

## Antitumor potential of *Castanopsis indica* (Roxb. ex Lindl.) A. DC. leaf extract against Ehrlich's ascites carcinoma cell

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Methanol extract of *C. indica* (MECI) leaves showed direct cytotoxicity on Ehrlich ascites carcinoma (EAC) cell in a dose dependant manner and there was significant decrease in the tumor volume, viable cell count, tumor weight and elevated the life span of EAC tumor bearing mice. Hematological profile and biochemical estimations were significantly restored to normal levels in MECI treated as compared to EAC control mice. MECI treatment significantly modulated the tissue antioxidant assay parameters as compared to the EAC control mice. The results revealed that MECI possesses significant dose dependent antitumor potential which may be due to its cytotoxicity and antioxidant properties.

**Keywords:** Antioxidant, Antitumor, *Castanopsis indica*, Cytotoxicity, EAC cell

Cancer continues to be one of the major devastating diseases. Cancer is a class of diseases in which a cell or a group of cells display uncontrolled growth, invasion and sometimes metastasis<sup>1</sup>. According to WHO estimates, globally 10 millions new cancer cases are diagnosed each year. It caused about 13% of all human deaths in 2007. It is estimated that by the year 2020, there will be 20 million new cancer cases with 12 million deaths<sup>2</sup>. Two major concerns with currently available anticancer drugs are their inability to discriminate between normal and tumor cells and hence unpleasant drug toxicity and development of resistance due to expression of drug transporters<sup>3</sup>.

Chemotherapy, radiotherapy and surgery are only three major existing modes of treatment in modern medicine. Unfortunately, currently available cancer chemotherapeutic agents insidiously affect the host cells especially bone marrow, epithelial tissues, reticule-endothelial system and gonads<sup>4,5</sup>.

Scientists are therefore also turning towards developing clues for treatment from natural resources. India is a rich source of medicinal plants and a number of plant extracts are used against diseases in various systems of medicine such as Ayurveda, Unani

and Siddha<sup>6</sup>. Plants derived natural products such as flavonoids, terpenes, saponin, glycoside and alkaloids have received considerable attention in recent years due to their diverse pharmacological properties including cytotoxic and cancer chemopreventive effects<sup>7</sup>. Several plant products have been tested for anticancer activity and some of them like vincristine, taxol and so on are now available as a drug of choice.

*Castanopsis indica* (Roxb. ex Lindl.) A.DC. (Family- Fagaceae) commonly known as Dhalne katus (Nepali) and Indian chestnut tree (English) is found throughout the Himalayan region of North East India, Bangladesh, Nepal, Sikkim, Bhutan, Thailand and Vietnam<sup>8</sup>. A decoction of the leaves is applied to treat stomach disorder and skin diseases. Powdered leaves are given to cure indigestion. A plant resin is given to treat diarrhea. A paste of leaves is applied for headache<sup>9</sup>. Bark paste is used to control chest pain<sup>10</sup>. Previous bioactivity studies have been revealed with its leaves on central nervous system depressant activity<sup>8</sup>. Ethanol (50%) extract from stem bark have been reported to have anticancer activity in KB cancer cell and phosphotidylserine (PS) targeting antibody system<sup>11</sup>. Previously isolated classes of constituents from the stem bark of *C. indica* are identified as psoralen,  $\beta$ -Sitosterol, angelicin, erythrodiol, ursolic acid, castanopsin, castanopson and castanopsol<sup>12</sup>. Antitumor activity of its leaves has not yet been

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explored scientifically; therefore an effort has been made to find out the antitumor potential of methanol extract of *C. indica* leaves (MECI) against Ehrlich ascites carcinoma (EAC) cell in Swiss albino mice.

