ANTICANCER ACTIVITY OF AEGLE MARMELOS ON HUMAN HEPG2 CELLS BY REGULATION OF MATRIX METALLOPROTEINASES EXPRESSION

Running title: Role of Aegle marmelos against liver cancer.

ABSTRACT:

Background: Aegle marmelos belong to the family rutaceae. It is commonly known as Bael. It is used in traditional medicine, as it has antidiarrheal, antimicrobial, antiviral, radio-protective, anticancer, chemo-preventive properties which are of great medicinal use. Liver cancer is the 5th most common type of cancer, Hepatocellular carcinoma is the most common form, which originates from the liver. Aegle marmelos is said to inhibit the proliferative action of cancer cells.

Objective: To investigate the role of Aegle marmelos against human liver cancer cells (HEP G2 cell line) by inhibiting the activity of matrix metalloproteinases which is responsible for spread of cancer.

Materials and methods: HEP G2 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 dunken's multiple test. Corresponding graphs are also plotted.

Results: The viability of the cells decreased from 100% to 50%. The mRNA expression of MMP-2 and MMP-9 decreased after the addition of the extract.

Conclusion: From this study we can conclude that Aegle marmelos, a novel innovative anticancer drug inhibited the proliferative action

of liver cancer cells by reducing the expression of MMP 2 and MMP 9 and possesses anti-cancer activity.

Key Words: Aegle marmelos, novel, innovative Hepatocellular carcinoma, mRNA expression, proliferative, viability.

INTRODUCTION

Cancer has now become one of the leading causes of death globally. Various researches on inhibiting cancer cell proliferation are carried out globally[1]**.Cancer begins when healthy cells are mutated and grow out of control-Metastasis.**[2][3]**. There are various types of liver cancers, hepatocellular carcinoma is one of which that originates in the liver unlike other types where the origin is not liver.**[4][5][6]

Primary liver cancer is of 3 types out of which hepatocellular carcinoma is the most common type (75%)[7]**.HEP G2 cell lines are liver cancer cell lines obtained first from an argentinian male who had hepatocellular carcinoma.**[8]**. These cells are epithelial in morphology, they are suitable in vitro polarized human hepatocytes for study purposes. These cells have high degrees of morphological and functional differentiation.**[9][10]

Aegle marmelos inhibit the in vitro proliferation of tumor cell lines like leukemia K562, T Lymphoid Jurkat, Melanoma col 038, this is compared to the anti tumor agent 5-fluorouracil.[11,12]**Aegle marmelos also possess chemopreventive potential and has antitumor effects.**[13]**.**[5]**Methanolic extracts of Aegle marmelos when evaluated against cisplatin which might induce renal toxicity in rats shows that this plant extract possesses nephroprotective and antioxidant property**[6][14]**.It also reduces murine ascites which reduce tumor volume and a viable tumor is prophylactically.**[15][16,17] Our team has extensive knowledge and research experience that has translate into high quality publications[18–22][23–27]

A Bael compound named skimmianine extracted from leaf and immature barks has anticancer property , analgesic property and even anti diuretic property.[28][29] **The**

fruits also have antiviral properties with essential vitamins like vitamin A, vitamin C, riboflavin niacin.[30]**. In the view of the wide ranging pharmacological activity this study was conducted to investigate the role of Aegle marmelos in prevention of liver carcinogenesis.**[31][16]

MATERIALS AND METHODS:

In order to test the anticancer property of aegle marmelos a cancer cell line is procured and it is treated with varied doses of the leaf extract of aegle marmelos, then the viability of cancer cells is analysed using MTT assay. A decrease in viability would indicate the effectiveness of the drug.The detailed explanation of the tests performed and chemicals used is described below.

Cell lines and cell culture:

The Human Liver cell line (Hep G2) 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% CO_2.

