Evaluation Of Pro-Apoptotic Effect of Aegle Marmelos Fruit Extract on Breast Cancer Cell Lines

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ABSTRACT

Background: One of the most significant therapeutic plants in Ayurveda is bael. Bael is regarded as a healing tree that fortifies the body and is known as a symbol of fertility. It is said that a sweet drink made from the pulp of ripe fruit can treat bacillary dysentery. Unripe Bael fruits are said to be effective in treating stomach aches, diarrhoea, and dysentery with periods of constipation. The roots are also a key component in the Ayurvedic medicine Dashamula, which is used as a treatment for colitis, dysentery, diarrhoea, flatulence, and fever. The leaves are said to lessen diarrhoea, dysentery, bleeding piles, dropsy (edoema), and digestive problems.

Breast cancer is not an infectious or contagious illness. Only gender (female) and age, the only known risk factors for breast cancer in women, account for almost half of all cases (over 40 years). Ageing, obesity, drinking too much alcohol, radiation exposure history, family history of breast cancer, reproductive history (such as age at first pregnancy and age at first menstruation), smoking, and postmenopausal hormone therapy are some variables that raise the risk of breast cancer.

Aim: The aim of the study is to evaluate the pro-apoptotic effect of Aegle marmelos fruit extract on breast cancer cells. **Materials and methods:** Cell line maintenance, Preparation of the Herbal Extract, Cell morphological analysis-inverted microscope, Cell migration analyzed by scratch wound healing assay, Statistical analysis-One way ANOVA followed by students-test using SPSS. **Results:** The chemical constituents found in the extract reveals the anti cancer properties of the extract as it suppresses the migration and proliferation of MCF-7 cells. In our study cells were treated with Aegle marmelos fruit extract 20µg/ml for 24 hours along with the control group. These results revealed that Aegle marmelos inhibited the proliferation of MCF-7 cells, changed the cytoplasmic morphology, reduced the invasion and migration ability of MCF-7 cells and exhibited pro apoptotic effects. **Conclusion:** The study concluded that the Aegle marmelos fruit extract processes pro-apoptotic effects on breast cancer cell lines.

KEYWORDS: Apoptotic effect, MCF7 cell lines, Herbal extract, Cytotoxicity.

INTRODUCTION

Cancer is a multifactorial genetic disease, causing uncontrolled division and proliferation of abnormal cells in the body and the spread of these abnormal cells into the other body tissues (1) (2). Breast cancer is the very often detected cancer among women worldwide and is also the dominant cause of death due to cancer in over 100 countries. Different areas of the breast might give rise to breast cancer. There are three basic components of a breast: connective tissue, ducts, and lobules. The glands that generate milk are called lobules (3). Milk travels through tubes called ducts to the nipple. The connective tissue, which is made up of fatty and fibrous tissue, envelops and holds everything in place. The ducts or lobules are where most breast cancers start. Blood and lymph vessels are two ways that breast cancer can travel outside of the breast. Breast cancer is said to have metastasized when it spreads to other body regions.

Breast cancer risk is higher in women who have specific gene mutations, such as a BRCA1 or BRCA2 mutation. These women also have a higher risk of developing ovarian cancer and could have a higher risk of developing other malignancies. A mutated gene linked to breast cancer increases the risk of breast cancer in men as well (4).

Patients with breast cancer can choose from a variety of treatments. Clinical trials are being conducted to test various treatments, some of which are standard (currently used treatments). A clinical trial for treatment is a scientific study aimed at finding out more about potential new treatments for cancer patients or to assist enhancing existing treatments (4,5) (5). A new treatment might replace the standard one if clinical trials reveal that it is superior to the current one. Patients might consider participating in a clinical trial. Only patients who have not begun treatment are allowed to participate in some clinical trials. Some common treatments opted by physicians are surgery, chemotherapy, radiotherapy, targeted therapy, hormone therapy, immunotherapy (6).

Our team has extensive knowledge and research experience that has translate into high quality publications (7–16). Although a number of anticancer drugs have been discovered, apart from being expensive they have some serious side effects as well. So, it is important to develop safe, effective and economical treatment of the disease (17). The use of herbal medicine for prevention and cure of various ailments has been practiced by humans since antiquity, and it was the main source of treatment before the evolution of modern allopathic, or synthetic medicine (18). Plethora of medicinal plants have attracted attention among the scientific communities for its therapeutic efficacies against a number of diseases including cancer (19–27,27–29). Aegle marmelos (common Bangla name, Bael) is a tree belongs to Rutacea family, which grows in most of the areas of Bangladesh, India, and Southeast Asia used for treatment of asthma, anemia, fractures, wounds healing, swollen joints, high blood pressure and jaundice (30).