### Materials and Methods

**Plant material and preparation of extract**—The leaves of the *C. indica* were collected from the Gangtok, Sikkim during September 2010 and authenticated by the Botanical Survey of India, Gangtok, India. A voucher specimen (No. SHRC-5/5/2010/Tech.276) has been preserved in Phytotherapy and Pharmacology Research Laboratory of Department of Pharmaceutical Technology, Jadavpur University Kolkata, India for future reference. The leaves were dried at room temperature for 7 days and powdered in a mechanical grinder. Finally powdered plant material (200 g) was successively extracted by petroleum ether (60-80 °C) followed by methanol using Soxhlet extraction apparatus. The solvent was completely removed under reduced pressure and stored in a vacuum desiccator. The yield of the petroleum ether and methanol extract was about 4 and 11% w/w respectively.

**Phytochemical analysis**—The preliminary phytochemical analysis of the methanol extract of *C. indica* was done for qualitative analysis by using the standard methods<sup>13</sup>.

**Chemicals**—Chemicals used were trichloroacetic acid (TCA), thiobarbituric acid (TBA), phenazine methosulphate (PMS), reduced nicotinamide adeninedinucleotide (NADH), nitroblue tetrazolium (NBT), trypan blue and dithionitro benzene (DTNB). They were obtained from Sigma-Aldrich, Kolkata, India. 5-fluorouracil (5-FU) was from MERCK Limited, Mumbai, India.

**Animals**—Swiss albino mice of about 8 weeks of age with an average body weight of 20-25 g were obtained from Indian Institute of Chemical Biology, Kolkata. The mice were grouped and housed in poly acrylic cages (38 cm × 23 cm × 10 cm) with not more than 6 animals per cage. The animals were maintained under standard laboratory conditions (25-30° C and 55-60% RH with 14:10 h L:D cycle) and were allowed free access to standard dry pellet diet and water *ad libitum*. The mice were acclimatized to laboratory conditions for 7 days before commencement of the experiment. All the procedures described were reviewed and approved by Jadavpur University Animal Ethics Committee (367001/C/ CPCSEA).

**Acute toxicity study**—The LD<sub>50</sub> value of MECI in male Swiss albino mice was determined<sup>14,15</sup> and it was found to be 250 mg/kg body weight, ip. One-tenth and one-fifth of the maximum safe dose were selected for the *in vivo* experiment.

**Assay for *in vitro* cytotoxicity**—*In vitro* short term cytotoxicity of MECI was assayed using EAC cell line. Briefly 1×10<sup>6</sup> EAC cells suspended in 100 µl of phosphate buffered saline (PBS, 0.2 M, pH 7.4) were mixed with 100 µl of various concentrations (1-100 µg/ml) of MECI and final volume was adjusted to 1 ml with PBS. The mixture was incubated at 37 °C for 30 min. Cell suspension in phosphate buffer saline without extract served as control. After the incubation, the viability of the cells was determined using trypan blue (0.4% in normal saline) dye as per Bala *et al*<sup>15</sup>. The percentage of cytotoxicity was determined by calculating % inhibition and IC<sub>50</sub> values of MECI.

**Assay for *in vivo* antitumor activity**—**Transplantation of tumor:** The Ehrlich tumor was initially described as a spontaneous murine mammary adenocarcinoma<sup>16</sup>. EAC cells were obtained from Chittaranjan National Cancer Institute, Kolkata, India. Ascitic fluid was drawn out from EAC tumor bearing mouse at the log phase (days 7–8 of tumor bearing) of the tumor cells. EAC cells were maintained *in vivo* in mice by ip transplantation of 2×10<sup>6</sup> cells per mouse after every 10 days. The viable EAC cells were counted (Trypan blue indicator) under the microscope and were adjusted at 2×10<sup>7</sup> cells/mL. EAC cells suspension (0.1 ml) was injected (ip) in each mouse.

**Treatment schedule**—Swiss albino mice (20-25 g) were divided in to five groups of 12 animals each. All the animals in each groups except Group-I received EAC cells (2 × 10<sup>6</sup> cells/mouse, ip). This was taken as day '0'. Gr. I served as normal saline control (5 ml/kg body weight, ip) and Gr. II served as EAC control. After 24 h of EAC transplantation animals in Grs III and IV received MECI (25 and 50 mg/kg body weight, ip respectively) and Gr. V mice received standard drug 5-FU (20 mg/kg body weight, ip) once daily for 9 consecutive days<sup>15</sup>. After administration of last dose, 6 mice from each group were kept fasting for 18 h and blood was collected by cardiac puncture for the estimation of hematological and biochemical parameters. The animals then sacrificed for the study of antitumor activity and tissue antioxidant assay parameters. Rest of the animals in each groups were kept alive with food and water *ad libitum* to check

percentage increase in life span of the tumor host to determine the mean survival time (MST).

