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# Evaluation of chemo-preventive efficacy of *Ficus religiosa* latex extract by flow cytometry analysis and gene expression studies performed by RT-PCR in various cell lines

L. Saida<sup>1</sup>, C. D. S. L. N. Tulasi<sup>2\*</sup> and M. Lakshmi Narasu<sup>2</sup>

## Abstract

**Background:** An extract of *Ficus religiosa* latex has been previously found to possess potent pharmacological activity with high antioxidant content phytochemical. The present research was conducted to investigate the chemo-preventive efficacy of latex extract on human breast adenocarcinoma MDA MB 231, human neuroblastoma IMR 32, and human colorectal HCT 116 cell lines.

**Results:** The results showed that the latex crude extract induced cytotoxicity in all the selected cell lines with IC<sub>50</sub> value  $4.8 \pm 1.13$  µg/ml against the IMR 32 cell line. The cell cycle analysis results indicated the arrest and accumulation of cells at G1 phase in case of MDA MB 231 cells and HCT 116 cells whereas in the case of IMR 32 cells the arrest was in G2/M phase. The clear bands of fragments observed in DNA ladder experiments showed that apoptosis is induced by extracts in the cell lines. This could be correlated with the gene level expression studies on selected pro-apoptotic (p53 and caspase-3) and anti-apoptotic (Bcl-2, AKT) genes, which got upregulated and downregulated, respectively.

**Conclusion:** Based on the experimental evidence, *Ficus religiosa* contains phytochemicals with potent antitumor activities.

**Keywords:** *Ficus religiosa*, Cytotoxicity, Apoptosis, Cell cycle arrest

## Background

The planet Earth has a treasure of medicinal plants with varied therapeutic properties used for treating ailments of the human race. The environmental factors and the genetic makeup of humans are inter-related, and any change or disturbance within these parameters would give rise to malignancy. Genetic changes can occur by gain or loss of nucleotides in the DNA (deoxyribonucleic

acid) sequence or by the errors during the mitosis, which will alter the final output, and this process is called mutation. This is the underlying mechanism in most of the prevailing cancers. It is a cluster of diseases involving abnormal cell growth due to mutant regulatory genes and gaining the potency to invade various body parts. There exists some chemotherapeutics as well radiology to cure cancer-affected tissues, but finally, they end up in inducing the malignancy in other parts of the body. In this preview, phytochemicals extracted from herbal plants possess therapeutic activity and will not have that inductive effect on the unaffected tissue.

\* Correspondence: [yrvlm.chidepudi@gmail.com](mailto:yrvlm.chidepudi@gmail.com)

<sup>2</sup>Centre for Biotechnology, Institute of Science and Technology, Jawaharlal Nehru Technological Institute, Kukatpally, Hyderabad, Telangana 500085, India

Full list of author information is available at the end of the article



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**Table 1** PCR cycle conditions

S. no	Steps	Apoptosis-related genes and $\beta$ -actin gene		
		Temperature	Duration	Cycles
1.	Initial denaturation	94 °C	3 min	1 cycle
2.	Denaturation	94 °C	45 s	35 cycles
	Primer annealing	52 °C	45 s	
	Synthesis	72 °C	1 min	
3.	Final extension	72 °C	7 min	1 cycle
4.	Hold at 4 °C			

*Ficus religiosa* Linn. belonging to the family Moraceae, universally known as peepal, is widely cultivated in Southeast Asia and has been known for its medicinal properties in traditional *unani* and folk medicine system. It has been used to treat respiratory disorders, hiccup, gout, ulcers, stomatitis skin diseases, allergies, arthritis, inflammatory disorders, diabetes, bone fracture, gynecological disorder, etc. [1, 2]. Its various therapeutic uses in folk medicine have encouraged in considering it for disease management such as brain-related disorders [3, 4], diabetes [5], kidney, and urinary disorders [6].

*Ficus religiosa* was reported to possess cytotoxic activity against HT29 and MDA MB-435S cancer cell lines [7]; further reports indicated its cytotoxic activity in MCF-7 cell line [8]. It is also known that a wide range of phytochemicals is distributed throughout the plant; however, the cytotoxicity can be attributed to flavonols including myricetin, quercetin, and phytosterols namely  $\beta$ -sitosterol and stigmasterol [9]. As a part of continuous screening of natural plants and medicinal plants with anti-cancer properties, the

present study included investigating the anti-cancer potential of *Ficus religiosa* latex extracts on human neuroblastoma cells (IMR 32), human breast adenocarcinoma (MDA MB 231), and human colorectal carcinoma (HCT 116).

## Methods

### Collection of plant materials and Solvent extraction

*Ficus religiosa* latex was collected from the bark of healthy plants by making an incision with a sharp sterile knife during the early hours of morning every day till sufficient volume of plant material is obtained and stored at  $-20\text{ }^{\circ}\text{C}$  for further use. Authentication of the plant material was done at the Department of Botany, Andhra University, Visakhapatnam, Andhra Pradesh, as the plant material was collected from the university during the fruiting season and deposited in the botany department herbarium with voucher number 22273. The thawed samples of latex was dried and macerated for extraction in solvents like methanol, ethanol, ethyl acetate, and acetone at a ratio of 1:10 (W/V—1 g in 10 ml) at room temperature for about 24 h. The extracted filtrate was filtered with Whatman No. 1 filter paper and then evaporated in a rotavapor till dry. The solvent extracts were dissolved in respective solvents for phytochemical screening, antioxidant activity, and anti-microbial activity, but for chemo-preventive activity, they were dissolved in DMSO (dimethyl sulfoxide), and in the culture medium, the DMSO concentration was maintained to be less than 0.2%. The dissolved solvent extracts were maintained sterile by filtration using 0.22- $\mu\text{m}$  membrane filter.

**Table 2** Primer sequences of apoptosis-related genes and  $\beta$ -actin as the control gene

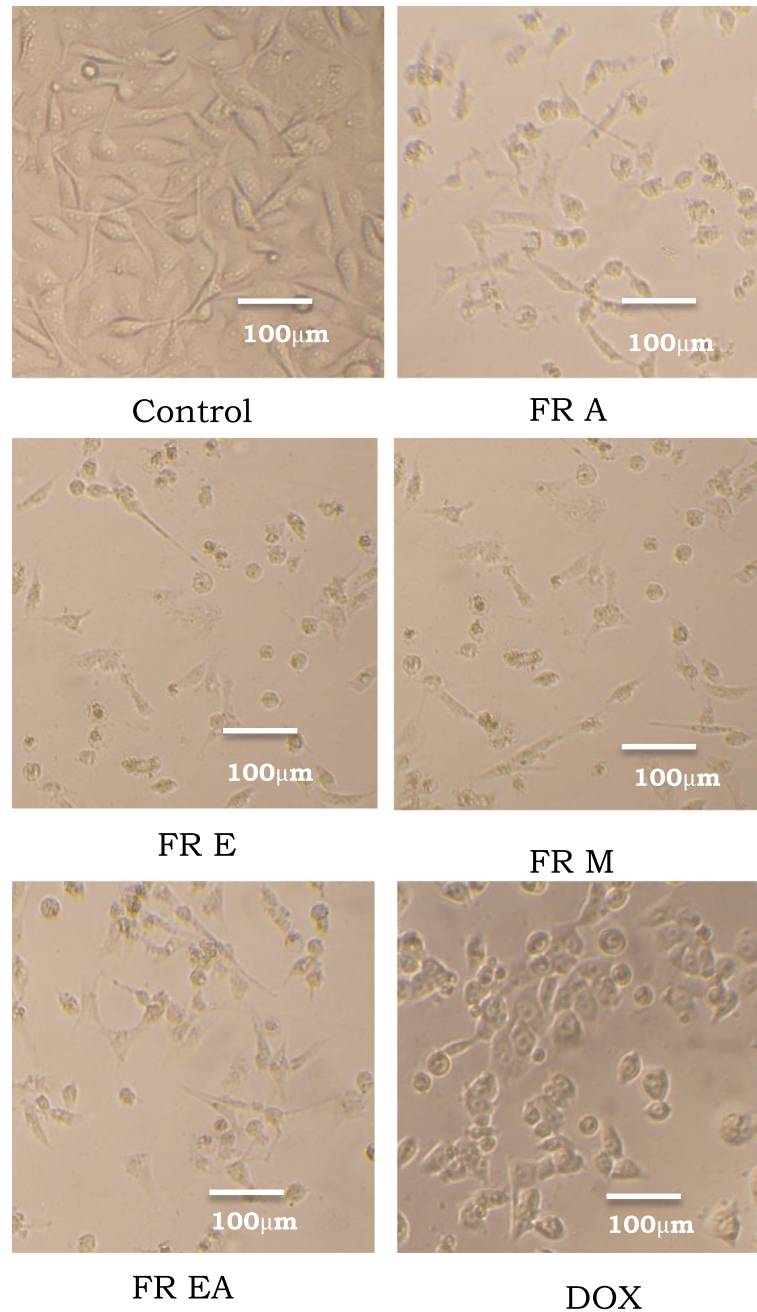
S. no	Gene name	Gene size (bp)	Primer sequence (forward and reverse)
1	$\beta$ -actin	135	5'-CACCATTTGGCAATGAGCGGTTC-3' 5'-AGGTCITTTGCGGATGTCCACGT-3'
2	p53	128	5'-CCTGAGCATCTTATCCGAGTGG-3' 5'-TGGATGGTGGTACAGTCAGAGC-3'
3	Caspase3	146	5'-GGAAGCGAATCAATGGACTCTGG-3' 5'-GCATCGACATCTGTACCAGACC-3'
4	Bcl2	127	5'-ATCGCCCTGTGGATGACTGAGT-3' 5'-GCCAGGAGAAATCAAACAGAGGC-3'
5	AKT	155	5'-TGGACTACCTGCACTCGGAGAA-3' 5'-GTGCCGCAAAAGGTCTTCATGG-3'
6	Nrf2	112	5'-CACATCCAGTCAGAAACAGTGG-3' 5'-GGAATGTCTGCGCCAAAAGCTG-3'
7	RelA	135	5'-TGAACCGAAACTCTGGCAGCTG-3' 5'-CATCAGCTTGCAGAAAGGAGCC-3'

### Chemo-preventive efficacy

#### Cancer cell lines

Cancer cell lines selected for the present study were human neuroblastoma cells (IMR 32), human breast adenocarcinoma (MDA MB 231), and human colorectal carcinoma (HCT 116) procured from NCCS, Pune. The cells were maintained in RPMI 1640 medium supplemented with 10%

fetal bovine serum, 1% 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin maintained at 37 °C in humid incubator with 5% CO<sub>2</sub>. According to their growth rates, the optimum plating density of each cell line was determined to be  $5-6 \times 10^3$  cells/well in a 96-well microtiter plate to maintain exponential growth throughout the experiment.



