ANTICANCER ACTIVITY OF METHANOL EXTRACT OF *IPOMOEA REPTANS* IN EAC BEARING MICE

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Summary

Cancer is one of the most hazardous and fatal diseases in the world. Scientists from the different countries worldwide are actively involved in control of this disease. Increasing interest and research on herbal medicine have revealed its importance in treating many diseases including cancer. The present study was carried out to evaluate the antitumor activity of methanol extract of *Ipomoea reptans* (MEIR) on Ehrlich's Ascites Carcinoma (EAC) model in mice. After inoculation of EAC cells into mice, treatment with MEIR (400 mg/kg b.wt) and standard drug 5-Fluorouracil were continued for 9 days. Evaluation of the effect of drug treatment was made by the study of tumor growth response including increase in life span, and study of hematological parameters, biochemical estimations, antioxidant assay of liver tissue and *in vitro* cytotoxicity. Experimental results revealed that *I. reptans* possesses significant cytotoxicity and further studies may establish it to be a potent antitumor agent.

Key words: *Ipomoea reptans*, EAC, Hematological studies, Biochemical estimations, Antioxidant assay, *in vitro* cytotoxicity.

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Introduction

Cancer is a leading cause of mortality worldwide. It is a disease of cells. A cancerous growth or tumor is an abnormal growth of cells which tend to proliferate in an uncontrolled way and in some cases, to metastasize. Despite of many therapeutic advances in the understanding of the etiology of carcinogenesis, overall mortality statistics are unlikely to get changed and hence there is a need of reorientation of the concepts for the use of natural products as new chemopreventive agents. Natural or semi synthetic compounds may be used to block or prevent the development of invasive cancers. Cellular carcinogenesis forms the biological basis for the identification of preventive products, the assessment of their activity and ultimately the success or failure of a therapy.

The Ehrlich ascites tumor is used as a transplantable tumor model to investigate the antineoplastic effect of the compounds. Following intraperitoneal inoculation of Ehrlich tumor cells, the ascitic volume and number of tumor cells increase progressively [1]. Ascitis is probably formed in consequence of tumor-induced inflammation, due to the increase in peritoneal vascular permeability [2]. Mice bearing the ascitic tumor die after a short period of time due to several factors: mechanical pressure exerted by the progressive increase of ascitic fluid, intraperitoneal hemorrhage and endotoxemia [3].

Traditional medicine and ethnobotanical information often play an important role in scientific research. For anticancer activities several plant products have been tested and some of them are now available as the drugs of choice. The vast natural resources of India can provide effective anticancer agents. One of the best approaches in this regard is the selection of plants based on traditional uses and testing the selected plants' efficacy and safety on the basis of modern science.

Ipomoea reptans (Linn) Poir belongs to the family Convolvulaceae. The aerial part of this plant is an edible, green leafy vegetable, available in all over India. Traditionally the aerial part of this plant has been found to be used in various pathological conditions. The present study was carried out to evaluate the antitumor activity of *Ipomoea reptans* aerial parts on EAC model in mice.

Materials and Methods

Plant material

The aerial parts of *I. reptans* was collected in March 2007, from Khardah, West Bengal, India and identified by the Botanical Survey of India, Howrah, India. A voucher specimen (PIR- 1) was retained in our laboratory for further reference.

Preparation of plant extract

The aerial parts were dried and powdered in a mechanical grinder. The powdered material was extracted by methanol using soxhlet apparatus. This extract was filtered and concentrated in *vacuo* and kept in a vacuum desiccator for complete removal of solvent. The yield was 17.25% w/w with respect to dried powder. Preliminary qualitative analysis showed the presence of polyphenols,

flavonoids and saponins in the methanol extract. Aqueous suspension of MEIR was prepared using 2 % (v/v) Tween-80 and used for the treatment.

Animals

Swiss albino male mice (20 ± 2 g) were used for the study. The animals were kept in polypropylene cages with sawdust bedding and maintained under standard laboratory conditions. Standard pellet diet (Hindustan Lever, Kolkata, India) and water were given *ad libitum*. The mice were acclimatized to laboratory condition for one week before commencement of experiment.

