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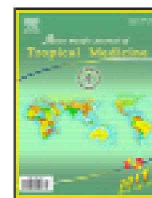

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Evaluation of antitumour activity of *Calotropis gigantea* L. root bark against Ehrlich ascites carcinoma in Swiss albino mice

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ABSTRACT

Objective: To investigate experimentally the possible antitumor effect of methanol extract (ME) of *Calotropis gigantea* L. (*C. gigantean*) root bark and its petroleum ether (PEF) and chloroform (CF) soluble fractions against Ehrlich ascites carcinoma (EAC) in Swiss albino mice. **Methods:** The effects of ME (10 and 20 mg/kg), PEF (40 and 80 mg/kg) and CF (20 and 40 mg/kg) on the growth of EAC and life span of EAC bearing mice were studied. Hematological profile and biochemical parameters (SALP, SGPT and SGOT) were also estimated. **Results:** Results of *in vivo* study showed a significant decrease in viable tumor cell count and a significant increase of life span in the ME and CF treated group compared to untreated one. The life span of ME and CF treated animals was significantly (P<0.05) increased by 43.90% (20 mg ME/kg) and 57.07% (40 mg CF/kg). ME and CF brought back the hematological parameter more or less normal level. ME and CF also restored the altered levels of serum alkaline phosphatase (SALP) and serum glutamate oxaloacetate transaminase (SGOT). **Conclusions:** Methanol extract (ME) of *C. gigantea* root bark and its chloroform soluble fraction (CF) possesses significant antitumor activity.

1. Introduction

One of the major limitations in the currently available treatment modalities for cancer is their side effects[1]. Hence alternate treatment for cancer is being tested. Plant derived natural products such as flavonoids, terpenes, alkaloids and so on have received considerable attention in recent years due to their diverse pharmacological properties including cytotoxic and cancer chemopreventive properties[2-3]. Information on the ethnopharmacologic use of plants has given important lead in the cancer drug development. One of the best approaches in search for anticancer agents from plant resources is the selection of plant based on ethno medical leads and testing the selected plant's efficacy and safety in light of modern science.

Calotropis gigantea L. (*C. gigantea*), locally known as Boro Akanda belongs to the Asclepiadaceae family and it grows in tropical region and most abundant in Bangladesh, India, Burma, Pakistan and in the sub Himalayan tract[4]. Traditionally extracts and preparations from roots and leaves of *C. gigantea* are used against abdominal tumours, boils, syphilis, tuberculous, leprosy, skin diseases, piles, wounds, rheumatism and insect-bites. Root bark of this plant is used in dysentery and as a purgative, alterative, diaphoretic and emetic[5]. Antipyretic, analgesic, anticonvulsant, anxiolytic, sedative, hepatoprotective, wound healing, antidiabetic, larvicidal, anti-inflammatory and anti-diarrhoeal activities of *C. gigantea* have been scientifically proven[6-14]. Several phytochemicals have been isolated from *C. gigantea* and they include cytotoxic cardenolides[15-17], antifeedant nonprotein amino acid[18], naphthalene and terpene derivatives[19], flavonol glycosides[20], pregnanes[21-23], ursane-type triterpenoids[5] and steroids[24-25]. In the present communication, the crude methanol extract of root bark of *C. gigantea* and its petroleum ether (40-60 ℃) and chloroform soluble fractions were evaluated for their antitumour activity against Ehrlich

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Ascites Carcinoma (EAC) cells in mice.

2. Materials and methods

2.1. Plant material

The root bark of *C. gigantea* was collected from the relevant area (Meherchandi) of Rajshahi University Campus, Rajshahi, Bangladesh in November, 2010. The plant was taxonomically identified by Professor A. T. M. Naderuzzaman, Department of Botany, Rajshahi University and a voucher specimen (No. 1A. Alam, Collection date 15.08.2004) was kept in the Department of Botany, Rajshahi University.

