq} and FR\textsubscript{et} inhibited lipid peroxidation

We used modified TBARS method to analyze the anti-lipid peroxidation capacity of both FR\textsubscript{aq} and FR\textsubscript{et} extracts. Animal tissue (liver) homogenate was mixed with different concentrations of the extracts and lipid peroxidation was induced with FeCl\textsubscript{3}. It was observed that the aqueous extract showed greater anti-lipid peroxidation (ALP) activity compared to the ethanolic extract (Figure 3.4), with IC\textsubscript{50} values of 29.06 and 34.39 μg/ml, respectively, for FR\textsubscript{aq} and FR\textsubscript{et}.
3.3.4 FR\textsubscript{aq} and FR\textsubscript{et} exhibited significant cytotoxic activity in cervical cancer cell lines

We evaluated the cytotoxic potential of both FR\textsubscript{aq} and FR\textsubscript{et} extracts in cervical cancer cell lines, SiHa and HeLa. It was observed that FR\textsubscript{aq} exhibited 100% viability in both SiHa and HeLa up to 160 μg/ml (Figure 3.5 A), beyond which it was cytotoxic to both the types of cell lines. However, FR\textsubscript{et} showed 100% survival till 160 and 80 μg/ml in SiHa and HeLa cell lines, respectively, beyond which it was cytotoxic to both the cell types (Figure 3.5 B). Thus, our results showed that besides antioxidant potential, 	extit{F. religiosa} aqueous and ethanolic bark extracts exhibited significant cytotoxic activity in both the cervical cancer cell lines.

Based on the antioxidant potential, polyphenol content and ALP activity, FR\textsubscript{aq} was selected for further experiments. Before evaluating its anticancer mechanism, we evaluated the cytotoxic potential of FR\textsubscript{aq} on C33A (HPV negative) cell line. Ficus did not induce any cytotoxicity up to 160 μg/ml concentration in C33A cells (Figure 3.5 C),

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure3.4.png}
\caption{\textbf{Anti-lipid peroxidation by TBARS Method.} The graph represents the inhibition of lipid peroxidation activity by the FR\textsubscript{aq} and FR\textsubscript{et} extracts expressed in percentage. (n=3 experiments)}
\end{figure}
which was similar to that observed in SiHa and HeLa. However, at higher concentrations, FR$_{aq}$ induced cytotoxicity in all the three cell lines.

Figure 3.5: Cytotoxic effect of (A)FR$_{aq}$ and (B)FR$_{et}$ extract in cervical cancer cell lines. SiHa and HeLa were treated with different concentrations (0-620 µg/ml) of extract for 24 h. The viability was measured by MTT assay. (C) Cytotoxic effect of FR$_{aq}$ on C33A (HPV negative) cell line after 24 h of treatment.

3.3.5 Phytochemical analysis of F. religiosa

The freshly prepared FR$_{aq}$ extract was qualitatively tested for the presence phytochemical and showed the presences of flavonoids, phenols, saponins, tannins and carbohydrates (Table 3.1).
**Table 3.1: Preliminary phytochemicals analysis of FR<sub>aq</sub> extract.**

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Phytochemical</th>
<th>Occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Flavonoids</td>
<td>+</td>
</tr>
<tr>
<td>2.</td>
<td>Phenols</td>
<td>+</td>
</tr>
<tr>
<td>3.</td>
<td>Saponins</td>
<td>+</td>
</tr>
<tr>
<td>4.</td>
<td>Tannins</td>
<td>+</td>
</tr>
<tr>
<td>5.</td>
<td>Carbohydrate</td>
<td>+</td>
</tr>
</tbody>
</table>

‘-’ = Absent ‘+’ = Present

The freshly prepared FR<sub>aq</sub> extract was qualitatively tested for the presence of flavonoids, phenols, saponins, tannins and carbohydrates using standard procedures of analysis (Khandelwal et al., 2005).

