RESEARCH PAPER

Effect of Aegle marmelos on the Expression of Matrix Metalloproteinase of Human HepG2 Cells

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ABSTRACT

Introduction

Aegle marmelos commonly known as Bael belongs to the Rutaceae family. It is used in the treatment of stomach ache, dysentery and cardiac ailments. It is known to have analgesic, antineoplastic and chemoprotective activity. Liver cancer is a type of cancer that starts from the liver cells (hepatocytes). The main aim is to evaluate the effect of Aegle marmelos on the expression of matrix metalloproteinase of human HepG2 cells of liver cancer cell line.

Materials and methods

HepG2 cells were procured from NCCS, Pune and were grown in Dulbecco's Modified Eagle Medium (DMEM). Different doses of plant extract were added and kept for 24 hrs for the cells to get trypsinized. Total RNA is then isolated from HepG2 cells. cDNA is then synthesized using reverse transcription by commercially available RT-PCR kit. Ten microliters of cDNA will be used for the amplification of MMPs using gene specific primers by commercially available RT-PCR kit. Untreated HepG2 cells were compared with cells treated with the extract of various concentrations (100-500 micrograms).

Results

On assessing the cell viability of HepG2 cells, it was found that as the concentration of the extract increased, the percentage of cell viability of HepG2 cells decreased. Down regulation of m-RNA expressions of MMP-3 and MMP-9 enzymes is found to be significant when the extract concentration is around 400-500 micrograms.

Conclusion

From this study, it was proven that Aegle marmelos has effects on MMP expression in HepG2 cells of the liver cancer cell line. Therefore it can be used in drug formulation to treat liver cancer as it has less side effects and is easily available.

KEYWORDS: Aegle marmelos, HepG2 cells, liver, cancer, cell viability, Innovative technology

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INTRODUCTION:

Aegle marmelos commonly known as Bael which belongs to the Rutaceae family. It is found in the Indian subcontinent and Southeast Asia. It is used in treatment of stomach ache, dysentery and cardiac ailments (1) (2). Liver cancer is a type of cancer that begins from the cells (hepatocytes). Liver cancer is classified into several types. Out of all types, "hepatocellular carcinoma" is the most common type of liver cancer which begins from hepatocytes (3) (4). Other forms of liver cancer include intrahepatic cholangiocarcinoma and hepatoblastoma. Causes for liver cancer can be due to mutations in DNA, chronic hepatitis infection, cirrhosis etc. Treatments for liver cancer include hepatectomy, liver transplant, chemotherapy, radiotherapy etc. *Aegle marmelos* are proved to possess pharmacological properties such as antidiarrheal, antimicrobial, antiviral, radioprotective, anticancer, antipyretic and ulcer healing properties (5) (6). *Aegle marmelos* is found to possess phytochemicals such as marmol , marmin, marmelosin , marmalade , psoralen etc. Extract of *Aegle marmelos* is prepared by boiling the bark, leaves or roots. The extract is found to contain analgesic, astringent, antidiarrheal, anti-inflammatory properties. The extract is also used in treating ophthalamia, deafness, inflammation, Diabetes etc. The fruits are used in treating diarrhea, dysentery and stomach ache. It is also found to possess antineoplastic, chemoprotective and chemopreventive properties which are used in treating

and preventing cancer (7). Anti Inflammatory property of *aegle marmelos* was proved by testing it on albino rats where the aqueous extract of root bark of Bilwa (*Aegle marmelos)* was prepared and used (8) (9). Hypoglycemic and antihyperglycemic activity of *Aegle marmelos* seed extract was proved in normal and diabetic rats (10) (11). *Aegle marmelos* is proven to have protective effects against gastrointestinal disorders as it contains biologically important phytochemicals such as limonene , linalool, cineole, citronell, marmelin etc (12) (13). Unripe fruit of *Aegle marmelos* is proven to possess antidiarrheal activity. Decoction of A.marmelos can control several types of diseases caused by EPEC(enteropathogenic *Escherichia coli*), LT(labile toxin) producing ETEC(enterotoxigenic *E.coli*), EIEC(enteroinvasive *E.coli*), V.Cholerae, S. flexneri and it also controls giardiasis and rotaviral infections to some extent (14) (15). *Aegle marmelos* is proven to possess antidiabetic activity and its relationship with its antioxidant properties where the methanolic extract of the herb was administered in diabetic rats (16) (17)
(18). The main aim is to evaluate the effect of *Aegle marmelos* on the expression of matrix metalloproteinase of human HepG2 cells of liver cancer cell line.
Our team has extensive knowledge and research experience that has translated into high quality publications (19–21) (22),(23)(24)(25–27)(25–29)
Thus by proving this effect of *Aegle marmelos*, we can introduce this in drug formulation and reduce the risks of side effects caused by various standard treatments such as chemotherapy and radiotherapy**.**

MATERIALS AND METHOD:
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 purchasedTAKARA (Meadowvale Blvd, Mississauga, ON L5N 5S2, Canada).

Cell lines and cell culture
Human liver cell line(Hep G2) was procured from 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% CO_2.

Cell viability by MTT assay
Cell viability was assayed using a modified colorimetric technique which is based on the ability of live cells to convert MTT, a tetrazolium compound into purple formazan crystals by mitochondrial reductases (Mosmann, 1983). Briefly, Hep G2 Human colon cell line(1×10^4/well) were exposed to different concentrations of *Aegle Marmelos* fruit extract(100-500µg) with Hep G2 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 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 Super Script 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 quality control purposes, melting curves were obtained for all the 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).

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.

