

STUDIES ON CHEMOTHERAPEUTIC POTENTIAL OF HYDROETHANOLIC LEAF EXTRACT OF AEGLE MARMELOS ON HUMAN LUNG CANCER CELL LINES

ABSTRACT-

Background: Lung cancer, one of the world's leading causes of cancer death. There is an improvement in mortality by screening of lung cancer by low dose computed tomography. Long time ago, lung cancer was a reportable disease which now became a commonest cause of death due to cancer. Discovery of medicine for various diseases is still an ongoing process. A lot of diseases are still remaining a threat without any treatment or medicine. Herbal derived medicines are preferred because of their low side effects on patients.

Objective: To investigate the role of Aegle marmelos against human lung cancer cell lines (A549 cell line).

Materials and methods: A549 cell lines were procured from NCCS (National center for cell sciences) Pune, India. It was cultured and viability of the cells before and after adding the extract was analysed using the MTT assay. mRNA amplification was done using real time PCR. Statistical analysis was done using ANOVA and Dunnett's multiple test. Corresponding graphs are also plotted.

Results: The viability of the cells decreased from 100% to 50%. The mRNA expression of wnt and β -catenin decreased after the addition of the extract.

Conclusion: The study concluded that Aegle marmelos possesses a good, novel, innovative, anti- cancer activity against the lung cancer cell lines A549.

KEYWORDS -

Lung cancer, drug, innovative , Aegle marmelos, Cell lines, cell viability.

Running title: Role of Aegle marmelos against liver cancer.

INTRODUCTION-

Cancer is the second leading cause for death following cardiovascular diseases in India. Herbal plants are usually used by many traditional healers to treat various symptoms and signs of diseases including fever, cold, headache, diabetes, diarrhea, and now even cancer. It is very clear that now plants have a very useful clinical effect and have an antitumor activity(1,2). WHO has given a statistic of 70-95% of the developing world's population relying on various traditional plants based medicine systems and healthcare. Cancer cells have the ability to escape apoptosis in many ways(3)(4). The main function of anticancer drugs is to prevent it from escaping apoptosis and stop it from proliferating more. These drugs are set to induce cell apoptosis in tumour cells by having cytotoxic effect and hence remains a vital treatment for cancer(5)(6,7). A few drugs that perform the above action are 5-fluorouracil, cisplatin, etoposide, VM26, etc.(8)(1). Thus the identification of potential chemotherapeutic agents using mechanism based studies holds great promise for elucidating mechanisms and devising more specific and effective treatments for cancer related diseases(9)(10)(1,2,11).

One such approach of discovery is the ethnomedical data approach, where plant selection is based on prior information on the folk medicinal use of plants. It is generally known that this data substantially increases the chance of finding active plants related to random approaches(12). The plant selected for present study – *Aegle marmelos*. There is no recorded data for cytotoxicity against cancer cell lines and normal cell lines for these plants(13,14). Previous studies have concluded that the solvent extracts of the above plant-*Aegle marmelos* were screened for in-vitro antioxidant, antiproliferative, anti-inflammatory and anticancer activities(15)(16). This research focuses on the chemotherapeutic potential of hydro ethanolic extract of *Aegle marmelos* on human lung cancer cell lines.

MATERIALS AND METHODS -

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, RPMI 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).

Study design : in vitro study.

Duration : 3 months

No ethical consideration involved as it is an in vitro study in the laboratory

Inclusion criteria : The study involved Human lung cancer cell lines for in vitro based studies and cell viability assays to evaluate anticancer activity of Aegle marmelos through Wnt-mRNA expression of A549 and β - Catenin-mRNA expression of A549

Exclusion criteria : The study excluded the involvement of aqueous and other solvent extracts for anticancer activity.

Cell lines and cell culture

The Human Lung cancer cell line (A549) was purchased from the National Centre for Cell Sciences (NCCS), Pune, India. Cells were cultured in RPMI 1640 medium (Thermo Fisher Scientific, CA, USA) containing 10% fetal bovine serum (Thermo Fisher Scientific, CA, USA), 100 U/ml penicillin and 100 μ g/ml streptomycin (Thermo Fisher Scientific, CA, USA) at 37°C with 5% CO₂.

Cell viability by MTT assay

Cell viability was assayed employing a modified colorimetric technique that supported the power of live cells to convert MTT, a tetrazolium compound into purple formazan crystals by mitochondrial reductases (Mosmann, 1983). Briefly, the cells (1 \times 10⁴/well) were exposed to different concentrations of Aegle marmelos extract (100-500 μ g/ml) with A549 cells for 48 h. At the end of the treatment, 100 μ l of 0.5 mg/ml MTT solution was added to each well and incubated at 37 °C for an hour. The formed formazan crystals were dissolved in dimethyl sulfoxide (100 μ l) and incubated in dark for an hour. Then the intensity of the colour developed was assayed employing a Micro ELISA plate reader at 570 nm. The number of viable cells was expressed because the percentage of control cells cultured in serum-free medium. Cell viability on top of things medium with none treatment was represented as 100%. The cell viability is calculated using the formula: red blood cell viability = [A_{570 nm} of treated cells/A_{570 nm} of control cells] \times 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 internal control purposes, melting curves were acquired for all samples. The specificity of the amplification product is decided by melting curve analysis for every primer pair. The data were analyzed by comparative CT method and the fold change is calculated by 2^{-ΔΔCT} method described by Schmittgen and Livak (2008) using CFX Manager Version 2.1 (Bio Rad, USA).

