

## Anti-tumor Activity of *Acanthospermum hispidum* DC on Dalton Ascites Lymphoma in Mice

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**Abstract** – The present study investigated the anti-tumor activity of ethyl acetate extract of *Acanthospermum hispidum* DC against daltons ascites lymphoma in mice. The extract was prepared by cold maceration with ethyl acetate for 3 - 7 days and evaporated in vacuum to dry. (Yield : 14.2 g, 1.42% w/w). The extract was fractionated by column chromatography by using gradient elution technique and the diterpenes fraction isolated (0.649 g). Both extract and the fraction were administered as oral suspension with tween 20 in water to tumor bearing mice (DAL) and changes in dead cell count, histopathology of tumor cells, hematological parameters and median survival time (MST) were examined and compared with that of tumor control or 5-Fluorouracil (5-FU). The results indicate that both ethyl acetate extract and fraction possess anti-tumor activity. The study suggests that *Acanthospermum hispidum* DC seems promising as a source of diterpenes for potential anti-tumor activity.

**Keywords** – *Acanthospermum hispidum* DC, anti-tumor activity, Daltons ascites lymphoma, ethyl acetate extract, diterpenes

### Introduction

The major thrust area of diseases attracting the attention of researchers on herbal medicines is cancer. Because currently available cancer chemotherapeutic agents are highly reactive leading to indiscriminate reaction with a wide range of cell constituents. A number of plants with anti-cancer property have been identified (Babu *et al.*, 1995; Subramoniam *et al.*, 1996; Devi *et al.*, 1996; Banerjee *et al.*, 1996; Rao *et al.*, 1997; Bhattacharya *et al.*, 1998; Prashar *et al.*, 1998). *Acanthospermum hispidum* DC (Family: Asteraceae N.O. Composite), native of South America is a hispid herb and widely distributed in other countries including India. This plant is traditionally used as anti-malarial in Burkina Faso (Summerfield *et al.*, 1998) and in infectious diseases in Benin (Sanon *et al.*, 2003). It has been documented for anti-plasmodial activity (Summerfield *et al.*, 1998; Sanon *et al.*, 2003), as abortive agent in Brazil (Lemonica *et al.*, 1994) and as diuretic, febrifuge, sudorific and in treatment of gonorrhoea in South America (Uphof *et al.*, 1968; Morton *et al.*, 1981) and for anti-microbial activity (Fleischer *et al.*, 2003). The plant has also been reported for the presence of terpenoids

and polyphenolic constituents with some of the former possessing invitro anti-neoplastic activity (Nair *et al.*, 1985; Jakupovic *et al.*, 1986). *Acanthospermum glabratum* DC another allied species has been reported for anti-tumor activity (saleh *et al.*, 1980) and also for the presence of acanthospermol- $\beta$ -galactoside, a class of diterpenes. Diterpenes have been documented to possess anti-tumor activity (Lee *et al.*, 2002). However, there are no reports on the anti-tumor activity of *Acanthospermum hispidum* DC which are earlier reported for the presence of diterpenes and hence in the present study the anti-tumor activity of the plant was investigated against daltons ascitic lymphoma (DAL) in mice and also an attempt was made to isolate the diterpenes and examine its role in anti-tumor activity.

### Experimental

**Plant material collection and extraction** – The whole plant excluding fruits of *Acanthospermum hispidum* DC were collected from rain forest area, Thirunelveli district, Tamilnadu, India, in the month of May-2003 and authenticated by Survey of Medicinal Plants Unit-Siddha, C.C.R.A.S., Government of India, Thirunelveli, Tamil Nadu, India. The plant material was shade dried and

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pulverized. The powder was extracted by cold maceration in an aspirated bottle with ethyl acetate for 3 - 7 days. The extract was concentrated under vacuum and dried in a desiccator (yield: 14.2 g; 1.42% w/w).

