Antitumor and antioxidant potentials of ethanolic extract of *Leonotis nepetaefolia* R.Br. against Ehrlich Ascites carcinoma cell lines.

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**ABSTRACT**

The present study is aimed at investigating the antitumor and antioxidant potentials of the ethanolic extract of *Leonotis nepetaefolia* R.Br (EELN) on Ehrlich Ascites Carcinoma cell lines. Tumor was induced intraperitoneally using Ehrlich Ascites Carcinoma cells (1X10^6 cells/mouse). Ethanolic extract of *Leonotis nepetaefolia* R.Br was administered at the dose level of 100, 200 & 300 mg/kg bw/day for 14 days to the experimental animals after 24h of tumor inoculation. The antitumor effect of EELN was assessed by studying the parameters such as Tumor volume, PCV, viable and non-viable cell counts, life span, Hematological and antioxidant profiles. Administration of EELN decreased the body weight, Ascites fluid volume, PCV and Viable cell counts and increased the mean survival time of tumor bearing animals. The EELN brought back the altered levels of hematological parameters, and antioxidant enzyme levels in dose dependent manner in EAC bearing mice. The results obtained were comparable with that of the standard drug 5-Flurouracil (20mg/kg bw.).

**Key words**: Ehrlich Ascites Carcinoma(EAC), Ethanolic extract of *Leonotis nepetaefolia* R.Br.(EELN), Mean survival time (MST), Tumor growth response, MDA, GSH, SOD, Catalase.

**INTRODUCTION**

Cancer continues to represent one of the major causes of mortality in the world and claims over 6 million death every year. An extremely promising strategy for cancer treatment today is chemotherapy. However most of the synthetic chemotherapeutic agents exhibit severe toxicity resulting in undesirable side effects. Plant derived products such as terpenes, flavones and alkaloids have received considerable attention in recent years due to their diverse pharmacological activity including cytotoxic and cancer preventive effects besides being human friendly. The rich and diverse plant sources of India is likely to provide potential anti cancer agents. Till date large number of natural products have been screened for their anticancer potential through various experimental models. This has resulted in the discovery of 30 effective anticancer drugs. This prompted us to develop a novel anticancerous drug from plant sources and a survey was conducted in and around Trichy to select a potential plant source. A common Lamiaceae member botanically identified as *Leonotis nepetaefolia* R.Br. was selected and its ethanol extract was subjected to preliminary anticancer screening.

Selected taxon is an under shrub with scarlet flowers-ovate leaves. Phytochemical analysis of the selected drug source revealed that it contains, labdanic acid the diterpene methoxynepetaefolin, the terpenic alcohols nepetaefolinol and leonotinine and a coumarin, characterized as 4,6,7-trimethoxy-5-methylchromen -2-one.

**MATERIAL AND METHODS**

**Plant collection and extraction**

The aerial parts of *Leonotis nepetaefolia* R. Br. were collected from in and around Trichy in the month of December 2007 and identified with the help of Flora of Presidency of Madras and authenticated with the specimens deposited at RAPINAT Herbarium, Department of Botany, St. Joseph’s college, Trichy. The plant material was shade dried and pulverized. About 500gms of plant material was soaked in the ethanol for 48hrs. The solvent was distilled off under reduced pressure at 50°C and dried in vacuum (yield: 6.5% w/w) and dissolved in isotonic normal saline and used for the studies.

**Experimental protocol**

Male Swiss albino mice (25± 2g) were procured from the Tamil Nadu Veterinary University, Chennai, and were housed in micron boxes in a controlled environment (temperature 25±2°C and 12h dark/light cycle). They were fed with standard laboratory diet and were given sterilized water ad libitum. The animals were maintained in standard Animal house CPCSEA approval no:79003/ ac/CPCSEA. The study was conducted after obtaining the necessary clearance from Institutional Animal Ethical Committee.

