ABSTRACT

Objective: The present investigation was performed to evaluate the antiproliferative and antioxidant activity of *Aegle marmelos* leaves in Dalton's Lymphoma Ascites (DLA)-bearing mice.

Materials and Methods: The DLA cells maintained in vivo in Swiss albino mice were used for developing ascitic tumor in mice by intraperitoneal transplantation. The standardized 50% ethanolic extract of *A. marmelos* leaves (AMEE) was administered intraperitoneally in dose levels 200 and 400 mg/kg, after 24 hours of tumor inoculation in mice for two weeks.

Results: The AMEE treatment significantly prevented \((P<0.001)\) the increase in body weight due to tumor cell growth and increased the mean survival time of the tumor-bearing mice as compared to the untreated DLA control mice. The treatment of DLA-bearing mice brought down the Alanine Aminotransferase (ALAT), Aspartate Aminotransferase (ASAT), and alkaline phosphatase to normal levels. The extract decreased the levels of hepatic lipid peroxidation and increased the levels of hepatic antioxidants Glutathione, Superoxide Dismutase (SOD), and catalase. All the changes observed with AMEE treatment were dose dependent.

Conclusion: The hydroalcoholic extract of *A. marmelos* exhibits strong antitumor and antioxidant activities in DLA-bearing mice.

KEY WORDS: *Aegle marmelos*, antioxidant activity, antiproliferative activity, Dalton's Lymphoma Ascites

Introduction

Oxidative stress is a known mediator of cancer.\(^1\) Reactive oxygen species such as organic and hydrogen peroxides have been shown to act as tumor promoters.\(^2\) Antioxidants have been demonstrated to serve as subcellular messengers of normal cell function and have a significant protective role against oxidative injury.\(^3\) Consequently, the search of anticancer agents possessing antioxidant potential is worthy in terms of combined benefits of inhibition of proliferation and scavenging the toxicants inducing oxidative stress in cancer.

*Aegle marmelos* (L) known as bael has been reported to possess anticancer activity in different human cell lines, including Leukemia K562, T-lymphoid Jurkat, B-lymphoid Kaji, Erythroleukemia HEL, Melanoma Colo 38, breast cancer MCF7, and MDA MB-231 cell lines,\(^4\) and it has also been observed to inhibit proliferation of transplanted Ehrlich Ascites Carcinoma in mice.\(^5\) The plant extract exhibited cytotoxicity against tumor cell lines in brine shrimp lethality assay and Methyl Thiazolyl Tetrazolium (MTT)-based assay.\(^6\) The *A. marmelos* leaf extract pretreatment in isoproterenol-treated rats increased the activities of antioxidant enzymes Superoxide Dismutase (SOD), catalase, and glutathione peroxidase in the heart tissue.\(^7\) However, so far no reports are available on antitumor and antioxidant activity of the plant in Dalton's Lymphoma Ascites (DLA)-bearing mice. Hence, the present study was undertaken to obtain an insight into the antitumor and antioxidant potential of 50% alcoholic extract of *A. marmelos* leaves.

Materials and Methods

Chemicals

Sodium Dodecyl Sulfate (SDS), trichloroacetic acid, Folin–Ciocalteu reagent (E.Merck, Mumbai), 5,5'-bithio-bis-nitrobenzoic acid (DTNB) (Sigma Lab, USA), Nicotinamide Adenine Dinucleotide (NADH), phenazonium methosulfate, Thiobarbituric Acid (TBA), Nitroblue Tetrazolium (NBT), Tris-hydrochloride, and EDTA (Otto chemicals, Mumbai) were procured from standard

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suppliers of chemicals. Other chemicals and reagents used were of analytical grade.