3.3.6 Ficus modulated the growth kinetics of cervical cancer cells

To test the effect of Ficus on the growth kinetics, SiHa and HeLa cells were treated with different concentrations of FR<sub>aq</sub> (0, 20, 40, and 80 µg/ml) and were grown for 24, 48 and 72 h. At the end of each treatment, the cells were stained with trypan blue, and the viable cells that excluded the dye were counted. It was observed that there was a dose-dependent decrease in the growth kinetics of FR<sub>aq</sub>-treated cells compared to the untreated control cells. It was observed that FR<sub>aq</sub> decreased the growth of the cells in a dose- and time-dependent manner.

In SiHa, FR<sub>aq</sub> decreased the cell growth at 80 µg/ml concentration by ~4.78-(p=0.008), ~4.72- (p=0.001) and ~3.42-fold (p=0.053) at 24, 48 and 72 h, respectively, compared to the untreated control cells (Figure 3.6 A). Similarly, at 80 µg/ml concentration of FR<sub>aq</sub>, HeLa cells exhibited ~5.53- (p≤0.001), ~5.94- (p=0.010) and ~6.37-fold (p=0.001) decrease in the cell growth at 24, 48 and 72 h, respectively, compared to the untreated control cells (Figure 3.6 B). This was further supported by colony formation and soft agar assays wherein a dose-dependent decrease in the number
of colonies was observed in both the cervical cancer cell lines (Figure 3.6 C and D, respectively). Interestingly, at 80 μg/ml concentration, FR\textsubscript{aq} significantly reduced the number of colonies in HeLa (~4.97 fold; p≤0.001) and SiHa (~2.95 fold; p≤0.001) compared to their respective untreated control cells (Figure 3.6 D). Thus, Ficus regulated the growth kinetics of cervical cancer cells in a significant manner.

![Graphs showing the effect of Ficus on cervical cancer cell lines](image)

**Figure 3.6:** Ficus regulated the growth of cervical cancer cells. SiHa (A) and HeLa (B) were treated with FR\textsubscript{aq} (0-80 μg/ml) for 24-72 h and the number of viable cells were counted using the trypan blue dye exclusion method. Data represent mean ± SD of three independent experiments. (C) The cervical cancer cell lines (SiHa and HeLa) were treated with FR\textsubscript{aq} (0-80 μg/ml) for one week. The colonies were stained with crystal violet and photographed. The experiments were repeated three times. (D) Both SiHa and HeLa (5x10^4) along with FR\textsubscript{aq} (0-80 μg/ml) were grown in soft agar for two weeks. Colonies were counted from at least 10 different areas and the average of each has been plotted. The data represents mean ± SD of 5 independent experiments.
3.3.7 Ficus induced cell cycle arrest in SiHa

To analyze the mechanism behind the Ficus mediated regulation of growth kinetics in cervical cancer cells, we investigated the cell cycle distribution in SiHa, HeLa and C33A. Flow cytometry analysis showed that in presence of FR$_{aq}$, SiHa exhibited an increase in G$_1$ population with a simultaneous decrease in S phase in a dose-dependent manner (Figure 3.7 A).

![FACS pictograms](image)

**Figure 3.7**: Ficus arrested the SiHa cell at G$_1$/S phase. Representative FACS pictograms of (A) SiHa, (B) HeLa and (C) C33A cells treated with FR$_{aq}$ (0-80 μg/ml) are shown. Cells were treated with different concentrations of FR$_{aq}$ (0-80 μg/ml) for 24 h. Enhanced accumulation of the cells in G$_1$ phase with a concomitant decrease in S-phase population was observed after treatment with Ficus in SiHa (as indicated by histograms). The data represents mean ± SD of three independent experiments.
Interestingly, at 80 µg/ml concentration, there was an increase in the percentage of cells in G₁ phase (from 59.88 to 72.33%) with a simultaneous decrease in the S phase population (from 15.98 to 8.50%; p<0.050). On the other hand, in HeLa (Figure 3.7 B), there was a significant increase in sub-G₀ population (from 3.65 to 87.38%; p<0.001) indicating apoptotic population. Interestingly, at non-toxic doses, FRₐq did not affect the growth of HPV negative C33A (Figure 3.7 C) cells.

