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Bioassay of *Eucalyptus* extracts for anticancer activity against Ehrlich ascites carcinoma (eac) cells in Swiss albino mice

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ABSTRACT

Objective: To evaluate the antineoplastic activity of *Eucalyptus* extract (EuE) against Ehrlich ascites carcinoma (EAC) in Swiss albino mice. **Methods:** Preliminary examination of four plant extracts (namely *Eucalyptus*, *Costus*, *Azadirachta*, *Feronia*) has been done by observing the reduction ability of number of EAC cells in previously inoculated Swiss albino mice. Among them as EuE showed maximum capability, the whole study has been conducted with EuE only. Important parameters *viz.* enhancement of life span, reduction of average tumor weight *etc.* have been studied. In addition the effects of EuE on hematological parameters in both normal and EAC inoculated mice have been measured. Effect of EuE on normal peritoneal cells has also been studied. **Results**: EuE reduced tumor burden remarkably. It reduced the tumor growth rate and enhanced the life span of EAC bearing mice noticeably. It reversed back the hematological parameters towards normal, reduced the trasplantability of EAC cells and enhanced the immunomodulatory effects in mice. The host toxic effect of EuE in mice is minimum and mostly reversible with time. All such data have been compared with those obtained by running parallel experiments with *bleomycin* at dose 0.3 mg/kg (*i.p.*). **Conclusions:** The *Eucalyptus* extract may be considered as a potent anticancer agent for advanced researches.

1. Introduction

Bangladesh has a rich and prestigious heritage of herbal medicines. More than 250 plants are being used for the treatment of various diseases. However, few of these plants have undergone chemical, pharmacological and toxicological studies[1-4]. In order to get anticancer agents from natural sources, we have primarily selected four plants [namely *Feronia limonia* (*F. limonia*), *Azadirachta indica* (*A. indica*), *Costus curvibracteatus* (*C. curvibracteatus*) and *Eucalyptus camaldulensis* (*E. camaldulensis*)] which are well known to possess therapeutic values in the Ayurvedic and Unani pharmacopoeia. Among these, the *Eucalyptus* extract (EuE) has been found to be more promising. The present work has therefore, been designed to study its anticancer activity *in vivo*.

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2. Materials and methods

2.1. Materials

The dried powder materials of the plants were extracted with petroleum ether (yield 9.25 %) by the procedure similar to that described elsewere[5,6]. The petroleum ether extract were distilled, evaporated, and dried in vacuum. The crude extracts were dissolved in dimethylsulfoxide (DMSO) for the experiments.

2.2. Chemicals and reagents

All the chemicals and reagents used throughout the investigation were of reagent grade.

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 *ad libitum*.

2.4. Tumor cells

Ehrlich ascites carcinoma (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. Ethical clearance

This research work was approved by Ethical Review Committee of Research Cell of Rajshahi Medical College, Bangladesh (ref. RMC/ER/2010-2013/01).

2.6. Preparation of stock solution of the test samples

For therapeutic treatment, stock solution of bleomycin was made by dessolving in distilled water at the concentration of 0.075 mg/mL. Petroleum ether extract of *C. curvibracteatus, A. indica, F. limonia* were dissolved in DMSO at the concentrations of 12.5 mg/mL, 25 mg/mL, 50 mg/mL, respectively and petroleum ether extract of *E. camaldulensis* (EuE) was dissolved in DMSO at the concentrations of 6.25 mg/mL, 12.5 mg/mL, 25 mg/mL.

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

The lethal dose, LD_{50} of extracts was evaluated following method as used earlier[7].

2.8. Study of anticancer activity

The procedures for evaluations of the anticancer activity were just described in our recent published papers[8-11].

2.9. Cell growth inhibition

Fourteen groups of Swiss albino mice (6 in each group) weighing 20-25 g were used. For therapeutic evaluation 136×10^4 EAC cells for every mouse were inoculated into each group of mice on day 0. Treatments were started after 24 hours of tumor inoculation and continued for five days. For *C. curvibracteatus, A. indica, F. limonia,* group one to nine received the petroleum ether extract at the doses of 50 mg/kg (*i.p.*), 100 mg/kg (*i.p.*), and 200 mg/kg (*i.p.*). For EuE, group ten to twelve received the petroleum ether extract at the doses of 25 mg/kg (*i.p.*), 50 mg/kg (*i.p.*), and 100 mg/kg (*i.p.*). Group thirteen received bleomycin at the dose of 0.3 mg/kg (*i.p.*) and group fourteen was used as control. Mice in each group were sacrificed on day six and the total intraperitoneal tumor cells were harvested by normal saline (0.98%). Viable cells were first identified by using trypan blue and then counted by a haemocytometer. Total numbers of viable cells in each animal of the treated groups were compared with those of control (EAC untreated) group.

