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Preventive effect of ethanol extract of *Alpinia calcarata* Rosc on Ehrlich's ascitic carcinoma cell induced malignant ascites in mice

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ABSTRACT

Objective: To explore cytotoxic activity of ethanol extract of *Alpinia calcarata* Rosc (EEAC) rhizome against Ehrlich ascites carcinoma (EAC) tumor bearing Swiss Albino mice. **Methods:** In the present study, its anti-neoplastic activity has been studied by monitoring parameters like tumor weight measurement, survival time, tumor cell growth inhibition, haematological characteristics *etc*. **Results:** It was found that EEAC at dose 8 mg/kg/day (*i.p.*) significantly decreased tumor weight (62.0%; $P<0.01$), increased life span (70.25%; $P<0.01$) and reduced tumor cell growth rate (85.7%; $P<0.01$) in comparison to those of EAC bearing mice. The plant extract also improved the depleted haematological parameters like RBC, WBC, Hb%, differential counts (*e.g.* lymphocytes, neutrophils, monocytes *etc*) of EAC bearing mice towards normal. The host toxic effects were not very high and recovered gradually towards normal within a few days after treatment. **Conclusions:** EEAC exhibits potent *in vivo* cytotoxic activity against EAC tumor bearing Swiss Albino mice. So, the plant can be considered as a probable new source of antitumor agents.

1. Introduction

Ayurveda, the South Asian system of medicine mainly uses plant-based drugs or formulations to treat various ailments including cancer. Plant derived compounds have a special place in anti-cancer therapy, and some of new chemotherapeutic agents are currently available for use in a clinical setting include paclitaxel, vincristine, podophyllotoxin and camptothecin, a natural product precursor for water soluble derivatives[1-3]. Due to lack of effective drugs, cancer is a fatal disease rating the top three cause of death. Many of the chemotherapeutic agents for the treatment of cancer are highly expensive, mutagenic, carcinogenic and teratogenic and marrow inhibition limits their applications[4]. Therefore the quest for effective anti-cancer drug is an active research field. Efforts are being made to identify naturally occurring anti-carcinogens, which would prevent, slow/reverse cancer development[5]. Plants, vegetables and herbs used in the folk and traditional medicine have been accepted currently as one of the main source of cancer chemoprevention drug development[6]. The plant *Alpinia calcarata* Rosc. belongs to the family, Zingiberaceae popularly known as Sugondha Boss (Bengali) has a widespread occurrence in Bangaldesh, India, Sri Lanka, Malaysia, China, and Timor. Compounds isolated from Zingiberaceae plants were found to have anticancer activity against various cell lines and also have strong antioxidant, anti-inflammatory activity[7-13]. Some novel compounds have DNA topoisomerase II poisoning activity and can induce apoptosis[14]. Here the plant selected is used in traditional medicine for the treatment of various ailments such as a warming digestive tonic, carminatve, stomachic, expectorant, stimulant and antifungal agent[15]. It is also used as tonic, aphrodisiac and diuretic, and in the treatment of headache, lumbago diabetes, chest pain, rheumatic pains, bronchitis, dyspepsia, sore throat, impotence and diseases of the kidney and liver. It is particularly reputed for its efficacy in chest complain[16]. Rhizome of the plant also possesses several diterpenoids, some of which are cytotoxic, and can induce cell cycle arrest such as Calcaratan D, Calcaratan E[17-19]. Alpinia calcarata Rosc. shows potent antibacterial activity against some pathogenic gram positive and gram negative bacteria[20]. The essential oils of the plant have significant repellant properties against *Periplaneta americana*[21]. It also posses antituberocolin

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property[22]. Hot water and ethanolic extracts of Alpinia *calcarata* rhizomes have dose dependent antinociceptive activity[23]. However, their anticancer activity has not been investigated so far. From this viewpoint the present study was carried out to evaluate the anti-tumor activity of ethanol extract of *Alpinia calcarata* Rosc (EEAC) against Ehrlich ascites carcinoma (EAC) in Swiss albino mice.

2. Materials and methods

2.1. Materials

The dried powder material of the rhizome of *Alpinia calcarata* Rosc was extracted with ethanol (yield 9.25%). The ethanol extract was then distilled, evaporated, and dried invacuum. The crude extract was dissolved in dimethylsulfoxide (DMSO) for the experiments. For therapeutic treatment, crude ethanol extract was dissolved in DMSO (10%) at the concentration of 0.5 mg/mL, 1 mg/mL and 2 mg/mL.

