Anti-tumor effect of *Euphorbia hirta* on Ehrlich's ascites carcinoma in mice

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SUMMARY

Anti-tumor activity of *Euphorbia hirta* (50 mg/kg and 100 mg/kg) has been evaluated against Ehrlich's ascites carcinoma (EAC) in Swiss albino mice. Intraperitoneal (*i.p*) administration of *Euphorbia hirta* was effective in reducing solid tumor mass development induced by EAC cells. It exhibited significant anti-tumor activity in mice, when used at the dose of 100 mg/kg/day *i.p.*, for 14 days. The administration of *Euphorbia hirta* (100 mg/kg/day *i.p.*) resulted in an increase ($P < 0.001$) of the life span (59.9%) of ascites tumor bearing mice as compared to the control group. After 14 days, on developed tumor masses, *Euphorbia hirta* administration brought about significant reduction in tumor volume and it reverse the changes in the hematological parameters, responding to tumor inoculation. The results are indicative of the anti-tumor activity of *Euphorbia hirta* against EAC induced tumor in a dose dependent manner.

Key words: *Euphorbia hirta*; Ehrlich's ascites carcinoma; Anti-tumor activity; Median survival time; Hematological parameters

INTRODUCTION

Cancer is the second leading cause of death after heart disease in the world. It involves fundamental biological process concerning disordered cell replication death, disorganization of organ structure (Polasa, 2000) and ability to invade various tissues to form malignant tumors (Cotran *et al.*, 1994, Workman and Koy, 2001). Plant products have been contributed several novel compounds promising anti-tumor activity. Many naturally occurring tested for anticancer activity on experimental animals in the present availability of some effective anticancer drugs (Refsnes *et al.*, 1974; Siegas *et al.*, 1999; Ram sevak *et al.*, 2000; Filleur *et al.*, 2001; Ramnath *et al.*, 2002). Herbs have been used as food and for medicinal purposes for centuries. Research interest has been focussed on various herbs that posses antiplatelet, anti-tumor, or immune- stimulating properties that may be useful adjuncts in helping reduce the risk of cardiovascular disease and cancer. Many herbs contain potent antioxidant compounds that provide significant protection against chronic diseases and may also have antiviral or anti-tumor activity. The volatile essential oils of commonly used culinary herbs, spices, and herbal teas inhibit mevalonate synthesis and thereby suppress cholesterol synthesis and tumor growth.

Euphorbia hirta (Family: Euphorbiaceae) is an

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erect or climbing ascending annual herb with hairy stems and opposite or long lanceolate leaves found throughout India (Ambasta, 1986). This plant is very well known for its various treatments in the ancient traditional system of medicine, it is administered in the form of liquid extract or tincture with lobelia or senega in the treatment of cough and asthma. It is also applied topically to ulcers, edemas, phelegmons, abscesses and inflammed glands (Kirtikar and Basu, 1975). Anti-diarrheal effect (Galvez *et al.*, 1993), anti-amoebic effect (Nadir *et al.*, 1981) ACE inhibitory effect (Williams *et al.*, 1997), anti-bacterial effect (Ajao *et al* 1985), diuretic effect (Johnson *et al.*, 1999), anti-inflammatory effect (Martinez - Vazquez *et al.*, 1999) of *E. hirta* have been reported earlier. The present study was undertaken to evaluate the anti-tumor effect of the ethanol extract of *Euphorbia hirta* against Ehrlich's ascites carcinoma in mice.

MATERIALS AND METHODS

Plant materials
Leaves of *Euphoria hirta,* which was reported to be used against cancer by folk doctors in TamilNadu and leaves, were collected in the month of June 2002. The twigs of the plant along with flowers were submitted and Chief botanist at Department of Botany, St Joseph's college, Trichirappalli, authenticated it. A voucher specimen (Voucher No: PPRL/HS/1/2005) was preserved in our laboratory herbarium, Department of Pharmaceutical Technology, Jadavppur University, Kolkata.

Preparation of plant extracts
The leaves were shade dried and powdered, passed in sieve 22. The powered material was extracted using 95% ethanol in Soxhlet apparatus and concentrated to dryness under reduced pressure by means of rotary-vacuum evaporator (Buchi, Mumbai, India). The dried ethanol extract of *Euphorbia hirta* (EEEH) was stored in a desiccator for the pharmacological and phytochemical screening.

