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Effects of ethanol extract of *Pisonia aculeata* Linn. on ehrlich ascites carcinoma tumor bearing mice

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In order to scientifically appraise some of the anecdotal, folkloric, ethno medical uses of *Pisonia aculeata* Linn. (Nyctaginaceae), the present study was undertaken to examine the antitumor activity of *Pisonia aculeata* leaves extract on Ehrlich Ascites Carcinoma (EAC) in mice. Tumor was induced in mice by intraperitoneal injection of Ehrlich Ascites Carcinoma cells ($1X10^6$ cells/mouse). Ethanol extract of *Pisonia aculeata* (EEPA) was administered to the experimental animals at the doses of 250 & 500 mg/kg/day, *p.o.* The antitumor effect of the extract was evaluated by using survival time, hematological parameters, increase in body weight, solid tumour volume and peritoneal cell count. Oral administration of EEPA increased the survival time and inhibits the weight gain of the tumor bearing mice. After 14 days of inoculation, the extract also reduces the solid tumor volume developed by the EAC cells. The findings of this study indicate that the EEPA possesses significant antitumor activity on dose dependent manner..

Key words: *Pisonia aculeata*, ehrlich ascites carcinoma, hematological parameters, survival time, peritoneal cell count and solid tumor

INTRODUCTION

The majority of the world's population in developing countries still relies on herbal medicines to meet their health needs in cases when synthetic medicine could not relieve patients who suffer from hard to cure illnesses like cancer. *Pisonia aculeata* Linn. is a large scandent shrub, which holds an important place in folklore medicine. It is extensively used by native medical practitioners and tribes for treating swelling, rheumatic pains, jaundice and tumors (Nadkarni, 2005; Anonymous, 2003). Preliminary phytochemical screening of the extract showed the presence of alkaloids, triterpenes, phenolic compounds, flavonoids and glycosides. However no studies to date have been able to demonstrate the pharmacological activities. The present study is focused on evaluation of the anticancer activity of the leaves of *Pisonia aculeata* against Ehrlich Ascites carcinoma in mice.

MATERIALS AND METHODS

Collection and extraction

The fresh leaves of *Pisonia aculeata* were collected in and around Kolli hills in Salem district, Tamilnadu, India, in the month of September 2006 and authenticated by Dr. Gopalan, Botanical Survey of India, Coimbatore, Tamilnadu, India. A voucher specimen (Voucher No. PCH 002) representing this collection has been retained in our laboratory for future reference.

The leaves were shade dried and pulverized. The powder was treated with petroleum ether for dewaxing and removal of chlorophyll. Later it was packed (250 g) in soxhlet apparatus and subjected to continuous hot percolation for 8 h using 450 ml ethanol (70% v/v) as solvent. The ethanol extract was concentrated under vacuum and dried in a dessicator (yield 12.5 g, 5 % w/w). Without any purification, aliquot portions of the crude extract were suspended in 5% gum acacia for use on each day of our experiment (Suffness and Douros, 1978).

The Phytochemical studies were performed as described by Wagner *et al*, 1984. The presence of alkaloids, glycosides, flavonoids, phenolic compounds, steroids and terpenoids were analyzed. The extract showed the positive test for alkaloids, glycosides, triterpenes, flavonoids and phenolic compounds.

Animals

Swiss albino mice (20-25 g) were procured from Venkateshwara Enterprises, Bangalore, Karnataka, India, and used throughout the study. They were housed in microlon boxes in a controlled environment (temperature 25±2 °C and 12 h dark/light cycle) with standard laboratory

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diet and water *ad libitum*. The study was conducted after obtaining Institutional Animal Ethical Committee clearance.

Acute Toxicity Studies (LD_{50})

The oral acute toxicity study of the extract was carried out in Swiss albino mice using the up and down procedure (OECD, 2001). This method was carried out in fifteen animals, three per treatment group and widely different dose ranges of 1, 2, 3, 4 and 5 g/kg, respectively and observed after 24 h. Based on the results the extract did not produce any mortality at the doses tested. To optimize the dose levels, 250 & 500 mg/kg body weight was selected for the evaluation.