2.2. Chemicals and reagents

All the chemicals and reagents used throughout the investigation were of reagent grade. Ethanol and sodium chloride were purchased from MERCK, Merck KGaA, 64271 Darmstadt, Germany; DMSO, hydrochloric acid (6M) from BDH Laboratory supplies, Roole, BH 15, England; bleomycin and cells counting fluids (WBC, RBC fluids) from Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany.

2.3. Animals

Adult Swiss albino male mice (20-25 g) were used through out this study. They were obtained from International Center for Diarrheal Diseases Research, Bangladesh (ICDDRB). Animals were fed with standard mouse-pellets (collected from ICDDRB) and water was given in *ad libitum*.

2.4. Tumor cells

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

2.5. Ethical clearance

Protocol used in this study for the use of mice as animal model for cancer research was approved by the Institutional Animal, Medical Ethics, Biosafety and Biosecurity Committee (IAMEBBC) for Experimentations on Animal, Human, Microbes and Living Natural Sources (225/320-IAMEBBC/IBSc), Institute of Biological Sciences, University of Rajshahi, Bangladesh.

2.6. Determination of median lethal dose (LD_{50})

An acute toxicity study relating to the determination of LD_{50} was performed[24]. The crude EEAC was dissolved in 2% DMSO and injected intraperitoneally to 8 groups of mice (each containing n=6) at different doses (100, 150, 200, 250, 300, 400, 500 and 600 mg/kg). LD_{50} was evaluated by recording mortality after 24 hours.

2.7. Cell growth inhibition

In vivo tumor cell growth inhibition was carried out by the method as described by Sur *et al*[25]. For this study, 5 groups of mice (6 in each group) were used. For therapeutic evaluation 14×10^5 cells/mouse were inoculated in to each group of mice on the first day. Treatment started after 24 h of tumor inoculation and continued for 5 days. Group 1 to 3 received the crude extract (EEAC) at the doses 2 mg/kg, 4 mg/kg and 8 mg/kg, respectively per day per mouse. In each case the volume of the test solution injected (*i.p.*) were 0.1 mL/day per mouse. Group 4 received bleomycin (0.3 mg/kg, *i.p.*) and finally the group 5 was treated with the vehicle (normal saline) and was considered as untreated control. The mice were sacrificed on the 6th day after transplantation and tumor cells were collected by repeated intraperitoneal wash with 0.9% saline. Viable tumor cells per mouse of the treated group were compared with those of control.

The cell growth inhibition was calculated by using the formula:

$$\% \text{ Cell growth inhibition} = \left(1-\frac{Tw}{Cw}\times 100\right)$$

Where, Tw = Mean of number of tumor cells of the treated group of mice and Cw = Mean of number of tumor cells of the control group of mice.

2.8. Bioassay of EAC cells (Transplantation ability of EAC cells)

The effect of EEAC on transplantability of EAC cells was carried out by the method as described in the literature[26]. In this experiment two groups of mice (n=4) were inoculated with 115×10^5 EAC cells. Group 1 was treated with the test compound at the dose of 8 mg/kg for 5 consecutive days and group 2 served as control. On day 7, tumor cells from the mice were harvested in cold (0.9%) saline, pooled, centrifuged and re-inoculated into two fresh groups of mice (n=4) as before. No further treatment was done on these mice. On day 5, they were sacrificed and viable tumor cells count/mice were estimated.

2.9. Average tumor weight and survival time (MST)

These parameters were measured under similar experimental conditions as stated in the previous experiment. Tumor growth was monitored daily by measuring weight change. The host survival time was recorded and expressed as MST in days and percent increase

of life span was calculated[27] as follows:

$$\text{MST} = \frac{\sum \text{Survival time (days) of eac h mouse in a group}}{\text{Total number of mice}} \times 100$$

$$\text{Percent increase of life span \%} = \left(\frac{\text{MST of treated group}}{\text{MST of control group}}\right) \times 100$$

2.10. Haematological studies

The haematological parameters *viz*, WBC, RBC, Hb content, differential counts *etc*. were determined by the standard methods using cell dilution fluids and haemocytometer[28,29]. For this purpose, blood was collected from the mouse by tail puncture method. Three groups of mice (with n=4) were taken for doses 2, 4 and 8 mg/kg of the extract. Treatment started after 24 hours of tumor transplantation and was continued for 10 consecutive days. On day 12, the blood parameters were assayed.

For normal mice, four groups (n=4) were taken for the purpose. The blood from the mice of group Ⅰ was assayed on day 0 (without any treatment). The second and third groups of mice were treated with extract at dose 8 mg/kg for 5 and 10 consecutive days, respectively and analyzed as before. The mice of 4th group were treated with 50 mg/kg for 10 consecutive days and assayed on day 25.

