ABSTRACT

One of the major achievements of medical science has been the control and management of infectious diseases. The present study was designed to evaluate the antitumor effect and antioxidant role of methanolic root extract of tissue cultured Pluchea indica. Antitumor activity and antioxidant status of this extract (100 and 300 mg/kg) were evaluated against Ehrlich Ascites Carcinoma (EAC) cells in mice. After 24 h of tumor inoculation, the root extract was administered orally daily for 14 days. After administering of the last dose followed by 18 h fasting, mice were then sacrificed for observation of anti-tumor activity. The effect of methanolic extract of P. indica (MEPI) on the growth of transplantable murine tumor and life span of EAC bearing hosts were estimated. Simultaneously the hematological values and liver biochemical parameters (lipid peroxidation, antioxidant enzymes) were estimated in treated animals. MEPI decreased mice weight, tumor volume, viable cell count and amounted to 19.25 g, 1.87 ml, and 24.00 × 10⁶ cells/ml, respectively when compared to EAC control. MEPI also showed an increase in the non-viable cell count (55.25 × 10⁶ cells/ml) and mean survival time of 26.50 days thereby increasing the life span by 62.27% more than EAC tumor-bearing mice. The hematological profile reverted to more or less normal levels in extract treated mice. Treatment with MEPI decreases the levels of lipid peroxidation and increases the levels of glutathione, super oxide dismutase and catalase. It was revealed from the study that the tissue cultured P. indica root extract possesses potent antitumor and antioxidant activities.

Keywords: biochemical parameter, EAC, hematological profile, MEPI

INTRODUCTION

For millennia, plants have been a valuable source of natural products for maintaining human health, and in the last decade, in particular there have been more intensive studies focusing on natural therapies. Plants should thus be thoroughly investigated to better understand their properties, safety and efficiency. Again plant tissue culture research is multi-dimensional and would no doubt revolutionize medicinal plant research. Pluchea indica (L.) Less. (Asteraceae) is a large, evergreen shrub found abundantly in salt marshes and mangrove swamps in Sunderbans (India), Bangladesh, Myanmar, China, Philippines, Malaysia, Tropical Asia and Australia. In Indo-China decocation of roots is prescribed for fevers as a diaphoretic and an infusion of the leaves is given internally to treat lumbago while the roots and leaves are used as an astringent and antipyretic (Kirtikar and Basu 1999). The plant is also used to cure rheumatoid arthritis (Chatterjee 1996). The root extract has anti-inflammatory (Sen and Nag Chowdhury 1991), antiulcer (Sen et al. 1999). The plant is also used to cure rheumatoid arthritis (Sen and Nag Chowdhury 1991) and two thiophene derivatives, besides two pentacyclic triterpenes of rare occurrence from roots (Chakravarty and Mukhopadhyay 1994) have been isolated from this plant. A pure compound (R/J/3) isolated from the roots of P. indica was found to be very effective against Entamoeba histolytica (Biswas et al. 2006). P. indica plant was tissue cultured with the purpose of obtaining more secondary metabolites. Tissue-cultured P. indica leaves have a diuretic effect (Pramanik et al. 2007). The main objective of this study was to evaluate the antitumor activity and antioxidant role of tissue-cultured P. indica against Ehrlich Ascites Carcinoma (EAC) in Swiss albino mice.

MATERIALS AND METHODS

In vitro tissue culture and micropropagation

The tissue culture preparation of Pluchea indica (L.) Less. was based on the protocol reported by Pramanik et al. (2007) and is briefly summarized here. P. indica roots, stems with internodes were excised from plant collected from Diamond Harbour region, West Bengal during the flowering stage. These explants cleaned and sterilized with 0.1% w/v HgCl₂ solution followed by repeated rinsing with sterile distilled water. The roots were then aseptically cut into 0.5 ± 0.2 cm² pieces and placed with their dorsal cut side on the Murashige and Skoog (MS, Murashige and Skoog 1962) basal medium containing 1.2% w/v agar. Maximum numbers of shoots were induced in Medium II (Medium II – MS + 0.437 µM IAA + 0.023 µM MBAP) while shoots were placed in Medium III (Medium III – MS + 0.437 µM IAA + 0.023 µM BAP + 1.73 µM GA₃). The roots with maximum length were placed in Medium I (Medium I – MS + 0.437 µM IAA). Cultures were maintained in

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tubes of 10 ml medium and kept at 22 ± 2°C maintaining with a relative humidity of 78% for 18 hours photoperiod (140–180 mmol/m²/s). After 30 days of experiment, the callus and regenerative shoots were transferred to the fresh media. The procedure was repeated for ten times to obtain their potential for root regeneration.

