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In vitro and *in vivo* anticancer activity of *Indigofera cassioides* Rottl. Ex. DC.

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ABSTRACT

Objective: To evaluate the antitumor, cytotoxic and antioxidant activities of methanolic leaf extract of *Indigofera cassioides* (MEIC) against transplantable tumors and human cancer cell lines. **Methods:** MEIC was investigated for its short term cytotoxicity on EAC and DLA cells by trypan blue dye exclusion method and *in vitro* cytotoxicity on HeLa, HEp-2, HEpG-2, MCF-7, HT-29, Vero and NIH 3T3 cells by MTT assay. *In vivo* antitumor activity was studied on EAC and DLA tumor bearing mice. Activity was assessed by monitoring the mean survival time, effect on hematological parameters, antioxidant enzyme levels and solid tumor volume. **Results:** MEIC exhibit potent *in vitro* cytotoxicity against all the tested cancer cell lines, but it was found to be safe on normal cells. The extract significantly ($P<0.001$) increase the mean survival time and also have a protective effect on the hemopoietic system at the tested dose levels (200 and 400 mg/kg). The extract prevented lipid peroxidation and restored the antioxidant enzymes catalase, superoxide dismutase, glutathione peroxidase and glutathione-s-transferase in the liver of tumor control animals. It also significantly ($P<0.01$) reduce the solid tumor volume. **Conclusions:** The results strongly support that MEIC shows potent antitumor and cytotoxic effects against EAC, DLA and human cancer cell lines. The extract prevents lipid peroxidation and promotes the enzymatic antioxidant defense system in tumor bearing animals which might be due to activities like scavenging of free radicals by the phytochemicals in MEIC.

1. Introduction

Cancer is one of the leading causes of human death. In modern medicine, chemotherapy, radiotherapy and surgery are the major treatments available for cancer. Intervention with chemo preventive agents in the early stage of carcinogenesis is theoretically more rational than attempting to eradicate fully developed tumors with chemotherapeutic agents. These agents have a narrow margin of safety and the therapy may fail due to drug resistance and dose-limiting toxicities, which may severely affect the host cells. Hence the use of natural products is an alternative in the control and eradication of cancer. Medicinal plants are playing an important role as a source of effective anticancer agents and it is significant that 60% of currently used anticancer agents are derived from natural sources including plants[1]. There is a growing interest in the pharmacological evaluation of various plants used in Indian traditional system of medicine.

Indigofera cassioides Rottl. Ex. DC. (Syn: *Indigofera pulchella* Roxb.) (*I. cassioides*) belonging to the family Fabaceae, is a large shrub, distributed throughout the hills of India. The flowers of the plant are reported to be antiscorbutic, diuretic and alternative. A decoction of the root is given for cough and its powder is applied externally for chest pain. The leaves and roots are used for swelling of the stomach [2, 3]. The leaves are used by tribes and native medical practitioners to treat diseases such as arthritis, inflammation, tumor and liver diseases. However, there is no phytochemical and pharmacological evaluation has been carried out on *I. cassioides*. Based on this evidence we have selected *I. cassioides* for the present study. The aim of the present study is to evaluate the antitumor properties of the methanol extract of *I. cassioides* (MEIC) against Ehrlich ascites carcinoma (EAC) and Dalton's lymphoma ascites (DLA) tumor models along with its antioxidant status.

2. Materials and methods

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2.1. Chemicals

5-Fluorouracil (5-FU) was obtained from Dabur Pharmaceutical Ltd (New Delhi, India). Trypan blue, thiobarbituric acid, trichloroacetic acid, ethylenediaminetetracetic acid (EDTA), RPMI-1640 media and 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltertazolium bromide (MTT) were procured from HiMedia (Mumbai, India). Dimethyl sulfoxide and methanol were obtained from Loba Chemie (Mumbai, India). All other chemicals used were of analytical grade.

2.2. Plant material and extraction

Leaves of *I. cassioides* were collected from the Yercaud hills in the month of November 2008. The plant was authenticated by Dr. G.V.S. Murthy, Joint Director, Botanical Survey of India, Coimbatore, Tamilnadu, India. A voucher specimen is preserved in our laboratory for future reference (Voucher No. P. Ch. IC 003/2008). The plant material was shade dried, pulverized and extracted (500 g) with 80% methanol at room temperature for 72 h. The extract was filtered and concentrated to dryness under reduced pressure and controlled temperature (40-50 ℃) in a rotary evaporator. The extract was a dark brown solid weighing 41 g (yield, 8.2 %) and was preserved in a vacuum desiccator at 4 ℃ until further use.

