



**RESEARCH ARTICLE**

***In -Vitro* Anticancer Activity of Ethanolic Extract of Leaves of *Aegle marmelos* in HT 29 Human Colon Cancer Cell Lines**

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**ABSTRACT**

The present study was designed to evaluate the in-vitro cytotoxicity activity of ethanolic extract of leaves of *Aegle marmelos* in HT 29 human cancer cell lines. In this study the extract was tested using human cancer cell lines HT-29 for its effects on cell viability, growth inhibition and cell morphology. Cell viability, inhibition were determined by XTT-assay. Morphology was studied by using DAPI staining technique. The results showed decreased cell viability and increased growth inhibition in a concentration dependant manner and also altered the cell morphology after treatment with the plant extract. The data demonstrated that ethanolic extract of leaves of *Aegle marmelos* has a potential cytotoxicity activity on HT29 cell lines.

**KEYWORDS**

*Aegle marmelos*, HT29 Human cell lines, anti cancer, XTT-assay, DAPI staining

**INTRODUCTION**

Starting from ancient period medicinal plants play a vital role in the treatment of several diseases. In traditional systems of medicine many plants were used to cure cancer. Colon cancer is the third most commonly diagnosed cancer and the second most common cause of cancer death in the US.<sup>1</sup> The incidence of colorectal cancer is higher in developed countries, with approximately half of the western population developing adenoma by the age of 70.<sup>2</sup> Medicinal plants possess valuable bioactive compounds that protects human from various complications. The World Health Organization (WHO) estimates that almost 75% of world's population has therapeutic experience with herbal drugs.

The potential of using the natural products as anti cancer drugs was recognized in 1950's by U.S Natural Cancer Institute (NCI) Since 1950 major contributions have taken for the discovery of naturally occurring anti-cancer drugs.<sup>3</sup> *Aegle marmelos* is one such plant that was used quite often in traditional system of medicine. It belongs to the family Rutaceae, and popularly known as "Bael tree". It is indigenous to India and found wild all over the Sub-Himalayan forests, in Central, and South India. The plant is reported to have multiple therapeutic properties such as anti-inflammatory, antipyretic and analgesic<sup>4,5,6</sup>, anti-diabetic<sup>7,8,9</sup>, antifungal<sup>10</sup>, antimicrobial, antibacterial and antiparasitic<sup>11</sup>, anticancer<sup>12</sup> and hepatoprotective<sup>13</sup> activity. Therefore, many folk remedies from plant source are used for the protection of cancer starting from ancient period. Hence the present work was undertaken to scientifically prove the anticancer activity of *Aegle marmelos* by an *in-vitro* study. The activity of *Aegle marmelos* leave

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extract was assessed by investigating cell viability, inhibition, observing morphological changes in cancer cells after treatment with the plant extract.

## **MATERIALS AND METHOD**

### **Collection of Plant Material**

The plant material, leaves of *Aegle marmelos* were collected from Rachakonda forest, Nalgonda district. T.S, India. The leaf of *Aegle marmelos* was authenticated by Dr. Vatsavaya S. Raju, the Botanist of Kakatiya University, Warangal. TS, India.

### **Preparation of Extract**

The collected plant material was shade dried and it was powdered using mixer grinder. Care was taken to avoid fungal contamination while drying. The dried plant material was subjected to cold extraction by soaking 100g of dry powder in 200ml of distilled ethanol for three days at room temperature with occasional shaking. Then it was filtered using whatman filter paper No. 1 (125mm) and the filtrate was evaporated to dryness in an evaporating dish at room temperature to obtain a semi solid substance<sup>14</sup>. Then the dried extract was weighed and stored in an air tight container for anti-colon cancer anticancer screening.

### **Drugs and Chemicals**

XTT Sodium salt [2,3-Bis(2-Methoxy-4-Nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide inner salt], Phenazine methosulphate (PMS) and Triton-X, (toxic substance to the cell lines as reference) were obtained from Sigma Chemical Co., St. Louis, MO, USA. DMEM (Dulbecco's Modified Eagles Medium), FBS (Foetal Bovine Serum) Medium was from Invitrogen, Carlsbad, CA, USA. All other reagents were used of analytical grade.

### **Cell Cultures**

HT 29 (human colon cancer cell lines), were obtained from Biotechnology department of Kakatiya University, Warangal, India. The cells were grown and maintained in Dulbecco's Modified Eagle media supplemented with LGlutamine, 10% v/v foetol bovine serum,

sodium carbonate in 75 square mm tissue culture flasks to the 60-70% confluence. The media also supplemented with 100 µg/ml streptomycin (Invitrogen) and 250 IU/ml penicillin (Invitrogen) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.<sup>15</sup>

### **XTT Cell Viability Assay**

To determine cell viability, XTT sodium salt [2,3- Bis(2-Methoxy-4-Nitro-5-sulphophenyl)-2H-Tetra zolium-5-carboxanilide inner salt] assay was performed according to the method previously described by Arung et. al.<sup>16</sup> The assay is based on the cleavage of the yellow tetrazolium salt XTT to form orange coloured formazan dye by viable cells.<sup>17</sup> Thus water soluble formazan is produced. The amount of water soluble formazan product generated from XTT is proportional to the number of living cells in the sample and can be quantified by measuring absorbance at wave length of 450nm by using microtitre plate reader (ELISA READER). Thus XTT assay provides a quantitative measurement of viable cells by determining the amount of formazan produced by metabolically active cells. In this assay cells were grown in 75square mm tissue culture flask to the 60-70 % confluency. The cells are trypsinised, counted and seeded (50 X10<sup>4</sup>/well) in 96 well microtitre plates in 100 µl of DMEM and incubated for 4 hrs at 37°C in 5% Co<sub>2</sub> for cell adherence. Then the cells were treated with predetermined concentrations of plant extract in triplicates. 0.1% Triton X treated cells were used as reference. After treatment, the plates are incubated for 24 hrs at 37°C in 5% CO<sub>2</sub>. After 24 hrs of incubation, each well was observed under microscope to see the morphological changes occurred in the cell structure. 50 µl of XTT-PMS solute was added to all the wells and incubated in a humidified atmosphere for another 4 hrs for color development. The absorbance of the color was measured at 450nm wave length using microplate reader. Percentage viability of the cells was calculated at corresponding concentrations of the sample with reference to untreated cells and also observed the viability of the cells treated with 0.1 % Triton X as reference.