2.10. Average tumor weight and survival time

For such determination, five groups of Swiss albino mice (6 in each group) were used. On day 0, 136×10^4 EAC cells per mouse were inoculated into each group. Treatment was started after 24 hours of tumor cell inoculation and continued for 10 days. Tumor growth was monitored by recording daily weight change and host survival was recorded and expressed as mean survival time in days and percent increase of life span was calculated by using the following formulae:

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

$$\text{Percent increase of life span (ILS) \%} = \left(\frac{\text{MST of treated group}}{\text{MST of control group}} - 1\right) \times 100$$

2.11. Bioassay of EAC cells surviving treatment with EuE

The procedure is very much alike to those used in our earlier works[8-11]. After 24 h of EAC cell inoculation, mice received either no drug treatment or *Eucalyptus* extract at dose 100 mg/kg (*i.p.*). On day 5, cells from each group (n=6) were harvested in cold (0.9%) saline, pooled, centrifuged and reinoculated (136×10^4 cells/mouse) into two groups of mice (n=6). On day 5 animals, from each group were sacrificed and viable tumor cells/ mouse counting were performed.

2.12. Hematological studies

The influence of EuE on the hematological parameters of EAC bearing and normal mice were carried out. Comparison was made amongst four groups (n=6) of mice on the 12th day after tumor transplantation. The four groups comprised group one, tumor bearing mice (without treatment), group two, tumor bearing mice treated with EuE extract (100 mg/kg/day, *i.p.* 10 days), group three and four were normal untreated and treated (100 mg/kg, *i.p.* 10 days) respectively. Blood was collected from each mouse by tail puncture and the total counts of white blood cell (WBC) and red blood cell (RBC) as well as hemoglobin (Hb) were determined by the standard method[12] using specific cell dilution fluids and hemocytometer.

2.13. Effect of EuE on normal peritoneal cells

Effect on normal peritoneal cells was observed with EuE by the procedure described earlier[13]. Two groups of normal mice (n=6) were used here. The first group of mice was treated with the extract at dose 25, 50, 100 mg/kg (*i.p.*) for three consecutive days. The second group was treated with vehicle and served as control. Total peritoneal exudates cells and number of macrophages were counted (with 1% neutral red) after 24 h of last treatment and compared with those of untreated control.

2.14. Brine shrimp lethality bioassay

Cytotoxicity of EuE was screened against *Artemia salina* in a 1-day *in vivo* assay according to published protocol[14]. A total of 3 mg of the extract 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[15].

2.15. Statistical analysis

The experimental results have been expressed as mean ±SEM. Data have been calculated by one way ANOVA followed by Dunnett '*t*' test using SPSS software of 10 version.

3. Results

Lethal dose (LD_{50} value) of EuE (1020 mg/kg) together with those of other plant extracts was found out and shown in Table 1. The data obtained for cell growth inhibition have also been furnished here.

Treatment with *C. curvibracteatus*, *A. indica*, *F. limonia* extracts resulted in significant cell growth inhibition at the doses 200 mg/kg (*i.p.*) and 100 mg/kg (*i.p.*), as evident from 84.97%, 75.99%, 71.72% and 72.75%, 61.70%, 63.47% reduction of tumor cells growth respectively. Treatment with EuE resulted in maximum cell growth inhibition of 96% at the dose 100 mg/kg (*i.p.*) which was 88% at dose 50 mg/kg. Treatment with bleomycin at dose 0.3 mg/kg showed cell growth inhibition by 88.00 % (Table 1).

Figure 1 shows the results obtained for the effect of EuE on life span of EAC bearing mice. The longevity of tumor bearing mice was found to be increased with the increasing doses. Obviously the highest result was obtained at dose 100 mg/kg (*i.p.*).

