

EVALUATION OF ANTICANCER EFFECT OF *Aegle marmelos* IN HUMAN BREAST CANCER CELLS BY *INVITRO* ANALYSIS

ABSTRACT:

Background: Cancer is uncontrolled division and proliferation of abnormal cells in the body. Nowadays, therapeutic Treatment of cancer has become a great clinical challenge and alternative medicines are being extensively studied to cure cancer. *Aegle marmelos* is one such plant Which has many pharmacological activities.

Aim: To study the anticancer activity of *Aegle marmelos* which promotes cell death in human cancer breast cells MCF7 by modulating wnt/beta catenin expression.

Materials and methods: Human Breast Cancer cell line (MCF-7) was purchased from NCCS. Cell viability was assayed using a modified colorimetric technique that is based on the ability of live cells to convert MTT. The specificity of the amplification product was determined by melting curve analysis for each primer pairs. The obtained data were analyzed statistically by one-way analysis of variance (ANOVA) and Duncan's multiple range test with a computer-based software.

Results: the viability of cancer cells after addition of *Aegle marmelos* has decreased from 100% to 50% with increase in concentration of the extract 100-600 micrograms/ml. It is also evident that there was a fold change in control over Wnt m-RNA expression of MCF7 that decreased significantly on the addition of *Aegle marmelos* extract. The results also showed that there was a fold change in control of the beta catenin m-RNA expression of MCF7, which decreased significantly in addition to *Aegle marmelos*.

Conclusion: The present study concluded an innovative finding that *Aegle marmelos* promotes cell death in MCF7 cells by modulating the pathway. The plant extract also possesses hepatorenal protective effect thus, it can be used as a novel and safe anti-cancer drug against breast cancer cells.

Keywords: *Aegle marmelos*, cell death, hepatoprotective, innovative, drug

INTRODUCTION:

Cancer is the second leading cause of death in the world. According to the survey reports of 2008 nearly 12.7 million new cases of cancer (56% from developing countries) and 7.6 million cancer deaths were reported((1,2).

Cancer is caused by changes to the DNA in the cells. Breast cancer being the most common form of cancer among females in the world(3).The origin of breast cancer is because of age, hereditary factor, reproductive factors, prolonged exposure to estero genes, lack of breastfeeding and other lifestyle related factors)(4). Along with these there can be environmental factors which are involved in development of breast cancer (4–6). On the basis of stage of cancer, location of the tumour along with health condition of a patient, it will be treated using methods such as surgery, ionising radiations and chemotherapy are employed. But when chemotherapy and radiations are done on cancer patients it causes deleterious effect on normal healthy cells due to lack of their specificity(7). As these cancer treatments are very expensive, most of the people living in developing countries in the world prefer a complementary and other alternative form of medicine for treating and managing the symptoms and pain of cancer(8). One such traditional and ancient form of medicine is Ayurveda which has been practiced from old days, its emphasis on prevention of diseases and promotion of good health(1,9).

The Ayurvedic remedies are mainly based on plants(10) and one such Ayurvedic plant which is extensively studied is “Aegle marmelos” a medium studied deciduous plant belonging to the Rutaceae family(11). It’s common name is stone apple or golden apple (12). Bael is considered as the “Emblem of fertility” and healing tree that strengthens the body (13, 14). It is usually found in South East Asia and several parts of India(15). It is known to have anti-proliferative, antipyretic, anti-oxidant, anti-diarrhoeal, anti- inflammatory, hypoglycemic, anti-cancer and anti-fungal activities (16). The phyto chemical properties of Aegle marmelos shows that the fruit contains compounds like carotenoids, phenolics, flavonoids, alkaloids, tannins, terpenoids(17,18). All these phytochemicals act as a primary antioxidant and free radical scavengers are found to be high in the alcoholic extract of the fruit pulp(19). Along with that some Other phytochemicals such as aegeline, aegelenine, marmelin, o-methyl halfordinol are some of the coumarins present in the fruit pulp of the Aegle marmelos(20). Leaf extract of Aegle marmelos is to be very effective for various tumor cell lines including breast cancer cell line MCF- 7(21). Wnt/ Beta catenin is a cascade of conserved evolutionary pathways(22). The

pathway plays an important role in embryonic development and helps in Wnt/ maintenance of adult tissue homeostasis by regulating the cell proliferation, migration, differentiation and as well as of the stem cells(23). Most of the studies say that some crucial molecules in the pathway signalling also possess a diagnostic value in anti cancer therapy(24). Hence the study was conducted to assess whether *Aegle marmelos* promotes cell death in the MCF7 cell line by modulating the Wnt/Beta catenin pathway(25).