### Cell Morphological Studies

To determine the morphological changes of cells DAPI staining was performed as described by Sandra et al.<sup>18</sup> Briefly the cells were seeded on glass slide and treated with EEAM for 24 hours. Untreated and treated cells were rinsed with phosphate buffered saline, fixed with ice- cold 10% tri chloroacetic acid and further washed with cold 70%, 80% and 90 % of ethanol. The cells were stained with 1 µg/ml 4- 6 – diamidino – 2 – phenylindole (DAPI) for 3 minutes cover slipped with 90% glycerol and observed under fluorescence microscope.

### Statistical Analysis

The CC50 (cytotoxic concentration) is the concentration of the toxic compound that reduces the biological activity by 50%. The CC50 value was obtained from XTT assay and calculated using Microsoft excel software. The values were expressed in geometric mean. Differences were considered to be a statically significant when  $P < 0.05$  and  $P < 0.01$ .

### RESULTS AND DISCUSSION

From MTT assay, after treatment with various concentrations of EEAM, parameters like cell Viability, growth inhibition and morphological changes were compared with untreated (control) cells. Decrease in cell viability & increase in growth inhibition by EEAM was observed on HT29 in dose dependent manner, but a significant growth inhibition was seen with 50 µg/ml of EEAM.

### Cell Morphology

Based on XTT assay results, DAPI staining was conducted to investigate EEAM induced changes in cell structures. Cells were incubated with EEAM, and morphological alterations were confirmed via fluorescence microscope. As shown in Figure 1,2,3 after 24 h of incubation with various concentrations of EEAM, many of the cells showed cytoplasmic shrinkage and loss of normal nuclear architecture, became detached and found floating in the medium. As a result, the number of cytotoxic cells increased with EEAM concentration, with the highest having the most

pronounced inhibitory effect on cell proliferation than the control.

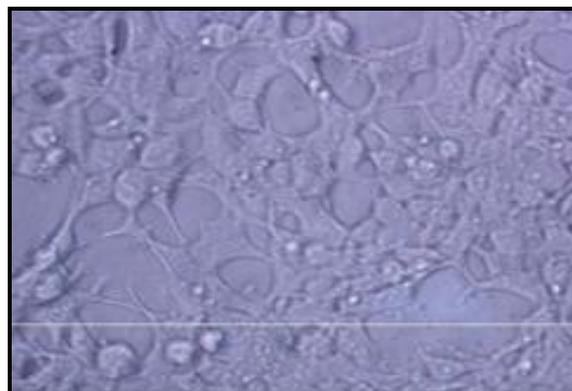


Figure 1: Untreated cells



Figure 2: Treated with 0.1% Triton-X

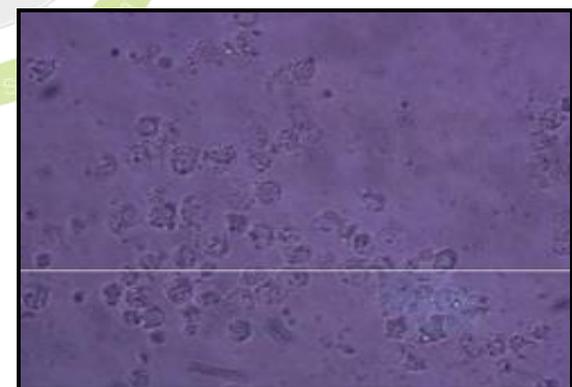


Figure 3: HT-29 cell treated with EEAM

Table 3: XTT Assay - Effect of EAM on HT29 Cell lines

Treatment	% Inhibition	% Viability
Control	0	100
Triton X	89.23	10.77

EEAM 50 µg/mL	62.45	37.55
EEAM 40 µg/mL	39.23	60.77
EEAM 30 µg/mL	10.21	89.79
EEAM 20 µg/mL	0	100.9
EEAM 10µg/mL	0	101.2

### DISCUSSION AND CONCLUSION

Various plant extracts have been evaluated and shown to have a cytotoxic effect in cancer cell lines; they include those of *Solanum iyratum* tested on human colon adeno carcinoma cell line.<sup>19</sup> *Annona globra* on human leukemia cell lines.<sup>20</sup> *Zynostemma penta phylum* on human lung cancer (A549),<sup>21</sup> 15 sukan wood and blumea babamitera on rat and human heptoma cellular carcinoma cells (MeA-RH7777 AND HepG2).<sup>22</sup> The results of our study show that *Aegle marmelos* leaf extract has a cytotoxic effect on human colon cancer cell lines (HT29), and Morphological studies also confirmed that the ethanolic extract of leaves of *Aegle marmelos* has got potential cytotoxic effect. From this we can conclude that, ethanolic extract of leaves of *Aegle marmelos* has the promising anti cancer activity. However, further study may still be necessary to elucidate the chemical nature and the active principle of the leaf which is responsible for its activity.

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