Antitumor activity of ethanol extract of *Gracilaria edulis* (Gmelin) Silva on Ehrlich ascites carcinoma-bearing mice

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**OBJECTIVE:** To evaluate antitumor activity of *Gracilaria edulis* in Swiss albino mice with Ehrlich ascites carcinoma (EAC).

**METHODS:** Tumors were induced in mice by intraperitoneal injection of EAC cells. Ethanol extract of *Gracilaria edulis* (EEGE) was administered to the experimental animals in different doses after 24 h of tumor inoculation. The antitumor effect of the EEGE was evaluated by assessing *in vitro* cytotoxicity, survival time, biochemical parameters and hepatic enzyme levels.

**RESULTS:** EEGE increased the life span of EAC-bearing mice compared with that of the model control mice (P<0.05 or P<0.01). EEGE treatment also converted the changes of biochemical parameters and hepatic enzyme levels in the EAC-bearing mice (P<0.05 or P<0.01). EEGE induced inhibition of tumor formation in EAC-bearing mice compared with that of the model control group (P<0.05 or P<0.01).

**CONCLUSION:** The present study scientifically proved the antitumor activity of marine algae *G. edulis* and the effect can be correlated with doses.

**KEYWORDS:** *Gracilaria*; carcinoma, Ehrlich tumor; antibody-dependent cell cytotoxicity; survival rate; mice

Cancer is one of the ten leading diseases which cause death, and advancing in rank year by year throughout the world. Cancer is the second largest killer disease in the developed countries and is currently responsible for about 25% of all deaths¹. Cancer is a group of diseases where cell growth is aggressive and abnormal, invasive, and/or metastatic many times leading to death². Carcinoma arises from epithelial cells invading surrounding tissues and organs and may metastasize, or spread, to lymph nodes and other sites³. The treatment of cancer has undergone major advances, which include benefits of combination chemotherapy as well as the incorporation of biologic therapy, and yielding significant improvements in survival over the past decade⁴.
Natural products from terrestrial and marine organisms provide a chemically diverse array of compounds, which is a limitation of synthetic chemistry techniques. Natural products that have been used traditionally as therapeutic agents have shown tremendous potentials in advanced treatment methods\(^{[3]}\). Marine red algae (red seaweeds) encompasses the largest and most diverse assemblage of the marine plants. It is important to the marine environment and used as raw materials for the extraction of valuable products\(^{[4]}\). *Gracilaria* is one of the red seaweeds used as an important source of agar extracts and related products. A number of natural products have been studied for anticancer activity on various experimental models and resulted in nearly 30 effective anticancer drugs\(^{[7]}\). The aim of this present study is to evaluate the anticancer activity of ethanol extract of the algae *Gracilaria edulis* (EEGE) in Ehrlich ascites carcinoma (EAC) bearing mice.

1 Materials and methods

1.1 Plant material collection and extraction

Fresh algae of *Gracilaria edulis* was collected from the Mandapam region, Rameswaram, Tamilnadu, India and taxonomically authenticated. The whole algae materials were shade dried and powdered. Nearly 400 g of the coarse powdered was extracted with adequate volume of a series of organic solvents in the order of increasing polarity followed by preliminary phytochemical screening\(^{[5]}\). The ethanol extract was found to contain alkaloid, flavonoid, sterol, terpenoids, protein, saponin, phenol, coumarin, tannin and glycosides. With this information, we decided to use the ethanol extract for further investigation in this study.

1.2 Reagent and apparatus

Dulbecco’s modified eagle medium (DMEM) and fetal bovine serum (FBS) were from Gibco (Gaithersburg, USA). 5-Fluorouracil (5-FU), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and all reagents were from Sigma Co. (St. Louis, USA). Incubator was the product of Thermo-Forma Company (Union City, USA). Spectrophotometer used in this study was from Shimadzu Corporation (Kyoto, Japan) and the Horiba ABX 80 Diagnostics (ABX Pentra Montpellier, France) was used for biochemical parameters.

1.3 EAC cell line

EAC cell lines were obtained as courtesy sample from Amala Cancer Research Center, Thrissur, India. The cells were maintained by weekly intraperitoneal inoculation of \(10^6\) cells per mouse\(^{[5]}\).

