

Anti-Cancerous Activity of *Albizia Amara* (Roxb.) Boivin using Human Breast Cancer Cells (MCF-7) by *In Vitro* Methods***Gopinath P¹, Sundara Vadivel D¹, Kamatchiammal S², Saroja V.²**

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ABSTRACT

Traditional medicinal plants in India are majorly used for treating many diseases in olden days. Based on that, the present study was aimed to investigate the anticancer effect of various extracts of leaves of *Albizia amara* on Human Breast Cancer Cells (MCF-7) by comparing with the 5-FU treated cells. The cytotoxicity of plant extracts were detected by MTT assay, among the three solvent extracts, the ethyl acetate extract showed effective cytotoxic action with median inhibitory concentration (IC₅₀) of 36.31µg/ml followed by ethanol extract with 57.54µg/ml and aqueous extract with 83.18µg/ml. The ethyl acetate extract treated cells showed significant signs of apoptosis such as cell shrinkage, membrane blebbing and nuclei fragmentation. The gene expression for cancer DNA markers and Interleukins were examined by real time PCR which showed that the expression of bcl-2 was decreased in cells treated with ethyl acetate extract and with 5FU when compared with control cells. The expression of p53 and TNF-α was expressed high in cells treated with ethyl acetate extract than with control cells but was low when compared with 5FU treated cells. The expression of IL4 and IL6 was low in cells treated with ethyl acetate extract and 5FU treated cells when compared with control cells. The expression of protein such as survivin and ORP150 decreased in cells treated with ethyl acetate extract than 5FU treated cells and control cells which were detected by Bio Analyzer. These results suggest that the ethyl acetate extract of *Albizia amara* has potential broad spectrum of anticancer activity.

Keywords: *Albizia amara*, apoptosis, cytotoxicity, ethyl acetate extract, gene expression, MCF-7

Received 03 July 2013

Received in revised form 16 July 2013

Accepted 18 July 2013

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INTRODUCTION

Breast cancer is the most commonly occurring cancer in females, rare in male. It accounts for 23% of newly occurring cancer worldwide and represents 13.7% of all cancer death. In both developed and developing countries, breast cancer is the most frequent cancer and most frequent cause of cancer deaths [1]. The report, by National Confidential Enquiry into Patient Outcome and Death (NCEPOD) have shown that more than 1 in 4 patients died from the side effect rather than from cancer and patients suffering from treatment related toxicity despite receiving other treatment to reduce chemotherapy side effects [2]. An another study discovered that breast cancer patients receiving chemotherapy, some

parts of their brain, responsible for learning and memory was affected when compared to untreated patients [3]. In search of new agents to treat cancer with fewer or less side effects a number of medicinal plant has been evaluated [4], because in pharmacology several active ingredients are found from medicinal plants [5,6]. Some of the successful and potential lead molecules isolated from medicinal plants are vincristine, vinblastine, taxol, camptothecin and podophyllotoxin [7]. Since the medicinal plants have phyto constituents, which protects the plant from oxidative damages and may have same role in humans. They have a wide range of action such as antitumor, antiviral, antibacterial,

antimutagenic etc. they may act in different stages of the development of malignant tumour by protecting the DNA from oxidative damages. They inactivate carcinogen by inhibiting the expression of mutagenic genes; they also inactivate the enzymes charged with activating procarcinogens and activate the systems responsible for the detoxification of xenobiotics [8]. It can also inhibit anti apoptotic gene as well as activate apoptotic gene [9, 10].

This research was aimed to identify the potential anticancer activity of widely available medicinal plant by investigated its effect at molecular level in Human breast cancer cell line (MCF-7). *Albizia amara* is a naturally occurring medicinal plant, locally known as Munja belonging to the family of *mimosideae*, it has been reported to have a number of biological activities in traditional medicine. Several investigation in leaves of *Albizia amara* indicated that it has potent antioxidant and antibacterial, antifungal, antimicrobial, larvicidal activity. There is no scientific data published regarding anticancer activity of it. Thus the study was carried out to determine the anticancer activity of *Albizia amara*.

MATERIALS AND METHODS

Cell Culture

MCF-7 (Human Breast Cancer Cell Line) was obtained from King Institute, Guindy,

Chennai, Tamilnadu. The cell line was maintained and sub cultured in 25 m² tissue culture flasks using 5 ml of minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 3% L-glutamine, penicillin (100 IU/ml), streptomycin (100 µg/ml), Amphotericin B (20 µg/ml), phenol red. The pH of the medium was adjusted to 7.2-7.4 with 7.5% sodium bicarbonate and all flasks were incubated at 37°C in a humidified 5% CO₂/95% O₂ incubator.