Cell viability by MTT assay:

Cell viability was analysed using a modified colorimetric technique that is based on the ability of living cells to convert MTT, a tetrazolium compound into purple formazan crystals by mitochondrial reductases which is present in every living cell (Mosmann, 1983). Briefly, the cells (1 ×104/well) were exposed to different concentrations of Aegle marmelos extract (100-500µg/ml) with HepG2 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 formazan crystals formed were dissolved in dimethyl

sulfoxide (100 µl) and incubated in the dark for an hour. Then the intensity of the colour developed was assayed using a Micro ELISA plate reader at 570 nm. The number of cells that were viable was expressed as the percentage of control cells cultured in serum-free medium. Cell viability of the cells in control medium without any treatment was represented as 100%. The cell viability can be calculated using the following formula: % cell viability = [A570 nm of treated cells/A570 nm of control cells] × 100.

Gene expression analysis by Real Time-PCR:

Samples obtained from each group were submerged in 2 ml Trizol (Invitrogen, Carlsbad, CA, USA) for the purpose of 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 checking quality control, melting curves were obtained for all samples. The specificity of the amplification product was determined by analysing the melting curve for each 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).

The following chemicals 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).

Table 1: Primer Sequence

S.No	Gene	Primer sequence
2	Human MMP-2	Forward: 5′-ACC TAC ACC AAG AAC TTC CG-3′ Reverse: 5′-TTG GTT CTC CAG CTT CAG GT-3′
3	Human MMP-9	Forward:5′-TCC CTG GAG ACC TGA GAA CC-3′ Reverse: 5′-TCC CTG GAG ACC TGA GAA CC-3′
4	Human β-actin	Forward:5'-CTACAATGAGCTGCGTGTGG -3' Reverse: 5'TAGCTCTTCTCCAGGGAGGA-3'

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:

From the study we can infer that the viability of 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 extract was 300 to 500 micrograms.(figure 1).The fold changes over control of the mRNA expression of MMP-2 decreases significantly on the addition of Aegle marmelos extract.(Figure 2). The fold changes over control of the mRNA expression of MMP-9, decreases significantly on the addition of Aegle marmelos extract.(figure 3)

