

Original Article

EVALUATION OF ANTIOXIDANT AND CHEMOPREVENTIVE POTENTIAL OF METHANOLIC EXTRACTS OF LEAF OF *AEGLEMARMELLOS* ATTRIBUTES TOWARDS DUCTAL CARCINOMA STUDIED IN MCF7 CELLS

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ABSTRACT

Objective: The objective of the present research work had been made to evaluate the antioxidant potential along with anti-cancer activity of methanolic extracts from the leaf of *A. marmelos*.

Methods: Standard methods for antioxidant potential in terms of DPPH and nitric oxide scavenging assay, and anticancer activity *in vitro* method (Cytotoxicity/MTT assay and % of cell viability) by using MCF7 cell line.

Results: Results of antioxidant efficacy revealed that the IC₅₀ value for DPPH and nitric oxide scavenging assay was considered to be 62.032%, and 20.69% respectively. The methanolic extract of *A. marmelos* was found to possess enhanced anticancer potential against MCF7 cells. Cytotoxicity activity of MCF7 cells, when treated with methanolic extract of *A. marmelos*, was found to be 43.42% at 25µg, 52.31% at 50µg, 56.31% at 75µg, 58.38% at 100µg, 62.25% at 125µg. The IC₅₀ value was found as 49.36µg. Toxicity was significantly increased with increased concentration and viability significantly decreased with the increased concentration of methanolic extract of leaf from *A. marmelos* for MCF7 cell when compared to cyclophosphamide.

Conclusion: From the studies, it was postulated that methanolic extract of leaf from *A. marmelos* has significant chemopreventive activity. These specific identities will be useful for the identification and authentication of raw drug.

Keywords: *Aeglemarmelos*, Antioxidants, Anticancer, DPPH, MCF7 Cells

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INTRODUCTION

Cancer is an important public health problem, with significant associated global mortality, disability, and the contribution of risk factors. It is the second leading cause of death in developed countries [1]. There are greater than 200 different types of cancer but, lung cancer, breast cancer, prostate cancer, and large bowel cancer accounts for more than half of all cases [2]. Use of traditional medicine is one of the common practices in India due to their wide spread of pharmacological activities [3]. Reservoir of bioactive compounds is provided in many species of plants. Only a small percentage of which have been examined and was still an important source of anticancer agents. Worldwide effects are on-going to determine novel anticancer compounds from plants. In recent years, resulting from the fear of side effects, people prefer more and more use of natural plant products for cancer [4]. *A. marmelos* is a perennial tree, wild in Sub Himalaya tract, central and South India. This plant is usually called as bael in hindi, vilvam in tamil and bilva in sanskrit. It belongs to the family *Rutaceae*. It is indigenous to India and is used in folk medicines. Ayurvedic practitioners utilize almost all of their parts but the greatest medicinal value ascribed to its fruit especially for its antioxidant pronounced effect [5-8]. Nevertheless, the antioxidant and anticancer activity of leaves from *A. marmelos* has never been investigated. Hence, this present communication attempt had been evaluated the effectiveness of antioxidant activity (DPPH and nitric oxide radical scavenging assay) along with *in vitro* anticancer activities (% of cell viability and cytotoxicity/MTT assay by using MCF7 cell) in methanolic extracts from leaf of *A. marmelos*.

MATERIALS AND METHODS

Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), and ascorbic acid were purchased from sigma chemical Co. (St., Louis, USA). Tertbutyl-4-

hydroxytoluene (BHT), folincioalceu reagent, dimethyl sulfoxide (DMSO) and methanol were purchased from Merck Co. (Germany). Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), MCF7 cells and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide(MTT). All the chemicals and reagents used were of analytical grade purchased from Sigma-Aldrich, India.

Cell lines

MCF7 Cell lines were purchased from the National Centre For Cell Sciences, Pune, India, through Biozone research technologies Lab, poes road, Teynampet, Chennai, India. The cells were cultured in Dulbecco's modified Eagle's medium containing L-glutamine and 25 mmol 4-[2-hydroxyethyl]-1-piperazine ethane sulfonic acid, penicillin (100µg/ml), streptomycin (50µg/ml) and 10% foetal bovine serum. Cells were maintained at 37 °C in a humidified incubator in an atmosphere of 5% CO₂.

