Ficus religiosa (Linn.) bark extract secondary metabolites bestow antioxidant property inducing cell cytotoxicity to human breast cancer cells, MDA-MB-231 by apoptosis involving apoptosis-related proteins, Bax, Bcl-2 and PARP

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Abstract

Ficus species, auspicious in many cultures worldwide are sources of novel bioactive secondary metabolites. The mechanism by which they exhibit antioxidant and apoptotic properties is scant. The main objective of the current study was to evaluate the properties of activity guided fractionated bark methanol extract of *Ficus religiosa*. As the methanol extract exhibited highest antioxidant activity it was evaluated for secondary metabolites and therapeutic properties. UPLC-MS analysis of the extract identified the 11 and five secondary metabolites including the rutin, 3-caffeoylquinic acid, luteolin 7-*O*-rutinoside, 6-C-glucosyl-8-C-arabinosylapigenin and kaempferol-3-*O*-rutinoside were reported. The MTT assay results identified minimal cytotoxicity for non-cancerous cell line (HEK 293 T) and maximum cell death for human breast cancer cells, MDA-MB-231 (EC_{50}, 91.32 ± 4.21μ $g.mL^{-1}$). A high degree of DNA fragmentation in MDA-MB-231 cells subjected to the extract was observed. A clear indication of apoptosis via chromatin condensation was visualized by CLSM. The apoptotic response to treatment was also apparent in the increase in BAX along with the proteolytic cleavage of PARP-1 and a decreased Bcl-2 levels as revealed by Western blot analysis. The MDA-MB-231 cells upon exposure to the extract (91μ $g.mL^{-1}$), stimulated cells to early apoptosis (32.5%) and apoptosis (61.6%) as evidenced by flow cytometer studies. Apoptotic cells being represented by a sub G0/G1 population (86.25%) seen to the left of the G0/G1 peak were recorded. The presence of novel bioactive compounds has uncovered possible therapeutic values by modulating antioxidant and apoptosis leading to the development of potential alternative anticancer drugs.

Introduction

One of the most diverse genera belonging to Moraceae family is the *Ficus* genus. They constitute to more than 800 species. Collectively known as fig trees, they are distributed in tropical and subtropical regions [1]. *Ficus religosa* (Linn.) (Family - Moraceae), commonly called ‘Dhainyaro’, is distributed above mean sea level (200–1800 m). Diverse ethnomedicinal value for the stems, leaves, barks and flowers have been reported. Various health benefits such as antioxidant, antimicrobial, antidiabetic, anticancer and antirheumatic activities have been attributed to most of the reported *Ficus* spp. [2, 3]. Drug formulations incorporate this plant, thus it edges the researchers to evaluate it and provide scientific validity for its application in medical treatment.

Traditionally the bark is used as an antibacterial, antiprotozoal, antiviral, astringent, antidiarrhoeal, in the treatment of gonorrhea, ulcers. The leaves are used for skin diseases, have reported antivenom activity and are reported to regulate the menstrual cycle. In Bangladesh, it has been used in the treatment of various diseases such as inflammation and infectious diseases [4]. In case of high fever, its tender branches are used. Fruits are used as laxatives [5]latex is used as a tonic and fruit powder is used to treat asthma [6].

Pharmaceutical industries and agriculture incorporate natural products research in development of high-value commodities thus it occupies a prominent position for use in human healthcare, nutrition and

therapeutics [7]. Natural product chemistry has also played a vital role in providing better substitutes for existing drugs [8], especially in dreaded diseases like cancer, a major cause of morbidity and mortality in developing and developed countries alike [9] and the World Health Organization (WHO) has described cancer as the second principal cause of death, accounting for approximately 16.66% of all deaths. There is a constant urge to search for new, alternative bioactive compounds with anticancer and antioxidant activities.

Breast cancer is the most common cancer as well as the second leading cause of cancer-related deaths in women across the world. One out of ten women over 55 years of age is frequently diagnosed with breast cancer. Dietary pattern has been identified as one of the major factors for the difference in breast cancer incidence. Major issues concerning conventional anti-cancer chemotherapy are the occurrence of side effects induced by the non-specific targeting of both normal and cancerous cells. Based on this, there has been growing interest in the use of naturally occurring molecules with chemo-preventive and chemotherapeutic properties in cancer treatment. Breast cancer cell lines are useful tools for studying the mechanism of new nutraceuticals, pharmaceuticals and drugs effects on mammalian cells. MDA-MB-231 cell line is a human breast cancer cell line known to be widely used in such studies.

Bark extractives retard wood decay and resin formation protects wounded tissues. The toxic and antifeedant compounds in the bark minimize insect and animal browsing through their poisonous, unpalatable or emetic properties. However, it is worth noting that these compounds may be toxic to the producing plant and the wide variety of phytochemicals produced may be part of a selection process to minimize plant toxicity while maximizing protection. These components endow woods with their many colors and hues, scents and beauty. Tropical and sub-tropical tree species typically contain greater amounts of extractives [10] in their bark wood that may be extracted with solvents in comparison to other parts of the tree [11]. They may be rare and structurally complicated metabolites that possess great medicinal value, such as the anticancer drug for this ailment, a growing burden [9].

With this thought in mind, in the present study we report activities of the extract from *F. religiosa* bark. The antiproliferative cell cytotoxicity capacity via apoptosis as the possible mechanism is detailed for the first time.

The secondary metabolite finger print by Ultra performance Liquid Chromatography-Mass Spectroscopy (UPLC-MS) analysis of bark methanol extract was carried out to identify confidently these in the extracts. Cell cytotoxicity studies in MDA-MB-231 (human breast cancer cells) and HEK 293 T (non-cancerous cell line) by MTT assay was carried out. The cell death by apoptosis was assessed by DNA fragmentation and accumulation of apoptotic subG0/G1 cells. Morphologically it was assessed by combined acridine orange and ethidium bromide staining for detecting incidence of cell death. The apoptotic response to treatment was also evaluated by incorporating studies of apoptosis-related proteins, BAX, Bcl-2 along with PARP by Western blot analysis.

Methods

Plants

The fresh bark (50 g) of test plant, *Ficus religiosa* (Linn.) was collected during winter (January, 2017-18) from Kigga region of Western Ghats in Chikmagalore District of Karnataka State, and a herbarium specimen was deposited (*F. religiosa* bark # IOE LP0024). Fresh bark was washed, shade-dried, ground to a powder and 100 g of powder were extracted three times by hexane, chloroform and methanol (1:10 w/v) at room temperature. The solvent was evaporated to yield dry extracts in SpeedVac (Savant SPD 2010, Thermo Scientific), and stored under dark at 4^{o}C until further use.

Chemicals and Reagents

Trypsin, chloramphenicol, acridine orange, quercetin (QE), beta-carotene, (+)-catechin, butylated hydroxytoulene (BHT), proteinase K, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 3-(4,5-dimethylthylthiazol-2-yl)-2,5-diphynyl tetrazolium bromide (MTT), Nonidet P-40, RNAse A and ethidium bromide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nonessential amino acid, sodium biocarbonate, Dulbecco'smodified Eagle's medium (DMEM), L-glutamine and gallic acid were purchased from HiMedia (India). StepupTM 1kb DNA ladder was purchased from Merck Biosciences, Bangalore (India). Fetal Bovine Serum (FBS) and trypsin-EDTA were purchased from GIBCO-BRL. MDA-MB-231 (human breast cancer cells) and HEK 293 T (non-cancerous cell line) were procured from the National Center for Cell Science (NCCS), Pune, India. Antibodies against Bax, Bcl-2, cleaved PARP-1 and β-actin were purchased from Cell Signaling Technology (Bedford, Massachusetts, USA). All other reagents and media components used were of analytical grade and were purchased from Sigma chemicals, Himedia, Merck or other local commercial sources unless otherwise mentioned.

Determination of total phenols and flavonoids content

Total phenolic content of the crude extract was determined by the Folin-Ciocalteu micro-method as described [12] which was expressed in µg of gallic acid equivalent per mg extract (µg GAE.mg^{-1} extract). The total flavonoid content was determined [13] and expressed in µg of quercetin equivalent per mg extract (µg QE. mg^{-1} extract).

beta-carotene/linoleic acid bleaching assay

The crude extract's ability to prevent beaching of beta-carotene, by its oxidation in the presence of O_2 molecule, was performed by earlier reported procedure with small modifications [14]. The absorbance values were measured at 470 nm to calculate the inhibitions percentages (I %) of the sample.