2.3. Preliminary phytochemical analysis

The extract was analyzed for the presence of various phytochemical constituents employing standard screening tests [4]. Conventional protocol for detecting the presence of steroids, alkaloids, tannins, flavonoids, glycosides, etc., was used.

2.4. Tumor cells and inoculation

Normal mouse embryonic fibroblast (NIH 3T3), normal African green monkey kidney Cells (Vero), human cervical cancer cells (HeLA), human laryngeal epithelial carcinoma (HEp-2), human liver cancer cells (HEpG-2), human breast cancer cells (MCF-7) and human colon cancer cells (HT-29) were obtained from National Centre of Cell Sciences (Pune, India). The cultures were maintained in Dulbecco's modified eagles medium (DMEM) containing 10 % inactivated calf serum and were grown in 25 cm^2 tissue culture flasks (Tarson Products Ltd, Kolkatta, India) until confluence and use for cytotoxic assays. EAC and DLA cells were supplied by Amala Cancer Research Centre (Trissur, Kerala, India). The cells were maintained *in vivo* in Swiss albino mice by intraperitoneal transplantation. Tumor cells aspirated from the peritoneal cavity of mice were washed with normal saline and used for further studies.

2.5. Short term cytotoxic activity

Short term cytotoxicity was assessed by incubating 1 $\times 10^6$ EAC or DLA cells in 1 mL phosphate buffered saline at varying concentrations (31.25 - 1000 μg/mL) of the MEIC at 37 ℃ for 3 h under a CO_2 atmosphere. The viability of the cells was determined by trypan blue dye exclusion method [5].

2.6. In vitro cytotoxicity on human cancer cell lines

Stock cells of normal mouse embryonic fibroblast (NIH 3T3), normal African green monkey kidney cells (Vero), human cervical cancer cells (HeLA), human laryngeal epithelial carcinoma (HEp-2), human liver cancer cells (HEpG-2), human breast cancer cells (MCF-7) and human colon cancer cells (HT-29) were cultured in RPMI-1640 and DMEM supplemented with 10 % calf serum, penicillin (100 IU/mL) and streptomycin (100 μg/mL) in a humidified atmosphere of 5 % CO_2 at 37 ℃ until confluent. The cells were dissociated with 0.2 % trypsin and 0.02% EDTA in PBS. The cytotoxic assay was carried out by adding 0.1 mL of cell suspension containing 10 000 cells to each well of a 96-well microtitre plate (Tarson, Kolkatta, India) and fresh medium containing various concentrations of extract was added at 24th h after seeding. Control cells were incubated without the extract and with DMSO. The microtitre plates were incubated at 37 ℃ in a humidified atmosphere with 5 % CO_2 for a period of 72 h. The percentage cytotoxicity was determined by the standard MTT assay method and IC_{50} was calculated [6].

2.7. Animals

Healthy male swiss albino mice weighing (25±2) g were obtained from Venkateshwara Enterprises, Banglaore, India. The mice were grouped and housed in polypropylene cages and maintained under standard conditions (25±2) ℃ with 12 h dark/light cycle. The animals were fed with standard animal pellet diet and water ad libitum. The experiment protocols received clearance from the Institutional Animal Ethical Committee (IAEC) and CPCSEA, Chennai, India (Proposal No: SVCP/IAEC/Ph.D/01/2008-09 dt 24.12.2008).

2.8. Acute toxicity studies

The oral acute toxicity study of MEIC was carried out in Swiss albino mice using the OECD guidelines 423 [7]. The animals received MEIC starting at 2 g/kg orally by gavage. The animals were observed for toxic symptoms and mortality continuously for first 4 h after administration. Finally, the number of survivors was noted after 24 h and these animals were then maintained for further 13 days with daily observations.

2.9. Antitumor studies

Antitumor activity of MEIC was evaluated by the procedure described by Senthil Kumar *et al*., [8]

2.9.1. Effect of MEIC on survival time

Healthy Swiss albino mice were divided into eight groups (I-VIII) each group consisting of six animals. Group I - IV were inoculated with EAC cells (1 x 10^6 cells/mouse) and Group V-VIII were inoculated with DLA cells (1×10^6 cells/mouse) on day '0' and treatment with MEIC started 24 h after inoculation. Group I and V served as tumor control which received the vehicle (CMC, 0.3 %). Group II and VI were treated with the standard drug (5-FU, 20 mg/kg) by intraperitoneal route. Group III, IV, VII and VIII received the plant extract at the dose of 200 mg/kg and 400 mg/kg by oral route, respectively. All the treatments were given for nine days. The median survival time and percentage

increase in life span was calculated.
% increase in life span = [T-C/C]× 100
Where, T= No. of days the treated animals survived
C= No. of days the control animals survived