MATERIALS AND METHODS:

Cell viability by MTT assay

Cell viability was assayed using a modified colorimetric technique that is based on the ability of live cells to convert MTT, a tetrazolium compound into purple formazan crystals by mitochondrial reductases (Mosmann, 1983). Briefly, the cells (1×10^4 /well) were exposed to different concentrations of *Aegle marmelos* extract (100-500µg/ml) with MCF-7 cells for 48 h. At the end of the treatment, 100 µl of 0.5 mg/ml MTT solution was added to each well and incubated at 37 °C for an hour. Then the formazan crystals formed were dissolved in dimethyl sulfoxide (100 µl) and incubated in the 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.

Gene expression analysis by Real Time-PCR

Samples from each group were submerged in 2 ml Trizol (Invitrogen, Carlsbad, CA, USA) for RNA extraction and stored at -80°C until further processed. cDNA synthesis was performed on 2 µg RNA in a 10 µl sample volume using Superscript II reverse transcriptase (Invitrogen) as recommended by the manufacturer. Real-time PCR array analysis was performed in a total volume of 20 µl including 1 µl cDNA, 10 µl qPCR

Master Mix 2x (Takara, USA) and 9 µl ddH₂O. Reactions were run on an CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA) using universal thermal cycling parameters (95°C for 5 min, 40 cycles of 15 sec at 95°C, 15 sec at 60°C and 20 sec at 72°C; followed by a melting curve: 5 sec at 95°C, 60 sec at 60°C and continued melting). For quality control purposes, melting curves were acquired for all samples. The specificity of the amplification product was determined by melting curve analysis for each primer pair. The data were analyzed by comparative CT method and the fold change is calculated by $2^{-\Delta\Delta CT}$ method described by Schmittgen and Livak (2008) using CFX Manager Version 2.1 (Bio Rad, USA).

The chemicals Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Pvt Ltd, USA. Trypsin-EDTA, fetal bovine serum (FBS), antibiotics-antimycotics, DMEM 1640 medium and phosphate buffered saline (PBS) were purchased from Gibco, Canada. (5,5,6,6-tetrachloro-1,1,3,3-tetraethyl benzimidazole carbocyanine iodide) and Real Time PCR kit was purchased TAKARA (Meadowvale Blvd, Mississauga, ON L5N 5S2, Canada).

Cell lines and cell culture

The Human Breast Cancer cell line (MCF-7) was purchased from the National Centre for Cell Sciences (NCCS), Pune, India. Cells were cultured in DMEM 1640 medium (Thermo Fisher Scientific, CA, USA) containing 10% fetal bovine serum (Thermo Fisher Scientific, CA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Thermo Fisher Scientific, CA, USA) at 37°C with 5% CO₂.

Table 1: Primer Sequence

S.No	Gene	Primer sequence
2	Human β -catenin	Forward: 5'-CTTACACCCACCATCCCACT-3' Reverse: 5'-CCTCCACAAATTGCTGCTGT-3'
3	Human Wnt	Forward:5'-GCCGTGTCATGCTCAGAA-3' Reverse: 5'-GTGGACTACCCCTGCTGATG-3'
4	Human β -actin	Forward:5'-CTACAATGAGCTGCGTGTGG -3' Reverse: 5'TAGCTCTTCTCCAGGGAGGA-3'

Statistical analysis

The obtained data were analyzed statistically by one-way analysis of variance (ANOVA) and Duncan's multiple range test with a computer-based software (Graph Pad Prism version 5) to analyze the significance of individual variations among the control and experimental groups. The significance was considered at $p < 0.05$ level in Duncan's test.

Results:

From the study we can infer that the viability of cancer cells after addition of *Aegle marmelos* has decreased from 100% to 50% with increase in concentration of the extract 100-600 micrograms/ml. It nearly reached 50% when the concentration of the extract was 300 to 500 micrograms (figure 1). It is also evident that there was a fold change in control over the Wnt m-RNA expression of MCF7 that decreased significantly on the addition of *Aegle marmelos* extract (figure 2). The results also showed that there was a fold change in control of the beta catenin m-RNA expression of MCF7, which decreased significantly in addition to *Aegle marmelos* (figure 3).

