



Effect of Aegle Marmelos Hydroethanolic Leaf Extract on Expression of Antiapoptotic Markers in Human Melanoma Cells

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Authors' contributions

This work was carried out in collaboration among all authors. Author SB did the literature search, survey, experimental data collection, analysis and manuscript writing. Authors GS, JS and SP did the study design, data verification and manuscript drafting. All authors read and approved the final manuscript.

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ABSTRACT

Background: Aegle marmelos commonly known as Bael is a herbal plant. It is from a family called Rutaceae. It has many medicinal uses: anti-diarrheal, anti-microbial, anti-viral, anti-cancer, chemopreventive, Ulcer healing and many others.

Aim: To study the effect of Aegle marmelos hydroethanolic leaf extract on expression of antiapoptotic markers in human melanoma cells.

Objective: The present study investigated the effect of Aegle marmelos hydroethanolic leaf extract on expression of antiapoptotic markers in human melanoma cells.

Materials and Methods: DMSO and MTT chemicals were purchased from Sigma chemical

Pvt Ltd. Trypsin EDTA, FBS, RPMI 1640 medium and PBS, Real time PCR kit was purchased from Canada. Human melanoma cell line (A375) was purchased from NCCS, Pune, India.

Results: The data was analysed statistically by ANOVA and Duncan's multiple range test with a computer based software (Graph Pad Prism version 5). The percentage of cell viability decreases with the increase in dosage of Aegle marmelos leaf extract.

Conclusion: The study concluded that Aegle marmelos hydro ethanolic leaf extract a novel and innovative herbal drug has a significant effect on the expression of antiapoptotic markers in human melanoma cell lines.

Keywords: *Aegle marmelos*; innovative; novel; human melanoma; RT-PCR; gene expression.

1. INTRODUCTION

Aegle marmelos (L.) Correa (A.marmelos) is commonly called "Bael". It is a kind of tree from the family "Rutaceae" which was widely used in as Indian traditional medicinal plant because of its various medicinal properties [1]. Bael is considered among chemical diversity which has a larger number of species of plants, animals, micro-organisms.on recent discovery in cellular regeneration [2-3]. Bael grows well in drained soil in the regions of tropical and subtropical parts of Indian subcontinent of Asia [4-6].

In the last 20 years, plant endophytes have gained more attention towards themselves because they act as a source of secondary metabolites with a new chemical skeleton [5,7]. They are very well known for their properties of anti-proliferative, antipyretic, anti-oxidant, [8] anti-diarrheal, anti-cancer [9-10]. Bael is found to have many bioactive compounds like flavonoid, quercetin, alkaloids, polysaccharides, citral, marmin, tannin and fagarine [11]. Our team has extensive knowledge and research experience that has translate into high quality publications [12–16].

The objective of the work reported in this article is to find out the expression of hydroethanolic leaf extract of Aegle marmelos on the human melanoma cell line [17-18]. This study targets tumor related genes like Bcl2, Bcl-xl, p53 genes [19-20].

2. 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 - tetraethylbenzimidazolocarbocyanine 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 Melanoma cell lines for in vitro based studies and cell viability assays to evaluate anticancer activity of Aegle marmelos through mRNA expression of Bcl2 and mRNA expression of p53

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

Cell lines and cell culture: The Human Melanoma cell line (A375) 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 µg/ml streptomycin (Thermo Fisher Scientific, CA, USA) at 37°C with 5% CO₂.

2.1 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 ×10⁴/well) were exposed to different concentrations of Aegle marmelos extract (100-500µg/ml) with A375 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 formed crystals were dissolved in dimethyl sulfoxide (100 µl) and incubated in the 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 = [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 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) [21]. 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 [22]. The data were analyzed by comparative CT method and therefore the fold change is calculated by 2^{-ΔΔCT} method described by Schmittgen and Livak (2008) using CFX Manager Version 2.1 (Bio Rad, USA).