**Isolation of diterpenes enriched fraction from ethyl acetate extracts** – The ethyl acetate extract was chromatographed on silica gel GF column (Merck-60 : 120 mesh) in hexane and eluted by gradient elution technique using hexane, benzene, chloroform and ethyl acetate. The eluate (20 ml each) was tested for diterpenes by thin layer chromatography using hexane-ethyl acetate (17 : 3) as mobile phase and 50% sulphuric acid as spray reagent followed by UV (254 nm) analysis (Harbone, 1984). The fraction (79 - 113) that answered for diterpenes were pooled and subjected to preparative TLC followed by UV, IR and HPTLC analysis.

**Animals** – Adult male swiss albino mice (20 - 25 g) were procured from King Institute, Chennai, India. They were housed in polypropylene cages in groups of six and had free access to food (Pellets obtained from Lipton, India) and water prior to as well as during experimentation. They were maintained under normal room temperature (28 - 30 °C) and acclimatized in the laboratory conditions for 3 days, prior to experimentation with 12/12 hour light/dark cycle. The experiments were conducted during the light period. The study was conducted after obtaining the institutional animal ethical committee clearance.

**Tumor cell line used** – Dalton's lymphoma in ascitic form was obtained through the courtesy of Amala Cancer Research Center, Thirussur, Kerala, India. They were maintained by weekly intraperitoneal inoculation of  $10^6$  cells/mouse (Gothoskar *et al.*, 1971). Tumor in mice was induced as follows:

The ascitic fluid of the DAL was drawn out from the donor mice carrying the tumor for 7 - 9 days. The freshly drawn ascitic fluid was diluted in phosphate buffer saline (pH 7.4) to a concentration of  $10^6$  cells/mL and aliquots of 0.3 - 0.5 mL of the diluted solution was injected intraperitoneally into the mice belonging to age group of 4 - 6 weeks. The development of tumor (7 - 9 days) was confirmed from the cells count of the ascitic fluid equivalent to  $10^6$  cells/mL.

**Drug Treatment** – The ethyl acetate extract or the isolated fraction was suspended in distilled water with tween-20 and used for the study. Four groups of tumor bearing mice (n = 6) were used for the study. Group I was treated with 300 mg/kg. p.o. of ethyl acetate extract, Group-II received 50 mg/kg. p.o. Of fraction, Group-II was treated with 12.5 mg/kg p.o. of 5-Fluorouracil (Biochem Pharmaceuticals, Mumbai, India). The untreated

Group IV was used as control. The dose for ethyl acetate extract and fraction was selected based on toxicity studies (16), which showed no toxicity up to 5 g/kg for both. Animal received drug treatments on 9 th and 18 th day and were examined for various parameters for anti tumor activity on 11 th and 20 th day post tumor inoculation.

**Effect of ethyl acetate extract and fraction on dead cell count** – 0.1 ml of the peritoneal fluid was aseptically withdrawn from the animal using a 1 ml syringe and diluted approximately with trypan blue solution and examined under microscope. The number of dead cells (Stained cells) randomly in every 200 cells were counted and the results were recorded as percent protection against tumor growth using the following calculation.

$$\text{Percent protection against tumor growth} = \frac{\text{No. of dead cells in treated groups} - \text{No. of dead cells in untreated groups}}{\text{No. of dead cells in untreated groups}} \times 100$$

**Effect of ethyl acetate extract and fraction on histopathology of tumor cells** – A small volume of the peritoneal fluid from the treated as well as untreated animals was withdrawn aseptically and stained with Maygrunwald's reagent and examined for histo- pathological changes.

**Effect of ethyl acetate extract and fraction on hematological parameters** – Blood was drawn from each animal from the retro-orbital flexes and the white blood cell count (WBC), red blood cell count (RBC), hemoglobin and differential leukocyte count (DLC) were determined. (D'Amour, 1965; Docie, 1958). The data were statistically analyzed by two way-ANOVA followed by Dunnett's 't' test.  $P < 0.05$  was considered as statistically significant.

**Effect of ethyl acetate extract and fraction on Median Survival Time (MST)** – The median survival time of the treated groups was determined and compared with that of the tumor control group using the following calculation.

$$\text{Percent increase in life span} = \frac{T - C \times 100}{C}$$

Where, T = Number of days the treated animals survived, C = Number of days tumor control animals survived. All the datas were statistically analyzed by one way ANOVA by Dunnet's 't' tests.  $P < 0.05$  was considered as statistically significant.

