

## ORIGINAL ARTICLE

# Inhibition of Ehrlich's ascites carcinoma by ethyl acetate extract from the flower of *Calotropis gigantea* L. in mice

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### Summary

As part of a scientific appraisal of some of the folkloric and ethnomedical uses of *Calotropis gigantea* L. (Family: Asclepiadaceae), the present study was designed to evaluate the antitumour activity of the *Calotropis gigantea* flower against Ehrlich's ascites carcinoma (EAC) by using a crude ethyl acetate extract from the flower of *Calotropis gigantea* (designated as EECF) in Swiss mice. EECF was administered intraperitoneally at doses of 50, 100 and 200 mg/kg body weight. Bleomycin (0.3 mg/kg) was used as a positive control. EECF treatment significantly decreased both viable tumour cells and body weight gain induced by the tumour burden and prolonged survival time. The haematological and biochemical (glucose, cholesterol, triglyceride, blood urea, ALP, SGPT and SGOT) parameters altered during tumour progression, were also significantly restored in EECF-treated mice. Among the three doses tested, the highest dose was the most potent and comparable with the standard drug bleomycin (0.3 mg/kg). In conclusion, EECF from the *Calotropis gigantea* flower has a potent inhibitory effect against EAC cells in a dose dependent manner.

**Key words:** *Calotropis gigantea*; Ehrlich's ascites carcinoma; flower extract; Swiss mice

### Abbreviations

- |  |  |
|--|--|
| 1. ALP (Alkaline phosphatase)  | 7. RBC (Red blood corpuscle)                         |
| 2. EAC (Ehrlich's ascites carcinoma)                                 | 8. ROS (Reactive oxygen species)                     |
| 3. EECF (Ethyl acetate extract of <i>Calotropis gigantea</i> flower) | 9. SEM (Standard error of mean)                      |
| 4. IICB (Indian Institute of Chemical Biology)                       | 10. SGOT (Serum glutamate oxaloacetate transaminase) |
| 5. ILS (Increase in life span)                                       | 11. SGPT (Serum glutamate pyruvate transaminase)     |
| 6. MST (Mean survival time)  | 12. WBC (White blood corpuscle)                      |

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### INTRODUCTION

Cancer is one of the leading causes of human death (Nitha et al. 2007). In modern medicine, chemotherapy, radiotherapy and surgery are the major treatments available for cancer (Gibbs 2000). Intervention with chemopreventive agents in the early stage of carcinogenesis is theoretically more rational than attempting to eradicate fully developed tumours

with chemotherapeutic drugs (Ajith and Janardhanan 2003). These agents have a narrow margin of safety and the therapy may fail due to drug resistance and dose-limiting toxicities, which may severely affect the host normal cells (Nitha et al. 2007). Hence the use of natural products is an attractive alternative in the control and eradication of cancer (Suffines and Pezzuto 1991).

Medicinal plants used as folk medicine have strong antitumour activity against the Ehrlich ascites carcinoma (EAC) cell line (Kwiecinski et al. 2008). The potent inhibitory effects of the flower extracts of different medicinal plants against EAC have also been reported in the literature (Nair et al. 1991, Latha and Panikkar 1998).

*Calotropis gigantea* L. (Family: Asclepiadaceae), popularly known as 'Boro Akanda', grows widely throughout the Indian subcontinent including Bangladesh and is used in folk medicine (Sastri 1950).

The roots and leaves of *Calotropis gigantea* are used traditionally for the treatment of abdominal tumours, boils, syphilis, leprosy, skin diseases, piles, wounds, rheumatism, insect-bites, ulceration and elephantiasis (Ghani 2003).

Various parts of this plant have been reported to have multiple therapeutic properties, such as: anti-inflammatory, analgesic, anticonvulsant, anxiolytic, sedative, anti-diarrhoeal and antipyretic (Chitme et al. 2004, 2005, Adak and Gupta 2006, Argal and Pathak 2006).

