RESEARCH PAPER

EFFECTS OF AEGLE MARMELOS LEAF EXTRACT OF INFLAMMATORY SIGNALLING MOLECULES IN COLON CANCER CELLS IN VITRO (HT-29)

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ABSTRACT

Aegle marmelos commonly used in India as Ayurvedic medicine. Medicinal plants like *Aegle marmelos* contain phytochemicals such as phenols, flavonoids, alkaloids, cardiac glycosides, saponins, etc. inhibit the proliferation of tumor cells, apoptosis is induced. Colon cancer affects all age groups majorly especially the older people who are more prone to this cancer. Risk factors of colon cancer are poor diet, tobacco smoking and heavy alcohol consumption. Often have no symptoms but can be detected by screening. Metastasis causes the majority of deaths. The parts of the plant have many therapeutic potentials such as anticancer, antimicrobial, antidiabetic, etc. The aim of the present study is to analyse the effects of *Aegle marmelos* leaf extract of inflammatory signalling molecules on colon cancer cell lines. The anticancer activity HT-29 human colon cancer cell line was studied by MTT assay. Effects of *Aegle marmelos* leaf extraction on Interleukin-6 mRNA expression and TNF-α mRNA expression was studied by real time PCR. Statistical analysis was done using Graphpad prism. $P<0.05$ was considered significant. It was found that there was a significant reduction of Interleukin-6 mRNA expression and TNF-α mRNA expression. It is concluded that *Aegle marmelos* has potential anticancer activity due to reduced expression of inflammatory cytokines.

KEYWORDS: Ayurvedic medicine, Interleukin-6 mRNA, TNF-α mRNA, apoptosis, inflammatory cytokines, Innovative techniques.

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INTRODUCTION :

Colon cancer affects all age groups majorly, especially the older people who are at high risk of this cancer (1)(2). Some risk factors of colon cancer are old age, poor diet, inflammatory intestinal conditions and inherent syndrome that increases the risk of colon cancer(3)(4). Cancer often shows no symptoms only detected by screening (5),(6). Typically this cancer is treated at an early stage by surgery (7). Colonoscopy and biopsy confirmation is the diagnosis of colon cancer (8).

Aegle marmelos commonly known as bilwa, bael, golden apple, wood apple, ect (9). English name is Stone apple (10) . The

nativity of this species is the Indian sub-continent and southeast Asia (11). This species belongs to the family Rutaceae (9). Since 800 BC bael has existed in India. "This species shows higher degree of variation in vegetative, reproductive, yield and phytochemicals characters (12). *Aegle marmelos* are exported to Malaysia and Europe (11). The therapeutic potential of Agele marmelos fruits have antioxidant and antifungal activity (13)(14)). The therapeutic potential of *Aegle marmelos* of leaves are antimicrobial, antidiabetic, hepatoprotective and cytoprotective (13)(15). The stem bark has antibacterial and antiproliferative properties. Antidiarrheal activity is possessed by dried fruit pulp of *Aegle marmelos.* The fruit pulp and callus has anti-inflammatory and antipyretic properties (16). Still more than 60 years old there is no permanent cure for cancer (17) (18). Many chemotherapeutic drugs of cancer lead to side effects though there is not even a permanent cure for cancer (19) (20).

Our team has extensive knowledge and research experience that has translated into high quality publications(21–23)(4,10,24–27),(28)(29),(30)(31),(32)(33)(2,6,34–36)

The aim of the present study is to analyse the effects of *Aegle marmelos* leaf extract of inflammatory signalling molecules on colon cancer cell lines.

MATERIAL 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).

Cell lines and cell culture

Human colon cell line(HT-29) was purchased from the National Centre for Cell Sciences (NCCS), Pune, India. Cells were cultured in DMEM 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.

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, HT-29 Human colon cell lines (1 ×104/well) were exposed to different concentrations of Aegle Marmelos leaf extract (100- 500µg) with HT-29 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. The crystals formed were dissolved in dimethyl sulfoxide (100 µl) 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.

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 (Tamara, USA) and 9µl ddH2O. Reaction 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−ΔΔCT method described bySchmittgen and Livak (2008) using CFX Manager Version 2.1 (Bio Rad, USA).

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 (Graphpad PadPrism 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.

RESULT:

Cell viability was studied by MTT assay. The result showed that there was maximum inhibition of cancer cells at 300 and 400 µg suggesting the cytotoxic effect (p <0.05) (Figure 1). Effect of *Aegle marmelos* leaf extract IL-6 mRNA and TNF-α mRNA expression result showed that extract has significantly decreased the mRNA expression of IL-6 mRNA and TNF-α mRNA in cancer cells (p <0.05) (Figure 2 and 3).

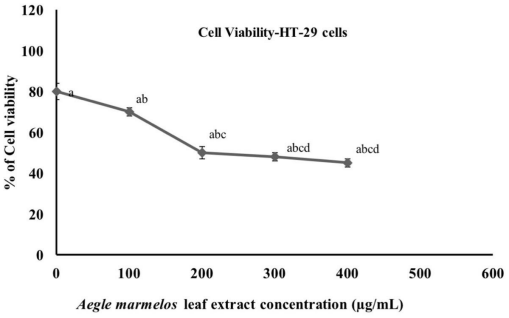

Figure 1: The graph represents the effect of *Aegle marmelos* leaf extract cell viability inHT-29cells. 'X' axis represents the percentage of cell viability and 'Y' represents the *Aegle marmelos* leaf extract concentration. Cell viability of HT-29 cells decreases with increase in concentration of the *Aegle marmelos* leaf extract. Each bar represents a mean ± SEM of 6 observations. Significance at p< 0.05, a-compared with untreated control cells, b- compared with 100µg treated HT-29 cells.c-compared with 200µg treated cells.