2.2. Extraction

The pulverized plant material (1.0 kg) was extracted with methanol (2.0 L) at room temperature for two weeks. The methanol extract was concentrated and yielded 38.6 g of a crude extract (ME). The crude extract (25.0 g) was suspended in H_2O and partitioned with petroleum ether (40-60 ℃) and chloroform successively to yield 13.8 g and 10.5 g petroleum ether (PEF) and chloroform (CF) soluble fractions, respectively[26].

2.3. Animals

Male Swiss albino mice (25-30 g) were procured from the Animal Research Branch of the International Centre for Diarrhoeal Diseases and Research, Bangladesh (ICDDR,B). They were used throughout the study and housed in iron cages in a controlled environment (temperature 25±2 ℃ and 12h dark/light cycle) with standard laboratory diet and water ad libitum. Experiments were carried out in accordance with the Ethical Committee Guidelines laid down by the local committee regarding the care and use of animals for experimental procedures.

2.4. Tumour cells

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

2.5. Acute toxicity studies

An acute toxicity study relating to the determination of LD_{50} was performed in mice by the method of Lorke[27]. For each sample, this method was carried out in twenty animals, four per treatment group and widely different dose ranges of 100, 200, 400, 800 and 1 600 mg/kg body weight. Then after 24 hrs,
the mortality number caused by each test sample was observed from which the median lethal dose (LD_{50}) of ME, PEF and CF was determined.

2.6. Cell growth inhibition

In vivo tumour cell growth inhibition was carried out by the method as described by Senthilkumar *et al*[28]. 2×10^5 EAC cells were inoculated into 8 groups of mice (6 in each) on day 0. The groups and the design of the experiment were as follows:

Group 1: EAC + 2% Dimethylsulfoxide (DMSO)
Group 2: EAC + ME (10 mg/kg b.wt; i.p.)
Group 3: EAC + ME (20 mg/kg b.wt; i.p.)
Group 4: EAC + PEF (40 mg/kg b.wt; i.p.)
Group 5: EAC + PEF (80 mg/kg b.wt; i.p.)
Group 6: EAC + CF (20 mg/kg b.wt; i.p.)
Group 7: EAC + CF (40 mg/kg b.wt; i.p.)
Group 8: EAC + Bleomycin (0.3 mg/kg b.wt; i.p.)

Treatment was continued for 5 days and on day 6 after tumour transplantation, animals were sacrificed. Tumour cells were collected by repeated washing with 0.9% saline and viable tumour cells per mouse of the treated group were compared with those of control.

2.7. Studies on survival time, hematological and biochemical parameters

Swiss Albino mice were divided into nine groups (n = 12). All the animals were injected with EAC cells (2×10^5 cells/mouse) intraperitoneally except for the normal control group. This was taken as day 0. Group 1 served as the normal control and group 2 served as the tumour control. These two groups received 2% DMSO. Group 3, 4, 5, 6, 7 and 8 were treated with methanol extract and its petroleum ether and chloroform soluble fractions at 10 and 20 mg/kg body weight, respectively. Group 9, which served as the positive control, was treated with bleomycin at 0.3 mg/kg body weight. All these treatment were given 24 h after the tumour inoculation, once daily for 10 days. Six mice from each group were sacrificed on 14th day after tumor inoculation for the study of hematological and biochemical parameters. Hematological parameters (Hemoglobin, RBC, WBC and Differential count of WBC) were measured from freely flowing tail vein blood of each mice of each group[29]. Then every mouse was sacrificed and blood was collected by heart puncture. The blood samples of each animal were allowed to clot for 45 min at 4 ℃. Serum was separated by centrifugation at 4 000 rpm for 10 minutes and analyzed for serum alkaline phosphatase (SALP), serum glutamate pyruvate transaminase (SGPT) and serum glutamate oxaloacetate transaminase (SGOT) in an Bioanalyzer (Microlab 200) using commercial kits (Atlas

Medica, UK). The rest of the animal groups were kept to check the survival time of EAC-tumor bearing mice.