Plant material

Fresh leaves materials of *A. marmelos* were collected from Chennai and Tiruvallur district of Tamilnadu during the month of July 2016. Based on organoleptic macroscopic examination the plant materials were identified and authenticated (Unisza/103/2015) by botanist Mr. Jayaraman West Tambaram, Chennai, using the Flora of Presidency of Madras [9-10] and the voucher specimen was deposited in the department of biochemistry, SRM Arts and Science College, Kattankulathur for further reference.

Preparation of extracts

The leaves of *A. marmelos* were collected and allowed to dry at room temperature in the laboratory for a period of 2 w in sunshade, coarsely powdered and weighed. Then it was soaked with methanol for 48 h, the extract was filtered, the filtrate was concentrated by distillation over boil water batty and the last traces of solvent were

removed under vacuum [11]. Dried crude extracts were stored in sterile amber coloured storage vials in refrigerator until used for antioxidant and cell line work.

Antioxidant assays

Radical scavenging activity (RSA) using DPPH

Radical scavenging activity of methanolic extract of leaf from *A. marmelos* was determined by using DPPH radical (1,1-diphenyl-2-picrylhydrazyl) according to the standard method with slight modification [12]. Stock solution of methanolic extract from the leaf of *A. marmelos* was prepared at the concentration of 10 mg/ml. Different concentrations of the extract (20, 40, 60, 80, 100µg) were added, to an equal volume of the methanolic solution of DPPH (0.1 mmol). Ascorbic acid was used as a standard control. Blank was of pure methanol and control sample was methanol along with DPPH solution. The DPPH ~ [100µl (0.1%)] with BTH 100µl (0.16%) was used as standard. DPPH (0.1%) freshly prepared were used as the control. The reaction mixture was shaken vigorously and covered with aluminium foil and incubated for 30 min in the dark at room temperature; the absorbance was recorded at 517 nm against a blank by using a spectrophotometer. The experiment was repeated for three times. The percentage Free radical scavenging activities of test samples and the positive control were expressed in terms of IC₅₀ values, which is the concentration of a sample required to decrease the absorbance at 517 nm by 50% compared to the control response. Annihilation activity of free radicals was calculated in percentage inhibition according to the following formula:

$$\% \text{ inhibition of DPPH radical scavenging activity} = \frac{A_0 - A_1}{A_0} * 100$$

Where A₀ is the absorbance of DPPH radical+methanol; A₁ is the absorbance of DPPH radical+leaf extract.

Nitric oxide scavenging activity

The nitric oxide assay was performed as described previously with slight modification [13]. Stock solutions of leaf extracts were prepared in the concentration of 1 mg/ml. Standard ascorbic acid was produced in the concentration of (25 µl) (0.16%). The reaction mixture consists of 2 ml of sodium nitroprusside (10 mmol) and 0.1 ml of phosphate buffered saline with different concentrations 25, 50 and 100µg of samples and incubated at 25 °C for 15 min. After incubation, 0.5 ml of the reaction mixture was mixed with 1 ml of 1% sulphanilamide and allowed to stand for 5 min for complete diazotization, to this 1 ml of 2% phosphoric acid and followed by 1 ml of 0.1% naphthyl-ethylene-di-amine di-hydrochloride was added, mixed well and allowed to stand for 30 min at 25 °C. Blank consists of all the reagents except for the extract or standard solution is

substituted with water. The absorbance of these solutions was measured at 540 nm against the corresponding blank solution using a spectrophotometer. The experiment was repeated for three times. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. Pink colored chromophore controls were expressed in terms of IC₅₀ values, which is the concentration needed to decrease nitric oxide induced by 50% in comparison to the control response. The annihilation activity of free radicals was calculated in percentage inhibition according to the following formula:

$$\% \text{ inhibition of nitric oxide radical scavenging activity} = \frac{A_0 - A_1}{A_0} * 100$$

Where A₀ was the absorbance of control; A₁ was the absorbance in the presence of extract and standard ascorbic acid.