Cells

EAC cells were originally obtained through the courtesy of Amala Cancer Research Center, Thrissur, Kerala, India. They were maintained by weekly intraperitoneal inoculation of 10^6 cells/mouse (Gothoskar and Ranadive, 1971).

Effect of EEPA on Survival Time

Animals were inoculated with 1×10^6 cells/mouse on day '0' and treatment with EEPA started after 24 hrs of inoculation, at doses of 250 and 500 mg/kg/day, *p.o.* The control group was treated with the same volume of 0.9% sodium chloride solution. All the treatments were given for nine days. The median survival time (MST) and average body weight changes of each group, consisting of 6 mice were noted. The antitumor efficacy of EEPA was compared with that of 5-fluorouracil (Dabur Pharmaceuticals, India; 5-FU, 20 mg/kg/day, *i.p.* for 9 days). The MST of the treated groups was compared with that of the control group using the following calculation.

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 (Sur and Ganguly, 1994).

Effect of EEPA on Normal Peritoneal Cells

Five groups of normal mice (n=5) were used for determining the effect of the EEPA on normal peritoneal cells. Group I was treated once with 250 mg/kg, *p.o.* of EEPA, Group II received the same treatment for two consecutive days, Group III was treated once with 500 mg/kg, *p.o.* of EEPA and Group IV received the same treatment for two consecutive days, respectively (Sur and Ganguly, 1994).

Effect of EEPA on Hematological Parameters

In order to detect the influence of EEPA on hematological status of EAC bearing mice, a comparison was made among four groups (n = 6) of mice on the 14th day after inoculation. The groups comprised of (I) Tumor bearing mice (II) Tumor bearing mice treated with EEPA (250 mg/kg/day, *p.o.* for 9 days) (III) Tumor bearing mice treated with EEPA (500 mg/kg/day, *p.o.* for 9 days) and (IV) Control mice (normal). Blood was drawn from each mouse by the retroorbital plexus method and the white blood cell count (WBC), red blood cells (RBC) hemoglobin, protein and packed cell volume (PCV) were determined (Sur and Ganguly, 1994, D'Amour, 1965; Lowry *et al*, 1951; Docie, 1958).

Effect of EEPA on Solid Tumor

Mice were divided into three groups (n = 6). Tumor cells (2×10^6 cells/mouse) were injected into the right hind limb of all the animals intramuscularly. The mice of group I were tumor control. Group II received EEPA (250 mg/kg/day, *p.o.*) and group III received EEPA (500 mg/kg/day, *p.o.*) for 5 alternative days. Tumor mass was measured from the 11th day of tumor induction. The measurement was carried out every 5th day for a period of 30 days. The volume of tumor mass was calculated using the formula $V = 4/3\ \pi r^2$, where 'r' is the mean of 'r^1' and 'r^2' which are the two independent radii of the tumor mass (Ramnath *et al*, 2002; Kuttan *et al*, 1990).

Statistical Analysis

All values were expressed as mean ± SEM. Statistical analysis was performed with one way analysis of variance (ANOVA) followed by Dunnett's '*t*' test. *P* values < 0.05 were considered to be statistically significant when compared to control.

RESULTS

Effect of EEPA on Survival Time

The effect of EEPA on the survival of tumor bearing mice is shown in Table 1. The MST of the control group was 21 ± 0.21 days, whereas it was 25 ± 0.3, 34 ± 0.21 and 38 ± 0.3 days for the groups treated with EEPA (250 & 500 mg/kg) and 5-FU (20 mg/kg) respectively (*P*<0.001 & 0.05). The increase in the life span of tumor bearing mice treated with EEPA and 5-FU was found to be 19.4%, 61.9% and 80.95% respectively.