Antitumor activity of MECI was assessed by observation of changes with respect to the following parameters as per Haldar *et al*<sup>16</sup>:

**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 and expressed in milliliter (mL).

**Tumor weight**—The tumor weight was measured by taking the weight of the mice before and after the collection of the ascetic fluid from peritoneal cavity and expressed in gram (g).

**Percentage increase life span (% ILS)**—The effect of MECI on tumor growth was monitored by recording the mortality of the experimental mice. Percentage increase in life span (% ILS) was calculated by the following formula:

$$\text{ILS (\%)} = [(\text{Mean survival time of the treated group} / \text{Mean survival time of the control group}) - 1] \times 100$$

$$\text{Mean survival time (MST) in days} = (\text{Day of the first death} + \text{Day of the last death}) / 2$$

**Tumor cell (viable/nonviable) count**—The ascitic fluid was taken in a WBC pipette and diluted to 20 times. Then a drop of the diluted cell suspension was placed on the Neubauer's counting chamber and the number of cells in the 64 small squares was counted.

The viability and nonviability of the cell were checked by trypan blue assay. The cells were 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 dye were nonviable. These viable and nonviable cells were counted.

$$\text{Cell count} = (\text{number of cells} \times \text{dilution factor}) / (\text{area} \times \text{thickness of liquid film})$$

**Hematological parameters**—Collected blood was used for the estimation of hemoglobin (Hb) content, red blood cell (RBC) count and white blood cell (WBC) count by standard procedures<sup>15</sup>.

**Biochemical parameters**—The blood samples were allowed to clot and the serum was separated by centrifugation at 5000 rpm for 10 min. Serum was used to estimate the biochemical parameters like total protein, serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT) and alkaline phosphatase (ALP). All the

analysis was performed by using commercially available kits from Span Diagnostics Ltd, Surat, India.

**Tissue antioxidant assay parameters**—The tissue antioxidant assay was performed with liver and kidney tissue and evaluation was carried out by measuring the level of total protein (Span Diagnostics kit), protein carbonylation (PC)<sup>17</sup>, lipid peroxidation<sup>18,19</sup>, the amount of enzymatic Catalase (CAT)<sup>20</sup> and superoxide dismutase (SOD)<sup>21</sup> and nonenzymatic antioxidant system such as reduced glutathione (GSH)<sup>22</sup>.

**Statistical analysis**—The data expressed as the mean  $\pm$  SE, were statistically analyzed by using one way analysis of variance (ANOVA) followed by Dunnett's post hoc test by Graph Pad Prism software, version 5.  $P < 0.05$  was considered as significant and  $P < 0.01$  as highly significant.

## Results

Preliminary phytochemical analysis of methanol extract of *C. indica* demonstrated strong positive test for steroid, triterpenoid, saponin, flavonoid and tannins.

In the *in vitro* cytotoxicity study, MECI showed direct cytotoxicity on the EAC cell line in a concentration dependent manner in the dose range of 1-100  $\mu\text{g/ml}$ . The  $\text{IC}_{50}$  value was found to be  $71.50 \pm 6.25 \mu\text{g/mL}$ .

Antitumor activity of MECI against EAC tumor bearing mice was assessed by tumor volume, tumor weight, cell count (viable and non-viable), mean survival time and % increase in life span. The tumor volume, tumor weight and viable cell count were found to be significantly increased and non-viable cell count was significantly decreased in EAC control animals when compared with normal control animals (Table 1). Administration of MECI at the doses of 25 and 50 mg/kg significantly decreased the tumor volume and viable cell count. Non-viable cell count was significantly higher in MECI treated animals when compared with EAC control animals. Further, the median survival time was increased to 28.5 (ILS = 50.00%) and 34 (ILS = 78.94%) on administration of MECI in a dose dependant manner.