1.4 Acute toxicity studies

The acute oral toxicity study of the extract was carried out as per the guidelines by the Organization for Economic Cooperation and Development\(^{[6]}\). This method was carried out in six animals, one per treatment group at widely different dose ranges of 100 to 1,000 mg/kg body weight and the animals were observed for 24 h. As the extract did not produce any mortality at the dose levels tested with this encouraging results we continued to optimize the dose levels, and 100, 200 and 300 mg/kg body weight were selected for the further evaluation.

1.5 Animals

Adult Swiss albino mice of weight approximately 25 to 30 g were used as experimental animals, procured from Tamilnadu Veterinary and Animal Science University, Chennai, India. The animals were maintained in well-ventilated cages and fed with commercial pelleted mice chow and water *ad libitum*. The animals were divided into six groups of six animals each. The study was conducted with necessary Animal Ethical Committee clearance (No. 790/03/ac/CPSEA). Group 1 was set as the normal control. Animals in groups 2 to 6 were injected with EAC cells (0.2 mL of \(2\times10^6\) cells per mouse) intraperitoneally. After 14 d of treatment, animals were sacrificed by retro-orbital plexus method. Blood was collected for biochemical and hepatic enzyme assays.

1.6 In vitro cytotoxicity

Dilutions of the drug extract were made with phosphate buffer saline and further adjusted to make stock solution up to 1,000 \(\mu\)g/mL. EAC cells were aspirated from peritoneal cavity of tumor-bearing mice and then the drug dilutions at 0, 50 and 100 \(\mu\)g/mL were then added to the EAC cells followed by incubation at 37°C for 3 h. Cell viability was determined by dye exclusion method using trypan blue\(^{[11]}\). Under identical conditions, 5-FU was used as positive.
drug control for antitumor activity. The percentage viability was calculated according to the following formula: viability (%) = (number of unstained cells/total number of cells) × 100%. 

1.7 Cell viability Cell viability was assessed by using MTT cytotoxicity method[15]. EEGE-treated cells were suspended in DMEM with 10% FBS; 180 µL of this cellular suspension was transferred to a 96-well microplate and incubated with 20 µL of a 5 mg/mL MTT solution for 3 h at 37 °C. The supernatant was removed by centrifugation and formazan crystals were dissolved in 200 µL of DMSO. Absorbance was read at 570 nm on a plate reader and viability was calculated as the proportion of absorbance of each sample compared to that of the controls.

1.8 Effects of EEGE on animal survival time All animals were inoculated with 2 × 10⁶ cells per mouse on day 0, except the group 1 (normal control group) and treatment with EEGE started at 24 h after inoculation, at doses of 100, 200 and 300 mg/kg per oral on groups 3, 4 and 5 animals respectively for 14 d. Group 6 animals were treated with the standard drug 5-FU (20 mg/kg). The normal control group was treated with the equal volume of normal saline. The mean survival time and average body weight changes of each group were observed. The percentage increase in life span was calculated from the median survival time (MST) by using the following formula: increase in life span (%) = (T - C)/C × 100%, where T=number of days the treated animals survived and C=number of days the control animals survived[10].

1.9 Effects of EEGE on biochemical parameters Serum collected from the experimental animals was used for the estimation of biochemical parameters such as total cholesterol, uric acid, glucose and protein[14]. Packed cell volume (PCV) was analyzed by Horiba ABX 80 Diagnostics.

1.10 Effects of EEGE on hepatic enzymes Blood was collected from the experimental animals; serum was separated and the level of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) were estimated[17].

1.11 Statistical analysis The mean ± standard error of mean was calculated for each parameter. Data were subjected to statistical analysis using analysis of variance followed by Bonferroni test.

Statistical analysis and was performed using SPSS for Windows (15.0) software (SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant when P<0.05.

2 Results

2.1 In vitro cytotoxicity studies The effect of EEGE in in vitro cytotoxicity studies is in a dose-dependent manner where the maximum activity was observed with the highest dose used in the study. At the minimum dose used, the extract was found to be decreasing the cell viability but at the doses at 50 and 100 µg/mL, the extract produced better activity, whereas with the 50 µg/mL the cell viability was reduced to 25% and with 100 µg/mL the cell viability reduced even more to 30%, indicating that the drug was able to produce significant response at the dose above 50 µg/mL.