Determination of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and reducing power activities

To determine the antioxidant activity (IC_{50}) of the extract, we used the stable radical DPPH according to Brand-Williams *et al*. [15]. With respect to the reductive potential, it was determined by the Fe^{3+} to Fe^{2+}

transformations in the presence of the crude extract, using the method of Gülçin *et al*. [16]. The BHT and quercetin were used as positive control.

Evaluation of *F. religiosa* extract induced cell cytotoxicity

a. Cell lines, culture conditions and MTT assay

In vitro experiments were done using human cell lines, MDA-MB-231 (human breast cancer cells) and non-cancerous cell line (HEK 293 T). They were grown in DMEM, supplemented with 10% FBS as per reported protocol [17]. The cells were plated at 3×10^4 cells/cm2, grown in humidified 5 % CO2 with 95 % air atmosphere at 37°C, and experiments were initiated 48h after plating. The culture medium was replaced two times a week. For the experiments, confluent cells were trypsinized and plated in 96-, 6-well plates, or into tissue culture dishes (6 mm).

The cell viability was tested using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). MTT (1 mg/mL) was dissolved in sterile phosphate buffer saline (PBS; 0.05 M phosphate buffer, pH 7.2, 0.8 % NaCl) at room temperature. The solution was further sterilized by passing through a 0.2-µm filter and stored at 4°C in the dark.

The cells were plated at 3×10^4 cells/cm2, grown in humidified 5 % CO2 with 95 % air atmosphere at 37 °C, and experiments were initiated 48 h after plating. Different concentrations of *F. religiosa* bark methanol extract in the range from 1 to 200µg.mL^{-1} into the respectively labeled wells. After 48h of incubation, MTT (10µL) was added to each well and the plates were incubated at 37°C for 4 h in the dark. The supernatants were aspirated from the wells and washed thrice with PBS. DMSO (100µL) and 25µL of 0.1M glycine buffer (pH 10.5) were added to each well. After an incubation period of 15min, the absorbance was measured at 570 nm using multimode plate reader (Varioskan Flash Top, Thermo Fisher Scientific, Germany). Controls consisting of same concentration of cells without *F. religiosa* extract were maintained. Any absorbance due to reaction of the extract with MTT in wells devoid of cells was subtracted from the readings. Triplicate wells were assayed for each condition.

b. DNA fragmentation assay

MDA-MB-231 cells ($5x10^4$cells.mL^{-1}) were treated with *F. religiosa* extract (91mg.mL^{-1}) and camptothecin (2mg.mL^{-1}) for 48h. Treated and untreated cells were harvested and re-suspended in phosphate-citrate buffer (40 µL containing 192 parts of 0.2M Na_2HPO_4 and 8 parts of 0.1M of citric acid, pH 7.8). They were incubated for 30 min at room temperature. The cells were then centrifuged (5 min) followed by addition of Nonidet P-40 (3µL of 0.25%), RNAse A (3µg) and incubated at 37°C. After 30 min, proteinase K (3 µg) was added and fragmented DNA was precipitated with the addition of ethanol overnight at −20°C followed by centrifugation. The DNA pellet obtained was dissolved in Tris-EDTA (TE; 10mM Tris, 1mM EDTA pH 8.0) buffer. Fragmentation was analyzed by agarose gel (1.8%) electrophoresis. The fragmented DNA ladder formation in MDA-MB-231 cells was visualized on a UV transilluminator after staining with ethidium bromide (5µg).

c. Apoptosis assessed by nuclear morphology

Apoptotic nuclear morphological changes of MDA-MB-231 cells after treatments with the extract were observed after dual staining with acridine orange/propidium iodide (AO/PI). MDA-MB-231 cells (5×10^4 cells/well) were seeded in six-well plates on 0.01% poly-L-Lysine coated cover slips (24mm) and were treated with *F. religiosa* (91mg.mL^{-1}) and camptothecin (2mg.mL^{-1}) for 48h. Following incubation for 48 h, cells were washed with phosphate buffered saline (PBS) twice and stained with AO/PI (1mg.mL^{-1}) mixture for 2–3min. Apoptotic nuclear morphological changes after treatments were observed by Confocal Laser Scanning Microscopy (CLSM) LSM 710 (Carl Zeiss, Germany).

d. Western Blot Analysis: Bax, Bcl-2 and cleaved PARP

Cytosolic fractions of MDA-MB-231 cells treated with *F. religiosa* bark methanol extract (45 and 91µg.mL^{-1}) and camptothecin for 48h and control cells (minus treatment) were compared for release of Bax, Bcl-2 and cleaved poly ADP ribose polymerase (PARP) by Western blot analysis. Cell cultures (5×10^4 cells) were washed, and cytosolic fractions were prepared as reported earlier [17]. Briefly, cell, *F. religiosa* bark methanol extract and camptothecin treated, and controls were lyzed by cold lysis 50 mM, Tris–HCl buffer (pH 7.5) containing NaCl (150mM), Nonidet P-40 (0.5 %), phenylmethylsulfonyl fluoride (PMSF; 1 mM), NaF (1 mM), dithiothreitol (DTT; 1 mM), and complete protease inhibitor cocktail (4 mg/ml). Protein content in the cell lysates were determined using Bradford method (Bio-Rad protein assay kit, Bio-Rad Hercules, CA). Total protein (50 µg) was separated by sodium docecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) in a 1-mm thick, 12 % polyacrylamide gel. Immediately after SDS-PAGE, the separated proteins were blotted [18] onto nitrocellulose membrane (Millipore) using Multiphor II (LKB, Pharmacia) electrophoretic transfer apparatus. The blots were blocked in fat-free milk [2 % in Tris-buffered saline; TBS (10 mM Tris–HCl, pH 8.0 containing 125mMNaCl)]. They were incubated with primary antibodies: Bcl-2 antibody (1:1000 dilution), Bax antibody (1:1000 dilution), cleaved PARP (1:1000 dilution) b-actin (1:5000 dilution) for 2 h at 37°C, diluted in TBS. After three washes with TBS, the blots were incubated with secondary antibodies; horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit immunoglobulin (Ig) G (Cell Signaling Technology Inc.) for 1 h at room temperature. After three washes, the blots were subjected to 3,3'-diaminobenzedine and hydrogen peroxide treatment staining peroxidase activity to visualize immunoreactive proteins.

e. Apoptosis analyzed by Annexin V FITC and Propidium (PI) Labeling and Flow Cytometer

After exposure to *F. religiosa* extract (91µg.mL^{-1}) for 48h, these cells and control (minus *F. religiosa* extract) were trypsinized and centrifuged at $10^3\times$g for 3 min. Cells ($5x10^4$) were resuspended in 200µL of PBS and incubated with annexin V FITC and PI for 15 min according to manufacturers' protocol (Beckman Coulter, U.S.A.). Theoretically, cells stained by annexin V were early apoptotic cells (lower right) and cells stained by PI were considered as necrotic (dead) cells (upper left). Viable cells were not stained (lower left). Late apoptotic cells were double stained (upper right). A fluorescent activated flow cytometer (Cell Lab QuantaTM, SC, Beckman Coulter, U.S.A.) was used to examine the cells (1×10^4).

f. Ficus religiosa bark methanol induces apoptosis by generation of subG0/G1 cells in cell cycle analysis

Apoptotic cells can be identified by certain characteristics like the loss of DNA from permeabilised cells due to DNA fragmentation. It results in a population of cells with a reduced DNA content. So when, stained with a DNA intercalating dye like propidium iodide (PI), then a DNA profile representing cells in G1, S-phase and G2M will be observed with apoptotic cells being represented by a sub G0/G1 population seen to the left of the G0/G1 peak.