Table 1:

Primer Sequence:

S.No	Gene	Primer sequence
2	Human β-catenin	Forward: 5'-CTTACACCCACCATCCCA CT-3' Reverse: 5'-CCTCCACAAATTGCTGCTGT-3'
3	Human Wnt	Forward:5'-GCCGTGTCATGCTCAGAA-3' Reverse: 5'-GTG GAC TAC CCC TGC TGA TG-3'

4	Human β -actin	Forward:5'-CTACAATGAGCTGCGTGTGG -3' Reverse: 5'TAGCTCTTCTCCAGGGAGGA-3'
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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 -

The viability of cancer cells which were 100% viable, after addition of Aegle marmelos extract decrease in viability based on the dosage was observed. It is observed to decreased to 50% viability when the concentration of the extract increased upto to 500 microgram(Figure 1).It was also observed that there was a fold change over control of the Wnt-mRNA expression of A549, which decreases significantly on the addition of Aegle marmelos extract(Figure 2). It was observed that there was a fold change over control of the β - Catenin-mRNA expression oh A549, which decreases significantly on the addition of Aegle marmelos extract(Figure 3).

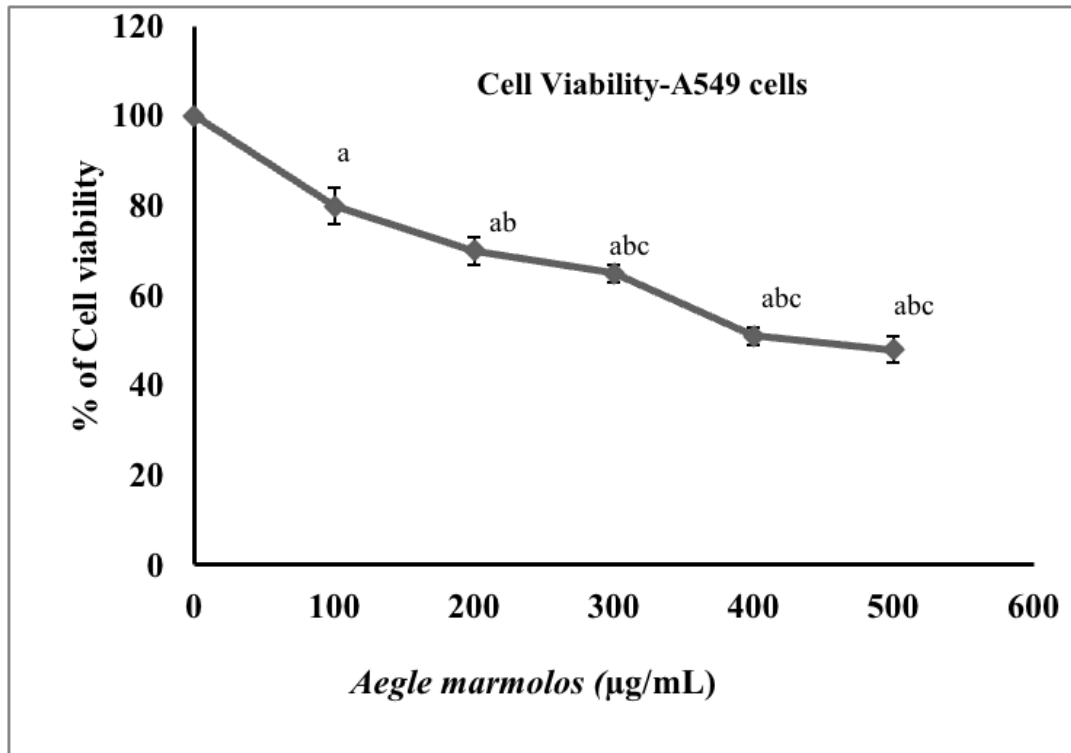


Figure 1 represents the effect of *Aegle marmelos* leaf extract on cell viability in A549 cells. Each bar represents a mean \pm SEM of 6 observations. Significance at $p < 0.05$, a-compared with untreated control cells, b-compared with 1nM treated A549 cells, c-compared with 300µg treated cells. X-axis represents the concentration of extract and Y-axis represents the percentage of cell viability. There is a significant decrease in cell viability.