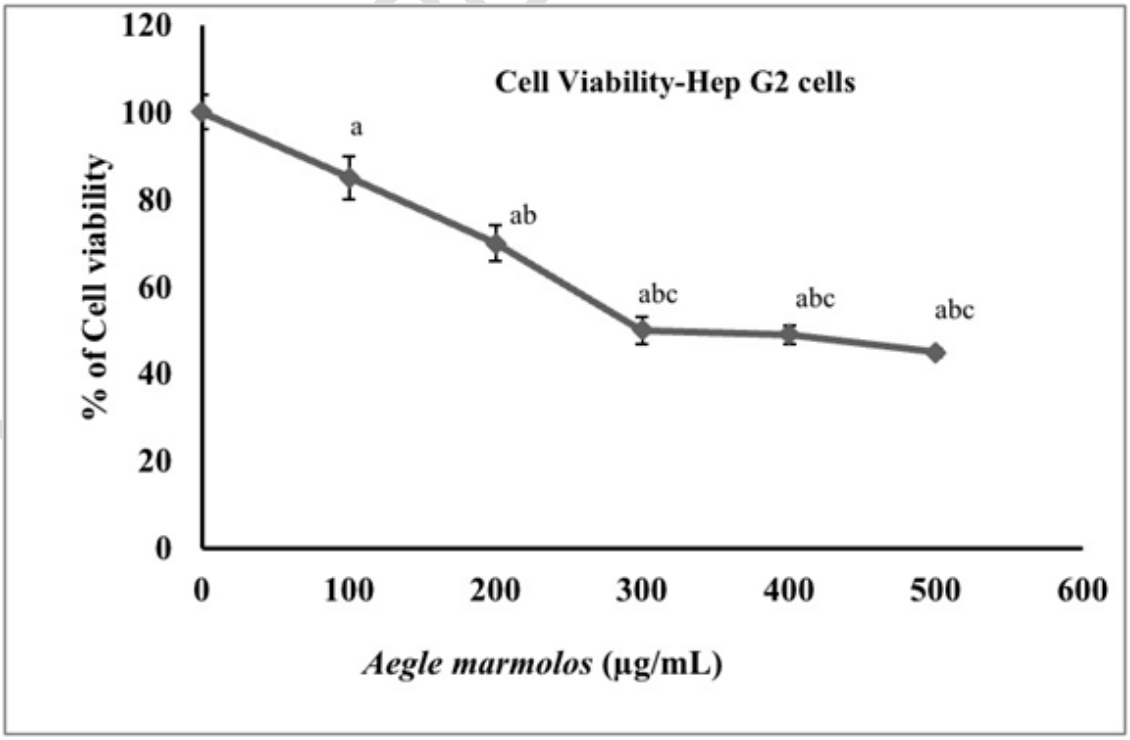

Figure 1 represents the effect of *Aegle marmelos* leaf extraction cell viability in HepG2 cells. Each bar represents a mean ± SEM of 6 observations. Significance at p< 0.05, a-compared with untreated control cells, b-compared with 100µg treated HepG2 cells. X axis represents the concentration of extract and Y axis represents the percentage of cell viability. There is a significant decrease in the cell viability based on the dosage.

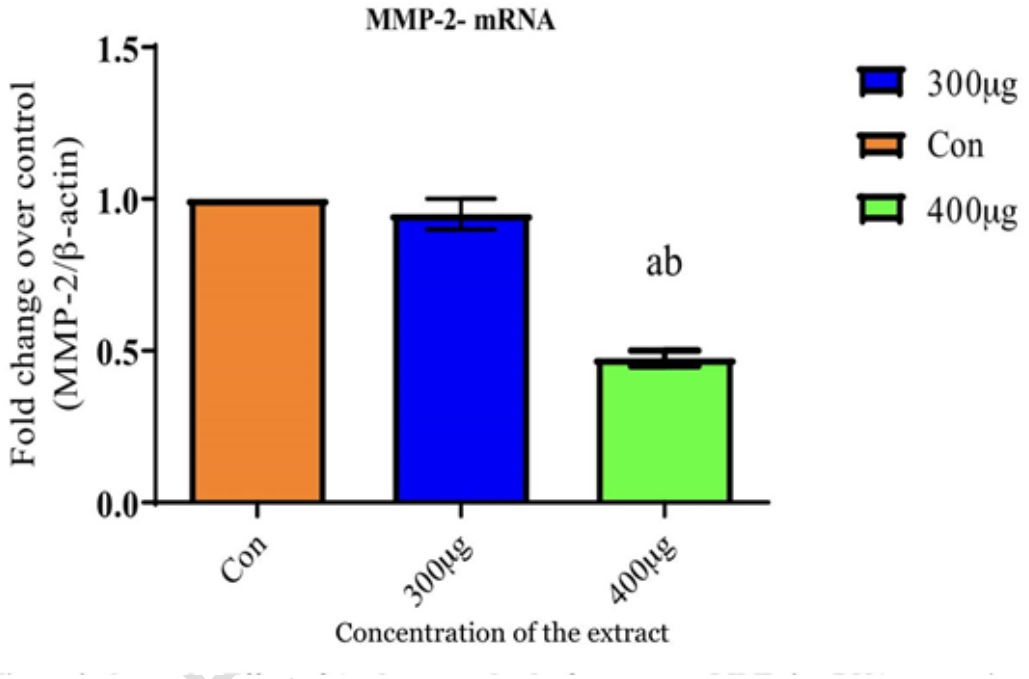

Figure 2 shows the Effect of Aegle marmelos leaf extract on MMP-2 mRNA expression in HepG2 cells. Each bar represents a mean ± SEM of 6 observations. Significance at p< 0.05, a-compared with untreated control cells, b-compared with 300µg treated cells.X axis represents the concentration of extract added and Y axis represents the fold change over control of the mRNA expression of MMP-2.There is a significant reduction in the mRNA expression of the MMP 2 based on the dosage.