2.8. Statistical Analysis

All values were expressed as mean ± SEM (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 of 12 version. $P<0.05$ were considered to be statistically significant when compared with control.

3. Results

The LD_{50} value of ME, PEF and CF was evaluated in Swiss albino mice and found to be 259.2, 905.1 and 482.5 mg/kg body weight, respectively. The average number of viable tumour cells per mouse of tumour control group was found to be $(5.12 \pm 0.18) \times 10^7$ cells/mL. Treatment with ME (10 and 20 mg/kg), PEF (80 mg/kg) and CF (20 and 40 mg/kg) decreased the viable cells significantly ($P<0.05$) (Figure 1A).

The effect of ME, PEF and CF on the survival of tumor-bearing mice is shown in Figure 1B and 1C. The mean survival time (MST) for the control group was 19.8±0.47 days, whereas it was 26.8±2.14, 28.5±1.62 ($P<0.05$), 24.1±0.92, 25.1±1.80, 26.5±1.43, 31.1±1.52 and 39.0±0.85 ($P<0.05$) days for the groups treated with ME (10 and 20 mg/kg), PEF (40 and 80 mg/kg) and CF (20 and 40 mg/kg) and bleomycin (0.3 mg/kg) respectively. Among the test samples, the highest increase in the lifespan of tumor-bearing mice treated with CF (40 mg/kg) was found to be 57.07% as compared to the control group whereas it was 96.97% for bleomycin (0.3 mg/kg). PEP did not show significant efficacy to increase the MST of EAC cell bearing mice.

Hematological parameters of tumor-bearing mice on day 14 showed significant changes when compared with the normal mice (Table 1). The total WBC count was found to increase with a reduction in the hemoglobin content of RBC. The differential count of WBC showed that the percentage of neutrophils increased ($P<0.05$) while that of lymphocytes decreased ($P<0.05$). At the same time interval, ME (10 and 20 mg/kg) and CF (20 and 40 mg/kg) treatment could change these altered parameters to near normal whereas no significant change was found for PEF.

Mice of EAC control group showed significant ($P<0.05$) increase in the activities of ALP and SGOT when compared with the respective normal values (Table 1). Significant ($P<0.05$) depletion in the activities of ALP and SGOT was found by treatment with ME (at 10 and 20 mg/kg) and CF (20 and 40 mg/kg). At 0.3 mg/kg dose, bleomycin significantly ($P<0.05$) decreases the activities of ALP and SGOT. However SGPT was not significantly altered by tumour growth in only EAC cell bearing mice but treatment of ME (at 20 mg/kg) and CF (at 40 mg/kg) increased SGPT ($P<0.05$) when compared with only EAC cell bearing mice.