2.11. Brine shrimp lethality bioassay

Cytotoxicity of the compound was screened against *Artemia salina* in a 1-day *in vivo* assay[30] according to published protocol. For the experiment 3 mg of the compound was dissolved in 0.6 mL (600 μL) of distilled water to get a concentration of 5 μg/μL and by serial dilution technique, solutions of varying concentrations such as 5, 10, 20, 40, 80 and 100 μg/mL were obtained. After 24 h of incubation, the vials were observed using a magnifying glass and the number of survivors in each vial were counted and noted. From this data, the percentage of mortality of the nauplii was calculated for each concentration and the LC_{50} value was determined using Probit analysis as described in the literature[31].

2.12. Statistical analysis

The experimental results have been expressed as the mean±SEM. Data have been calculated by one way ANOVA followed by Dunnett 't' test using SPSS software of 10 version. Value at P<0.05 was considered as significant.

3. Results

Maximum cell growth inhibition (85.7%) was found after treatment with dose 8 mg/kg whereas 69.3% and 34.5% cell growth inhibition were observed at dose 4 mg/kg, and 2 mg/kg, respectively. Bleomycin at dose 0.3 mg/kg on the other hand inhibited the cell growth by 93.7%. EAC cell growth inhibition by EEAC was quite comparable to the standard anticancer drug bleomycin.

The transplantability of EAC cells were remarkably inhibited by EEAC since the reduction in EAC cell growth as high as 54.78% was observed, when 6 days treated EAC cells were reinoculated into fresh mice (viable cells counts were performed on day 5).

In vivo MST of the untreated tumor bearing mice was (20.5± 1.4) days. With the treatment of EEAC, this value increased markedly. About 70.25% enhancement of MST was found at dose 8 mg/kg. EEAC at dose 4 mg/kg and 2 mg/kg increased the MST by 57.3% and 28.4%, respectively; whereas bleomycin at dose 0.3 mg/kg increased the MST value by 88.4%.

The treatment with EEAC also reduced the rate of tumor growth. It was found to be only 28% at dose 8 mg/kg after 20 days as against 92% without any treatment. With bleomycin (0.3 mg/kg) however the growth rate reduced to 21% in 20 days (Figure 1).

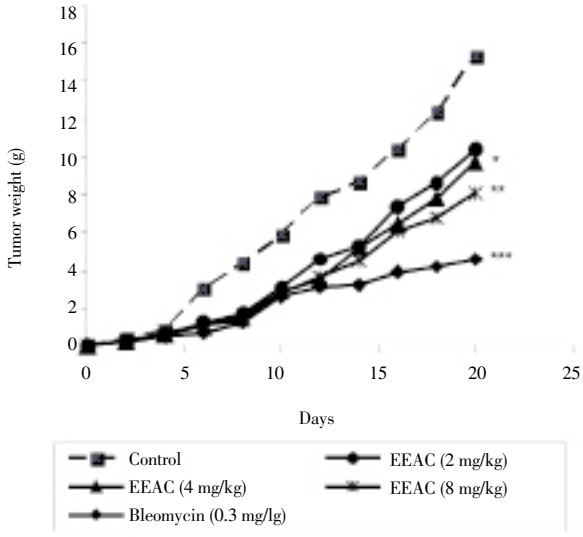

Figure 1. Effect of EEAC on tumor weight in mice.
*P<0.05,** P <0.01 and*** P <0.001 compared with control group.

The haematological parameters of both treated and non-treated mice were examined. In EAC cell bearing mice all the parameters such as haemoglobin, WBC, RBC and differential counts (monocytes, lymphocytes, neutrophil, monocytes) were significantly changed as compared to those of the normal mice. These parameters reverted towards normal values when treated with the extract at dose 8 mg/kg. In case of parallel treatment of normal mice, these parameters were slightly changed from normal values and after 25 days of the initial treatment they were restored to almost normal values (Table 1 and 2). The extract at dose 8 mg/kg also enhanced both the peritoneal cells and the number of macrophages to some extent in normal mice (Table 3).

LD_{50} value in Swiss albino mice was 250 mg/kg. The brine shrimp lethality bioassay was done to assess the *in vitro* cytotoxic effect of the compound. Medium lethal concentration (LC_{50}) of brine shrimp lethality was 14.07 μg/mL.

Table 1

Effect of crude EEAC on blood parameters of tumor bearing and normal swiss albino mice on day 12 of tumor inoculation (mean ± SEM).