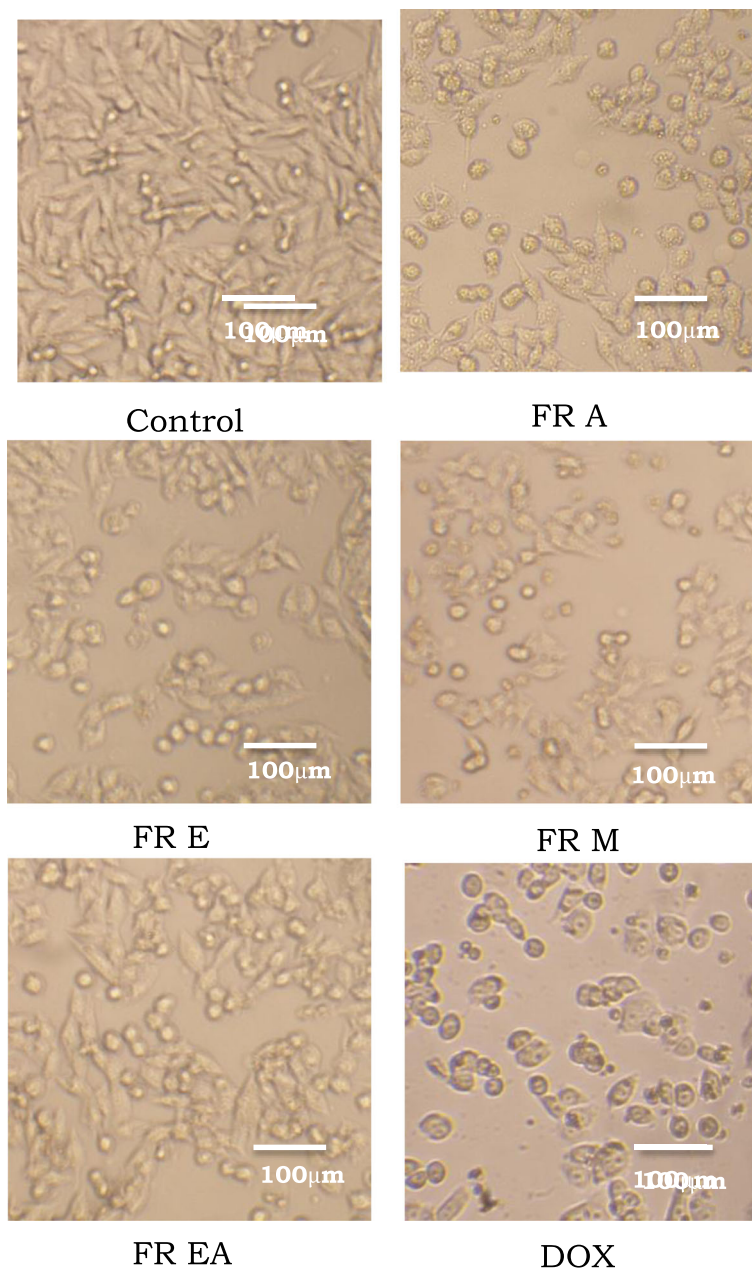
**Fig. 1** Morphological changes observed in MDA MB 231 cell line when treated with IC<sub>50</sub> concentrations of *F. religiosa* latex solvent extracts along with control (MDA MB 231) and doxorubicin (DOX) treated cells. A—acetone (111.86 µg/ml); E—ethanol (50.86 µg/ml); M—methanol (145.5 µg/ml); EA—ethyl acetate (107.25 µg/ml)

### Visualization of cells' morphological changes under light microscope

The effect of *F. benghalensis* and *F. religiosa* latex extracts on the morphology of cells after treating and incubating for 24 h was visualized under Olympus IX71 phase contrast light microscope. The consumed medium was removed, PBS (phosphate buffer saline) was added, and the cells were observed and photographed with an Olympus camera attached to the microscope.

### Propidium iodide staining

Propidium iodide staining technique was often used to detect the apoptotic morphological changes taking place in the cell after treating with plant extract. According to Rima and Mangamoori's [10] method, all the cell lines were treated with IC<sub>50</sub> concentrations of plant extracts; untreated cells served as control in a 12-well microtiter plate. After incubation for 24 h, cells were washed with cold PBS, fixed with absolute alcohol and refrigerated for 30 min at 4°C, then rehydrated with room



**Fig. 2** Morphological changes observed in IMR 32 cell line when treated with IC<sub>50</sub> concentrations of *F. religiosa* latex solvent extracts along with control (IMR 32) and doxorubicin (DOX)-treated cells. A—acetone (102.84 µg/ml); E—ethanol (4.8 µg/ml); M—methanol (146.01 µg/ml); EA—ethyl acetate (39.24 µg/ml)

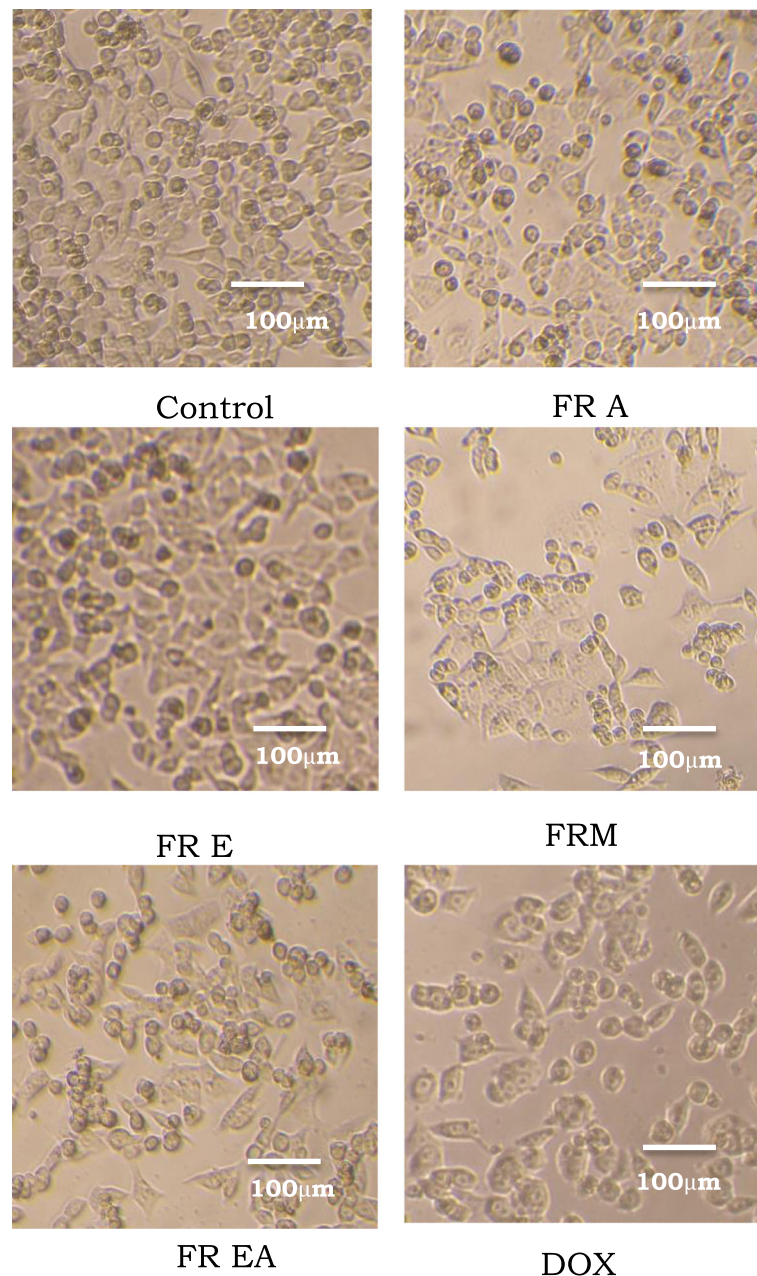


temperature PBS. Propidium iodide solution (25  $\mu\text{M}$ ), 100  $\mu\text{l}$  to each well, was added and incubated at 37  $^{\circ}\text{C}$  for 5 min. Photographs were taken using fluorescent microscope at  $\times 200$  magnification.

#### MTT assay for cell viability

For the assay, cells grown in T<sub>25</sub> tissue culture flask when reached optimum confluency were washed with phosphate buffer saline (PBS), treated with 0.025 %

trypsin-EDTA (2 ml), and flushed with medium for the uniform cell detachment from the T-flask. The cell suspension with all the viable cells were counted by typhan blue exclusion using a haemocytometer and then diluted with medium to give the cell density of  $5\text{--}6 \times 10^3$  cells/well. The cells were then seeded by adding 100  $\mu\text{l}$ /well of diluted cell suspension to the 96-well microtiter plate and incubated at 37  $^{\circ}\text{C}$  for 24 h ensuring proper and uniform cell attachment to the bottom of the plate. After



**Fig. 3** Morphological changes observed in HCT 116 cell line when treated with IC<sub>50</sub> concentrations of *F. religiosa* latex solvent extracts along with control (HCT 116) and doxorubicin (DOX)-treated cells. A—acetone (120.71  $\mu\text{g/ml}$ ); E—ethanol (24.27  $\mu\text{g/ml}$ ); M—methanol (122.89  $\mu\text{g/ml}$ ); EA—ethyl acetate (101.56  $\mu\text{g/ml}$ )

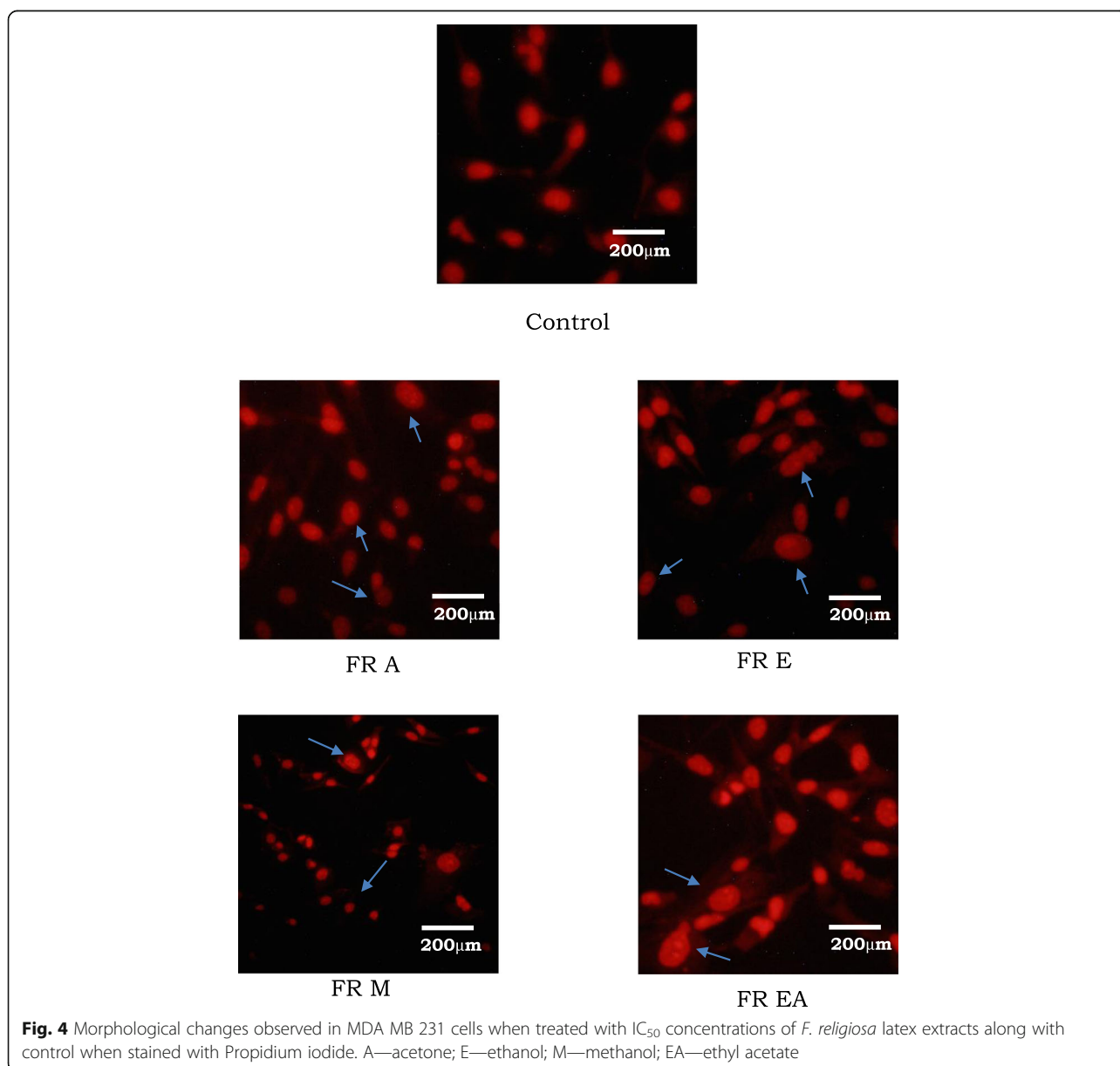
the incubation period, the cells were treated with extracts which were already dissolved in DMSO stock. The latex extracts were further diluted with medium to make five different concentrations. One hundred microliters of each concentration was loaded in the well in triplicates. Positive control, doxorubicin, was used at concentrations 0.25 µg/ml, 0.5 µg/ml, 0.75 µg/ml, 1 µg/ml, and 1.5 µg/ml. The final dilution used for treating cells was made up to 0.4% of the initial solvent which was used as negative control. The plates were incubated for 24 h. The media was removed after incubation, and cell viability was assessed with MTT dye (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay

by adding 100 µl of 0.1 mg/ml stock solution [11]. The cells were incubated with MTT reagent for 1 h so that the viable cells will form purple-colored MTT formazan crystals. After incubation period, 100 µl of DMSO was added to dissolve the formed crystals and the absorbance was measured with UV-visible spectrophotometer at 540 nm.