## Results and Discussion

The eluates obtained from column chromatography of

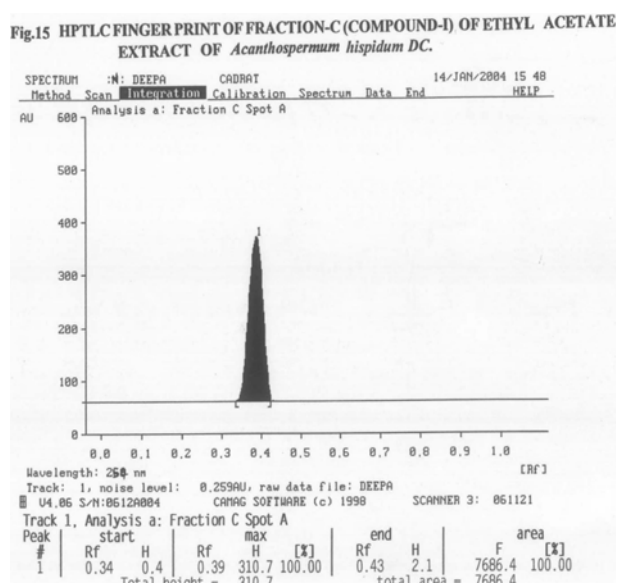
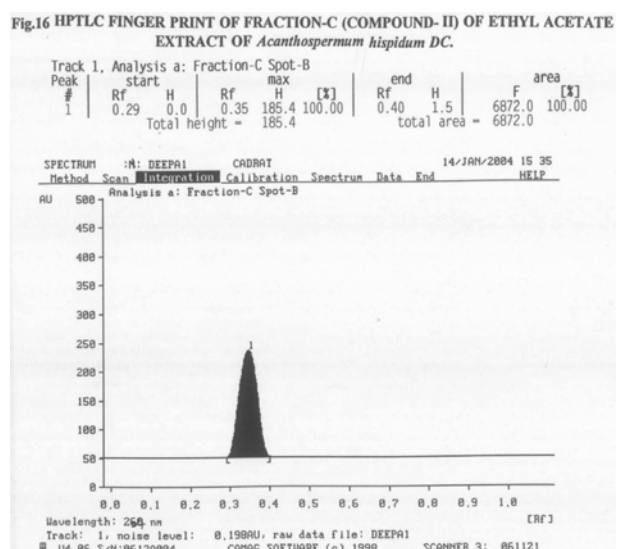
**Table 1.** IR Spectral analysis of component I and II

Description	Wave number (Cm <sup>-1</sup> )	Stretching
Component-I	1710	Carbonyl (–C = O)
	1450	–C-H deformation (alkane)
	1350	Carboxylic
	1170	Acetate (–C–O–)
	1100	–SO <sub>2</sub> –NH <sub>2</sub>
Component-II	1680	Carbonyl (–C = O)
	1520	–C-H deformation (alkene)
	1100	–SO <sub>2</sub> –NH <sub>2</sub>

**Table 2.** Dead cell count of DAL

Sl. No.	Group	% of dead cells		% protection against tumor	
		11th day	20th day	11th day	20th day
1.	Control (DAL)	25	35	–	–
2.	5-FU (Standard)	35	49	40	40
3.	Ethyl acetate extract	32	48	28	37.14
4.	Fraction	38	54	52	54.29

Each value represents the mean of six experiments.

**Fig. 1.** HPTLC of compound I of fraction C of ethyl acetate extract.**Fig. 2.** HPTLC of compound II of fraction C of ethyl acetate extract.