2.2 Effects of EEGE on survival time The effect of EEGE on the survival of tumor-bearing mice (Table 1) obtained at the dose level of 300 mg/kg was highly significant (P<0.01) and not different from the standard drug used in positive drug control group.

**Table 1 Effects of EEGE treatment on the survival of EAC-bearing mice**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>MST (d)</th>
<th>Increase in Emax (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model control</td>
<td>5</td>
<td>17.00±1.31</td>
<td></td>
</tr>
<tr>
<td>EEGE 100 mg/kg</td>
<td>5</td>
<td>23.16±1.42</td>
<td>36.23</td>
</tr>
<tr>
<td>EEGE 200 mg/kg</td>
<td>5</td>
<td>26.33±1.21</td>
<td>54.88</td>
</tr>
<tr>
<td>EEGE 300 mg/kg</td>
<td>5</td>
<td>29.51±1.05</td>
<td>73.32</td>
</tr>
<tr>
<td>5-Fluorouracil 20 mg/kg</td>
<td>5</td>
<td>33.01±2.00</td>
<td>74.11</td>
</tr>
</tbody>
</table>

*P<0.05, **P<0.01, vs model control group. EEGE: ethanol extract of G. australis; EAC: Ehrlich ascites carcinoma; MST: median survival time.

2.3 EEGE-induced tumor apoptosis Phenotypically, apoptosis is characterized by cell shrinkage, chromatin compaction, plasma membrane blebbing, DNA fragmentation and collapse of the cell into small intact fragments (apoptotic bodies). We observed that EEGE treatment caused cell death in EAC. The nature of cell death may be further confirmed by specific apoptosis tests; we utilized cytolysis assay method in which trypan blue stained the dead cells (Figures 1A and 1B).

![Figure 1 Detection of EEGE-induced cell death](image)

A: Normal control group (group 1); B: EEGE-treated group. Cells were stained with trypan blue and visualized under a light microscope (×40). EEGE: ethanol extract of G. australis.
2.4 Effects of EEGE on biochemical parameters
The biochemical parameters of tumor-bearing mice showed significant changes compared to the normal control mice ($P<0.01$). Glucose level in the model control mice was low and with administration of the drug the glucose level reached to normal level in group 5 and group 6 ($P<0.01$). Urea, cholesterol, total protein and uric acid levels of the EAC model control mice were significantly higher than the normal control mice ($P<0.01$), and 300 mg/kg of EEGE and the standard drug showed similar effect in correspondence to urea level. The dose level of 300 mg/kg was highly significant and comparable to that of the standard drug. Similar pattern of result was obtained for PCV (Table 2).

2.5 Effects of EEGE on hepatic enzymes
Hepatic enzymes AST, ALT, ALP and LDH were elevated in the EAC-bearing model control mice ($P<0.01$). Hepatic enzymes in the animals of groups 5 and 6 were similar to normal animals, indicating the recovery of EAC toxicity in the liver ($P<0.01$). The dose level of 300 mg/kg was highly significant and comparable to that of the standard drug (Table 3).

### Table 2. Effects of EEGE on biochemical parameters of EAC-bearing mice

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Glucose (mg/L)</th>
<th>Urea (mg/L)</th>
<th>Cholesterol (mg/L)</th>
<th>Protein (mg/L)</th>
<th>Uric acid (mg/L)</th>
<th>PCV (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>6</td>
<td>915.0±18.7</td>
<td>360.0±14.1</td>
<td>930.0±14.1</td>
<td>65.0±1.4</td>
<td>24.8±1.5</td>
<td>0.20±0.06</td>
</tr>
<tr>
<td>Model control</td>
<td>6</td>
<td>618.3±17.2△△</td>
<td>611.6±23.2△△</td>
<td>1608.3±17.2△△</td>
<td>92.0±1.6△△</td>
<td>45.2±1.9△△</td>
<td>2.80±0.8△△</td>
</tr>
<tr>
<td>EEGE 100 mg/kg</td>
<td>6</td>
<td>715.0±21.4*</td>
<td>555.0±18.7*</td>
<td>1351.6±11.7*</td>
<td>86.5±1.9*</td>
<td>43.8±2.0*</td>
<td>1.50±0.24*</td>
</tr>
<tr>
<td>EEGE 200 mg/kg</td>
<td>6</td>
<td>805.0±16.4**</td>
<td>455.0±16.8**</td>
<td>1376.6±16.3**</td>
<td>75.0±2.2**</td>
<td>40.7±2.2**</td>
<td>0.90±0.75**</td>
</tr>
<tr>
<td>EEGE 300 mg/kg</td>
<td>6</td>
<td>835.0±15.5**</td>
<td>400.0±13.9**</td>
<td>1078.3±20.4**</td>
<td>67.1±2.3**</td>
<td>31.2±2.8**</td>
<td>0.30±0.66**</td>
</tr>
<tr>
<td>5-Flourouracil 20 mg/kg</td>
<td>6</td>
<td>860.0±14.1**</td>
<td>405.0±20.1**</td>
<td>1005.0±18.7**</td>
<td>70.5±0.1**</td>
<td>27.0±1.6**</td>
<td>0.20±0.90**</td>
</tr>
</tbody>
</table>