Chemicals and Reagents

5-flourouracil (Sigma), MTT dye (Sigma), acridine orange (Sigma), ethidium bromide (Sigma), DMSO (Dimethyl sulfoxide), **GeNei** TRIsoln, cDNA kit (Qiagen), Primers (both forward and reverse), SYBR green RT mix (Qiagen), RNase, TPVG (Qiagen).

Plant material

Fresh disease free leaves of *Albizia amara* were collected in the month of July 2012 at kalakadu, Tirunelveli Dist., Tamilnadu, India. Leaves were washed thoroughly 2-3 times with tap water and once with sterile distilled water, shade dried, grounded in to coarse powdered form and used for extraction. The leaves were examined and authenticated by Mr. V. Chelladurai, Research officer-Botany (Scientist-C) (retd.), Central Council for Research in Ayurveda & Siddha, Govt. of India.



Figure 1: Collected Leaves of *Albizia Amara*

Preparation of solvent extract

Fifty grams of coarse powdered leaves of *Albizia amara* was filled in the thimble and extracted with 250 ml of petroleum ether, ethyl acetate, ethanol, and water

successively using a soxhlet apparatus (Ausco). Each of the solvent extract was concentrated separately under reduced pressure using rotary flash evaporator

(VirTis advantage plus) and stored for further testing.

Preparation of stock solution

The dried plant extract was dissolved in 1ml of DMSO (0.1% v/v) and made up to 10 ml with complete media (MEM) to give stock solution of extract 10mg/ml.

Extract dilution

Stock solution was diluted with complete media to obtain the concentrations of 10, 20, 40, 60, 80, 100 µg/ml. All were stored in air tight container until tested.

Cytotoxicity assay

Growth inhibition of MCF-7 cells by *Albizia amara* was determined by MTT assay [11, 12]. Briefly, cells were harvested and seeded at a density of 10000 cells/well in a flat bottom 96 well plates. The cells were incubated for 24 hours at 37°C in 5% CO₂

Percentage of cell inhibition = 100 -

$$\frac{\text{OD of treated cells}}{\text{OD of untreated cells}} \times 100$$

Microscopic analysis

Inverted Light microscopy:

A flask of sub cultured cells were treated with IC₅₀ concentration of ethyl acetate extract and incubated for 48 hours in 5% CO₂ Incubator. After incubation, flask was taken out and confirmed that there was no contamination detected. The flask was observed under inverted light microscope (Nikon Eclipse). Cells were identified as apoptotic if they displayed condensed nuclear, fragmented nuclei and/or membrane blebbing. The morphology of untreated cells was served as control.

Florescent microscopy:

A flask of sub cultured cells were treated with IC₅₀ concentration of ethyl acetate extract and another flask left untreated. The entire used medium was decanted and about 1ml of washing medium, PBS was added in both the flasks and discarded it. About 1ml of TPVG was added and left for 5 minutes for cell detachment. All the cells from both flasks were transferred into separate sterile tubes and centrifuged for 5 minutes at 1000 rpm. The supernatant was discarded and pellet was resuspended with phosphate buffer saline and mixed with equal mixture of acridine orange/ethidium bromide (each dye was dissolved in 100µg/ml of PBS). 10µl of mixture were placed on a glass slide and covered by cover

Incubator (R S Biotech- galaxy B) for cells attachment prior to addition of the drug. Various concentrations of leaves extract and standard 5FU were added to the cells and incubated for 48 hours. Each concentration was tested in triplicates. After incubation, the medium was replaced with phenol red and FBS free medium and 15 µl of MTT (5mg/ml) dye was added per well. The plate was wrapped with aluminium foil and incubated for additional 4 hours. Without disturbing MTT crystals, medium was removed carefully and 100 µl of DMSO were added to each well to solubilize the formazan crystals. The OD was measured at the wavelength of 570 nm by an ELISA reader. The percentage of cell inhibition was determined by following formula,

slip and viewed under fluorescent microscope. The differential uptake of these two dyes allowed the identification of viable (green color) and non viable cells (red color).

