

INDUCTION OF CASPASE-3 DEPENDENT APOPTOSIS, CELL CYCLE ARREST AND CYTOTOXICITY IN BREAST CANCER CELLS BY *ABRUS PRECATORIUS*

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ABSTRACT

Objective: To evaluate the leaf extract of *Abrus precatorius* as a potential therapy for breast cancer treatment.

Methods: Aqueous leaf extracts of *A. precatorius* was prepared by the process of maceration. The collected filtrate was further partitioned successively into five solvent fractions starting from water, hexane, chloroform, ethyl acetate and butanol by solvent fractionation method using separating funnel. Finally, all the five fractions were subjected to cytotoxic activity assay. The molecular mechanism underlying cytotoxicity was determined by using various approaches viz., 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, Fluorescence-activated cell sorting (FACS) analysis, Western blot analysis, casp-glow assay and Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis.

Results: Among five fractions only ethyl acetate fraction of *A. precatorius* (EAF-AP) showed significant cytotoxic activity on MDA-MB-231 cells, with an IC₅₀ value of 47.3 µg/ml. Apoptosis was confirmed by the appearance of Sub G0/G1 (apoptotic) peak by FACS analysis. Western blotting results clearly indicated cleavage of Caspase-3 and PARP. Casp-glow assay confirmed activation of caspase-3, an important mediator of apoptosis. Semi-quantitative RT-PCR analysis showed an up-regulation of pro-apoptotic genes (*p21*, *p53* and *Bax*) and down-regulation of the anti-apoptotic *Bcl-2* gene, which is an important hallmark of an apoptosis.

Conclusion: All these results indicate that EAF-AP, induced apoptosis in MDA-MB-231 cells, making *A. precatorius* a potentially good candidate for anticancer drug development.

Keywords: *Abrus precatorius* L., Cytotoxicity, Breast cancer cells, Apoptosis

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INTRODUCTION

Cancer is a multifaceted devastating disease that arises as a result of a multistep process called carcinogenesis affecting millions of people per year and poses both economic and psychological challenges [1-3]. It is a major public health problem that continues to be a second leading cause of death in humans all over the world. It has been reported around 14.1 million people were detected with cancer and 8.2 million people died from cancer [4]. Precisely, by 2025 nearly 80% of an increase in the number of all cancer deaths will occur in developing regions of the world and reach close to 25 million over the next two decades [5]. Among cancer, breast cancer (malignant breast neoplasm) is the most frequently diagnosed disease and second leading cause of cancer-related deaths in women [6]. Worldwide breast cancer mortality among females was 23 % (1.38 million) of the total new cancer cases and 14% (458,400) of the total cancer deceases recorded in 2008 [7].

Despite significant advances toward targeted therapy and screening techniques, breast cancer cells subsequently survive and gain resistance to the treatment [8]. Thus, identification and characterization of novel agents that are relatively safe and can suppress the growth of metastatic human breast cancers is highly desirable. In recent years, there has been growing interest in alternative therapies and therapeutic use of natural products; especially those derived from plants [9]. Many chemotherapeutic agents currently in clinical use have originated from plants or are analogs of plant-derived compounds [10]. Over 60% of the current anticancer drugs have their origin in one way or another from natural sources [11, 12]. Medicinal plant-derived compounds have played an important role in the development of several clinically useful anticancer agents. Anticancer properties of these compounds include regulation of cancer-associated gene expression, inhibition of enzyme activity, induction of apoptosis or cell cycle arrest [14].

Abrus precatorius which is commonly known as Indian liquorice, rosary pea, jequirity bean belongs to the family Leguminosae (Fabaceae). Its seeds, roots, vines and leaves have been used as a folk medicine since ancient times [15]. The leaf is commonly chewed or sucked to obtain its sweet taste and is also used traditionally for the treatment of various health issues [16, 17]. Many previous studies have shown that *A. precatorius* has several biologically active molecules that show effective cytotoxic activity against various cancer cell lines [18-20]. So far the emphasis has been given to the seed extracts; whereas very few studies have been carried out to evaluate the anticancer potential of leaf extracts. A thorough scientific screening of *A. precatorius* for its anticancer properties has not been reported. Hence in the present study, the leaves of *A. precatorius* were fractionated into various solvent systems, in order to isolate the maximum number of cytotoxic compounds. Therefore, efforts were made for isolating and identifying the key anticancer molecules present in *A. precatorius* leaves and to further confirm an exact molecular mechanism behind the cytotoxic and apoptotic effect of *A. precatorius* on MDA-MB-231 breast cancer cells.