2.9.2. Effect of MEIC on hematological parameters

In order to detect the influence of MEIC on hematological status of tumor bearing animals, a comparison was made among eight groups (n=5 animals per group) of mice on the 14th day after inoculation. Group I and V served as normal control which received the vehicle (CMC, 0.3 %). Group II and VI served as tumor control for respective cell lines. Group III, IV, VII and VIII were treated with the plant extract at the dose of 200 mg/kg and 400 mg/kg by oral route for nine days. Blood was drawn from each mouse by retro orbital plexus method after anaesthetized slightly with anesthetic ether. The hematological parameters like hemoglobin (Hb), red blood cells (RBC), white blood cells (WBC), platelets (PLT), lymphocytes (LYM), hematocrit (HCT), hemoglobin (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width (RDW) and MID cells (less frequently occurring and rare cells correlating to monocytes, eosinophils, basophils etc.,) were determined by using a blood automatic analyzer (Celldyn, Abbot Inc. USA). After blood collection, animals were sacrificed by cervical dislocation. The liver from each mouse was excised and rinsed in ice cold normal saline solution. A 10 %w/v liver homogenate was prepared in ice cold 10 % KCl solution and was centrifuged for 15 min at 4 ℃. The supernatant, thus was obtained and used for the estimation of lipid peroxidation (LPO) [9], catalase (CAT) [10], superoxide dismutase (SOD) [11], glutathione peroxidase (GPx) [12] and glutathione S-transferase (GST) [13]. Ascitic fluid of all group animals, except normal, were smeared and stained with Lieshman stain solution for cytological studies.

2.9.3. Effect of MEIC on solid tumor volume

Mice were divided into six groups with each group consisting of six animals. Group I - III were injected with EAC cells (2×10^6 cells/mouse) into the right hind limb of the animals intramuscularly. In the same way, the animals from Group IV - VI were injected with DLA cells (2×10^6 cells/mouse) into the right hind limb, intramuscularly. Group I and IV served as tumor control. Group II, III, V and VI were treated with MEIC at the dose of 200 mg/kg and 400 mg/kg by oral route, respectively, for five alternative days. From the 15th day onwards, tumor diameter was measured every fifth day and recorded up to 30 days by using vernier calipers. The tumor volume was calculated by using the formula $V = 4/3\ \pi r^2$, where 'r' is the mean of r1 and r2 which are the two independent radii of the tumor mass.

2.10. Statistical analysis

All the values were expressed as mean ± SEM. The data were statistically analyzed by one-way ANOVA, followed by Tukey multiple comparison test and data for solid tumor volume were analyzed by Dunnett test. P values <0.05 were considered as significant.

3. Results

3.1. Preliminary phytochemical analysis

The preliminary phytochemical screening revealed that the extract contains terpenoids, alkaloids, saponins, glycosides, flavonoids and phenolic compounds.

3.2. Short term cytotoxicity assay

The IC_{50} of MEIC was found to be 47.10 μg/mL for EAC cells and 72.63 μg/mL for DLA cells.

3.3. In vitro cytotoxic activity

In the *in vitro* cytotoxicity studies, the cells were treated with various concentrations of the extract (31.25-500 μg/mL). The percentage cytotoxicity progressively increased in a concentration dependent manner. The IC_{50} of MEIC was found to be below 100 μg/mL on all the tested human cancer cell lines, but it was found to be very high for the normal cells. This indicated that MEIC possess selective cytotoxicity against the cancer cells, but is safer towards the normal cells (Table 1).

3.4. Acute toxicity studies

In acute toxicity studies, animals treated with MEIC did not show any toxic symptoms or mortality when dosed up to 2 000 mg/kg body weight by oral route. This indicated that the extract was found to be safe at the tested dose level. Hence 1/10th (200 mg/kg) and 1/5th (400 mg/kg) of this dose were selected for the *in vivo* studies.

Table 1
In vitro cytotoxicity studies of methanol extract of *I. cassioides* against human cancer cell lines by MTT assay method.

Cell lines studied	IC_{50} (μg/mL)*
NIH 3T3 (Normal mouse embryonic fibroblast)	292.40
Vero (Normal African green monkey kidney cells)	280.30
HeLa (Human cervical cancer)	55.67
HEp-2 (Human laryngeal epithelial carcinoma)	68.45
HEpG2 (Human liver cancer)	46.88
MCF-7 (Human breast cancer)	87.50
HT-29 (Human colon cancer)	50.84

*Average of three determinations, three replicates; IC_{50}: Drug concentration inhibiting 50% cellular growth following 72 h of drug exposure.