**Animals:** Male Swiss albino mice (25± 2g) were procured from the Tamil Nadu Veterinary University Chennai, and were housed in micron boxes in a controlled environment (temperature 25±2°C and 12h dark/light cycle). They were fed with standard laboratory diet and were given sterilized water ad libitum. The animals were maintained in standard Animal house CPCSEA approval no:79003/ ac/CPCSEA. The study was conducted after obtaining the necessary clearance from Institutional Animal Ethical Committee.

**Cells:** EAC cells obtained through the courtesy of Amla Cancer Research Centre, Thrissur, maintained and were used in the present work.

**Short time In-Vitro cytotoxicity:** Short-term cytotoxicity was assessed by incubating 1 X10^6 EAC cells in 1 ml phosphate buffer saline at 37°C for 3 hrs in CO_2 atmosphere with varying concentrations of EELN. The viability of the cells were determined by the trypan blue exclusion method.

**Survival time**

The percentage increase in life span was calculated as follows.

\[ \text{ILS} \% = \left( \frac{\text{Mean survival time of treated group}}{\text{Mean survival time of control group}} \right) - 1 \times 100 \]

MST = (Day of first death + day of last death)/2

**GROUP I** - Normal control

**GROUP II** - Ehrlich Ascites Carcinoma cells (1X10^6 cells/mouse)

**GROUP III** - Ehrlich Ascites Carcinoma cells (1X10^6 cells) treated with 100mg/kg bw of the ethanolic extract of *Leonotis nepetaefolia* R.Br. (EELN)

**GROUP IV** - Ehrlich Ascites Carcinoma cells (1X10^6 cells) treated with 200mg/kg bw of the ethanolic extract of *Leonotis nepetaefolia* R.Br. (EELN)

**GROUP V** - Ehrlich Ascites Carcinoma cells (1X10^6 cells) treated with 300mg/kg bw of the ethanolic extract of *Leonotis nepetaefolia* R.Br. (EELN)

**GROUP VI** - Ehrlich Ascites Carcinoma cells (1X10^6 cells) treated with 5 - Fluouracil (5-FU) (20mg/kgbw.)(Standard Drug)

Treatment started after 24hrs of EAC inoculation. The plant extract was administered orally for 14 days. After 14days of treatment animals from each group were sacrificed by cervical decapitation method to evaluate the antitumor and antioxidant potential.
Body weight:
Body weights of the experimental mice were recorded both in the treated and control group at the beginning of the experiment and at the end of the experiment.

Tumor volume:
The mice were dissected and the ascitic fluid was collected from the peritoneal cavity. The volume was measured using a graduated centrifuge tube and packed cell volume was determined by centrifuging at 1000 rpm for 5 min.

Tumor cell count:
The ascitic fluid was taken in a WBC pipette and diluted 100 times. Then a drop of the diluted cell suspension was placed on the Neubauer counting chamber and the number of cells in the 64 small squares were counted.

Viable/non-viable tumor cell count:
The cells were then stained with trypan blue (0.4% in normal saline) dye. The cells that did not take up the dye were treated as viable and those that took up the stain were treated as nonviable. Thus viable and nonviable cells were counted.

\[ \text{Cell count} = \frac{\text{No of cells} \times \text{X Thickness of liquid film}}{\text{Area X Thickness of liquid film}} \]

Hematological studies:
Blood was collected and used for the estimation of Hemoglobin (Hb) levels, red blood cell counts (RBC) \(^a\), white blood cell counts (WBC) \(^b\). WBC differential counts were determined using Leishman stained blood smear method \(^b\).

Antioxidant studies:
The liver was excised out from the sacrificed animals rinsed in ice cold normal saline followed by a wash in cold 0.15m Tris-HCl (pH7.4) and dried. A 10%(w/v) homogenate was prepared in 0.15 M Tris-HCl buffer. From this one portion of the homogenate was utilized for the estimation of lipid peroxidation \(^a\) and the second portion was used for the estimation of glutathione after precipitating proteins with TCA \(^b\), and the rest of the homogenate was centrifuged at 1500rpm for 15 min at 4°C. The Supernatant was used for the estimation of superoxide dismutase(SOD) and Catalase \(^a\), \(^b\).