EXTRACTION OF DNA RNA and PROTEIN

Cells treated with IC₅₀ concentration of ethyl acetate extract and 5-FU for 48 hours were collected and subjected to extraction of RNA, DNA and protein using **GeNei™** TRIzoln according to manufacturer's instructions. TRIzoln is a monophasic solution in which the samples were lysed, addition of chloroform results in three phase separation, aqueous phase, predominantly of RNA, an interphase, predominantly of DNA and organic phase, predominantly of protein. The extracted RNA was used for real time RT-PCR, the DNA for fragmentation study and proteins for protein analysis.

DNA fragmentation

Briefly, the DNA were precipitated from an interphase obtained from phase separation by addition of 0.3ml of 100% ethanol and washed the DNA pellet with 1ml of 0.1M sodium citrate in 10% ethanol twice. After centrifugation at 5000 rpm for 10 minutes at 2-8°C the pellet was suspended in 2ml of 75% ethanol and kept for 20 minutes at room temperature and centrifuged at 5000 rpm for 10 minutes at 2-8°C then the pellet

was air dried and resuspended with 300µl of 8mM NaOH and incubated at room temperature for 15-20 minutes. The resultant DNA was quantified using NanoDrop apparatus (Thermo scientific) and used for electrophoresed in 1.2% agarose gel containing 3µl of ethidium bromide [13, 14]. After electrophoresis, the gel was photographed under UV light.

Extraction of RNA

Briefly, 0.5ml of isopropanol added to the aqueous phase obtained from phase separation centrifuged at 12000 rpm for 10 minutes at 2-8°C. The pellet obtained was washed with 75% ethanol and centrifuged at 10000 rpm for 10 minutes at 2-8°C. The pellet was air dried and resuspended with 100µl of RNase free water. The resultant RNA was quantified and used for RT-PCR.

Reverse Transcriptase PCR (RT-PCR)

The extracted RNA from treated and non treated cell lines were detected its expression qualitatively by reverse transcribed into complementary DNA or cDNA. The reaction consists of 10µl of the extracted RNA, 5µl of 5X buffer, 2µl of 10mM dNTPs, 1.5µl of hexamer primer and kept the tubes in thermal cycler at 70°C for 5 minutes and cooled immediately using ice. Then 1.5µl of MuLV

Reverse transcriptase, 1µl of DTT and 4µl of RNase free water was added for a total of 25µl. Samples were spun once before loading into PCR. The RT-PCR program was performed as follows: 25°C for 5 minutes for binding of hexamer, 42°C for 45 minutes for cDNA synthesis and 85°C for 5 minutes for denaturation of remaining/unconverted RNAs. The cDNA was stored at -20°C in deep freezer until its use.

Real time PCR (qPCR)

The cDNA obtained from RT-PCR was used as template to examine the expression level of cancer DNA markers (p53, Bcl-2, TNF-α) and immune response markers (IL4, IL6) in the presence of housekeeping gene primers (GAPDH). The real time reaction consists of 25µl SYBR green RT mix, 5µl of cDNA, 2µl of 10pM/µl forward primer, 2µl of 10pM/µl reverse primer (Table: 1), 16µl of PCR grade water for a total of 50 µl. Placed the plate in real time PCR and set the program as follows, pre denaturation at 95°C for 1 minute, denaturation at 95°C for 15 seconds.; 40 cycles of 95°C for 15 seconds, annealing at 60°C for 15 seconds and extension at 72°C for 45 seconds. Data acquisition performed during the extension step. This reaction was performed using step 1 plus ABI system.

Table 1: Oligo-Nucleotide Primer Sequences Used In This Study

S.NO.	PRIMER NAME	PRIMER SEQUENCE
1	p53	AGGGATACTATTTCAGCCCCGAGGTG ACTGCCACTCCTTGCCCCATTC
2	Bcl-2	ATGTGTGTGGAGAGCGTCAACC TGAGCAGAGTCTTCAGAGACAGCC
3	TNF-α	TCTCTAATCAGCCCTCTGGCC TGGGCTACAGGCTTGTCCTC
4	IL4	CTATTAATGGGTCTCACCTCCCAACT CATAATCGTCTTTAGCCTTTCCAAG
5	IL6	GCCTTCGGTCCAGTTGCCTT GCAGAATGAGATGAGTTGTC
6	GAPDH	ATTGACCACTACCTGGGCAA GAGATACTTCAACTTTGACCT

Data analysis

The data set of both samples and control of real time PCR was analyzed with appropriate bioinformatics and statistical program for estimation of relative expression of genes using Real Time PCR and the results normalized to GAPDH gene (Reference

gene). The data were statistically evaluated, interpreted and analyzed using step 1 plus ABI software.