Tumor cells

Ehrlich ascites carcinoma (EAC) cells were obtained from Chittaranjan National Cancer Institute (CNCI), Kolkata, India. The EAC cells were maintained in Swiss albino mice, by intraperitoneal (i.p.) transplantation on every 9th days [4]. The ascitic fluid was collected by syringe and the tumor cell count was performed in a Neabauer hemocytometer and 2×10^7 cells/ml were obtained by dilution with normal saline [5]. Tumor cell suspension showing more than 90 % viability (checked by trypan blue dye (0.4%) exclusion assay) was used for transplantation.

Treatment Schedule

The mice were weighed and divided into four groups (n=12). EAC cells (2×10^6 cells/ mouse) were injected i.p. to each mouse of each group except normal saline group. This was taken as Day 0. Extract and reference drug treatment were continued for subsequent 9 days starting from Day 1. On 10^{th} day, 24 h after the last dose six mice were sacrificed from each group and the rest were kept for the life span study of the tumor hosts. After sacrificing the animals, blood was collected to evaluate the hematological and biochemical parameters. Liver tissue was collected from the animals for the evaluation of *in vivo* antioxidant status.

The groups and the design of the experiment were as follows:

Group I : Normal saline (5ml (0.9% w/v)/kg b.wt, i.p.)
Group II : EAC ($2x10^6$ cells/mice) + Normal saline (5ml/kg b.wt, i.p.)
Group III : EAC ($2x10^6$ cells/mice) + MEIR (400mg/kg b.wt, i.p.)
Group IV : EAC ($2x10^6$ cells/mice) + 5-fluorouracil (20mg/kg b.wt, i.p.)

Tumor growth response

The effect of MEIR on tumor growth and host's survival time were examined by studying the following parameters such as tumor volume, tumor cell count, viable tumor cell count, nonviable tumor cell count, median survival time and increase in lifespan.

Tumor volume

The mice were dissected and the ascitic fluid was collected from the peritoneal cavity. The volume was measured by taking it in a graduated centrifuge tube.

Tumor cell count

The ascitic fluid was taken in a WBC pipette and diluted 100 times. Then a drop of the diluted cell suspension was placed on the Neubauer counting chamber and the numbers of cells in the 64 small squares were counted.

Viable and nonviable tumor cell count

The cells were then stained with trypan blue (0.4% in normal saline) dye. The cells that did not take up the dye were viable and those that took the stain were nonviable. These viable and nonviable cells were counted.

Percentage increase in life span

The effect of MEIR on tumor growth was monitored by recording the mortality daily for 6 weeks and percentage increase in life span (%IMST) was calculated. An enhancement of life span by 25% or more was considered as effective antitumour response [6, 7].

IMST (%) = [(Median survival of treated group/Median survival of control group)-1] x100
Median Survival Time (MST) = [1^{st} Death + Last Death]/ 2

Hematological studies

RBC, WBC counts and estimation of hemoglobin was done by standard procedures from the blood obtained intracardially [8, 9].

Hemoglobin estimation

0.1ml of heparinized blood was taken in Sahli's Hemoglobinometer and diluted with 0.1N HCl until the color matched with standard. The reading was then taken from the graduated cylinder and expressed as g/100ml of blood.

Counting of erythrocytes

The blood sample was diluted (1:2000) with the diluting fluid using Thoma pipette. After vigorous mixing, a drop of resultant mixture was discharged under the cover glass of Neubauer hemocytometer and the corpuscles were allowed to settle for 3 minutes. The number of erythrocytes in 80 small squares was counted under light microscope. The number of cells in 1 cumm of undiluted blood was calculated.

Total count of leukocytes

Blood was diluted 1:20 with a diluting fluid. The Neubauer hemocytometers were filled with the mixture and the number of cells in four corner blocks (each block subdivided into 16 squares) was determined and the total leukocyte count per cumm of blood was calculated.