Plant Material Collection and Extraction
The A. marmelos leaves were collected from Natham area, Dindigul, Tamil Nadu, and authenticated by Dr. Stephen, Department of Botany, American College, Madurai. They were cleaned to remove contaminants and shade dried. The dried leaves were then powdered coarsely and extracted with 50% ethanol by maceration at room temperature for one week. The macerate was concentrated by vacuum distillation and freeze dried (Martin Christ, Alpha 2-2) for 48 hours. The dry hygroscopic extract (12% w/w) of A. marmelos Leaves Ethanol Extract (AMEE) was stored in a desiccator until use. Tannins, flavonoids, and saponins are the phytoconstituents present in the extract. The flavonoid content of the extract was estimated by the method of Chia-Chi Chang et al., 1996 which was 0.98% ± 0.07% w/w equivalent to rutin as the standard flavonoid.

Experimental Animals
The protocol used in this study for use of mice as animal model for cancer was approved by the Institutional Animal Ethical Committee, Ultra College of Pharmacy, Madurai [UCP/IAEC/2007/010].

Swiss albino mice of age about 8 weeks, with an average body weight of 24 ± 2 g were used in the experiment. The animals were bred and brought up in our laboratory facility with 12-hour cycles of light and dark at 23°C. They were fed standard laboratory diet (AMRUT Rat and Mice feed) and were given Reverse Osmosis (RO) purified water ad libitum.

DLA-induced Ascitic Antitumor Studies
The DLA cells were supplied by Amala Cancer Research Centre, Trissur, Kerala. The cells were maintained in vivo in Swiss albino mice by intraperitoneal (i.p.) transplantation. The tumor cells aspirated by phosphate buffer saline from the peritoneal cavity of the mice were administered intraperitoneally to all animals to develop ascitic tumor, except those in the normal group.

Antitumor activity was carried out in Swiss albino mice (8-10 weeks). Five groups of Swiss albino mice (24 ± 2 g), six animals in each group were taken. All groups of mice, except those from the normal group, were injected DLA cells in the intraperitoneal cavity and the treatment was started after 24 hr of tumor inoculation. The animals had free access to water and food.

The designation of the animal groups and treatment details are as follows:

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal control</td>
</tr>
<tr>
<td>II</td>
<td>DLA control (injected i.p. with sterile physiological saline 0.9% w/v of NaCl)</td>
</tr>
<tr>
<td>III</td>
<td>DLA bearing AMEE treated (Dose-I, 200 mg/kg - i.p. administration)</td>
</tr>
<tr>
<td>IV</td>
<td>DLA bearing AMEE treated (Dose II, 400 mg/kg - i.p. administration)</td>
</tr>
<tr>
<td>V</td>
<td>Standard control (Vincristine 20 μg/kg - i.p. administration)</td>
</tr>
</tbody>
</table>

All the treatments were started 24 hr after tumor inoculation and continued for 14 days (2 weeks). The body weight of animals in all groups during the treatment was noted daily. After the last dose and on the next day after 18 hr fasting, blood samples were collected by cardiac puncture from three animals in each group, after euthanasia effected by cervical dislocation under thiopentone sodium anesthesia. The remaining animals were kept to check the survival time of DLA-bearing mice.

Tumor Growth Response
The antitumor activity of the alcoholic extract of A. marmelos was measured in DLA-bearing mice with respect to the parameters viz. body weight, ascites tumor volume, Packed Cell Volume (PCV), Mean Survival Time (MST), and Percentage Increase in Life Span (PILS).

\[ \text{PILS} = \left( \frac{\text{MST of treated group}}{\text{MST of control group}} - 1 \right) \times 100 \]

\[ \text{MST} = \frac{\text{Day of first death} + \text{Day of last death}}{2} \]

Hematological and Serum Biochemical Parameters Estimation
The Hemoglobin (Hb) content, Red Blood Cell (RBC) count, total WBC count, and WBC differential count of the collected blood samples were estimated. The sera were analyzed for Alanine Aminotransferase (ALAT), Aspartate Aminotransferase (ASAT), Alkaline Phosphatase (ALP), and total protein content.

Statistical Analysis
The mean values of all parameters were compared for statistical significance by one-way Analysis Of Variance (ANOVA) followed by the Tukey–Kramer Multiple comparison test.

Results
Phenols, tannins, flavonoids, and saponins are the phytoconstituents present in AMEE. The flavonoid content of the extract is 0.98% ± 0.07% w/w equivalent to rutin as the standard flavonoid.