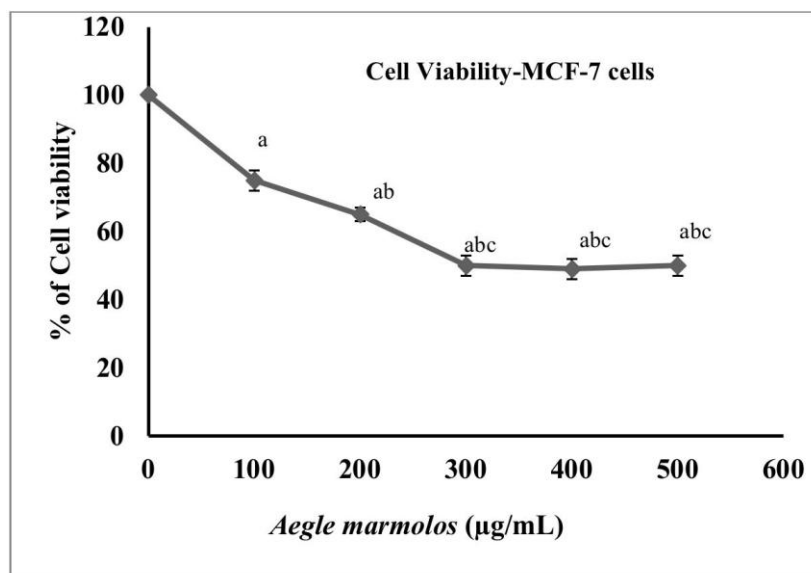


Figure 1-Effect of Aegle marmelos leaf extract on cell viability in MCF-7 cells.

Each bar represents the mean $\pm$ SEM of 6 observations. Significance at $p < 0.05$, a-compared with untreated control cells, b-compared with 1nM treated MCF-7 cells. X-Axis represents the concentration of *Aegle marmelos* extract and Y-axis represents the % of the cell viability in human breast cancer cells(MCF 7)

Wnt mRNA expression (Fold change over control)

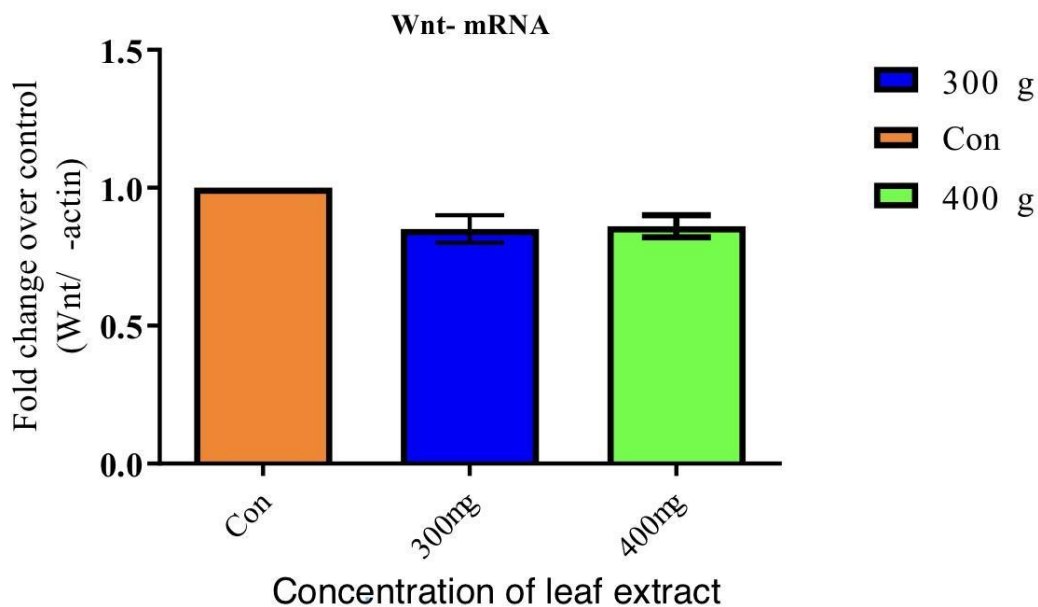


Figure -2 Effect of Aegle marmelos leaf extract on Wnt mRNA expression in MCF-7 cells.

Each bar represents a mean $\pm$ SEM of 6 observations. Significance at $p < 0.05$. X-Axis represents the concentration of the Aegle marmelos extract against the cell lines and Y-axis represents the fold change over control on the Wnt-mRNA expression.

