

## ANTI-TUMOR ACTIVITY AND ANTI-OXIDANT STATUS OF EUPHORBIA THYMIFOLIA LINN AGAINST EHRlich ASCITES CARCINOMA IN SWISS ALBINO MICE

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### ABSTRACT

**Aim:** To study Anti-tumor effect and anti-oxidant status of *Euphorbia thymifolia* Linn **Materials & Methods:** The present study deals with life span of Ehrlich ascites carcinoma bearing hosts, peritoneal cell count, hematological profile, solid tumor mass and biochemical parameters such as lipid peroxidation, reduced glutathione, superoxide distmutase and catalase activities. **Results:** *Euphorbia thymifolia* at oral dose of 200mg/kg prolongs the life span of Ehrlich ascites carcinoma-tumor bearing mice and causes significant increase in number of peritoneal cell count and significant decrease in volume of solid tumor mass compared to Ehrlich ascites carcinoma bearing control animals. Hematological profile including Red blood cells count, White blood cells count, Hemoglobin and protein estimation

were found to be nearly normal levels in extract treated mice compared to tumor bearing control mice. *Euphorbia thymifolia* significantly decreased the levels of lipid peroxidation, reduced glutathione and significantly increased the levels of superoxide distmutase and catalase in treated group of animals. **Conclusion:** The results of the present study suggest that ethanolic extract of *Euphorbia thymifolia* is an effective anti oxidant and anti tumor activity against Ehrlich ascites carcinoma tumor in mice.

**KEY WORDS:** Anti-oxidants, Ehrlich ascites carcinoma, Euphorbia thymifolia.

## INTRODUCTION

Cancers figure among the leading causes of death worldwide, accounting for 8.2 million deaths in 2012. <sup>[1]</sup> An extremely promising strategy for cancer prevention today is chemoprevention, which is a way to prevent or delay the development of cancer by taking medicines, vitamins or other agents in humans. <sup>[2]</sup> Many chemotherapeutic drugs used in cancer treatment are derived from plants. They are developed after rigorous clinical trials for safety and efficacy. There is a growing interest in the pharmacological evaluation of various plants used in Indian traditional system of medicine.

The plants Euphorbia thymifolia Linn belongs to family Euphorbiaceae popularly known as Chhoti dudhi, is a small prostrate, hispidly pubescent, annual weed, which is commonly found in India & tropical countries. It is believed to possess diuretic, laxative & detumescent, anti-malarial, anti-diarrheic, anti-rash, anti-dysentery, anti-carbuncle detoxification and anti-hemorrhoidal activity. <sup>[3]</sup> Charaka prescribed Dudhika as an ingredient of vegetable soup for diarrhoea and painful bleeding piles. <sup>[4]</sup> Euphorbia thymifolia possess anti-oxidant and anti-viral activities. <sup>[5]</sup> The plant is also used to treat eye swelling and discharge. <sup>[6]</sup>

Plant derived natural products such as tannins, flavonoids, terpenoids and steroids etc have received considerable attention in recent years due to their diverse pharmacological properties including anti-oxidant and anti-tumor activity. <sup>[7-8]</sup> Antioxidants play an important role in inhibiting and scavenging radicals, thus providing protection to humans against infection and degenerative diseases. <sup>[9]</sup> From this view point the present study was carried out to evaluate the antitumor activity, lipid peroxidation and antioxidant status of Euphorbia thymifolia against Ehrlich ascites carcinoma [EAC] in swiss albino mice.

## MATERIALS AND METHODS

### Plant material

The whole plant of Euphorbia thymifolia was collected around Bangalore city and was identified by Prof. Jawahar Raveendran, in charge-Raw Drug Repository, FRLHT, Bangalore, Karnataka, where a voucher specimen [FRL/CRJ/RDRNO/90/2008-09] is deposited.

### **Extraction**

The plant was shade dried, powdered and extracted by using Soxhlet apparatus with ethanol. After extraction, the filtrate was concentrated under reduced pressure.

### **Phytochemical screening**

Preliminary phytochemical screening of *Euphorbia thymifolia* revealed the presence of steroids, glycosides, flavonoids, tannins, tri terpenoids & phenolic compounds. [10-11]

### **Tumor cells**

EAC cells were obtained from the courtesy of department of Radiology, Kasturba Medical College, Manipal, India. The EAC cells were maintained by intra peritoneal inoculation of  $1 \times 10^6$  cells/mouse.