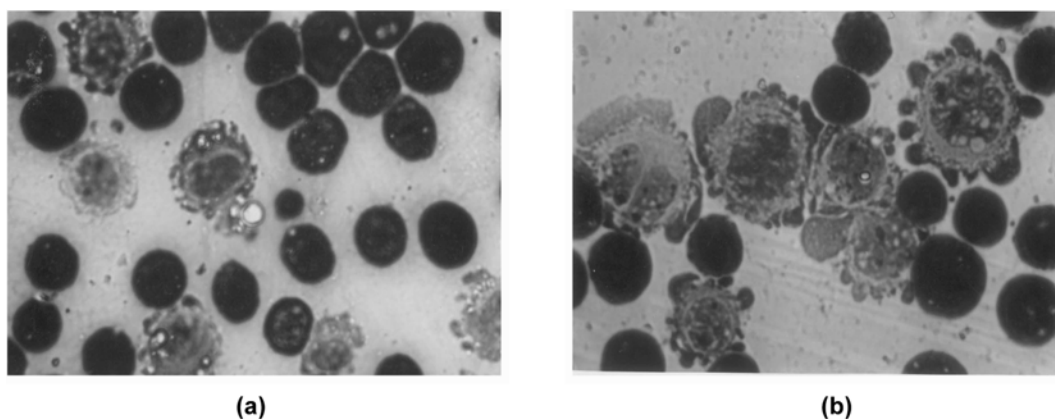
the ethyl acetate extract was brownish green and answered for the presence of diterpenes under uv (254 nm) analysis.

The yield of the fraction was 0.649 g (12.88% w/w) which may be diterpenes. TLC of the fraction showed presence of two components with R<sub>f</sub> values 0.39 and 0.35 (component I and II respectively) which may be diterpenes. The absorbance maxima (λ max) in uv-visible spectroscopic analysis for component I and II were 437 and 670 nm, respectively. Both components I and II of the fraction showed similar stretching pattern for different functional groups as shown in Table 1. HPTLC of components I and II established the R<sub>f</sub> values which akin to that observed in TLC. (Fig 1, 2).

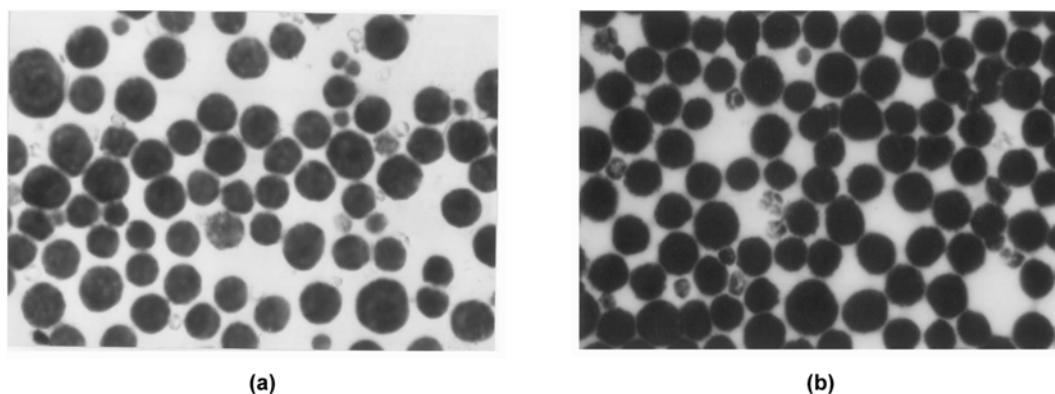
**Effect of ethyl acetate extract and fraction on dead cell count** – The effect of ethyl acetate extract and fraction on dead cell count is shown in Table 2. The percent protection against tumor for ethyl acetate extract and fraction were 28 and 52% respectively on 11<sup>th</sup> day post tumor inoculation and whereas on 20<sup>th</sup> day post tumor inoculation, the percent protection against tumor were 37.14 and 54.29% for ethyl acetate extract and fraction respectively. 5-FU treated groups showed 40% protection on both 11<sup>th</sup> and 20<sup>th</sup> day post tumor inoculation. As compared to 5-FU treated groups, the diterpenes fraction showed higher degree of protection against tumor on both 11<sup>th</sup> and 20<sup>th</sup> day post tumor inoculation. In contrast, ethyl acetate extract showed lesser protection as compared to 5-FU. However, on 20<sup>th</sup> day post tumor inoculation, the extract showed the protection closer to that of 5-FU.