There was increased level of WBC and decreased level of hemoglobin (Hb) and RBC in EAC control group as compared to normal control group (Table 2). After treatment with MECI at the doses of

Table 1—Effect of MECI on tumor volume, tumor weight, total cell count, viable and nonviable cell count, median survival time (MST) and percentage increase life-span (%ILS) in EAC bearing mice. [Values are mean  $\pm$  SE from 6 observations in each group]

Parameters	EAC control	EAC + MECI (25 mg/kg)	EAC + MECI (50 mg/kg)	EAC + 5-FU
Tumor volume (ml)	2.85 $\pm$ 0.13	1.62 $\pm$ 0.18 <sup>#</sup>	0.98 $\pm$ 0.12 <sup>*</sup>	0.55 $\pm$ 0.05 <sup>*</sup>
Tumor weight (g)	3.11 $\pm$ 0.10	1.17 $\pm$ 0.07 <sup>#</sup>	0.93 $\pm$ 0.09 <sup>*</sup>	0.51 $\pm$ 0.04 <sup>*</sup>
Total cell ( $\times 10^7$ cell/ml)	9.30 $\pm$ 0.43	5.25 $\pm$ 0.21 <sup>*</sup>	3.90 $\pm$ 0.15 <sup>*</sup>	3.81 $\pm$ 0.09 <sup>*</sup>
Viable cell ( $\times 10^7$ cell/ml)	8.92 $\pm$ 0.42	3.78 $\pm$ 0.31 <sup>#</sup>	1.04 $\pm$ 0.08 <sup>*</sup>	0.60 $\pm$ 0.08 <sup>*</sup>
Nonviable cell ( $\times 10^7$ cell/ml)	0.38 $\pm$ 0.04	1.47 $\pm$ 0.09 <sup>#</sup>	2.86 $\pm$ 0.08 <sup>*</sup>	3.21 $\pm$ 0.09 <sup>*</sup>
Viable cell (%)	95.91	72.00	26.67	15.75
Nonviable cell (%)	4.09	28.00	73.33	84.25
MST (days)	19.00	28.50	34.00	39.00
ILS (%)	00	50.00	78.94	105.26

One way ANOVA between EAC control group vs treated groups followed by Dunnett's test. *P* values: <sup>#</sup>< 0.05; <sup>\*</sup>< 0.01.

Table 2—Effect of MECI on hematological parameters in EAC bearing mice. [Values are mean  $\pm$  SE from 6 observations in each group]

Parameters	Normal control	EAC control	EAC + MECI (25 mg/kg)	EAC + MECI (50 mg/kg)	EAC + 5-FU
RBC (cell $\times 10^6/\mu$ l)	5.29 $\pm$ 0.20	2.93 $\pm$ 0.11 <sup>a,*</sup>	4.01 $\pm$ 0.22 <sup>b,#</sup>	4.89 $\pm$ 0.20 <sup>b,*</sup>	5.19 $\pm$ 0.20 <sup>b,*</sup>
WBC (cell $\times 10^3/\mu$ l)	4.98 $\pm$ 0.32	7.97 $\pm$ 0.48 <sup>a,*</sup>	6.13 $\pm$ 0.33 <sup>b,#</sup>	5.08 $\pm$ 0.25 <sup>b,*</sup>	5.09 $\pm$ 0.33 <sup>b,*</sup>
Hemoglobin (g/dL)	11.48 $\pm$ 0.38	4.80 $\pm$ 0.29 <sup>a,*</sup>	8.19 $\pm$ 0.43 <sup>b,#</sup>	9.69 $\pm$ 0.50 <sup>b,*</sup>	10.35 $\pm$ 0.46 <sup>b,*</sup>

One way ANOVA between EAC control group and the treated groups followed by Dunnett's test. <sup>a</sup>EAC control group vs normal group; <sup>b</sup>treated groups vs EAC control group. *P* values: <sup>#</sup>< 0.05; <sup>\*</sup>< 0.01.

