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AEGLE MARMELOS AND *IN VITRO* ANTICANCER MODELS - AN OVERVIEW

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ABSTRACT

Tremendous progress has been made in basic cancer biology and in the development of novel cancer treatments; cancer remains a leading cause of death in the world. Over the last few years, researchers have aimed at identifying and validating plant derived substances for the treatment of various diseases. There have been vast discoveries of potent cytotoxic agents attributed to Asian and Ayurvedic Indian traditional medicine. By increasing research into herbal drugs, there is a hope to identify the most promising agents and understand their many actions. The present review aims to explore medicinal values of *Aegle Marmelos,* against cancer and various diseases and also suggest some methods for screening anticancer agents.

Keywords: *Aegle Marmelos*, Cancer, Cytotoxic agents, *in vitro* anticancer activity.

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INTRODUCTION

Cancer is a general term applied to series of malignant diseases that may affect different parts of the body. These diseases are characterized by a rapid and uncontrolled formation of abnormal cells; which may mass together to form a growth or tumor or proliferate throughout the body.

Terrestrial plants have been used as medicines in Egypt, China, India and Greece from ancient time and an impressive number of modern drugs have been developed from them. The first written records on the medicinal uses of plants appeared in about 2600 BC. Medicinal plants represent a vast potential source for anticancer compounds. These compounds are extremely complex molecular structures, which would

be difficult to synthesize (conceptualize) in the laboratory. The antitumour activity of medicinal plant derived compounds may result via a number of mechanisms, including effects on cytoskeletal proteins which play a key role in cell division, inhibition of DNA topoisomerase enzymes, antiprotease or antioxidant activity, stimulation of the immune system, etc. Plants can delay or even prevent cancer onset. Plants can support the immune system, thus improving body resistance to the disease and its treatments. Plants can prevent and decrease side effects of conventional treatments. Plants can provide nutritional, as well as psychological support (1-5).

Bael (*Aegle Marmelos* Linn), family Rutacae, is also known as Bale fruit tree, is a moderate sized , slender, aromatic tree, 6.0 -7.5 m in height, and 90 to 120 cm in girth, with a somewhat fluted bole of 3.0-4.5 meter growing wild throughout the deciduous forests of India, ascending to an altitude of 1200 meter in the western Himalayas and also occurring in Andaman island. This is generally considered as sacred tree by the Hindus, as its leaves are offered to Lord Shiva during worship. According to Hindu mythology, the tree is another form of Lord Kailashnath. Leaves, fruit, stem and roots of this tree at all stages of maturity are used as ethno medicine against various human ailments. The different parts of Bael are used for various therapeutic purposes, such as for treatment of Asthma, Anaemia, Fractures, Healing of Wounds, Swollen Joints, High Blood Pressure, Jaundice, Diarrhoea Healthy Mind and Brain Typhoid Troubles during Pregnancy. The ripe fruit is a good and simple cure for dyspepsia. The pulp of unripe fruit is soaked in gingelly oil for a week and this oil is smeared over the body before bathing. This oil is said to be useful in removing the peculiar burning sensation in the soles. The roots and the bark of the tree are used in the treatment of fever by making a decoction of them. The leaves are made into a poultice and used in the treatment of opthalmia. The leaf part of the plants have been claimed to be used for the treatment of inflammation, asthma, hypoglycemia, febrifuge, hepatitis and analgesic (6-10).

Biological Uses of *Aegle Marmelos*

Anti diabetic activity

Aqueous extract of *Aegle marmelos* leaves, was evaluated for hypoglycemic and antioxidant effect by Upadhya *et al*, by using alloxon induced diabetes in male albino rats and proposed AML may be useful in the long-term management of diabetes (5).

Hepatoprotective activity

Singanan *et al* worked on *Aegle Marmelos* leaf extract on alcohol induced liver injury in albino rats and presented data of excellent hepatoprotective effects (6).

Antimicrobial activity

Maheshwari *et al*, studied on ethnolic ectract of dried fruit pulp of *Aegle Marmelos* against various intestinal pathogens i.e. *Shigella boydii, S. sonnei & S. Flexneri* and proposed that certain phytochemicals including Phenols, Tannins and Flavonoids were effective against all (7).

Analgesic, anti-inflammatory and antipyretic activity

Arul *et al*, presented anti-inflammatory, antipyretic and analgesic properties of serial extract of leaves of *Aegle Marmelos*, and presented that most of the extract caused a significant inhibition of the carrageenan-induced paw oedema and cotton-pellet granuloma in rats (8).

Antifungal activity

Patil reported the antifungal activity of ethanolic extract of the *Aegle marmelos* leaves including antidiarrhoeal, and antimicrobial activities (9).