**Effect of ethyl acetate extract and fraction on histopathology of tumor cells** – Ethyl acetate extract treated animals (Fig. 3) showed both vacuolization and necrosis of tumor cells on both 11<sup>th</sup> and 20<sup>th</sup> day as compared to tumor control (Fig. 4) and the degree of necrosis was marked on 20<sup>th</sup> day. Diterpenes fraction treatment also produced similar results to that of ethyl acetate extract (Fig. 5) as compared to 5-FU treatment (Fig. 6). Both ethyl acetate extract and diterpenes fraction showed higher degree of vacuolization and necrosis.

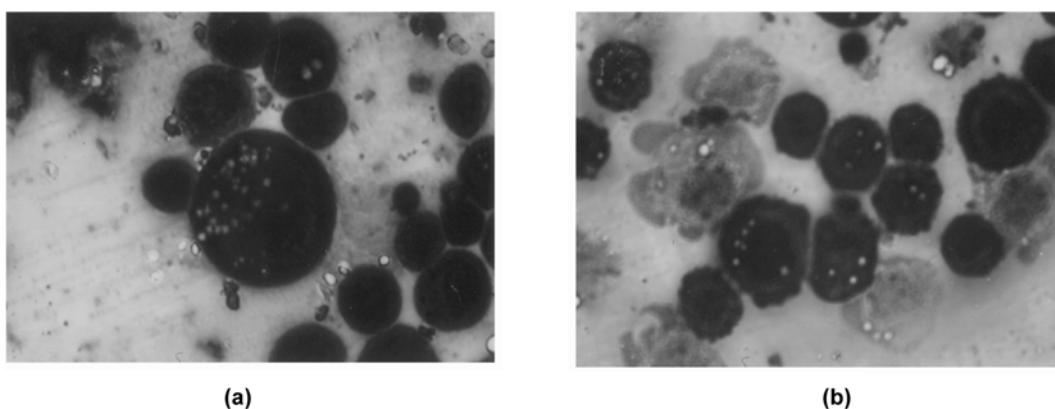
**Effect of ethyl acetate extract and fraction on hematological parameters** – Tumor bearing mice showed



**Fig. 3. (a):** Ethyl acetate extract treated animals on 11<sup>th</sup> day post tumor inoculation, **(b):** Ethyl acetate extract treated animals on 20<sup>th</sup> day post tumor inoculation.



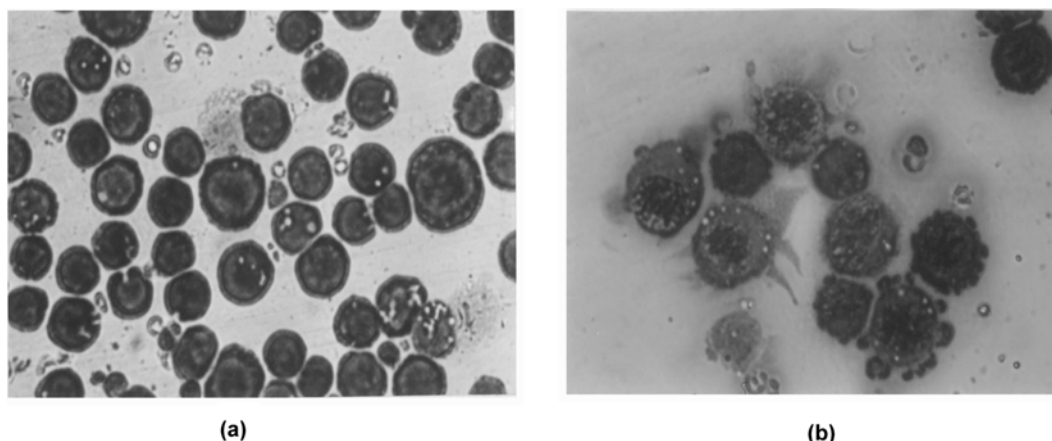
**Fig. 4. (a):** Tumor control animals on 11<sup>th</sup> day post tumor inoculation, **(b):** Tumor control animals on 20<sup>th</sup> day post tumor inoculation.