3.5. Effect on mean survival time

In both EAC and DLA tumor bearing the animals, the extract significantly (P<0.001) increased the mean survival time and percentage increase in life span in dose dependent manner. The results were almost comparable to that of 5-FU, the standard drug (Table 2).

3.6. Effect on hematological parameters

Hematological parameters of both EAC and DLA tumor bearing mice on day 14 were found to be significantly altered from normal group. There was a significant decrease in hemoglobin, RBC and lymphocytes in tumor bearing animals, accompanied by an increase in WBC, hematocrit (HCT), MID cells and protein. At the same treatment interval, MEIC at the dose of 200 and 400 mg/kg changed these altered

parameters significantly closer to normal levels. All these results suggest the anticancer nature of the extract (Table 3). The EAC and DLA tumor cells from the ascitic fluid of different treatment groups were stained with Lieshman stain. They showed marked cytological changes and cytolytic activity when compared to the respective tumor control cells (Figure 1-6).

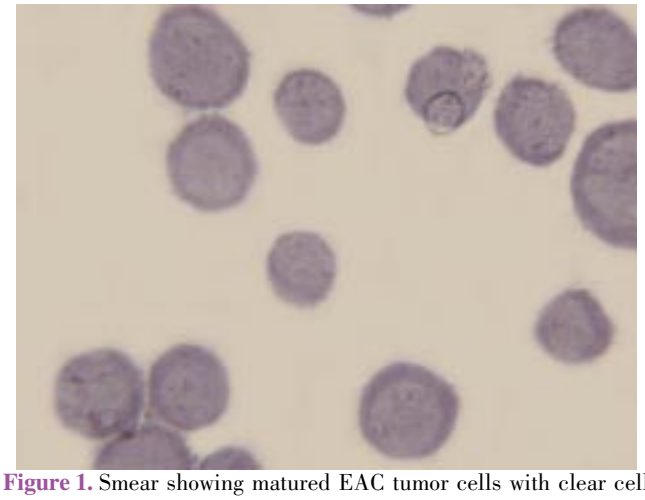

Figure 1. Smear showing matured EAC tumor cells with clear cell wall without degeneration.

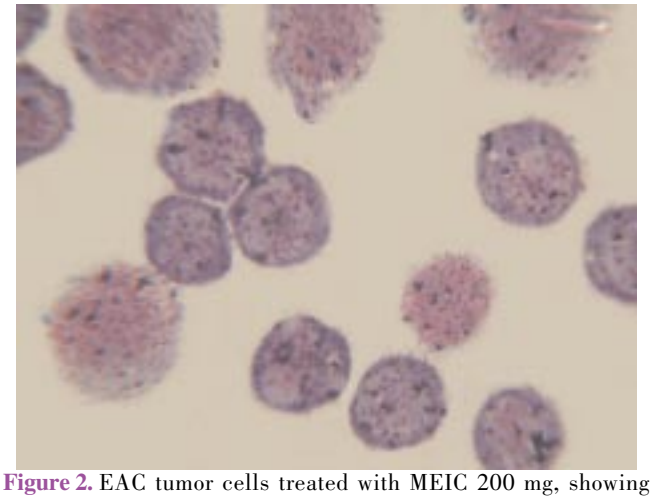

Figure 2. EAC tumor cells treated with MEIC 200 mg, showing degenerative changes in the form of membrane blebbing.

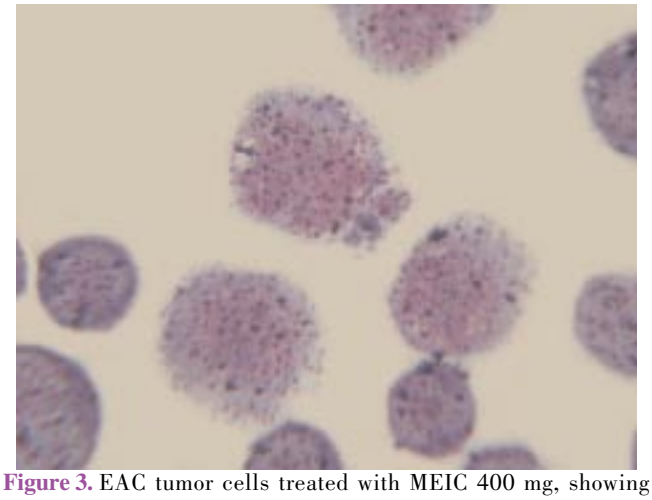

Figure 3. EAC tumor cells treated with MEIC 400 mg, showing degenerative changes like membrane blebbing, vacuolated cytoplasm and a reduction in the staining intensity.