Biochemical estimation

The changes in the biochemical parameters of EAC bearing mice due to the treatment of MEIR were evaluated. Serum biochemical enzymes such as serum glutamic oxaloacetic (SGOT) and glutamic pyruvic transaminase (SGPT) activities were estimated by the method of Reitman and Frankel [10].

***In vivo* antioxidant assay**

The antioxidant assay was performed with liver tissue and evaluation was carried out by measuring the level of lipid peroxidation [11] and the amount of enzymatic (CAT) and nonenzymatic antioxidant system (GSH) by the methods of Luck [12] and Ellman [13] respectively.

Assay for *in vitro* cytotoxicity

The *in vitro* short term cytotoxicity of MEIR was assayed using EAC cell lines. Briefly $1x10^6$ viable cells of cell line suspended in 0.1 ml of phosphate buffered saline (PBS) (0.2 M, pH 7.4) with various concentrations of extracts (100-500 µg/ml) and phosphate buffer in a final volume of 1 ml were incubated at 37°C for 30 minutes. Cell suspension in phosphate buffer without extract served as control. After the incubation, the viability of the cells was determined using trypan blue by the method of Boyse *et al*. [14]. The percentage of cytotoxicity was determined by calculating % inhibition and IC_{50} values.

Statistical Analysis

Values were presented as mean ± S.E.M. Data were statistically evaluated by one-way analysis of variance (ANOVA) followed by post hoc Dunnett's test using SPSS software. $P< 0.05$ was considered as statistically significant.

Results

Treatment with MEIR significantly reduced tumor volume and viable cell count compared to those of EAC control mice, while nonviable cell count was found to be significantly high in the treated groups. These results were summarized in Table.1. The effect of MEIR on the survival time of EAC bearing mice was shown in Table.1, which implies that MEIR can significantly increase the survival time with respect to the EAC control mice.

Fig.1 shows the effect of MEIR on hematological profile in EAC treated mice. All the hematological systems were altered in EAC bearing mice from the normal values. Hemoglobin

content and RBC count significantly decreased and the total WBC count increased in EAC control group mice, whereas MEIR treatment significantly increased the hemoglobin content and RBC count and reduced WBC count as those of the reference drug treated group.

Biochemical estimation as shown in Fig.2 indicates the elevated level of liver functional enzymes in serum in EAC treated group with respect to normal animals, while these were significantly reduced to near normal value in the drug treated groups.

As shown in Table.2, level of lipid peroxide in EAC bearing mice was significantly high with respect to normal control group, while it was found to be reduced significantly in the extract as well as reference drug treated groups. Both of the enzymatic (CAT) and nonenzymatic (GSH) antioxidant systems of EAC control groups were reduced compared to normal animals, while these levels were found to be restored to near normal in the treated groups, as found in Table.2.