Animals
Adult Swiss male albino mice (5 - 6 weeks) weighing, 20 - 24 g procured from Venkateswara Enterprises, Bangalore were housed in microlon boxes with controlled temperature ($22 \pm 2°C$) on a 12:12 h light/dark cycle, animals had free access to standard laboratory diet and water *ad libitum.*

Reagents and chemicals
5-fluorouracil (5-FU) was purchased from Biochem Pharmaceuticals Ltd (Biochem, Mumai). Trypan blue was purchased from Sigma Chemicals (Sigma, U.S.A.). All other chemicals used were of analytical grade.

Cell lines
Ehrlich's ascites carcinoma (EAC) cells were obtained from the courtesy of Amala Cancer Research Centre, Thrissur. EAC cells maintained by weekly intraperitoneal (*i.p.*) inoculation of 10^6 cells/mouse (Clarkson *et al.*, 1965; Gothoskar *et al.*, 1971).

Treatment schedule
Effect of EEEH on tumor growth and host survival were determined by evaluation tumor volume, tumor cell count and percentage increase in lifespan (% ILS) of the tumor hosts, respectively. For calculating the survival time four groups of mice were used and given food and water *ad libitum.* Tumor was induced to all the groups by injecting 0.2 ml of 2.3×10^5/ml of EAC cells in to the peritoneal cavity of mice, except normal group. This was taken as day '0'. On the first day the normal saline (0.9% w/v, NaCl 5 ml/kg/mouse/day) administered in to normal group. Two different doses of EEEH (50 mg/kg and 100 mg/kg *i.p.*) and standard drug 5-flurouracil (5-FU, 20 mg/kg *i.p.*) were subsequently administered for 9 days. On the 9th day , after the last dose and 18 h fasting six from each group were sacrificed for the anti-tumor activity and the rest of the animals were kept for check the mean survival time (MST) and increase in the lifespan of the tumor bearing mice.

(Sivakumar *et al.*, 2005).

Tumor growth response

Antitumor growth effect of EEEH was assessed by observation of changes with respect to ascitics tumor volume, packed cell volume, viable and nonviable tumor cell count, MST and percentage increase in the lifespan (% ILS). Transplantable murine tumor was carefully collected with the help of s sterile 3 ml syringe and measured the tumor volume and the ascetic fluid with was withdraw in a graduated centrifuge tube and packed cell volume was determined by centrifuging at $1,000 \times g$ for 5 min. viable and nonviable cell count of asciatic cell were stained by the tryban blue dye (0.4% in normal saline) exclusion test and count was determined in a neubauer counting chamber. The effect of EEEH on tumor growth was monitored daily by recoding the mortality and percentage increase in life span (5 ILS) was calculated using the following formula ILS (%) = {Mean survival of treated group/Mean survival of control group) - 1 × 100} (Raikapoor *et al.*, 2003).

Hematological studies

On the 14 day after tumor inoculation blood was obtained through tail vein from normal mice, tumor bearing mice and tumor bearing mice treated with EEEH (100 mg/kg/mouse/day *i.p.* for the 9 days. For the total count blood was drawn into RBC or WBC pipettes, diluted and counted in a neubauer counting chambers. Sahli's Hemoglobinometer was used for the determination of hemoglobin concentration. Differential count of leukocytes was done on a freshly drawn blood film using Leishman's stain. Hemoglobin content (D' Amour *et al.*, 1965), RBC and WBC count (Wintrobe *et al.*, 1961) and differential leukocyte count (Dacie and Lewis, 1958) was estimated from the peripheral blood of normal, EAC control and treated groups.

Measurement of normal peritoneal cells

Three groups of normal mice (n = 6) were used for this study. One group was treated with 100 mg/kg, *i.p.* of EEEH only once for a single day, while the second group received the same treatment for two consecutive days. The untreated third group was used as control and treated with equivalent volume of normal saline. After 24 h intraperitoneal fluid was collected by repeated intraperitoneal wash with cold normal saline and intraperitoneal cells were counted using hemocytometer in each of the treated groups and compared with untreated control group (Hudson and Hay, 1989; Sur and Ganguly, 1994).