Material

The roots of tissue cultured *P. indica* were separated, washed, oven-dried at 60°C, powdered by micro pulverizer and sieved through 100 meshes (1/100 inch diameter). Fibers and unwanted debris were discarded after sieving. The powdered root material was preserved in an airtight container for further use.

Extraction

The pulverized root powder (500 gm) was soaked overnight with 1.5 liters petroleum ether (60-80°C for synthesis, Merck) and then dried it and extracted with methanol (AR Grade, Purity 99.8%, Supplied by local Supplier) using a Soxhlet extractor to obtain the methanolic extract of *P. indica* (MEPI). Then the solvent was evaporated under reduced pressure using a rotary evaporator to obtain a semisolid residue. The yield of the extract was 8.7% w/w.

The extract was suspended in 2% v/v aqueous Tween 80 solution prior to the experiment and used.

Chemicals

All the chemicals were of analytical grade. 1-Chloro-2,4-dinitrobenzene (CDNB), bovine serum albumin (Sigma chemical, USA), Thiobarbituric acid, Nitrobiuletrazolium chloride (NBT) (Loba Chemical), 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) (SICCO Research Laboratory, Bombay). EAC cells were obtained from Chittaranjan National Cancer Institute, Kolkata, India. The EAC cells were maintained by intraperitoneal inoculation of 2 × 10⁶ cells/mouse.

Animals

In vivo studies were carried out on Swiss albino mice weighing 18 ± 0.25 g of either sex. The animals were kept under standard conditions of 12:12 h light and dark cycle in polypropylene cages and fed with standard laboratory diet and water ad libitum. All in vivo procedures described were reviewed and approved by the University Animal Ethical Committee.

Antitumor activity

The antitumor activity of *P. indica* root extract was assessed using male Swiss albino mice divided into 5 equally sized groups (n=12). The tumor becomes palpable in the mice after nine days of inoculation of EAC cells. At the day zero, EAC cells were taken from these inoculated mice and injected (0.2 ml of 2x 10⁶ cells/mouse) to all the groups intraperitoneally except the normal group. From the first day, normal saline 5 ml/kg mouse/day was administered to normal and EAC control groups respectively for 14 days period intraperitoneally. Similarly MEPI at different doses (100 and 300 mg/kg) and standard drug 5-fluorouracil (20 mg/kg) were administered in groups 3, 4, and 5 respectively. After the administration of last dose followed by 18 h fasting, 6 mice from each group were sacrificed for the study of antitumor activity, hematological and liver biochemical parameters. The remaining animals in each of the groups were kept to check the mean survival time (MST) of the tumor-bearing hosts.

Antitumor effect of MEPI was assessed by observing the changes with respect to body weight, ascitic tumor volume, viable and non-viable tumor cell count, MST and percentage increase in life span (% ILS). MST of each group containing six mice were observed by recording the mortality count daily for six weeks and % ILS was calculated using following equation (Gupta et al. 2004). MST = [(Mean survival time of treated group/mean survival time of control group)-1] × 100.

Hematological studies

Hematological studies were performed by recording the hemoglobin content, red blood cell (RBC) and white blood cell (WBC) counts from freely flowing tail vein blood (Gupta et al. 2004). Differential WBC leukocyte counts were carried out from leishman stained blood smears of normal, EAC control, and MEPI treated groups respectively.

Estimation of biochemical parameter

After the collection of blood samples the mice were sacrificed and the liver were removed, rinsed in ice-cold normal saline followed by cold 0.15 mol/L Tris-HCL buffer (pH 7.4), blotted dry, and weighed. Liver was homogenated (10% w/v) in 0.15 mol/L Tris-HCL buffer in a potter homoginizer and a portion of supernatant was employed in spontaneous lipid peroxidation (Ohkawa et al. 1979). The remaining portion of the same after precipitating proteins with TCA was used for the estimation of glutathione (Ellman 1979). The homogenate was centrifuged at 1500 rpm for 15 min at 4°C. The supernatant thus obtained was used for the estimation of superoxide dismutase, catalase, and protein content (Gupta et al. 2004).

Statistical analysis

The experimental results were expressed as mean ± SEM. Data were assessed by the method of analysis of one way ANOVA followed by Dunnett’s t-Test. *P*<0.05 was considered as statistically significant.

RESULTS

Table 1 shows the mean survival time of the EAC control group was 16.33 days, while mean survival time increased to 22.00 and 30.50 days at the dose of (100 mg/kg) and (300 mg/kg) respectively in MEPI-treated groups. The group treated with the standard drug 5-fluorouracil (20 mg/kg) showed 34.16 days for the same. Treatment with MEPI at the two different doses reduced the body weight, tumor volume and viable tumor cell count in a dose-dependent manner in comparison to that of EAC control group. Further, nonviable tumor cell counts at different doses of MEPI were increased when compared with the EAC control.