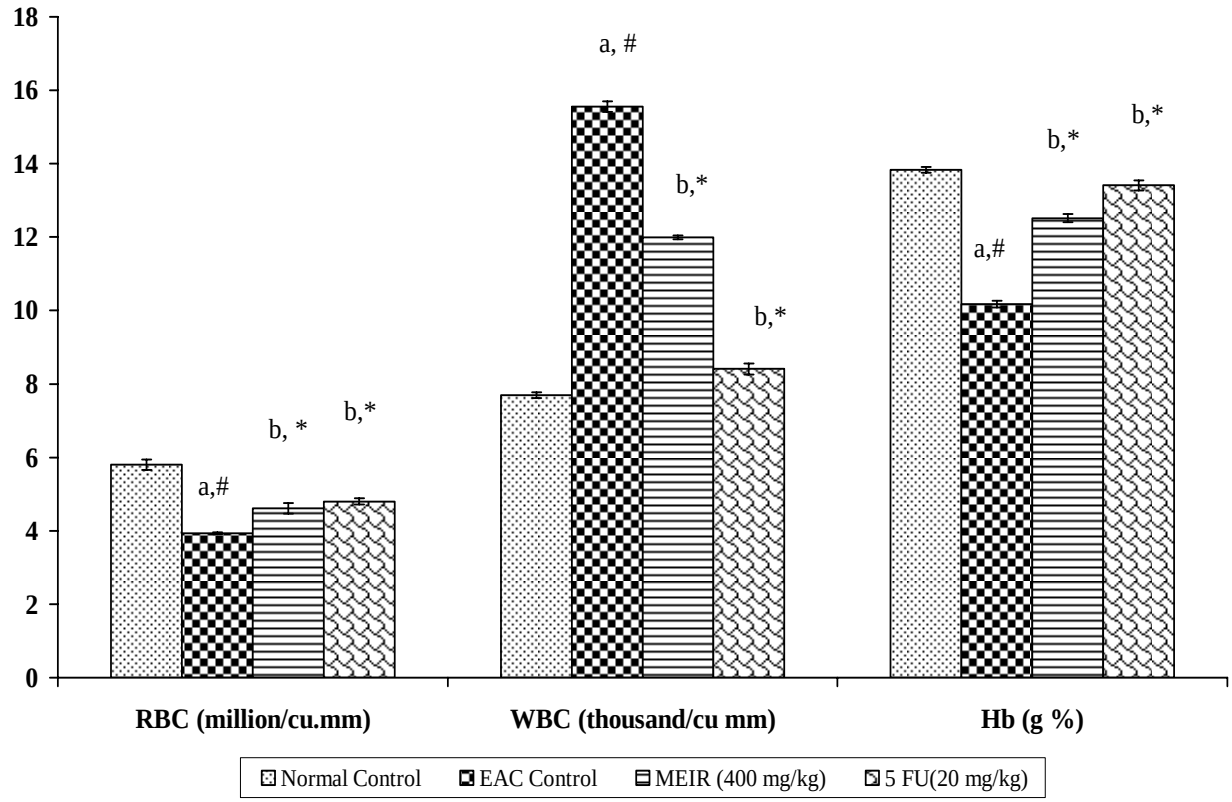
In the *in vitro* cytotoxicity study, MEIR showed direct toxicity on the EAC cell line, in a concentration dependent manner in the dose range of 100-500 µg/ml, as was revealed in Fig.3 and the IC_{50} value was found to be 281.15 µg/ml.

Table.1. Effect of Methanol extract of *I.reptans* (MEIR) on Tumor volume, Viable and nonviable cell count, and MST (Median survival time) of EAC bearing mice

Groups	Tumor Volume (ml)	Viable cell count (%)	Nonviable cell count (%)	MST (days)	% IMST
EAC Control	3.85±0.18	85.61±2.08	14.39±2.08	17	0
MEIR (400 mg/kg)	1.72±0.13*	44.12±1.34*	55.88±1.34*	29.5	73.53
5Fluorouracil (20 mg/kg)	0.74±0.09*	36.32±1.28*	63.68±1.28*	34	100