3.3.8 Ficus altered the expression of cell cycle regulating protein in SiHa

We investigated the mechanism of G₁/S phase arrest in SiHa by evaluating the expression of G₁ checkpoint proteins such as p53, pRb, phospho Rb (ppRb) and p21. There was a significant increase in the expression of p53 (Figure 3.8 A and C) as well as its downstream effector, p21 (Figure 3.8 B and D) after treatment of the cells with FRₐq. The expression of pRb was analyzed since dephosphorylated pRb is known to form complexes with E2F to repress the transcription of cell proliferative genes (Giacinti et al., 2006). FRₐq significantly increased the expression of pRb (Figure 3.8 A and C) with a simultaneous decrease in the levels of ppRb (Figure 3.8 B and D) in a dose-dependent manner. These results suggest that Ficus induced G₁/S arrest in SiHa by modulating the expression of the cell cycle regulatory proteins.
Figure 3.8: *Ficus* modulated the expression of cell cycle regulatory proteins. Western blot shows the expression levels of p53 and pRb (A) as well as p21 and ppRb (B). Tubulin was used as a loading control. (C, D) Densitometric analysis of the western blot showing fold change in protein levels upon FR\textsubscript{aq} treatment. The bands were quantified by densitometry scanning using Image J 1.44p (Wayne Rasband, National Institutes of Health, USA, [http://imagej.nih.gov/ij](http://imagej.nih.gov/ij)). The data represents mean ± SD of three independent experiments.

### 3.3.9 Ficus induced apoptosis in HeLa

We found that in HeLa, *Ficus* treatment resulted into increase in the number of cells in sub-G\textsubscript{0} phase, indicative of apoptotic population (Figure 3.7 B). On staining with Annexin V-FITC, the cells showed a dose-dependent increase in both early as well as late apoptotic cell population (Figure 3.9 A). Interestingly, at 80 μg/ml FR\textsubscript{aq} concentration, there was ~4.4-fold (p≤0.050) and ~5.5-fold (p≤0.050) increase in both early as well as late apoptotic cell population, respectively, compared to the untreated
control cells. On the other hand, no apoptosis was observed in FRaq treated SiHa (Figure 3.9 B) or C33A (Figure 3.9 C) cells.

Figure 3.9: Ficus induced apoptosis in HeLa. Representative FACS pictograms of (A) HeLa, (B) SiHa and (C) C33A cells treated with FRaq (0-80 μg/ml) are shown. Percent of annexin V-positive (early-apoptotic cells, lower right quadrant) and Annexin V/PI-double-positive cells (late-apoptotic cells, upper right quadrant) are indicated. The data represents mean ± SD of three independent experiments.

3.3.10 Ficus increased intracellular calcium and decreased mitochondrial membrane potential in HeLa

We studied Ca\(^{2+}\) signaling mechanism in cells treated with FRaq and observed that it induced a dose-dependent increase in the intracellular calcium levels (Figure 3.10 A). Ionomycin was used as a positive control. Interestingly, the increase in intracellular
calcium resulted into disruption of the mitochondrial membrane potential (ΔΨm) that was observed by decrease in red fluorescence intensity, after staining the cells with JC-1 dye (Figure 3.10 B). There was ~3-fold reduction in the red fluorescence intensity (p≤0.001) at 80 μg/ml concentration of FRaq. FCCP was used as a positive control in the study.

Figure 3.10: Ficus increase intracellular calcium and decreased mitochondrial membrane potential. (A) Flow cytometric analysis of the rapid calcium release in HeLa cells after treatment with FRaq (0-80 μg/ml) has been shown. Ionomycin was used as a positive control. The data represents mean ± SD of three independent experiments. (B) FACS analysis following JC-1 staining of HeLa showed alteration of the mitochondrial membrane potential after FRaq (0-80 μg/ml) treatment compared to untreated control cells. The data represents mean ± SD of three independent experiments.