Anticancer activity

In vitro assay for cytotoxicity (MTT assay)

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay [14] is based on the ability of living but not dead cells to reduce a yellow tetrazolium dye to a purple formazan product. Cells were maintained in DMEM, supplemented with 10% FBS, at 37 °C in a humidified atmosphere with 5% CO₂. MCF7 cells were plated in 96-well flat bottom tissue culture plates at a density of approximately 1.2 x 10⁴ Cells/well and allowed to attach overnight at 37 °C. Methanolic leaf extract of *Aeglemarmelos* 1 mg/ml was prepared as a stock. The medium was later discarded and cells were incubated with different concentrations of the samples (25, 50, 75, 100 and 125 µl) for 24h. After the incubation, the medium was discarded and 100 µl fresh medium was added with 10 µl of MTT (5 mg/ml). After 4h, the medium was discarded and 100 µl of DMSO was added to dissolve the formazan crystals. Then, the absorbance was read at 570 nm in a microtitre plate reader. Cyclophosphamide was invoked as a positive control. Medium along with cells (untreated) serves as a control. Cell survival was calculated by the following formula: Viability % = (Test OD/Control OD) X 100. Cytotoxicity % = 100-Viability%.

RESULTS AND DISCUSSION

DPPH: Radical scavenging activity (RAS) using 1,1-diphenyl-2-picrylhydrazyl was a widely used method to evaluate the free radical scavenging ability of various samples including plant extract. The principle of the DPPH method is based on the reduction of DPPH in the presence of a hydrogen donating antioxidant owing to the formation of diphenylpicrylhydrozine. Extracts reduced the colour of DPPH owing to the power of hydrogen donating ability [15].

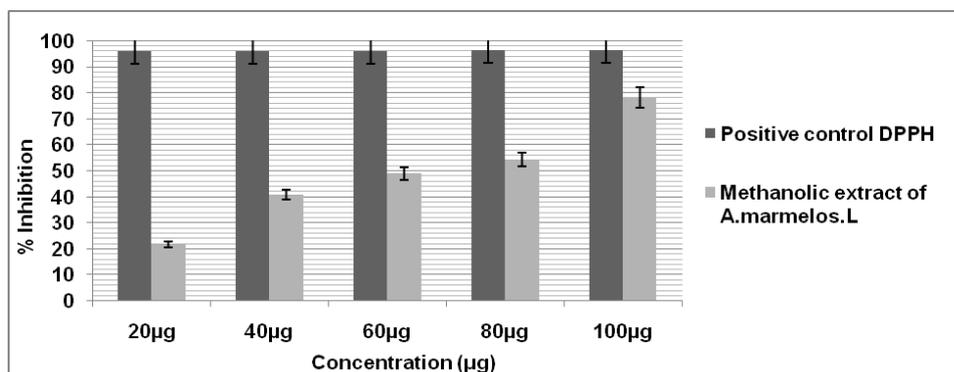


Fig. 1: DPPH radical scavenging of methanolic extract of *A. marmelos*, n=number of determinations (2), SD = Standard Deviation (mean±SD), for different concentration in each group. Statistical significance p value (<0.05) compared with standard

Fig. 1 showed DPPH anion scavenging power of extracts. Discolouration of violet with DPPH to Yellow clearly demonstrated that the methanolic extract of leaf from *A. marmelos* has excellent antioxidant activity. The percentage of inhibition against the

concentration of methanolic extract of leaf from *A. marmelos* was found to be 21.77% at 20µg/ml, 40.82% at 40µg/ml, 48.88% at 60µg/ml, 54.30% at 80µg/ml and 78.11% at 100µg/ml respectively when compared to that of standard BTH. The half maximal

inhibitory concentration IC_{50} was found to be 62.032%. This result shows that the maximum free radical scavenging potential was obtained after 30 min for methanolic extract of leaves from *A. marmelos*. It is evident that the extract did show proton donating ability and hence it could serve as a free radical inhibitor and primary antioxidant. Usually higher total phenol and flavonoids contents lead to better DPPH-scavenging activity [16-19]. In the present study the DPPH radical scavenging activity of methanolic extract of leaves of *A. marmelos* shows significant scavenging activity, it may be due to the presence of high amount of phenolic compounds, flavonoids, and tannins. Hence the result empathise that methanolic extract of leaves from *A. marmelos* has the significant

anti-oxidant property it may be due to potential hydrogen donating ability.