* $P<0.05$, ** $P<0.01$, vs model control group; △△ $P<0.01$, vs normal control group. EEGE: ethanol extract of Gracilaria edulis; EAC: Ehrlich ascites carcinoma; PCV: packed cell volume.

### Table 3. Effects of EEGE on hepatic enzymes of EAC-bearing mice

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>AST</th>
<th>ALT</th>
<th>ALP</th>
<th>LDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>6</td>
<td>35.83±1.17</td>
<td>57.33±1.63</td>
<td>96.50±1.40</td>
<td>310.83±1.47</td>
</tr>
<tr>
<td>Model control</td>
<td>6</td>
<td>101.50±1.87△△</td>
<td>157.50±1.88△△</td>
<td>162.50±1.87△△</td>
<td>501.67±2.16△△</td>
</tr>
<tr>
<td>EEGE 100 mg/kg</td>
<td>6</td>
<td>96.00±1.98*</td>
<td>127.50±1.97*</td>
<td>145.00±1.41*</td>
<td>449.00±2.61**</td>
</tr>
<tr>
<td>EEGE 200 mg/kg</td>
<td>6</td>
<td>64.50±1.20**</td>
<td>91.50±1.23**</td>
<td>129.33±2.12**</td>
<td>364.50±2.43**</td>
</tr>
<tr>
<td>EEGE 300 mg/kg</td>
<td>6</td>
<td>52.16±1.33**</td>
<td>65.17±1.94**</td>
<td>107.83±1.47**</td>
<td>322.50±1.87**</td>
</tr>
<tr>
<td>5-Flourouracil 20 mg/kg</td>
<td>6</td>
<td>41.16±1.47**</td>
<td>60.50±1.87**</td>
<td>92.00±1.53*</td>
<td>317.17±2.32**</td>
</tr>
</tbody>
</table>

* $P<0.05$, ** $P<0.01$, vs model control group; △△ $P<0.01$, vs normal control group. EEGE: ethanol extract of Gracilaria edulis; EAC: Ehrlich ascites carcinoma; ALT: aspartate aminotransferase; AST: alanine aminotransferase; ALP: alkaline phosphatase; LDH: lactate dehydrogenase.

### 3 Discussion
This study was carried out to evaluate the antitumor activity of EEGE in EAC-bearing mice and from literature we learned that marine algae such as G. edulis, G. foliifera, G. verrucosa was found to be potent antitumor agents\[18-21\]. These algae has been studied for their influence on biochemical and pathological conditions in human and other animal models. In this study we selected a marine alga, which is predominant in the coastal areas of Tamilnadu, to screen for its antitumor activity. G. edulis was selected for reasons including less information on the antitumor activity and also this alga was reported to be antioxidant\[20\]. Presence of conjugated polyenoic fatty acids in these seaweeds and the role of the fatty acids as a possible mechanism for the putative antitumorigenic were factors which influenced the selection of this marine organism\[20\]. The seaweeds were authenticated and subjected to anticancer screening against EAC cell lines. The ethanol extract of G. edulis was prepared and both in vitro and in vivo studies were carried out.

