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Anticancer Activity of Leaf Hydro Ethanolic Extract of *Aegle marmelos* in Human Lung Cancer Cell Mediated through Caspase-3 and Caspase-9 mRNA Expression

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Authors' contributions

This work was carried out in collaboration among all authors. Author RS managed the literature searches, collected experimental data and analysed. Author RS managed the survey and wrote the first draft of the manuscript. Authors GS and JS designed the study, verified data, drafted of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Background: *Aegle marmelos* (AE) is a medicinal plant that comes under the rutaceae family and the plant was used in the past for treating many diseases and illness symptoms. The plant has many effects such as anti-diarrhoeal, antimicrobial, antiviral, radioprotective, anticancer, chemopreventive, antipyretic, ulcer healing, antigenotoxic, diuretic, antifertility and anti-inflammatory properties.
Aim: To know the anticancer activity of hydroethanolic leaf extract of *Aegle marmelos* over lung cancer cells treated with caspase 3 and caspase 9 mRNA expression.
Materials and Methods: The required chemicals were collected mainly from Canada. The lung

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cancer cells (A549) were collected from NCCS pune and then RNA was extracted from the cells and then the study was conducted after treating it with caspase 3 and caspase 9 mRNA expression. The cells were treated with many dosage of hydroethanolic extract of *Aegle marmelos* and the cell viability was noted.

Results: The study reported that extract of *Aegle marmelos* has a great anticancer activity about 1 fold change over rate of 1.7 for cells treated with caspase 3 and a fold change over of 1 in caspase 9 treated lung cancer cells.

Conclusion: The study concluded an innovative finding that the hydroethanolic leaf extract of *Aegle marmelos* has a great anticancer activity against lung cancer cells treated with caspase 3 and caspase 9 mRNA expression.

Keywords: Aegle marmelos; anticancer activity; caspase expression; hydroethanolic extraction; innovative.

1. INTRODUCTION

Cancer is one of the deadliest diseases that is more prevalent over the growing world and the death due to cancer increases each and every day and there are many solutions available for this deadly disease but as we get cured from the cancer we get affected by the side effects of the treatments [1]. There should be some alternative for these side effects causing medicines and the natural answers for this question [2].

Nature never fails to fascinate us with its power, and nature gives us everything we need, in the same way we get the solution for cancer from nature itself [3,4]. In nature we have many medicinal herbs and trees that we did not discover until now and if we do that we would get almost solutions for all the problems we face in the present days [5,6]. One such medicinal herb is *Aegle marmelos* commonly known as bael tree, contains many medicinal effects such as antidiarrheal [7], antimicrobial, antiviral, radioprotective, chemopreventive, antipyretic, ulcer healing, antigenotoxic, diuretic, antifertility and anti-inflammatory properties [8].

This old herb was used in olden days by our ancestors to cure many diseases [9]. Now interestingly this plant is found to have anticancer properties also and if this property of the plant is used wisely we could create a potential anticancer drug [9,10]. The aim of this study is to know the anticancer of *Aegle marmelos* over lung cancer cells treated with caspase 3 and caspase 9 mRNA expression [11,12].

Aegle marmelos was used in the countryside part of India as dried pulp in summer drinks as it helps in overcoming sunstroke. The Bale leaves are also used in the preparations of salads. Bale fruit absorbs toxins produced by bacteria and other pathogens in the intestine and hence helps in treating dysentery [13]. The bale leaves are also used in ayurvedic medicine to treat loss of appetite. Our team has extensive knowledge and research experience that has translate into high quality publications [14–18,19–23].

The oil extracted from its fruits and leaves are used to cure respiratory disorders. As it had an anti-inflammatory effect the fruit was used for the cure of tuberculosis [14].