Gene expression analysis -

Wnt mRNA expression (Fold change over control)

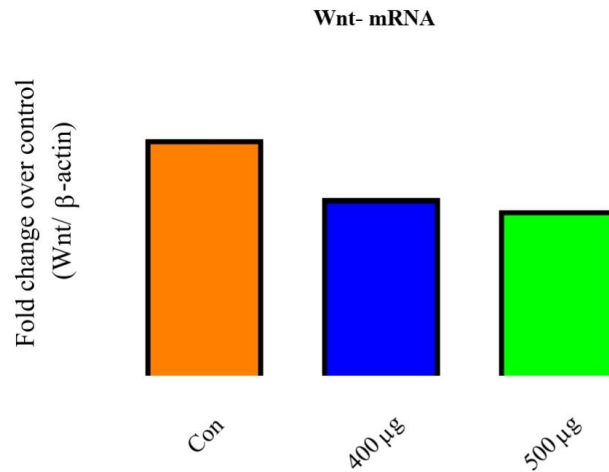


Figure 2 shows the effect of *Aegle marmelos* leaf extract on Wnt mRNA expression in A549 cells. Each bar represents a mean \pm SEM of 6 observations. Significance at $p < 0.05$, a-compared with untreated control cells. X-axis represents the concentration of extract added and Y-axis represents the fold change over control of the mRNA expression of A549 cell lines.

β - Catenin- mRNA expression (Fold change over control)

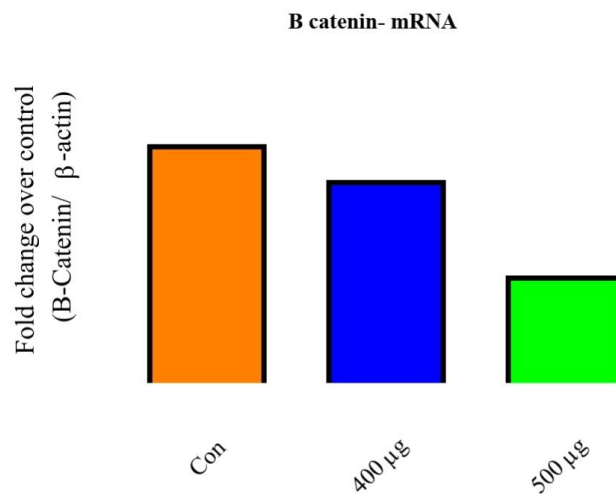


Figure 3 represents the effect of *Aegle marmelos* leaf extract on β - Catenin mRNA expression in A549 cells. Each bar represents a mean \pm SEM of 6 observations. Significance at $p < 0.05$, a-compared with untreated control cells, b-compared with 400 μ g treated cells. X-axis represents the concentration of extract added and Y-axis represents the fold change over control of the mRNA expression of A549 cell lines.

DISCUSSION-

Many commercially proven anti cancer drugs used in modern times were initially used in crude form in traditional or folk healing practises or for other purposes(17)(18). An estimate states that 75% of 120 active plant derived compounds currently used worldwide have been through follow up studies to verify authenticity of data from folk and ethnomedical uses(19). There is a great potential in discovery of traditional plant uses(20).The extract with one of its phytochemicals called lupeol has increased the era gene expression in MDA – MB-231 (Era - negative breast cancer cells) thus inhibiting cell proliferation.

Plant phenols may be considered as potential compounds for selective blocking signal transduction pathways, this is in accordance with the current study where the Aegle marmelos blocks the MAPK pathway which inhibits the maturation of the Wnt-mRNA and beta-catenin mRNA(21)(22)(23).The phytochemical profile of Aegle marmelos show pharmacological activity and hepatoprotective activity(24)(6).

Derivatives of Aegle marmelos were found to be exhibiting strong activity in inhibiting in vitro cell growth of human K562 cells(25)(26).

Aegle marmelos important pharmacological activities such as antidiabetic, antioxidant, antimicrobial, hepato-protective, anticancer and anti proliferative activity(27)(28).

Extensive experimental and clinical studies prove that Aegle marmelos possess various properties which helped to play a role in prevention and treatment of many diseases(29).

The hydro alcoholic extract of Aegle marmelos exhibits strong antitumor and antioxidant activities on DLA-bearing mice(30)(31).

Plant research has increased all over the world and a large body of evidence has been collected to show immense potential of medicinal plants used in various traditional systems(32)(33).Aegle marmelos has properties that are medicinal and is a hypoglycaemic extract(34,35).

Limitations of this study is, only when the study is done in in vivo conditions, exact results could be achieved but before advancements are needed to be developed , various steps of drug testing need to be approved. In vitro results have proven to be positive and this study also suggests a future study concentrating on the high significance of the extract against Aegle marmelos.

CONCLUSION-

Hence, taking the entire result parameters into account, it can be concluded that Aegle marmelos hydroethanolic leaf extract possesses chemotherapeutic potential by suppressing the lung tumour growth rate. It also has hepato-renal protective effects, and thus can be targeted as a novel and safe anticancer drug against lung cancer. Hence some of the extracts of the compound, if structurally identified and characterised, may be a potent candidate for anticancer drug development.

❖ **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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