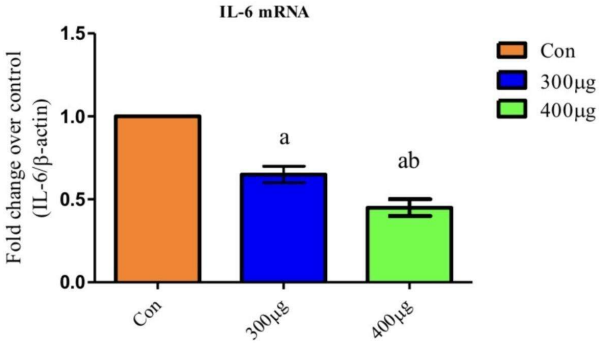

Figure 2: The graph represents the effect of *Aegle marmelos* leaf extract IL-6 mRNA expression in HT-29cells. 'X' axis represents the Fold change over control and 'Y' represents the *Aegle marmelos* leaf

extract concentration. Orange colour denotes control, Blue color denotes 300µg and green denotes 400µg. Each bar represents a mean ± SEM of 6 observations. Significance at p< 0.05, a-compared with untreated control cells, b-compared with100µg treated HT-29 cells.

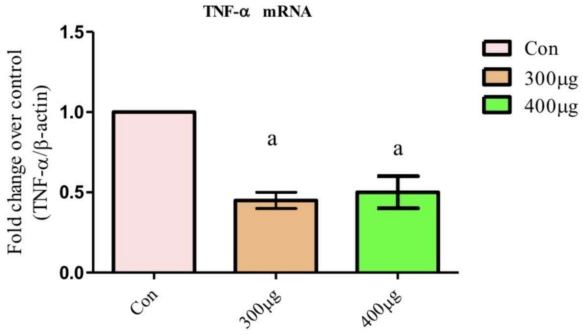

Figure 3: The graph represents the effect of *Aegle marmelos* leaf extract TNF-α mRNA expression in HT-29 cells.'X' axis represents the Fold change over control and 'Y' represents the *Aegle marmelos* leaf extract concentration. Pink colour denotes control, Sandal color denotes 300µg and green denotes 400µg. Each bar represents a mean ± SEM of 6 observations. Significance at p<0.05, a-compared with untreated control cells, b-compared with 100 µg treated HT-29 cells.

DISCUSSION:

Colon cancer typically affects older adults. Early causes can begin as non-cancerous polyps. Common treatments include surgery to remove the cancer, chemotherapy and radiation therapy. The present study was investigated to analyse the anticancer effect of *Aegle marmelos* leaf extract. The present findings clearly indicate that *Agele marmelos* leaf extract significantly reduces the expression of inflammatory cytokines such as TNF-α and Interleukin-6 and this study suggested that *Agele marmelos* has potential anticancer activity. This might be due to the bioactive compounds in the leaf extract which might reduce the inflammation mediated signaling in colon cancer cells. In this regard it has been recorded that *Agele marmelos* has anticancer activity against various cancers. Leaf extract of *Aegle marmelos* has the ability to induce apoptosis in human colon cancer cell line HT-29 (18) (37).

Previously it has been studied that *Aegle marmelos* have a molecular mechanism of eugenol-induced apoptosis in human colon cancer cells (38) which is similar to the present study. *Allium sativum* root extract and *Camellia sinensis* leaf extract induce cell apoptosis by two different mechanisms. *Allium sativum* causes inhibition of PI3K/Akt pathway upregulation of PTEN downregulation of Akt and pkt expression. Previously various plants have been studied to understand its anti cancer effect. *Camellia sinensis* leaf extract involved in attenuation of COX-2 expression and modulation of NFκB, AP-1, CREB and NF-IL-6 and hence possessed an anticancer effect (39,40)(41).

Many inflammatory cytokines that prevent the tumour induced cells from causing cancer. NF-κB is an inflammatory cytokine which, on expression of its own repressor, IκBalpha, inhibits NF-κB and forms an auto feedback loop(31)(32) . Tumor proliferation stops by blocking NF-κB, becoming more sensitive to the action of anti-tumor (39).

Biofunctionalization of SNPs employing bioactive compounds found in the plant extract can improve the anticancer effects of *G. sylvestre* bioactive compounds without compromising their therapeutic characteristics (42)(43). Pomegranate fruit indicates the anticancer activity induced by Akt by inhibiting the expression of TNF-α-inducing proteins (Tipα) in the COX-2 pathway in cancer cells (44). This study was similar to our present findings that increased concentration showed reduced TNF-α mRNA expression .This research was done only on leaf extract of *Aegle*

marmelos to show inflammatory signalling molecules. Further studies required to prove which part of the plant has a potential effect on colon cancer.

CONCLUSION:

It is concluded from the present findings that *Aegle marmelos* has potential anticancer activity in colon cancer cells as a result of reduced expression of inflammatory cytokines and hence the *Aegle marmelos* can be used as a therapeutic natural drug for the treatment of colon cancer.

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CONFLICT OF INTEREST:

All the authors declared that there was no conflict of interest in the present study.

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AUTHORS CONTRIBUTION

Vaishali : Literature search, data collection, Analysis, manuscript drafting.

Gayatri Devi : Data Verification, Manuscript drafting.

Jothi Priya: Data verification,manuscript drafting.

Selvaraj: Data verification,manuscript drafting.

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