Statistical analysis

All the values are expressed in mean ± SEM. The data were statistically analyzed using one way analysis of ANOVA followed by Dunnett's multiple comparison test using Graphat Instat Software (San Diego, U.S.A.) and student's *t*-test (Armitage *et al.*, 1971). Difference in means were considered to be significant when $P < 0.01$.

RESULTS

Effect of EEEH on survival time of tumor bearing mice

The effect EEEH on the survival of tumor bearing mice is shown in Table 1 and Fig. 1. Median survival time for the control group was 19.7 ± 1.0 days, while it was 27.0 ± 0.9, 31.5 ± 1.1, and 36.1 ± 0.8, days for the groups treated with Euphorbia hirta 50 mg/kg, 100 mg/kg and 5 FU 20 mg/kg, respectively. Significant ($P < 0.001$) increases in the lifespan of

Table 1. Effect of EEEH treatment on the survival of EAC bearing mice

Experiment	Median survival time (days)	Increase in lifespan (%)
EAC Control	19.7 ± 1.0	
EEEH (50 mg/kg)	27.09 ± 0.9*	41.6%
EEEH (100 mg/kg)	31.5 ± 1.1*	59.9%
5 FU (20 mg/kg)	36.1 ± 0.8*	83.2%

Values are expressed as mean ± SEM (n = 10). Days of drug treatment = 9; *$P < 0.001$, Significantly differ from control group.
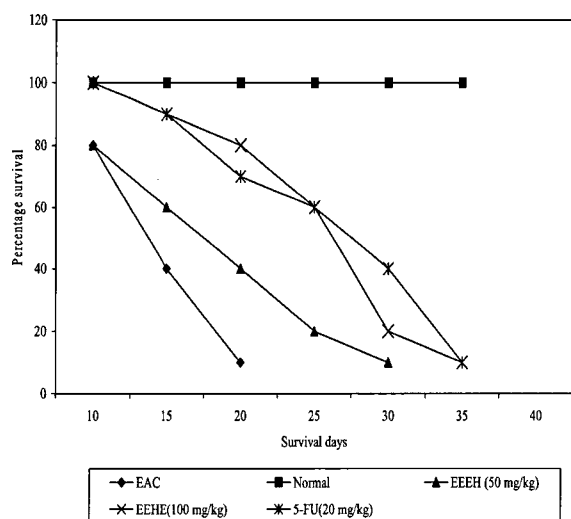
Fig. 1. Effect of EEEH and 5-FU on the percentage survival days of mice treated with EAC cells.

tumor bearing mice treated with EEEH 50, 100 mg/kg and 5 FU- 20 mg/kg was found to be 41.6%, 59.6% and 83.2%, respectively, as compared to the control group.

Effect of EEEH on normal peritoneal cells

The average number of peritoneal exudates cells per normal mouse was found to be 5.98 ± 0.1. EEEH (100 mg/kg/day *i.p.*) treatment increased the number of peritoneal cells as shown in Table 2. Single treatment with EEEH (100 mg/kg) enhance the number to 8.2 ± 0.1, while two consecutive treatments enhanced the number to 8.9 ± 0.1 ($P < 0.001$).

Effect of EEEH on tumor growth

Intraperitoneal administration of EEEH (100 mg/kg) to tumor bearing mice was found to be effective in

Table 2. Effect of EEEH (100 mg/kg/day *i.p.*) on peritoneal cells

Experiment	Number of peritoneal cells ($\times 10^6$) per mouse
Control	5.98 ± 0.1
Once treated	8.2 ± 0.1*
Treated two consecutive days	8.9 ± 0.1*

Values are expressed as mean ± SEM (Number of animals used = 6 in each groups).
*$P < 0.001$ as compared to control group

controlling the tumor volume development was shown in Table 3. The untreated control mice showed a tumor volume of 4.0 ± 0.09 ml. A tumor volume of 1.8 ± 0.14 ml, 1.6 ± 0.1 ml and 1.4 ± 1.12 ml (induced by EAC cells) were recorded for the animals treated with the dose of EEEH 50 mg/kg, 100 mg/kg, and 5 FU 20 mg/kg, respectively. Tumor volumes in mice for the treated groups were lesser as compared to controls and statistically it was significant ($P < 0.001$).