MATERIALS AND METHODS

Reagents and chemicals

MTT reagent (Sigma, St. Louis, MO, USA), Propidium Iodide (Invitrogen, New York, NY, USA), Caspase-3 and PARP antibody, Caspase-Glow assay Kit (Promega Corporation, Madison WI, USA), RNase (Sigma, St. Louis, MO, USA), proteinase K (Sigma, St. Louis, MO, USA), ethidium bromide (Invitrogen, New York, USA), Reverse Transcription kit (Applied Biosystems, Inc., California, San Francisco), protease inhibitor cocktail (Calbiochem), polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica MA, USA), HRP-conjugated secondary antibodies (Sigma, St. Louis, MO, USA), β-actin (Sigma, St. Louis, MO, USA).

Plant material

Fresh, disease-free leaves of *A. precatorius* were collected from Danvantrivanna, Bangalore University campus, Bangalore, Karnataka, India. The plant material was identified and authenticated by an expert taxonomist. The collected plant material was brought to the laboratory, washed thoroughly under running tap water in order to remove dirt, germs and other contaminants, shade dried then powdered and used for extraction. An authenticated voucher specimen of the plant (BU/MKS-MDNBL/AP-1) was deposited in the herbarium of Molecular Diagnostics and Nanobiotechnology Laboratories, Department of Microbiology and Biotechnology, Bangalore University, Bangalore for future reference.

Preparation of extract and solvent fractionation

Aqueous leaf extracts of *A. precatorius* was prepared by the process of maceration. Dried plant sample was suspended in double distilled water at the rate of 10 gms in 100 ml, sonicated for 15 min and mixed with a magnetic stirrer for overnight. The extract was filtered through two layered cheesecloth. The collected filtrate was further partitioned successively into five solvent fractions by separating funnel starting from water, hexane, chloroform, ethyl acetate and butanol (21). The partitioning scheme was shown in (fig. 1). Finally, all the five fractions were dried under reduced pressure, dissolved in DMSO and subjected to cytotoxic activity assay.

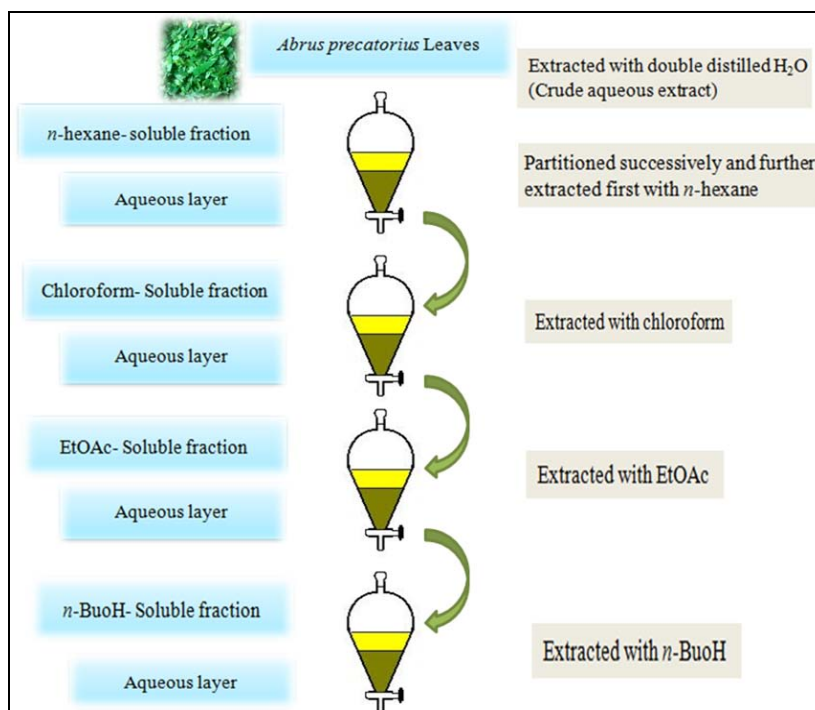


Fig. 1: Partitioning scheme of water, hexane, chloroform, ethyl acetate and butanol fractions obtained from the *A. precatorius* leaf extract

Culturing of MDA-MB-231 cells

Human breast cancer cell line MDA-MB-231 were obtained from American Type Culture Collection (ATCC), which was maintained in Dulbecco's Modified Eagle's medium (DMEM) with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C. Cells were treated with the extract once the culture would reach around 80% confluence.