Earlier it has been reported that green sea algae has been effective in treating the EAC-bearing mice\[22\] and we observed that the algal extract is toxic to the EAC cells as there is an increase in the number of cells taking the trypan blue dye with increase in the dose of EEGE in the in vitro cytotoxicity study. The dependable principle for assessing the importance of any anticancer drug includes the prolongation of the life span of animal\[23\]. It is evident that EEGE increased the life span of the EAC-bearing mice by restricting the activity of the EAC cells (Table 1).

Cell death induced by EEGE was observed with the trypan blue-stained cells, and the mechanism of the cell death may be due to apoptosis, which was seen as the morphological characteristics of the dead cells. An assessment of the cytotoxic activity is necessary to ensure the antiproliferative property. Inoculation of EAC cell lines leads to an alteration in the biochemical profiles of the disease
control animals such as glucose\textsuperscript{[24]}, protein\textsuperscript{[25]}, urea and uric acid\textsuperscript{[26]}. Decrease in glucose level, resulting in a hypoglycemic condition, is a characteristic feature of all types of cancer\textsuperscript{[27]}. This may be attributed to the multiplication of cells requiring more glucose to produce energy and Warburg effect in glucose metabolism in cancer\textsuperscript{[28]}. Further uncontrolled gene expression in cancer cells leads to an increase in the protein concentration\textsuperscript{[29]}. Due to an increase in the cell turn-over, the level of uric acid increases in EAC-bearing mice. Treatment with EEGE restored all these biochemical parameters to normal level with treatment with 300 mg/kg. This is the indirect method of evaluating the antitumor activity of algal extract on EAC cells.

Hepatocytes are severely damaged in the animals with EAC cells\textsuperscript{[30]} and this can lead to an increase in the levels of ALT, AST, ALP and LDH in the serum of EAC-bearing mice\textsuperscript{[31]}. Reduced level of these hepatic enzymes in serum is one of the indications of the antitumour potential\textsuperscript{[22]} and EEGE was successful in lowering the enzymes on EAC cells. The action of this plant extract was effective on PCV and was also found to be similar to the positive control drug. The PCV was significantly lower in mice treated with EEGE when compared to the model control group. These results could indicate either a direct cytotoxic effect of EEGE on tumor cells or an indirect local effect, which may involve macrophage activation and vascular permeability inhibition\textsuperscript{[32]}. Analysis of in vitro cytotoxicity, survival time, biochemical parameters and hepatic enzymes assay clearly revealed that EEGE possesses antitumor activity. Preliminary phytochemical screening showed the presence of alkaloids and flavonoids in EEGE. The cytotoxic and antitumor properties of EEGE may be due to these compounds. Further in-depth studies leading to the identification and characterization of these chemical molecules may result in the development of effective anticancer drug from bioresources.

4 Acknowledgements

The authors thank Dr. D. J. Sridharan, Department of Botany, National College, Trichy, India for identification and authentication of the algae.

5 Competing interests

The authors declare that they have no competing interests.

REFERENCES


18 Devi GK, Manivannan K, Thirumaran G, Rajathi FA,
帚状江蓠乙醇提取物对小鼠 Ehrlich 腹水癌的抑制作用

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目的:探讨帚状江蓠乙醇提取物对小鼠 Ehrlich 腹水癌的抑制作用。

方法:通过向小鼠腹腔注射 Ehrlich 腹水癌细胞建立肿瘤模型。为了解帚状江蓠乙醇提取物的口服急性毒性, 在造模后 24 h, 给予实验小鼠不同剂量的帚状江蓠乙醇提取物, 观察小鼠存活时间。其次, 通过检测 Ehrlich 腹水癌荷瘤小鼠生存时间、体外细胞毒性、血清生物学指标以及肝脏酶水平等来评估帚状江蓠的抗肿瘤作用。

结果:与模型对照组相比, 帚状江蓠乙醇提取物不仅抑制了肿瘤的形成, 也延长了 Ehrlich 腹水癌荷瘤小鼠的生存时间(P<0.05 或 P<0.01)。帚状江蓠乙醇提取物可逆转 Ehrlich 腹水癌荷瘤小鼠各项生物学指标及肝脏酶水平的变化。

结论:本研究表明, 海藻类植物帚状江蓠具有抗肿瘤作用, 其抗肿瘤的效果可能与服用剂量相关。

关键词:江蓠属; 腹水癌; Ehrlich 腹水癌; 抗体依赖细胞细胞毒性; 存活率; 小鼠