

HEPATOPROTECTIVE EFFECTS OF ETHANOL EXTRACT OF ALPINIA CALCARATA ROSC (EEAC) ON EHRlich ASCITES CARCINOMA (EAC) INDUCED CARCINOGENESIS IN EXPERIMENTAL MICE

Farhadul Islam¹, Rasheda Perveen² and Tanzima Yeasmin^{1*}

¹Department of Biochemistry and Molecular Biology, University of Rajshahi, Rajshahi, Bangladesh

²Department of Microbiology, Gono University, Savar, Dhaka, Bangladesh

Abstract

A large number of plants belonging to the Zingiberaceae family are well known to possess strong anti-tumor properties. Thus the objective is to determine the protective effects and host toxic nature of ethanol extract of *Alpinia calcarata* Rosc (EEAC) Rhizome, on male Swiss albino mice. Here the gross general observation such as body weight changes, salivation, diarrhea, muscular numbness, biochemical parameters like serum GPT (glutamate pyruvate transaminase), GOT (glutamate oxaloacetate transaminase), ALP (alkaline phosphatase), serum glucose, cholesterol, urea, triglyceride and creatinine of normal and EAC bearing mice treated with the extract were studied. Histopathology of liver, kidney, lung, heart, spleen and brain were also investigated of both normal and EAC bearing mice. In normal mice there was a modest increase in all the above parameters during the treatment period (14 consecutive days at the 8.0 mg/kg/day). After treatment the enhanced values gradually decreased to normal levels. In case of EAC bearing mice, the toxic effects induced by EAC cells were found to be nullified by the treatment EEAC. No significant abnormalities in histology of the various organs of normal mice were detected owing to such treatment. Thus, the plant can therefore, be considered as a safe and probable new source of potent antitumor agents with significant hepatoprotective features.

Key words: Anticancer agent, Host toxicity, *Alpinia calcarata* Rosc, histopathology, subacute toxicity, Hepatoprotective Effects.

Introduction

Natural products play important role in cancer chemotherapy and some plants derived compounds such as paclitaxel, vincristine, podophyllotoxin, camptothecin are now available for use in a clinical setting [1-3]. Cancer is fatal diseases and one of the top three causes of death due to lack of effective treatment modalities. The available chemotherapeutic agents sold for the treatment of cancer are highly expensive and they also mutagenic, carcinogenic, teratogenic and causes anemia. This major side effect limits their applications[4]. So, to find out effective anticancer drug, research in this field going on all over the world and plants, vegetables, herbs used in the folk and traditional medicine have been considered as one of the main source of cancer chemoprevention for future[5]. The plant *Alpinia calcarata* Rosc. belongs to the family, Zingiberaceae popularly known as Sugondha Boss (Bengali) has a widespread occurrence in Bangladesh, 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[6-8]. Some novel compounds have DNA topoisomerase II poisoning activity and induce apoptosis[9]. Here the plant we selected is used in traditional medicine for the treatment of various ailments such as a warming digestive tonic, carminative, stomachic, expectorant, stimulant and antifungal agent[10]. 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. Rhizome of the plant also possesses several diterpenoids, some of which are cytotoxic, induce cell cycle arrest such as Calcaratan D, Calcaratan E [11-12]. *Alpinia calcarata* Rosc. shows potent antibacterial activity against some pathogenic gram positive and gram negative bacteria [13]. It also possesses antituberculin property [14]. Hot water and ethanolic extracts of *Alpinia*

Corresponding Author

Tanzima Yeasmin,

Professor, Department of Biochemistry and Molecular Biology, University of Rajshahi, Rajshahi-6205, Bangladesh

E-mail: yeasmin_bio@yahoo.com

calcarata rhizomes have much dose dependent antinociceptive activity [15]. However, their anticancer activity has not been investigated so far. From this viewpoint the anticancer activity of ethanol extract of *Alpinia calcarata* Rosc (EEAC) against Ehrlich ascites carcinoma (EAC) in Swiss albino mice has been carried out to evaluate its anti-tumor potency. EEAC exhibit significant anticancer property by inhibiting tumor cells proliferation, reducing tumor burden and also by enhancing survival of tumor bearing mice [16].