MTT (3, 4, 5-dimethylthiazol-2-yl)-2-5-diphenyl tetrazolium bromide) assay

MTT method was used to evaluate the cytotoxic activity of the EAF-AP [22]. MTT assay was extensively used for quantifying cell death that was based on the capacity of mitochondrial enzymes of viable cells to reduce the yellow soluble salt MTT to purple-blue insoluble formazan precipitate which was quantified spectrophotometrically [23]. After 24 h cells were treated with different concentrations of EAF-AP for the specified time interval. 20µl of MTT (5 mg/ml) reagent was added to each well and then incubated for 3 h at 37 °C in CO₂ incubator. After 3 h medium was removed and 200µL of DMSO to solubilize formazan was added and mixed. Absorbance was recorded at 595 nm with a multiwell plate reader.

Cell cycle analysis

Cell cycle analysis was performed by propidium iodide (PI) based measurements of DNA content of the cells by flow cytometry. Cells were plated in a six-well plate at the rate of 4 X 10⁵ cells per ml and

incubated for 24 h. Cells were treated with EAF-AP for another 24 h at its IC₅₀ value of 47.3 µg/ml. The treated cells were trypsinized, washed with DPBS and fixed with 70% cold methanol and the mixture was kept in -20 °C overnight. The cells were centrifuged for 5 min at 2000 rpm at 4 °C, the pellet was treated with 2 mg/ml RNase-A at 37 °C and stained with 50 µg/ml propidium iodide. The sample was then subjected to FACS analysis and the percentage of cells in each stage of the cell cycle was determined using Cell-Quest software (Becton Dickinson, CA, USA).

Western blot analysis

The cells were seeded in a 6 well plate and incubated for 24 h. The cells were treated with EAF-AP at its IC₅₀ concentration (42.7µg/ml). For immunoblot analysis, total cell lysates were collected in lysis buffer (50 mmol HEPES-KOH, pH 7.5, 1% Triton X-100, 150 mmol NaCl) and protease inhibitor cocktail. The extracts were centrifuged at 12,000 rpm for 10 min and then the clear supernatant was collected. The protein content was determined by Bradford method. Proteins (100 µg) were resolved on 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel and electrotransferred to polyvinylidene fluoride (PVDF) membrane (Immunobilon-P, 0.45 µm). The membranes were blocked with 5% (w/v) non-fat dry milk and the membranes were incubated with specific antibodies to PARP (1:1000 dilution) and Caspase-3 (1:2000 dilution) overnight at 4 °C. Blots were then washed with TBST and incubated with HRP-conjugated secondary antibodies. Detection of proteins was then performed by using femto-lucent substrate and X-ray film. β-actin was used as an internal control.

Caspase-3 activity assay

The cells were placed in a six-well plate and then treated with EAF-AP for 24 h at 47.3 $\mu\text{g/ml}$ concentration. After treatment individual cells were trypsinized to make a single cell suspension and neutralized with spend media. Fluorescein isothiocyanate (FITC) conjugated (Z-VAD-FMK) (Promega, Madison, USA) was diluted in the ratio 0.5 μl in 250 μl of the complete medium. Cells without FITC were kept as control. Both treated and control cells were incubated for 90 min in a CO_2 incubator with intermittent mixing. After the incubation period, the cells were centrifuged at 3000 rpm for 5 min. The pellet was then re-suspended in 0.3 ml of washing buffer. The cells were kept on ice and taken for FACS analysis.

Gene expression analysis by semi-quantitative RT-PCR

Total RNA was extracted from the cells by Trizol[®] reagent according to the manufacturer's protocol. After extraction, total RNA was quantified by measuring absorbance using a nano-drop. Before performing reverse transcription, RNA was treated with 10^{-5} U deoxyribonuclease I (DNase-I) for 30 min at 37 °C. DNase-I treated RNA was reverse transcribed by ABI Reverse Transcription kit following the manufacturer's protocol. The cDNA equivalent to 20 ng of total RNA was used for PCR reactions. For expression analysis, gene-specific primers as listed in (table 1) were used. *RPL35a* (a ribosomal protein coding RNA) was used as an internal control and PCR products were analyzed on 1% agarose gels.