The cytotoxic activity of the latex extract was calculated as percentage of cell growth inhibition by using following formula

$$\% \text{ Growth inhibition (\%)} = [(A_{\text{Control}} - A_{\text{Sample}}) / A_{\text{Control}}] \times 100$$

where  $A_{\text{Control}}$  is the absorbance of control and  $A_{\text{Sample}}$  is the absorbance of treated cells at different



concentration.  $IC_{50}$  values (the concentration of sample required for inhibition of 50 % of cell growth) were obtained from the regression line.

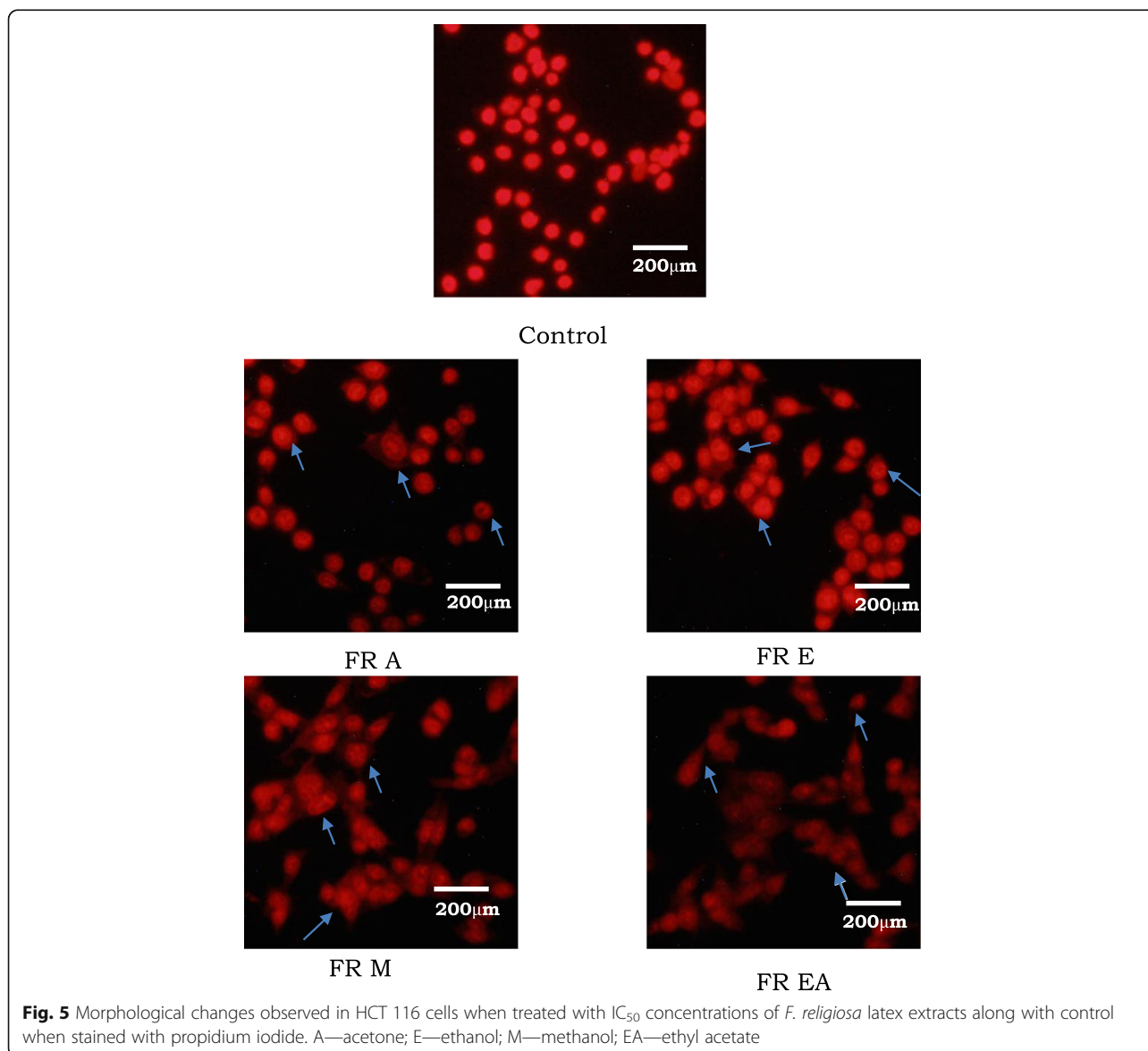
#### Isolation of human peripheral blood mononuclear cells (lymphocytes)

Lymphocytes were isolated from the blood (5 ml) of a healthy adult volunteer collected after explaining in detail the purpose of the experiment as well as after accomplishing a verbal consent. The protocol was performed according to the guidelines of Helsinki. The blood sample was centrifuged by diluting it with equal volume of PBS (pH 7.4) over Histopaque 1077 solution at 400g for 30 min [12]. The interface suspended with lymphocytes was carefully extracted and centrifuged with PBS as well with RPMI 1640 repeatedly ensuring

the platelet removal and was finally suspended in 500  $\mu$ l of RPMI 1640 media. Typhan blue exclusion was carried out to assess the viability of isolated lymphocytes, and it was found to be 90%. The final cell density was adjusted to about  $2 \times 10^5$  cells/ml.

#### Effect of plant extracts on lymphocytes viability

The isolated viable lymphocytes were seeded and then immediately treated with the plant extract of 200  $\mu$ g/ml concentration. The treated lymphocytes were incubated in incubator for 24 h at 37 °C. After the incubation period, the morphological changes were observed and then the lymphocytes were subjected to centrifugation at 800g for 10 min at 4 °C, and cell viability was calculated using MTT assay (3-(4,5-dimethylthiazole-2-yl)-2-5-diphenyltetrazolium bromide) [11]. The formazan crystals



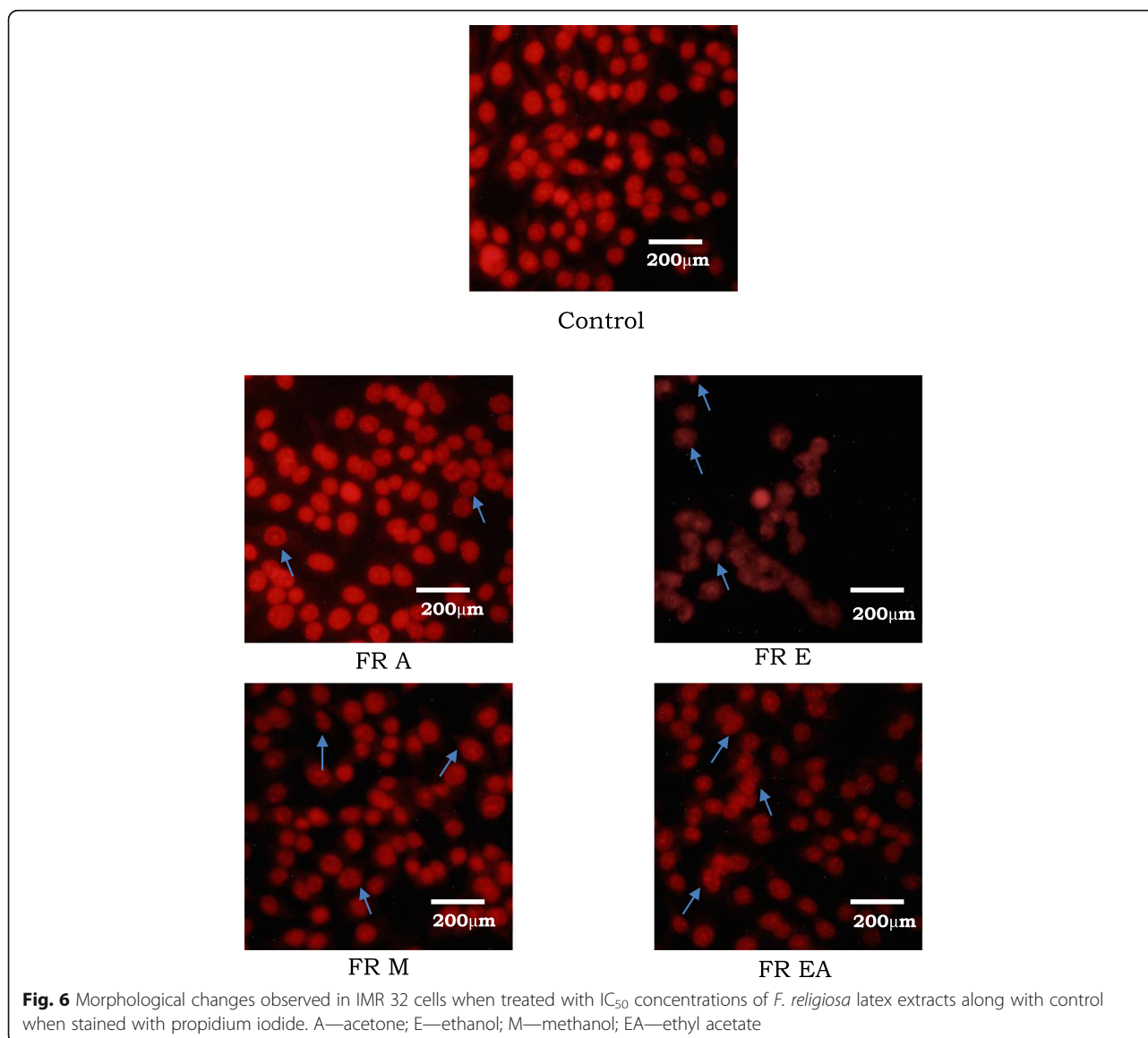
color was measured by an Elisa plate reader at 540 nm, and the percentage of viable lymphocytes was calculated.

#### Analysis of DNA fragmentation by DNA ladder assay

The conventional DNA ladder assay is known to have certain drawbacks like loss of DNA fragments during processing steps as well as require expensive reagents. The present protocol is cost-effective, easy to perform, sensitive, and a rapid method for detection and analysis of apoptotic DNA fragments. It is a time and cost-effective method, reducing the laborious steps of extraction and assay time as large number of samples can be handled at a time with accurate sensitivity.