As to identify effective host friendly anticancer drugs from nature, we here report the host toxic and hepatoprotective effects of EEAC with the aim of determining whether the extract while functioning as antitumor agent can also exert any unacceptable toxic side effects to the host. For this purpose, different biochemical parameters as well as histopathological investigation of mice organs were studied which may help for further research to develop an effective chemotherapeutic agent.

Materials and Methods

Materials: The collected rhizome of *Alpinia calcarata* Rosc were shade dried and reduced to coarse powder. The dried powder was extracted with ethanol (yield 9.25 %) at room temperature for 10 days. The ethanol extract was then distilled, evaporated and dried in vacuum and stored in a vacuum desiccator for further use. The crude extract was dissolved in dimethyl sulfoxide (DMSO) for the experiments.

Chemicals and reagents: All the chemicals and reagents used throughout the investigation were of reagent grade and from BDH, England, E'MERK, Germany and Sigma Aldrich, USA.

Test animals: Adult male Swiss albino mice, six to eight weeks old (25 ± 5 gm body weight) were collected from animal resource branch of the International Centre for Diarrheal Disease Research, Bangladesh (ICDDR'B) and used throughout the studies. Animals were housed in polypropylene cages containing sterile paddy husk as bedding material under hygienic conditions with a maximum of ten animals in a cage. They were maintained under controlled conditions (12:12 h light-dark), temperature ($22 \pm 5^\circ\text{C}$). The mice were fed with standard mice food-pellets (collected from ICDDR'B) and water was given in ad libitum.

Cell lines: Ehrlich Ascites Carcinoma (EAC) cells were obtained by the courtesy of Indian Institute of Chemical Biology (IICB), Kolkata, India. The cells were maintained as Ascites tumour in swiss albino mice by intraperitoneal inoculation (bi-weekly) of 2×10^6 cells/mouse.

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.

Preparation of stock solution of the extract: 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.

Determination of median lethal dose (LD_{50}): An acute toxicity study relating to the determination of LD_{50} was performed [17]. The crude ethanol extract of *Alpinia calcarata* Rosc (EEAC) was dissolved in 10 % dimethyl sulfoxide (DMSO) and injected intraperitoneally (i.p) to eight 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 which was found to be 250 mg/kg (i.p.).

Grouping and administration of the sample: The effects of EEAC on both normal and EAC bearing mice were studied. For this purpose, four groups of mice each containing 24 animals were used. Group 1 containing normal mice treated with EEAC, group 2 EAC bearing mice treated with EEAC. Group 3 and 4 served as tumor control (EAC bearing without treatment) and normal control (normal mice without treatment) respectively. Group 1 (normal mice) were treated with EEAC at the dose 8.0 mg/kg/day (i.p.) for 14 consecutive days. For tumor bearing mice (group 2) similar treatment were done after 24 hours of EAC cell inoculation (2×10^6 cells/mouse).

Gross general observation after treatment: The mice were observed daily, very keenly to notify the general features such as behavior, central nervous system (CNS) excitation, CNS depression, food intake, salivation, diarrhea, muscular weakness and reflexes. The body weight of each of the mice of four groups was measured before administration of EEAC and at the completion of the treatment prior to sacrifice the animals. The weights of individual mice were compared.

Measurement of biochemical parameters: The parameters viz. serum GPT (glutamic pyruvic transaminase), GOT (glutamic oxaloacetic transaminase), ALP (alkaline phosphatases), serum glucose, cholesterol, urea, creatinine, triglyceride etc were determined for both normal and EAC bearing mice. For this experiment, on day 5, 10, 15 and 25, six (06) mice from each group were sacrificed. Blood was collected from heart in plastic centrifuge tubes. These were then allowed to clot at room temperature for half an hour and centrifuged at 4000 rpm for 15 minutes using a WIFUNG centrifuge LABOR-50M. The clear straw colored serum was then collected from the upper part of the tubes in vials with a Pasteur pipette. All the parameters were determined according to the procedures [18] established earlier.