Table 1: Primer sequences of *p53*, *p21*, *BAX*, *Bcl-2* and *Rpl35a* genes

| S. No. | Gene | Amplicon size | Primer sequence |
|--------|---------------|---------------|---|
| 1. | <i>p53</i> | 146bps | RT-FORWARD:-GCTCGACGCTAGGATCTGAC RT-REVERSE:-CAGGTAGCTGCTGGGCTC |
| 2. | <i>p21</i> | 140 bps | RT-FORWARD:-GCCATTAGCCATCACAGT |
| 3. | <i>BAX</i> | 108 bp | RT-REVERSE:-ACCGAGGCACTCAGAGGAG |
| 4. | <i>Bcl-2</i> | 141 bp | RT-FORWARD:-GGAGGAAGTCCAATGTCCAG |
| 5. | <i>Rpl35a</i> | 141bps | RT-REVERSE:-TCTGACGCAACTTCAACTG RT-FORWARD:-CCAGAGGAGGAGGTAGGGAC RT-REVERSE:-TGATGTGAGTCTGGGTGAG RT-FORWARD:-CTGGTTTTGTTGGTTTGCC RT-REVERSE:-AAGGGAGCACACAGCTCTTC |

Statistical analysis

The data represent the mean \pm SD of three independent experiments each in a triplicate. The significance between control and treated groups was analysed by student's t-test and *p* values less than 0.05 were taken as significant by GraphPad Prism 5.0 software (GraphPad Software Inc., CA, USA).

RESULTS

MTT assay for cytotoxicity

Among the five fractions of *A. precatorius*, only ethyl acetate fraction of *A. precatorius* (EAF-AP) shows significant cytotoxic activity in a dose-dependent manner on MDA-MB-231 cells (fig. 2a). Other four fractions did not show much cytotoxic activity, suggesting that the active cytotoxic

compounds of *A. precatorius* leaf extract are fractionated in an ethyl acetate fraction only. Treatment of MDA-MB-231 cells for 48 h with an EAF-AP showed a very significant and higher cytotoxic activity with an IC_{50} value of 47.3 $\mu\text{g/ml}$ (fig. 2b). These results indicated that EAF-AP has a potent cytotoxic activity on MDA-MB-231 breast cancer cells.

Morphological studies

Microscopic examination of MDA-MB-231 cells treated with EAF-AP showed significant morphological changes such as shrinkage and detachment from the surface (fig. 3). Owing to the loss of cells, differences in the cell number can also be visualized in a culture dish, when it was treated with EAF-AP for 48 h in comparison to the control dish. These results show that *A. precatorius* has a potent cytotoxic activity on breast cancer cells.

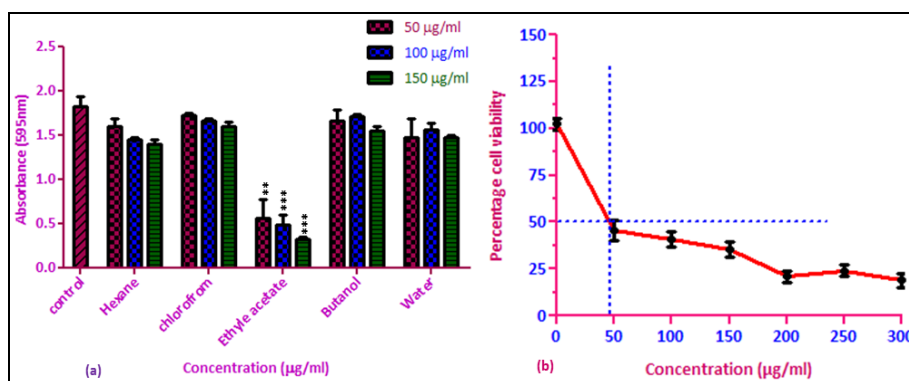


Fig. 2: Cytotoxic activity of various solvent fractions on MDA-MB-231 cells (a) ethyl acetate fraction of *A. precatorius* (EAF-AP) showed significant cytotoxic activity at concentration (50-150 $\mu\text{g/ml}$) after 48 h of treatment (b) Dose dependent cytotoxic activity of EAF-AP on MDA-MB-231 cells was observed, with an IC_{50} value of 47.3 $\mu\text{g/ml}$. Values are mean \pm SEM of at least three independent experiments, each in triplicates. Values are mean \pm SEM of at least three independent experiments, each in triplicates. Significant cytotoxic activity (*p* value \leq 0.05) between the groups