2. MATERIALS AND METHODS

2.1 Cell Viability by MTT Assay

Cell viability was assayed with a modified colorimetric technique that is based on the power 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 A549 cells for48 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 formed crystals were dissolved in dimethyl sulfoxide (100 µl) and incubated in the dark for an hour. Then the intensity of the color developed was assayed with a Micro ELISA plate reader at 570 nm. The number of viable cells was expressed in the form of percentage of control cells cultured in serum-free medium. Cell viability over the control medium with no treatment was represented as

Table 1. Primer sequence

S. No	Gene	Primer sequence
1	Human Caspase-3	Forward: 5′-TTCAGAGGGGATCGTTGTAGAAGTC -3′ Reverse: 5′-CAAGCTTGTCGGCATACTGTTTCAG-3′
2	Human Caspase-9	Forward:5′-ATGGACGAAGCGGATCGGCGGCTCC-3′ Reverse: 5′-GCACCACTGGGGGTAAGGTTTTCTAG-3′
3	Human β-actin	Forward:5'-CTACAATGAGCTGCGTGTGG -3' Reverse: 5'TAGCTCTTCTCCAGGGAGGA-3'

100%. The cell viability is calculated using the formula: percentage of cell viability = [A570 nm of treated cells/A570 nm of control cells] × 100.

2.2 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 ddH_2O. 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 the purpose of quality control, melting curves were acquired for all samples. The specificity of the amplification product was 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 bySchmittgen and Livak (2008) using CFX Manager Version 2.1 (Bio Rad, USA).

2.3 Chemicals and Other Requirements

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, DMIM 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 purchasedTAKARA (Meadowvale Blvd, Mississauga, ON L5N 5S2, Canada).

2.3.1 Cell lines and cell culture

Human Lung cancer cell line (A549) 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% CO_2.

2.4 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.

3. RESULTS

The cell viability of the lung cancer cells (A549) when treated with hydro ethanolic leaf extract of *Aegle marmelos* was discussed in Fig. 1. The cell viability showed a decrease with increase in concentration of the leaf extract of AE and at 400 and 500 microgram of leaf extract the cell viability reduced notably. The effect of *Aegle marmelos* on the caspase 3 mRNA treated lung cancer cells in the form of fold changeover was discussed in Fig. 2. It showed that as the expression is positive, there is an increase in the fold change as the cell viability decreases. The effect of *Aegle marmelos* on caspase 9 mRNA expression treated lung cancer cells in the form of fold over change was discussed in Fig. 3. It showed that as the expression is positive the fold change increases as the cell viability decreases.