In ancient India and other parts of South Asia, Bael was one of the most revered herbs used in ayurvedic medicine. Bael has been used as a food and medicine since

5000 B.C., and it was known to humans even while the renowned Sanskrit epic-poem Ramayana was being written (31). Bael was acknowledged as a vital component of ayurveda treatment in the renowned book Charaka Samhita, which is a thorough compendium of all the pertinent ayurvedic knowledge. The tree is fragrant, and each portion has significant therapeutic value. Ayurvedic and folk medicine systems use fruits, leaves, bark, roots, and seeds to treat a variety of illnesses(32).

Previous research has shown that administration of the ethanolic fruit extract from Aegle marmelos significantly reduced the levels of urea, uric acid, and creatinine. The observed decrease in serum levels of kidney biomarkers was suggestive of a renal protective effect of ethanolic fruit extract from Aegle marmelos. Through the regeneration of the injured area of the kidney, the phenols and flavonoids in the ethanolic fruit extract from Aegle marmelos may be to blame for the observed renal protective action (33).

This study aims to evaluate the pro-apoptotic effect of Aegle marmelos fruit extract on breast cancer cells.

MATERIALS AND METHODS-

Reagents

DMEM (Dulbecco's Modified Eagle Medium), Phosphate Buffered Saline (PBS), Trypsin-EDTA, Fetal bovine serum (FBS), were purchased from Gibco, Canada. Acridine orange (AO), ethidium bromide (EtBr), Dimethyl sulfoxide (DMSO), [3-(4,5-dimethythiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT), DAPI, AO/EtBr were purchased from Sigma Chemical Pvt Ltd, USA. All other chemicals used were extra pure of molecular grade and were purchased from SRL, India.

Cell line maintenance

Estrogen dependent (MCF-7) breast cancer cell lines were obtained from the NCCS, Pune. The cells were grown in T25 culture flasks containing DMEM supplemented with 10% FBS and 1% antibiotics. Cells were maintained at 37◦C in a humidified atmosphere containing 5% CO2. Upon reaching confluency, the cells were trypsinized and passaged.

Preparation of the Herbal Extract

Fruit powder of Aegle marmelos obtained from IMPCOPS (Chennai, India) was used for the present study. About 50g of Aegle marmelos powder was soaked in 500 mL of 95% ethanol and kept at room temperature for 3 days in a static condition. Then the solution was filtered with crude filter paper followed by whatmann paper. Fine filtrate was subjected to rota evaporation after that 3g of the material was obtained. The total ethanol extract was concentrated in a vacuum evaporate and immediately stored at 4˚C.

Cell viability (MTT) assay

The cell viability of Aegle marmelos extract treated MCF-7 cells was assessed by MTT assay. The assay is based on the reduction of soluble yellow tetrazolium salt to insoluble purple formazan crystals by metabolically active cells. MCF-7 cells were plated in 96 well plates at a concentration of 5x103 cells/well 24 hours after plating, cells were washed twice with 100µl of serum-free medium and starved by incubating the cells in serum-free medium for 3 hours at 37ºC. After starvation, cells were treated with fruit extract of different concentrations for 24 hours. At the end of

treatment, the medium from control and fruit extract treated cells were discarded and 100µl of MTT containing DMEM (0.5 mg/ml) was added to each well. The cells were then incubated for 4 hours at 37ºC in the CO2 incubator.

The MTT containing medium was then discarded and the cells were washed with 1x PBS. Then the formazan crystals formed were dissolved in dimethyl sulfoxide (100µl) and incubated in dark for an hour. Then the intensity of the color developed was assayed using a Micro ELISA plate reader at 570 nm. The number of viable cells was expressed as the percentage of control cells cultured in serum-free medium. Cell viability in the control medium without any treatment was represented as 100%. The cell viability is calculated using the formula: % cell viability = [A570 nm of treated cells/A570 nm of control cells]×100.

Morphology study

Based on MTT assay we selected the optimal doses (IC-50: 20µg/ml) for further studies. Analysis of cell morphology changes by a phase contrast microscope. 2×105 cells were seeded in 6 well plates and treated withAegle mar also fruit extract (concentration for MCF-7 cells) for 24h. At the end of the incubation period, the medium was removed and cells were washed once with a phosphate buffer saline (PBS pH 7.4). The plates were observed under a phase contrast microscope. Determination of mode of cell death by acridine orange (AO)/ethidium bromide (EtBr) dual staining

The effects of Aegle marmelos fruit extract in MCF-7 cell death were also determined by AO/EtBr dual staining as described previously (Cury-Boaventura et al.,2004). The cells were treated with Aegle marmelos fruit extract for 24 h and then the cells were harvested, washed with ice-cold PBS. The pellets were resuspended in 5 µl of acridine orange (1 mg/mL) and 5 µl of EtBr (1 mg/mL). The apoptotic changes of the stained cells were then observed by using a fluorescence microscope.