Anticancer activity

Leticia evaluated the anticancer potential of folk medicine used in Bangladeshi and used extracts of *Aegle marmelos* for cytotoxic action using brine shrimp lethality assay; sea urchin eggs assay, and MTT assay using tumor cell lines. The extract of *Aegle marmelos* was found to exhibited toxicity on all used assays (10).

Antiulcer activity

Goel reported that oral administration of pyranocoumarin isolated from the seeds of *Aegle marmelos* Correa, showed significant protection against pylorus-ligated and aspirin-induced gastric ulcers in rats and cold restraint stress-induced gastric ulcers in rats and guinea pigs. The antioxidative properties of phenolic compounds (e.g. phenolic acids, flavonoids, quinones, and tannins) have been well

established. Polyphenols are natural antioxidants assumed to function as terminators of free radical chains or as chelators of redox-active metal ions capable of catalyzing lipid peroxidation (11).

***In vitro* models for assessing cytotoxic, antioxidant, and apoptotic activities**

MTT assay

After 48h of incubation, to each well 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added and incubated at 37° C for 4h. The medium with MTT was flicked off and the formed formazan crystals were solubilized in 100µl of DMSO. Using micro plate reader the absorbance was measured at 570 nm. The % cell inhibition was determined using the following formula (12).

% Cell Inhibition = [100- Abs (sample)/Abs (control)] x100

Calcein-AM release assay

CL-6, HepG2, and HRE cells were plated in 96-well culture plates (1 ×104 cells/well). After 24 hours of incubation, cells were incubated with various concentrations of ethanolic extract of ginger (1.95, 3.90, 7.81, 15.62, 31.25, 62.5, 125, and 250 µg/ml) at 37°C for 24 hours. 5-FU (at concentrations of 3.90, 7.81, 15.62, 31.25, 62.5, 125, 250, and 500 µg/ml) was used as positive control drug. Cells were then resuspended in complete medium and washed three times with PBS and incubated with 15 µM calcein-AM at 37°C for 30 min with occasional shaking. Calcein cellular fluorescence was read directly using a plate reader machine at excitation and emission wavelengths of 490 and 530 nm, respectively (12).

Hoechst 33342 assay

Inhibition of proliferation of CL-6, HepG2, and HRE cells by the ethanolic extract of ginger was measured by Hoechst 33342 assay. Wells of H342 plates were washed twice with 250 µl PBS and tapped dry on a tissue paper. Plates were wrapped up and stored at -20 °C for DNA quantification with Hoechst 33342. An amount of 100 µl of 0.01% SDS solution was added to each well. The plates were shaken for 30 min and then refrozen at -70 °C. The plates were thawed and 100 µl of a H342 solution (2 µg/ml Hoechst 33342, 10 mM Tris–HCl pH 7.4, 1 mM EDTA, 1 M NaCl) were added and plates were shaken at 37°C for 1 hour (in the dark). The fluorescence intensity was monitored in a plate reader machine at excitation and emission wavelengths of 355 and 460 nm, respectively (13).

Trypan blue exclusion method

Trypan blue dye assay method was carried out to evaluate the in vitro cytotoxicity potentials of both alcohol and aqueous extracts of *D. indica* L. EEDI and AEDI were dissolved in distilled water. Different concentrations (5, 10, 50, 100, 150 and 250mcg/ml) of both extracts were prepared. In a test tube, 100µl of plant extract was mixed with 800µl of phosphate buffer saline and 100µl (1X106 in 1ml) of Dalton's Ascitic Lymphoma (DAL) was added. Similar method was followed with Ehrlich Ascitic Carcinoma (EAC) cell line also. Each concentration of the extracts was tested in triplicate. All the samples were incubated at 37°C in an incubator for 30min. About 100µl of tryphan blue dye was added to all the test tubes and the number of dead cells was counted in a haemocytometer under a compound microscope. Percentage of cytotoxicity was calculated by the following formula. (14)

% dead cells = Number of dead cells/Sum of dead cells and living cell × 100

Lactate Dehydrogenase (LDH) leakage assay

LDH leakage assay was carried out using LDH cytotoxicity detection kit by Sigma Aldrich Inc., USA, according to protocol in the user's manual. To determine IC50, different concentrations of herbal extracts were incubated with 100 µl of DAL and EAC cell suspensions having 1x 106 cell /ml in 96 well plates and incubated at 37°C for 4 hrs in 5% CO_2 atmosphere. All the control and test substances were tested in triplicates and mean ± SEM of the absorbance values were recorded to calculate the cytotoxicity. LDH leakage (%) related to control wells containing cell culture medium without extracts was calculated by

[A] test / [A] control X100

Where [A] test is the absorbance of the test sample and [A] control is the absorbance of the control sample (14).