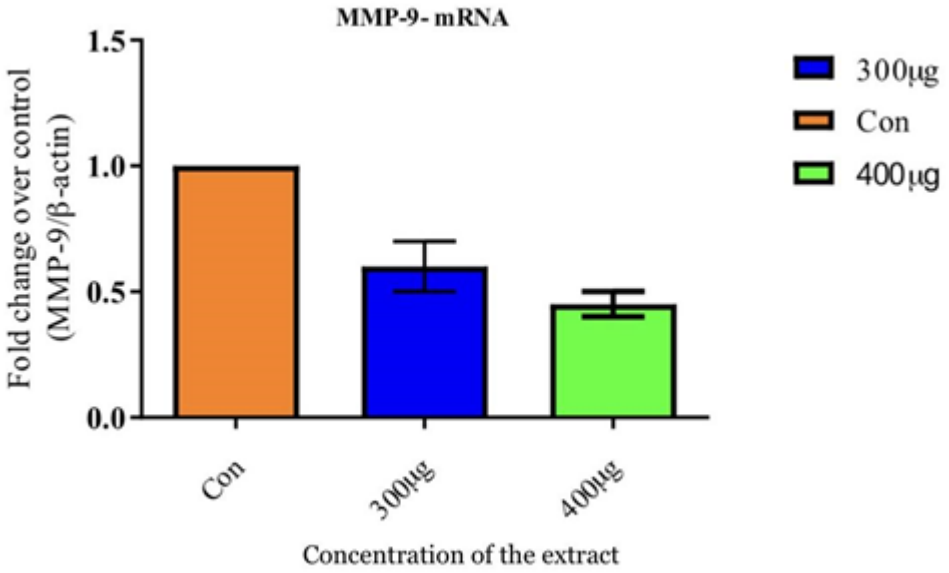

Figure 3 represents the effect of Aegle marmelos leaf extract on MMP-9 mRNA expression in HEPG2 cells. Each bar represents a mean ± SEM of 6 observations. Significance at p< 0.05, a-compared with untreated control cells. a-compared with untreated control cells, b-compared with 300µg treated cells. X axis represents the concentration of extract added and Y axis represents the fold change over control of the mRNA expression of MMP-9. There is a significant reduction in the mRNA expression of the MMP 9 based on the dosage.

DISCUSSION

Hepatocellular carcinoma is placed fifth in overall worldwide cancer rates and third cause of death in the world[32]**.Anticancer substances present in the diet is an attractive strategy to inhibit various cancers including liver cancer.**[33]**.Aegle marmelos fruit, stem and leaf extracts in diet can also have similar effects.**[34]

Plant phenols may be considered as potential compounds for selective blocking signal transduction pathways, this is similar to this study where the Aegle marmelos extract blocks the MAPK pathway which inhibits the maturation of the MMPs.[35]

Several medicinal plants prescribed as a constituent of liver protective herbal drugs have been shown to inhibit chemically induced hepatic carcinogenicity in experimental animals.[36]**. Aegle marmelos inhibit the in vitro proliferation of tumor cell lines like leukemia K562,**[37,38]**breast cancer cells, and jurkat cells similar to the findings of this study where it inhibits the human HEPG2 cell lines**[39]**.**

Aegle marmelos also have chemopreventive potential, antitumor , reducing tumour volume and size.They also possess nephroprotective and antioxidant activity.[40]**The phytochemical profile of Aegle marmelos show pharmacological activity and hepatoprotective activity.**[41]

In this study Aegle marmelos plant extract inhibit the MMPs which causes degradation of proteins in the extracellular matrix,[37]**degrades outer membrane of cells, degrades basement membrane so that cancer cells easily enter lymphatic and blood vessels , release growth factors for easy proliferation of cancer cells.**[42]**This effect is similar to the findings of**[43] **which discusses prevention of cancer by Aegle marmelos.**[44]

Limitation of this study is, only when done invivo it will give accurate results but before that advanced research needs to be done, various steps of drug testing needs to be approved. In vitro results have proven to be positive and the future scope of this study is of high significance against cancer treatment.

CONCLUSION;

From this study we can conclude that Aegle marmelos help in inhibiting the proliferation of HEP G2 cell line of hepatocellular carcinoma. It prevents the

maturation of MMPs which reduces the viability of cancer cells. The mRNA expression of MMPs has decreased in folds after being treated with the plant extract. Liver cancer can be treated using Aegle marmelos extract.

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