Phytochemical investigations of the plant have shown the presence of cytotoxic cardenolide glycosides (Kiuchi et al. 1998, Mueen et al. 2005, Lhinhatrakool and Sutthivaiyakit 2006), pregnanes (Kitagawa et al. 1992, Shibuya et al. 1992, Zhu-Nian et al. 2008), a nonprotein amino acid (Pari et al. 1998), terpenes (Gupta and Ali 2000), flavonoids (Sen et al. 1992) and steroids (Habib et al. 2007). In addition, *Calotropis gigantea* flowers are stomachic, digestive and analgesic (Kartikar and Basu 1994, Pathak and Argal 2007).

Powdered flowers, in small doses, are useful in the treatment of colds, coughs, asthma, catarrh, indigestion, inflammatory diseases and loss of appetite (Ghani 2003). Moreover, phytochemicals with anticancer and cytotoxic properties have been isolated from the root and leaves (Kiuchi et al. 1998, Lhinhatrakool and Sutthivaiyakit 2006, Zhu-Nian et al. 2008).

With the potential of these ethnopharmacological properties in mind, the present study was designed to evaluate the antitumour activity of ethyl acetate extract from *Calotropis gigantea* flower against EAC in Swiss mice.

## MATERIAL AND METHODS

### *Plant material*

The flowers of *Calotropis gigantea* were collected in March, 2008 from the Meherchandi area of Rajshahi University campus and authenticated by Professor A. T. M. Naderuzzaman, Botany Department, Rajshahi University. A voucher specimen (No. 1A. Alam, Collection date 15.08.2004) was preserved in the Botany Department, Rajshahi University, Bangladesh.

### *Extraction*

The collected flowers were shade dried and reduced to coarse powder. The powder was extracted with ethyl acetate at room temperature for 7 days, after which the solvent was completely removed by rotary vacuum evaporator and the crude ethyl acetate extract of *Calotropis gigantea* flower (EECF) stored in a vacuum desiccator for further use.

### *Animals*

Male and female Swiss albino mice (20–25 g) were collected from the Animal Research Branch of the International Centre for Diarrhoeal Diseases and Research, Bangladesh (ICDDR,B). The mice were grouped and housed in iron cages with eight animals per cage and maintained under standard laboratory conditions (temperature  $25 \pm 2^\circ\text{C}$ ; humidity  $55 \pm 5\%$ ) with 12 hrs dark/light cycle. The mice were allowed free access to standard dry pellet diet (Collected from ICDDR,B) and water *ad libitum*, and were acclimatized to laboratory conditions for 10 days before the beginning of the experiment. The permission and approval for animal studies were obtained from the Animal Ethics Committee of the Science Faculty, University of Rajshahi.

### *Tumour cells*

EAC cells were obtained from the Indian Institute for Chemical Biology (IICB), Kolkata, India, and were maintained by weekly intraperitoneal (ip.) inoculation of  $10^5$  cells/mouse in the laboratory.

### *Acute toxicity study ( $LD_{50}$ )*

The acute toxicity study was conducted by the method of Lorke (1983) to determine the  $LD_{50}$  value of EECF in mice. This method was carried out by a single intraperitoneal injection in thirty six animals (6 in each group) at different doses (100, 200, 400, 800, 1600 and 3200 mg/kg body weight).  $LD_{50}$  was evaluated by recording mortality after 24 hours.

### *Cell growth inhibition*

*In vivo* cell growth inhibition was carried out as described by Sur and Ganguly (1994). For this study

the mice were divided into five groups (8 mice in each group) and for therapeutic evaluation the mice of all groups were inoculated with  $1.5 \times 10^5$  cells/mouse on the first day. Treatment was started after 24 hours of tumour inoculation, and continued for 5 days. The mice in group 1 were given 2% v/v dimethylsulfoxide (DMSO) and considered as an untreated tumour control. EECF (50, 100 and 200 mg/kg body weight) and the standard drug, bleomycin (Biochem Pharmaceutical, India) at 0.3 mg/kg body weight were administered intraperitoneally (ip.) to groups 2, 3, 4 and 5, respectively. The mice were sacrificed on the 6<sup>th</sup> day after transplantation and tumour cells were collected by repeated intraperitoneal wash with normal saline (0.9% NaCl). Viable tumour cells were counted (Trypan blue test) with a haemocytometer and the total number of viable tumour cells per mouse of the treated group were compared with those of the control.