Statistical Analysis:
Data of the results obtained were subjected to statistical analysis. Values were recorded as mean ± S.E.M., n=6. P<0.05 is considered as statistically significant value.

### Table 1. Short time In-Vitro cytotoxic effect of EELN

<table>
<thead>
<tr>
<th>Concentration of EELN (µg/ml)</th>
<th>Viable cells</th>
<th>Viable cells (%)</th>
<th>No. of dead cells</th>
<th>Dead cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>92</td>
<td>94.84</td>
<td>5</td>
<td>5.16</td>
</tr>
<tr>
<td>10</td>
<td>84</td>
<td>88.42</td>
<td>11</td>
<td>11.52</td>
</tr>
<tr>
<td>50</td>
<td>81</td>
<td>82.65</td>
<td>17</td>
<td>17.35</td>
</tr>
<tr>
<td>100</td>
<td>60</td>
<td>63.15</td>
<td>35</td>
<td>36.84</td>
</tr>
<tr>
<td>200</td>
<td>41</td>
<td>42.70</td>
<td>55</td>
<td>57.30</td>
</tr>
</tbody>
</table>

### Table 2 : Effect of EELN on body weight and MST

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Body weight of the animals(gms)</th>
<th>Mean survival Time MST (days)</th>
<th>Increase in Life span (ILS) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group II</td>
<td>34.17±0.79</td>
<td>19.3±0.92</td>
<td>-</td>
</tr>
<tr>
<td>Group III</td>
<td>32.98±0.91*</td>
<td>22.5±1.19</td>
<td>16.58*</td>
</tr>
<tr>
<td>Group IV</td>
<td>31.8±0.86*</td>
<td>27.5±1.2*</td>
<td>42.48*</td>
</tr>
<tr>
<td>Group V</td>
<td>30.55±0.86*</td>
<td>33.5±0.88*</td>
<td>73.57*</td>
</tr>
<tr>
<td>Group VI</td>
<td>29.4±0.62*</td>
<td>35.5±10*</td>
<td>85.7*</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M., n=6
*P<0.05 statistically significant when compared to EAC control group (Group II)

RESULTS
Effect of EELN on In-Vitro cytotoxic studies:
From the Table 1 it is evident that the death rate of Ehrlich Ascites Carcinoma cells increased with increase in concentration of ethanolic extract of Leonotis nepetapholia (EELN).

### Table 3: Effect of EELN on tumor growth

<table>
<thead>
<tr>
<th>Group</th>
<th>Tumor volume (mm³)</th>
<th>Packed cell volume (mm³)</th>
<th>Viable cells (%)</th>
<th>Non-viable cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group II</td>
<td>3.5±0.11</td>
<td>1.9±0.02</td>
<td>7.9±0.19</td>
<td>0.53±0.04</td>
</tr>
<tr>
<td>Group III</td>
<td>2.6±0.05*</td>
<td>1.3±0.02*</td>
<td>5.3±0.37*</td>
<td>0.69±0.07*</td>
</tr>
<tr>
<td>Group IV</td>
<td>1.7±0.04*</td>
<td>1.0±0.04*</td>
<td>4.0±0.51*</td>
<td>0.85±0.18*</td>
</tr>
<tr>
<td>Group V</td>
<td>1.1±0.06*</td>
<td>0.5±0.11*</td>
<td>3.2±0.17*</td>
<td>0.97±0.19*</td>
</tr>
<tr>
<td>Group VI</td>
<td>0.8±0.11*</td>
<td>0.4±0.14*</td>
<td>2.2±0.11*</td>
<td>0.99±0.57*</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M., n=6
*P<0.05 statistically significant when compared to EAC control group (Group II)

Viable cells: Not Stained with Trypan blue
Non-viable cells: Stained with Trypan blue