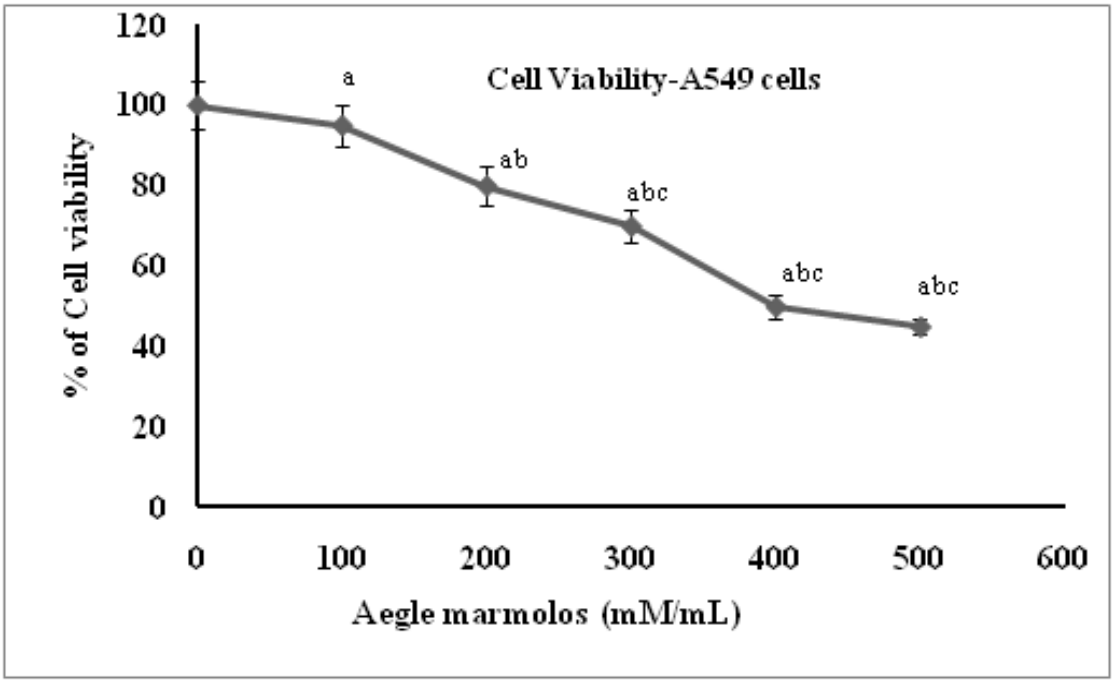

Fig. 1. x axis represents the concentration of *Aegle marmelos* and the y axis represents the percentage of cell viability. Effect of*Aegle marmelos* leaf extract on cell viability in A549 cells. Each bar represents a mean ± SEM of 6 observations. Significance at p< 0.05, a-compared with untreated control cells, b-compared with 1nM treated A549 cells

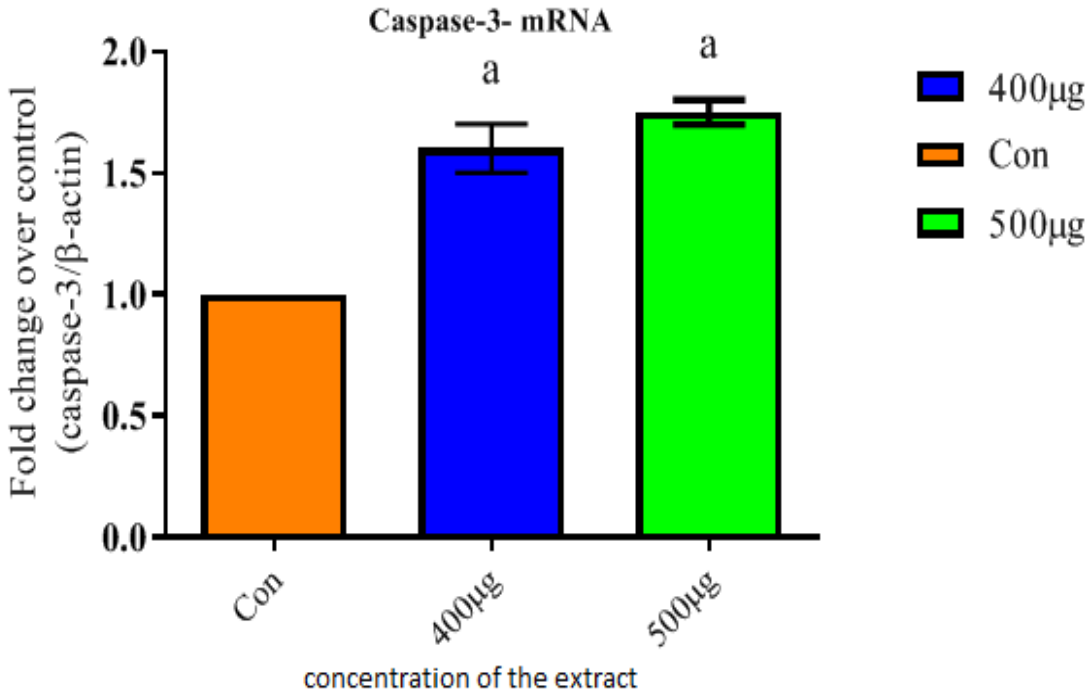

Fig. 2. The x axis represents the concentration of the leaf extract and the y axis represents the fold changeover control.Effect of *Aegle marmelos* leaf extract on caspase-3 mRNA expression in A549 cells. Each bar represents a mean ± SEM of 6 observations. Significance at p< 0.05, a-compared with untreated control cells