### **Animals**

Swiss albino mice were procured from Raghavendra Enterprises, Bangalore. The mice were grouped and housed in polyacrylic cages (38 X 23 X 10 cm) with not more than ten animals per cage and maintained under standards laboratory conditions [temperature  $25 \pm 20$  °C ] with dark/light cycle [14/10h] They were allowed free access to standard dry pellet diet and water ad libitum. The mice were acclimatized to laboratory conditions for 10 days before commencement of the experiment. All procedures described were reviewed and approved by the institutional animal ethical committee CPCSEA No. (997/C/06/CPCSEA).

### **Acute toxicity study**

The acute oral toxicity study was carried out as per guidelines set by organization for Economic Co-operation and Development [OECD] Guidelines No. 423. [12] Healthy adult Swiss albino mice weighing between 25 to 35 gm were used for the study. Animals were divided into four groups of three animals each and kept fasted for overnight. The different doses like 5, 50, 300, 2000 mg/kg body weight were administered. Based on the result, the extract did not produce any mortality at the doses tested. To optimize the dose levels  $1/10^{\text{th}}$  of  $LD_{50}$  [200 mg/kg body weight] ethanolic extract was selected for the evaluation.

### **Antitumor activity**

Male Swiss albino mice were then divided into three groups containing ten mice. All the groups were injected with EAC cells [ $1 \times 10^6$  cells/mouse] intraperitoneally except the normal group. This was taken as day zero.

**Survival time**

Group I: EAC bearing mice, treated with 0.9% normal saline, Group II: EAC bearing mice, treated with ethanolic extract of *Euphorbia thymifolia* 200 mg/kg, p. o. Group III: Tumor bearing mice, treated with 5- fluorouracil 20 mg/kg, i. p. The antitumor efficacy of ethanolic extract of *Euphorbia thymifolia* was compared with that of 5-fluorouracil. The mean survival time of the treated groups was compared with that of the control group using the following calculation:

$$\text{Increase in lifespan} = \frac{T - C}{C} \times 100$$

Where T = average number of days the treated animals survived, C = average number of days control animals survived.

**Effect of ethanolic extract of *Euphorbia thymifolia* on normal peritoneal cells**

Animals were divided into three groups. Each group consisted of six animals. Group I: Vehicle control, Group II: Mice + treated with of ethanolic extract of *Euphorbia thymifolia* 200 mg/kg, p.o. only once for a single day, Group III: Mice + treated with ethanolic extract of *Euphorbia thymifolia* 200 mg/kg, p.o. for two consecutive days. Peritoneal cells were collected after 24 h treatment by repeated intraperitoneal wash with normal saline and peritoneal cells were counted using neuber's chamber and compared the result of treated groups with those of the untreated group. Total no. of cells = total no. of cells count into neuber's chambers X D.F X 104

**Hematological parameters**

Group I: treated with 0.9% normal saline, Group II: EAC bearing mice, treated with 0.9% normal saline, Group III: EAC bearing mice, treated with ethanolic extract of *E.thymifolia* 200 mg/kg, p.o. On 14<sup>th</sup> day after tumor inoculation blood was drawn from each mouse by the tail vein and the white blood cell count, red blood cell count, hemoglobin content, protein estimation and differential count were determined. [13] Compared the result of treated groups with those of the untreated group. Total no. of cells = total no. of cells count into neuber's chambers X D.F X 104

**Biochemical assay**

After the collection of blood samples, the mice were sacrificed, then their liver was excised, rinsed in ice, cold normal saline and used [14] for estimation of lipid peroxidation, reduced glutathione, [15] Superoxide dismutase, [16] & Catalase. [17]

**Effect of ethanolic extract of Euphorbia thymifolia on solid tumor**

Mice were divided into two groups. Each group consists of eight animals. Tumor cells ( $1 \times 10^6$  cells/mice) were injected into the right hind limb (thigh) of all experimental animals intramuscularly. Group I: EAC bearing mice, treated with 0.9% normal saline, Group II: EAC bearing mice, treated with ethanolic extract of Euphorbia thymifolia 200 mg/kg, p.o for 05 alternative days. Tumor mass was measured from the 11<sup>th</sup> day of tumor induction. The measurement was carried out every 5<sup>th</sup> day for a period of 30 days. [18] The volume of tumor mass was calculated using the formula:  $V = 4/3\pi r^2$  Where r is radius of the tumor mass

**RESULTS****Effect of ethanolic extract of Euphorbia thymifolia on survival time of EAC bearing mice**

The effect of ethanolic extract of Euphorbia thymifolia on the survival of EAC bearing mice was showed in Table 1. The mean survival time for the control group was  $16.9 \pm 0.64$  days, whereas for the treated group with Euphorbia thymifolia was  $28.10 \pm 0.58$  days. The increased in life span of EAC bearing mice treated with Euphorbia thymifolia and 5-Fluorouracil was found to be 66.63% and 96.75% respectively as compared to vehicle control group.