Effect of AMEE on Body Weight and Tumor Parameters
The mean body weight of the DLA group mice (33 g) increased as compared to that of normal mice (24 g). AMEE treatment in two dose levels (200 and 400 mg/kg) significantly \((P<0.001)\) inhibited this increase in body weight in DLA mice [Table 1]. There was also significant \((P<0.001)\) reduction in tumor volume and PCV in the AMEE-treated DLA mice group as compared to that in the DLA control group [Table 1]. Mean survival time and life span of the DLA-bearing mice were increased in AMEE-treated groups in a dose-dependent manner [Table 1].

Effect of AMEE on Hematological Parameters of DLA-Bearing Mice
The hemoglobin content and RBC count were reduced in DLA-bearing mice as compared to normal animals. The reduction in the Hb content was inhibited by AMEE treatment significantly \((P<0.05)\) at both dose levels (200 and 400 mg/kg),
which is comparable to that of standard vincristine treatment. The reversal of the change in RBC count by AMEE treatment was observed with a greater significance ($P<0.001$). There was an increase in WBC count in DLA control mice as compared to that in normal mice. AMEE treatment in DLA-bearing mice inhibited this increase, as shown by the restoration to normal values. Reduction in lymphocytes count and rise in the number of polymorphs were the changes noticed in DLA-bearing mice when compared to normal mice. These changes were reversed to normal by AMEE treatment significantly ($P<0.001$) at the doses 200 and 400 mg/kg [Table 2].

**Effect of AMEE on Serum Biochemical Parameters**

The levels of ALAT, ASAT, and ALP were increased in DLA control group as compared to that in normal animals, whereas the protein content in serum of DLA control animals was decreased [Table 2]. AMEE treatment at 200 and 400 mg/kg produced significant reduction ($P<0.001$) in the levels of ALAT, ASAT, and ALP as compared to that in DLA control [Table 2].

**Effect of AMEE on Lipid Peroxidation and GSH in DLA‑bearing Mice**

The lipid peroxidation in liver tissues of the DLA control group was significantly increased ($P<0.001$) as shown by higher values of Thiobarbituric Acid Reactive Substances (TBARS [Malondialdehyde (MDA)]) as compared to that in normal mice. Treatment with AMEE at 200 and 400 mg/kg significantly ($P<0.001$) reduced the MDA levels when compared with DLA control animals [Table 3]. The GSH levels in livers of tumor-bearing mice were significantly ($P<0.001$) decreased when compared with those in the normal group. The reduced level of GSH was found to increase on administration of AMEE when compared with the DLA control group [Table 3]. Total protein levels in AMEE-treated groups as well as standard treated groups increased as compared to significant reduction ($P<0.001$) in DLA control animals [Table 3].

**Effect of AMEE on Antioxidant Enzymes of DLA‑bearing Mice**

There was significant ($P<0.001$) reduction in the levels of liver SOD and catalase in DLA control animals. Administration of AMEE at two different dose levels (200 and 400 mg/kg) resulted in a significant increase ($P<0.001$) in the levels of SOD and catalase as compared with that noted in DLA control animals [Table 3].

**Discussion**

The quantitative criteria used in the present antiproliferative screening in DLA-bearing mice are retardation of primary tumor growth and extension of survival time of the tumor-bearing animals.

The body weight of DLA-bearing mice increased due to increase in ascitic tumor volume and inhibition of this increase in body weights by AMEE treatment shows its protective role in prevention of tumor growth. During the two-week period of the study after DLA inoculation, the ascitic fluid collection was rapid in untreated mice, indicating active secretion and accumulation of ascitic fluid in the abdomen due to excess tumor accumulation of ascitic fluid in the abdomen due to excess tumor.