The viable tumor cell counts were shown in Table 3. The viable tumor cell counts for the control group 8.4 ± 0.13, while it was 5.77 ± 0.13, 3.51 ± 0.22 and 2.5 ± 0.28 for the groups treated with EEHE 50 mg/kg, 100 mg/kg and 5 FU 20 mg/kg, respectively. The reduction of viable tumor cell count was found to be effective ($P < 0.001$) as compared to the control group.

Effect of EEEH on hematological parameters

Hematological parameters of tumor bearing mice on 14th day were found to be significantly altered from group was shown in Table 4. The total WBC count, protein and PCV were found to be increased along with the hemoglobin content of RBC.

Table 3. Effect of EEEH on tumour growth and tumor cell count induced by EAC

Treatment	Tumor volume (ml)	Viable tumor cell count ($\times 10^7$ cells/ml)
EAC control	4.0 ± 0.09	8.4 ± 0.13
EEEH (50 mg/kg/day *i.p*)	1.8 ± 0.14*	5.7 ± 0.13*
EEEH (100 mg/kg/day *i.p*)	1.6 ± 0.1*	3.5 ± 0.22*
5FU (20 mg/kg/day *i.p.*)	1.4 ± 1.12*	2.5 ± 0.28*

Results are expressed as mean ± SEM. Days of treatment = 7; *$P < 0.001$ as compared to control group.

Table 4. Effect of EEEH (100 mg/kg/day, *i.p.*) on hematological parameters

Hematological Parameters	Normal	Tumor bearing mice (14 days)	EEEH Treated Tumor bearing mice
Hb (%)	14.6 ± 0.3	8.9 ± 0.1*	12.8 ± 0.3*
RBC Cells/ml × 10^{10}	3.9 ± 0.3	7.7 ± 0.2*	2.8 ± 0.3**
WBC Cells/ml × 10^{10}	7.5 ± 0.2	16.7 ± 0.2*	10.8 ± 0.2**
Protein (g%)	8.4 ± 0.3	13.5 ± 0.3*	0.8 ± 0.2**
PCV (mm)	16.9 ± 0.3	25.8 ± 0.3*	18.9 ± 0.2**
Neutrophil	69.7 ± 0.3	25.8 ± 0.2*	56.2 ± 0.2**
Lympocytes	30.1 ± 0.3	72.2 ± 0.2*	42.7 ± 0.3**
Monocytes	2.33 ± 0.42	1.33 ± 0.33[a]	1.17 ± 0.31[b]

Days of drug treatment = 14, n = 6 animals in each group. *$P < 0.001$ as compared to normal mice; *$P < 0.01$, **$P < 0.001$ as compared to tumor bearing mice. [a]Non significant as compared to normal group; [b]Non significant as compared to control group.

Differential counts were significantly reduced by EEEH treatment and monocytes counts were not significant when compared with the control group (tumor treated groups). At the same time interval, EEEH (100 mg/kg/day *i.p*) treatment could change altered hematological parameters to near normal.

DISCUSSION

The present study was carried out to evaluate the anti-tumor effect of *Euphorbia hirta* on Ehrlich's ascites carcinoma bearing mice in *in vivo*. Various reports on the mechanism behind the anti-tumor activity of various plant extracts indicate that different plant extracts exhibited their anti-tumor activities through different mechanism in the host (Sakagami *et al.*, 1987; Sllchenmyer *et al.*, 1991; Tanaka *et al.*, 1996; Chaudhuri *et al.*, 1998; Rosangkima *et al.*, 2002; Das *et al.*, 2004). In the anti-tumor effect of tea plant (*Camallia sinensis* var assamica, Theaceae) root extract, the activity of superoxide dismutase, a free radical scavenger, was found to be increased in the serum of tumor bearing mice, suggesting the involvement of the tea root extract in the enhancement of the defense mechanism (Chaudhuri *et al.*, 1998).