Histopathology: The major body organs like brain, liver, kidney, heart, lung and spleen, were collected from the experimental animals (group 1 and 4) on 15th day and processed by standard methods [19] to prepare slides of tissues by hematoxylin and eosin staining. The slides were viewed under Motic advanced system microscope (B, series) with the help of Motic J. 1 software in a Macintosh computer. Drug induced hepatotoxicity, nephrotoxicity and spleen toxicity, neuro-toxicity, cardio-toxicity and lung toxicity were observed.

Statistical Analysis: The experimental results have been expressed as the mean \pm SEM (Standard Error of Mean). Data have been calculated by one way ANOVA followed by Dunnett's t test using SPSS software of 10 version.

Results

During the whole experimental period, general behavior, CNS excitation, CNS depression, reflexes, muscular weakness, salivation, diarrhea and food intake of all the mice were observed. The control group (group 4) and group 1 (EEAC treated) mice did not show any abnormalities and their food intake was also observed to be normal. No muscular numbness of the hind and fore legs, salivation or diarrhea was observed. But EAC bearing mice (group 3) showed some noticeable signs such as tremor, convulsion and reflex abnormalities, muscular numbness of the hind and fore legs, salivation or diarrhea due to the tumorigenesis while mice of group 2 (EAC bearing mice treated with EEAC) minimize such toxicities a great extent owing to antitumor activity of EEAC. Table 1 shows the average body weights of all the mice before and after the treatment and the data were compared. No significant changes in body weights of group 1 and 4 mice were observed. However, the body weights of EAC bearing mice (group 3) increase remarkably due to tumor growth which is also limited to the mice treated with EEAC (group 2). Almost 61 % body weight increment were found in EAC bearing mice on day 25 but EAC bearing mice receiving EEAC inhibit this increment significantly ($P < 0.001$) up to only 22 % and on day 15 body weight of EAC bearing mice having EEAC were obtained only 14 % ($P < 0.05$) but it were found to be 40 % in case of EAC bearing control mice. On the other hand body weight changes of normal mice treated with EEAC were found to be 12 % and this value is quite insignificant when compared to the control mice (7 % increment) on day 25.

Days of experiment	EEAC treated (Group 1)		Control (Group 4)		EAC +EEAC (Group 2)		Control EAC (group 3)	
	Body weight(gm) n = 6, Mean± SEM	% change	Body weight n = 6, Mean± SEM	% change	Body weight(gm) n = 6, Mean± SEM	% change	Body weight(gm) n = 6, Mean± SEM	% change
0	30.47±1.69		33.23±2.72		32.9±3.08		30.30±2.0	
5	31.9±1.44	4.69	32.95±3.08	0.84	33.2±3.41	2.06	33.5±3.2	10.56
10	33.8±1.66	10.92	33.12±3.41	0.33	35.1±3.37	6.68	37.5±2.2	23.76
15	33.05±2.01	8.46	34.08±3.21	2.55	37.65±3.93	14.43**	42.4±4.4	39.93
25	34.35±1.81	12.73	35.71±3.37	7.46	40.33±4.28	22.58***	48.7±5.6	60.72

Table 1. Effects of EEAC on body weight of experimental and normal mice

Treatment was continued for 14 consecutive days at dose 8.0 mg/kg (i.p.) (number of mice in each day = 6). For tumor bearing mice, similar treatment was started 24 hours of EAC cell transplantation (2×10^6 cells/mouse). Treatment was discontinued after 14 days from the start.

Results are shown as mean \pm SEM, where significant values are ** $P < 0.01$ and *** $P < 0.001$ when compared with control (EAC Bearing mice).

Effects of the extract on the enzyme activities (GPT, GOT and ALP) have been presented in figures 1-3. With normal mice, these activities were found to be moderately increased during the treatment period (14 consecutive days at dose 8.0 mg/kg/day i.p.) and after which these were found to gradually return to the more or less normal level. For EAC bearing untreated mice, all such values increased almost linearly with time while EAC bearing mice treated with EEAC however, diminished GPT, GOT and ALP values significantly. After treatment, the GPT values returned to normal levels with time (Fig. 1) while the ALP values remained almost the same with some backdrop (Fig. 2). In case of GOT, the test compound partially reduced the rate of its increment and reverse it back to near normal (Fig. 3).