Table 1: Effect of EEPA on median survival time and average increase in body weight of EAC tumor bearing mice

Design of treatment	MST (in days)	Increase in life span T/C%	Average increase in body weight (g)
Tumor control	21 ± 0.21	-	13.3 ± 0.21
5-FU (20 mg/kg, i.p)	38 ± 0.3[a]	80.95	4.5 ± 0.22[a]
EEPA (250 mg/kg, p.o)	25 ± 0.3[b]	19.04	8.67 ± 0.21
EEPA (500 mg/kg, p.o)	34 ± 0.21[a]	61.9	5.3 ± 0.36[a]

N = 6 animals in each group; [a]*P* <0.001; [b]*P* <0.05 when compared with control; Values are expressed as mean ± SEM.

The effect of EEPA on the inhibition of average increase in body weight is shown in Table 1. The average weight gain of tumor bearing mice was 13.33 ± 0.21 g, whereas it was 8.67 ± 0.21, 5.3 ± 0.36 and 4.5 ± 0.22 g for the groups treated with EEPA (250 & 500 mg/kg) and 5-FU (20 mg/kg) respectively ($P< 0.001$).

Effect of EEPA on normal peritoneal cells

The average number of peritoneal exudate cells per normal mouse was found to be 5.6 ± 0.4 X 10^6. Single treatment with EEPA (250 & 500 mg/kg) does not increase the peritoneal cell count significantly (Table 2). But two consecutive treatments with EEPA (500 mg/kg) significantly enhanced the number to 12.2 ± 0.24 ($P< 0.001$).

Effect of EEPA on hematological parameters

Hematological parameters of tumor bearing mice on the day 14 were showed significant changes when compared to normal mice (Table 3). The total WBC count, protein and PCV were found to increase with a reduction in the hemoglobin content of RBC. The differential count of WBC showed that the percentage of neutrophils increased while that of lymphocytes decreased ($P< 0.001$ & 0.01 when compared to normal mice). At the same time interval, EEPA (250 & 500 mg/kg) treatment could change these parameters near to normal. Maximum alteration occurred in the EEPA treatment at the dose of 500 mg/kg ($P< 0.001$, 0.05 & 0.01 when compared to tumor control).

Effect of EEPA on solid tumor

There was significant reduction in the tumor volume of mice treated with EEPA (250 & 500 mg/kg, p.o.). Tumor volume of control animals was 12.12 ± 0.4 ml whereas it was 7.34 ± 0.33 ml and 6.05 ± 0.1 ml for the groups treated with EEPA 250 & 500 mg/kg respectively ($P< 0.001$ & 0.01) (Table 4).

Table 2: Effect of EEPA on peritoneal cell count of EAC tumor bearing mice

Design of treatment	No. of peritoneal cells/mouse X 10^6
Normal	5.6 ± 0.4
250 mg/kg (treated once)	7.6 ± 0.24
250 mg/kg (treated twice)	8.2 ± 0.19
500 mg/kg (treated once)	8.8 ± 0.19
500 mg/kg (treated twice)	12.2 ± 0.24[a]

N = 5 animals in each group, [a]P <0.001 when compared with control, Values are expressed as mean ± SEM.

DISCUSSION

Murine type tumors are common malignant tumors. The primary therapy for those tumors includes surgery, radiation therapy and chemotherapy. Although these therapies have been very successful in the treatment of early carcinoma, the prognosis for advanced and recurrent diseases remains very guarded. Some treatments produce serious side effects.

The reliable criteria for judging the value of any anticancer drug are prolongation of life span, inhibition of gain in average body weight and decrease of WBC from blood (Clarkson and Burchenal, 1965; Oberling and Guerin, 1954). The results of the present study showed an antitumor effect of EEPA against EAC in Swiss albino mice. A significant ($P<0.001$ & 0.05) enhancement of MST, decrement of gain in average body weight and enhancement of peritoneal cell count was observed.

The effect of EEPA treatment on the peritoneal exudates cells of normal mice is an indirect method of evaluating its inhibitory effect on tumor cell growth. Normally, a mouse contains about 5 X 10^6 peritoneal cells, 50% of which are macrophages. EEPA treatment was found to enhance the peritoneal cell count on dose dependent manner. These results demonstrate the indirect inhibitory effect of EEPA on EAC cells, which is probably mediated by the enhancement and activation of either macrophage or cytokine production.