Extraction of protein

Briefly, to the supernatant obtained from phase separation added 1.5ml of isopropanol to precipitate protein. Washed the pellet

with wash buffer, 0.3M guanidium hydrochloride in 95% ethanol, centrifuged at 10000 rpm for 5 minutes at 2-8°C. Vacuum dried the pellet and resuspended with 1%SDS by incubating at 50°C. The resultant protein was quantified and used for protein analysis.

Protein analysis

The extracted protein from treated and non treated cell lines were used to detect the expression levels of survivin and ORP150 in Agilent 2100 Bioanalyzer using Agilent high sensitivity protein 250 kit contains chip and reagents. Opened the chip priming station, pipette 12µl of gel mix in each of the wells marked G, pipette 12µl de-staining solution in the well marked DS, pipette 6 µl of heat denatured sample into the wells marked 1...10, pipette 6 µl of heat denatured ladder into well marked with ladder symbol. Placed the chip in bio analyzer and the run was started within 5 minutes after loaded into the wells. The incoming raw signals were displayed in the Instrument context of 2100 expert software screen.

RESULTS AND DISCUSSION

In this study, the anticancer effects of various extracts of leaves of *Albizia amara*

has been investigated using standard MTT cytotoxicity test. From the result obtained, the ethyl acetate extract showed effective cytotoxicity hence used for further investigations such as apoptosis using inverted light and fluorescent microscopy after stained with AO/EtBr dyes, regulation of gene expression by real time RT-PCR and protein expressions by Bio Analyzer.

MTT assay, also called cell viability assay is an internationally accepted *in vitro* method for anticancer drug screening. The principle of this assay is to distinguish the viable and non viable cells. The advantage of this assay is that it can be run on microtiter dishes on hundreds of cells sample at one time so that the various concentrations of each plant extracts can be used to get an idea of the dose response relationship from which IC₅₀ concentration was determined. From the results obtained, the ethyl acetate extract showed effective cytotoxicity with median inhibitory concentration (IC₅₀) of 36.31µg/ml (Figure 3) followed by ethanol extract with 57.5431µg/ml, aqueous extract with 83.1831µg/ml, and it was 1.2µg/ml with 5FU (Figure 2).

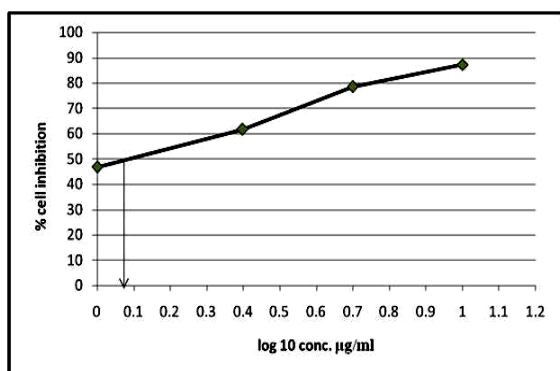


Figure 2: Graphical Representation of Concentration Dependent Cell Inhibition of 5-Fluorouracil

Albizia amara inhibited the cell growth and decreased the cell survival through induction of cell death. Cells treated with high concentration of extract showed significant

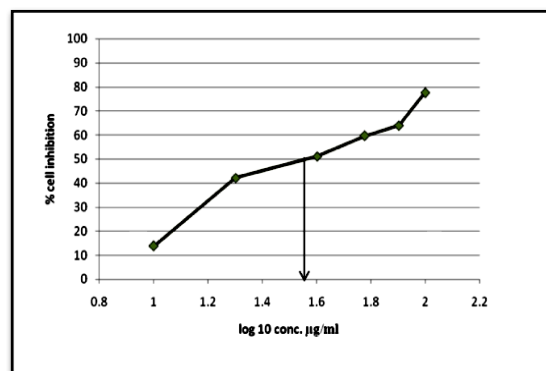
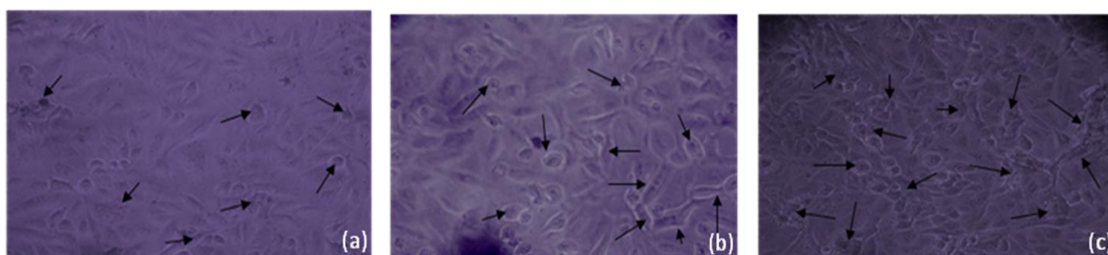