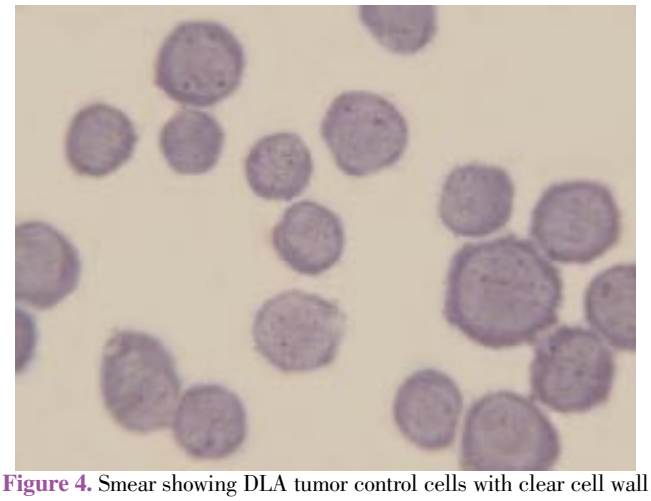

Figure 4. Smear showing DLA tumor control cells with clear cell wall and definite structure of tumor cells.

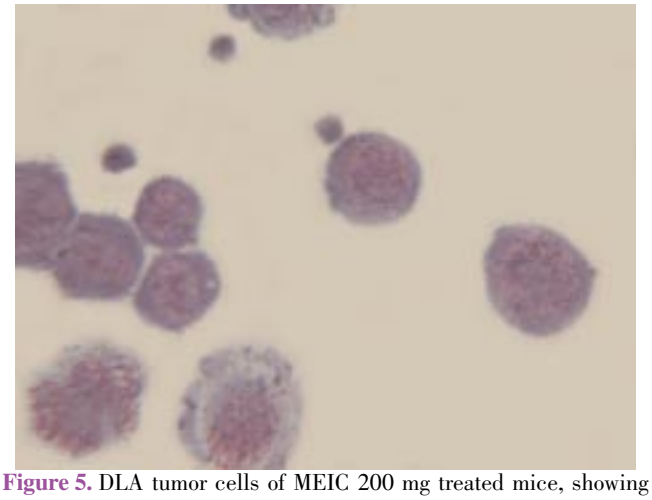

Figure 5. DLA tumor cells of MEIC 200 mg treated mice, showing cell wall destruction and degradation of tumor cells.

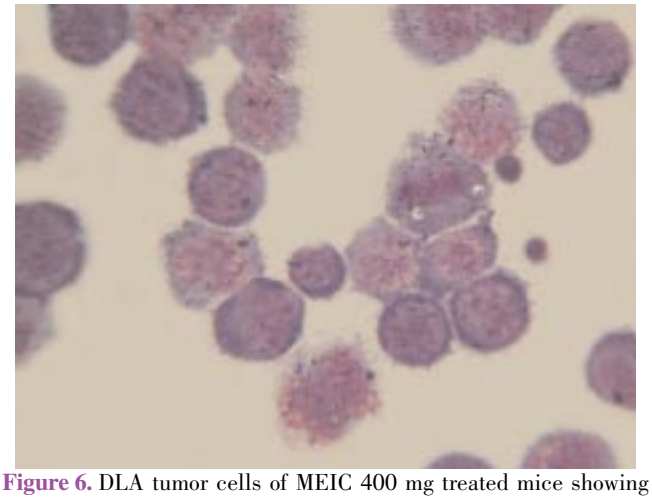

Figure 6. DLA tumor cells of MEIC 400 mg treated mice showing complete destruction of cell wall, degenerative changes like vacuolated cytoplasm, membrane blebbing and low staining intensity.

3.7. Effect on antioxidant parameters

The levels of lipid peroxidation in liver tissue were significantly increased in EAC and DLA tumor control groups as compared to the normal group ($P<0.001$). After administration of MEIC at different doses to tumor bearing mice, the levels of lipid peroxidation were significantly ($P<0.001$) reduced as compared to tumor control groups. Inoculation with the tumor cells drastically increased

the GST and GPx content in both tumor control groups as compared with normal group. Administration of MEIC restored the levels of GST and GPx when compared to tumor control group. Decreased levels of superoxide dismutase (SOD) and catalase (CAT) in the livers of the EAC and DLA tumor bearing mice was observed (P<0.001) when compared with normal group. After treatment with MEIC at the tested doses, the levels of SOD and catalase were bringing back to near normal (Table 4).