**Fig. 5 (a):** Fraction-C of ethyl acetate extract treated animals on 11<sup>th</sup> day post tumor inoculation, **(b):** Fraction-C of ethyl acetate extract treated animals on 20<sup>th</sup> day post tumor inoculation.

significant changes in hematological parameters when compared with normal mice (Table 3). The WBC count and neutrophil count significantly increased. While, RBC count, lymphocyte count and Hb was significantly lowered in tumor control mice as compared to normal mice

( $P < 0.001$ ). Ethyl acetate extract treatment showed significant reduction in WBC count ( $P < 0.001$ ) and neutrophil count and slight increase in RBC count, significant increase in Hb and reduction in lymphocyte count ( $P < 0.001$ ) on 20<sup>th</sup> day post tumor inoculation as



**Fig. 6. (a):** 5-FU treated animals on 11<sup>th</sup> day post tumor inoculation, **(b):** 5-FU treated animals on 20<sup>th</sup> day post tumor inoculation.

**Table 3.** Changes in haematological parameters induced by DAL

Description	Day	WBC $\pm$ SEM	RBC $\times 10^6 \pm$ SEM	Hb g/dL $\pm$ SEM	Lymphocytes $\pm$ SEM	Neutrophils $\pm$ SEM	Mono cytes
Normal mice	–	5000 - 7000	8.7 - 12.5	10.2 - 16.2	70 - 75	23 - 28	0.1 - 3.5
Tumor Control (DAL)	11	15,175 $\pm$ 1.86*	3,495 $\pm$ 0.01*	6.9 $\pm$ 0.97*	57.10 $\pm$ 0.86*	38.25 $\pm$ 1.13*	2
	20	16,633 $\pm$ 2.04*	2.153 $\pm$ 0.02*	6.46 $\pm$ 0.08*	28.66 $\pm$ 0.81*	58.66 $\pm$ 0.81*	2
5-FU (Std 12.5 mg/kg.p.o)	11	12,7375 $\pm$ 2.05	2.40 $\pm$ 0.08	5.8 $\pm$ 0.98	48.06 $\pm$ 0.41	56 $\pm$ 0.82	2
	20	11,600 $\pm$ 2.03	2.61 $\pm$ 0.03	7.2 $\pm$ 0.04	54.98 $\pm$ 0.81	34.98 $\pm$ 0.03	2
Ethyl acetate extract (300 g/kg.p.o)	11	12,066.66 $\pm$ 1.92**	2.39 $\pm$ 1.16**	6.6 $\pm$ 0.29**	45.33 $\pm$ 2.05**	51.66 $\pm$ 0.40**	2
	20	9,166.66 $\pm$ 1.87**	3.28 $\pm$ 0.07**	9.63 $\pm$ 0.25**	57.30 $\pm$ 0.16**	30.35 $\pm$ 2.05**	2
Fraction (50 mg/kg.p.o)	11	12,670 $\pm$ 2.12**	2.49 $\pm$ 0.37**	6.9 $\pm$ 0.04**	43.25 $\pm$ 0.82**	52.53 $\pm$ 1.64**	2
	20	8,250 $\pm$ 1.98**	4.02 $\pm$ 0.04**	11.98 $\pm$ 0.16**	63.66 $\pm$ 0.12**	28.16 $\pm$ 0.76**	2

n = 6 animals in each groups, \* P < 0.001 vs normal mice, \*\* P < 0.001 vs tumor control. Values are expressed as mean  $\pm$  SEM.

compared to tumor control. The diterpene enriched fraction treatment (50 mg/kg.p.o) altered the change in WBC count, hemoglobin and neutrophil count to near normal except the RBC and lymphocyte count, which were however significantly elevated as compared to tumor control (Table 3) (P < 0.001). The results of ethyl acetate extract are comparable with that of standard (5-FU) on changes in all the hematological parameters on both 11<sup>th</sup> and 20<sup>th</sup> day except RBC count (P < 0.001) on 11<sup>th</sup> day and lymphocyte count on 20<sup>th</sup> day. Fraction treatment showed comparable changes with standard on all parameters on 20<sup>th</sup> day, while comparable results were observed with Hb content and neutrophil count (P < 0.001). There was no significant variation in WBC, RBC and lymphocyte count on 11<sup>th</sup> day (Table 3).