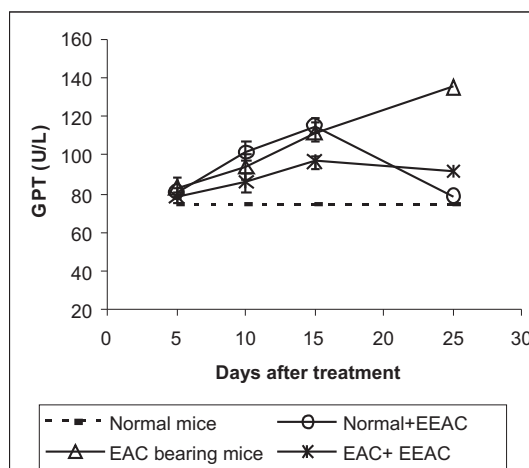


Figure 1. Effects of EEAC on serum GPT level in experimental mice

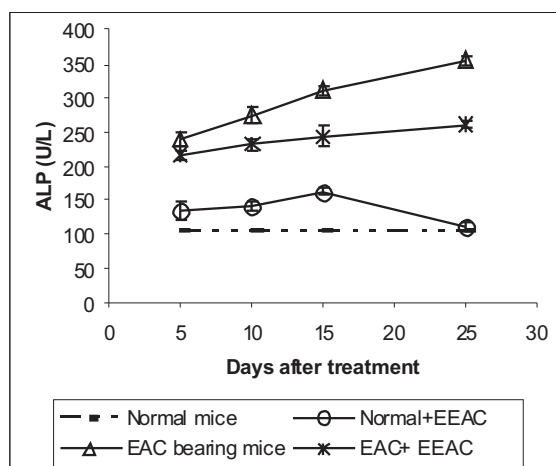


Figure 2. Effects of EEAC on serum ALP level in experimental mice

Treatment was continued for 14 consecutive days at dose 8.0 mg/kg (i.p). For tumor bearing mice, similar treatment was started after 24 hours of EAC cell transplantation (2×10^6 cells/mouse). Treatment was discontinued after 14 days from the start. EEAC=Ethanol Extract of *Alpinia calcarata* Rosc
EAC= Ehrlich Ascites Carcinoma GPT=Glutamic Pyruvic Transaminase Results are shown as mean \pm SEM.

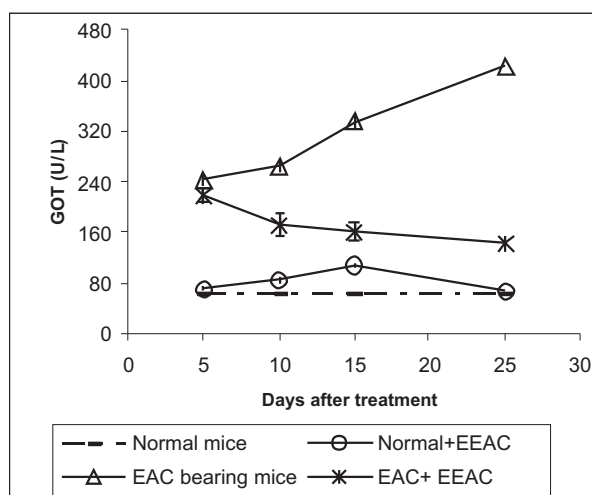


Figure 3. Effects of EEAC on serum GOT level in experimental mice

Treatment was continued for 14 consecutive days at dose 8.0 mg/kg (i.p). For tumor bearing mice, similar treatment was started after 24 hours of EAC cell transplantation (2×10^6 cells/mouse). Treatment was discontinued after 14 days from the start.

EEAC=Ethanol Extract of *Alpinia calcarata* Rosc

EAC= Ehrlich Ascites Carcinoma

GOT=glutamic oxaloacetic transaminase

Results are shown as mean \pm SEM.