3.8. Effect on solid tumor volume

The solid tumor volumes of EAC & DLA tumor bearing mice was presented in Table 5. In EAC tumor bearing animals, the lower dose group animals does not show significant activity, but higher dose level significantly (P<0.01) reduces the tumor volume when compared to tumor control groups. In DLA tumor bearing animals, the extract significantly reduce the solid tumor volume in dose dependent manner.

Table 2
Effect of methanol extract of *I. cassioides* on survival & increase in life span of EAC & DLA tumor bearing mice (n=6).

Design of treatment	EAC		DLA	
	MST	Increase in life span (%)	MST	Increase in life span (%)
Tumor control	23.83±1.14	0.00	20.50±0.76	0.00
5-FU	45.50±1.18 [a]	90.93	40.83±0.60 [a]	99.17
MEIC 200	37.17±0.70 [a,b]	55.97	27.17±0.70 [a,b]	32.53
MEIC 400	42.50±0.76 [a]	78.35	33.70±1.02 [a,b]	64.39

MST: Mean survival time. Data were expressed as Mean±SEM, a P<0.001 *vs.* tumor control; b P<0.001 *vs.* 5-FU. Data were analyzed by using one way ANOVA followed by Tukey Kramer Multiple comparison test.

Table 3
Effect of methanol extract of *I. cassioides* on hematological parameters of EAC/DLA tumor bearing mice (n=5).

Parameters	EAC				DLA			
	Normal	Tumor control	MEIC 200	MEIC 400	Normal	Tumor control	MEIC 200	MEIC 400
HGB (g/dL)	15.22±0.33	6.84±0.26[a]	9.12±0.26[a,d]	10.88±0.35[a,d]	12.56±0.58	6.46±0.33[a]	8.26±0.37[a,e]	10.98±0.29[d]
RBC (M/uL)	5.79±0.36	3.32±0.18[a]	3.69±0.09[a]	5.21±0.36[e]	7.16±0.22	4.04±0.09[a]	4.54±0.21[a]	5.88±0.32[c,d]
HCT (%)	17.92±0.69	29.92±1.51[a]	28.08±1.00[a]	23.52±0.49[b,d]	17.66±0.57	30.16±1.76[a]	21.06±0.62[d]	18.78±1.09[d]
MCV (fL)	47.44±0.63	37.66±1.96[a]	41.16±1.44[c]	41.40±1.75	47.06±0.82	41.80±1.47[c]	45.50±0.40	45.40±0.72
MCH (pg)	16.48±0.39	18.18±1.23	16.70±0.29	15.96±0.37	16.72±0.38	16.68±0.88	17.36±0.74	17.24±0.19
MCHC (g/dL)	34.92±0.26	36.06±0.82	35.10±0.75	34.64±0.39	34.88±0.46	37.28±1.11	34.52±0.37	36.40±0.77
RDW (%)	24.96±0.69	17.46±0.50[a]	17.98±0.46[a]	23.28±1.10d	17.36±0.70	18.58±0.44	18.14±0.51	17.94±0.85
PLT (K/uL)	635.00±25.42	755.20±13.67[b]	713.00±15.29	613.60±26.74[e]	703.20±8.45	639.00±17.33[c]	687.20±14.56	727.80±9.89[d]
WBC (K/uL)	6.00±0.24	17.62±0.70[a]	13.04±0.82[a,d]	9.62±0.48[b,d]	4.60±0.15	8.40±0.40[a]	6.32±0.19[a,d]	5.06±0.16[d]
LYM (%)	68.40±1.95	45.98±1.58[a]	48.28±1.89[a]	57.70±0.44[b,d]	62.38±4.10	18.36±1.89[a]	44.04±1.45[a,d]	49.92±1.50[c,d]
MID (%)	16.06±0.93	38.00±3.48[a]	29.20±1.65[a,f]	23.30±1.25[d]	14.16±1.61	62.72±2.71[a]	27.92±0.80[a,d]	25.44±1.42[b,d]
Granulo (%)	15.54±1.82	1 6.04±2.30	22.52±0.62	19.00±1.63	24.30±2.82	18.80±2.40	28.04±1.56	24.64±2.85
Total proteins (g/dL)	5.72±0.23	14.38±0.78[a]	8.18±0.62[c,d]	6.64±0.43[d]	5.35±0.11	14.27±0.42[a]	10.70±0.35[a,d]	6.44±0.29[d]

Data were expressed as Mean±SEM; Hematological parameters (total red blood cell = RBC, white blood cells = WBC, lymphocytes = LYM, platelet = PLT, hematocrit = HCT, hemoglobin = HGB, mean corpuscular volume = MCV, mean corpuscular hemoglobin = MCH, mean corpuscular hemoglobin concentration = MCHC, red blood cell distribution width = RDW and MID cells = less frequently occurring and rate cells correlating to monocytes, eosinophils, basophils etc., aP<0.001; bP<0.01; cP<0.05 *vs.* Normal; dP<0.001; eP<0.01; fP<0.05 *vs.* tumor control. Data were analysed by One way ANOVA followed by Tukey Kramer Multiple comparison test.