Cell cycle analysis

As EAF-AP has a potent cytotoxic activity on MDA-MB-231 cells, it was necessary to investigate the nature of cell death in these cells. FACS analysis is conventionally performed to assess whether a compound induces cell cycle arrest or apoptosis in a cell. As shown in fig. 4, FACS analysis indicates that treatment of MDA-MB-231 cells

with EAF-AP induces apoptosis in these cells. A significant increase (from 11.8% to 53.46%) in sub G0/G1 population of cells is observed upon treatment with EAF-AP in comparison to control cells (table 2). Further, not much change in the cell population of S-phase or G2 was observed, suggesting that EAF-AP may not have a cytostatic activity. These results show that EAF-AP has a pro-apoptotic activity on MDA-MB-231 cells.

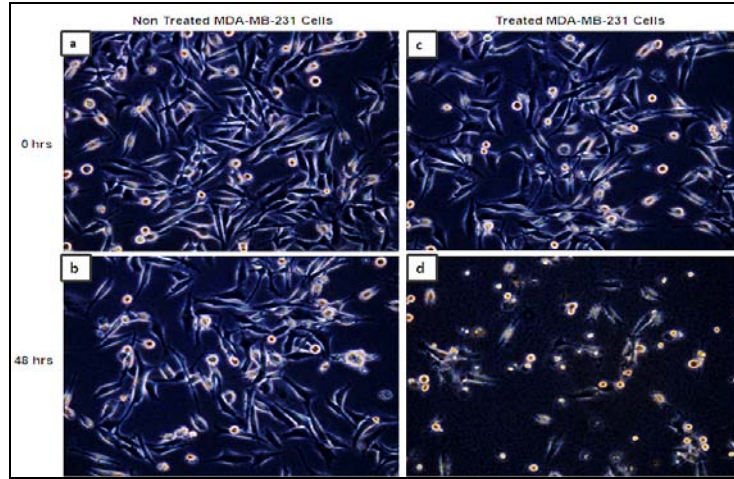


Fig. 3: Microscopic view of human breast cancer MDA-MB-231 cells (10X magnification) (a) Non-treated control cells at 0 h and (b) 48 h; (c) Treated cells at 0 h and (d) 48 h at with IC₅₀ concentration (47.3 µg/ml)

Table 2: Percentage of cells in the different stages of the cell cycle after treatment with *A. precatorius* extract

| Cell cycle stage | MDA-MB-231 Cells (%) | |
|----------------------------|----------------------|----------------|
| | Non treated | EAF-AP treated |
| G1 (M1) | 61.53±5 | 23.91±3 |
| S (M2) | 10.48±2 | 13.26±1 |
| G ₂ M (M4) | 4.27±2 | 4.47±2 |
| Apoptotic (Sub-G0-G1) (M1) | 11.8±2 | 53.46±8 |

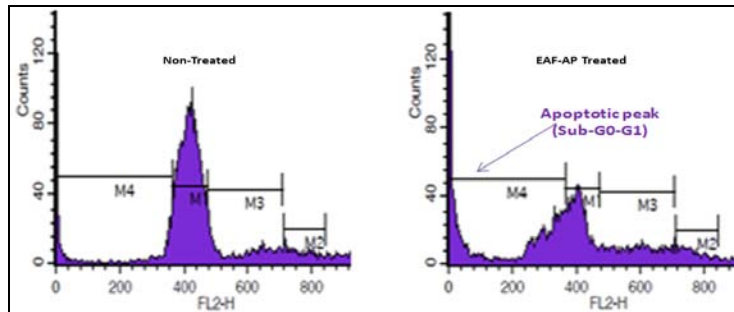


Fig. 4: Cell cycle phase analysis by flow cytometry in MDA-MB-231 cells treated with *A. precatorius* extract. Histograms show a number of cells (vertical axis) vs. DNA content (horizontal axis). A notable increase in the sub-G0/G1 population can be seen in the treated sample