Briefly, human breast cancer cell lines ($5x10^4$cells.mL^{-1}) cells were cultured in culture flasks ($25cm^2$) in the presence of extract (91µg.mL^{-1}) for 48 h. After treatment, the cells were washed and centrifuged. The cells were resuspended and fixed with 70% ethanol at 4°C for 2 h. After fixing, the cells were washed with PBS and centrifuged again. The pellet was broken up by vortexing and then resuspended in PBS (250 µl) containing propidium iodide (PI; 20 µg.mL^{-1}), RNase A (20 µg.mL^{-1}) and Triton X-100 (0.1%). After incubation for further 30 min in the dark, cells were washed two times with buffer. The cells ($1×10^4$ counts) were read in Flow Cytometer, Cell Lab Quanta SCTM flow cytometer (Beckman Coulter) as per manufacturers' protocol.

LC analysis of methanol extract in negative mode

Synapt G2 (UPLC/MS separations with QuanTof) along with the Agilent zorbax SB-C18 (15

cm, 3.5 µm) column was coupled to an HCTultra ion trap MS detector was used for the qualitative analysis of the metabolites [19, 20] according to manufacturers protocol. The nebulizer pressure was 60 psi at a drying temperature of 350°C and the nitrogen flow rate 10 L.min^{-1} was used. The bark methanol extract was filtered (0.2 micron syringe filters, Millipore, U.S.A) and an aliquot (15µL) was injected into the system with the dwell time as 420 msec, the flow rate as 0.4 mL.min^{-1} and finally the temperature of the column as 25^oC. The mobile phase consisted of (A) water + 0.1% acetic acid (v/v) and (B) acetonitrile containing 0.1% acetic acid (v/v). The used linear gradients included 10%, 50%, 95% (repeated twice), and 100% (repeated twice) (v/v) for the A solution and 0%, 25%, 45%, 55%, 60% for the B solution. The washing time was 75 min applying 0.3 mL/min at all the times. The ESI-MS spectra in negative ionization mode mass spectra were acquired by electrospray, ESI ion source (Bruker Daltonik GmbH, Bremen, Germany). The collision gas helium was used for the fragmentation in the ion trap of the isolated compounds. The detection conditions were as follows: drying gas (N2) was 300^oC at a dry gas flow rate of 35 mL.min^{-1}, nebulizing pressure (N2) of 30 psi and capillary voltage 4 V. Control samples were prepared by diluting separate analytic stock solutions.

Statistical analysis

All experiments and measurements were made in triplicate. The values are expressed as the mean ± Standard deviation (SD). The results were subjected to analysis of variance followed by Tukeys test to

analyse differences between the *F. religiosa* bark methanol extract and control samples. Statistically significant differences (*P* value < 0.001) were shown.

Results

The hexane and chloroform extracts of *Ficus religiosa* bark extracts exhibited very negligible or no antioxidant activities. Only methanol extracts of bark showed interesting and consistent results (results not shown). This might be because of wide soluble properties of low molecular and polar substances including the antioxidant active phenol compounds in methanol extract [21]. Hence, methanol extracts alone were selected for further studies.

LC analysis of *Ficus religiosa* bark methanol extract in negative mode

Polyphenol extraction was achieved using methanol as a solvent by applying sequential–liquid extraction. The UPLC-MS chromatogram obtained is shown in Figure 1, and peaks were labeled according to the order of their retention time. Structural characterization was performed using the retention time of standards and published data [19, 20]. (+)-catechin was used as standards in this experiment. Presence of phytoconstituents, rutin, 3-caffeoylquinic acid, Luteolin 7-*O*-rutinoside, 6-C-glucosyl-8-C-arabinosylapigenin and Kaempferol-3-*O*-rutinoside were identified. Table 1 and Figure 1 summarize the LC-MS data and phytoconstituents of *F. religiosa* extract details.

Total secondary metabolites contents (phenols and flavonoids) in *Ficus religiosa* bark methanol extract

Total phenol and flavonoid in *F. religiosa* results were expressed as Gallic acid equivalents (GAE; $mg.g^{-1}$ of the extract) and Rutin equivalents (mg/g of the extract). The extracts reported a total phenol content of 163.91 ± 0.211 (GAE; $mg.g^{-1}$ of extract) and flavonoid content of 64.15 ± 0.41 (Rutin equivalents; $mg.g^{-1}$ of extract).

Antioxidant activity of *Ficus religiosa* bark methanol extract

To evaluate the antioxidant activity of *F. religiosa* crude extract, several tests were performed. The IC_{50} of crude extract in comparison to those of BHT and quercetin were presented (Table 2). The result showed that *F. religiosa* extract exhibited free radical-scavenging activity with IC_{50} value of (15.28 ± 1.22) $\mu g.mL^{-1}$ by DPPH assay. The IC_{50} values for BHT and quercetin were 4.32 ± 1.08 and 1.13 ± 0.18 $\mu g.mL^{-1}$ (Table 2).

The next test performed was β-carotene acid bleaching test, IC_{50} value of the crude extract was less effective than BHT and more effective than quercetin. Furthermore, the β-carotene/linoleic acid assay IC_{50} values were significantly near to BHT than quercetin, the synthetic antioxidant agents (Table 2).

The fractionated bark methanol extract showed a high value of phenolic and flavonoid contents with (16.32 ± 0.19) µg $GAE.mg^{-1}$ extract and (12.51 ± 0.15) µg $QE.mg^{-1}$ extract respectively.

Animal Cell culture experiments

Ficus religiosa bark methanol extract induced cell cytotoxicity to MDA-MB-231 cells

EC_{50} is a useful parameter for quantification of drug effect on cell survival. The effect of increasing concentration of extracts on the cell cytotoxicity to MDA-MB-231 (human breast cancer cells) and HEK 293 T (non-cancerous cell line) was measured by MTT assay.

The minimal cytotoxic activity and cell death on non-cancerous cell line (HEK 293 T) makes the *F. relgiosa* extract safe for isolation of anticancer compounds and for use in pharmaceutical industries (Figure 2a). Maximum cytotoxic activity and cell death was obtained with human cervical cancer cell line MDA-MB-231 (EC_{50}, 91.32 ± 4.21 $mg.mL^{-1}$) (Figure 2b). The positive control, camptothecin assayed for cytotoxicity under similar conditions exhibited IC_{50} of 2.75 ± 2.34 $mg.mL^{-1}$ (Table 3).

Ficus religiosa bark methanol extract induced DNA fragmentation in to MDA-MB-231 cells

DNA fragmentation assay results revealed a high degree of fragmentation (Figure 3) as a smear with nonspecific DNA degradation, a clear indication of apoptotic inducing capacity of this extract on MDA-MB-231 cells [22].

Ficus religiosa bark methanol extract induces cell cytotoxicity to MDA-MB-231 cells was visualized by acridine orange (AO) – propidium iodide (PI) staining for morphological observation of cell structure by Confocal Laser Scanning Microscopy (CLSM)

To confirm apoptosis, MDA-MB-231 cells were stained with acridine orange (AO) and propidium iodide (PI) dual stain wherein an emission of green and orange fluorescent wavelengths by AO and PI respectively were captured by CLSM. Control cells did not exhibit significant morphological changes and were green (Figure 4a). However, the cells subjected to treatment with *F. religiosa* bark methanol extract ($91mg.mL^{-1}$) and camptothecin ($2mg.mL^{-1}$) groups showed the signs loss of cell morphology and an uptake of PI indicating a loss of cell membrane morphology. An increase in orange fluorescing nuclear stain indicating apoptosis leading to late apoptotic cells with chromatin condensation for *F. religiosa* (Figure 4b) and camptothecin (Figure 4c) in comparison to controls (Figure 4a) visualized green (indicating live cells) was observed. Chromatin condensation confirms MDA-MB-231 cell cytotoxicity by *F. religiosa* extract to be an apoptotic event.