Nitric oxide

The available nitric oxide radical is linked with various carcinomas and inflammatory conditions [20]. Nitric oxide can react rapidly in the intracellular environment to form nitrate, nitrite, and S-nitrosothiols. These metabolites play a key role in mediating many xenotoxic effects such as DNA damage. Nitric oxide causes DNA damage via peroxynitrite. In addition to reactive oxygen species, nitric oxide is also involved in inflammation, cancer and other pathological conditions [21].

Table 1: Nitric Oxide scavenging activity of methanolic extract of *A. marmelos*

| Concentration (μ g) | % of Inhibition | |
|--------------------------|------------------------|--|
| | Standard ascorbic acid | Methanolic extract of <i>A. marmelos</i> |
| 20 | 70.34 | 49.92 |
| 40 | 71.89 | 56.76 |
| 60 | 74.38 | 65.52 |
| 80 | 79.45 | 77.53 |
| 100 | 90.80 | 80.21 |

n=number of determinations (2), SD = Standard Deviation (mean \pm SD), for different concentration in each group. Statistical significance p value (<0.05) compared with standard.

Nitric Oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. The extract directly competes with oxygen to react with nitric oxide and thereby inhibits the nitrite formation. In the present study, the % of nitric oxide scavenging activity of methanol extract of leaf of *A. marmelos* was found to be increased with increased in concentration of extract significantly when compared with the standard. The table 1 shows that the percentage of nitric oxide radical inhibition of the sample with concentration ranging from 20, 40, 60, 80 and 100 μ g/ml was found to be 49.92%, 56.76%, 65.52%, 77.53% and 80.21% respectively. The half maximal inhibitory concentration IC_{50} was found to be 20.69%. This result shows that the methanolic extract of leaves from *A. marmelos* had significant antioxidant property. In the present study, the nitric oxide scavenging ability of a methanolic extract of leaves of *A. marmelos* is highly significant when compared with standard ascorbic acid. This positive result confirmed that theethanolic extract of leaves of *A. marmelos* was capable of scavenging nitric oxide ion due to the presence of phenolic compounds, tannin contents. Hence this result emphasizes that methanolic extract of leaves of *A. marmelos* may have potential anticancer and anti-inflammatory property [22]. Several concentrations ranging from 20-100 μ g/ml of methanolic extract of leaf from *A. marmelos* were tested for their antioxidant activity using *in vitro* models. Hence the result obtain emphasize that leaf of *A. marmelos* may have significant antioxidant or free radical scavengers property. It was observed that

free radicals were scavenged by the test compounds in concentration-dependent manner in both methods. This result may provide scientifically support for the use of plant materials for the treatment of cancer related diseases in traditional medicine especially for ductal carcinoma.

MTT assay for cytotoxicity of MCF7 cells

The anticancer activity of samples on MCF7 cells were determined by the MTT assay was used to measure the cytotoxicity [23]. Cells (1×10^5 /well) were plated in 0.2 ml of medium/well in 96-well plates. In MTT assay the medium from the wells was removed carefully after incubation. Each well was washed with MEM (w/o) FCS for 2-3 times and 200 μ l of MTT (5 mg/ml) was added. The plates were incubated for 6-7 h in 5% CO_2 incubator for cytotoxicity. After incubation, 1 ml of DMSO (solubilizing reagent) was added to each well and mixed well by micropipette and left for 45 sec. Presence of viable cells was visualized by the development of purple color due to the formation of formazan crystals. The suspension was transferred to the cuvette of a spectrophotometer and the OD (optical density) values were read at 595 nm by using DMSO as a blank. Measurements were performed and the concentration required for 50% inhibition of viability (IC_{50}) was determined graphically standard graph was plotted by taking, the concentration of the drug in X-axis and relative cell viability in Y-axis. Cell viability (%) = Mean OD/Control OD x 100%