Table 2 illustrates the hemoglobin content and RBC count in the EAC control group was decreased when compared to normal group. Treatment with MEPI at the dose of 100, and 300 mg/kg increased the hemoglobin content and RBC count to more or less normal levels. The total WBC counts and protein were found to be increased in EAC control group when compared with normal group. Administration of MEPI at the dose of 100, and 300 mg/kg in EAC bearing mice reduced both WBC count and protein as compared with EAC control. MEPI increased the monocyte and lymphocyte count to 1.29% and 65.60% respectively and simultaneously reduced the neutrophils count 31.70% to normal value in comparison to EAC control group.

Table 3 illustrates that the levels of lipid peroxidation in liver tissue were increased in EAC control group as compared to the normal group (*P*<0.01) and MEPI decreases it to more or less normal level. GSH content were decreased in EAC control group compared to normal group. But administration of MEPI (100 and 300 mg/kg) reduces the GSH to normal level. The levels of superoxide dismutase (SOD) and catalase in the liver of EAC bearing mice decreased in comparison with normal group. MEPI has increased the SOD and catalase levels to normal at the dose of 100 and 300 mg/kg body weight.

DISCUSSION

Among currently available drugs, synthetic drugs do have potential adverse reactions and which can be minimized to greater extent through natural compounds as supported by Vinca alkaloids and taxol. The MEPI treated animals at the
Table 1 Effect of methanol root extract of tissue-cultured P. indica on body weight, mean survival time, % ILS, tumor volume, viable and non-viable tumor cell count of EAC-bearing mice. Body weight of normal mice: 18.25 ± 0.25 g.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>EAC control (2 × 10^6 cells/mouse)</th>
<th>MEPI (100 mg/kg) +EAC</th>
<th>MEPI (300 mg/kg) +EAC</th>
<th>Standard 5-fluorouracil (20 mg/kg) +EAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>22.50 ± 0.43</td>
<td>21.13 ± 0.68</td>
<td>19.25 ± 0.24</td>
<td>18.00 ± 0.57</td>
</tr>
<tr>
<td>Mean survival time (d)</td>
<td>16.33 ± 0.76</td>
<td>22.00 ± 0.77</td>
<td>30.50 ± 0.84</td>
<td>34.16 ± 0.94</td>
</tr>
<tr>
<td>Increase life span (%)</td>
<td>-</td>
<td>34.72</td>
<td>86.77</td>
<td>109.18</td>
</tr>
<tr>
<td>Tumor volume (mm^3)</td>
<td>3.50 ± 0.20</td>
<td>2.30 ± 0.16</td>
<td>1.87 ± 0.42</td>
<td>-</td>
</tr>
<tr>
<td>Viable tumor cell count (10^6 ml^-1)</td>
<td>58.50 ± 1.50</td>
<td>40.75 ± 0.47</td>
<td>24.00 ± 0.70</td>
<td>9.23 ± 0.25</td>
</tr>
<tr>
<td>Non viable tumor cell count (10^6 ml^-1)</td>
<td>19.90 ± 1.04</td>
<td>38.25 ± 0.47</td>
<td>55.25 ± 0.47</td>
<td>-</td>
</tr>
</tbody>
</table>

N = 6. Mean ± SEM. ^<0.01 vs EAC control group.

Table 2 Effect of methanol root extract of tissue-cultured P. indica on hematological parameters of EAC-bearing mice.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal</th>
<th>EAC control (2 × 10^6 cells/mouse)</th>
<th>MEPI (100 mg/kg) +EAC</th>
<th>MEPI (300 mg/kg) +EAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (g%)</td>
<td>13.78 ± 0.05</td>
<td>10.18 ± 0.10</td>
<td>11.41 ± 0.08</td>
<td>12.56 ± 0.15</td>
</tr>
<tr>
<td>RBC/10^6 L^-1</td>
<td>6.17 ± 0.07</td>
<td>3.94 ± 0.01</td>
<td>4.77 ± 0.02</td>
<td>5.18 ± 0.06</td>
</tr>
<tr>
<td>WBC/10^6 L^-1</td>
<td>4.20 ± 0.01</td>
<td>13.03 ± 0.05</td>
<td>8.83 ± 0.03</td>
<td>6.29 ± 0.03</td>
</tr>
<tr>
<td>Monocyte (%)</td>
<td>1.37 ± 0.02</td>
<td>1.05 ± 0.01</td>
<td>1.18 ± 0.002</td>
<td>1.29 ± 0.004</td>
</tr>
<tr>
<td>Lymphocyte (%)</td>
<td>67.60 ± 0.72</td>
<td>34.33 ± 0.43</td>
<td>41.60 ± 0.41</td>
<td>56.60 ± 0.50</td>
</tr>
<tr>
<td>Neutrophil (%)</td>
<td>21.20 ± 0.37</td>
<td>52.15 ± 0.37</td>
<td>44.50 ± 0.59</td>
<td>31.70 ± 0.51</td>
</tr>
</tbody>
</table>

N = 6. Mean ± SEM. ^<0.01 vs normal group, ^<0.01 vs EAC control group.