#### Studies on survival time

The animals were divided into five groups, with 8 mice in each group, and inoculated with  $1.5 \times 10^5$  cells/mouse on day zero. The control group was treated only with 2% DMSO solution. Treatment (ip.) with EECF was started after 24 hours of inoculation, at doses of 50, 100 and 200 mg/kg/mouse/day (except the control group) and continued for 10 days. The antitumour efficacy of EECF was compared with that of bleomycin (0.3 mg/kg/mouse/day; ip. for 10 days). The average body weight of each group was noted. The survival time was recorded and expressed as mean survival time (MST) in days and the percent increase of life span (%ILS) was calculated (Raj Kapoor et al. 2003) as follows:

Percent increase of life span (% ILS)

$$ILS (\%) = \frac{MST \text{ of treated group}}{MST \text{ of control group}} \times 100 - 100$$

where mean survival time (MST)

$$MST = \frac{\sum \text{Survival time days of each mouse in a group}}{\text{Total number of mice}}$$

#### Haematological and biochemical studies

In order to detect the effect of EECF on the haematological and biochemical parameters of EAC cell bearing mice, a comparison was made among six

groups (n = 8) of mice on the 15<sup>th</sup> day after inoculation (Senthilkumar et al. 2008). All the groups except the normal group were injected intraperitoneally on day zero with EAC cells (0.1 ml of  $1.5 \times 10^5$  cells/mouse). After one day of inoculation, normal saline (5 mg/kg/mouse/day) and 2% DMSO (5 mg/kg/mouse/day) were administered intraperitoneally to the normal (group 1) and EAC control (group 2), respectively, for 10 days. EECF at 50, 100 and 200 mg/kg/mouse/day and bleomycin at 0.3 mg/kg/mouse/day, were administered in groups 3, 4, 5 and 6, respectively, as above. The haemoglobin, RBC, WBC and differential count of WBC were measured on 15<sup>th</sup> day after tumour inoculation from the freely flowing tail vein blood of every mouse in each group (Mukherjee 1988). Then every mouse was sacrificed and blood was collected by heart puncture. Serum was separated by centrifugation at 4000 rpm for 10 minutes and analyzed for glucose, total cholesterol, urea, triglyceride, alkaline phosphatase (ALP), serum glutamate pyruvate transaminase (SGPT) and serum glutamate oxaloacetate transaminase (SGOT) in an Bioanalyzer (Microlab 200) using commercial kits (Atlas Medica, UK).

#### Sub-chronic toxicity studies

To determine sub-chronic toxicity, healthy Swiss albino mice were divided into four groups of 8 animals in each. Mice in group 1 received (ip.) 2% DMSO at 5 ml/kg/mouse/day and group 2, 3 and 4 received (ip.) EECF at 50, 100 and 200 mg/kg/mouse/day, respectively for 14 days. At 24 hours after the last treatment and following 18 hrs fasting, the mice were sacrificed. The haematological and biochemical parameters (glucose, total cholesterol, urea, triglyceride, ALP, SGPT and SGOT) were determined as described above.

