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ANTICANCER AND ANTIOXIDANT ACTIVITIES OF ETHANOL EXTRACT OF *INDIGOFERA CORDIFOLIA* ROTH. ON EHRLICH ASCITES CARCINOMA TUMOUR BEARING MICE

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ABSTRACT

Indigofera cordifolia Roth. belongs to Fabaceae, is an Indian medicinal plant. It has been used in traditional medicine to treat various disorders including cancer. Objective of the present work is to evaluate the anticancer and antioxidant potential of ethanol extract of *I. cordifolia* (EIC) against EAC tumour bearing mice. Antitumour activity was assessed by monitoring the mean survival time, percentage increase in life span, effect on hematological parameters, antioxidant enzyme levels, lipid peroxidation and solid tumour volume. 5-fluorouracil (5-FU, 20 mg/kg/i.p) was used as a standard. EIC at the dose of 200 and 400 mg/kg, significantly increase the mean survival time, exerted a protective effect on the hemopoietic system, prevented lipid peroxidation and restored the antioxidant enzymes catalase, superoxide dismutase, glutathione peroxidase and glutathione-*s*-transferase in the liver of tumour control animals. It also significantly reduces the solid tumour volume. The results obtained strongly support that EIC shows potent anticancer activity against EAC cells. The extract also prevents lipid peroxidation and increase the antioxidant enzymes level in tumour bearing animals which might be due to the presence of a variety of phytochemicals in EIC. The present study supports the ethnomedical use of *Indigofera cordifolia*.

Key words: Anticancer, Antioxidants, Lifespan, *Indigofera cordifolia* Roth., Tumour volume.

INTRODUCTION

Over the past few years, cancer has remained a major cause of death and the number of individuals affected with cancer is continuing to expand. Hence, a major portion of the current pharmacological research is devoted to anticancer drug design customized to fit new molecular targets [1]. Due to enormous propensity of plants, which synthesize a variety of structurally diverse bioactive compounds, the plant kingdom is a potential source of chemical constituents with antitumour and cytotoxic activities [2, 3]. India is a rich source of medicinal plants and a number of plant extracts have been used in various systems of medicines such as Ayurveda, Siddha and Unani to cure a range of diseases. Only a few of them have been scientifically explored. The rich and diverse plant sources of India are likely to provide effective anticancer agents. Plant derived natural products such as flavonoids, terpenoids and alkaloids have received significant attention in recent years due to their varied pharmacological properties including cytotoxic and cancer chemopreventive effects. One of the best approaches in the search of anticancer agents from plant sources is the selection of plants based on ethnomedical leads [4].

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Several members of the species of *Indigofera* like *Indigofera trita, Indigofera aspalathoides* etc. are used traditionally for a wide variety of ethnomedical properties such as antitumour, hepatoprotective, antioxidant, anti-inflammatory and analgesic [5-8]. Among them, *Indigofera cordifolia* (Fabaceae) is a small trailing, much branched annual or biennial herb, distributed throughout India. It has long been used by tribes and native medical practitioners to treat rheumatism, arthritis, inflammation, tumour and liver diseases [9]. Earlier studies performed in our laboratory on this plant exhibited potent in vitro antioxidant and anticancer activities [10]. In continuation of our previous findings and based on the ethno pharmacological literatures, *Indigofera cordifolia* was selected to prove scientifically having anticancer property on experimental animal model by using transplantable tumours.

MATERIALS AND METHODS

Chemicals

5-Fluorouracil (5-FU) was obtained from Dabur Pharmaceutical Ltd (New Delhi, India). Thiobarbituric acid and trichloroacetic acid were procured from HiMedia (Mumbai, India). Dimethyl sulfoxide and ethanol were obtained from Loba Chemie (Mumbai, India). All other chemicals used were of analytical grade.

Plant material and Extraction

Entire plants of *I. cordifolia* were collected from Tiruchengode, Tamilnadu. 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. Col. IC 006). The plant material was shade dried, pulverized and extracted (500 g) with ethanol (95% v/v) using soxhlet apparatus for 72 h. The prepared extract was filtered and concentrated to dryness under reduced pressure and controlled temperature in a rotary evaporator. The extract was then stored in a refrigerator until further use.

Preliminary phytochemical screening

Prepared ethanolic plant extract of *I. cordifolia* was analyzed for the presence of various phytochemical constituents employing standard procedures [11-13]. Conventional protocol for detecting the presence of steroids, alkaloids, tannins, flavonoids, glycosides, etc., was used.

Tumour cells and inoculation

EAC cells were obtained from Amala Cancer Research Centre (Trissur, Kerala, India). The cells were maintained *in vivo* in Swiss albino mice by intraperitoneal transplantation. Tumour cells aspirated from the peritoneal cavity of mice were washed with normal saline and were used for further studies.