### Table 4: Effect of EELN on hematological parameters

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Hb(g/dl)</th>
<th>RBC count (10⁶/µl)</th>
<th>WBC count (10⁶/µl)</th>
<th>Lymphocytes (%)</th>
<th>Neutrophils (%)</th>
<th>Monocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>13.2±0.14</td>
<td>5.2±0.05</td>
<td>5.8±0.34</td>
<td>68±0.14</td>
<td>29±0.97</td>
<td>1.8±1.5</td>
</tr>
<tr>
<td>Group II</td>
<td>10.8±0.27*</td>
<td>3.9±0.14*</td>
<td>10.0±0.47*</td>
<td>28±0.24*</td>
<td>66±0.12*</td>
<td>2.1±0.5*</td>
</tr>
<tr>
<td>Group III</td>
<td>11.9±0.06*</td>
<td>4.2±0.02*</td>
<td>8.6±0.57*</td>
<td>41±1.1*</td>
<td>52±0.62*</td>
<td>1.71±0.84*</td>
</tr>
<tr>
<td>Group IV</td>
<td>12.5±0.03*</td>
<td>4.6±0.13*</td>
<td>7.6±0.38*</td>
<td>57±1.5*</td>
<td>57±0.47*</td>
<td>1.58±0.39*</td>
</tr>
<tr>
<td>Group V</td>
<td>13.5±0.01*</td>
<td>5.1±0.15*</td>
<td>6.2±0.72*</td>
<td>67±0.18*</td>
<td>29±0.9*</td>
<td>1.34±0.47*</td>
</tr>
<tr>
<td>Group VI</td>
<td>13.4±0.37*</td>
<td>4.9±0.39*</td>
<td>5.4±0.06*</td>
<td>69±1.5*</td>
<td>30±1.1*</td>
<td>1.7±0.8*</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M., n=6
*P<0.05 statistically significant when compared with normal group
**P<0.05 statistically significant when compared with EAC group

### Table 5: Effect of EELN on enzymatic and non-enzymatic antioxidants

<table>
<thead>
<tr>
<th>Particulars</th>
<th>LPO/µmol (MDA·mg-protein)</th>
<th>Glutathione mg·g⁻¹ (wet tissue)</th>
<th>SOD U·mg⁻¹ (protein)</th>
<th>Catalase U·mg⁻¹ (protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>0.9±0.02†</td>
<td>2.3±0.09</td>
<td>4.9±0.35</td>
<td>28±0.09</td>
</tr>
<tr>
<td>Group II</td>
<td>3.9±0.23**</td>
<td>0.4±0.41**</td>
<td>1.2±0.76*</td>
<td>10±0.76**</td>
</tr>
<tr>
<td>Group III</td>
<td>2.9±0.26**</td>
<td>0.9±0.73**</td>
<td>2.6±0.89**</td>
<td>12±0.96**</td>
</tr>
<tr>
<td>Group IV</td>
<td>2.7±0.38**</td>
<td>2.6±0.33**</td>
<td>3.3±0.43**</td>
<td>18±0.54**</td>
</tr>
<tr>
<td>Group V</td>
<td>1.3±0.03**</td>
<td>2.3±0.37**</td>
<td>4.1±0.22**</td>
<td>23±0.60**</td>
</tr>
<tr>
<td>Group VI</td>
<td>1.2±0.01**</td>
<td>2.9±0.22**</td>
<td>4.2±0.61**</td>
<td>25±0.77**</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M., n=6
*P<0.05 statistically significant when compared with normal group
**P<0.05 statistically significant when compared with EAC group

Effect of EELN on Body weight and MST:
Life span of the tumor bearing animal increased upto 16.58%,42.48%,73.57% respectively in drug treated group compared to Mean survival time of Tumor bearing animals (Table 2). The body weight of the tumor bearing animal also reduced indicating the tumor inhibiting property of the EELN.

Tumor response:
Elevated levels of Tumor volume, Packed cell Volume, and Viable cell counts in tumor bearing animals significantly decreased in the drug treated groups (Table 3) which further provided supporting scientific evidences for the anti-tumor potentials of the test drug (EELN).