Figure 3: Graphical Representation of Concentration Dependent Cell Inhibition of Ethyl Acetate Extract of *Albizia Amara*

decrease in the number of viable cells. The concentration dependent cytotoxicity of ethyl acetate extract in MCF-7 cell line is depicted in the (Figure 4).



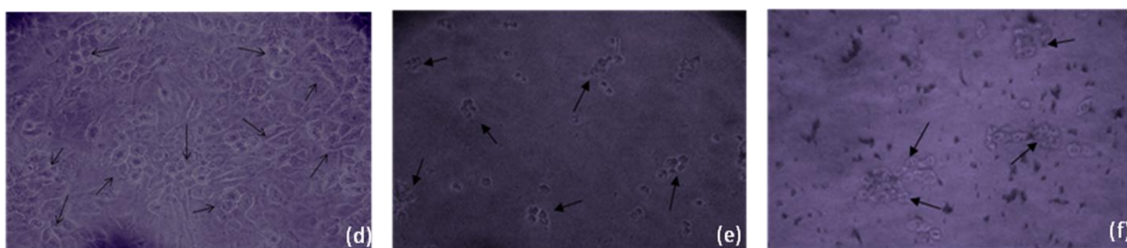


Figure 4: Cytotoxic Action of Different Concentration of Ethyl Acetate Extract of *Albizia Amara* on MCF-7 Cell Line. (A) 10 μ g/ML, (B) 20 μ g/ML, (C) 40 μ g/ML, (D) 60 μ g/ML, (E) 80 μ g/ML, (F) 100 μ g/ML.

Apoptosis has been reported as one of the most fundamental biological process in eukaryote that plays a key role in both physiological and patho physiological conditions. The apoptosis is characterized by distinct morphology. During the process of apoptosis the cell shrinks, detaches from the neighboring cells followed by chromatin condensation and nuclear fragmentation into multiple chromatin bodies, leading to

formation of apoptotic bodies which are phagocytosed by neighboring cells. These morphology changes are observed using light and fluorescent microscope [12]. After the cells treated with ethyl acetate extract of *Albizia amara*, cell shrinkage, membrane blebbing and disorganized cell structures were observed under inverted light microscope but not in untreated cells. [Figures 5 (a) & (b)]

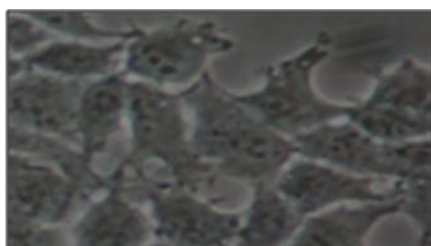


Figure: 5 (A) MCF-7 Control Cells without Any Treatment

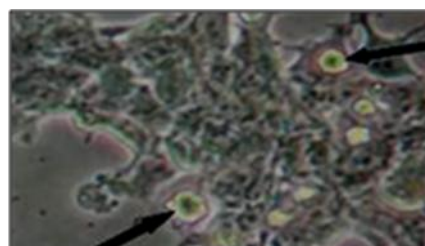


Figure: 5 (B) Breast Cancer Cells Treated with 50 Concentration of Ethyl Acetate Extract of *Albizia Amara*. Appearance of Membrane Blebbs, Decreased Number of Cells And Disorganised Cell Structure Signified That Apoptosis Had occurred