Preparation of extract suspension

EIC was suspended in distilled water using sodium carboxy methyl cellulose (CMC, 0.3%) and administered orally to the animals with the help of an intragastric catheter to study *in vivo* antitumour activity.

Animals

Healthy male Swiss albino mice (20-25 g) were utilized all the way through the study. They were housed in standard microlon boxes and were given standard laboratory diet and water *ad libitum*. The study was conducted later than obtaining Institutional Animal Ethical Committee clearance.

Acute toxicity studies

The oral acute toxicity study of EIC was carried out in Swiss albino mice using the OECD guidelines AOT 423 [14]. Healthy, young and non-pregnant female mice were randomly selected and kept in their cages for at least 5 days prior to the study. The overnight fasted animals were weighed and administered the plant extract (in 0.3 % sodium CMC) as a single dose (2000 mg/kg) by oral route using oral intubation canula. After administration of the extracts, the animals were observed for toxic symptoms and mortality continuously for first 4 hours. Finally the number of survivors were noted after 24 hours and the observation made daily for a period of 14 days.

Antitumour Studies

Effect of EIC on survival time of EAC Tumour Bearing Mice

Healthy Swiss albino mice were divided into four groups (I-IV) each group consisting of six animals. Group I – IV were inoculated with EAC cells (1 X 10^6 cells/mouse) on day '0' and treatment with EIC started 24 h after inoculation. Group I served as tumour control which received the vehicle (CMC, 0.3 %). Group II animals were treated with the standard drug (5-FU, 20 mg/kg) by intraperitoneal route. Group III and IV 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 using the following formula [15].

Increase of life span = [T-C/C]X100

Where T = number of days treated animals survived and C = number of days control animal survived.

Effect of EIC on hematological parameters of EAC Tumour Bearing Mice

In order to detect the influence of EIC on hematological status of tumour bearing animals, a comparison was made among four groups (n=5 animals per group) of mice on the 14^{th} day after inoculation [15]. Group I served as normal control which received the vehicle (CMC, 0.3 %). Group II served as tumour control,

group III &, IV 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 total red blood cell (RBC), white blood cells (WBC), lymphocytes (LYM), packed cell volume (PCV) and hemoglobin (HGB) were analyzed using Celldyn cell analyzer. Total protein was also an analyzed by using autoanalyzer.

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 °C. The supernatant, thus obtained was used for the estimation of lipid peroxidation (LPO) [16], catalase (CAT) [17], superoxide dismutase (SOD) [18], glutathione peroxidase (GPx) [19] and glutathione S-transferase (GST) [20].

Effect of EIC on solid tumour volume

Mice were divided into three groups and each group consisting of six animals. All animals were injected EAC cells (2 x 10^6 cells/mouse) into the right hind limb intramuscularly. Group I served as tumour control. Group II & III were treated with EIC at the dose of 200 mg/kg and 400 mg/kg by oral route, respectively, for five alternative days. From the 15^{th} day onwards, tumour diameter was measured every fifth day and recorded up to 30 days by using vernier calipers. The tumour volume was calculated by using the formula $V = 4/3\ \pi r^2$, where 'r' is the mean of r_1 and r_2 which are the two independent radii of the tumour mass [15].

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 tumour volume were analyzed by Dunnett test. P values <0.05 were considered significant.

RESULTS

Phytochemical Analysis

Preliminary phytochemical screening of the plant extract showed the presence of various phytochemical constituents. The ethanolic extract of *I. cordifolia* (EIC) showed the presence of a variety of phytochemicals such as terpenoids, phytosterols, alkaloids, amino acids, flavonoids, saponins, glycosides, tannins, phenolic compounds, proteins and amino acids. Gums and mucilage were found to be absent in the extract. The results are displayed in table 1.

Acute Toxicity Studies

The ethanolic extract of *I. cordifolia* (EIC) was subjected to study the acute toxicity studies. In the acute toxicity study, the ethanol extract of *I. cordifolia* did not cause any mortality in mice up to 2000 mg/kg. None of the doses tested produced any gross apparent effect on general motor activity, muscular weakness, fecal output, feeding behaviour etc. during the period of observation. This indicated that the extract was found to be safe at the tested dose level. Hence $1/10^{th}$ (200 mg/kg) and $1/5^{th}$ (400 mg/kg) of this dose were selected for the *in vivo* studies.

***In vivo* antitumour studies**

Antitumour activity of ethanol extract of *I.cordifolia*(EIC)

Effect of EIC on mean survival time

There was a significant (P<0.001) increase in mean survival time and percentage increase in life span on EAC tumour bearing mice in a dose dependent manner. The results were almost comparable to that of 5-FU, the standard drug (Table 2).