The cells were allowed to grow till they reach  $2.8-3 \times 10^6$  cells confluency in the tissue culture flask, which

were considered as a starting material for isolation of DNA. The cells were washed with PBS and treated with trypsin, flushed with medium for the uniform cell detachment from the T-flask. The cell suspension with all the viable cells were counted by typhan blue exclusion using a hemocytometer and then diluted with medium to give the cell density of  $1 \times 10^5$  cells/ well. The cells were then seeded by adding 500  $\mu$ l/well of diluted cell suspension to the 12-well microtiter plate and incubated at 37°C for 24 h ensuring proper and uniform cell attachment to the bottom of the plate. After incubation, cells were treated with IC<sub>50</sub> concentrations of plant extracts and again incubated for 24 h. Cells were extricated, washed with PBS, and stored as pellets at -20°C overnight. The pellets were added directly with 100  $\mu$ l of DMSO followed by vortexing, and an equal volume of



**Fig. 6** Morphological changes observed in IMR 32 cells when treated with IC<sub>50</sub> concentrations of *F. religiosa* latex extracts along with control when stained with propidium iodide. A—acetone; E—ethanol; M—methanol; EA—ethyl acetate



TE buffer (pH 7.4) with 2% SDS was added and mixed, and the resultant solution was centrifuged at 12,000g for 20 min at 4 °C. The supernatant was transferred into a fresh Eppendorf tube, which contains isolated DNA [13]. The purity of isolated DNA was analyzed by recording absorbance at 260 nm and 280 nm and calculating it by A260/A280. The supernatant was then loaded in 1.5% agarose gel and the ladder formation was evident by bands formation in the gel, observed under UV light in Gel documentation system (Bio-rad laboratories, Segrate, Italy).

### RT-PCR for gene expression studies

#### Extraction and Isolation of RNA

The total RNA was isolated and extracted from all the cancer cells lines ( $5 \times 10^6$ ) treated with  $IC_{50}$  values of plant ethanol extract along with control (untreated cells). After incubation for 24 h at 37 °C in incubator with 5% CO<sub>2</sub>, the cells were treated with 1 ml of TRI-ZOL reagent as per the manufacturer's protocol. The dissociated cells were collected into a fresh sterile tube and left at room temperature for 5 min in order to facilitate the complete nucleoprotein dissociation. To this mixture, 100 µl of 1-bromo-3-chloropropane was added for 1 ml of TRIZOL reagent added initially, vortexed gently, and centrifuged for 15 min at 12000g, 4 °C. The top aqueous layer out of three layers was carefully transferred into new Eppendorf tube, and 500 µl of 2-propanol was added and tilted for 20 s, kept at room temperature for 10 min, and centrifuged for 15 min at 12000g, 4 °C. The supernatant was discarded, and the pellet was washed with 1.0 ml of 75% of ethanol twice by centrifugation at 4 °C, 12000g for 10 min and decanting the supernatant. The RNA pellet was air dried and finally dissolved in 20 µl of nuclease free water.

#### Quantification of isolated RNA

The absorbance of the isolated RNA solution was recorded at 260 nm and 280 nm using a dual-beam UV-visible spectrophotometer and the concentration of RNA as well as its purity was calculated by

$$\text{RNA ng/}\mu\text{l} = \text{Absorbance at 260 nm} \times \text{Dilution factor} \times 40$$

The ratio of absorbance at 260 nm and 280 nm when obtained to be 1.8–2.0 depicts the purity of RNA as high quality with minimal contaminating proteins. The purity was also checked on 1.0% agarose gel indicating the presence of three bands corresponding to 28s, 18s, and 5s RNA.

#### cDNA synthesis

The RNA isolated was taken as template for the synthesis of cDNA using the manufacturer's protocol of Revert Aid cDNA synthesis kit. The final concentration of RNA

**Table 3**  $IC_{50}$  (µg/ml) values of *F. religiosa* latex extracts on human cancer cell lines exhibiting best activity

S. no	Cell lines	<i>Ficus religiosa</i> latex extracts	$IC_{50}$ (µg/ml)
1.	MDA MB 231	Ethanol	<b>50.86 ± 0.9*</b>
		Methanol	145.5 ± 4.5
		Acetone	111.86 ± 1.56
		Ethyl acetate	107.25 ± 4.12
		Doxorubicin	0.82 ± 0.13
2.	IMR 32	Ethanol	<b>4.8 ± 1.13*</b>
		Methanol	146.01 ± 1.7
		Acetone	102.84 ± 0.9
		Ethyl acetate	39.24 ± 3.21*
		Doxorubicin	0.37 ± 0.05
3.	HCT 116	Ethanol	<b>24.27 ± 0.66*</b>
		Methanol	122.89 ± 4.01
		Acetone	120.71 ± 3.01
		Ethyl acetate	101.65 ± 1.6
		Doxorubicin	0.48 ± 0.3

Each value is the average of three analyses ± standard deviation of 3 separate experiments in three selected cancer cell lines.

\*The  $IC_{50}$  values of the extract in comparison with control drug among all the cell lines were statistically significant ( $p < 0.001$ ;  $t$  test).

considered to synthesize cDNA was 2 µg/µl. To this, 1 µl of oligo-nucleotide (dT)<sub>15</sub> primers was added and made up to 12 µl with nuclease free water (DEPC treated), incubated at 65 °C for 5 min in a thermocycler, Eppendorf thermocycler (personal). The reaction mixture was chilled by placing the Eppendorf tubes on ice, and further 5X reaction buffer (4 µl), ribolock RNase inhibitor (20 U/µl) (1 µl), 10 mM dNTP mix (2 µl), and revertaid reverse transcriptase enzyme (M-MuLV RT – 200 U/µl) (1 µl), was added making the final volume to 20 µl. The above mixture was subjected to reverse transcription at 45 °C for 60 min and then termination of synthesis at 70 °C for 5 min. The synthesized cDNA was stored at – 80 °C for further amplification.

#### Amplification of cDNA with gene primers by reverse transcriptase-PCR

RT-PCR was performed by taking 1 µl of cDNA synthesized, added with 1 µl forward and 1 µl reverse primer

**Table 4** % Cell viability of lymphocytes against *F. religiosa* latex extract

S. no	Solvent extract (200 µg/ml)	<i>F. religiosa</i>
1	Ethanol	70%
2	Methanol	66%
3	Acetone	65%
4	Ethyl acetate	74%

(BioServe) of each gene, 7  $\mu$ l of nuclease free water (DEPC treated), 10  $\mu$ l of PCR master mix provided in the kit as per the manufacturer's protocol making up the final volume to 20  $\mu$ l. PCR cycle conditions like temperature profile was followed as per each gene selected for the present study and is mentioned in Table 1. The amplified cDNA sample was separated by electrophoresis on 1.5% agarose gel stained with ethidium bromide, and the bands were visualized under UV in Bio-Rad gel documentation system. The quantity of amplified cDNA in treated cells was compared with that of untreated cells (control) by measuring the peak area and the relative gene expression of all these apoptosis-related genes (Table 2) was determined using ImageJ software (LOCI, University of Wisconsin) with respect to  $\beta$ -actin gene.

#### Analysis of cell cycle arrest by flow cytometry (FACS)

The uni-variant analysis of DNA content by staining with propidium iodide (PI) and deconvolution of the cellular DNA content at various stages of cell cycle represented on frequency histograms was generated by flow cytometer. This reveals the distribution of cells undergoing different phases of cell division like  $G_1$ , S, and  $G_2/M$  and facilitates the detection of apoptotic cells. The treated cells with  $IC_{50}$  values of ethanol extract as well as the untreated cells that served as controls were trypsinized and centrifuged to collect the pellet, which was washed twice with PBS and finally suspended in 100  $\mu$ l of 70% ethanol and 900  $\mu$ l ice-cold PBS, stored at  $-20^\circ\text{C}$  overnight. The ethanol suspended cells were centrifuged, and the pellets were rehydrated in PBS by centrifugation for 10 min. The rehydrated pellets were suspended in 1 ml of propidium iodide (PI) staining solution prepared by 0.1% (v/v) Triton X 100, 100  $\mu$ g/ml RNase

A, and 20  $\mu$ g/ml PI in PBS and incubated in dark at  $37^\circ\text{C}$  or 30 min. The cell fluorescence was measured in the FACS flow cytometry (BD LSR Fortessa cell analyzer) with a total of 10,000 cells/samples at once. The maximum excitation of DNA bound with PI dye is at 536 nm whereas emission is recorded at 617 nm. The relative distribution of cells at various phases of cell division was recorded as peaks on frequency histogram.

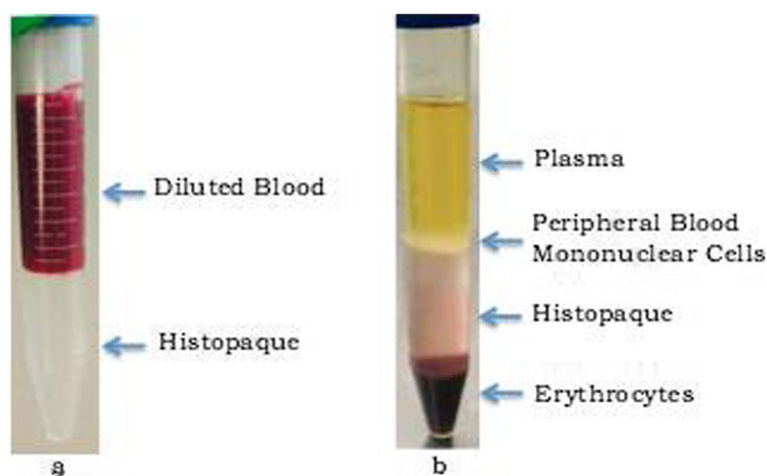
#### Statistical analysis

The 50% cytotoxic concentrations ( $IC_{50}$ ) of plant extracts were estimated from concentration-effect curves after linear regression analysis and were represented as mean  $\pm$  standard error of the mean values of three different experiments. The  $t$  test is carried out to obtain the  $p$  value ( $p < 0.001$ ) for statistical significance analysis.

## Results

### Chemo-preventive efficacy of *F. religiosa* latex extract Visualization of cells' morphological changes under light microscope

The phenotypic changes in the cancer cells treated with Ficus plant latex solvent extracts were observed morphologically under inverted phase contrast microscope. Prominent morphological aberrations were clearly noticed, and this is indicative of cancer cell growth inhibition and cell death after 24 h of extract treatment when compared to the untreated (control) cell of each cell line. The control as well as treated cells are represented in Fig. 1 (MDA MB 231), Fig. 2 (IMR 32), and Fig. 3 (HCT 116). The major abnormalities in morphology of treated cells were loss of shape as well as structure that indicate detachment of cells and entering cell death stage characterized by disruption of nuclear membrane into discrete fragments and budding of the whole cell into membrane