Further study was done to observe the changes at nuclear level such as chromatin condensation and nuclear fragmentation under fluorescent microscopy after the cells stained with acridine orange (AO) and ethidium bromide (EtBr) dyes. Acridine orange (AO) is a membrane permeable cationic dye that binds to nucleic acids of viable cells and causes a green fluorescence. Ethidium bromide (EtB) is impermeable to intact membranes but readily penetrates the membranes of nonviable cells and binds to DNA or RNA, causing orange fluorescence. The untreated cells showed number of viable cells whereas treated cells showed fragmented nuclei, membrane blebbing and cell shrinkage [Figures 6 (a) & (b)]

One of the commonly used techniques for confirmation of apoptosis is identification of DNA ladders which occurred due to activation of nuclease during apoptosis that cuts the DNA into oligonucleosome fragments [12]. This unique ladder composed of nucleotide fragments at an interval of 180-200 base pairs can be visualized by DNA agarose gel electrophoresis. In the present study, DNA ladders appeared in both standard 5 FU and ethyl acetate extract treated cells but not in control cells (Figure7) which proves that the apoptosis induced by ethyl acetate extract of *Albizia amara* was by fragmenting the DNA.

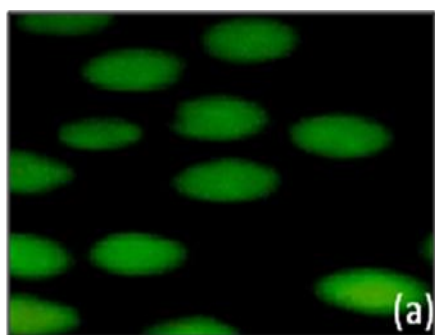


Figure 6: (A) MCF-7 Control Cells Without Any Treatment. Acridine Orange Was Taken By Live Cells and Fluoresced As Green Colour

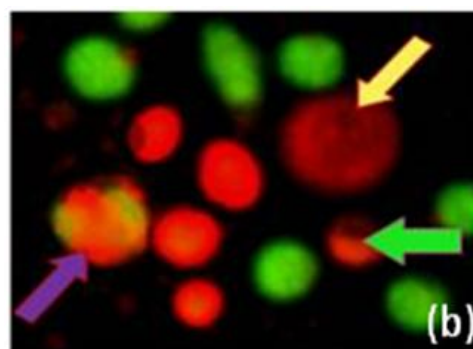


Figure 6: (B) Breast Cancer Cells Treated With I_{c50} Concentration Of Ethyl Acetate Extract Of *Albizia Amara*. Ethidium Bromide Was Taken By Dead Cells And Fluoresced As Orange Colour. Membrane Blebbing (Yellow Arrow), Nuclei Fragmentation (Purple Arrow) And Cell Shrinkage (Green Arrow) were Observed

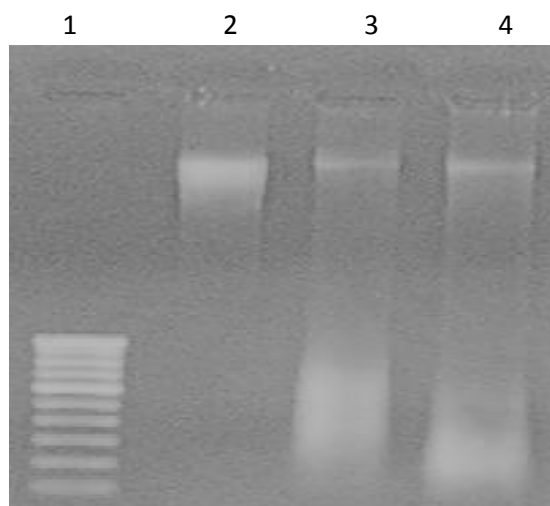


Figure 7: Fragmentation of DNA, Lane 1:100 BP DNA Marker; Lane 2: MCF-7 Cells Untreated (Control); Lane 3: MCF-7 Treated with Standard 5-Fluorouracil; Lane 4: MCF-7 Treated with Ethyl Acetate Extract of *Albizia Amara*

The results obtained from Real time PCR for the genes Bcl-2, p53, TNF- α , IL4 and IL6 are presented in Figure 8. The expression of p53 was found to be up regulated in test extract (RQ-4.43) treated breast cancer cells when compared with control (RQ-2.88), but slightly lesser than 5-FU (RQ-6.43). Under normal growth conditions, p53 is a short-lived protein and is expressed at relatively low basal levels within the cell. In response to various cellular stresses, including exposure to DNA-damaging agents, UV and irradiation, hypoxia, and nucleotide depletion, p53 is rapidly induced and functions as a transcriptional activator. In

response to genotoxic stress as observed after treatment with anticancer agents, p53 can act to arrest cell cycle progression and, in so doing, help to preserve the integrity of the cellular genome or may directly activate the process of programmed cell death.