EELN on Hematological profiles:
Hematological parameters (Table 4) of tumor animals such as levels of Hemoglobin and counts of RBC and WBC reverted back to near normal and Differential counts of Lymphocytes, Neutrophils and Monocytes, which were also altered reverted back to normal value after the test drug administration.

Effect Of EELN On Non-Enzymatic & Enzymatic Antioxidant Profile:
From the Table 5 it is evident that the Increased levels of LPO and reduced levels of Glutathione, SOD and Catalase were restored to normal level in the test drug administered animals.

DISCUSSION:
Besides surgery and radiation therapy, chemotherapeutic agents were used as adjuvants in the management of cancer. At present more than 25% of drugs used to treat cancer are derived from plants. In the present study Leonotis nepetapholia R.Br. a traditional drug source identified and collected from in combat this serious killer disease.
One of the reliable criteria for judging the merit of any anticancer drug is prolongation of life span of the tumor bearing animals (15). In the present study it is observed that EELN (the drug under study) increased the life span of tumor bearing animal by about 16.58%,42.48%,& 73.57% and is dose dependent. The change in body weights of the animals depicted the tumor growth inhibiting property of EELN in a dose-dependent manner.

Tumor bearing mice revealed increased peritoneal cell counts and ascites fluid. Ascites fluid is the direct nutritional source for tumor cells and fluid volume and will directly represent the tumor growth (16). Treatment with EELN at various dose levels decreased the Ascites fluid volume as well as peritoneal cell counts.

Usually, in cancer chemotherapy the major problems that are often encountered are myelosupression and anemia. The anemia encountered in cancer animals are mainly due to reduction in RBC and Hemoglobin content which may be due to iron deficiency or hemolysis (17,18). Treatment with EELN brought back the Hemoglobin, RBC and WBC contents more or less to near normal. This suggested that EELN has a protective action on the hematopoietic system.

Excessive production of free radicals will result in oxidative stress, leading to damage of macromolecules such as lipids and can induce lipid peroxidation (19,20). Increased lipid peroxidation causes degeneration of tissues and lipid peroxide formed in the primary site are transferred through circulation and can provoke further damage to the cells. MDA end product of lipid peroxidation will be higher in cancerous tissue than normal tissue. Higher MDA level in cancerous tissue was brought back to normal after the test drug administration in a dose dependent manner.

Glutathione is a potent inhibitor of neoplastic process (21). Free radical scavenging potential of the Herbal drug under study increased the level of glutathione which in turn facilitated the inhibition of neoplastic process and contributed towards the increase of life span.

SOD represent the first line of protection against superoxide anions because it catalyzes dismutation of superoxide anions into Hydrogen peroxide and molecular oxygen. (22). A decrease in SOD and catalase activity in EAC bearing mice might be due to loss of Mn-SOD activity in EAC cells and a loss of mitochondria. The administration of EELN at different doses increased the activities of SOD and catalase.

The data of the results were comparable with that of the standard drug 5-fluorouracil (20mg/kg bw). It is observed that 300mg/Kg bw. is the most effective dose. Thus present findings clearly depicted the anti tumor and antioxidant potentials of the ethanolic extract of Leonotis nepetaphila R.Br (EELN).

CONCLUSION

The present study is aimed at to investigate the antitumor and antioxidant potentials of the ethanolic extract of Leonotis nepetaphila R.Br (EELN) on Ehrlich Ascites Carcinoma cell lines. Tumor was induced intraperitoneally using Ehrlich Ascites Carcinoma cells (1X10^6/cells/mouse). Ethanolic extract of Leonotis nepetaphila R.Br was administered for 14 days to the experimental animals after 24h of tumor inoculation at the dose level of 100, 200 & 300 mg/kg,bw./day. The drug treatment increased the lifespan and restored the altered levels of hematological parameters and enzymatic and non-enzymatic anti oxidants in the liver tissue of EAC bearing animals. It is also noted that of the various doses administered 300mg/Kg,bw. was the most effective dose and the results were comparable with that of the standard drug 5-fluorouracil.

REFERENCES


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