Ficus religiosa bark methanol extract dose-dependently activated BAX and PARP-1 and decreasing Bcl-2 mRNA regulating BAX/Bcl-2 signaling in MDA-MB-231 cells

For a better understanding of mechanism of apoptosis, a dose dependent experiment involved two concentrations *F. religiosa* bark methanol extract (45 and $91mg.mL^{-1}$) was incorporated and the results are presented. All bands were densitometrically analyzed using Scion Image and band densities in samples were normalized versus β-actin gene expression. In Figure 5, treatments markedly decreased the

expression of Bcl-2, *F. religiosa* bark methanol extract (45mg.mL^{-1}; 0.09 fold; 91mg.mL^{-1}; 0.12 fold) and camptothecin (2mg.mL^{-1}; 0.09 fold) compared to non-treated control cells (0.86 fold). An increased the expression of cleaved PARP in treatments *F. religiosa* bark methanol extract (45mg.mL^{-1}; 0.79 fold; 91mg.mL^{-1}; 0.9 fold) and camptothecin (2mg.mL^{-1}; 0.89 fold) when compared to non-treated control cells (0.58 fold). In addition, the treatment of *F. religiosa* bark methanol extract (91mg.mL^{-1}) and camptothecin (2mg.mL^{-1}) considerably increased the BAX in a dose dependent manner (1.1 and 1.12 folds respectively) compared to control cells (1.01 fold). The *F. religiosa* bark methanol extract could exhibit similar mechanism of cell cytotoxicity via apoptosis as the anti-cancer drug, camptothecin included in the present study evaluated by incorporation of apoptosis-related protein.

Ficus religiosa bark methanol extract - mediated suppression of MDA-MB-231 cell growth correlated with apoptosis, the mode of cell death, as evidenced by Flow cytometer

To gain further insights into the mode of cytotoxicity mediated by *F. religiosa* bark methanol extract, cells were labeled with annexin V and propidium iodide (Figure 6). Cells treated with 91µg.mL^{-1} *F. religiosa* extract for 48 h presented a decrease in percentage of non-labeled alive (lower left) cells (6.47%; Figure 6b) compared to control (100%; Figure 6a). The percentage of cells labeled only with annexin V representing early apoptotic stage (lower right) increased upon extract treatment (32.50%) relative to control (0%). An increase in apoptotic cells to 61.60% (upper right) in comparison to control (0%) was presented by flow cytometer analysis (Fig. 6b).

Ficus religiosa bark methanol extract induced apoptosis to MDA-MB-231 cells as evidenced by presence of sub-G0/G1 phase cells

Apoptotic cells being represented by a sub G0/G1 population were seen to the left of the G0/G1 peak when human breast cancer cell lines (MDA-MB-231 cells) were exposed to *Ficus religiosa* bark methanol extract. A total of 86.25% cells (Figure 6B, e, f) recorded in sub G0/G1 phase in this experiment in comparison to control (1.86%) in this experiment (Figure 6B, d, f).

Discussion

The critical step in the employment of plants as medicinal with therapeutic properties is the proper separation and identification of bioactive secondary metabolites [19, 20]. Accordingly, the bark methanol extract of *Ficus religiosa* (Linn.) was subjected to bioassay guided fractionation and further the most active fraction, methanol extract was analyzed by Ultra performance Liquid Chromatography-Mass Spectroscopy (UPLC-MS). The qualitative analysis of non-volatile compounds such as phenolics and flavonoids is usually carried out by this analytical technique [23]. In the present study the coupling of UPLC with MS aided to improve and facilitate the detection of metabolites in a better resolution and fast run time providing excellent selectivity and sensitivity [24]. The retention time (RT) responsible for measuring the time taken for a compound to pass chromatography from injection time until the detection time of the particular compounds was employed to identify major constituents present in the said extract

providing the separation of compounds into stationary and mobile phases. Figure 1 and Table 1 represents the major compounds in *F. religiosa* extract that have been separated through chromatography in the present study.

A representative chromatogram (Figure 1) provided identifies five metabolites based on RT. They have reported antioxidant and anti-tumorogenic properties viz., rutin [25], 3-caffeoylquinic acid [26], Luteolin 7-*O*-rutinoside [27], 6-C-glucosyl-8-C-arabinosylapigenin [28] and Kaempferol-3-*O*-rutinoside [29]. Potential natural quorum sensing inhibitors viz., Kaempferol-3-*O*-rutinoside and rutin [20] were identified in the current studies. However, there were still several unknown compounds present that were not identified in this study. Thus, more work is required to identify these compounds that will be taken up in the future.

Phenols and flavonoids in the plant extracts exhibit antioxidant activity [30, 31] with the ability to scavenge free radical because they contain hydroxyl groups as they easily donate an atom to the unstable free radical [32]. In this study, the DPPH assay was used to evaluate antioxidant properties by estimating free radical scavenging activity. The DPPH free radical has the ability to accept an electron from an antioxidant compound. The scavenging of radicals for *F. religiosa* extract was found with the 50% inhibitory concentration (IC_{50} value) of 15.28 ± 1.22µg/mL. High antioxidant activity of the extract could have a positive relationship with the total secondary metabolite content [11].

To obtain maximum biological activity from the bark extract, optimization of physico-chemical parameters is essential [33]. Thus, cytotoxic activity by MTT assay of the bark extract was evaluated on human breast cancer cell line MDA-MB-231 and a non cancerous cell line, HEK 293 T. A maximum cell death was recorded for MDA-MB-231 and a minimal cytotoxic activity with cell death for HEK 293 T. Thus, this result makes the *F. religiosa* bark extract safe for isolation of anticancer compounds and for its use in pharmaceutical industries.

We next assessed the mode of cell cytotoxicity of bark extract. The ultimate fate of cancer cell death could be by apoptosis, autophagy or programmed necrosis. The presence of an intricate interrelationship between them has been postulated. However, apoptosis is the preferred mode of cancer cells death as it is programmed wherein the cellular constituents are tightly packed in organelles that are subsequently phagocytosed by macrophages and other cells. They are then degraded within phagolysosomes. As this process does not initiate an inflammatory reaction researchers study the mode of cancer cell death to ascertain its apoptotic mechanism.

The induction of apoptosis by the extract in MDA-MB-231 cells by DNA fragmentation was analyzed. To assess the degree of apoptosis, we analyzed the formation of fragmented DNA by agarose gel electrophoresis. MDA-MB-231 cells treated with the extract for 48 h. It showed the presence of inter-nucleosomal DNA fragments similar to DNA ladder mimicking the typical DNA fragmentation pattern. The high degree of DNA fragmentation, without appearance of nonspecific DNA degradation, which would be otherwise be seen as DNA smear, is a clear indication of apoptotic inducing ability of this extract [22].

An in-depth study was also carried out by acridine orange(AO)/ propidium iodide (PI) double staining to decipher the mechanistic aspect of cancer cell death and to confirm apoptosis. The emission of green and orange fluorescent wavelengths by AO and PI respectively, forms the basis of this assay. PI is a cell impermeable nucleic acid stain and internalized only when the membrane is compromised. MDA-MB-231 cells were stained with AO and PI dual stain wherein an emission of green and orange fluorescent wavelengths by AO and PI respectively were recorded by Confocal laser scanning microscopy. An increase in orange fluorescing nuclear stain (Figure 4b, c) indicating apoptosis with chromatin condensation for *F. religiosa* extract and camptothecin in comparison to controls (Figure 4a) visualized green (indicating live cells) was observed. Chromatin condensation confirms MDA-MB-231 cell cytotoxicity by extract to be an apoptotic event. Chromatin condensation and DNA fragmentation by caspase-activated endonucleases are characteristics of apoptosis [34, 35]. These events are observed during late apoptosis following activation of caspases and degradation of nuclear DNA into nucleosomal units [36, 37].

In the present study, Bcl-2, BAX, cleaved PARP-1, apoptosis–related proteins were incorporated as they form intrinsic component of the pathway. An enhanced expression of BAX and cleaved PARP-1 mRNA, as a response to escalating concentration of *F. religiosa* bark methanol extract was observed sensitizing C to undergo apoptosis. A decreased Bcl-2 level was observed.