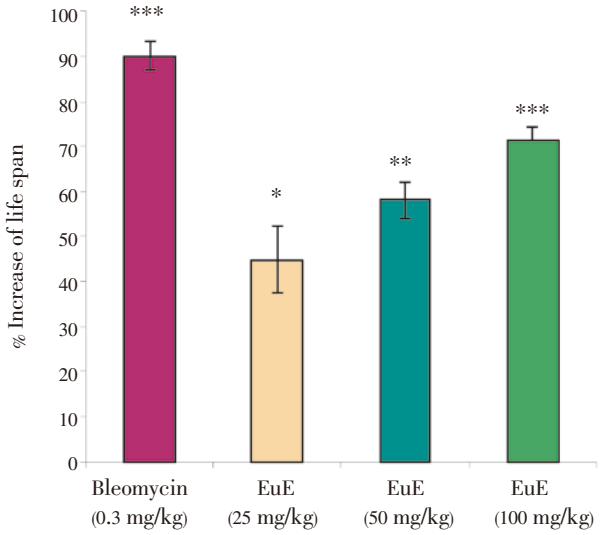

Figure 1. Percent increase of life span of EAC bearing mice treated with EuE and bleomycin.

136×10^4 EAC cells in every mouse were inoculated into each group of mice on day 0. Treatments were started after 24 hours of tumor inoculation and continued for five days. Mice were sacrificed on day six and the total intraperitoneal tumor cells were counted by a haemocytometer. Total numbers of viable cells in each animal of the treated groups were compared with those of control (EAC untreated) group.

The results were shown in mean ± SEM (n=6). *P<0.05,**P<0.01 and***P<0.001 when compared with control.

Effect of EuE at doses 25 mg/kg (*i.p.*), 50 mg/kg (*i.p.*), 100 mg/kg (*i.p.*) and bleomycin (0.3 mg/kg) on the tumor weight due to tumourgenesis is shown in Figure 2. Treatment of EuE previously inoculated with EAC cells, resulted in the inhibition of tumor growth. In the case of control (EAC bearing) group, the body weight was increased by 75.34% on day 20 when compared to the normal. Mice treated with the

Table 1

Effect of plant extracts on EAC cell growth inhibition (*in vivo*) (n=6) (mean ± SEM).

Treatment groups	Dose (mg/kg/day, *i.p.*)	No. of EAC cells in mouse on day 6 after tumour cell inoculation	% of cell growth inhibition	LD_{50} values (mg/kg)
C. curvibracteatus petroleum ether extract	50 mg/kg	$(3.25 \pm 0.85) \times 10^{7*}$	52.13	1040
	100 mg/kg	$(1.85 \pm 0.24) \times 10^{7**}$	72.75	
	200 mg/kg	$(1.02 \pm 0.21) \times 10^{7***}$	84.97	
A. indica petroleum ether extract	50 mg/kg	$(3.96 \pm 0.54) \times 10^{7}$	41.67	955
	100 mg/kg	$(2.60 \pm 0.18) \times 10^{7*}$	61.70	
	200 mg/kg	$(1.63 \pm 0.21) \times 10^{7**}$	75.99	
F. limonia petroleum ether extract	50 mg/kg	$(3.95 \pm 0.29) \times 10^{7}$	41.80	1150
	100 mg/kg	$(2.48 \pm 0.38) \times 10^{7**}$	63.47	
	200 mg/kg	$(1.92 \pm 0.56) \times 10^{7**}$	71.72	
E. camaldulensis petroleum ether extract	25 mg/kg	$(2.43 \pm 0.52) \times 10^{7*}$	63.15	1020
	50 mg/kg	$(0.89 \pm 0.12) \times 10^{7**}$	88.00	
	100 mg/kg	$(0.28 \pm 0.11) \times 10^{7***}$	96.00	
Standard bleomycin	0.3 mg/kg	$(0.81 \pm 0.36) \times 10^{7***}$	88.00	–
Control (EAC cell bearing mice)	–	$(6.79 \pm 0.53) \times 10^{7}$	–	–

*P<0.05, **P<0.01,***P<0.001, when compared with control.

Table 2

Effect of EuE on blood parameters of tumor bearing mice on day 12 of tumor inoculation (n=6) (mean ± SEM).