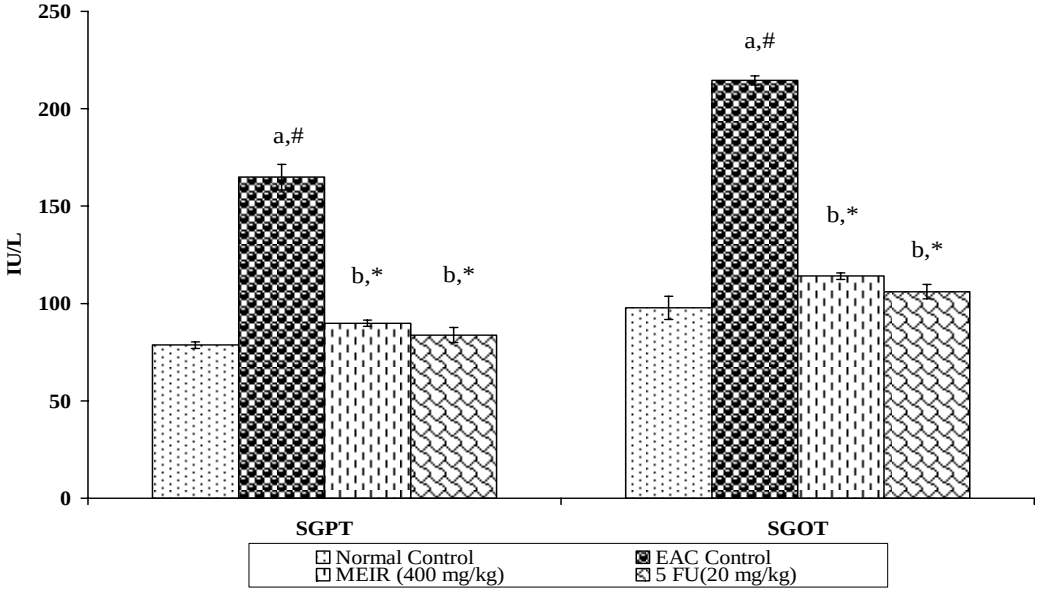
% IMST = Percentage increase in MST
Values are Mean±S.E.M.; n=6 in each group. Drug treatment was done for 9 days.
* $p < 0.05$, when compared to EAC Control; where the significance was performed by Oneway ANOVA followed by post hoc Dunnett's test.

Fig.1. Effect of Methanol extract of *I.reptans* (MEIR) on hematological parameters of EAC bearing mice



Values are Mean±S.E.M.; n=6 in each group. Drug treatment was done for 9 days.
[a] EAC control group *vs* normal control group, [#] $p < 0.05$;
[b] Treated group *vs* EAC control group, [*] $p < 0.05$; where the significance was performed by Oneway ANOVA followed by post hoc Dunnett's test.

Fig.2. Effect of Methanol extract of *I.reptans* (MEIR) on biochemical parameters of EAC bearing mice



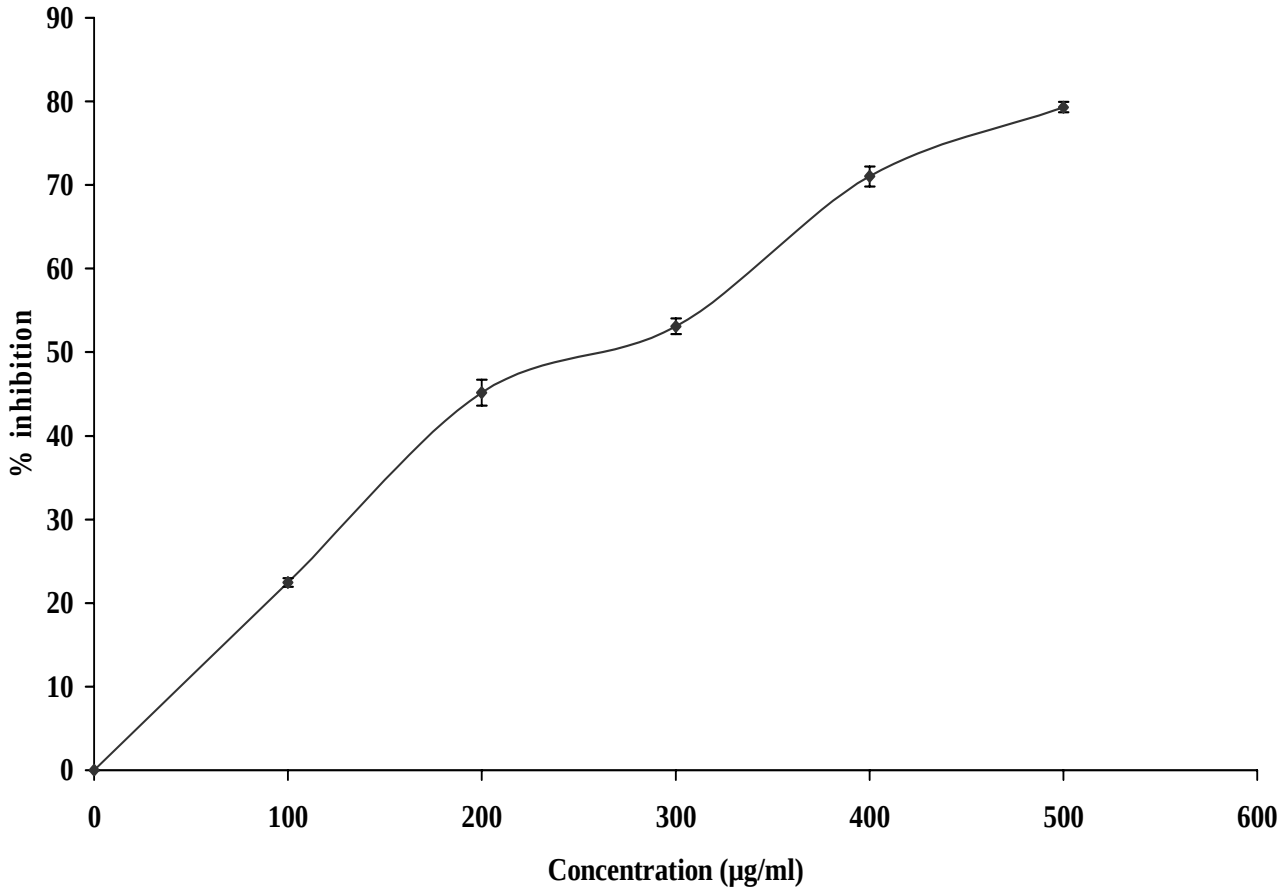
Values are Mean±S.E.M.; n=6 in each group. Drug treatment was done for 9 days.
[a] EAC control group *vs* normal control group, [#] $p < 0.05$;
[b] Treated group *vs* EAC control group, [*] $p < 0.05$; where the significance was performed by Oneway ANOVA followed by post hoc Dunnett's test.