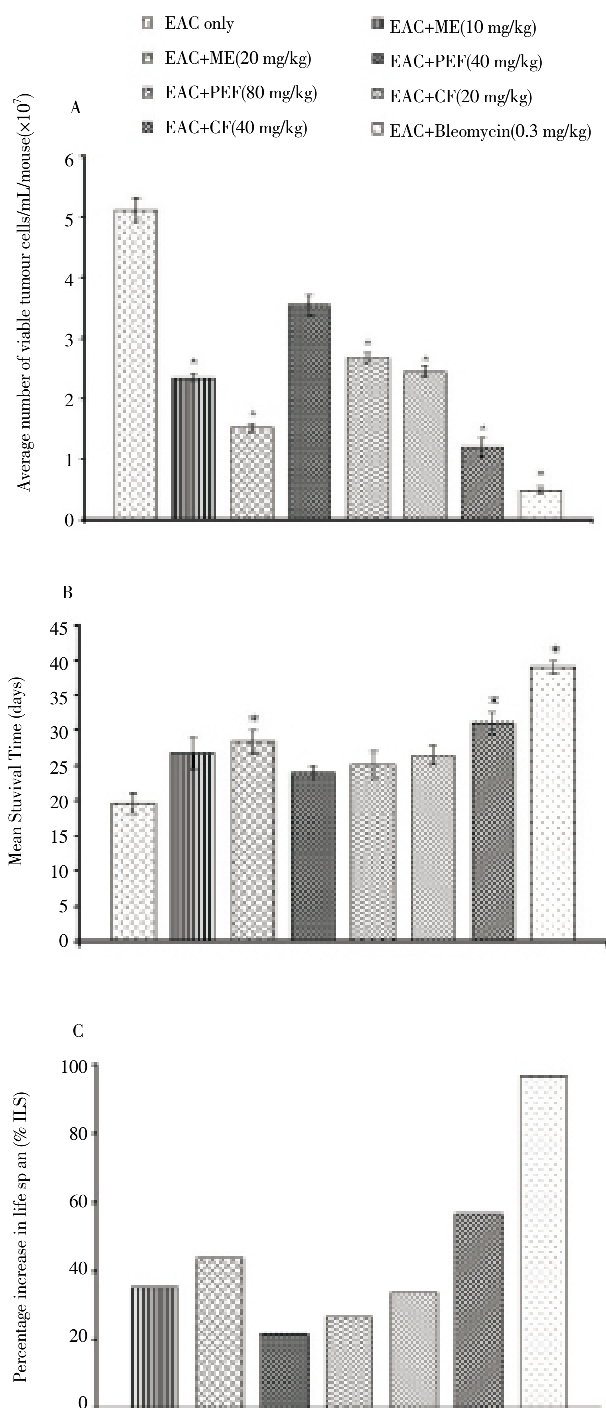

Figure 1. Effect of ME, PEF and CF on EAC cell bearing mice.
A: Viable EAC cells on day 6 after tumor cell inoculation.
B: Mean survival time
C: % Increase in life span
Data are expressed as mean ± S.E.M (n = 6); *$P<0.05$: between EAC control and treated group.

Table 1
Effect of ME, PEF and CF on hematological and biochemical parameters of EAC cell bearing mice.

Parameters	Treatment (mg/kg body weight)								
	Normal	EAC + Vehicle	EAC + ME (10 mg/kg)	EAC + ME (20 mg/kg)	EAC + PEF (40 mg/kg)	EAC + PEF (80 mg/kg)	EAC + CF (20 mg/kg)	EAC + CF (40 mg/kg)	EAC + Bleomycin (0.3 mg/kg)
Hgb (g/dL)	12.05 ±0.34	6.61 ±0.11*	8.16 ±0.21t	9.78 ±0.19t	6.21 ±0.29	6.73 ±0.16	9.51 ±0.24t	10.15 ±0.25t	14.37 ±0.25t
RBC($\times 10^9$ cells/mL)	6.10 ±0.05	2.98 ±0.04*	3.76 ±0.03t	4.24 ±0.03t	2.97 ±0.04	3.28 ±0.09	4.01 ±0.05t	4.56 ±0.04t	4.90 ±0.09t
WBC($\times 10^6$ cells/mL)	5.83 ±0.53	17.5 ±0.99*	12.3 ±0.66t	10.2 ±0.98t	17.6 ±1.22	16.5 ±0.61	11.3 ±0.71t	8.16 ±0.60t	9.37 ±0.59t
Lymphocytes (%)	69.5 ±0.76	35.3 ±1.17*	42.2 ±1.30t	46.6 ±0.49t	34.2 ±1.24	38.6 ±1.33	45.8 ±1.49t	53.1 ±1.55t	68.25 ±0.90t
Neutrophils (%)	26.6 ±1.28	60.5 ±1.33*	53.6 ±1.62	49.0 ±1.06t	62.5 ±1.64t	55.8 ±1.70	50.3 ±1.40t	43.6 ±1.22t	28.87 ±0.93t
Monocytes (%)	2.5 ±0.3	2.6 ±0.33	2.7 ±0.34	2.5 ±0.42	2.0 ±0.44	3.0 ±0.36	2.5 ±0.22	2.1 ±0.42	2.00 ±0.27
ALP (U/L)	113.1 ±1.35	293.0 ±1.70*	253.6 ±1.38t	168.5 ±1.24t	287.2 ±1.66	266.6 ±3.62	187.3 ±1.57t	163.7 ±1.25t	133.1 ±0.72t
SGPT (U/L)	56.3 ±0.52	51.3 ±1.12	53.0 ±1.85	79.1 ±2.93t	51.5 ±1.84	56.3 ±0.70	62.4 ±1.47	83.3 ±1.16t	71.3 ±0.36t
SGOT (U/L)	45.1 ±1.10	290.3 ±1.00*	205.2 ±1.55t	154.3 ±1.95t	287.9 ±1.94	277.9 ±1.58	206.1 ±0.92t	184.7 ±1.51t	80.0 ±0.78t