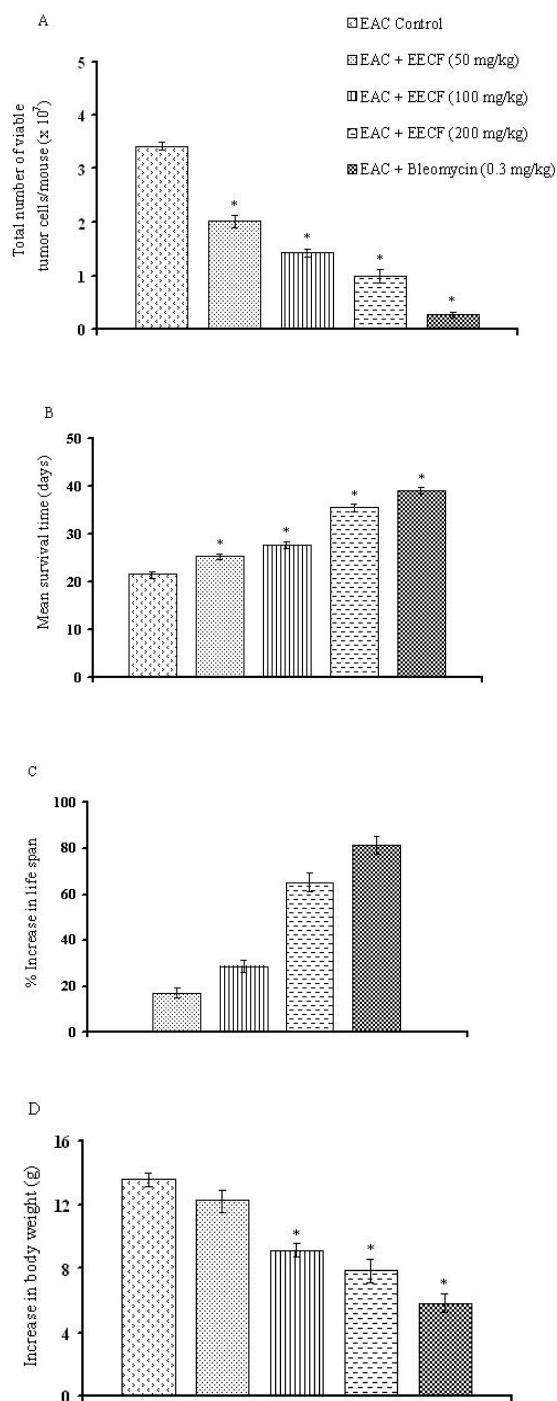
#### Statistical analysis

All values were expressed as mean  $\pm$  S.E.M (Standard error of mean). Statistical analysis was performed with one way analysis of variance (ANOVA) followed by Dunnett's 't' test using SPSS statistical software version 10. The significance level was  $2\alpha=0.05$ .

## RESULTS

#### Acute toxicity study

Intraperitoneal administration of graded doses of EECF to Swiss albino mice, in our toxicity study produced a LD<sub>50</sub> of 2225.0 mg/kg body weight, respectively.



**Fig. 1. Effect of EECF on EAC cell bearing mice.**  
A: Viable EAC cells on day 6 after tumour cell inoculation; B: Mean survival time; C: % increase in life span; D: increase in body weight on day 10. Data are expressed as mean  $\pm$  S.E.M (n = 8); \* statistically significant versus EAC control and EECF-treated group

#### Effect of EECF on cell growth inhibition

The effect of EECF on EAC cell growth is shown in Fig. 1A. Treatment with EECF at three doses (50, 100 and 200 mg/kg) and bleomycin (0.3 mg/kg) showed significantly fewer viable tumour cells (Fig. 1A). EECF at 200 mg/kg dose, showed the highest inhibition (71.24%) of EAC cell growth. The EAC cell growth inhibition for bleomycin was 92.37%.

#### Effect of EECF on survival time

The animals of the tumour control group inoculated with EAC cells survived for a period of 21.5 days. Treatment with EECF at 50, 100 and 200 mg/kg and with bleomycin at 0.3 mg/kg increased the mean survival time (MST) by  $25.25 \pm 0.47$ ,  $27.7 \pm 0.63$ ,  $35.5 \pm 0.86$  and  $39.0 \pm 0.85$  days, respectively (Fig. 1B). The EECF at 200 mg/kg was found to be the most potent in inhibiting the proliferation of EAC with a percentage increase in life span (ILS) of 65.1% (Fig. 1C). As shown in Fig. 1D, the EECF treatment at 100 and 200 mg/kg doses significantly inhibited the body weight gain when compared to the tumour control.