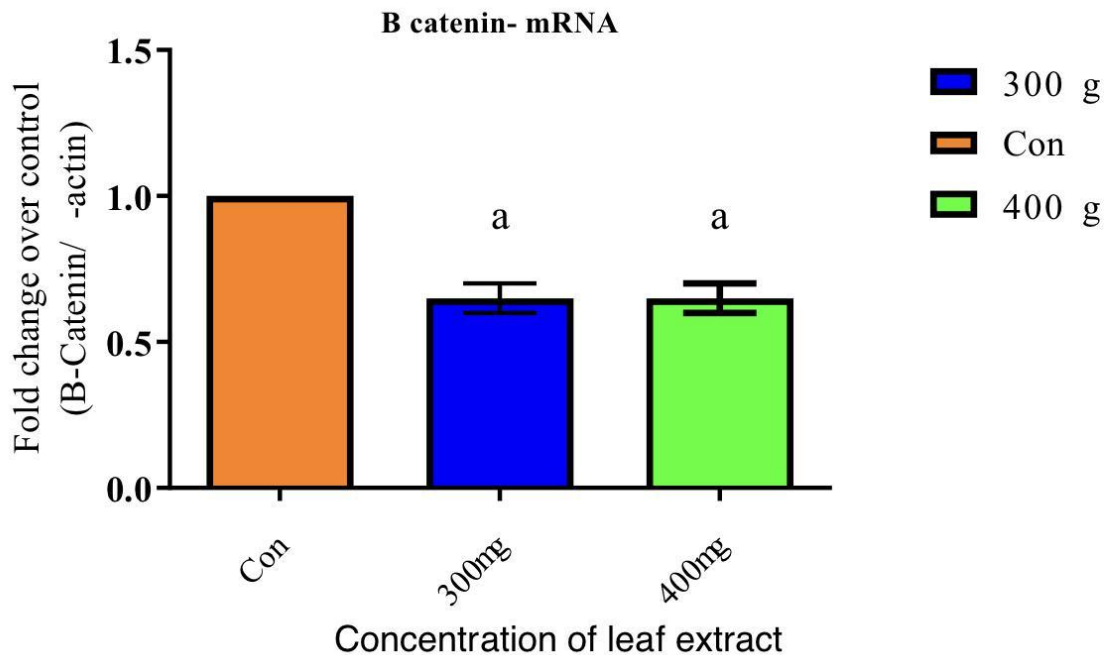


Figure-3 Effect of *Aegle marmelos* leaf extract on B- Catenin mRNA expression in MCF-7 cells. Each bar represents a mean $\pm$ SEM of 6 observations. Significance at $p < 0.05$, a-compared with untreated control cells. The X-axis represents the concentration of the aegle marmelos extract on the MCF-7 cells and Y-axis represents the fold change over control on the beta-catenin m-RNA expression.

DISCUSSION:

The cell viability of the breast cancer cells(MCF 7) in *Aegle marmelos* was determined by the MTT method(26). This method is based on the enzymatic cleavage of the tetrazolium salts into purple coloured formazan by cellular mitochondrial dehydrogenase enzymes present in the viable cells(27).

In the present study, *Aegle marmelos* had anticancer cell death activity in the MCF7 cells, other studies also reported that cytotoxic activity of bael leaf ethanolic extract can also act on the SKBR3 breast cancer cells(28).

The results are in line with the previous studies done by the several researchers who stated the anti-inflammatory and anti-cancerous properties of the *Aegle marmelos* plant extract(29). The decreased expression of $\text{TNF-}\alpha$ was also reported after treatment with *Aegle marmelos* ethanolic fruit extract in rat model as the same was observed in Human breast cancer cells(30).

Previous studies related to anti cancer activity cited that the hydro alcoholic extract exhibits strong antiTumor and antioxidant activities on DLA-bearing mice(31). Plant research has increased all over the world and a large body of evidence has been collected to show the immense potential of medicinal plants used in various traditions(32). These positive results on related cancers leave a trace of hope for discovering its action on breast cancer cells(33).

This paper allows us to identify antitumor compounds including cisplatin,chromomycin,cytosine,arabioside and 5-fluorouracil(34).Genetic and epigenetics deregulation of Wnt/beta catenin signalling contributes to human cancer,which has led to the development of extensive approaches targeting Wnt/Beta catenin signalling as cancer therapy(34,35).

Limitation of this study is, only when done in invivo it will give accurate results but before that advanced research needs to be done, various steps of drug testing needs to be approved. In vitro results have proven to be positive and the future scope of this study is of higher significance against cancer treatment.

CONCLUSION

From the above research done and with the supporting articles, we can say that *Aegle marmelos* has a therapeutic anti cancer activity on human breast cell line MCF7 with some bio active components like flavonoid, alkaloids, polysaccharides, citral, marmin, tannin,Gagarin etc. Not only anticancer activity but also other medicinal values that could be used in the future generation. In a clear view, the *Aegle marmelos* has a positive effect on breast cancer cells. Thus, this drug can be used for further study and can be formulated of as an anti cancer drug which will be useful for cancer patients.

NOTE:

The study highlights the efficacy of "ayurveda" which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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