Table.2. Effect of Methanol extract of *I.reptans* (MEIR) on antioxidant status of EAC bearing mice

Groups	LPO (nM/mg wet tissue)	GSH (μg/mg wet tissue)	CAT (μM of H_2O_2 decomposed/min/mg wet tissue)
Normal Control	255.61+4.19	34.92+0.64	0.729+0.08
EAC Control	642.70+7.10[a,#]	10.16+0.92[a,#]	0.327+0.04[a,#]
MEIR (400 mg/kg)	328.14+5.47[b,*]	29.92+1.72[b,*]	0.653+0.02[b,*]
5-Fluorouracil (20 mg/kg)	305.32+2.53[b,*]	28.74+1.03[b,*]	0.627+0.03[b,*]

LPO: Lipidperoxide; GSH: Reduced Glutathione; CAT: Catalase.
Values are Mean±S.E.M.; n=6 in each group. Drug treatment was done for 9 days.
[a] EAC control group *vs* normal control group, [#] $p < 0.05$;
[b] Treated groups *vs* EAC control group, [*] $p< 0.05$; where the significance was performed by Oneway ANOVA followed by post hoc Dunnett's test.

Fig.3. Effect of Methanol extract of *I.reptans* (MEIR) on *in vitro* EAC cell lines



Values are Mean ± S.E.M.; where n=3.

Discussion

The present study was carried out to evaluate the antitumor effect and antioxidant property of MEIR against EAC bearing mice. The tumor volume was significantly higher in EAC bearing control group. The Ehrlich ascitic tumor inoculation induced a local inflammatory action, with increasing vascular permeability, which leads to an intense oedema formation, cellular migration and a progressive ascitic fluid formation [15]. Moreover the ascitic fluid is very much essential for growth of tumor cells since this fluid is direct nutrition source for tumor cells [16].

In the MEIR treated group, the tumor volume was significantly reduced compared to EAC control group. The reduction of tumor volume probably by reducing the ascitic nutritional fluid volume and vascular permeability.