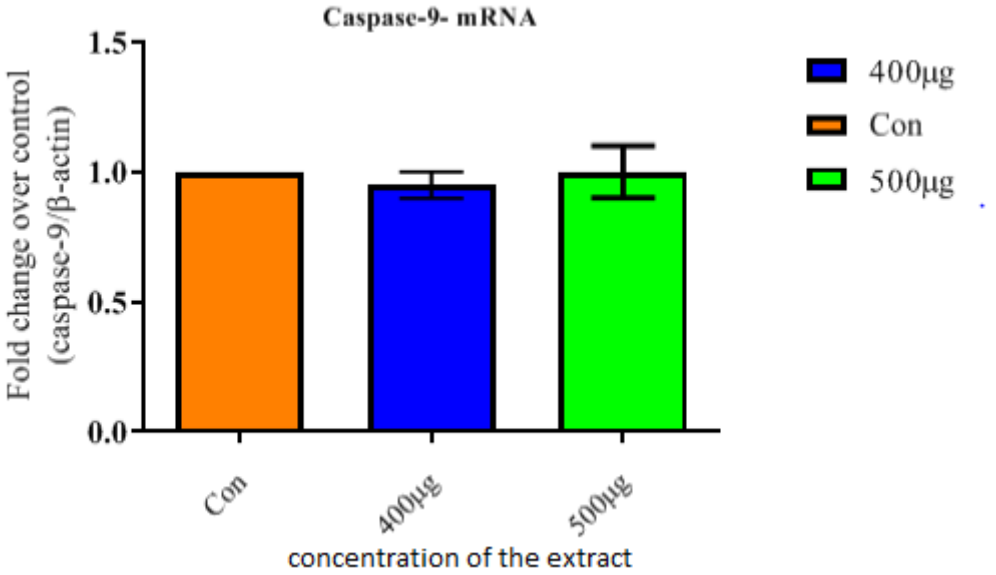

Fig. 3. the x axis represents the concentration of the leaf extract and the y axis represents the fold change over control. Effect of *Aegle marmelos* leaf extract on caspase-9mRNA expression in A549 cells. Each bar represents a mean ± SEM of 6 observations. Significance at p< 0.05, a- compared with untreated control cells

4. DISCUSSION

From the results obtained within the limit of study it could be seen that the hydroethanolic leaf extract has anticancer activity over lung cancer cells(A549) treated with caspase 3 and caspase 9 mRNA expression [24,25]. Phytochemical studies shows that fruits and leaves of the medicinal herb *Aegle marmelos* contains many phytochemical compounds like flavonoids, tannins and carotenoids which are the main reason behind the anticancer and other medicinal properties of the *Aegle marmelos [26][27]*. The anticancer activity is not only seen toward the lung cancer cells but also many other cells such as breast cancer cell lines (MCF7) and melanoma cancer cells [28][29] The anticancer activity of the *Aegle marmelos* could be even seen in the Swiss albino mice [30–33].

The anticancer activity of the plant is mainly due to the free radical scavengers that occur in the phytochemical aspects [24,25,34]. The lung cancer cells at initial stage remain actively dividing and then when the hydroethanolic extract of the *Aegle marmelos* is added the cell viability starts to decrease slowly as the over proliferation of the cell is stopped by the plant extract [35]. The cell viability reduces to the most at the concentration of 400 and 500 micrograms of the hydroethanolic extract and this dosage was taken as the dosage for the conduction of the MTT assay [27,36].

The anticancer activity of the *Aegle marmelos* now is tested only with the in vitro samples for now but in future the hydroethanolic extract of *Aegle marmelos* could be tested with the in vivo samples and if positive results are acquired [35,37], it could be used as an effective anticancer drug [30–32].

The anticancer drugs used at present in chemotherapy even though give a great result, it also gives many side effects that could not be avoided [38,39]. This could be stopped by bringing in a nearly effective natural remedy that could solve the problems nearly as effectively as the present synthetic drugs but does not give any side effects [40,41]. The anticancer activity of the natural plants that are found could be used to solve this problem [42]. The study was time consuming and costly hence it was hard to conduct and complete the research [43].

5. CONCLUSION

From the results gathered from the study and experiment it is clear that the hydroethanolic extract of the *Aegle marmelos* has anticancer activity against the lung cancer cells (A549) mediated through caspase 3 and caspase 9

mRNA expression. This property could be used in future with further in vivo studies and experiments.

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CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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