Table 3 Effect of methanolic root extract of P. indica on lipid peroxidation, glutathione content and antioxidant enzymes in the liver of EAC-bearing mice.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal</th>
<th>EAC control (2 × 10^6 cells/mouse)</th>
<th>MEPI (100 mg/kg) +EAC</th>
<th>MEPI (300 mg/kg) +EAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid peroxidation (nmol MDA.mg^-1)</td>
<td>0.85 ± 0.01</td>
<td>1.92 ± 0.008</td>
<td>1.33 ± 0.01</td>
<td>0.90 ± 0.01</td>
</tr>
<tr>
<td>Glutathion content (mg.g^-1)</td>
<td>2.35 ± 0.07</td>
<td>1.50 ± 0.08</td>
<td>1.88 ± 0.01</td>
<td>2.24 ± 0.05</td>
</tr>
<tr>
<td>Superoxide dismutase (U.mg^-1)</td>
<td>4.29 ± 0.01</td>
<td>2.78 ± 0.05</td>
<td>3.50 ± 0.01</td>
<td>4.02 ± 0.03</td>
</tr>
<tr>
<td>Catalase (U.mg^-1)</td>
<td>28.50 ± 0.05</td>
<td>12.40 ± 0.25</td>
<td>17.60 ± 0.20</td>
<td>21.50 ± 0.05</td>
</tr>
</tbody>
</table>

N = 6. Mean ± SEM. ^<0.01 vs normal group, ^<0.05 vs EAC control group.

doses of 100 and 300 mg/kg inhibited the body weight, tumor volume, and tumor cell count and also brought back the hematological parameters to more or less normal levels. The Tissue cultured extract also showed the hepatic lipid peroxidation and free radical scavenging enzyme GSH as well as antioxidant enzymes such as SOD and CAT in tumor-bearing mice to near normal levels. A decrease in tumor volume and viable tumor cell count as mentioned above finally reduced the tumor burden and enhanced the life span of EAC-bearing mice.

In cancer chemotherapy the major problem are of myelosuppression and anemia (Gupta et al. 2004). The anemia observed in tumor bearing mice is mainly due to reduction in RBC or low hemoglobin percentage. This may occur either due to iron deficiency or due to hemolytic/myelopathic conditions (Gupta et al. 2004). Treatment with MEPI brought back the hemoglobin content; RBC and WBC cell count near to normal values. This indicates that MEPI possesses protective action on the hematopoietic system.

Plant derived extracts containing antioxidant principles showed cytotoxicity towards tumor cells (Gupta et al. 2004). Excessive production of free radicals resulted in oxidative stress, which leads to damage of macromolecules such as lipids can induce lipid peroxidation in vivo (Yagi 1991). Increased lipid peroxidation would cause degeneration of tissues. Lipid peroxide formed in the primary site would be transferred through the circulation and provoke damage by propagating the process of lipid peroxidation (Gupta et al. 2004). MDA, the end product of lipid peroxidation was reported to be higher in carcinomatous tissue than in non-diseased organs (Yagi 1991). Glutathione, a potent inhibitor of neoplastic process plays an important role as an endogenous antioxidant system that is found particularly in high concentration in liver and is known to have key function in the protective process (Gupta et al. 2004). MEPI reduced the elevated levels of lipid peroxidation and increased the glutathione content in EAC bearing mice.

On the other hand the free radical scavenging system, SOD and catalase are present in all oxygen metabolizing cells and their function is to provide a defense against the potentially damaging reactivities of superoxide and hydrogen peroxide. Sun et al reported a decrease in SOD activity in EAC bearing mice, which might be due to loss of SOD activity in EAC cells and the loss of mitochondria, leading to a decrease in total SOD activity in the liver (Gupta et al. 2004). The inhibition of SOD and CAT activities as a result of inhibition of tumor growth was also reported (Gupta et al. 2004). Similar findings were observed in the present investigation with EAC bearing mice.

Previous many studies have shown that steroidal molecules have got the antitumor properties, especially β-sitosterol (Hullatti et al. 2006). Some of the triterpenoid compounds from plant sources have shown the inhibitory action on cancer cell lines (Hullatti et al. 2006). The phytochemical investigation of P. indica root extract has shown the presence of steroids, β-sitosterol and triterpinoids in methanolic extract (Biswa et al. 2005). Hence the observed antitumor property of tissue cultured P. indica root extract may be due to the presence of steroidal and triterpinoind molecules in the methanolic extract. These results clearly indicate the further details work on this extract may provide us with an effective non-toxic antitumor agent.

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