**Effect of ethanolic extract of Euphorbia thymifolia on normal peritoneal cell count**

The average number of peritoneal cells per normal mouse was found to be  $5.32 \pm 0.28$ . Single day treatment with Euphorbia thymifolia enhanced peritoneal cells to  $7.08 \pm 0.17$ , while two consecutive treatments groups it was enhanced the number to  $9.072 \pm 0.23$ .

**Effect of ethanolic extract of Euphorbia thymifolia on hematological parameters of EAC bearing mice**

Red blood cells count and hemoglobin content in EAC bearing control group was significantly decreased compared to vehicle control group. Treatment with Euphorbia thymifolia at the dose of 200 mg/kg significantly increased the RBC count and hemoglobin content compared to EAC bearing mice. The total WBC count and total protein was found to be increased significantly in the EAC bearing control group when compared to normal control. Administration of Euphorbia thymifolia extract in EAC bearing mice significantly reduced the WBC count and total protein as compared with EAC bearing control group. In differential count of WBC, the presence of neutrophils increased, while lymphocytes count decreased in the EAC bearing control group. While in the Euphorbia thymifolia treated group

changed those altered parameters more or less to the normal values. Results are explained in Table 3.

#### Effect of ethanolic extract of *Euphorbia thymifolia* on biochemical estimation

Antioxidant enzyme level in EAC bearing mice were altered during diseased condition and all the enzymes brought back to normal level after the treatment with *Euphorbia thymifolia*. The antioxidant potential of *Euphorbia thymifolia* was evaluated by estimating the antioxidant enzymes like superoxide dismutase, lipid peroxidation, reduced glutathione, and Catalase from the liver tissues of EAC bearing mice. *Euphorbia thymifolia* treated group significantly reversed the altered enzymes levels as compared to EAC bearing control group. Results are represented in Table 4.

#### Effect of ethanolic extract of *Euphorbia thymifolia* on solid tumor

There was markedly reduction in the tumor mass of *Euphorbia thymifolia* treated of EAC bearing mice. The tumor mass of EAC bearing control on 37<sup>th</sup> day was  $2.94 \pm 0.26$ , whereas in the extract treated group it was reduced upto  $1.16 \pm 0.14$ . Results are presented in Table 5.

**Table: 1. Effect of ethanolic extract of *Euphorbia thymifolia* on the survival time of EAC bearing mice**

Treatment	Dose (mg/kg, p.o.)	MST (in days)	% Increased in life span
EAC bearing control	0.1 ml/10 gm (0.9 % normal saline)	$16.9 \pm 0.64$	-
<i>Euphorbia thymifolia</i>	200	$28.10 \pm 0.58^{**}$	66.63
5-Fluorouracil	20	$33.25 \pm 1.62^{**}$	96.75

Values are expressed as mean  $\pm$  SEM (n=10), \*\*P<0.01 when compared with EAC bearing control group.

**Table: 2. Effect of ethanolic extract of *Euphorbia thymifolia* on normal peritoneal cell count in normal mice**

Treatment	Dose (mg/kg, p.o.)	No of peritoneal cells/mouse $\times 10^6$
Normal mice		$5.32 \pm 0.28$
<i>Euphorbia thymifolia</i> treated once for a day	00	$7.08 \pm 0.17^{**}$
<i>Euphorbia thymifolia</i> treated once for two days	00	$9.07 \pm 0.23^{**}$

Values are expressed as mean  $\pm$  SEM (n=6), \*\*P<0.01 when compared with normal mice