Ascites fluid is the direct nutritional source to tumor cells and the faster increase in ascites fluid with tumor growth would be means to meet the nutritional requirement of tumor cell (Prasad *et al.*, 1994). The reliable criteria for judging the value of

any anticancer drug are the prolongation of life span of animals and disappearance of leukemia cells from blood (Oberling *et al.*, 1994; Mazumdar *et al.*, 1997). The results of the present study demonstrate that EEEH treatment suppresses the EAC cells growth, by reducing the tumor volume and viable tumor cell count (Table 2). EEEH- treated animals at the dose of 200 mg/kg significantly inhibited the tumor volume, packed cell volume, tumor cell count and brought the hematological parameters near to normal values.

The cytotoxicity of oxygen free radicals can cause several diseases (Emerit *et al.*, 1994). Body cells and tissues are continuously threatened by free radicals and reactive oxygen species, which are produced during normal oxygen metabolism or are induced by exogenous damage (De Groot, 1994; Grace, 1994). Reactive oxygen species (ROS) can damage DNA and division of cells with unpaired or mispaired damage leads to mutation. If these changes appear in critical genes, initiation or progression may result. ROS can interfere directly with cell signaling and growth. The cellular damage caused by ROS can induce mitosis, increasing risk that damaged DNA will lead to mutations, and can increase the exposure of DNA to mutagens. Free radicals can attract various inflammatory mediators; contribute to a great inflammatory response and tissue damage.

All these data point to the possibility of developing EEEH as a potential agent in the area of cancer

chemotherapy. Preliminary phytochemical screening indicated that presence of alkaloids and flavonoids in *Euphobia hirta* (Baslas *et al.*, 1980; Galvez *et al.*, 1993). Flavonoids have been shown to possess free radical scavenging antimutagenic and anti-malignant effects (Brown *et al.*, 1980; Husain *et al.*, 1987; Rebak *et al.*, 1988; Hirano *et al.*, 1989). More over flavonoids have chemopreventive role in cancer through effects on signal transduction in cell proliferation and angiogenesis (Weber *et al.*, 1996; Fotis *et al.*, 1997). Flavonoids can also prevent injury caused by free radicals in various ways. One way is the direct scavenging of free radicals. Flavonoids are oxidized by radicals, resulting in a more stable, less reactive radical. In other words, flavonoids stabilize the reactive oxygen species by reacting with the reactive compound of the radical. Because of the high reactivity of the hydroxyl group of the flavonoids, radicals are made inactive, according to the following equation: Flavonoid (OH) + R > flavonoid (O) + RH, where R is a free radical and O˙ is an oxygen free radical (Korkina *et al.*, 1997) It has been stated that flavonoids, as antioxidants, can inhibit carcinogenesis (Stefani *et al.*, 1999). Specific flavonoids are known to chelate iron thereby removing a casual factor for the development of free radicals.

The cytotoxicity and anti-tumor properties of the extract of *Euphobia hirta* may be due do its quercetin nature (Khandiya *et al.*, 2002). Quercetin in particular is known for its iron chelating and iron stabilizing properties. Direct inhibition of lipid peroxidation is another protective measure. Quercetin and apigenin also inhibited melanoma growth and influenced the invasive and meatastatic potential in mice. A large clinical study suggested the presence of an inverse association between flavonoid intake and the subsequent incidence of lung cancer (Hollman *et al.*, 1997). This effect was mainly ascribed to quercetin, which provided > 95% of the total flavonoid intake in that particular study. Quercetin and apigenin inhibited melanoma growth and influenced the invasive and metastatic potential in

mice (Caltagirone *et al.*, 2000). Quercetin, in particular, inhibits both cyclo-oxgenase and lipo-oxygenase activities, thus diminishing the formation of these inflammatory metabolites (Robak *et al.*, 1996; Kim *et al.*, 1998).

The exact mode of action of EEEH is not known. As far as present findings are concerned, the exact mechanism of action the plant extracts against Ehrlich's ascites carcinoma may not be elucidated in detail at present. However, the increase of lifespan and reduced tumor volume, viable tumor cell count by EEEH treatments seems to be playing a significant role in mediating cytotoxic and anti-tumor activity against Ehrlich's ascites carcinoma.

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