The expression of Bcl-2 was found to be down regulated in test treated cells (RQ-2.58) which was slightly lowered than 5-FU (RQ- 2.89) treated breast cancer cells, while up regulation was found in untreated (RQ-5.21) breast cancer cell. The obtained results agree with that obtained by Yadav et al., who reported that a large portion of these nutraceuticals show great potential

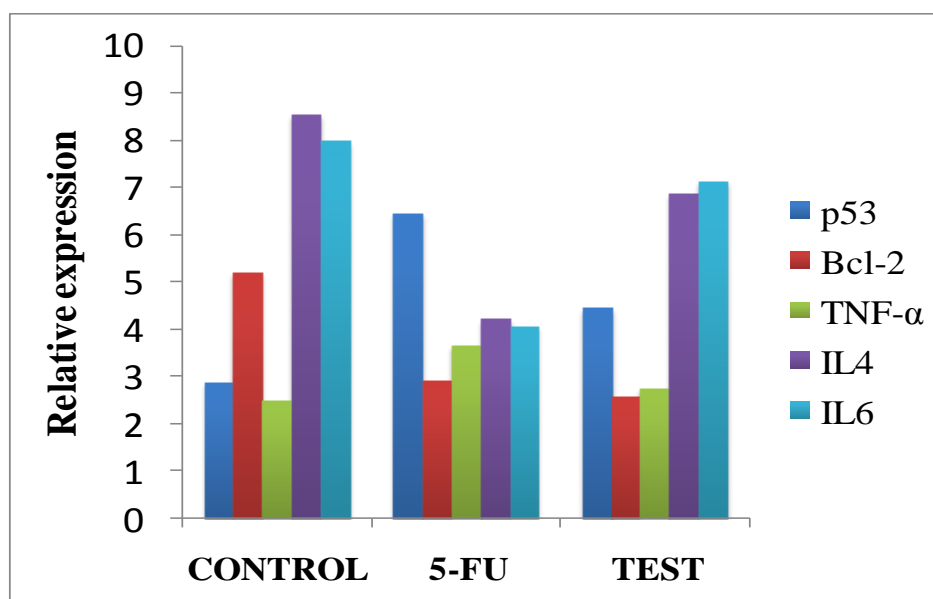


Figure 8: Comparative Expression Levels of P53, Bcl-2, TNF- α , IL-4, IL-6 Genes In The Breast Cancer Cell Line (MCF-7) Treated With 5FU, Ethyl Acetate Extract and Control

for targeting cancer through various mechanisms such as the anti-apoptotic proteins (e.g., bcl-2, bcl-xL) [15]. Over expression of anti-apoptotic members of the Bcl-2 family such as Bcl-2 and Bcl-XL have been implicated in cancer chemo resistance. The role of Bcl-2 in cancer derived cell line, Yang *et al.*, have reported that cisplatin resistance was associated with the over expression of anti-apoptotic protein Bcl-2 [16]. Doxorubicin has been shown to up regulate the expression of Bax protein and down regulate the expression of antiapoptotic Bcl-2 proteins [17]. Similar results are also observed with paclitaxel which causes a decrease in Bcl-2 expression and increase in Bax expression [18].

The expression of TNF- α was increased in both 5-FU treated breast cancer cells (RQ-3.67) and test treated cells (RQ-2.75) but down regulation was seen in control cells (RQ-2.48). TNF- α is a multifunctional cytokine playing a key role in apoptosis and cell survival as well as inflammation and immunity. TNF- α targets the tumor associated vasculature by inducing hyper permeability and destruction of vascular cell lining. This results in an immediate effect of selective accumulation of cytotoxic drugs inside the tumor and a late effect of destruction of the tumor vasculature [19].

The interleukins IL4 and IL6 expression was lower in cells treated with 5-FU compared with control cells. On the other hand, the expression levels in cells treated with test extract was lower than the control cells but higher in 5-FU treated cells. The obtained results agree with that obtained by Alshehri and Elsayed *et al.*, who reported the expression of interleukins, IL4, IL6, IL2 in MCF-7 treated with cichorium endivia, L [20]. Innate immune cells can constitute a substantial proportion of the cells within the tumor microenvironment and have been associated with tumor malignancy. Interleukins are a group of cytokines released in the body from numerous cells in response to various stimuli.