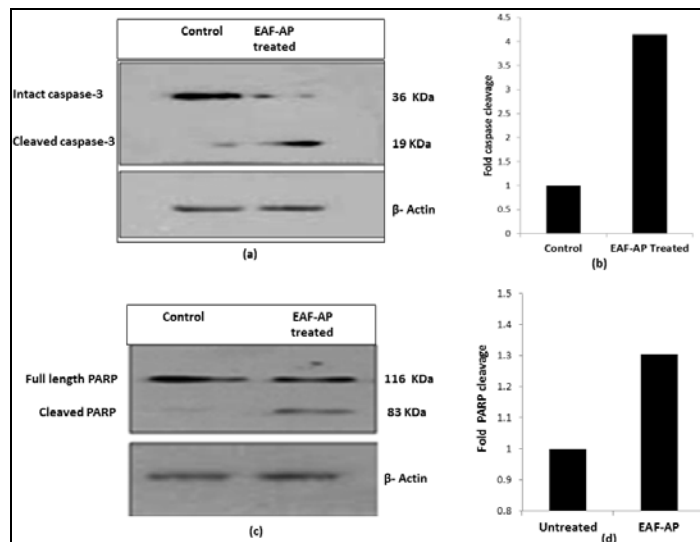


Fig. 5: Cleavage of caspase-3 and inactivation of PARP in MDA-MB-231 treated cells (a) Western blot and (b) densitometry of western blot bands. (c) PARP cleavage and (d) relative fold change of PARP cleavage

Western blot analysis

When a cell undergoes apoptosis, caspase-3 gets cleaved that leads to its activation. Western blotting analysis was performed to analyze cleavage and the activation of caspase-3 upon EAF-AP treatment of MDA-MB-231 cells. Treatment of MDA-MB-231 cells with EAF-AP results in proteolytic cleavage of caspase-3 as revealed by the appearance of 19 kDa cleaved fragment of caspase-3 (fig. 5a). The densitometry analysis of the western blot indicated 4 fold increase in cleavage of caspase-3 upon treatment with EAF-AP (fig. 5b). Activation of caspases leads to degradation of several target proteins and enzymes involved in cell cycle progression and DNA repair. A well-known target of caspases is PARP; an enzyme involved in repairing the DNA damage in a cell. Western blot analysis shows that EAF-AP treatment of MDA-MB-231 cells leads to cleavage and hence inactivation

of PARP. This was revealed by the appearance of 83 kDa cleaved fragment of PARP in the western blot (fig. 5c). The densitometric analysis indicated around 1.33 fold increase in the cleavage of PARP upon treatment with EAF-AP in MDA-MB-231 cells (fig. 5d).

Caspase-3 activity assay

To confirm the activation of Caspase-3, fluorescence-based caspase-glow assay was also performed. Flow cytometric analysis shows that the treatment of MDA-MB-231 cells with EAF-AP results in a significant shift in the peak towards the right, suggesting activation of caspase-3 (fig. 6.). The fluorescence intensity shift was recorded from 1.76% (control cells) to 54.27% (treated cells). The result shows that EAF-AP treatment leads to cleavage and activation of caspase-3 in MDA-MB-231 cells.

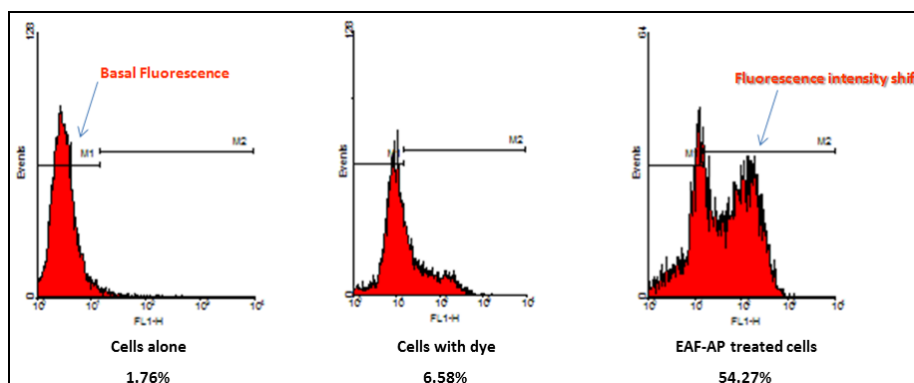


Fig. 6: Fluorescence-based Casp-glow assay shows activation of caspase-3 in MDA-MB-231. In comparison to control cells, a shift towards the right in the peak is observed in cells treated with *A. precatarius* extract