Apoptosis is controlled prominently by the members of Bcl-2 family, of which BAX and Bcl-2 have been identified as major regulators. BAX translocates to the mitochondria, inserts into the outer mitochondrial membrane allows the release of cytochrome c and initiates mitochondrial mediated apoptosis. In contrast, Bcl-2 binds to the outer mitochondrial membrane and forming a heterodimer with BAX resulting in neutralization of its proapoptotic effects. Thus, Bcl2 play an important role in resistance of cancer cells to chemotherapy or radiation therapy and can promote the expansion of neoplastic cell population. High expression of Bcl2 in various human cancers mediates the resistance of these to a wide range of chemotherapeutic drugs and γ-irradiation. Therefore, the blocking of Bcl2 can restore the apoptotic process in cancer cells. Thus, in response to a variety of stimuli including anticancer drugs, higher levels of BAX expression is beneficial. Therefore, the balance between the levels of BAX and Bcl-2 with increased BAX/Bcl-2 ratio has been reported to be critical in apoptotic fate of cells. In lieu with this requirement, results obtained in the present study demonstrated that *F. religiosa* treatment increased BAX levels and downregulated Bcl-2 levels resulting higher ratio of BAX/Bcl-2, promoting apoptosis.

The normal function of poly (ADP-ribose) polymerase-1 (PARP-1) a 113 kDa nuclear enzyme is to initiates the routine repair of DNA damage in response to a variety of cellular stresses. It is cleaved in fragments of 89 and 24 kDa during apoptosis. It is also suggested that cleavage of PARP occurs to prevent depletion of energy (NAD and ATP) required in later stages of apoptosis. PARP cleavage is thought to serve in preventing futile repair of DNA strand breaks during apoptosis**.** This cleavage has become a useful hallmark of apoptosis. In the present study, MDA-MB-231 cells when subjected to *F. religiosa* and camptothecin induced enhanced level of cleaved PARP-1 supporting apoptotic mode of cell cytotoxicity.

Flow cytometer analysis incorporated in the present study identifies increased early apoptotic (32.50%) and apoptotic cells (61.60%) cells upon extract treatment relative to control (0%). Further, apoptotic cells represented as sub G0/G1 population (86.25%) seen to the left of the G0/G1 peak was reported when MDA-MB-231 cells were exposed to *F. religiosa* bark methanol extract.

Thus, the knowledge of the signaling pathways along with physiological responses to the natural product including activity guided fractionated medicinal plant extract, partially or characterized natural molecules, synthesized molecular entities and / or therapeutic drugs defines the essentials to understanding the mechanism of toxicity. Thus these studies were incorporated in analysis of therapeutic potential of *F. religiosa* extract.

Hence, the excellent apoptotic and antioxidant activity demonstrated by the extract of *F. religiosa* could indicate the potential of fig species as pharmaceutical source.

Conclusion

The present study was carried out to explore the antioxidant and cell cytotoxicity potential of *Ficus religiosa* bark methanol extract. After physicochemical optimizations, antioxidant activity and cell cytotoxicity activity were studied. The extract was able to induce apoptosis in MDA-MB-231 cell line indicating programmed cell death with apoptosis of cancer cells as observed by DNA fragmentation, AO/PI staining and flow cytometer analysis. Isolation and purification of bioactive compounds from the bark is underway to get novel compounds which could be exploited in the treatment of cancer.

Declarations

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Conflicts of interest/Competing interests The authors have no conflicts of interest to disclose.

Ethics approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to participate The corresponding author on behalf of the coauthors agrees to accept the informed consent of compliance with ethical standard. No Human participation and/or Animal have been used in this study.

Consent for publication: All the authors have read and approved the manuscript.

Authors' contributions: SS developed the concept and experimental design. PS performed activity guided fractional, antioxidant and reducing assays. KMMS and KRK assessed UPLC-MS data. SS was involved in animal cell culture experiments. PS, KMMS, KRK and SS have contributed towards the preparation of figure, preparation of the manuscript.

Availability of data and material: Not applicable

Code availability: Not applicable

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Tables

TABLE- 1: UPLC-MS fingerprint and phytoconstitutents identified from *Ficus religiosa* bark methanol extract (n=3)

Compound with molecular formula	$[M-H]^-$	Retention time (min) *	References
Rutin $C_{27}H_{30}O_{16}$	609.1520	6.0	Elhawary et al, 2018
3-caffeoylquinic acid $C_{25}H_{24}O_{12}$	353	10.8	Lin and Harnly [13]
Luteolin 7-*O*-rutinoside $C_{23}H_{30}O_{15}$	595	13.76	Lin and Harnly [20]
(+)-Catechin $C_{15}H_{14}O_6$	290.26	14.92	standard
unknown		16.07	Lin and Harnly [21]
unknown		17.94	Lin and Harnly [21]
6-C-glucosyl-8-C-arabinosylapigenin $C_{26}H_{28}O_{14}$	563.102	20.82	Choi et al [13]
Kaempferol-3-*O*-rutinoside $C_{27}H_{30}O_{15}$	593.1304	33.57	Elhawary et al, 2018
unknown		45.34	Lin and Harnly [21]
unknown		47.81	Choi et al [13]
unknown		69.28	Choi et al [13]

* indicates $P < 0.001$, one-way ANOVA followed by Tukeys' test.

TABLE 2: IC_{50}* values of crude extract, BHT and quercetin

Antioxidant tests	*F. religiosa* extract ($mg.mL^{-1}$)	BHT ($mg.mL^{-1}$)	Quercetin ($mg.mL^{-1}$)
DPPH assay	15.28 ± 1.22	4.32 ± 1.08	1.13 ± 0.18
Reducing power assay	6.32 ± 0.22	7.31 ± 0.15	2.36 ± 0.15
beta-carotene/linoleic acid assay	5.14 ± 0.64	4.32 ± 0.26	1.05 ± 0.28

Values represent mean ± SD for triplicate experiments (n=3): *indicates $P < 0.001$, one-way ANOVA followed by Tukeys' test.

TABLE -3: Cell Cytotoxicity studies on MDA-MB-231 cells by MTT assay

Samples	EC_{50}*
Ficus religiosa bark methanol extract	91.32 ± 4.21 $\mu g.mL^{-1}$
Camptothecin	2.75 ± 2.34 $\mu g.mL^{-1}$

Values are expressed as the mean ± Standard Deviation (n=3): *indicates $P < 0.001$, one-way ANOVA followed by Tukeys' test.