The expression of proteins such as Survivin and 150 KDa oxygen regulated protein (ORP 150) in breast cancer cell lines from both treated and untreated were determined by bioanalyzer and the results are shown in Figures 9-10. Survivin expression in control cells was 97.4 whereas in standard treated 34.6 and test treated 10.5. The expression was reduced in drug treated MCF-7 cell line. Oxygen regulated protein expression in control cells was 1.4 whereas in standard treated 0.6 and test treated 0.2. The expression was reduced in drug treated MCF-7 cell line.

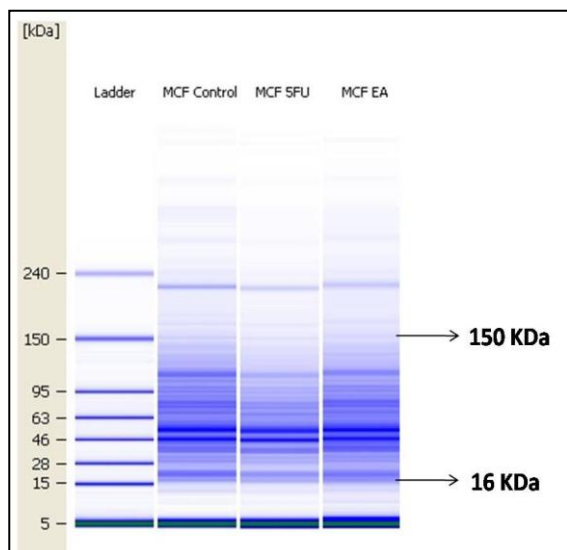


Figure 9: Gel Analysis of Protein

Survivin is a protein with molecular weight of 16 KDa plays multiple roles in malignancy including inhibition of apoptosis, stimulation of proliferation and promotion of angiogenesis, and is up regulated in malignant tissues. Moreover, many studies indicated aberrant expression of survivin is associated with poor prognosis and drug/radiation resistance [21]. Since survivin minimally expressed in normal, terminally differentiated adult tissue, targeting it for cancer treatment has less or no toxicity to normal cells [22].

Oxygen regulated protein with molecular weight of 150 KDa will over expressed in cells undergo stress or hypoxia to make the cells survive. It has wide role such as promotes angiogenesis by induce expression of vascular endothelial growth factor, VEGF, antiapoptotic, etc [23].

CONCLUSION

In conclusion, the present study indicated that *Albizia amara* treatment inhibited cell growth and proliferation of human breast cancer cells and induced apoptosis. *Albizia amara* inhibited the cell survivability by depleting the protein levels of survivin and ORP150. It also regulated the genes of Bcl-2, TNF- α and IL6 which may suggest that *Albizia amara* might have reduced chemo resistance, anti angiogenic and anticachectic action respectively.

This broad activity of *Albizia amara* can be confirmed with *in-vivo* animal models for the future perspective to develop *Albizia*

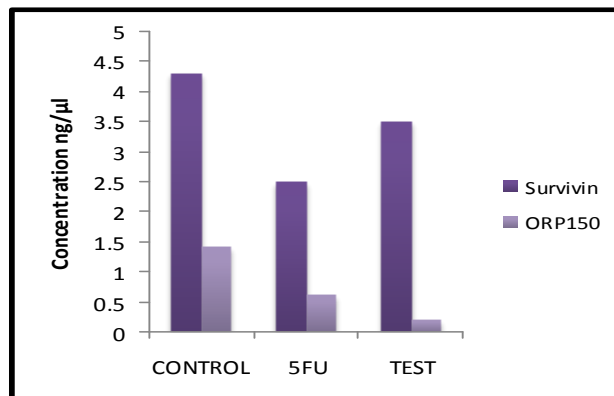


Figure 10: Comparative Expression Levels Of Survivin And Orp150 Protein In The Breast Cancer Cell (Mcf-7) Treated With 5fu, Ethyl Acetate Extract And Control Cells.

amara as an effective plant source in treatment of cancer.

ACKNOWLEDGEMENT

The authors are thanks to CSIR-NEERI (Chennai Zonal Laboratory) and CBT Anna University, Taramani, Chennai for the facilities provided during the research work.

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