Statistical analysis

All data obtained were analyzed by One way ANOVA flowed by Students-t-test using SPSS, represented as mean ± SD for triplicates. The level of statistical significance was set at $p<0.05$

RESULTS

Effect of *Aegle marmelos* fruit extract on cell viability of osteosarcoma cell line

The assessment of the cytotoxic potential of *Aegle marmelos* fruit extract in osteosarcoma cells line was done using MTT assay. The cells were treated with different concentrations of 10, 20, 30, 50, 100 and 200 µg of *Aegle marmelos* fruit extract for 24h. *Aegle marmelos* fruit extract treatment significantly decreased the viability of MG63 cancer cells compared to control at 24 h time point (Figure-1). The percentage of cell viability reduced gradually with increase in the concentration. The 50% growth inhibition was observed at 40 µg/ml concentration. For the further experiments IC^{50} dose 20 µg/ml was considered.

The Effect of Aegle marmelos on Cell Morphology

The cell morphological analysis of Aegle marmelos fruit extract treated lung cancer cells was observed in an inverted phase contrast microscope. The MCF-7 cells were

treated with Aegle marmelos fruit extract (20 µg/ml) for 24 h, compared with the untreated cells, treated cells showed significant morphological changes, which are characteristic of apoptotic cells, such as cell shrinkage and reduced cell density were observed in the Lippia nodiflora leaf extract treated cells (Fig. 2). Cells undergoing apoptosis also displayed other types of morphological changes such as rounded up cells that shrink and lose contact with neighboring cells. Some sensitive cells were even detached from the surface of the plates.

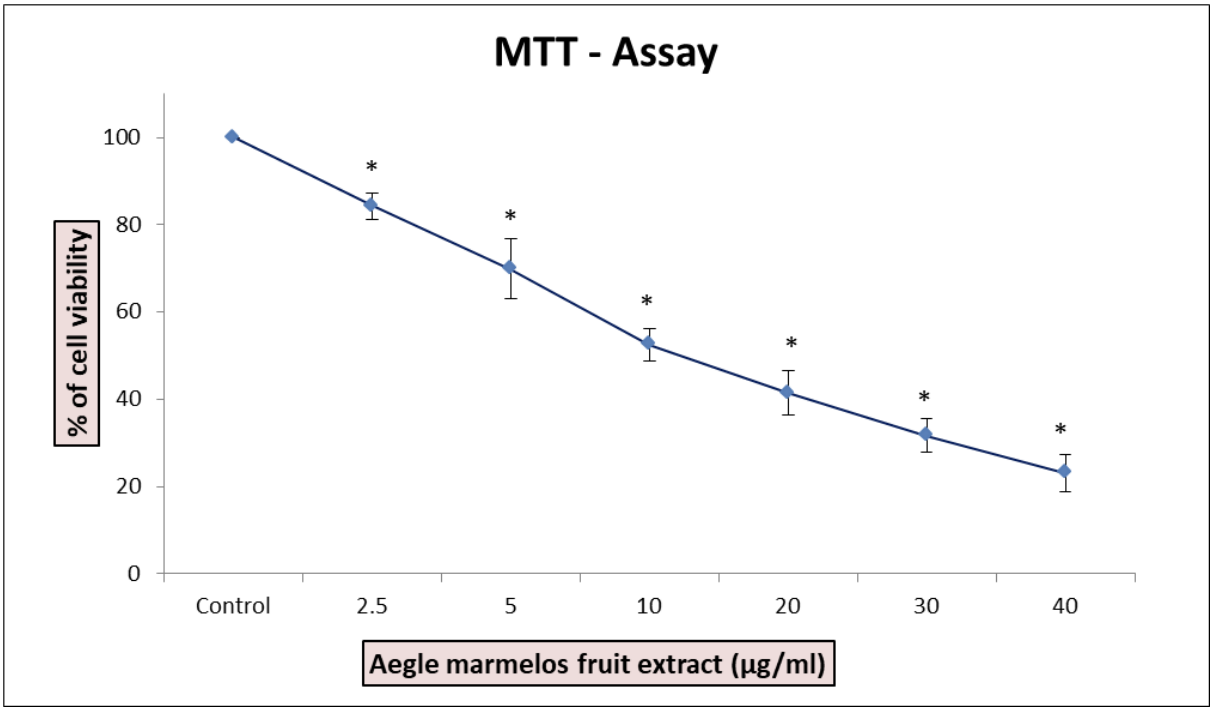

Fig 1: The cytotoxic effects of aegle marmelos on fibroblast cells. Cells were treated with aegle marmelos (10, 20, 30, 50, 100 and 200 µg) for 24 h, and cell viability was evaluated by MTT assay. Data are shown as means ± SD (n = 3). * compared with the control blank group, p< 0.001.