Table 4
Effect of methanol extract of indigofera cassioides on antioxidant enzyme levels of EAC/DLA tumor bearing mice (n=5).

Design of treatment	EAC					DLA				
	LPO	SOD	CAT	GPx	GST	LPO	SOD	CAT	GPx	GST
Normal	7.16±0.45	0.48±0.02	35.08±1.42	16.80±0.44	0.19±0.01	5.26±0.19	0.42±0.02	32.30±1.16	16.60±0.83	0.18±0.01
Tumor control	32.80±1.12[a]	0.17±0.01[a]	18.20±1.45[a]	52.16±1.39[a]	0.45±0.02[a]	32.70±1.41[a]	0.15±0.02[a]	14.58±0.72[a]	47.50±1.84[a]	0.39±0.01[a]
MEIC 200	22.80±0.98[a,c]	0.25±0.02[a,e]	20.90±0.55[a]	31.48±1.56[a,c]	0.36±0.01[a,d]	19.17±0.71[a,d]	0.24±0.01[a,e]	22.00±1.30[a,e]	26.91±1.64[a,d]	0.24±0.01[c,d]
MEIC 400	13.02±0.89[b,c]	0.34±0.01[a,c]	26.02±1.41[a,d]	24.04±0.53[b,c]	0.28±0.01[b,c]	12.66±1.02[a,d]	0.37±0.02[d]	25.10±1.35[b,d]	23.87±0.86[b,d]	0.20±0.01[d]

n=5; Data were expressed as mean±SEM, aP<0.001; bP<0.01; cP<0.05 *vs.* Normal; dP<0.001 *vs.* Tumor control; Data were analyzed by Tukey-Kramer multiple comparison test. LPO, μ moles of MDA/min/mg protein; SOD, units/min/mg protein; CAT, μ mole of H_2O_2 consumed/min/mg protein; GPx, μ moles of GSH oxidized/min/mg protein; GST, μ moles of CDNB conjugation formed/min/mg protein.

Table 5
Effect of methanol extract of *I. cassioides* on solid tumor volume of EAC/DLA tumor bearing mice (cm^3) (n=6).

Design of Treatment	Tumor volume (cm^3)							
	EAC				DLA			
	15th day	20th day	25th day	30th day	15th day	20th day	25th day	30th day
Tumor control	1.53±0.07	1.81±0.28	2.23±0.18	3.93±0.35	1.72±0.09	1.85±0.09	2.25±0.09	3.00±0.19
MEIC 200	1.14±0.1[a]	1.60±0.18	2.04±0.18	3.15±0.34	1.37±0.13[b]	1.54±0.12[b]	1.93±0.13[b]	2.38±0.18[b]
MEIC 400	1.06±0.05[a]	1.10±0.16b	1.73±0.28	2.33±0.24[a]	1.30±0.05[a]	1.42±0.03[a]	1.68±0.07[a]	1.90±0.05[a]

n=6, Data were expressed as Mean±SEM aP<0.01; bP<0.05 *vs* Tumor Control, Data were analyzed by using one way ANOVA followed by Dunnett's test.

4. Discussion

The effect of methanol extract of *I. cassioides* has been studied on several human cancer cell lines and normal cell lines as well as in murine tumor models. Our results show that the extract has potent cytotoxic and anticancer potential *in vitro* and *in vivo*. The extract treatment showed concentration dependent inhibition of cell growth of human cancer cell lines studies. The IC_{50} value varied depending upon the cell line. The effect was more pronounced against HEpG2 cell lines. This demonstrated the difference in sensitivity of cancer cell lines to phytochemicals containing in *I. cassioides*. This may also be due to different molecular characteristics of these cells. Further, the differential cytotoxicity of the extract against different human cancer cell lines shows that its use against different types of cancers might present positive results. At the same time, the IC_{50} value for normal cell lines were found to be very high which indicated that the selectivity of the plant extract towards the cancer cells.