Figures

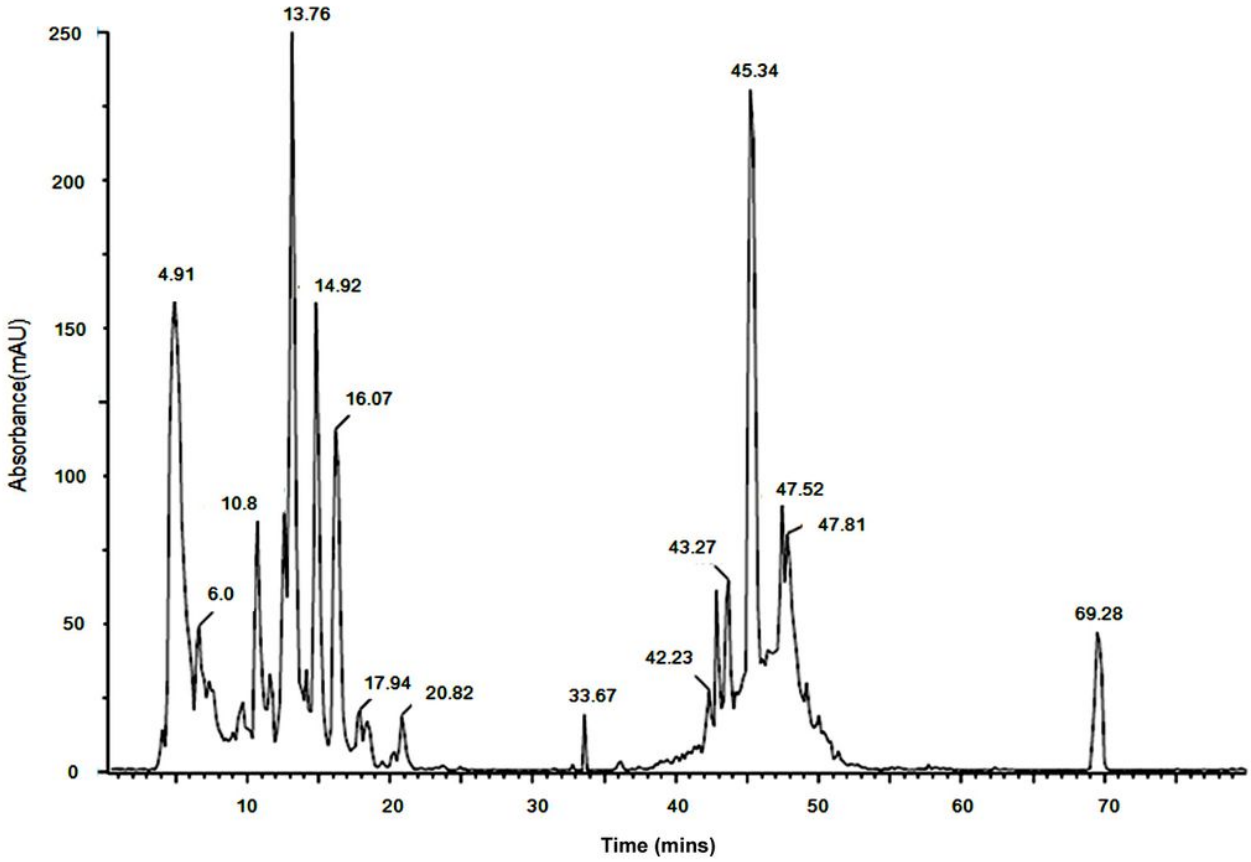

Figure 1

Figure 1

UPLC-MS analysis of Ficus religiosa bark methanol extract in negative mode.

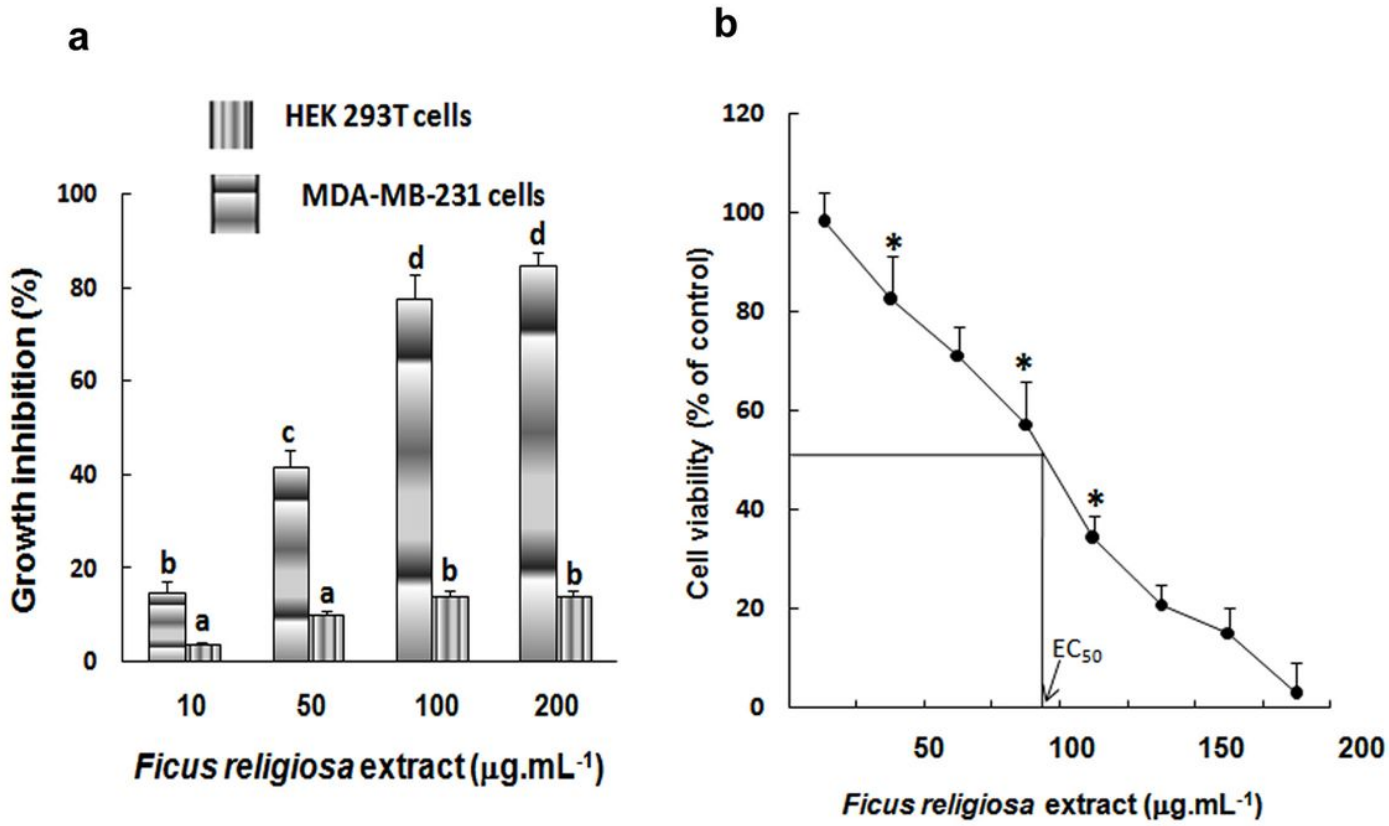

Figure 2

Figure 2

Ficus religiosa bark methanol extract induced cell cytotoxicity inducing growth inhibition (%) in comparison to control (untreated cells). a) Cell growth inhibition of MDA-MB-231 (human breast cancer cells) and non-cancerous cell line (HEK 293 T) by the Ficus religiosa bark methanol extract was evaluated. Cells were treated with indicated concentrations of the extract for 48 h and the inhibitory effects of the extract on cell growth were determined by assessing the cell viability using MTT assay. Same letters mean no statistical differences among the values according to the Tukey test ($P < 0.001$).

Values with different letters within the columns are significantly different according to Tukey test (P < 0.001). b) Cytotoxic effect of escalating concentrations of Ficus religiosa bark methanol extract (1–200 µg.mL-1) to MDA-MB-231 cells when exposed for 48h. Similar conditions of growth, like, 5 % CO2 at 37°C were maintained for these two experiments. Cell viability was estimated by MTT assay and EC50 value was determined. *Significant according to the Tukey test (p<0.001) versus control cells.