**Fig. 7** Blood sample collected for isolation of lymphocytes. **b** Different layers separated after centrifugation

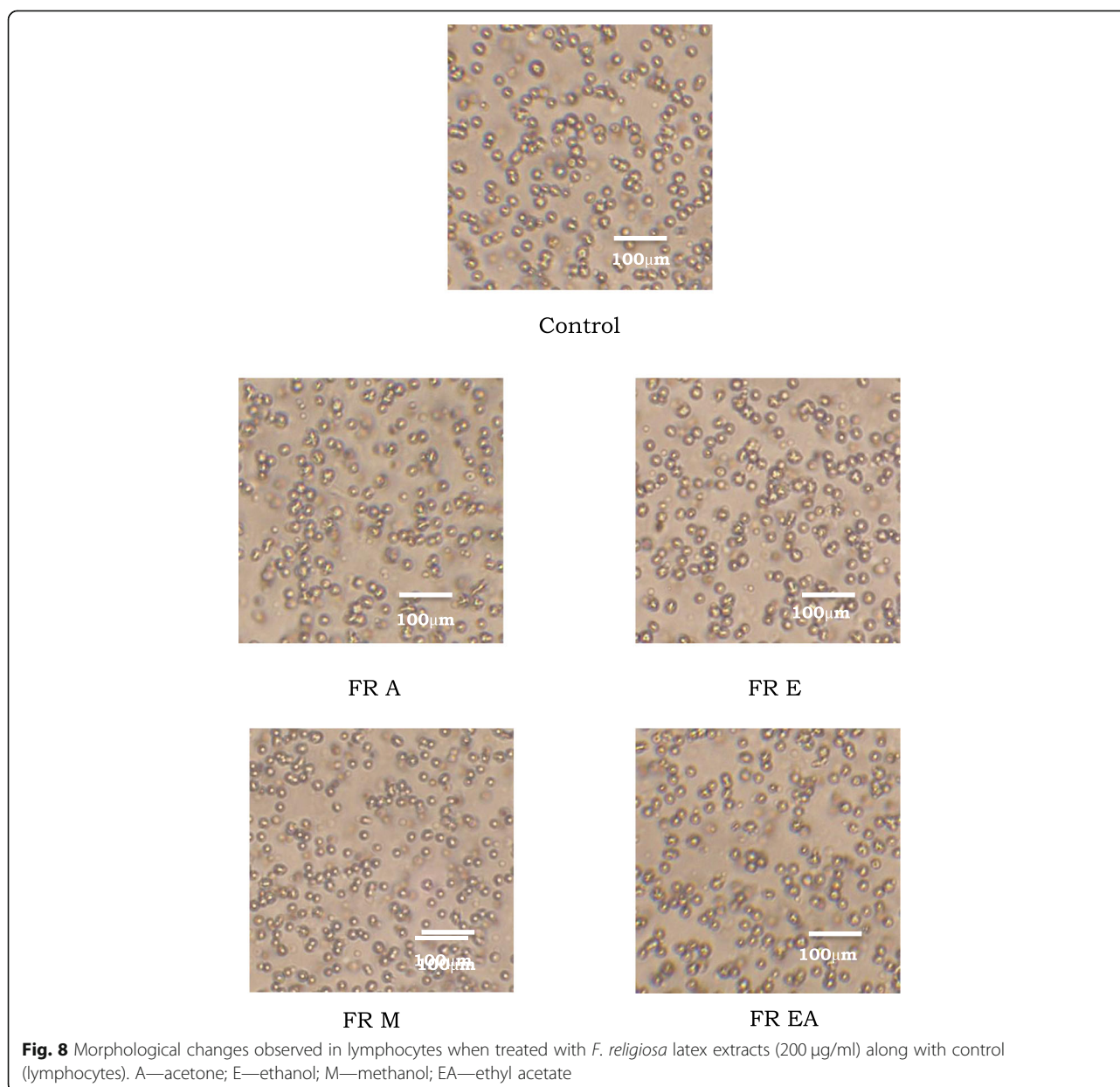
bound apoptotic bodies. This eventual detachment of cells from cell culture plate bottom is the usual feature of apoptosis, and it can be similar to the parting of apoptotic cells from normal cells in cancer tissue. Examining cells with trypan blue ruled out the cell death due to necrosis phenomenon, and it was observed that the dye was excluded by 90% of rounded and detached cells.

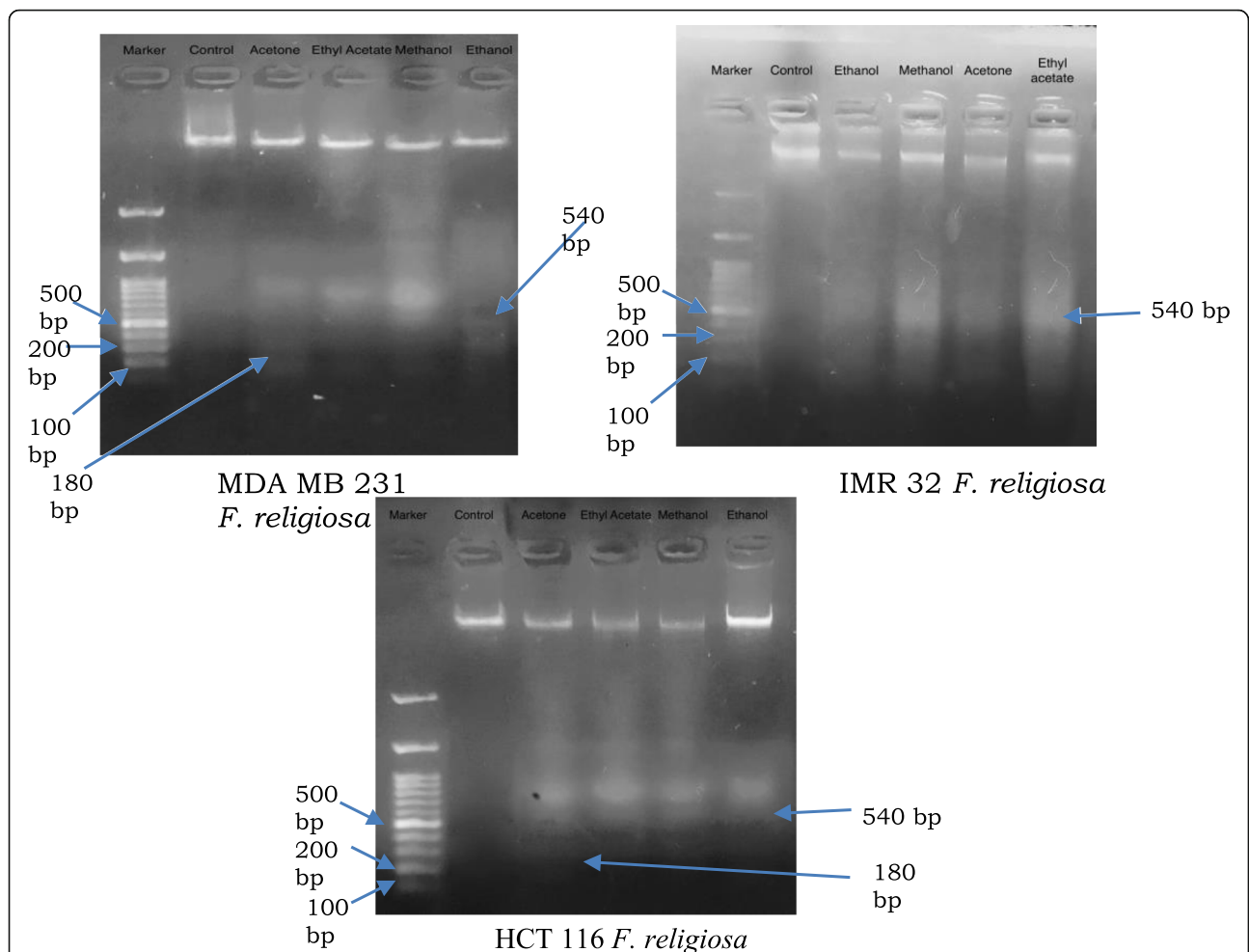
#### **Propidium iodide staining**

The PI staining is often used to distinguish between viable and non-viable cells. The dye penetrates into cell through the cell membrane of viable cells. The early apoptotic cells exclude the PI stain. Only late apoptotic cells can take up the stain. Early apoptotic

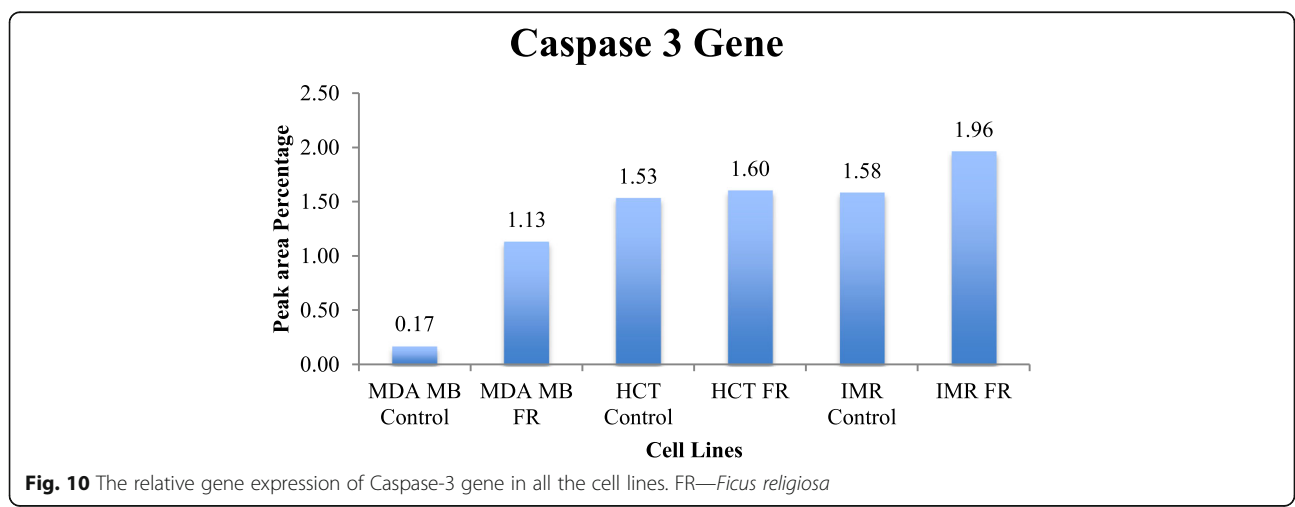
cells are considered as reproductively dead cells but yet marked as live cells in this assay because when DNA replication is at halt the cell grows in size as well as mitochondrial mass and activity were also increased [14].

The staining of treated and untreated cells with propidium iodide would portray the condensation and fragmentation of the chromatin material (DNA) and cytoplasm, irregular shapes like shrinkage and various other morphological changes which correspond to apoptosis-like formation of apoptotic bodies. The cells were treated with IC<sub>50</sub> values of each solvent extract, and the control cells also were stained in order to sort out the morphological changes induced by plant extracts clearly



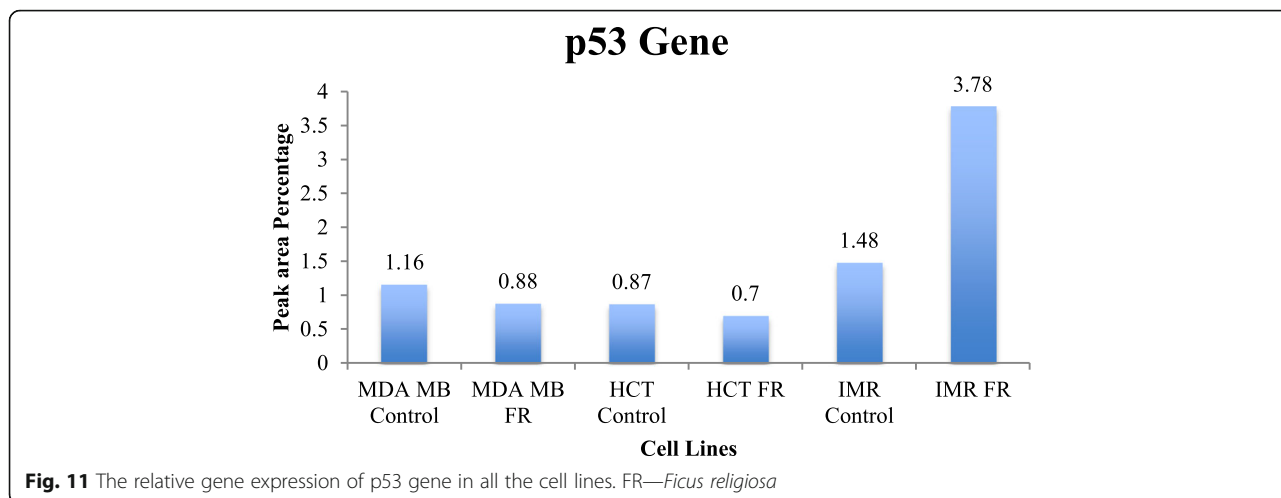


**Fig. 9** DNA fragmentation in the form of DNA ladder was analyzed on 1.5 % agarose gel electrophoresis. A 100-bp marker is loaded in first well followed by control and treated cells by IC<sub>50</sub> concentrations of *Ficus religiosa* latex in MDA MB 231 methanol (145.5 µg/ml), ethanol (50.86 µg/ml), acetone (111.86 µg/ml), and ethyl acetate (107.25 µg/ml) extracts; IMR 32 methanol (146.01 µg/ml), ethanol (4.8 µg/ml), acetone (102.84 µg/ml), and ethyl acetate (39.24 µg/ml); HCT 116 methanol (122.89 µg/ml), ethanol (24.27 µg/ml), acetone (120.71 µg/ml), and ethyl acetate (101.65 µg/ml) extracts



**Fig. 10** The relative gene expression of Caspase-3 gene in all the cell lines. FR—*Ficus religiosa*





after 24 h of treatment (Figs. 4, 5, and 6). Control cells of each cell line revealed a typical nuclear morphology characterized by intact chromatin structure, large vesicular nuclei, and noticeable nucleoli.