A reliable criterion for assessing the potential of any anticancer agent is the prolongation of the life span of the tumor bearing animals. An increase in life span of ascites bearing animals by 25% is considered as an indicative of significant drug activity. Our results show an increase in life span accompanied by a reduction in WBC count in MEIC treated mice. The plant extract also inhibited the accumulation of ascitic fluid in the peritoneal cavity of the tumor bearing animals. This suggests that the cytotoxic activity of MEIC on the transplantable tumor cells might be due to mechanisms including cytolytic effect. Cytological studies of leishman stained tumor cell streaks have revealed a decrease in the number of mitotic cells following MEIC treatment when compared with that of control. Degenerative changes in treatment group have been observed in the form of membrane blebbing, vacuolated cytoplasm and a reductive in the staining intensity.

The most common problems encountered in cancer chemotherapy are myelosuppression and anemia. Similar results were observed in the present study in animals of the EAC and DLA tumor control group. This is mainly due to reduction in RBC or hemoglobin production and this may occur either due to the iron deficiency or to hemolytic or other myelopathic conditions [14]. Treatment with MEIC brought back the hemoglobin content, RBC and WBC counts closer to normal level. This indicates that the extract have a protective effect on the hemopoietic system.

Cell proliferation, a characteristic feature of cancer, is inversely proportional to lipid peroxidation and cancer itself imparts an oxidative stress on the host. This is illustrated by the rapid elevation of lipid peroxidation and impaired antioxidant status of mice with various types of tumors. Several studies have demonstrated that tumor bearing animals can experience a systemic change of antioxidant enzymes in organs distant from the tumor. The tumor cells produce substantial amount of hydrogen peroxide, which may be released into circulation and then transported to the liver for detoxification [15]. Further, growing tumors sequester essential antioxidants from the host tissues and meet their demand [16]. So, the observed elevated levels of LPO in the liver of EAC and DLA tumor bearing animals may be due to the excessive generation of hydrogen peroxide by peritoneal cells that has been transferred to liver for detoxification along with the sequestration of antioxidant by tumor cells [17]. Treatment with MEIC significantly decreases the levels of LPO and shows its protective role on the liver by decreasing the tumor burden. GST and GPx are metabolic enzymes involved in the detoxification of free radicals, carcinogens and peroxides by conjugating these toxic substances with GSH, ultimately protecting the cells and organs from the oxidative stress. Treatment with MEIC decrease the tumor burden, hence decrease the oxidative stress, which in turns results in the restored levels of these enzymes.

SOD and catalase are considered as primary antioxidant enzymes, since they are involved in the direct elimination of the reactive oxygen species [18]. Catalase depression in liver is a well established phenomenon with tumor burden and decreased SOD activities were also seen in many organs of tumor bearing animals. Our results are in agreement with the above findings and treatment with MEIC effectively reduces the oxidative stress in tumor bearing animals and restored the activities of enzymic antioxidants.

Plant derived extracts containing antioxidant principles showed cytotoxicity towards tumor cells and antitumor activity in experimental animals. Antitumor activity of these antioxidants is either through induction of apoptosis or by inhibition of angiogenesis. The involvement of free radicals in tumors is well documented. The lowering of lipid peroxidation, GST, GPx and increase in levels of SOD and catalase in MEIC treated group indicates its potential as an inhibitor of tumor induced intracellular oxidative stress.

MEIC treatment decreased the volume of solid tumor and it may concluded that the extract, by a direct cytotoxic effect arresting the tumor cell growth. The results were also supported by the cytotoxic effects of the extract towards EAC

and DLA cell lines.

The exact mechanism by which *I. cassioides* mediates its antitumor activity is unknown. However, some compounds present in the extract such as flavonoids, terpenoids and saponins [19, 20] could explain these results. These compounds have been mentioned as antioxidants and consequently involved in antitumor activities. Flavonoids have been found to possess antimutagenic and antimalignant effect. Moreover, they have a chemo preventive role in cancer through their effects on signal transduction in cell proliferation and inhibition of neovascularization [21-25]. Saponins have been found beneficial targeted on inhibition of tumor angiogenesis by suppressing its inducer in the epithelial cells of blood vessels and then on adhering, invasion and metastasis of tumor cells. They also exhibit the antitumor effect by cell cycle arrest and apoptosis [20]. We propose that the additive and synergistic antioxidant activity of phytochemicals such as flavonoids, polyphenols and terpenoids present in *I. cassioides* are responsible for its potent antitumor activity which can be inferred from the increased life span of tumor bearing mice and the inhibition of solid tumor growth.

In conclusion, the MEIC treatment was effective in inhibiting the tumor growth in vitro and in vivo models. Further investigations are in progress in our laboratory to identify the active principles and the mechanisms involved in this antitumor activity.

Conflict of Interest statement

We declare that we have no conflict of interest.

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