Table 2 shows the effects of EEAC on serum glucose, cholesterol, urea, triglyceride and creatinine content of both normal and EAC bearing mice. All these parameters except glucose were increased in both normal and EAC bearing mice but they regain to its normal range in EEAC treated normal mice after the treatment period whereas these values return back to more or less normal level in EAC bearing mice treated with EEAC. The glucose content of normal mice was found to be increased up to 182.5 ± 7.2 on day 5 of treatment, after which it slowly reversed back towards normal and reached at 147 ± 7.5 on day 25 of initial treatment. On the other hand, in EAC bearing mice, the glucose content was found to be reduced abruptly with time and it was found to be only 54.3 ± 2.9 on day 25 of experiment. The treatment with EEAC increased the value close to normal (124.4 ± 3.7). Serum urea level of EAC bearing mice were found to be increased drastically with time and the value was 93 ± 3.1 on day 25 whereas in EAC bearing mice treated with EEAC it was found to be 36.2 ± 3.3 which was almost normal (37.2 ± 4.2). The level of serum triglyceride of EAC bearing mice having EEAC was increased only up to 148.2 ± 1.3 but the value of tumor bearing was much higher (231 ± 3.1) than the control mice (110 ± 2.5) on day 25. Furthermore, we also found that the creatinine level of EAC bearing mice was 3.2 ± 1.2 whereas the value reduced to 1.3 ± 1.0 in cancer bearing mice treated with EEAC.

Name of Exp.	Days	Serum glucose mg/dl blood	Serum cholesterol mg/dl blood	Serum urea mg/dl blood	Serum triglyceride mg/dl blood	Serum creatinine mg/dl
Normal mice (Group 4)	0	152.3 ± 1.3	142.7 ± 3.5		110 ± 2.5	0.26 ± 0.21
Normal + EEAC (Group 1)	5	182.5 ± 7.2	159.9 ± 5.3	24 ± 4.2	122.4 ± 3.2	0.32 ± 0.1
	10	179.2 ± 8.4	212 ± 5.2	27 ± 2.6	142.6 ± 6.1	0.36 ± 0.15
	15	177.7 ± 9.6	$242 \pm 4.3^*$	29.4 ± 3.5	$148.4 \pm 4.3^*$	0.42 ± 0.2
	25	147 ± 7.5	152.3 ± 2.4	32 ± 2.4	102 ± 4.5	0.25 ± 0.1
EAC control (group 3)	5	$87 \pm 2.3^*$	152 ± 3.7	$64.1 \pm 1.8^*$	188.6 ± 6.2	0.4 ± 0.2
	10	$73 \pm 3.7^{**}$	156.7 ± 2.6	$77 \pm 0.8^*$	192 ± 3.1	1.1 ± 0.31
	15	$65 \pm 2.4^{**}$	165 ± 3.7	$84.1 \pm 1.2^{**}$	$205 \pm 5.2^{**}$	2.7 ± 1.1
	25	$54.3 \pm 2.9^{**}$	$183 \pm 3.8^{**}$	$93 \pm 3.1^{***}$	$231 \pm 3.1^{***}$	$3.2 \pm 1.2^{**}$
EAC+ EEAC (group 2)	5	92.5 ± 2.5	152 ± 8.3	45.3 ± 4.3	158 ± 2.2	0.81 ± 0.4
	10	96.2 ± 7.1	162.2 ± 7.6	49.2 ± 1.2	172.1 ± 8.2	0.92 ± 0.2
	15	120 ± 2.6	168 ± 4.5	45.2 ± 2.5	178 ± 1.2	1.0 ± 0.5
	25	124.4 ± 3.7	172 ± 8.1	36.2 ± 3.3	148.2 ± 1.3	1.3 ± 1.0

Table 2. Effect of EEAC on biochemical parameters in experimental mice

Treatment was continued for 14 consecutive days at dose 8.0 mg/kg (i.p.) (number of mice in each day = 6). For tumor bearing mice, similar treatment was started 24 hours of EAC cell transplantation (2×10^6 cells/mouse). Treatment was discontinued after 14 days from the start. Results are shown as mean \pm SEM, where significant values are * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ when compared with normal mice).

Histology of liver, kidney, heart, lung, spleen and brain were performed to observe any changes in the cellular structures (infiltration, inflammation, congestion, degradation and regeneration etc) of the mice receiving the test compound at a dose of 8.0 mg/kg/day for 14 consecutive days with respect to control group (normal mice).