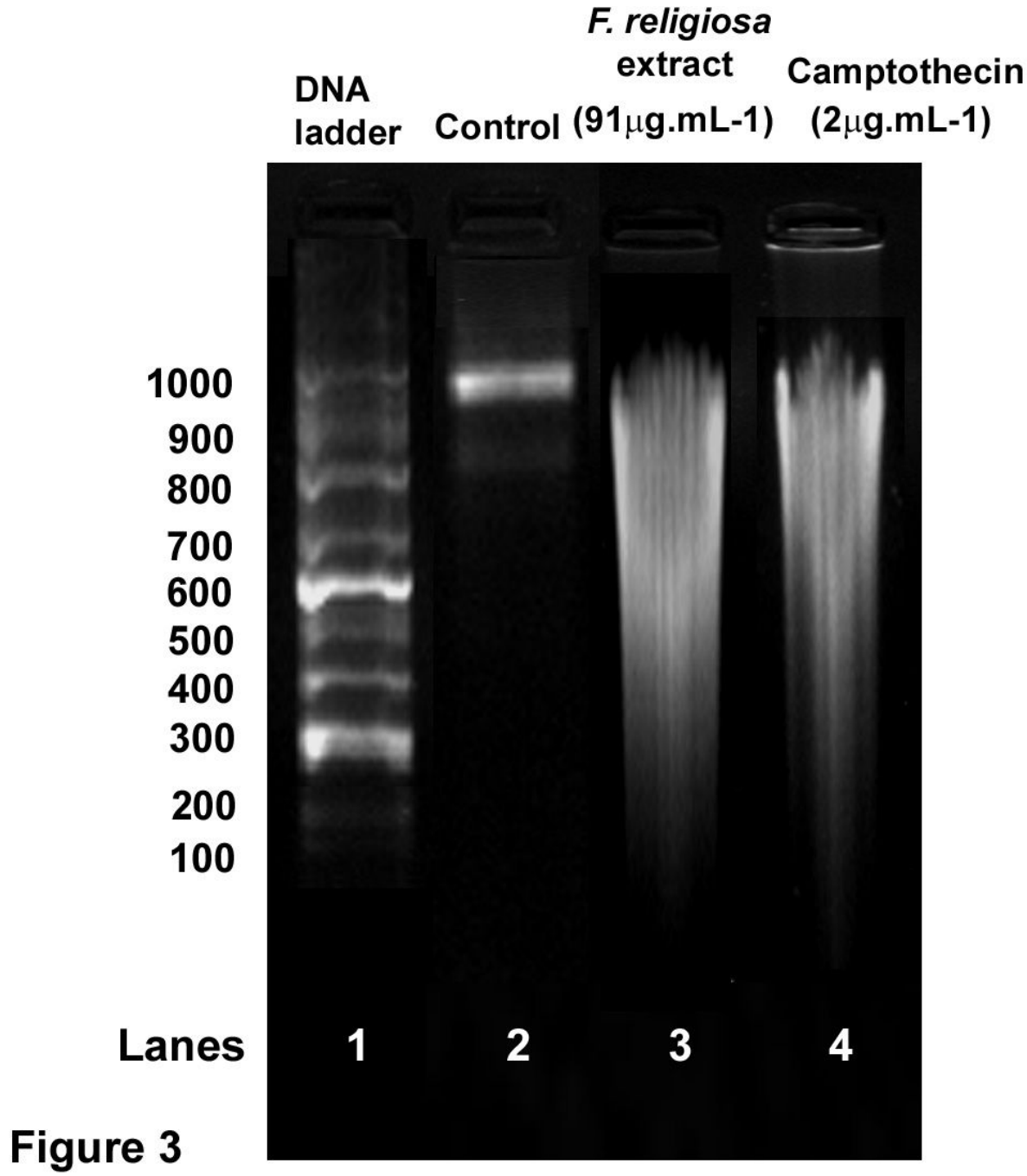

Figure 3

Ficus religiosa bark methanol extract (91µg.mL-1) and camptothecin (2µg.mL-1) induced DNA fragmentation in MDA-MB-231 cells. DNA was extracted and the fragmentation was determined by agarose gel electrophoresis as detailed in materials and methods. Lanes: 1, 1000bp ladder; 2, Control, untreated cells; 3, Ficuss religiosa bark methanol extract (91µg.mL-1); 4, camptothecin (2µg.mL-1)

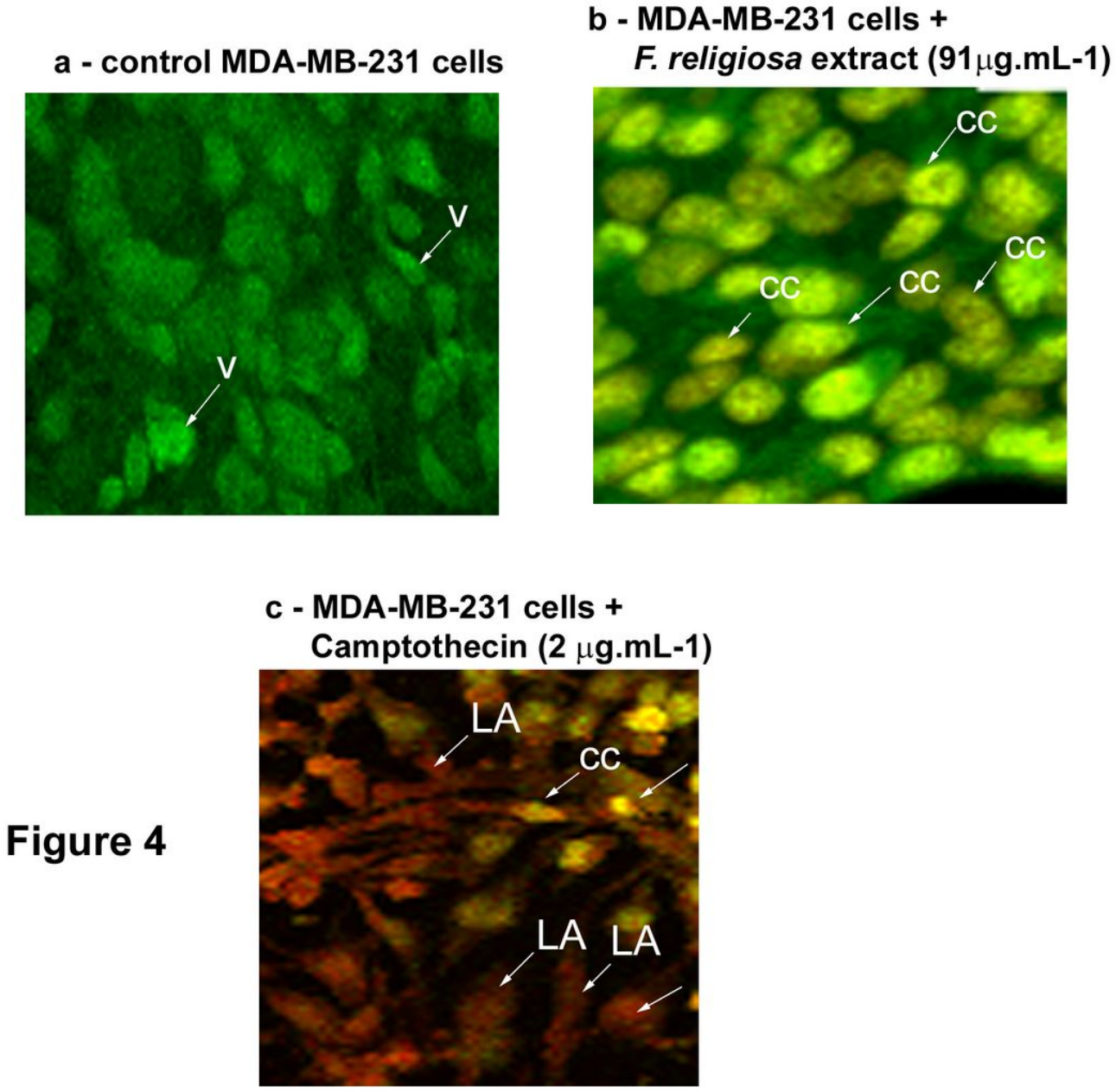

Figure 4

Figure 4

Ficus religiosa bark methanol extract and camptothecin inhibited cell growth and induction of apoptotic nuclear morphology in MDA-MB-231 cells. MDA-MB-231 cells were treated with the indicated

concentrations of the extract and camptothecin. They were stained with AO/PI dual staining. V, viable cells; CC, chromatin condensation; LA, late apoptotic cells are indicated in the representative images (n=3).