3.3.11 Ficus increased p53, caspase 3 and cytosolic cytochrom-c expression in HeLa

The mitochondrial membrane depolarization was associated with a dose-dependent increase in the cytosolic cytochrome c (Figure 3.11 A and C) that was accompanied by an increase in the expression of caspase 3 and p53 (Figure 3.11 B and D). These results indicate that Ficus induced apoptosis in HeLa through mitochondrial dependent pathway.
Figure 3.11: Ficus altered p53, caspase 3 and cytosolic cytochrome-c expression in HeLa (A) Western blot shows the expression of cytochrome c from cytosolic fraction. Tubulin was used as a loading control. (B) Total protein was isolated and analysed for expression of p53 and caspase 3 by immunoblotting. Tubulin was used as a loading control. (C and D) Densitometric analysis of the western blot showing fold change in protein levels. The bands were quantified by using Image J 1.44p (Wayne Rasband, National Institutes of Health, USA, [http://imagej.nih.gov/ij](http://imagej.nih.gov/ij)).

3.3.12 Ficus decreased invasion and migration of SiHa and HeLa

Wound healing assay was performed in both the cell lines and it was observed that Ficus effectively inhibited the migration of both SiHa (Figure 3.12 A) and HeLa (Figure 3.12 B) in a dose- and time-dependent manner compared to the untreated control cells. After 16 h, the untreated SiHa and HeLa cells were able to cover up ~82% of the wound, whereas at 80 µg/ml of FR_{aq} treatment, the cells covered up the wound by ~33%
(p<0.001) and 22% (p<0.001), respectively (Figure 3.12 C). At this particular dose, Ficus reduced the invasive capability of both SiHa and HeLa by ~2.45- (p≤0.001) and ~3.8-folds (p≤0.001), respectively, compared to the untreated control cells (Figure 3.12 D).

Figure 3.12: Ficus regulated invasion and migration of cervical cancer cells. Analysis of cell migration in SiHa (A) and HeLa (B) treated with FRaq (0-80 μg/ml) was measured by wound-healing assay. The upper panel of the image shows the wound made at 0 h. The lower panel shows the migration of cells corresponding to the distance travelled at 16 h. (C) Graphical representation of wound closure in SiHa and HeLa cells at 16 h after FRaq treatment has been shown. Values were represented as the percent wound closure and expressed as mean ± SD for three independent experiments. (D) Cell invasion assay showing the percentage of cells invaded per field in the presence or absence of FRaq. The invaded cells were counted in ten random fields and the values have been expressed as mean ± SD for three independent experiments.
3.3.13 Ficus reduced the expression of MMP-2 and HER-2 expression

It is well known that increased expression of MMPs in tumor tissues is associated with cancer cell matrix degradation, invasion as well as metastasis (Deryugina et al., 2006). We observed that FRaq significantly down-regulated the expression of MMP-2 in both SiHa and HeLa cells (Figure 3.13 A) compared to the untreated control cells.

![Image of gelatin zymography and Western blot analysis showing downregulation of MMP-2 and HER-2 expression.](image)

**Figure 3.13:** Ficus reduced the expression of MMP-2 and HER-2 expression. (A) Gelatin zymography showing downregulation of MMP-2 expression in FRaq (0-80 μg/ml) treated SiHa and HeLa. (B) Western blot analysis showing decrease in HER-2 expression in SiHa and HeLa treated with FRaq (0-80 μg/ml). Tubulin was used as a loading control. (C) Densitometric analysis of the western blot showing fold change in HER-2 protein levels in SiHa and HeLa. The bands were quantified by densitometry using Image J 1.44p (Wayne Rasband, National Institutes of Health, USA, [http://imagej.nih.gov/ij](http://imagej.nih.gov/ij)).

HER2/neu has been reported to enhance the metastatic potential of cancers cells (Yu et al., 1994) and is positively correlated with MMP-2 expression (Pellikainen et al., 1994). We found that FRaq decreased the expression of HER-2 in a dose-dependent
manner in both SiHa and HeLa (Figure 3.13 B and C). The data suggest that Ficus reduced the migration as well as invasion of cervical cancer cells by modulating the expression of HER-2 and MMP-2 proteins.