#### Effect of EECF on haematological and biochemical parameters of EAC cell bearing mice

On day 15, the haematological and biochemical parameters of only the mice bearing the EAC cell were changed significantly when compared to normal mice. The WBC count, neutrophils, cholesterol, triglyceride, blood urea, ALP and SGOT were found to be increased with a reduction in haemoglobin, RBC count, lymphocytes, glucose and SGPT (Table 1). Treatment with EECF at all doses, restored the altered haemoglobin level, RBC count, WBC count, percentage lymphocytes and neutrophils, glucose level, total cholesterol, triglycerides and serum urea to more or less normal levels. The enzymatic activities of ALP, SGPT and SGOT were also restored to normal levels in EECF-treated mice. At 0.3 mg/kg, the standard drug bleomycin significantly restored all haematological and biochemical parameters to normal.

#### Effect of EECF on normal mice (Sub-chronic toxicity)

Administration of EECF at 50, 100 and 200 mg/kg/mouse/day for 14 days, did not influence the body weight of the mice. The weight of liver, kidney, lung and spleen were also not altered by the treatment. Haematological and biological parameters remained unaltered in EECF-treated mice at 50 and 100 mg/kg/mouse/day but haemoglobin, RBC, WBC, glucose, and cholesterol were changed significantly at 200 mg/kg/mouse/day (Table 2).

Table 1. Effect of EECF on haematological and biochemical parameters of EAC cell bearing mice.

Parameters	Treatment (mg/kg body weight)					
	Normal	EAC + Vehicle	EAC + EECF (50)	EAC + EECF (100)	EAC + EECF (200)	EAC + Bleomycin (0.3)
Hgb (g/l)	154.8 ± 2.2	73.5 ± 2.0*	81.0 ± 1.4	83.7 ± 1.5 <sup>t</sup>	106.6 ± 1.5 <sup>t</sup>	143.7 ± 2.5 <sup>t</sup>
RBC (×10 <sup>12</sup> cells/l)	5.67 ± 0.10	2.27 ± 0.06*	2.64 ± 0.05 <sup>t</sup>	2.76 ± 0.08 <sup>t</sup>	3.84 ± 0.07 <sup>t</sup>	4.90 ± 0.09 <sup>t</sup>
WBC (×10 <sup>9</sup> cells/l)	8.75 ± 0.53	25.40 ± 1.19*	22.60 ± 0.84	20.20 ± 0.73 <sup>t</sup>	16.30 ± 0.57 <sup>t</sup>	9.37 ± 0.59 <sup>t</sup>
Lymphocytes (%)	75.5 ± 1.36	33.8 ± 1.35*	36.6 ± 1.13	41.3 ± 1.09 <sup>t</sup>	56.3 ± 0.91 <sup>t</sup>	68.2 ± 0.90 <sup>t</sup>
Neutrophils (%)	19.6 ± 1.38	63.7 ± 1.04*	60.0 ± 1.05	55.7 ± 1.10 <sup>t</sup>	41.1 ± 0.72 <sup>t</sup>	28.8 ± 0.93 <sup>t</sup>
Monocytes (%)	1.87 ± 0.40	1.50 ± 0.38	1.75 ± 0.25	1.70 ± 0.31	1.62 ± 0.26	2.00 ± 0.27
Glucose (mg/dl)	138.9 ± 1.15	54.3 ± 0.99*	60.6 ± 0.64 <sup>t</sup>	91.9 ± 0.77 <sup>t</sup>	111.3 ± 0.99 <sup>t</sup>	134.1 ± 1.21 <sup>t</sup>
Cholesterol (mg/dl)	103.8 ± 0.85	153.5 ± 0.78*	149.9 ± 1.27	148.3 ± 0.32 <sup>t</sup>	130.2 ± 0.49 <sup>t</sup>	112.4 ± 0.71 <sup>t</sup>
Triglyceride (mg/dl)	105.5 ± 1.02	186.8 ± 0.75*	182.5 ± 1.32	179.2 ± 1.67 <sup>t</sup>	160.6 ± 0.82 <sup>t</sup>	126.8 ± 1.28 <sup>t</sup>
Blood urea (mg/dl)	24.5 ± 0.44	64.1 ± 0.88*	58.9 ± 1.01 <sup>t</sup>	43.7 ± 0.75 <sup>t</sup>	36.1 ± 0.40 <sup>t</sup>	32.2 ± 0.52 <sup>t</sup>
ALP (U/l)	106.4 ± 0.74	238.9 ± 0.66*	232.6 ± 1.20 <sup>t</sup>	203.4 ± 0.97 <sup>t</sup>	165.8 ± 0.89 <sup>t</sup>	133.1 ± 0.72 <sup>t</sup>
SGPT (U/l)	71.9 ± 0.52	61.6 ± 0.49*	60.9 ± 0.46	65.2 ± 1.68	66.3 ± 0.70 <sup>t</sup>	71.3 ± 0.36 <sup>t</sup>
SGOT (U/l)	43.7 ± 0.84	242.6 ± 0.93*	236.0 ± 0.92 <sup>t</sup>	174.9 ± 0.72 <sup>t</sup>	140.5 ± 0.80 <sup>t</sup>	80.0 ± 0.78 <sup>t</sup>