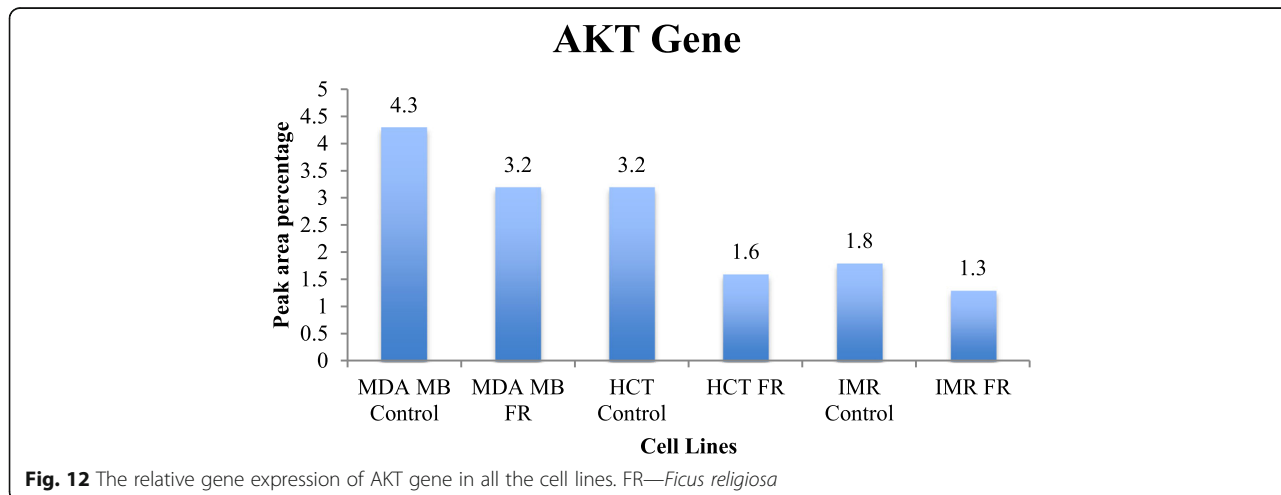
**Effect of Ficus latex extracts on the growth inhibition of cell lines**

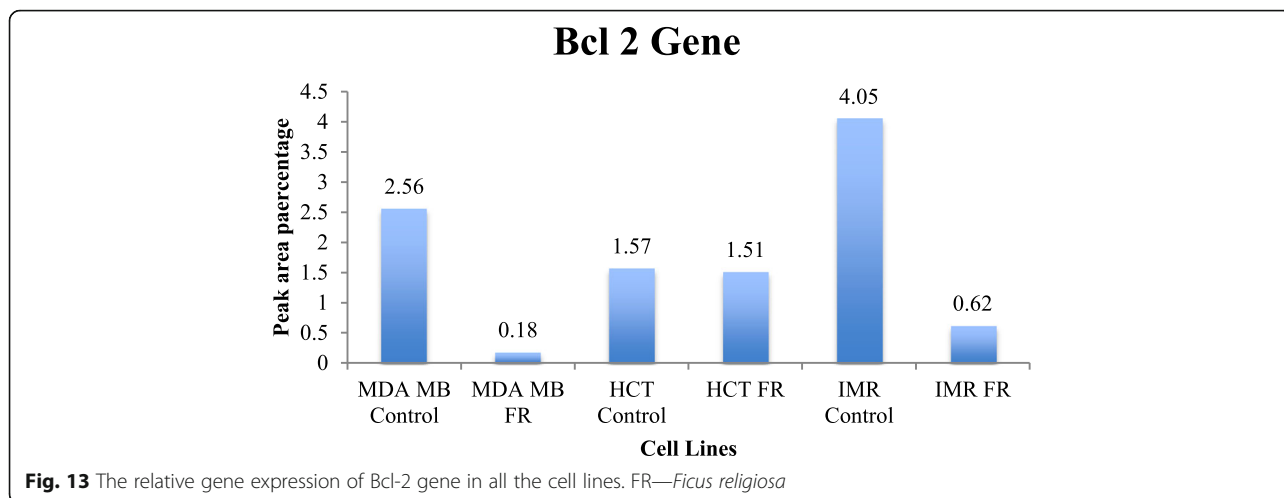
The principle underlying MTT assay is the reduction of MTT molecules by mitochondrial dehydrogenase of viable cells, producing blue color formazan molecules whose absorbance is measured spectrophotometrically at 540 nm. The anti-proliferative effect of *F. religiosa* solvent extracts on MDA MB 231, IMR 32, and HCT 116 was observed with MTT assay. The growth inhibition of the cancer cells was observed in a dose-dependent manner where 90% inhibition was observed at highest concentration, 200 µg/ml. The IC<sub>50</sub> value, 50% inhibition of cell proliferation, was calculated from the basic graphing in Microsoft excel (Table 3). The potent activity of *F. religiosa* was also seen with ethanolic extract against IMR 32

followed by HCT 116 and finally MDA MB 231 with IC<sub>50</sub> values 4.8 ± 1.13 µg/ml, 24.27 ± 0.66 µg/ml, and 50.86 ± 0.9 µg/ml, respectively. The positive control, doxorubicin, had shown its potency against all the cell lines chosen in the present study with IC<sub>50</sub> values of 0.82 ± 0.13 µg/ml against MDA MB 231, 0.37 ± 0.05 µg/ml against IMR 32, and 0.48 ± 0.3 µg/ml against HCT 116. The Ficus extract has shown best potency in inhibiting the cell growth in MDA MB 231, HCT 116, and IMR 32.

**Effect of plant extracts on viability of lymphocytes**

The lymphocytes were isolated and were treated with a concentration of 200 µg/ml of *F. religiosa* latex extracts. This is the concentration at which the growth of cancer cells was found to be inhibited 90%. The aim of this assay was to establish that this concentration would be less toxic to the normal cells like lymphocytes in order to reaffirm the non-toxic nature of this extract to normal cells. In this study, the cell viability was calculated using





MTT assay and the results are shown in Table 4 and Figs. 7 and 8.

**Analysis of DNA fragmentation by DNA ladder assay**

In the present study, the cells were treated with IC<sub>50</sub> values of latex extracts and the pellets were subjected to DNA extraction and isolation. The gel electrophoresis results depicted apoptosis visualized as DNA ladder. In case of present study, *F. religiosa* latex extract has shown bands at 500bp, 300bp, and 200bp in HCT 116, IMR 32, and MDA MB 231 (Fig. 9) when compared with marker ladder DNA, whereas the control lane has only one single band near to well indicating the viable cells and no DNA smear or fragments were observed.

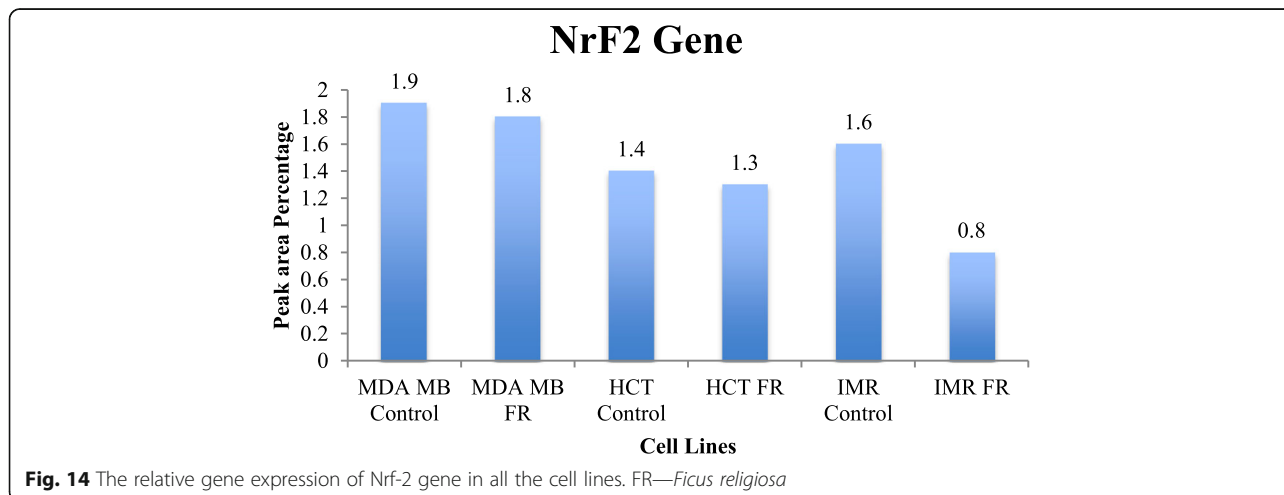
**Apoptotic gene expression studies**

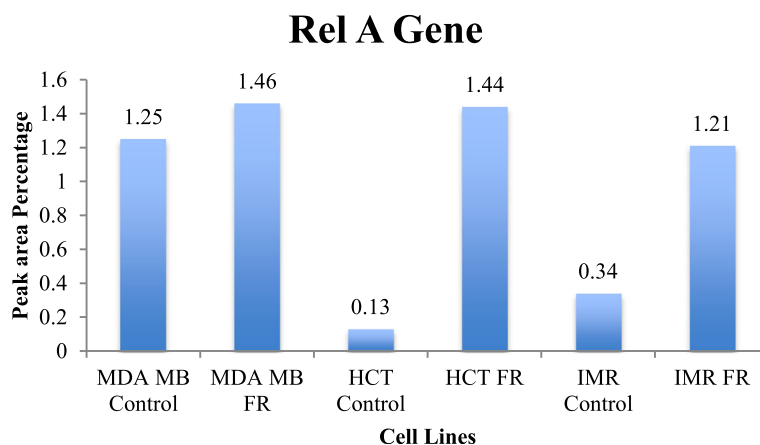
The expression of apoptotic genes like Caspase-3, p53 (pro-apoptotic), and Bcl-2 (anti-apoptotic) were studied. Under-expression of pro-apoptotic genes and over-expression of anti-apoptotic genes are the

characteristics of cells, which undergo continuous cell division and lack of cell death, a typical characteristic of cancer cells. β-actin gene was used as the control. Ficus solvent extracts were treated with IC<sub>50</sub> concentrations. RNA was isolated and transcribed to cDNA after which the expression of caspase-3, p53, and Bcl-2 were studied. In the present study, there was an up-regulation in the caspase-3 gene (Fig. 10) indicating apoptosis as the major pathway of cell death in treated cells.