3.3.14 Ficus reduced the expression of viral oncoproteins E6 and E7

Since, Ficus exhibited significant antineoplastic potential in both HPV16 (SiHa) and HPV18 (HeLa) positive cell lines, we investigated the expression of the viral proteins E6 and E7 in the treated and untreated cells. It was observed that, FR\textsubscript{aq} significantly reduced the expression of E6 and E7 oncoproteins in both SiHa and HeLa (Figure 3.14 A and B, respectively).

**Figure 3.14: Ficus decreased the expression of E6 and E7 proteins.** The expression of E6 and E7 oncoproteins was determined by immunoblotting with E6 and E7 antibodies in SiHa (A) and HeLa (B) treated with FR\textsubscript{aq} (0-80 μg/ml). Tubulin was used as a loading control. Densitometric analysis of the western blot showing fold change in E6 and E7 protein levels upon FR\textsubscript{aq} treatment.
in SiHa (C) and HeLa (D). The bands were quantified by densitometry using ImageJ 1.44p (Wayne Rasband, National Institutes of Health, USA, http://imagej.nih.gov/ij).

At 80 μg/ml FR<sub>aq</sub> concentration, the expression of E6 and E7 proteins were decreased by ~3.0- (p≤0.001) and 3.7-folds (p≤0.001), respectively, in SiHa (Figure 3.14 A and C) and by ~3.2- (p≤0.001) and 4.0-folds (p≤0.001), respectively, in HeLa compared to the untreated control cells (Figure 3.14 B and D). Thus, Ficus decreased the expression of the viral oncoproteins E6 and E7, which potentiates its therapeutic significance in cancer regulation.

3.4 Discussion

In the present study, we have investigated the antioxidant potential of aqueous (FR<sub>aq</sub>) and ethanolic (FR<sub>et</sub>) extracts of <i>F. religiosa</i> bark. We have further elucidated the anti-neoplastic activity of the aqueous extract of <i>F. religiosa</i> bark (FR<sub>aq</sub>), based upon its higher antioxidant potential, polyphenol content and ALP activity compared to FR<sub>et</sub> extract. FR<sub>aq</sub> regulated the growth kinetics of the cervical cancer cells lines in a statistically significant manner and thus, Ficus exhibited a promising anticancer potential.

p53, a master tumor suppressor, is the most frequently mutated gene in almost all kinds of human cancers (Rivlin et al., 2011). Moreover, loss of p53 function is responsible for the progression to more aggressive cancer phenotype (Muller et al., 2011). In cervical cancer, E6 from high-risk HPV types (16 and 18) initiates degradation of p53 and thus, restoration of its function could be an effective therapeutic approach (Scheffner et al., 1991). Reactivation of p53 in cervical cancer cells can lead to inhibition of cell proliferation as well as induction of apoptosis (Kochetkov et al., 2006). Most of
the chemopreventive drugs regulate the growth of cancer cells either by arresting them at G₁/S or G₂/M phase or by induction of apoptosis by p53-dependent or independent mechanisms (Sa et al., 2008). In our studies, we found that FRaq exerted its anti-proliferative activity in each of the cervical cancer cell line by different mechanisms. In HPV-16 positive SiHa cells, FRaq induced G₁/S phase arrest through increase in the expression of p53 and p21 with a simultaneous decrease in the phosphorylation of pRb tumor suppressor protein. p21^{WAF1/CIP1}, a cyclin-dependent kinase inhibitor, is a p53-inducible protein that blocks the cell cycle progression in the G₁/S phase (Niculescu et al., 1998). Thus, up-regulation of p21 by FRaq might have resulted into activation of downstream effectors of p53-dependent G₁/S arrest. The hypophosphorylated form of retinoblastoma protein (pRb), a tumor suppressor, forms a complex with E2F transcription factor resulting into repression of cell proliferative genes (Henley et al., 2012). The viral E7 oncoprotein is known to inactivate the complex formation between pRb and E2F, thereby resulting into destabilization of pRB that eventually leads to deregulation of the cell cycle (Jones et al., 1997). In our studies, we observed that Ficus reduced the levels of ppRb that might have resulted into increased expression of pRb as well as eventual arrest of cells in G₁/S phase.