**Table: 3. Effect of ethanolic extract of Euphorbia thymifolia on hematological parameters of EAC bearing mice**

Treatment	Dose (mg/kg, p.o.)	RBC (million/mm <sup>3</sup> )	WBC (10 <sup>3</sup> cells/m <sup>3</sup> )	HB (gm %)	Differential counts (%)			Total Protein
					L	N	M	
Vehicle control	0.1 ml/10 gm (0.9 % normal saline)	4.958 ± 0.40	9.556 ± 0.54	13.02 ± 0.22	65.60 ± 2.50	32.40 ± 2.76	0.8 ± 0.37	5.482 ± 0.41
EAC bearing control	0.1 ml/10 gm (0.9 % normal saline)	2.612 ± 0.20 <sup>a</sup>	24.438 ± 1.13 <sup>a</sup>	8.5 ± 0.43 <sup>a</sup>	37.02 ± 2.05 <sup>a</sup>	61 ± 2.96 <sup>a</sup>	0.8 ± 0.58 <sup>ns</sup>	15.69 ± 1.25 <sup>a</sup>
Euphorbia thymifolia	200	3.566 ± 0.08 <sup>**</sup>	14.434 ± 0.57 <sup>**</sup>	10.77 ± 0.33 <sup>**</sup>	59.60 ± 1.24 <sup>**</sup>	39.20 ± 0.73 <sup>**</sup>	1.2 ± 0.58 <sup>ns</sup>	9.44 ± 1.07 <sup>**</sup>

Values are expressed as mean ± SEM (n=6), a = <sup>\*\*</sup>P<0.01 when compared to vehicle control, <sup>\*</sup>P<0.05 & <sup>\*\*</sup>P<0.01 when compared with EAC bearing control mice, ns = statistically not significant

**Table: 4. Effect of ethanolic extract of Euphorbia thymifolia on biochemical parameters of EAC bearing mice**

Treatment	Dose (mg/kg, p.o.)	MDA nM/gm wet tissue	GSH mM/gm wet tissue	SOD U/mg protein	Catalase U/mg protein
Vehicle control	0.1ml/10 gm (0.9 % normal saline)	28.18 ± 0.74	4.37 ± 0.20	1.83 ± 0.02	10.56 ± 1.31
EAC bearing control	0.1 ml/10 gm (0.9 % normal saline)	46.47 ± 1.09 <sup>a</sup>	5.96 ± 0.09 <sup>a</sup>	0.46 ± 0.05 <sup>a</sup>	3.23 ± 0.93 <sup>a</sup>
Euphorbia thymifolia	200	32.71 ± 1.79 <sup>**</sup>	4.63 ± 0.21 <sup>**</sup>	1.41 ± 0.05 <sup>**</sup>	8.52 ± 1.02 <sup>**</sup>

Values are expressed as mean ± SEM (n=6), a = <sup>\*\*</sup>P<0.01 when compared with vehicle control, <sup>\*</sup>P<0.05, <sup>\*\*</sup>P<0.01 when compared with EAC bearing control mice

**Table: 5. Effect of ethanolic extract of Euphorbia thymifolia on solid tumor of EAC bearing mice**

Treatment	Dose (mg/kg, p.o.)	Solid tumor mass (cm <sup>2</sup> ) (days)					
		12 <sup>th</sup>	17 <sup>th</sup>	22 <sup>th</sup>	27 <sup>th</sup>	32 <sup>th</sup>	37 <sup>th</sup>
EAC bearing control	ml/10 gm (0.9% normal saline)	0.25 ± 0.025	0.42 ± 0.03	0.73 ± 0.05	1.19 ± 0.07	2.03 ± 0.14	3.05 ± 0.29
Euphorbia thymifolia	200	0.13 ± 0.008	0.22 ± 0.02	0.47 ± 0.02	0.93 ± 0.05	1.02 ± 0.060	1.24 ± 0.13**

Values are expressed as mean ± SEM (n=6), on 37<sup>th</sup> day p value was < 0.001 compared to EAC bearing control mice.

## DISCUSSION

In Ehrlich ascites carcinoma bearing mice, a regular rapid increase in ascites tumor volume was noted. Ascites fluid is the direct nutritional source for tumor cells and a rapid increase in ascites fluid with tumor growth would be a means to meet the nutritional requirement of tumor cells. [19] Treatment with extract of Euphorbia thymifolia inhibited the tumor masses and the reliable criteria for judging the value of any anticancer drug are the prolongation of the life span of animals.