In mice of the treated group, no abnormalities in the histopathology of kidney, heart, spleen, lung and brain were detected in comparisons with control group under microscope. Liver tissues showed very little infiltration with no central vein dilation, fatty generation or nodule formation in normal mice whereas major organs of EAC bearing mice showed significant cellular degeneration/regeneration. The hepatotoxicity generated by EAC cells were partially nullified by the protective effects of EEAC treatment (Fig. 4).

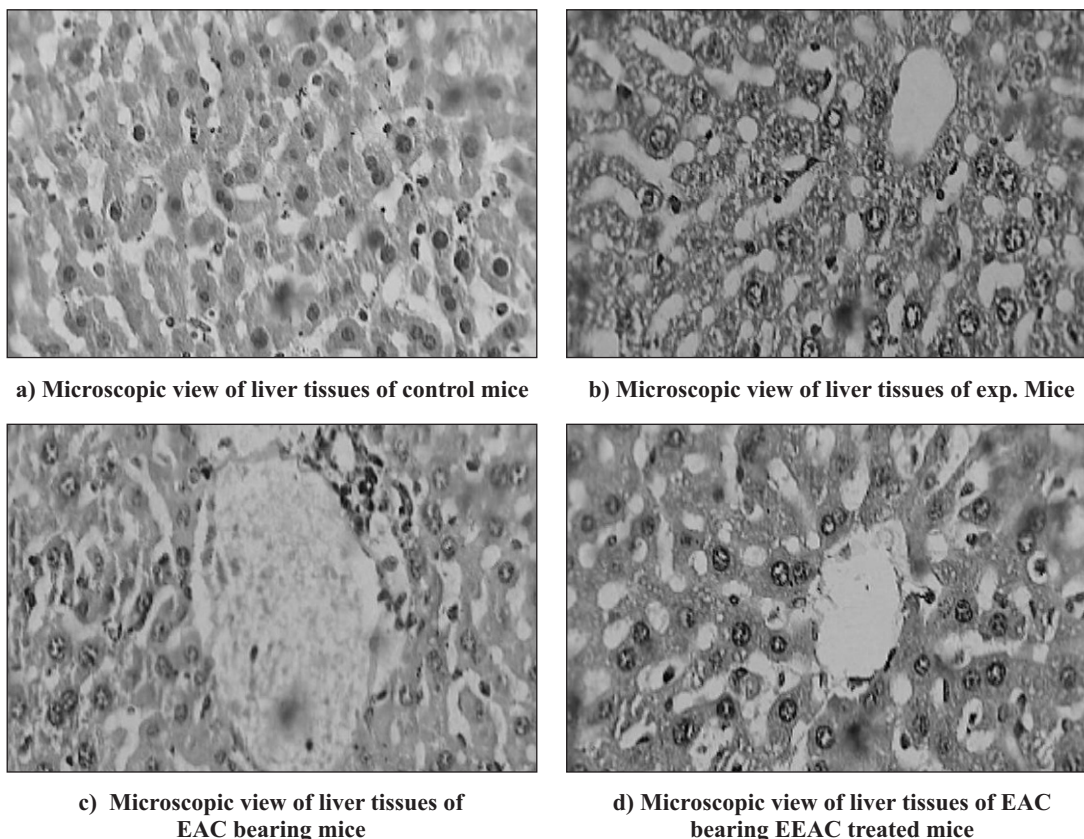


Figure 4. Histopathological examinations of experimental mice.

Treatment was continued for 14 consecutive days at dose 8 mg/kg (i.p). Treatment was discontinued after 14 days from the start. a) Liver tissues from control mice with no abnormality. b) Liver tissue from EEAC treated normal mice shown very little infiltration with no central vein dilation, fatty generation or nodule formation. c) Liver tissues from untreated EAC bearing mice with necrosis, central vein dilation and d) Liver tissues from EAC bearing EEAC treated mice with very little necrosis and no central vein dilation.

Discussion

All the results mentioned before showed that the crude extract EEAC at dose 8.0 mg/kg/day for consecutive 14 days on Swiss albino mice have no significant abnormalities in comparison to control. Normal mice receiving EEAC did not show any tremor, convulsion, reflex abnormalities or muscular disorder. It shows only insignificant changes of body weight due to normal growth and development of the animals. Whereas, the extract all-most nullified the physiological abnormalities (tremor, salivation, diarrhea, muscular problem etc) of EAC bearing mice, which is produced owing to the toxicities of carcinogenesis. As we reported earlier [16],

the tumor burden of EAC bearing mice is remarkably reduced by EEAC which is ratifying here as we find very limited body weight gain of EAC bearing mice treated with EEAC (Table 1).