Apoptosis is an important mechanism to kill the tumor cells and (Korbakis et al., 2012) can be induced by increase in the mitochondrial calcium that results into loss of membrane potential (ΔΨm), expansion of the matrix and rupture of the outer mitochondrial membrane (Fulda et al., 2010). This results into release of cyt c into the cytosol, either by inhibition of anti-apoptotic factors or activation of pro-apoptotic proteins leading to the activation of caspase 3/9 (Elmore et al., 2007). During cell death, mitochondria are known to accumulate Ca^{2+}, resulting into activation of the permeability transition pore (PTP) that leads to transient mitochondrial depolarization (Duchen et al.,
This leads to release of cyt c along with a large number of other factors from the inter-membrane space (Giorgi et al., 2012). In HPV-18 positive HeLa cells, FR_{aq} induced p53-dependent apoptosis through increase in intracellular calcium and depolarization of mitochondrial membrane potential that lead to release of cytosolic cyt c and increase in caspase 3 expression. Interestingly, at non-cytotoxic dose, Ficus did not induce either arrest or apoptosis in C33A (HPV-negative, p53 mutated), thereby suggesting alternate mechanisms of cell death.

The observed dichotomy in the regulation of growth in SiHa and HeLa could be due to variation in p53 activation that may decide the fate of a cell to either initiate apoptosis or undergo cell cycle arrest. It has already been reported that low levels of p53 induce cell cycle arrest whereas high levels of p53 induce apoptosis (Vousden et al., 2012; Zhang et al., 2000). Our results show that p53 was activated more in HeLa compared to SiHa in response to FR_{aq} treatment, thereby, resulting into apoptosis in the former and cell cycle arrest in the latter. The other reason for the altered response of SiHa and HeLa towards Ficus treatment could be the difference in their genetic make-up that includes their HPV status as well as the viral copy number (Meissner et al., 1999). For example, SiHa (squamous cell carcinoma) contains around 1-2 integrated copies of HPV 16 genome whereas HeLa (adenocarcinoma) has around 10-50 integrated copies of HPV 18 (Meissner et al., 1999; Bravo-Cuellar et al., 2010). Moreover, the rate of replication is also different in both the cell types.

HER2/neu oncogene is frequently amplified in cervical cancer and can be considered as a potent therapeutic target (Mitra et al., 1994; Chavez-Blanco et al., 2004). Its overexpression has been found to be associated with up-regulation of MMP-2 and MMP-9 that play an important role in cancer cell invasion and metastasis (Pellikainen et al., 2004). Interestingly, Ficus significantly reduced the expression of both HER-2 and
MMP-2 that might have resulted in the observed decrease in the migration as well as invasion of cervical cancer cells.

E6 and E7 are the two viral oncoproteins known to induce cervical cancer by inactivating the tumor suppressor proteins, p53 and pRb, respectively (Scheffner et al., 1991). p53 gene is mutated irreversibly in most of the cancers; however, cervical carcinomas and cell lines have been reported to retain wild-type p53 and pRb genes whose function gets masked by the viral E6 and E7 proteins (Abdulkarim et al., 2002). We observed that Ficus decreased the expression of E6 and E7 in both the cervical cancer cell lines. The down regulation of E6 and E7 oncoproteins might have led to the restoration of tumor suppressor functions of p53 and pRb proteins, respectively. This might have led to the activation of downstream signaling molecules resulting into either cell cycle arrest or apoptosis. Even though a direct effect of E6/E7 on HER-2 has not been reported, however, their coexpression has been demonstrated to be critical for induction of head and neck squamous cell carcinomas (HPV positive) (Al Moustafa et al., 2004) as well as breast cancer (Woods et al., 2005). These data suggest that F. religiosa has the potential to target HPV E6 and E7 proteins that could have a significant therapeutic potential in cervical cancer.

Thus, our findings provide a strong basis for further exploration of F. religiosa as a therapeutic drug against cervical cancer, either alone or as an adjuvant to standard chemotherapeutic agents.