The expression of p53 gene was variable in MDA MB 231-treated cells *F. religiosa*; ethanol extract has down-regulated the expression of gene. The expression of p53 in HCT 116 was downregulated by ethanol extracts when compared to control cells, and in the case of IMR 32 cells, *F. religiosa* ethanol extract upregulated the gene expression (Fig. 11).

The results of the present study with respect to AKT expression in all the treated cell lines indicated a downregulation when compared to control cells,





**Fig. 15** The relative gene expression of Rel A gene in all the cell lines. FR—*Ficus religiosa*

which is in accordance with the literature (Fig. 12). The AKT gene also termed as RAC kinase present in the cell naturally inhibits apoptosis and helps in cell survival [15–19]. This clearly depicts that AKT exerts anti-apoptotic effect and also phosphorylates the pro-apoptotic genes like caspase-9 [20] and BAD [21] and inactivates them.

Expression of Bcl-2 as well as Nrf2 were found to be downregulated after treatment with *Ficus* solvent extracts for 24 h against cell lines (Figs. 13 and 14). The role Nrf2 in cell is to induce Bcl-xL gene, which will enhance the rate of cell survival. Prior studies also reported the role of Nrf2:INrf2 in regulating anti-apoptotic factor like Bcl-2 [22–24]. During stress situations, both Nrf2 and Bcl-2 are produced from INrf2 (inhibitor of Nrf2) leading to stabilization of these genes. Bcl-2 stabilization leads to reduction in the rate at which cell participating in apoptosis. Nrf2 on stabilization will penetrate into the nucleus through a nuclear membrane and activates a series of cytoprotective proteins including all the anti-apoptotic genes like Bcl-2 and thereby decreases the cell death due to apoptosis. This clearly depicts that Bcl-2 stabilization as well as Nrf2-mediated activation of Bcl-2 will ultimately lead to reduced apoptosis and safeguard the cell during stress environment by maintaining the homeostasis.

The present study also showed the increased levels of Rel A gene expression in the treated cells of all the cancer cell lines which indicates that the *Ficus* latex extracts possess the ability of inducing apoptosis and inhibiting the cell proliferation (Fig. 15). The NF- $\kappa$ B proteins, family of transcriptional factors, play a vital role in regulating the apoptotic process in cell [25, 26]. Rel A (P65), the most common form of NF- $\kappa$ B, is generally rendered stable in cytoplasm by I- $\kappa$ B, an inhibitory protein [27]. Any

stimulus to the cell will trigger the degradation of the I $\kappa$ B and allow the NF- $\kappa$ B translocation into the nucleus and directly binds to multi-target genes promoter sequence [28]. This sort of stimulation of NF- $\kappa$ B pathway induces p53-mediated cell death [29], and the expression of Rel A in isolated form will stimulate apoptosis [30].

#### Analysis of cell cycle arrest by flow cytometry (FACS)

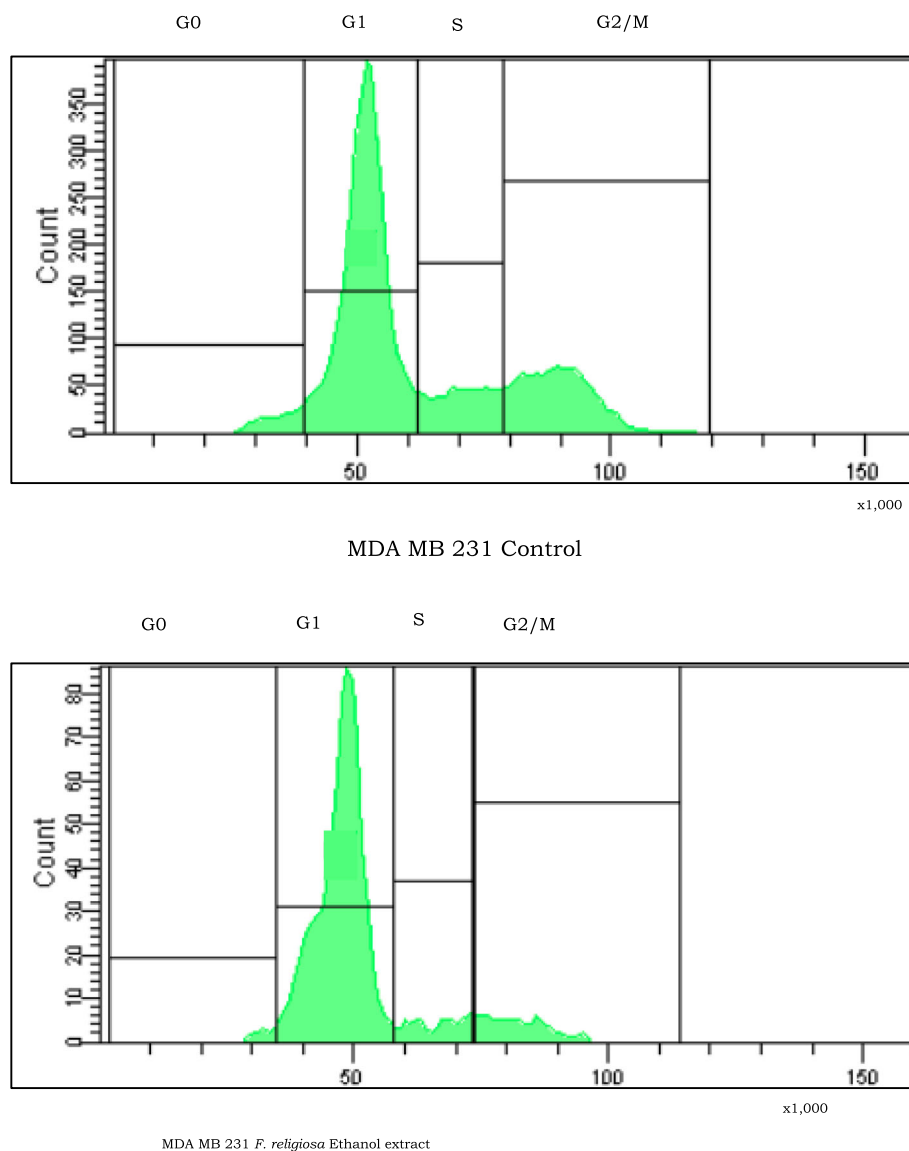
The three cancer cell lines were treated with *F. religiosa* ethanolic extracts as they have shown high potency in cell viability assay (MTT assay). The IC<sub>50</sub> concentrations were treated for 24 h, and the cells were subjected to FACS by staining with propidium iodide (PI). The cell cycle arrest was observed in all the cell lines when treated with plant extracts. The accumulation of cells at a particular phase was reported when compared to the distribution of cells in control cell line.

In case of MDA MB 231 cells, the arrest was observed in G1 phase of *F. religiosa* ethanolic extract when compared to that of control MDA MB 231 cells (Table 5 and Fig. 16).

When IMR 32 cells were observed, cells got accumulated in G2/M phase in both *F. religiosa* when compared to that of control IMR 32 cells (Table 6 and Fig. 17).

**Table 5** The % cell population of MDA MB 231 cells at different phases of cell cycle

Cell cycle phases	MDA MB 231 % cell population	
	Control	<i>F. religiosa</i> (ethanol)
G0	3.4	1.7
G1	61.6	80.2
S	12.4	7.1
G2/M	22.1	10.7



**Fig. 16** Analysis of cell cycle arrest of MDA MB 231 cells when treated with IC<sub>50</sub> concentration of *F. religiosa* ethanol extract (50.86 µg/ml) for 24 h along with control MD MB 231 cells

In case of HCT 116 cell line, it was observed that *F. religiosa* ethanol extract has cell cycle arrest in G1 phase when compared to control HCT 116 cells (Table 7 and Fig. 18).

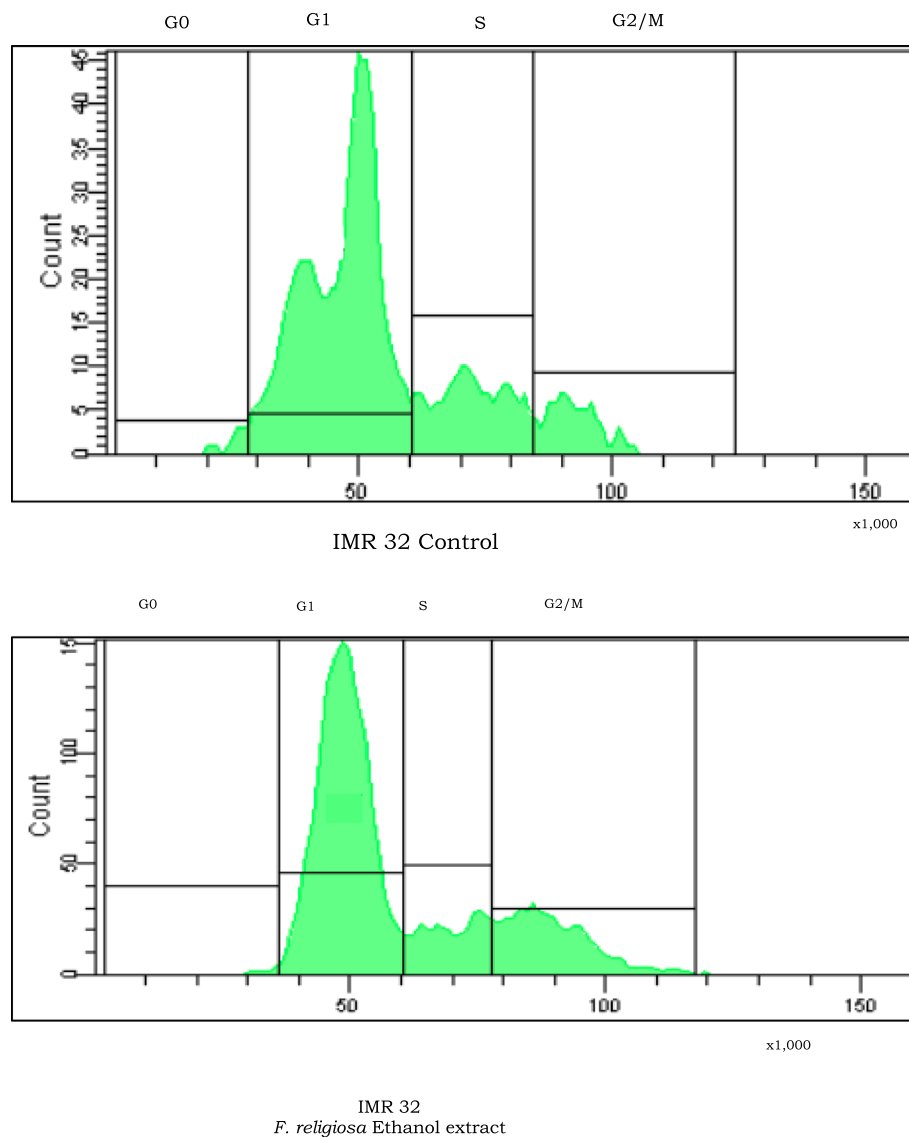
**Table 6** The % cell population of IMR 32 cells at different phases of cell cycle

Cell cycle phases	IMR 32 % cell population	
	Control	<i>F. religiosa</i> (ethanol)
G0	1.7	0.3
G1	69.0	65.5
S	19.2	13.2
<b>G2/M</b>	<b>10.0</b>	<b>20.6</b>