Treatment groups	RBC (cells/mL)	WBC (cells/mL)	% of Hb (gm/dL)
Normal mice	$(5.55 \pm 0.20)\times10^{9}$	$(10.70 \pm 1.40)\times10^{6}$	12.60 ± 0.40
Control mice (EAC bearing)	$(2.30 \pm 0.30)\times10^{9}$	$(26.80 \pm 0.60)\times10^{6}$	4.90 ± 0.75
EAC + EuE (100 mg/kg)	$(5.00 \pm 0.45)\times10^{9}$	$(15.00 \pm 0.44)\times10^{6}$	13.00 ± 0.12
Normal mice + EuE (100 mg/kg)	$(4.85 \pm 0.63)\times10^{9}$	$(6.23 \pm 0.13)\times10^{6}$	7.20 ± 0.82

Results were compared with normal (without EAC bearing mice) and control (EAC bearing mice).

Table 3

Effect of EuE on the enhancement of normal peritoneal cells in mice (n=6) (Mean ± SEM).

Treatment groups	Dose (mg/kg)	Macrophages	Total peritoneal cells
Control (Normal)	–	$(1.233 \pm 0.881)\times10^{6}$	$(5.50 \pm 1.15)\times10^{6}$
Normal + EuE	25	$(1.350 \pm 0.751)\times10^{6}$	$(6.45 \pm 1.81)\times10^{6}$
	50	$(1.670 \pm 0.854)\times10^{6}$	$(6.94 \pm 2.14)\times10^{6}$
	100	$(1.966 \pm 0.384)\times10^{6*}$	$(7.80 \pm 1.51)\times10^{6}$

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

Table 4

LC_{50} values after probit transformations of the mortality data of EuE and bleomycin.

Compounds	LC_{50} (μg/mL)	95% Confidense Limit (μg/mL) Lower–Upper	Regression equation	λ^2	df
EuE	20.59	14.39–29.46	Y=2.985+1.602X	1.548	3
Gallic acid	4.53	3.33–6.15	Y=3.93+1.62X	1.250	2

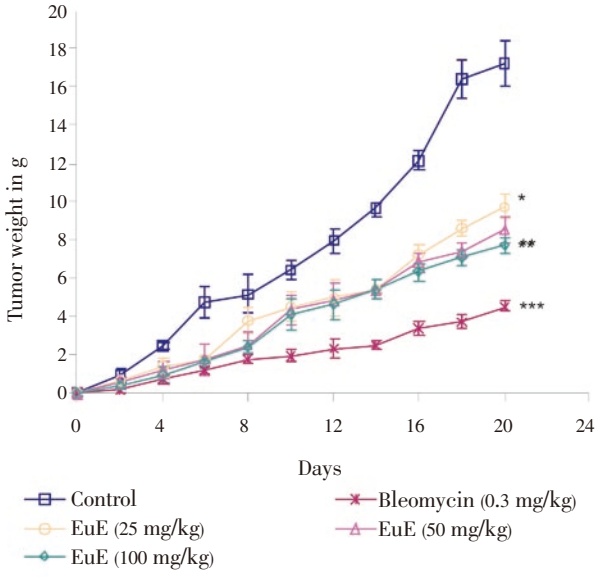

Figure 2. Tumor weight of EAC bearing mice treated with EuE and bleomycin.

136 ×10^4 EAC cells per mouse were inoculated in to each group of mice on day 0. Treatment was started after 24 hours of tumor cell inoculation and continued for 10 days. Tumor growth was monitored by recording daily weight change and host survival was recorded.

The results were shown in mean ± SEM (n=6). *P<0.05,**P<0.01, ***P<0.001 compared with the control.