Data are expressed as mean ±S.E.M. for eight animals in each group. *$P<0.05$: against normal group and t$P<0.05$: against EAC control group.

4. Discussion

In EAC tumor bearing mice, a regular rapid increase in ascitic tumor volume was observed. Ascitic fluid is the direct nutritional source for tumor cells and a rapid increase in ascitic fluid with tumor growth would be a means to meet the nutritional requirement of tumor cells[30-36]. In this investigation, treatment with ME and CF decreased the viable tumor cell count and increased the life span of the tumor bearing mice. The reliable criteria for judging the value of any anticancer drug are the prolonga-tion of the life span of animals[37]. ME and CF by decreasing the nutritional fluid volume and arresting the tumor growth increases the life span of EAC-bearing mice. Thus, ME and CF has potent antitumor activity against EAC bearing mice.

Myelosupression is a frequent and major complication of cancer chemotherapy[38]. In this study, ME and CF treatment and subsequent tumor inhibition resulted in appreciable improvements in hemoglobin content, RBC and WBC counts. These observations assume great significance, as anemia is a common complication in cancer and the situation aggravates further during chemotherapy since a majority of antineoplastic agents exert suppressive effects on erythropoiesis[39] and thereby limiting the use of these drugs. The improvement in hematological profile of the tumor bearing mice following the treatment with ME and CF could be due to the action of the different phytoconstituents present in the extract and fraction.

Numerous studies on the enzymes of carbohydrate metabolism in cancer showed that actively dividing neoplastic tissues require more energy than normal cells[40]. The consequent display of a high rate of glycolysis in malignant conditions is clinically manifested in the increased activity of several serum enzymes[41,42]. In our study, fourteen (14) days of inoculation with EAC brought the significant elevation in the levels of SGOT and SALP. Treatment with ME and CF restored the elevated biochemical parameters more or less to normal range thereby indicating the protective effect on the tumour induced complications. However some extent of hepatotoxicity was associated with the treatment of ME (20 mg/kg) and CF (40 mg/kg) as indicated by the elevation in the levels of SGPT. Some phytochemicals present in ME and CF may be responsible for this elevation.

The result of the present investigation is quite encouraging and it explores *in vivo* the potent anticancer activity of ME and CF of root bark of *C. gigantea* L. probably because of its direct cytotoxic effect. No significant results were obtained for PEF. *In vitro* some phytochemicals with potent cytotoxic effect have already been reported from the root bark of *C. gigantea*[23,15]. Our preliminary thin layer chromatography (TLC) screening also showed that ME and CF contained flavonoid, glycosides, saponin, steroids and terpenoids type compounds. Many such type of compounds are known to possess potent antitumor properties[43]. Further investigations are in progress in the laboratory to identify the active principles involved in this antitumor activity and investigate the mechanism of inhibition.

Conflict of interest statement

We declare that we have no conflict of interest.

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