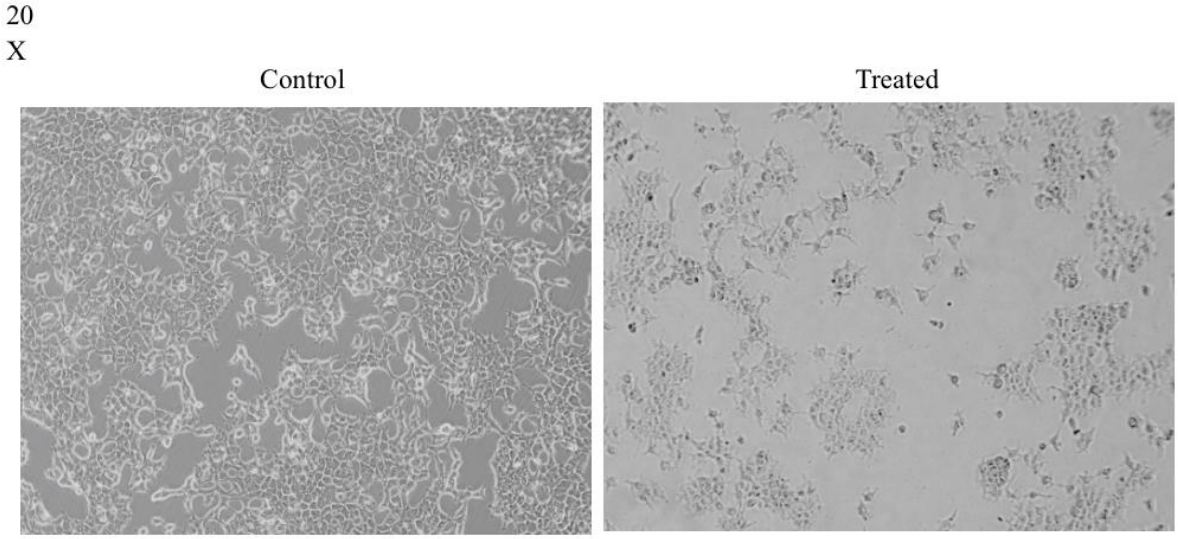

Fig 2: Cell morphological analysis by phase contrast inverted microscope. Cells were treated with aegle marmelos for 24 h along with the control group. Images were obtained using an inverted Phase contrast microscope.

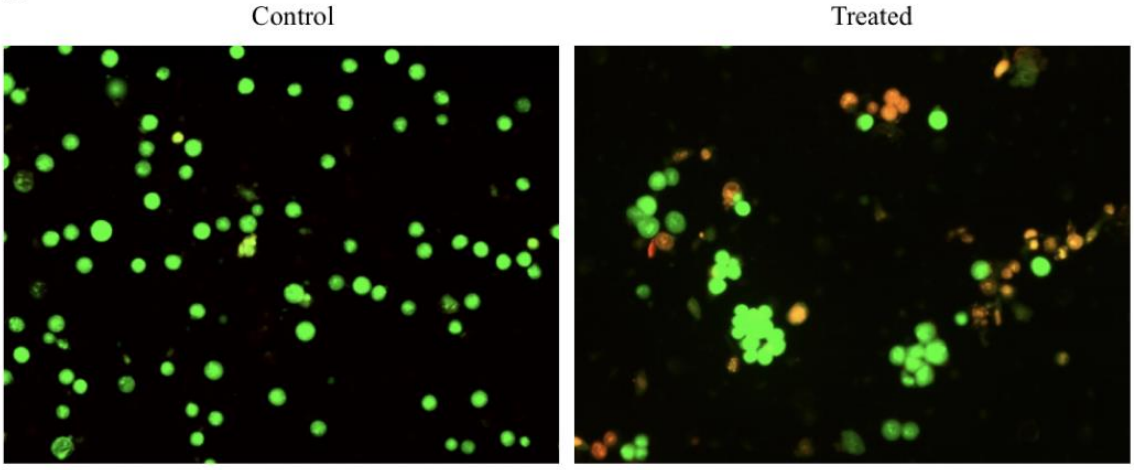

Fig 3: The induction of apoptosis in *Aegle marmelos* fruit extract-treated breast cancer cells was confirmed by Acridine orange/Ethidium bromide (AO/EtBr) dual staining. Cells were treated with aegle marmelos extract for 24 h along with the control group. Images were obtained using an inverted Flourescence Phase contrast microscope.