2.3 Primer Sequence

Table 1. Represents the gene primer sequence analysis in vitro by the method of RT_PCR for different genes of human Human Bcl2, Human BclxL, Human p53.

S.No	Gene	Primer sequence
2	Human Bcl2	Forward: 5'- GGCTGGGATACTTTTGTGGA -3'
		Reverse: 5'- AAGAGTGAGCCCAGCAGAAC -3'
3	Human Bcl xL	Forward:5'- TTGGGAAGTTTCAAATCAGC -3'
		Reverse: 5'- TGCATTCTTGGACGAGGG- 3'
4	Human p53	Forward:5'- CCTCAGCATCTTATCCGAGTGG-3'
		Reverse: 5'- TGGATGGTGGTACAGTCAGAGC-3'

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

From the study we can infer the following. the viability of cells, cancer cells which were 100% viable, after addition of Aegle marmelos extract decreased in viability based on the dosage.It almost reached 50% when the concentration of the extract 400 to 500 micrograms.(Fig. 1).The fold changes over control of the mRNA expression of Bcl2 shows that there is a significant reduction in the mRNA expression of Bcl2 based on dosage.(Fig. 2)The fold changes over control of the mRNA expression of Bcl xL shows that there is no significant reduction in the mRNA expression of the Bcl xL based on the dosage(Fig. 3).The fold change over control of the mRNA expression of p53 shows that there is no significant reduction in the mRNA expression of the p53 based on the dosage (Fig. 4).

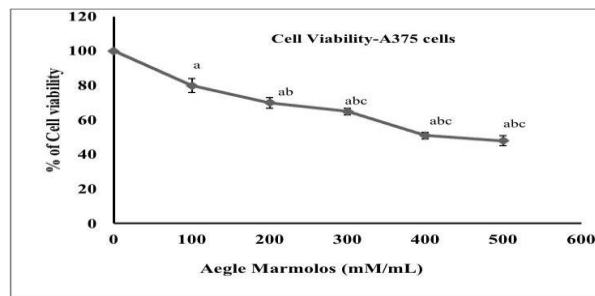


Fig. 1. Represents the effect of *Aegle marmelos* leaf extract on cell viability in A375 cells. Each bar represents a mean \pm SEM of 6 observations. Significance at $p < 0.05$, a-compared with untreated control cells, b-compared with 1nM treated A375 cells. X-axis represents the dosage of the leaf extract, Y-axis represents the percentage of cell viability. There is a significant decrease in the cell viability based on the dosage

3.1 Gene Expression analysis

Bcl2-mRNA expression (Fold change over control)

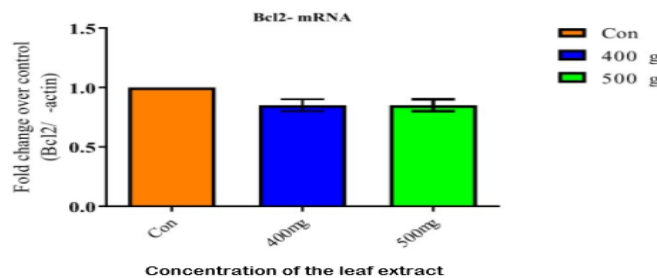


Fig. 2. Represents the effect of *Aegle marmelos* leaf extract on Bcl2 mRNA expression in A375 Cells. Each bar represents a mean \pm SEM of 6 observations. Significance at $p < 0.05$, a-compared with untreated control cells. X-axis represents the concentration of the dosage, Y-axis represents the fold change over control of the mRNA expression of Bcl2. There is a significant reduction in the mRNA expression of Bcl2 based on dosage

Bcl xL- mRNA expression (Fold change over control)