Literature survey [20] reveals that progression of tumor was accompanied by the following hematological changes compared to normal gradual decrease in hemoglobin content, erythrocyte count and gradual increase in leukocytes which was observed in control mice. The RBC count was almost restored back to normal range on treatment with EEAC which is described earlier [16]. It could also improve the WBC count efficiently. The hemoglobin levels were in the normal range in the EEAC treated group. Recovery of the hemoglobin content, RBC and WBC cells count in the experimental mice indicates the protective action of EEAC on the hemopoietic system.

It is well known that there are significant elevations in the levels of serum GPT, GOT and ALP in liver diseases and disorder in hepatocellular damage caused by a number of agents. An increase in these enzyme levels is also observed in patients with cardiac damage due to myocardial infarction and with liver disorders [21].

Biochemical measurements of these parameters in normal mice treated with EEAC showed some extent of increase due to mild hepatotoxicity during treatment period but they become normal after completion of treatment schedule. The slight host toxic effects observed in mice during treatment time are mostly reversible. This means that, the treatments of the extract do not cause any acute or permanent damage to the liver. But in case of tumor bearing mice, these parameters were found to be increase more drastically with time due to the acute and permanent toxicities induced by EAC cells on host. After treatment with EEAC in the EAC bearing mice these values remain near to the normal range in the treated group (fig 1-3). From this it follows that the damage generated by EAC was prevented by EEAC supplementation.

Treatment of normal mice with EEAC slightly changed blood glucose, cholesterol, urea, triglyceride and creatinine level which also rectified more or less to normal after treatment (Table 2). This indicates that after short term treatment the extract did not cause any extreme abnormality at the dose used in this study.

The development of hypoglycaemia and hyperlipidaemia in experimental animals with carcinoma has been previously reported [22]. In this experiment, the reduced glucose level and elevated cholesterol, triglycerides and serum urea were returned to more or less normal levels in EEAC-treated mice, thereby indicating a potent antitumour efficacy of EEAC (Table 2). And the anticancer activity presented here is only for the active ingredients present in it and not for the solvent [23] DMSO.

The histopathology studies of major organs also revealed the relatively less toxic nature of EEAC as compared to control group when viewed under microscope. The histopathology of kidney tissues of EEAC treated mice did not show any cellular and glomerular infiltration and there is no sign of tubular necrosis, casts and glomerular congestion. Tissues from brain and lung did not shown any cellular degeneration or regeneration in the treated mice and this is why they have no signs of neurotoxicity and pulmonary toxicity. Treated mice also have not any change in the splenic architecture. The histology of liver showed very little infiltration (inflammation) with no central vein dilation, fatty generation or nodule formation and due to this mild hepatotoxicity some biochemical parameters were deteriorate during treatment period which become normal after closing treatment whereas tissues from EAC bearing mice showed major abnormalities and it is interesting that the hepatic damage induced by EAC cells were nullified by EEAC supplementation (fig 4).

All these slight host toxic effects observed in mice during treatment time are mostly reversible and so treatment with EEAC do not cause any acute or permanent damage to the host.

Conclusion

The aim of this study was to determine the hepatoprotective and sub-acute toxicity of the extract to find out less host toxic potential anticancer agents and did not attempt to identify the specific mechanism involved. Almost in all cases the toxic effects of EAC cells on biomolecules have been found to be nullified by such treatment. In most cases antagonistic effects have been found instead of additive effects. Further elevation of glucose levels of EAC bearing mice by the treatment of the extract probably indicates their partial recovery from tumor growth.

As the major organs of the treated mice do not show any histopathological abnormalities, these findings in conjunction with those obtained from the measurement of hematology and serum biomolecules definitely give

positive support to conclude that EEAC is an effective antineoplastic agent with comparatively less toxic effects in our experimental model. However, further chronic toxicological studies and its anti-tumor activity should be carried out against other tumor cell lines which may bring promising results in cancer chemotherapy.

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