Gene expression analysis by semi-quantitative RT-PCR

Semi-quantitative RT-PCR analysis revealed a significant up-regulation of pro-apoptotic genes *p53*, *p21* and *Bax* when treated with EAF-AP for 24 h (fig. 7a). In addition, down-regulation of anti-apoptotic gene *Bcl-2* was also observed. The graphs below shows the quantitation of the change in expression levels of the respective

genes (fig. 7b). Treatment of MDA-MB-231 cells with EAF-AP leads to approximately 3.5 fold up-regulation in *p53*, 4.7 fold up-regulation in *p21* and 2.2 fold up-regulation in *Bax* gene expression level. Likewise, 1.61 fold down-regulation of anti-apoptotic gene *Bcl-2* was observed in EAF-AP treated MDA-MB-231 cells. These results indicated that EAF-AP leads to a change in the expression of various cell cycle regulators and hence affects the survival of these cells.

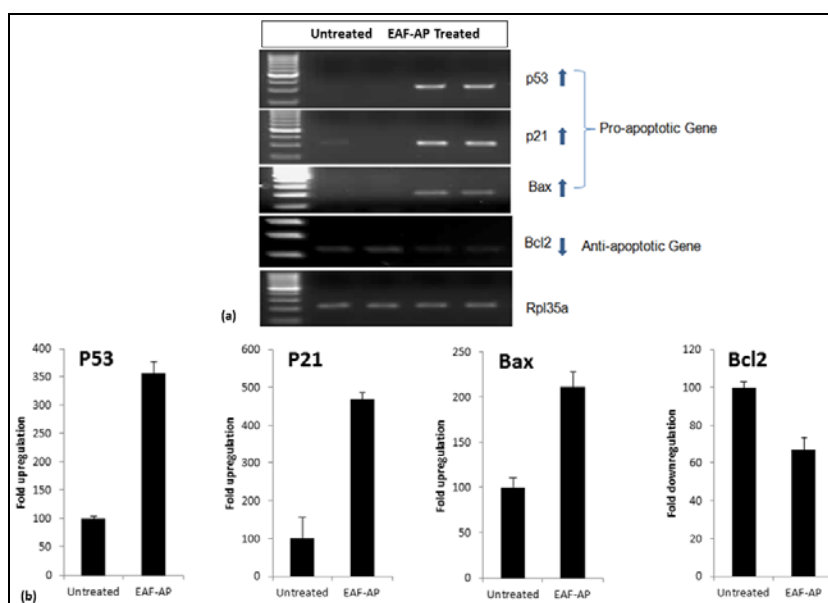


Fig. 7: Expression analysis of various pro-apoptotic and anti-apoptotic genes by PCR in treated and untreated MDA-MB-231 cells with *A. precatarius* extract (a) Lane 1=100 bp marker; Lane 2, 3= cells without treatment; Lane 4-5= cells treated with *A. precatarius* extract at (47.3 µg/ml) for 24 h. *Rpl35a* (a ribosomal protein coding RNA) as an internal control (b) Respective graphs show quantitation of the RT PCR band