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**Table 1:**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>Group V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>33.00 ± 0.63</td>
<td>26.83 ± 0.59**</td>
<td>26.33 ± 0.56**</td>
<td>25.50 ± 0.48**</td>
</tr>
<tr>
<td>Mean survival time (days)</td>
<td>18.00 ± 1.37</td>
<td>22.16 ± 0.98*</td>
<td>24.16 ± 0.60**</td>
<td>29.00 ± 0.45**</td>
</tr>
<tr>
<td>Percentage increase in life span (PILS)</td>
<td></td>
<td>23.0</td>
<td>34.2</td>
<td>61.1</td>
</tr>
<tr>
<td>Tumor volume (ml)</td>
<td>7.50 ± 0.18</td>
<td>4.5 ± 0.18**</td>
<td>3.16 ± 0.28**</td>
<td>2.16 ± 0.11**</td>
</tr>
<tr>
<td>Packed cell volume (ml)</td>
<td>4.16 ± 0.28</td>
<td>1.80 ± 0.11**</td>
<td>0.60 ± 0.13**</td>
<td>0.17 ± 0.04**</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM (n=6). **$P<0.001$, *$P<0.05$ when compared to DLA control group. DLA = Dalton’s Lymphoma Ascites, AMEE = Aegle marmelos ethanolic extract.

**Table 2:**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I Normal</th>
<th>Group II DLA control</th>
<th>Group III AMEE 200 mg/kg</th>
<th>Group IV AMEE 400 mg/kg</th>
<th>Group V Vincristine (20 μg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>12.70 ± 0.89</td>
<td>10.35 ± 0.07*</td>
<td>11.35 ± 0.07*</td>
<td>12.21 ± 0.69*</td>
<td>12.80 ± 0.73*</td>
</tr>
<tr>
<td>White blood cells (1 × 10³/mm³)</td>
<td>9.75 ± 0.11</td>
<td>13.75 ± 0.11**</td>
<td>11.50 ± 0.22**</td>
<td>9.50 ± 0.22**</td>
<td>10.50 ± 0.22**</td>
</tr>
<tr>
<td>Red blood cells (1 × 10³/mm³)</td>
<td>4.30 ± 0.31</td>
<td>2.00 ± 0.04*</td>
<td>4.06 ± 0.12**</td>
<td>4.30 ± 0.13**</td>
<td>4.50 ± 0.17**</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>63.00 ± 0.89</td>
<td>31.33 ± 0.21**</td>
<td>57.00 ± 0.45**</td>
<td>61.50 ± 0.22**</td>
<td>63.50 ± 0.67**</td>
</tr>
<tr>
<td>Polymorphs</td>
<td>37.00 ± 0.89</td>
<td>68.50 ± 0.22**</td>
<td>43.00 ± 0.45**</td>
<td>38.50 ± 0.22**</td>
<td>37.00 ± 0.63**</td>
</tr>
<tr>
<td>Aspartate aminotransferase (U/ml)</td>
<td>95.00 ± 0.73</td>
<td>187.33 ± 56.80**</td>
<td>125.50 ± 0.67**</td>
<td>98.00 ± 75.13**</td>
<td>122.66 ± 21.30**</td>
</tr>
<tr>
<td>Alanine aminotransferase (U/ml)</td>
<td>47.50 ± 0.67</td>
<td>90.50 ± 2.91**</td>
<td>80.50 ± 0.22**</td>
<td>68.00 ± 3.13**</td>
<td>73.00 ± 2.63**</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/ml)</td>
<td>95.00 ± 0.45</td>
<td>150.00 ± 8.94**</td>
<td>98.00 ± 3.58**</td>
<td>99.00 ± 0.89**</td>
<td>87.00 ± 0.89**</td>
</tr>
<tr>
<td>Total protein (g/dl)</td>
<td>6.70 ± 0.09</td>
<td>4.95 ± 0.11**</td>
<td>12.00 ± 0.45**</td>
<td>11.00 ± 0.89**</td>
<td>6.20 ± 0.36**</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM (n=6) after 14 days of treatment. **$P<0.001$, *$P<0.05$ as compared to either DLA control or Normal. The values of DLA group were compared with those of normal group. The values of treated group were compared with those of DLA group.
The significant lowering of tumor ascitic volume by AMEE treatment clearly indicates that AMEE has the potential to retard tumor growth.