25 and 50 mg/kg in EAC bearing mice significantly increased the RBC count, Hb content and significantly reduced the WBC count as compared with the EAC control group.

The total protein content was found to be significantly decreased in the EAC control group as compared with the normal control group. Administration of MECI significantly increased the total protein content as compared with the EAC control mice (Fig. 1A). Biochemical parameters like SGOT, SGPT and ALP in the EAC control group were significantly increased as compared to the normal control group. Treatment with MECI in EAC bearing mice significantly decreased the SGOT, SGPT and ALP level in a dose dependant manner as compared to EAC control groups (Fig. 1b, c and d).

Total protein and protein carbonylation significantly increased in both liver and kidney in EAC control mice when compared to normal control mice. After administration of MECI, the protein carbonylation of both the tissues were significantly reduced (Fig. 2a and b) in a dose dependant manner as compared to EAC control mice.

Reactive oxygen species (ROS) formed in cancer liver and kidney tissues results in lipid peroxidation and subsequently increase in malondialdehyde (MDA) level. In the present study the levels of

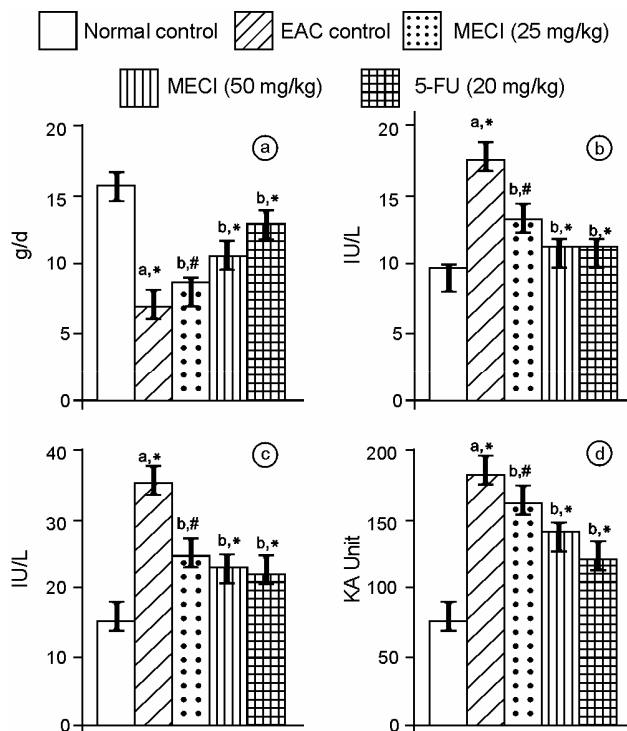


Fig. 1—Effect of methanol extract of *C. indica* leaves on serum biochemical parameters. Total protein count (A), SGOT (B), SGPT (C), ALP (D). Values are represented as mean  $\pm$  SE from 6 observations in each group. <sup>a</sup>EAC control group vs normal group; <sup>b</sup>treated groups vs EAC control group. *P* values: x = not significant; <sup>#</sup>< 0.05; <sup>\*</sup>< 0.01.