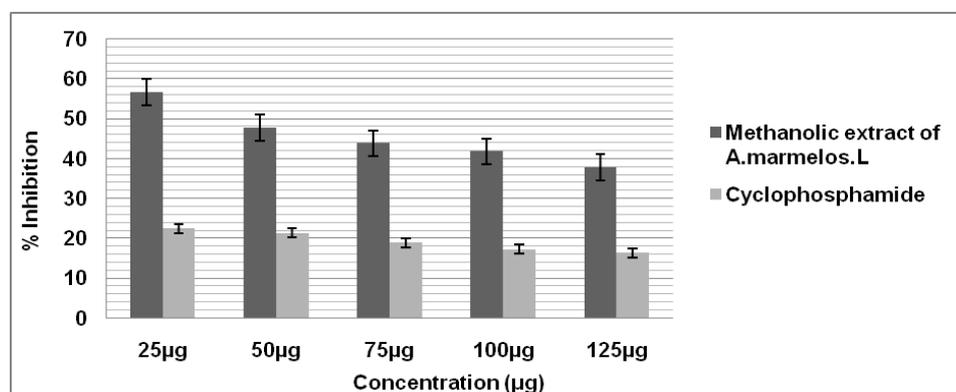


Fig. 2: Percentage of cell viability against a methanolic extract of *A. marmelos*, n=number of determinations (2), SD = Standard Deviation (mean \pm SD), for different concentration in each group. Statistical significance p-value (<0.05) compared with standard

The fig. 2 shows that the viability of MCF7 cell when treated with the methanolic extract of *A. marmelos* was found to be 56.58% at 25µg; 47.69% at 50µg, 43.69% at 75µg, 41.62% at 100µg, 37.75% at 125µg. Viability of MCF7 cell was significantly decreased with the increase concentration of the methanolic extract of leaf from *A.*

marmelos at 125µg when compared to cyclophosphamide. The IC₅₀ value of the given sample is 49.36µg. It was formerly reported that extract from *A. marmelos* has an anti-proliferative effect on cell line MCF7 [24], inhibits the proliferation of transplanted ehrlich ascites carcinoma bearing swiss albino mice [25].

Table 2: Percentage of cytotoxicity against a methanolic extract of *A. marmelos*

| Concentration (µg) | % of inhibition | |
|--------------------|------------------|--|
| | Cyclophosphamide | Methanolic extract of <i>A. marmelos</i> |
| 25 | 78.17 | 43.42% |
| 50 | 79.18 | 52.31% |
| 75 | 81.29 | 56.31% |
| 100 | 82.18 | 58.38% |
| 125 | 84.17 | 62.25% |

n=number of determinations (2), SD = Standard Deviation (mean±SD), for different concentration in each group. Statistical significance p value (<0.05) compared with a standard.

The table 2 shows that cytotoxicity activity of MCF7 cells, when treated with the methanolic extract of *A. marmelos*, was found to be 43.42% at 25µg, 52.31% at 50µg, 56.31% at 75µg, 58.38% at 100µg, 62.25% at 125µg. Toxicity significantly increased with the increased concentration of the methanolic extract of leaf from *A. marmelos* at 125µg when compared to Cyclophosphamide. The IC₅₀ value of the given sample is 125µg. Early report said that methanol, ethyl acetate and *n*-hexane extract of leaves of *Aeglemarmelos* Bael, showed potent cytotoxic activity with LC₅₀ values of 4.482, 5.278 and 5.278 µg/ml respectively whereas the standard vincristine sulfate showed 3.364µg/ml. The three extracts of leaves of *A. marmelos* possessed significant antioxidant and cytotoxic potentials in this experiment [26]. From this, *in vitro* cell line studies it was found that methanolic extract of leaf from *A. marmelos* was found to be non-toxic to normal cells, probably anti-proliferative and cytotoxic effect against MCF7 cells.

CONCLUSION

Result obtains from this research work emphasize that leaf of *A. marmelos* may have significant antioxidant or free radical scavengers properties along with the significant chemo-preventive activity. Hence in conclusion utilization or increased consumption of this plant product will be advantageous to mankind, and it will contribute to the prevention of chronic lifestyle along with several kinds of oxidative degenerative disease such as ductal carcinoma. Further investigations on health-promoting aspects in animal models in the future will be made.

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AUTHORS CONTRIBUTIONS

The Corresponding author, B. Arirudran supervised the experimental work and wrote the manuscript. B. Janani took place in the laboratory work and data collections. US Mahadeva Rao reviewed the manuscript and put necessary efforts to improve the quality of the manuscript.

CONFLICT OF INTERESTS

We declare that there were no conflicts of interest

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