Other Parameters (13)

Cell proliferation assay

Cell proliferation assay was carried out by seeding a total of 1 × 104 cells per well in a 96-well plate. After 36 h of incubation, various concentrations of fruit

extracts were added to the wells to obtain final concentrations of 1, 10, 100 and 500 µg/ml, respectively. Control groups were mixed with DMSO to obtain a final concentration of 1 %. Doxorubicin was used as positive control. The cells were incubated for an additional 48 h, 50 µl of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (1 mg/ml, Sigma) in phosphate buffered saline (PBS) was added to each well, and incubated for 4 h at 37 °C. The medium was removed and formazan was dissolved in DMSO and the optical density measured at 590 mm using a bioassay reader (Biorad, USA)

Cell viability assay

The cells were suspended in a 96-well plate at the same concentration as was used for the cell proliferation assay and then incubated for 24 h at 37 °C with 5 % CO_2. Various concentrations of the fruit extract, as indicated above, were added to each well, with DMSO as control. After 48 h of incubation, the cells were trypsinized and viable cell counting was performed by enumerating cells which excluded trypan blue dye using a haemocytometer

Cell cycle analysis

To determine cell cycle, 1 × 106 cells were suspended in each tissue culture dish and treated with the fruit extract at final concentrations of 80 and 160 µg/ml. For MDA-MB435, cells were treated with the fruit extract at final concentrations of 60 and 120 µg/ml. DMSO (1%) was added to the control group. After treatment with fruit extract for 48 h, the cells were collected and incubated with reagents as described in the protocol of Cycle Test™ Plus DNA reagent kit (Becton Dickinson Immunocytometry System). The DNA content of the cells was measured by flow cytometry. All samples were analysed within 3 hours by FACS Carlibur using Cell Quest software (10).

Tumor growth response

The effect of MECM on tumor growth and host's survival time were examined by studying the following parameters such as tumor volume, packed cell volume, tumor cell count, viable tumor cell count, nonviable tumor cell count, median survival time and increase in lifespan.

Tumor volume and Packed cell volume

The mice were dissected and the ascetic fluid was collected from the peritoneal cavity. The volume was measured by taking it in a graduated centrifuge tube. Packed cell volume was determined by centrifuging the ascetic fluid at 1000 rpm for 5 min.

Tumor cell count

The ascetic fluid was taken in a WBC pipette and diluted 100 times. Then a drop of the diluted cell suspension was placed on the Neubauer counting chamber and the numbers of cells in the 64 small squares were counted.

Viable and nonviable tumor cell count

The cells were then stained with trypan blue (0.4% in normal saline) dye. The cells that did not take up the dye were viable and those which took the stain were nonviable. These viable and nonviable cells were counted.

Percentage increase in life span

The effect of MECM on tumor growth was monitored by recording the mortality daily for 6 weeks and percentage increase in life span (%IMST) was calculated. An enhancement of life span by 25% or more was considered as effective antitumour response.

IMST (%) = [(Median survival time of treated group/ Median survival time of control group)-1]X100

Median Survival Time (MST) = [Day of first death + Day of last death]/ 2

Hematological studies

RBC, WBC counts and estimation of hemoglobin was done by standard procedures from the blood obtained intracardially

Hemoglobin estimation

0.1ml of heparinized blood was taken in Sahli's Hemoglobinometer and diluted with 0.1N HCl until the color matched with standard. The reading was then taken from the graduated cylinder and expressed as g/100ml of blood (14).

Counting of erythrocytes

The blood sample was diluted (1:200) with the diluting fluid using Thoma pipette. After vigorous mixing, a drop of resultant mixture was discharged under the cover glass of Neubauer hemocytometer and the corpuscles were allowed to settle for 3 minutes. The number of erythrocytes in 80 small squares was

counted under light microscope. The number of cells in 1 cumm of undiluted blood was calculated (15).

Total count of leukocytes

Blood was diluted 1:20 with a diluting fluid. The Neubauer hemocytometers were filled with the mixture and the number of cells in four corner blocks (each block subdivided into 16 squares) was determined and the total leukocyte count per cumm of blood was calculated (16).

CONCLUSION

It is evident from this review that *Aegle marmelos* contains a number of phytoconstituents which reveals its uses for various therapeutic purposes. To date, crude drugs have displayed an important role in the development of new anticancer drugs. However, evaluation of crude drugs up until now has been narrowly focused on the plants and seafood. Ultimately the vast potential of this class of agents were needed to improve cancer therapy. This review helps researchers to develop anticancer therapy of herbal drugs by using various *in vitro* methods, which has been discussed.

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