RESULT:
On assessing the cell viability of HepG2 cells, it was found that as the concentration of the extract increased (from 100-400**µ**g), the percentage of cell viability of HepG2 cells decreased (from 80-40%)(Figure 1). Down regulation of m-RNA expressions of MMP-3

and MMP-9 enzymes is found to be significant when the extract concentration is around 400-500 micrograms (Figure 2, Figure 3).

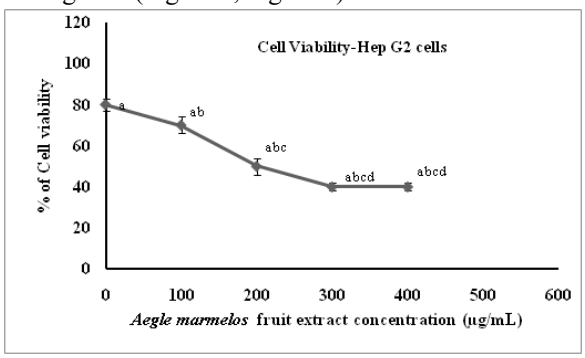

Figure 1 represents assessment of cell viability: Effect of *Aegle marmelos* fruit extraction cell viability of HepG2 cells. Each bar represents a mean SEM +/- of 6 observations. X axis denotes the concentration of extract and Y axis denotes the percentage of cell viability. Significance at P<0.005 , a-compared with untreated control cells , b-compared with 100 micrograms treated HepG2 cells , c-compared with 200 micrograms treated cells.

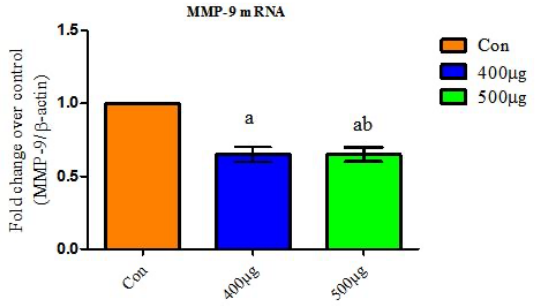

Figure 2 represents MMP-9 mRNA expression (fold change over control).Orange colour denotes control, Blue color denotes 400**µ**g, Green colour denotes 500**µ**g. X axis denotes the group and the y axis denotes the MMP -9 mRNA expression. Effect of *Aegle marmelos* fruit extraction on MMP-9 mRNA expression in HepG2 cells. Each bar represents mean +l- SEM of 6 observations . Significance at P<0.005, a-compared with untreated control cells , b-compared with 100 micrograms treated HepG2 cells.

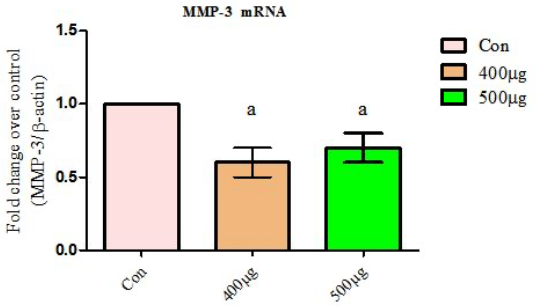

Figure 3: Bar graph represents MMP-3 mRNA expression (fold change over control).Pink colour denotes control, Brown color denotes 400**µ**g,Green colour denotes 500**µ**g**.** X axis denotes the group and the y axis denotes the MMP-3 mRNA expression. Effect of *Aegle marmelos* fruit extraction on MMP-3 mRNA expression in HepG2 cells . Each bar represents +/- SEM of 6 observations . Significance at P<0.005 , a-compared with untreated control cells , b-compared with 100 micrograms treated HepG2 cells.

DISCUSSION

Aegle marmelos induced cell growth and down regulation of MMP expression in HepG2 cells might be due to the presence of bioactive compounds present in the plant extract. In accordance with the present study the plant extract has been shown to have anticancer activity in other types of cancers such as colon cancer, breast cancer, prostate cancer etc.

Previous study carried out on effects of methanol leaf extract of *Costus speciosus*, it was found that the decrease in percentage of cell viability is significant when the concentration of the extract is 100 microgram/ml (30) (31), whereas in the present study the decrease in the percentage of cell viability is significant when the concentration of the extract is around 300-400 micrograms/ml. Previous study on apoptosis in Hepg2 cells using *Evolvulus alsinoides* (methanolic extract) the Ic50 value obtained was 80 microgram/ml (32)(33),whereas in the present study the Ic50 value obtained was 200 microgram/ml. Antitumor activities against HepG2 cells by some selected desert plants it was found that the Ic50 value of *O.Dayi* is 1mg/ml and Ic50 value of *O.baccatus* is 1.5mg ml (34) (35) whereas in this study the Ic50 value obtained was 200 micrograms/ml. Antitumor effect on vernolide, vernolipin and vernolide of *Vernonia extensa* plant , the Ic50 value obtained was at the range of 0.91-13.84 microM (36) (37), whereas in this study the Ic50 value obtained was 200 micrograms/ml.

The limitation of this study is anticancer and antiproliferative activity of the fruit extract alone was analysed. Furthermore studies can be done to check the benefits of other parts of the plant.

CONCLUSION:

From this study, conclude that *Aegle marmelos* have effects on MMP expression in HepG2 cells of the liver cancer cell line and hence can be used as an anticancer drug. Since it is nontoxic and has less side effects it can be used in drug formulation to treat liver cancer instead of other treatments such as chemotherapy and radiotherapy which exhibit a wide range of side effects such as nausea, fatigue and hair loss.

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Authors contributions:
Sadhana.k :literature research, data collection , analysis , manuscript drafting.
Gayatri Devi : Data verification ,manuscript drafting.

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