Group	RBC($\times10^9$ cells/mL)	WBC($\times10^6$ cells/mL)	Hb (g/dL)	% of lymphocyte	% of neutrophil	% of monocyte
Normal mice	6.11±0.40	10.40±1.20	11.60±1.20	72.00±0.30	23.00±0.20	5.00±0.65
EAC bearing mice	2.32±0.20	25.70±0.40	5.00±0.70	51.00±0.65	36.00±1.20	13.00±0.60
EAC+ 2 mg/kg	3.20±0.35	19.50±0.65*	11.00±0.21***	46.00±0.54	39.00±0.41	15.00±0.21
EAC+ 4 mg/kg	4.50±0.14	17.00±0.31*	11.80±0.36***	67.00±0.24***	30.00±0.54*	3.00±0.18***
EAC+ 8 mg/kg	5.20±0.31***	15.00±0.34**	12.50±0.11**	70.00±0.25***	30.00±0.45**	2.00±0.54***

*P<0.05,** P<0.01 and*** P<0.001 compared with control group.

Table 2

Effects of crude EEAC on blood parameters in normal mice on days, 0, 5, 10 and 25 at dose 8 mg/kg body weight(mean ± SEM).

Group		RBC($\times10^9$ cells/mL)	WBC($\times10^6$ cells/mL)	Hb(g/dL)	% of lymphocyte	% of neutrophil	% of monocyte
Normal mice	0	6.11±0.40	10.40±1.20	11.60±1.20	72.00±0.30	23.00±0.20	5.00±0.65
Mice treated with extract	5 mg/kg	4.00±0.71**	5.10±0.91***	5.20±0.10***	61.00±2.00**	32.00±0.97**	7.00±0.85
	10 mg/kg	5.20±0.82**	7.20±0.21**	7.30±0.19***	62.00±3.10**	29.00±1.20**	4.00±1.20
	25 mg/kg	5.33±0.54	8.30±1.10**	11.60±0.13	71.00±2.10	21.00±0.92	8.00±1.40

*P<0.05,** P <0.01 and*** P <0.001 compared with normal group.

Table 3

Effect of the EEAC on the enhancement of normal peritoneal cells in mice (mean ± SEM).

Group	Dose (mg/kg)	Macrophages($\times10^6$)	Total peritoneal cells($\times10^6$)
Control (Normal)	–	1.67±0.31	7.97±0.40
Normal + EEAC	2	2.00±0.50*	8.00±0.34*
	4	2.50±0.40*	8.90±0.44*
	8	3.40±0.55*	9.00±0.43*

*P<0.001 compared with control group.

4. Discussion

The results showed that the EEAC at dose 8 mg/kg can inhibit cell growth of tumor bearing mice satisfactorily, reduce tumor growth rate markedly and increase life span dramatically. These are important and promising in justifying the potency of a compound in cancer chemotherapy[32]. One of the major problems usually encountered in cancer chemotherapy is myelosuppression followed by anemia[33,34] due to the reduction of RBC and haemoglobin contents. This is probably owing to the deficiency of iron of haemolytic or myelopathic condition[35].The treatment with EEAC can also reverse all depleted haematological parameters back towards normal. The host toxic effect of EEAC is not very high. In addition the treatment in normal mice increases the macrophages and peritoneal cells which is also considered as a very vital event in acquiring self destroying activity of the living being towards cancer cells[36]. Enhancement of macrophages might produce some cytokines such as tumor necrosis factors, interleukins *etc* inside the peritoneal cavity, which in turn may be responsible for killing of tumor cells[37]. The transplantability of EAC cells shows a significant reduction of viability after the treatment with EEAC. In addition, since LD_{50} value of extract is 250 mg/kg as compared with the dose (8 mg/kg) used during treatment, the plant could be an source of effective anticancer agent with low toxicity at doses (up to 8 mg/kg).

Alpinia genus possess antioxidant properties[38]. Plant extracts have vital roles in balancing the intracellular oxidant species and antioxidant capability, produces reactive oxygen species which are involved in a variety of different cellular process ranging from apoptosis and necrosis to cell proliferation and carcinogenesis[39]. Natural compounds with antioxidants effects are important treatment of cancer[40]. More over, antisense glutaminase and inhibition decrease glutathione antioxidant capacity and increases apoptosis in Ehrlich ascetic tumour cells[41]. This may be attributed to the phytoconstituents with antioxidative activity.

However, the information obtained from the present investigation are insufficient for the crude EEAC. And more investigation have to be carried out with crude extract and purified compounds using various other cancer cell lines and higher animal models, in order to confirm the plant as a potent source of anticancer agent.

Conflict of interest statement

We declare that we have no conflict of interest.

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