The number of viable cells decreased and nonviable cells were significantly increased in EAC treated mice by MEIR compared to EAC control group. This may be due to the direct contact of the active constituent of the extract with the tumor cell in peritoneal cavity and therefore effectively arrested the growth and multiplication of the tumor cells.

One of the most reliable criteria for judging the value of any anticancer agent is the prolongation of life span of animals. A decrease in tumor volume and viable tumor cell count as mentioned above finally reduced the tumor burden and enhanced the life span of EAC bearing mice. There are number of reports that many plant extracts having anticancer activity were found to inhibit the development of ascitic tumor growth and prolongation of the life span of tumor bearing mice [17].

A literature survey [18] reveals that tumorigenesis and its progression have been accompanied by the following changes compared with normal cytogenesis (1) gradual decrease in hemoglobin content, erythrocyte count and bone marrow cellularity, (2) gradual increase in leukocytes, thrombocytes and splenic cellularity, and (3) reversal of the lymphoid-myeloid ratio in the differential WBC count. As found in the present study, it was observed that there was significant decrease in the hemoglobin level and erythrocyte count in EAC control group; however this reduction was effectively improved by treatment with the extract. The leukocytes were drastically increased in the tumor bearing mice and were significantly reduced towards normal level with the treatment of MEIR. Thus, the results indicate that MEIR do not adversely affect haematopoiesis rather improve it, and hence the common side effect anemia, associated with the most of the anticancer drug therapy is not present in the present case.

Significant elevation in the levels of SGOT, SGPT reflects the hepatocellular damages caused by a number of agents. Biochemical measurements of these parameters showed that to some extent hepatotoxicity was associated after 9 days of inoculation with EAC. Treatment with the MEIR restored the elevated biochemical parameters more or less to normal range, indicating the protection of the tumor cell induced hepatotoxicity by MEIR.

Oxidative stress occurs when there is an imbalance between the free radical production and the endogenous antioxidant defense and leads to various diseases including cancer. Number of studies have indicated that tumor growth can cause antioxidant disturbance in certain tissues of the tumor host [19].

Lipid Peroxidation (LPO) is an important process in cellular damage, which is mediated through the free radical metabolites, affecting the antioxidant system of the cells [20]. In EAC bearing mice the level of lipid peroxide in liver was significantly elevated after 9 days of tumor inoculation, which was however reduced to near normal level in the MEIR treated group animals.

GSH is the major cellular sulfhydral compound that serves as an effective reductant by interacting with numerous electrophilic and oxidizing compounds. It can act as a nonenzymatic antioxidant by direct interaction of SH group with ROS or it can be involved in the enzymatic detoxification reaction for ROS, as a cofactor or coenzyme [21]. The depletion of glutathione in cancer bearing mice may be due to its utilization by the excessive amount of free radicals. Treatment with MEIR increased GSH content in the liver as compared to EAC control animals.

CAT is a hem containing enzymes and plays pivotal role in the oxidative stress. In EAC control group the level of CAT was significantly reduced when compared to normal group. This may be attributed to its inactivation by superoxide radical through converting it to the ferroxy and ferryl states of the enzyme [22]. Iron has an important role in catalase activity. There are number of reports of association between the increase body iron during tumor growth. Mono [23] reported that serum iron level was increased with decreased total iron binding capacity and transferrin level in tumor bearing animal. The total iron binding capacity and transferrin are responsible for the formation of catalase [24]. By the treatment with MEIR, CAT level was increased which may be due to the scavenge of the superoxide radical, or through the reduction of the conversion of its ferroxy and ferryl states or increase the total iron binding capacity and transferrin.

Cytotoxicity is one of the main targets by chemicals to produce antitumor activity, number of anticancer drugs possess significant cytotoxic activity [25]. In present study of *in vitro* EAC cell line, MEIR also has shown to possess direct cytotoxic activity, when compared to EAC control group.

Thus the present study disclosed the cytotoxic effect of MEIR along with its potential as an inhibitor of EAC induced intracellular oxidative stress.

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