Data are expressed as mean ± S.E.M. for eight animals in each group.

\* statistically significant versus controls; <sup>t</sup> statistically significant versus EAC control group.

Table 2. Effect of EECF on haematological, biochemical parameters and body weight of normal mice.

Parameters	Treatment (mg/kg body weight)			
	Vehicle control (5 ml/kg)	Normal + EECF (50)	Normal + EECF (100)	Normal + EECF (200)
Hgb (g/l)	127.0 ± 2.0	120.7 ± 1.8	133.6 ± 1.9	139.3 ± 1.5*
RBC (×10 <sup>12</sup> cells/l)	6.11 ± 0.13	6.25 ± 0.63	6.05 ± 0.05	6.79 ± 0.03*
WBC (×10 <sup>9</sup> cells/l)	7.87 ± 0.52	7.37 ± 0.38	9.50 ± 0.46	10.12 ± 0.44*
Lymphocytes (%)	73.5 ± 1.21	72.0 ± 0.71	69.1 ± 0.77	70.25 ± 0.59
Neutrophils (%)	23.6 ± 1.05	25.7 ± 0.45	27.7 ± 0.90	26.3 ± 1.31
Monocytes (%)	1.87 ± 0.35	1.62 ± 0.32	2.12 ± 0.35	2.12 ± 0.23
Glucose (mg/dl)	149.2 ± 0.58	151.9 ± 0.73	150.0 ± 0.48	155.2 ± 0.90*
Cholesterol (mg/dl)	108.4 ± 0.70	106.3 ± 1.65	105.5 ± 0.94	101.5 ± 0.93*
Triglyceride (mg/dl)	98.3 ± 1.29	97.9 ± 0.73	99.7 ± 0.56	99.5 ± 1.28
Blood urea (mg/dl)	28.8 ± 0.34	30.4 ± 0.47	30.2 ± 0.42	28.2 ± 0.76
ALP (U/l)	109.5 ± 0.70	108.2 ± 0.72	105.1 ± 1.17	106.7 ± 1.51
SGPT (U/l)	68.5 ± 0.82	67.4 ± 0.79	65.3 ± 0.89	70.4 ± 0.77
SGOT (U/l)	51.2 ± 0.77	51.6 ± 0.80	52.5 ± 0.55	56.8 ± 1.71
Body weight gain (g) after 15 days	5.52 ± 0.31	5.25 ± 0.19	5.86 ± 0.30	5.11 ± 0.29

Symbols as in Table 1.