The analysis of the hematological parameters showed minimum toxic effect in mice treated with EEPA. After 14 days of transplantation, EEPA was able to reverse the changes in the hematological parameters consequent to tumor inoculation.

The reduction of tumor volume of EEPA treated mice shows dose dependent reduction, which was observed on 30th day. The maximum inhibition produced by EEPA at the dose of 500 mg/kg. The reduction in solid tumor volume indicated that EEPA plays a direct role in killing the tumor cells and enhance the curative effect of tumor chemotherapy.

The antitumour activity of EEPA was comparable to that

Table 3: Effect of EEPA on hematological parameters

Design of treatment	Hb (Gm %)	RBC 10^6 cells/mm^3	WBC 10^3 cells/mm^3	Protein mg %	PCV (mm)	Differential Count (%)		
						Lymphocytes	Neutrophils	Monocytes
Normal	16.33 ± 1.1	4.4 ± 0.2	6.5 ± 0.1	8.5 ± 0.22	16.6 ± 0.21	70.7 ± 1.1	30.3 ± 0.21	1 ± 0
Tumor control	8.1 ± 0.21[a]	2.23 ± 0.21[a]	15.8 ± 0.26[a]	13.5 ± 0.22[a]	25.17 ± 0.17[b]	24.3 ± 0.21[a]	67.33 ± 0.21[a]	1 ± 0
EEPA (250mg/kg)	12.3 ± 0.21[†]	3.71 ± 0.3[†]	11.6 ± 0.3	10.33 ± 0.21	20.5 ± 0.22	62.34 ± 0.21[†]	36.3 ± 0.36	1 ± 0
EEPA (500mg/kg)	14.4 ± 0.21[†]	3.86 ± 0.21[†]	9.42 ± 0.1[†]	8.33 ± 0.3[§]	17.3 ± 0.22[§]	65.5 ± 0.21[†]	27.8 ± 0.25[†]	1 ± 0

N = 6 animals in each group, [a] P<0.001; [b]P<0.01 when compared with normal mice, [†]P<0.001; [‡]P<0.05; [§]P<0.01 when compared with tumor control , Values are expressed as mean ± SEM.

Table 4. Effect of EEPA on solid tumor volume

Design of treatment	Solid tumor volume (ml)			
	15th day	20th day	25th day	30th day
Tumor control	6.7 ± 0.51	8.28 ± 0.3	10.88 ± 0.3	12.12 ± 0.4
EEPA (250 mg /kg)	4.33 ± 0.33[b]	5.56 ± 0.3[b]	7.12 ± 0.1[a]	7.34 ± 0.33[b]
EEPA (500 mg /kg)	5.63 ± 0.21[a]	5.88 ± 0.16[a]	5.88 ± 0.21[a]	6.55 ± 0.1[a]

N = 6 animals in each group, [a]*P <0.001*; [b]*P <0.01* when compared with control, Values are expressed as mean ± SEM.

of 5-fluorouracil which is commonly used as an active antitumour agent in vast series of preclinical and clinical studies (Tomlinson *et al*, 1990).

Preliminary phytochemical screening of the extract showed the presence of triterpenes, phenols, flavonoids and glycosides. These compounds have great antiseptic, anti-bacterial and greatly stimulating therapeutic properties. Treatment with phenolic compounds resulted in an inhibition of tumor proliferation and the progression of inflammation (Jin *et al*, 2001). Flavonoids have been shown to possess antimutagenic and antimalignant effects (Brown, 1980; Hirano *et al*, 1989). Moreover, flavonoids have a chemo preventive role in cancer through their effects on signal transduction in cell proliferation (Weber *et al*, 1996) and angiogenesis (Fotis *et al*, 1997). The antitumor properties of the extract may due to these compounds. The present study points to the potential anticancer activity of *Pisonia aculeata* in a dose dependent manner and might be a promising chemotherapeutic agent against murine tumors. Further studies to characterize the active principles and elucidate the mechanism of the action of EEPA are in progress.

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