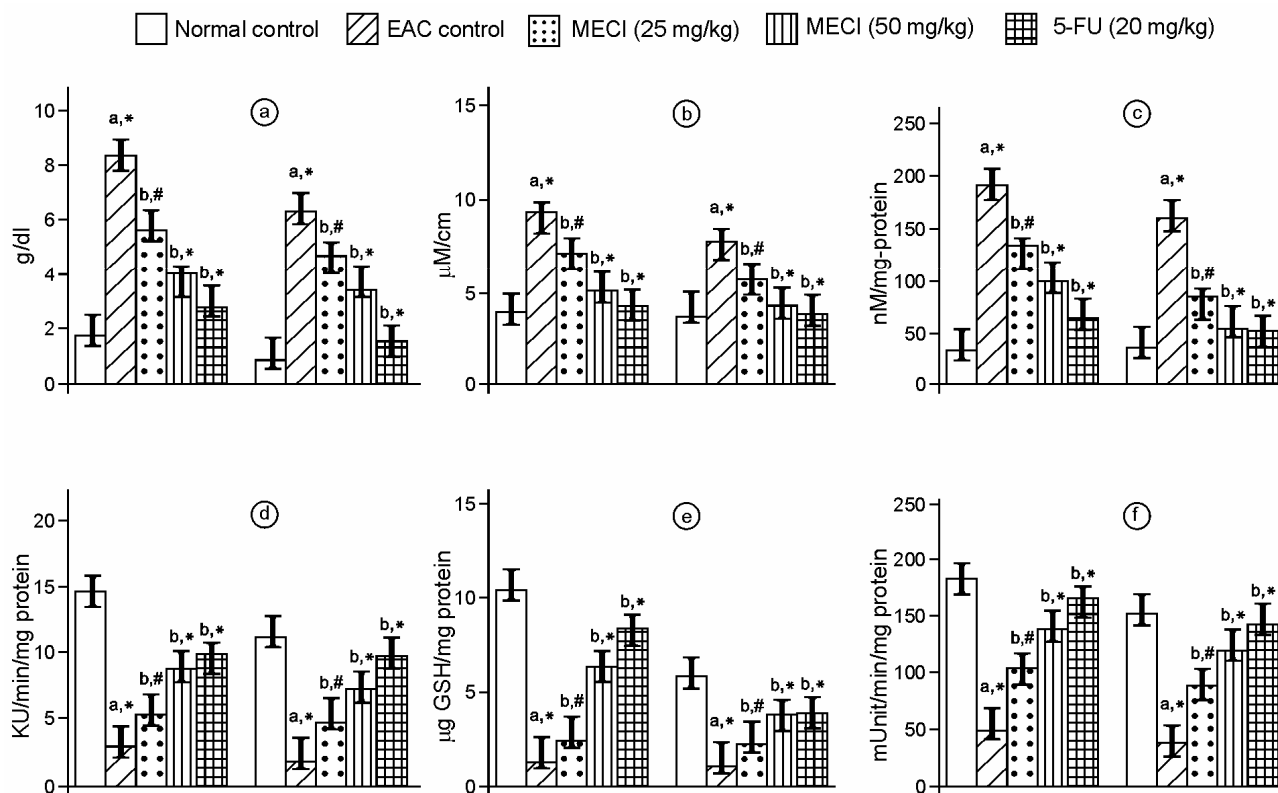


Fig. 2—Effect of methanol extract of *C. indica* leaves on tissue antioxidant defense parameters. Total protein count (A), Protein oxidation (B), Lipid peroxidation (C), Catalase (D), Reduce glutathione (E), and Superoxide dismutase (F). Values are represented as mean  $\pm$  SE from 6 observations in each group. <sup>a</sup>EAC control group vs normal group; <sup>b</sup>treated groups vs EAC control group. *P* values: x = not significant; # < 0.05; \* < 0.01.

MDA were significantly increased in EAC control animals when compared with normal control animals. Treatment with MECI significantly reduced the MDA levels as compared with EAC control group (Fig. 2c).

The levels of CAT, reduced GSH and SOD level were significantly decreased in EAC control group when compared with normal control group. Treatment with MECI in a dose dependent manner significantly increased the CAT, reduced GSH and SOD levels as compared with EAC control animals (Fig. 2d, e and f).

## Discussion

Synthetic anticancer drugs cause nonspecific killing of cells, whereas natural products offer protective and therapeutic actions to all cells with low cytotoxicity and are beneficial in producing nutrient repletion to compromised people<sup>23</sup>. Therefore, there is a need for new prototypes, new templates, to use in the design of potential chemotherapeutic agents<sup>24</sup>. The present study was undertaken to evaluate

the antitumor activity of MECI at the doses 25 and 50 mg/kg in EAC tumor bearing mice.

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<sup>25</sup>. Treatment with MECI decreased the tumor volume, tumor weight, 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 prolongation of the life span of animals<sup>18</sup>. It may be concluded that MECI increases the life span of EAC-bearing mice by decreasing the nutritional fluid volume and arresting the tumor growth.