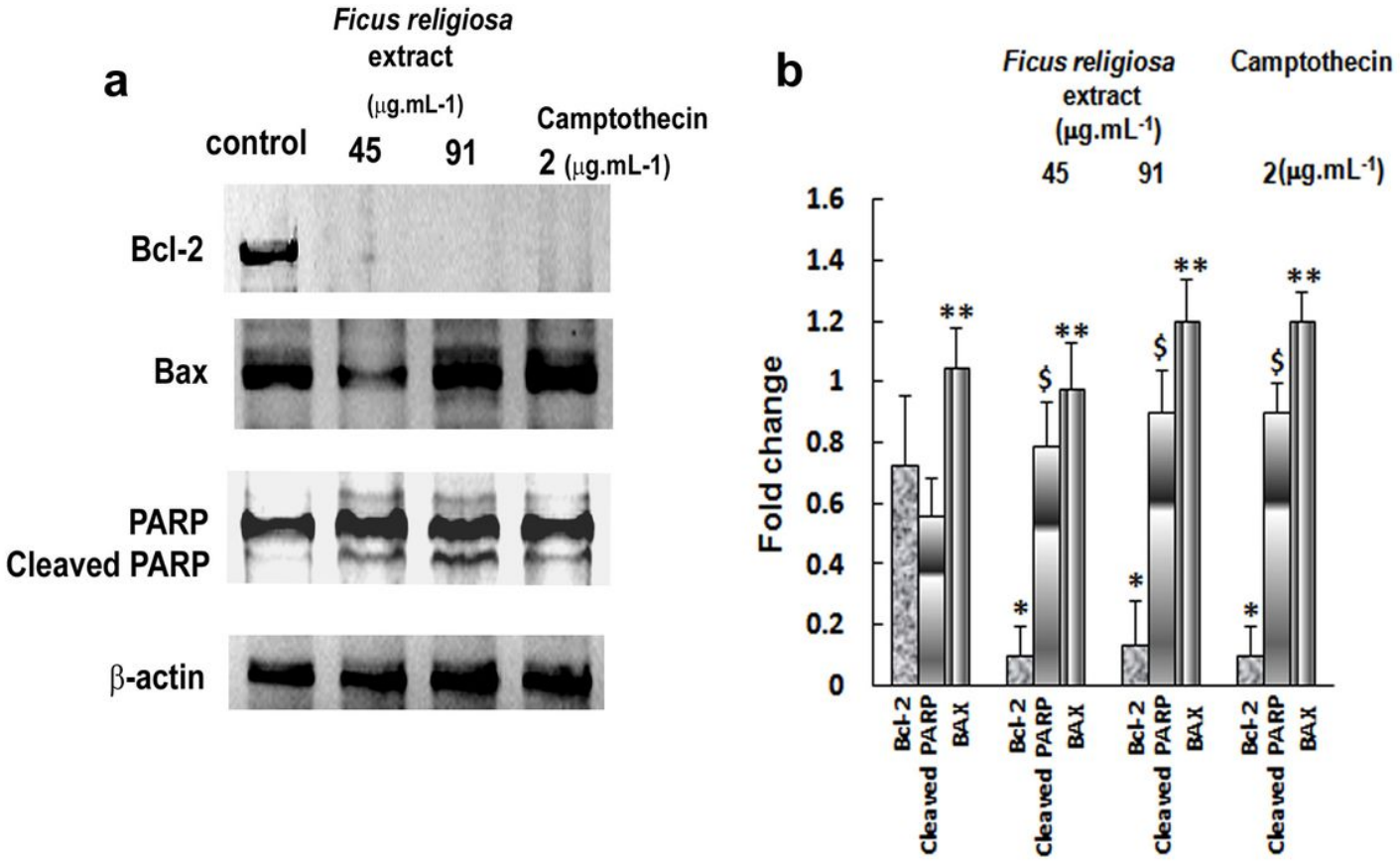

Figure 5

Figure 5

Ficus religiosa bark methanol extract induced apoptosis to in MDA-MB-231 cells and apoptosis-related proteins, Bcl-2, Bax and PARP were evaluated in the present study. a) MDA-MB-231 cells were incubated with the extract (45 and 91µg.mL-1) and campothecin (2µg.mL-1) for 48h. Equal amount of cell lysates

(50µg) were resolved in SDS-PAGE. The separated proteins were transferred to nitrocellulose membreane and probed with specific antibodies as indicated in materials and methods. β-actin was used as internal control. The results presented are representative on three independent experiments (n = 3). b) All bands were densitometrically analyzed using Scion Image and band densities in samples were normalized versus β-actin gene expression. ** indicates significant increase in BAX as a dose-dependent phenomenon in Ficus religiosa bark methanol treatment in comparison to control cells. $ indicates significant increase in cleaved PARP in Ficus religiosa bark methanol treatment in comparison to control cells. * indicates significant decrease in Bcl-2 in F. religiosa treatment in comparison to control cells.

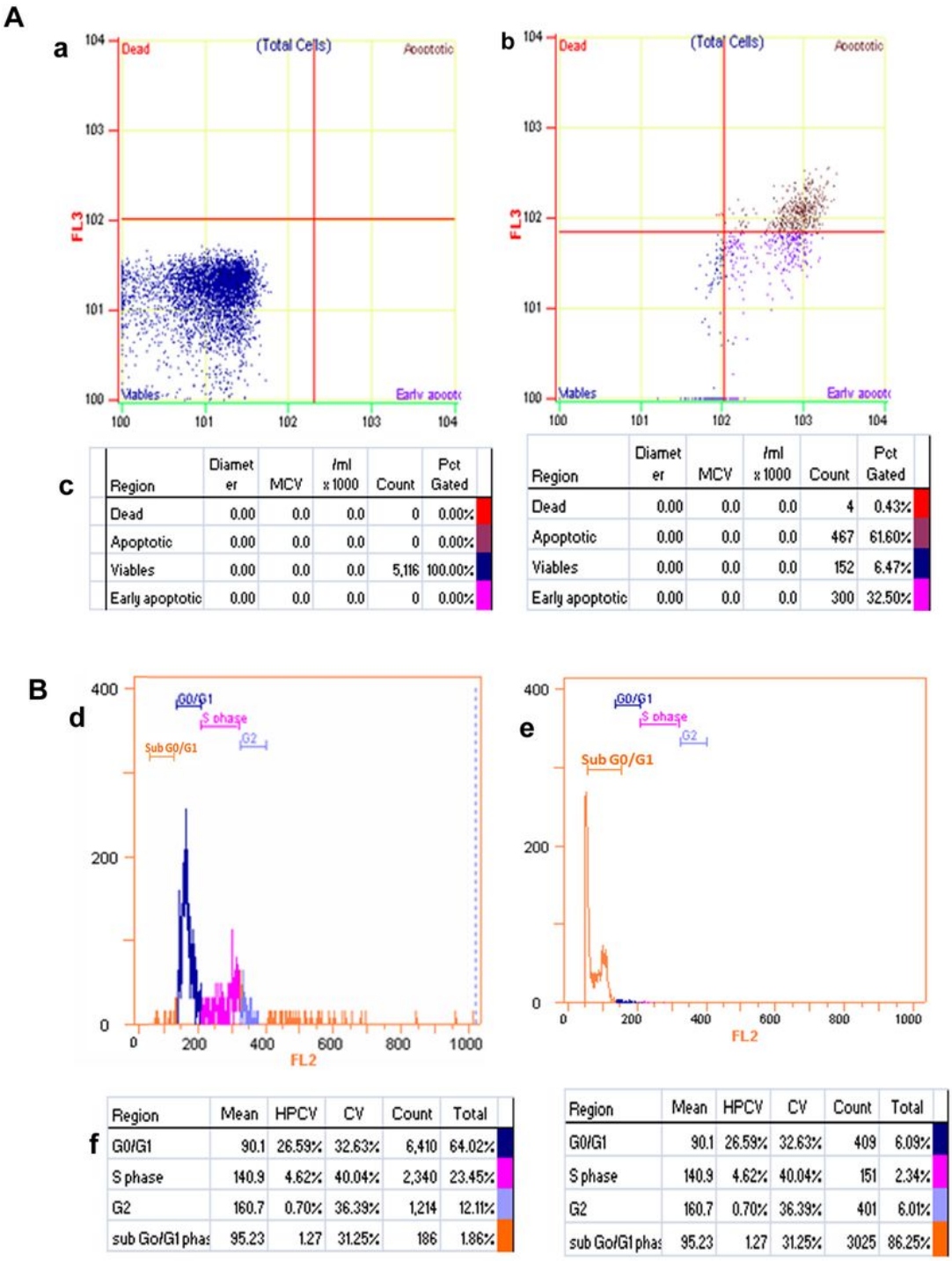

Figure 6

Figure 6

A. Ficus religiosa bark methanol extract sensitizes MDA-MB-231 cells to undergo apoptosis. The mode of cell death when these cells were treated with F. religiosa bark methanol extract was assayed by flow cytometry with annexin V and propidium idodide (PI) labeling. Flow cytometer results of control cells (a) and of F. religiosa bark methanol extract (91µg.mL-1) -treated cells (b) are presented in the figure (n=3). The different quadrants: lower left viable cells, upper left dead cells, upper right apoptotic cells, lower right

early apoptotic cells. The percentage of cells counted in each quadrant is shown below the dot plot graphs (c). B. Ficus religiosa bark methanol extract sensitizes MDA-MB-231 cells to undergo apoptosis by inducing DNA fragmentation resulting in accumulation of subGo/G1 apoptotic cells (n=3). The cells stained with a DNA intercalating dye like propidium iodide (PI), then a DNA profile representing control cells with G1, S-phase and G2M will be observed (d) and apoptotic cells are represented by a sub G0/G1 population seen to the left of the G0/G1 peak (e). Representative cell cycle analysis of three independent experiments carried out thrice is presented (f).