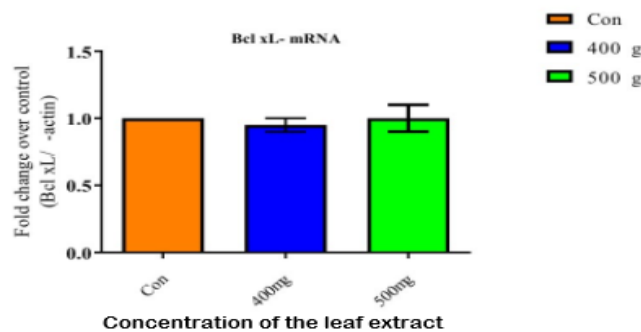


Fig. 3. Represents the effect of *Aegle marmelos* leaf extract on Bcl xL mRNA expression in A375 cells. Each bar represents a mean \pm SEM of 6 observations. Significance at $p < 0.05$, a-compared with untreated control cells. X-axis represents the concentration of the leaf extract, Y-axis represents fold change over control of the mRNA expression of Bcl xL. There is no significant reduction in the mRNA expression of the Bcl xL based on the dosage

p53- mRNA expression (Fold change over control)

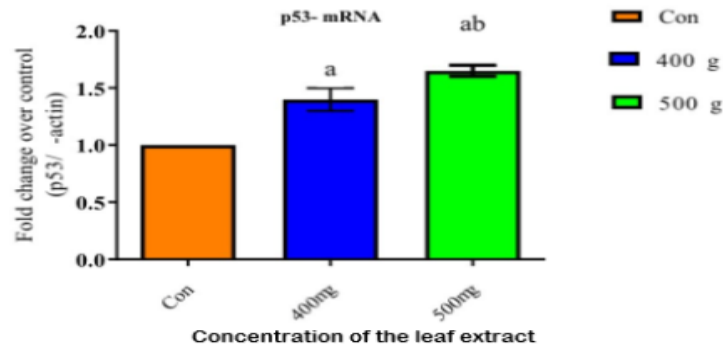


Fig. 4. Represents the effect of *Aegle marmelos* leaf extract on p53 mRNA expression in A375 Cells. Each bar represents a mean ± SEM of 6 observations. Significance at p< 0.05, a- compared with untreated control cells. X-axis represents the concentration of the leaf extract, Y-axis represents the fold change over control of the mRNA expression of p53. There is no significant reduction in the mRNA expression of the p53 based on the dosage

4. DISCUSSION

The cell viability of Bael leaf against A375 was determined by the MTT method. This method is based on the process of enzymatic cleavage of the tetrazolium salt into purple formazan by cellular mitochondrial dehydrogenases present in viable cells [23][24]. A proportion of people in some countries depend upon traditional medicines for their health needs [25-27]. Novel property of extracts of *Aegle marmelos*, medicinal plants which are of great use with both roots, leaves and fruits in various medicinal ways [28].

Previous studies of *Aegle marmelos* leaf extract were on, in vitro proliferation of K562, [29] Melanoma Colo38 [30], Breast cancer MCF7 [31], and MDA-MB-231 [32] human cell lines. Previous papers allow us to identify antitumor compounds including cisplatin, chromomycin, cytosine arabinoside and 5-fluorouracil [[24,33].

As the outcome was positive, we could observe from the present study that *Aegle marmelos* leaf extract can be used for treating cancer, not only skin cancer but also all other cancers [34]. From the results observed above, we say that as the concentration of the drug increases there is a significant decrease in the cell viability of the cancer cells. This leaf extract acts on p53 mRNA expression [35,36]. Thus this drug can be further assessed in the future study and with clear approval it can be used on cancer patients. These

natural medicines have no or less side effects in the long term effect on human life [37-38].

5. CONCLUSION

From the above study and the supporting articles, we can conclude that *Aegle marmelos* has an effect on anti apoptotic markers in human melanoma cell lines (A375), not only anticancer activity, but also other medicinal values that could be used in the future generation. In a clear view, the hydroethanolic leaf extract of *Aegle marmelos* on the expression of anti apoptotic markers in human melanoma cell lines has a positive effect. Thus this drug could be used for further study and can be made as an anti-cancer drug which will be useful for cancer patients.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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