In case of ascites tumors, the measurement of the total amount of tumor cell material in the ascitic fluid can be done in terms of total cell volume or PCV or ascitocrit. \(^{[13]}\) PCV may be considered comparable to that volume of solid tumors. Ascitocrit has been observed to be low in AMEE-treated group as compared to DLA control, and this inhibition of infiltration of cells was observed to be dose dependent.

AMEE treatment prolonged the life span of tumor bearing mice, which may have resulted because of the inhibition of tumor proliferation.

The hematological changes in cancer include anemia, \(^{[14]}\) and the association of increased WBC count with lymphocyte predominance is common in ascites tumor. \(^{[15]}\) AMEE treatment in DLA-bearing mice could normalize the blood cell profile, as shown by the increased hemoglobin content and RBC count with reduced WBC count [Table 2]. This clearly indicates the protective action of AMEE on the hemopoietic system.

The total protein content in both extract-treated group mice increased as compared to that in the DLA control group mice. The standard treated group showed almost near values as the normal group. The serum levels of ALT, AST, and ALP were sharply increased in DLA control, as these enzymes are the indicators of liver inflammation caused by peritoneal tumor ascites collection. Treatment with AMEE at the doses of 200 and 400 mg/kg produced almost normal values, thus signifying the reversal of tissue damage.

The liver proteins of the treated group are significantly higher than the untreated DLA control, but not elevated to normal levels similar to standard treatment [Table 3]. The prolongation of the treatment with AMEE may normalize the liver proteins.

The liver of EAC tumor-bearing mice, during the early phase after tumor implantation, has been found to be subjected to an oxidative stress with increased steady state levels of peroxyl radicals and reduced activities of antioxidant enzymes SOD and glutathione peroxidase. \(^{[16]}\) The same observation has been made in the present study as indicated by a significant reduction in SOD, catalase, and GSH levels of livers of DLA tumor-bearing mice. The first-line defense component of the antioxidant system includes SOD and catalase. SOD mainly acts as the normal group. The values of treated group were compared with those of normal group. The values of treated group were compared with those of DLA group. TBARS = Thiobarbituric acid reactive substances, MDA = Malondialdehyde, DLA = Dalton’s Lymphoma Ascites.

### Table 3:

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I Normal</th>
<th>Group II DLA control</th>
<th>Group III AMEE (200 mg/kg) treated DLA</th>
<th>Group IV AMEE (400 mg/kg) treated DLA</th>
<th>Group V Vincristine (20 µg/kg) treated DLA</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBARS (nM (MDA)/mg protein)</td>
<td>1.76 ± 0.06</td>
<td>3.25 ± 0.07*</td>
<td>1.43 ± 0.03*</td>
<td>1.35 ± 0.05*</td>
<td>1.65 ± 0.02*</td>
</tr>
<tr>
<td>Glutathione (mg/g wet tissue)</td>
<td>2.35 ± 0.06</td>
<td>1.55 ± 0.02*</td>
<td>1.80 ± 0.04*</td>
<td>1.85 ± 0.02*</td>
<td>2.03 ± 0.03*</td>
</tr>
<tr>
<td>Superoxide dismutase (U/mg protein)</td>
<td>0.45 ± 0.01</td>
<td>0.28 ± 0.01*</td>
<td>0.42 ± 0.00*</td>
<td>0.34 ± 0.01*</td>
<td>0.47 ± 0.00*</td>
</tr>
<tr>
<td>Catalase (U/mg protein)</td>
<td>5.58 ± 0.17</td>
<td>3.30 ± 0.04*</td>
<td>6.05 ± 0.67*</td>
<td>6.65 ± 0.06*</td>
<td>7.08 ± 0.03*</td>
</tr>
<tr>
<td>Total Protein (g/dl)</td>
<td>5.72 ± 0.03</td>
<td>1.38 ± 0.05*</td>
<td>2.00 ± 0.04*</td>
<td>2.40 ± 0.04*</td>
<td>4.50 ± 0.13*</td>
</tr>
</tbody>
</table>

Values are expressed as mean±S E M (n=6) after 14 days of treatment. 

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