The extract of Euphorbia thymifolia decreased the ascites fluid masses and increased the percentage of life span. It may be concluded that, extract of Euphorbia thymifolia by decreasing the nutritional fluid volume and arresting the tumor growth, finally increased the life span of tumor bearing mice. [20]

Usually, in cancer chemotherapy the major problems that are being encountered are of myeloid-suppression and anemia. [21-22] The anemia encountered in tumor bearing mice is mainly due to reduction in RBC or hemoglobin content, and this may occur either due to iron deficiency or due to hemolytic or myelopathic conditions. [23] Treatment with Euphorbia thymifolia brought back the hemoglobin content, RBC and WBC count more or less to normal levels. This indicates that extract of Euphorbia thymifolia possesses protective action on the hemopoietic system.

The improper balance between ROMs (Reactive Oxygen Metabolites) and antioxidant defenses results in “oxidative stress”, which deregulates the cellular functions leading to various pathological conditions including cancer. [24] ROMs overproduction induced by

different exogenous and endogenous mechanism may exhaust the antioxidant system of cells and contribute to a number of destructive processes and diseases, including cancer. [25] Epidemiological studies have suggested that high endogenous level of oxidative adducts and deficiencies in antioxidant levels are likely to be important risk factors for cancer. [26]

Lipid peroxidation, an autocatalytic free radical chain propagating reaction, is known to be associated with pathological conditions of a cell. Malondialdehyde was the end product of lipid peroxidation was reported to be higher in cancer tissues than in non-diseased organ. [27] It was also reported that the presence of tumors in the human body or in experimental animals was known to affect many functions of the vital organs, especially in the liver, even when the site of the tumor does not interfere directly with organ function. [28] The extract of *Euphorbia thymifolia* significantly reduced the elevated levels of lipid peroxidation and glutathione content in EAC-treated mice. The anti-tumorigenic effect of *Euphorbia thymifolia* may be due to the antioxidant and the free radical quenching property of the phytoconstituents of *Euphorbia thymifolia*.

Cells are also equipped with enzymatic antioxidant mechanisms that play an important role in the elimination of free radicals. SOD, CAT is involved in the clearance of superoxide and hydrogen peroxide ( $H_2O_2$ ). SOD catalyses the diminution of superoxide into  $H_2O_2$ , which has to be eliminated by glutathione peroxidase and/ or catalase. [29] Consistent with this, it has been reported that a decrease in SOD activity in tumor bearing mice may be due to loss of  $Mn^{2+}$ -containing SOD activity in Ehrlich ascites carcinoma cells and the loss of mitochondria, leading to a decrease in total SOD activity in the liver. [30] A small amount of catalase in tumor cells was reported. [30] The inhibition of SOD and CAT activities as a result of tumor growth were also reported. [31] Similar findings were observed in the present investigation with EAC-bearing mice. The administration of ethanolic extract of *Euphorbia thymifolia* at dose 200 mg/kg significantly increased the SOD and CAT levels.

The effect of *Euphorbia thymifolia* treatment on the peritoneal cells of normal mice was an indirect method of evaluating its inhibitory effect on tumor cell growth. Normally, a mouse contains about  $5.13 \times 10^6$  peritoneal cells, 50% of which are macrophages. The extract of *Euphorbia thymifolia* treatment was found to enhance the peritoneal cells count. These results demonstrate that indirect inhibitory effect of extract of *Euphorbia thymifolia* on Ehrlich ascites carcinoma cells, which is probably mediated by the enhancement and activation of macrophages. [18] It was reported that plant-derived extracts containing antioxidant principles

showed cytotoxicity towards tumor cells <sup>[32]</sup> and antitumor activity in experimental animals. <sup>[33]</sup> Antitumor activity of these antioxidants is either through induction of apoptosis <sup>[34]</sup> or by inhibition of neovascularization <sup>[35]</sup>, the implication of free radicals in tumors is well documented. <sup>[36-37]</sup>

Plant derived natural products viz., tannins, flavonoids, terpenoids and steroids etc have received considerable attention in recent years due to their diverse pharmacological properties including anti-oxidant and anti-tumor activity. <sup>[7-8]</sup> Preliminary phytochemical screening of *Euphorbia thymifolia* revealed the presence of steroids, glycosides, flavonoids, tannins, tri terpenoids & phenolic compounds. The above parameters are responsible for the antitumor and anti-oxidant activities of *Euphorbia thymifolia*. Further investigations are in progress to identify the active principles involved in this antitumor and antioxidant activity.

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