The nuclear morphology of apoptotic cells is assessed with AO/EtBr dual staining. *Aegle marmelos* fruit extract (20 µg/ml) was treated to the cells for 24 hours. Following treatment, the cells were stained for 20 minutes with both AO/EtBr dye and examined under fluorescence microscopy. The results revealed that AO stained both living and dead cells, but EtBr exclusively stained those that had lost membrane integrity. Green staining indicates healthy cells, yellow staining indicates early apoptotic cells, and orange staining indicates late apoptotic cells. Control cells in this study were uniformly green, but *Aegle marmelos* fruit extract-treated cells displayed yellow and red signals (Fig.3). According to these findings, *Aegle marmelos* fruit extract causes apoptosis in breast cancer cells.

DISCUSSIONS

According to a recent report by the International Agency for Cancer Research, cancer is the second leading cause of death worldwide, accounting for roughly 12.7 million new cases (56 percent of which occurred in developing nations) and 7.6 million deaths (63 percent of which occurred in less developed nations) in 2008 (34–36). According to projections, the incidence of cancer will triple by the year 2020, with developing nations experiencing a disproportionate increase in cancer diagnoses and fatalities due to a lack of resources to combat the issue (37).

Cancer may be treated with surgery, ionizing radiation, or chemotherapy, depending on the stage, tumor location, and patient's condition. However, combining 2 or all of the therapy methods is frequently used when the prognosis is poor in order to achieve effective control and palliation. Unfortunately, the use of chemotherapy,

ionizing radiation, and their combination have harmful side effects because of their lack of specificity, which causes cytotoxic effects on normal, healthy cells (37,38). Due to the high cost of these conventional treatments, many patients in developing nations prefer to employ complementary and alternative medicine to treat and manage their cancer symptoms and suffering.

One of the earliest medical systems is ayurveda, the conventional Indian medical system. Ayurveda places a strong emphasis on maintaining a healthy lifestyle, avoiding disease, and promoting disease prevention by using therapeutic techniques that will rejuvenate the body. The majority of the therapeutic and preventative Ayurvedic treatments come from plants (39–42). Numerous regularly used Ayurvedic botanicals have the potential to be utilized by humans in the future, according to preclinical research on experimental animals. One such plant, the medium-sized deciduous Aegle marmelos, has been thoroughly researched (Rutaceae family).

Recent research has also revealed that the Bael (Aegle marmelos) methanolic extract (25 and 50 mg/kg body weight) was successful in preventing the 2-acetyl aminofluorene and diethylnitrosamine induced hepatocarcinogenesis in Wistar rats. The incidence of liver tumors was reduced when Bael was co-administered compared to the carcinogen alone (without Bael) treated controls (43). Mechanistic studies unequivocally demonstrated that pretreatment with Bael extract (25 and 50 mg/kg body weight) prevented 2-acetyl aminofluorene-induced oxidative stress by restoring the levels of antioxidant enzymes and detoxification enzymes and by concurrently reducing ornithine decarboxylase activity and DNA synthesis. Some of the phytochemicals found in Bael, including lupeol, eugenol, limonene, citral, rutin, and anthocyanins, have been demonstrated in studies to have chemopreventive properties. These substances may have contributed to the symptoms seen as a result of the extract(44).

Previous mechanistic studies reveal that Bael's radioprotective properties are multifactorial and are caused by an increase in glutathione levels that acts as an immunomodulator, a free radical scavenger, an antioxidant, and a decrease in lipid peroxidation (44,45). In in vitro systems of investigations, the leaf extract is found to be a robust scavenger of both reactive oxygen species and reactive nitrogen species as well as a good iron chelator.

CONCLUSION:

The chemical constituents found in the extract reveals the anti-cancer properties of the extract as it suppresses the migration and proliferation of MCF-7 cells. Thus the result of the study concluded that the Aegle marmelos fruit extract processes a pro apoptotic effect on breast cancer cell lines.

CONFLICTS OF INTEREST:

The authors declare that there are no conflicts of interest in the present study.

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