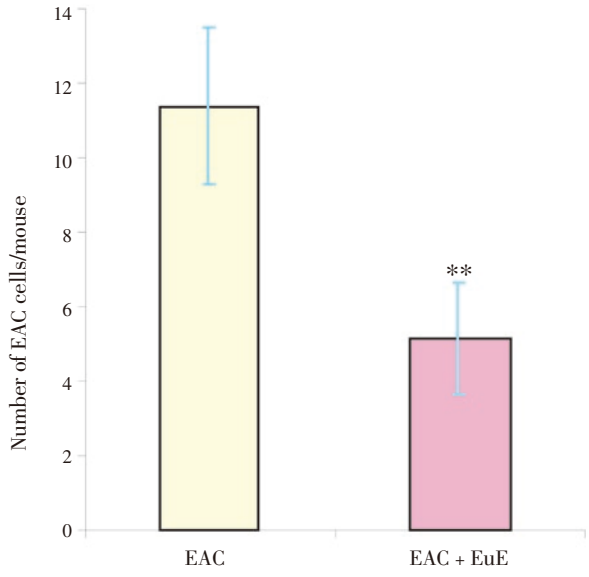

Figure 3. Effect of EuE on transplantability of EAC cells.

13.6×10^4 treated EAC cells were inoculated into mice on day 0. Mice were sacrificed on day 5 and cells were counted with haemocytometer. The results were shown as mean ± SEM (n=6). **P<0.01.

EuE at doses 25 mg/kg (*i.p.*), 50 mg/kg (*i.p.*), 100 mg/kg (*i.p.*) the body weight increased by 52.80%, 35.48%, 22.50% on day 20. In contrast the use of bleomycin as standard at the dose of 0.3 mg/kg (*i.p.*) the body weight was increased by 22% on day 20.

The effect of EuE on transplantability of tumor cells were observed by the reduction of intraperitoneal tumor burden in mice inoculated with treated EAC cells, with respect to control. 45% reduction of tumor burden was observed with EuE (Figure 3).

Hematological parameters were found to be altered from normal values along with the growth of tumor. Hemoglobin (Hb %) and RBC count were found to be decreased with the increase of WBC in the control mice. After treatment with

EuE at dose 100 mg/kg (*i.p.*), these parameters were found to be restored towards normal levels. Normal mice when treated with the EuE showed modest toxicity. Results are shown in Table 2.

The average number of peritoneal exudates cells per normal mouse were found to be $(5.5 \pm 1.5)\times 10^6$ of which the macrophage counts were $(1.233 \pm 0.881)\times 10^6$. Treatment with EuE for three consecutive days significantly enhanced the number of macrophages. Results are shown in Table 3. Finally the data for brine shrimp lethality bioassay were represented in Table 4.

4. Discussion

The work presented in this study was the screening of medicinal plant extracts as anticancer agents. Among the four extract varieties the *Eucalyptus* extract exhibited the highest activity. Results presented in this study showed that EuE (at different dose 25, 50 and 100 mg/kg) inhibited the growth of EAC cells significantly in mice in comparison with others. The order of potency (shown as percent of reduction ability) can be arranged as *Eucalyptus* (96%) >*Costus* (84%) > *Azadirachta* (75%) >*Feronia* (71%). The rest of the work has therefore been done with EuE only. EuE remarkably reduced the tumor growth rate and enhanced the life span of EAC bearing mice. The enhancement ability of life span of tumor bearing mice has been considered as a very important criterion of an anticancer drug. The bioassay experiment showed reduction of transplantability of EAC cells treated with EuE, indicating loss of viability of the treated cells.

To evaluate whether EuE indirectly inhibits tumor cells growth, effect of EuE treatment on the peritoneal exudates cells of normal mice was observed. In our experimental model each normal mouse contains about $(5.5\pm1.5)\times 10^6$ intraperitoneal cells, 20% of which are macrophages. Treatment with EuE enhanced the number of macrophages significantly. Enhancement and activation of macrophages might produce cytokine products such as tumor necrosis factor (TNF), interleukins (IL) etc inside the peritoneal cavity, which in turn might be responsible in killing of tumor cells[16].

Perturbations of hematological parameters in tumor bearing animals are partly responsible for the toxic effects produced in them. EuE treatment inhibited tumor cells growth, enhanced survival of treated mice and restored the haematological parameters. The high value of LC_{50} indicates the low toxic effect of EuE.

The observations described above show the efficacy of EuE with the dose mentioned, having little adverse side effect to the host. From these observations it can be concluded that the EuE may contain some compounds possessing potential antitumor activities. Further researches on cytotoxic activity, specific components of EuE against different cell lines, their structures, mechanism of action of bioactivity etc. are needed in order to elucidate novel anticancer drugs in future.

Conflict of interest statement

We declare that we have no conflict of interest.

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