**Discussion**

Ullman et al. reported the first scientific investigation on the activity of fig latex in the 1940s [31–33]. The previous studies have shown the cytotoxic activity of *Ficus racemosa* hot ethanol bark extract against calu-6 cells using XTT colorimetric assay in a concentration-dependent manner. The cytotoxic effect of plants is predominantly contributed by the presence of secondary metabolites like alkaloids, steroids,



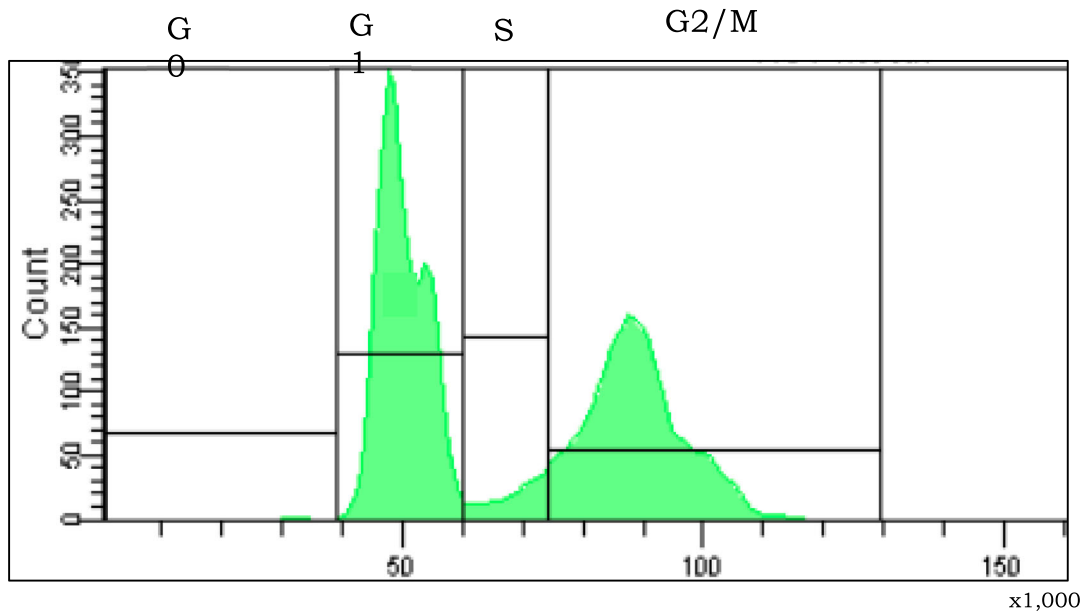


**Fig. 17** Analysis of cell cycle arrest of IMR 32 cells when treated with IC<sub>50</sub> concentration of *F. religiosa* ethanolic extract (4.8 µg/ml) and along with control IMR 32 cells

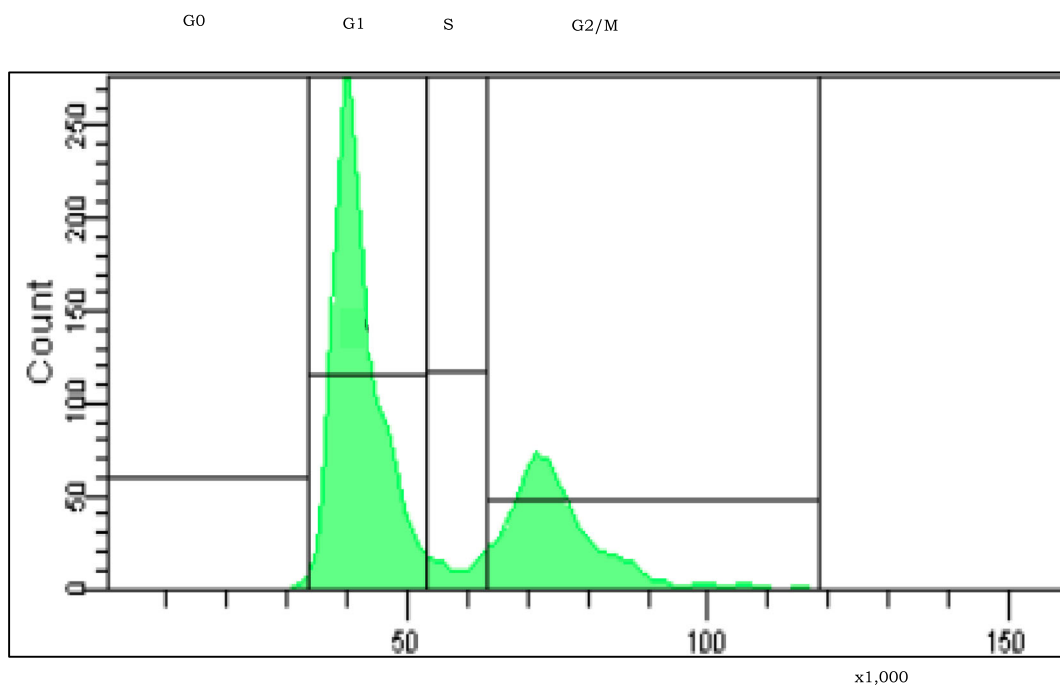
**Table 7** The % cell population of HCT 116 cells at different phases of cell cycle

Cell cycle phases	HCT 116 % cell population	
	Control	<i>F. religiosa</i> (ethanol)
G0	0.2	0.2
<b>G1</b>	<b>50.3</b>	<b>64.4</b>
S	4.3	4.1
G2/M	45.0	31.2

glycosides, terpenoids, tannins, and flavonoids in their extract [34, 35]. Iloki-Assanga et al. [36] demonstrated that extraction solvents have an effect on phenolic and flavonoid contents. This correlates with the present study as preliminary phytochemical screening has shown abundant phenolics and flavonoids which are responsible for antioxidant and anti-microbial activities as well contribute to the cytotoxic activity of the plant extracts against selected cancer cell lines [37]. Hashemi et al. [38] have studied the effect of *F. carica* latex in different concentrations and observed that 5 mg/ml concentration has shown potent inhibition against stomach cancer cell line growth. The cytotoxicity of leaf and fruit extract as well latex on HeLa cell line has shown that the percentage viability of cells at concentration 2 µg/ml was



HCT 116 Control Control

HCT 116 *F. religiosa* Ethanol extract

**Fig. 18** Analysis of cell cycle arrest of HCT 116 cells when treated with  $IC_{50}$  concentration of *F. religiosa* ethanolic extract (24.27  $\mu\text{g/ml}$ ) and along with control IMR 32 cells

least and it is dose dependent [39]. Rubnov et al. [40] have isolated 6-O-acyl-beta-D-glucosyl-beta-sitosterol from fig latex, which was found to be an effective cytotoxic agent and when subjected to in vitro proliferation studies against various cancer cell lines has shown potent activity. According to American Cancer

Institute, a crude extract will have potent cytotoxicity if the  $IC_{50}$  values are lower than 30  $\mu\text{g/ml}$  in order to discover and develop potential anticancer natural compounds [41]. Based on this criterion, the ethanolic extract is potential to be developed as new anticancer drug.

Lymphocytes results indicate that the  $IC_{50}$  concentration of the extract could show least toxicity at the highest concentration and the lymphocyte viability was more than 60% as previous studies indicated that in humans, a decline in total lymphocyte count by more than 40% was clinically significant [42, 43].

DNA fragmentation analysis is a qualitative method to assay the cell death using the DNA fragments formed by agarose gel electrophoresis. The hallmark feature of apoptosis is cell shrinkage and fragmentation as well condensation of chromatin (DNA). This DNA fragmentation analysis by agarose gel electrophoresis for detection of apoptosis is most frequently used as it easily distinguishes between the non-apoptosis (necrosis), and the apoptosis as the cleavage of DNA into fragments yields the characteristic hallmark of this assay "DNA ladder" [44, 45]. The plant solvent extracts of *Ficus religiosa* against all the cancer cell lines has given the clear bands appearing as DNA ladder which is nothing but DNA strands nicked after histone particle giving bands of molecular weight a multiples of 180 bp.

The gene analysis studies were carried out with ethanol extract of *Ficus religiosa* as it has shown potent cytotoxic activity of which the results are in accordance with previous studies where it was ascertained that downstream genes of p53-induced apoptosis in a p53-independent pathway [46]. However, in MDA MB 231 cells [47], p53 is mutated, and in 60% of human cancers, there is accumulation of the mutated non-functional protein. The ethanol extract of *Ficus religiosa* induced apoptosis by regulating Bcl 2 resulting in stimulation of Cytochrome c release which initiated the caspase cascade leading to apoptosis. Extract treatment caused up-regulation of caspase 3 and decreased expression of Bcl-2, which pointed out the involvement of the intrinsic pathway (mitochondrial pathway) in inducing apoptosis that can be targeted for cancer chemoprevention. Phytoconstituents or chemo-preventive agents were considered as apoptosis inducing agents as well as molecular targets in the mechanism of cell cycle arrest. They act as NF-kB inhibitors, as caspase activators, as AP-1 inhibitors, as ROS inducers, and as cell cycle inhibitors, which are involved in inducing apoptosis and restricting the further cell division. The anti-proliferative and apoptotic potential of ethanol extract is clearly depicted in the present study correlating with that of the literature [48]. AKT exerts its anti-apoptotic effect by producing NO (nitric oxide), which is directly linked to apoptotic regulatory pathway [49, 50]. This gene along with NF-kB (nuclear factor-kB) is involved in fork-head transcription pathway, yielding its anti-apoptotic signals [51–55]. Bcl-2 family proteins play a key role in regulation of apoptosis. It can be divided into three sub classes of which Bcl-2 belongs to pro-survival sub class

members along with Bcl-XL, MCL1, BFL1/A1 and Bcl-W. The other two sub classes are pro-apoptotic mediator members (Bak and Bax) and BH3 only sub-family members [56].

Apoptosis is a mechanism to kill cells that have lost the regulatory mechanism in cell division, and it is the pathway the cell chooses to kill the tumor cells [57]. Several chemo-preventive drugs exert their action by inducing p53-dependent or p53-independent apoptosis and by accumulating cells at G1/S or G2/M phase [58]. In a case study, *F. religiosa* aqueous extract was studied for its anti-proliferative activity on a cervical cancer cell line and was observed to induce cell cycle arrest at G1/S phase in HPV-16 (+) SiHa cell line by inducing the expression of p21 and p53 as well inhibiting phosphorylation of pRb tumor suppressor protein. p21 activates the downstream effectors of p53, which induces G1/S cell cycle arrest, and this could be the mechanism of inhibition by *F. religiosa* aqueous extract in this case study [59].

## Conclusion

All the extracts were found to be cytotoxic in the MDA MB 231, IMR 32, and HCT-116 cell lines. The gene studies and cell cycle analysis were carried out with ethanol extract as it has shown potent cytotoxic activity against all the cancer cell lines. IMR 32 cell line was inhibited with greater potency by ethanol extract of *Ficus religiosa*. Further, in vivo studies in animal models should be carried out, which would pave way towards clinical trials for the use of *Ficus* phytochemicals as a safe with no side effect drugs in cancer therapy.

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## Authors' contributions

MLN and LS designed the entire work and revised the manuscript. CDSLNT carried out the research work after an extensive literature review as well as has written the complete manuscript. The authors have read and approved the final manuscript.

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## Availability of data and materials

All the materials and data are available upon request

## Ethics approval and consent to participate

Not applicable

## Consent for publication

Not applicable

**Competing interests**

The authors declare that they have no competing interest

**Author details**

<sup>1</sup>Centre for Biotechnology, Institute of Science and Technology, Jawaharlal Nehru Technological Institute, Hyderabad, Telangana 500085, India. <sup>2</sup>Centre for Biotechnology, Institute of Science and Technology, Jawaharlal Nehru Technological Institute, Kukatpally, Hyderabad, Telangana 500085, India.

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