## DISCUSSION

In our study, the number of viable EAC cells in the peritoneum of EECF-treated mice were significantly lower when compared to the tumour control group (Fig. 1A). EECF also inhibited the increase in body weight due to the tumour burden (Fig. 1D) and prolonged the average life span of the animals (Fig. 1B and Fig. 1C). The Ehrlich tumour is a rapidly growing carcinoma with very aggressive behaviour (Segura et al. 2000) and able to grow in almost all strains of mice (Ahmed et al. 1988). The Ehrlich ascitic tumour implantation induces *per se* a local inflammatory reaction with increasing vascular permeability, which results in an intense edema formation, cellular migration and a progressive ascitic fluid formation (Fecchio et al. 1990). The ascitic fluid is essential for tumour growth, since it constitutes a direct nutritional source for tumour cells (Shimizu et al. 2004). The inhibition of EAC cells in this study could indicate either a direct cytotoxic effect of EECF on tumour cells or an indirect local effect, which may involve macrophage activation, vascular permeability inhibition and nutritional fluid deficiency.

In cancer chemotherapy the major problems are myelosuppression and anaemia (Maseki et al. 1981). The anaemia encountered in tumour bearing mice is mainly due to a reduction in RBC or haemoglobin percentage and this may occur either due to iron deficiency or due to haemolytic or myelopathic conditions (Hogland 1982). Recovery of the haemoglobin content, RBC and WBC cell count in the experimental mice indicates the protective action of EECF on the haemopoietic system. The development of hypoglycaemia and hyperlipidaemia in experimental animals with carcinoma has been previously reported (Silverstein et al. 1988, Killington et al. 1991). In this experiment, the reduced glucose level and elevated cholesterol, triglycerides and serum urea were returned to more or less normal levels in EECF-treated mice, thereby indicating a potent antitumour efficacy of EECF (Table 1).

It is well known (Sonnenwirth and Jarett 1980) that there are significant elevations in the levels of SGPT, SGOT and ALP in liver diseases and disorders and in hepatocellular damage caused by a number of agents. An increase in these enzyme levels is observed in patients with cardiac damage due to myocardial infarction and with liver disorders (Sonnenwirth and Jarett 1980). Biochemical measurements of these parameters showed that some extent of hepatotoxicity was associated after 15 days inoculation with EAC. After treatment with EECF values remain near the normal range in the treated

groups (Table 1). From this it follows that the tumour cell was to some extent inducing hepatotoxicity and that the damage was prevented by EECF supplementation.

In the short term toxicity study, treatment of normal mice with EECF (only at 200 mg/kg) slightly changed the glucose, cholesterol, haemoglobin, RBC and WBC counts (Table 2). This indicates that after short term treatment the extract did not cause any extreme abnormality at the three doses used in this study.

The potential antitumour activity of EECF at 200 mg/kg is comparable to that of bleomycin (0.3 mg/kg), which is commonly used as an active antitumour agent in a vast series of preclinical and clinical studies (Tannock and Richard 1998). The preliminary phytochemical studies in our laboratory indicated the presence of flavonoids, triterpenes, glycosides, steroids, heterocyclic and phenolic compounds in the EECF. Many such compounds are known to possess potent antitumour properties (Kintzois 2006). In addition, both *Calotropis gigantea* and *Calotropis procera* plants possess hepatoprotective and antioxidant properties (Ramachandra et al. 2007, Lodhi et al. 2009). Many natural compounds of plant-derived extracts have vital roles in balancing the intracellular redox status and antioxidant function. Imbalance between the production of the cellular oxidant species and antioxidant capability, produces reactive oxygen species (ROS) which are involved in a variety of different cellular processes ranging from apoptosis and necrosis to cell proliferation and carcinogenesis. Natural compounds with antioxidants effects are important therapeutic prospects for cancer (Matés et al. 2008, 2009). Moreover, antisense glutaminase inhibition decreases glutathione antioxidant capacity and increases apoptosis in Ehrlich ascetic tumour cells (Lora et al. 2004). This evidence seems to indicate that the antitumour properties of EECF may be due to the presence of phytoconstituents with antioxidative activity. Finally the present study demonstrates the *Calotropis gigantea* flower merits further investigation in isolating its active constituents and our future research plan is oriented in this direction.

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