**Effect of ethyl acetate extract and fraction on Median Survival Time (MST)** – The MST of tumor control was 20  $\pm$  0.35 days. Whereas, it was 55  $\pm$  0.22 and 57  $\pm$  0.28 days for ethyl acetate extract and diterpene enriched fraction respectively (Table 4). The increase in

**Table 4.** Median survival time (MST)

Groups	No. of Days Alive $\pm$ SEM	Mean Survival time (%)
Tumor control (DAL)	20 $\pm$ 0.35	100
5-FU (Standard)	24 $\pm$ 0.24*	120
Ethyl acetate extract	55 $\pm$ 0.22*	275
Fraction	57 $\pm$ 0.28*	285

n = 6 animals in each group, \*P < 0.001 vs control. Values are expressed as mean  $\pm$  SEM

the life span of tumor bearing mice treated with the extract and the fraction was found to be 175% and 185% respectively (P < 0.001) as compared to the tumor control group. Both Ethyl acetate extract and fraction increased the life span greater than that of 5-FU treatment, which was found to be 20% increase in life span as compared to tumor control (P < 0.001). The reliable criteria for judging the value of any anti-cancer drug are prolongation of life span (Obeling *et al.*, 1954) and decrease of WBC from the blood (Carlson *et al.*, 1965). In the present study both

ethyl acetate extract and the fraction of *Acanthospermum hispidum* DC significantly extended the MST and reduced WBC count as compared to tumor control and 5-FU treated groups and these effects were more pronounced in fraction treated groups. Besides, the decrease in RBC, observed in the tumor control groups was significantly reduced by the extract and more significantly by the fraction treatment particularly on 20<sup>th</sup> day and the degree of reduction in WBC count was marked in the extract and fraction treated groups as compared to 5-FU treated groups. Similarly, the other hematological parameters such as Hb, lymphocyte and neutrophils were significantly altered by extract and fraction treatment as compared to tumor control or 5-FU treatment. An increase in dead cell count is indicative of anti-tumor property. Both extract and fraction treatment significantly increased the dead cell count as compared to tumor control. Fraction appears to be having more effect than the extract or 5-FU treatment or tumor control and further, fraction treatment maintained the increased effect on dead cell count both on 11<sup>th</sup> and 20<sup>th</sup> day. Additionally, vacuolization and necrosis of tumor cells was observed in the extract as well as fraction treated groups. Thus the results of the study indicate the potential anti-tumor effect of the ethyl acetate extract and the fraction on Dalton's ascites lymphoma in mice. Phytochemical investigation of the isolated fraction from ethyl acetate extract by TLC, UV, IR and HPTLC analysis revealed the presence of two components with distinct  $R_f$  value 0.35 and 0.39 which may be diterpenes. The role of diterpenes from other plants in eliciting the anti-tumor activity has earlier been reported (Lee *et al.*, 2002) and the same observed in the present study too and so the anti-tumor activity of ethyl acetate extract may be attributed to the presence of diterpenes in the extract. These diterpenes act by inhibiting the activation of transcription factor NF Kappa B, the central mediator of apoptosis and immune response by directly targeting DNA binding activity of gene P<sub>50</sub> (Lee *et al.*, 2002). Diterpenes are also reported to have cytotoxic effect by down regulation and expression of mRNA of MDR cells (Ganguly *et al.*, 2001). They also possess non cytotoxic anti-tumor affect through inhibition of Farnesyl Protein Transferase (FPT) and Histone-deacetylase (Ganguly *et al.*, 2001). The fore said mechanisms may be operative in the anti-tumor activity of *Acanthospermum hispidum* DC observed in the present study; however it needs to be investigated in detail. Hence conclusively from the present study on the plant *Acanthospermum hispidum* DC it is promising that the plant is a source for diterpenes that possess anti-tumor property.

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