Usually, in cancer chemotherapy the major problems that are being encountered are of myelosuppression and anemia. The anemia encountered in tumor bearing mice is mainly due to reduction in RBC or hemoglobin percentage and this may occur either due to iron deficiency or hemolytic or myelopathic

conditions<sup>26</sup>. Treatment with MECI brought back the hemoglobin (Hb) content, RBC and WBC count towards the normal levels. These are clearly indicated that MECI possess protective action on the hemopoietic system.

Enzymes in serum have been studied for many years as possible early indicators of neoplasia and as aids following the progression and regression of disease<sup>27</sup>. Hepatotoxicity may occur due to cytotoxic agent itself or its toxic metabolites. If there is rapid cell destruction with extensive purine catabolism, urates may precipitate in the renal tubules and cause kidney damage<sup>24</sup>. In the present study, EAC control group exhibited increased levels of tissue enzymes such as SGOT, SGPT, ALP and the levels of total protein were decreased. Treatment with the MECI restored the elevated biochemical parameters more or less to normal range, indicating the protection of the tumor cell induced hepatotoxicity by MECI.

Carbonylation of protein often leads to a loss of protein function, which is considered a widespread marker of severe oxidative stress, damage and disease-derived protein dysfunction<sup>28</sup>. PC is found in various types of cancers including hematological malignancies and neurodegenerative diseases as well as in human cell lines<sup>29</sup>. Present results demonstrate that the PC levels were high in EAC control mice and it indicates the high free radical generation in the hematopoietic cells. High levels of free radicals may cause the oxidative stress in hematopoietic cells if the antioxidant defense system is not effective. Treatment with MECI restored the elevated PC levels more or less to normal range, indicating the antioxidant and free radical scavenging property of MECI.

Malondialdehyde (MDA) is formed during oxidative degeneration as a product of free oxygen radicals which is accepted as an indicator of lipid peroxidation<sup>30</sup>. MDA, the end product of lipid peroxidation was reported to be higher in cancer tissues than in non-diseased organ. The present findings indicate that MDA levels in the EAC control group were higher than those in normal control. This emphasizes the reduction in free radical yield and the subsequent decrease in harm and damage to the cell membrane and decrease in MDA production<sup>31</sup>.

GSH, a potent inhibitor of neoplastic process plays an important role as an endogenous non-enzymatic antioxidant system that is found particularly in high concentration in liver and is known to have key function in the protective process<sup>32</sup>. The level

of reduced glutathione was depleted in cancer bearing mice which may be due to its utilization by the excessive amount of free radicals. The results showed that the antitumor activity of MECI was accompanied with the enhancement in non-enzymatic antioxidant protection.

Cells are also equipped with enzymatic antioxidant mechanisms that play an important role in the elimination of free radicals. SOD and CAT are involved in the clearance of superoxide and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). SOD catalyses the diminution of superoxide into H<sub>2</sub>O<sub>2</sub>, which has to be eliminated by glutathione peroxidase and/or catalase<sup>33</sup>. Consistent with this, it has been reported that a decrease in SOD activity in EAC-bearing mice may be due to loss of Mn<sup>2+</sup> containing SOD activity in EAC cells and the loss of mitochondria, leading to decrease in total SOD activity in the liver and kidney. The inhibition of SOD and CAT activities as a result of tumor growth was also reported<sup>34</sup>. The administration of MECI significantly increased the SOD and CAT levels in a dose dependent manner. These findings suggest that MECI may inhibit tumorigenesis through the enhancement of cellular antioxidant system.

Plant derived extracts containing antioxidant principles showed cytotoxicity towards tumor cells and antitumor activity in experimental animals<sup>35</sup>. Antitumor activity of these antioxidants is either through induction of apoptosis or by inhibition of neovascularization<sup>36</sup>. The free radical hypothesis supported the fact that the antioxidants effectively inhibit the tumor and the observed properties may be attributed to the antioxidant and antitumor principles present in the MECI<sup>37</sup>.

## Conclusion

The present study demonstrates that the methanol extract of *C. indica* leaves has remarkable antitumor activity against Ehrlich's ascites carcinoma cells treated mice. Further investigations are in progress to identify the active principle(s) involved in antitumor activity.

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