DISCUSSION

Medicinal plants are candidates for chemoprevention of cancer because they possess chemopreventive agents with inhibitory effects on the initiation, promotion and progression of carcinogenesis. For these reasons, plants have always been an important source of drugs and are still playing a rapidly increasing role in the lead-finding of drug candidates for the development of chemotherapeutic agents [24; 25]. In order to identify and characterize the active cytotoxic compounds in leaf extract of *A. precatorius*, the aqueous fraction was further fractionated into five different solvent fractions. In the present investigation, for the first time, it was demonstrated that EAF-AP strongly inhibits the growth of MDA-MB-231 cells, with an IC₅₀ value of 47.3µg/ml. More than 90% of cell death was achieved at higher concentrations after 48 h of treatment. Earlier studies had shown that various plant extracts and phytochemicals isolated from medicinal plants have shown significant cytotoxic and apoptotic activity on different cancer cell lines [26]. Treated cells showed prominent morphological changes due to a dose-dependent inhibition of cells proliferation. Also to understand the exact molecular mechanism behind cell death, various apoptotic assays were performed. Most of the cytotoxic anticancer drugs in current use have been shown to induce apoptosis in susceptible cells [27; 28]. To gain insights into the mechanism by which cell reduction was achieved, the effect on cell cycle distribution by FACS analysis was done. Flow cytometric analysis showed that *A. precatorius* has a pro-apoptotic activity on MDA-MB-231 cells. Cell cycle analysis by flow cytometer exhibited a sub-G₀/G₁ peak in EAF-AP treated MDA-MB-231 cells. As compared to control, treated cells showed an accumulation of cells in G₀/G₁ phase and thus inhibited the transition of cells into S phase, followed by increases in the proportion of cells in the sub-G₁ phase. This increase of cells in sub-G₁ was taken to reflect the induction of apoptosis. The agents that alter the cell cycle have been of particular interest since cell cycle regulation was the basic mechanism underlying cell fate, i.e. proliferation, differentiation or apoptosis [29]. These results are in accordance with the previous results [30, 31].

Further, activation of caspase-3 was involved in the induction of apoptosis upon EAF-AP treatment of MDA-MB-231 cells. Caspase are molecules that play a central role in the process of apoptosis. Caspases get activated upon a signal, which in turn act on their target molecules finally leading to cell death. Caspase-3 is a very important key of apoptosis and has been recognized as crucial executioner caspase responsible for the activation and cleavage of over 100 substrates that finally will lead to DNA fragmentation and apoptosis. Caspase-3 cleaves DNA repair enzyme, poly-ADP-ribose polymerase (PARP) that finally will lead to DNA fragmentation and apoptosis [34]. Both caspase-3 and PARP are considered as significant markers of cells undergoing apoptosis. In this study, both of these markers showed positive alteration in the EAF-AP treated cells. Western blotting results clearly indicated cleavage of caspase-3 and PARP, which are the main indications of activation of caspase-3 and deactivation of PARP important mediators of apoptosis. Activation of caspase-3 was further confirmed by casp-glow assay by flow cytometry. Consistent with this result, various previous studies indicated phytochemicals induced apoptosis in cancerous cells through the activation of caspase-3 and PARP cleavage [35].

Cell cycle and apoptosis involve several regulators and mediators. Under normal conditions, cells divide and express these regulators and mediators at a steady state level. However, upon various stress conditions, cell cycle progression will be halted and various inhibitors of cell cycle progression are induced. If the damage is not repaired, then the cells will undergo apoptosis under normal conditions [37]. The *p53* tumour-suppressor is one such critical regulator of cellular response to DNA damage and apoptosis. EAF-AP treatment leads to the up-regulation of *p53* in addition to other cell cycle regulators *p21* and *Bax*. The balance between apoptotic and anti-apoptotic genes are known to be important in determining whether cells die or survive [38]. The results also show a down-regulation of anti-apoptotic gene *Bcl-2*, upon treatment of MDA-MB-231 cells with EAF-AP. All these studies suggest that EAF-AP treatment leads to the induction of apoptosis in MDA-MB-231 cells.

Some of the dietary agents that are also known to modulate apoptotic genes include curcumin, resveratrol, EGCG, indole-3-carbinol and silibinin [39]. EAF-AP is known to consist of cytotoxic compounds that are very effective against metastatic breast cancer MDA-MB-231 cells.

CONCLUSION

All these results suggest that EAF-AP acts as an effective pharmacological agent in treating breast cancer. Further, this data will help in the isolation, identification and characterization of the specific anticancer bioactive principle present in EAF-AP that are mainly responsible for the activity. In conclusion, it can be inferred that *A. precatorius* possess a profound anticancer activity and hence provide an alternative option for breast cancer treatment.

AUTHORS CONTRIBUTIONS

Dr Mohammad Shafi Sofi is the main author of the whole manuscript, all the experiment and statistical analysis were done by Dr Mohammad Shafi Sofi.

CONFLICTS OF INTERESTS

Declared none

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