Effect of EIC on hematological parameters

Hematological parameters of EAC tumour 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 tumour bearing animals, accompanied by an increase in WBC, packed cell volume (PCV), differential counts and protein. At the same treatment interval, EIC at the dose of 200 and 400 mg/kg changed these altered parameters significantly to near normal. All these results suggest the anticancer nature of the extract (Table 3).

Effect of EIC on antioxidant parameters

The levels of lipid peroxidation in liver tissue were significantly increased in EAC tumour control groups as compared to the normal group (P<0.001). After administration of EIC at different doses to EAC bearing mice, the levels of lipid peroxidation were significantly (P<0.001) reduced as compared to tumour control groups. Inoculation with the tumour cells drastically increased the GST and GPx content in both tumour control groups as compared with normal group. Administration of EIC at the tested doses decreased GST and GPx levels as compared with the tumour control group (P<0.001).

The levels of superoxide dismutase (SOD) in the livers of the EAC tumour bearing mice decreased significantly (P<0.001) when compared with normal group. After administration of EIC at the tested doses, increased levels of SOD as compared with the tumour control groups were observed (P<0.001). The catalase (CAT) levels in EAC tumour control group decreased as compared with normal group. Treatment with EIC increased catalase levels as compared to that of tumour control groups (Table 4).

Effect of EIC on solid tumour volume

The solid tumour volumes of EAC tumour

bearing mice are presented in Table 5. In the EAC tumour models, the extract significantly (P<0.01) reduces the tumour volume in dose dependent manner when compared to tumour control groups.

Table 1. Preliminary phytochemical analysis of ethanol extract of *I. cordifolia*

Name of the Phyhtochemical Constituents	Name of the Extract
	Ethanol Extract of *Indigofera cordifolia* (EIC)
Carbohydrates	+
Phytosterols	+
Alkaloids	+
Glycosides	+
Terpenoids	+
Proteins & aminoacids	+
Saponins	+
Tannins	+
Flavonoids	+
Fixed oils & Fats	+
Gums & Mucilages	-

Note: (+) Presence (-) Absence

Table 2. Effect of Ethanol Extract of *Indigofera cordifolia* on Mean Survival Time and Average Increase in Body Weight of EAC Tumour Bearing Mice

Design of Treatment	Mean Survival Time (in days)	Increase in Life Span (%)	Increase in Body weight (g)
Tumour control	17.3 ± 0.68	------	12.6 ± 2.07
5-FU	33 ± 1.12[a]	90.75	4.2 ± 0.97[d]
EIC 200	22.6 ± 1.45[b,c]	30.64	9.6 ± 1.17[e]
EIC 400	30 ± 1.27[a]	73.41	6.3 ± 0.83[e]

N=6; Values were expressed as mean ± SEM; [a]P<0.001; [b]P<0.05 vs Tumour Control; [c]P<0.001; [d]P<0.01;[e]P<0.05 vs 5-FU; Data were analysed by one way ANOVA followed by Tukey Kramer multiple comparison test.

Table 3. Effect of Ethanol Extract of *Indigofera cordifolia* on Hematological Parameters of EAC Tumour Bearing Mice

Design of Treatment	Hemoglobin (g/dL)	RBC (M/uL)	WBC (K/uL)	Packed Cell Volume (mm)	Total Protein (g/dL)	Lymphocytes (%)
Normal Control	10.94 ± 0.48	5.78±0.34	5.86±0.33	14.76±1.06	4.5±0.60	76.24±4.41
Tumour Control	3.6±0.28[a]	2.74±0.27[a]	15.44±0.84[a]	27.11±1.75[a]	8.8±0.72[a]	43.11±3.45[a]
EIC 200	7.62±0.50[f]	4.04±0.84[f]	12.44±0.62[f]	20.48±1.14[c]	5.31±0.2[d]	57.36 ±2.57 [b,e]
EIC 400	9.96±0.44 [e]	5.22±0.46[e]	7.62±0.38[d]	15.61±1.26[f]	4.62±0.34[d]	71.04±5.63[d]

N=5; Data were expressed as Mean ± SEM; [a]P<0.001; [b]P<0.01; [c]P<0.05 *vs* Normal; [d]P<0.001; [e]P<0.01; [f]P<0.05 *vs* Tumour Control. Data were analysed by One way ANOVA followed by Tukey Kramer Multiple comparison test.

Table 4. Effect of ethanol extract of *Indigofera cordifolia* on antioxidant enzymes and lipid peroxidation levels of EAC Tumour Bearing Mice

Design of Treatment	SOD	CAT	GPx	GST	LPO
Normal Control	0.45 ± 0.06	39.6 ±1.16	18.86 ± 0.52	0.24 ± 0.02	9.16± 1.12
Tumour Control	0.14 ±0.03[a]	16.3 ±0.96[a]	43.3 ± 1.67[a]	0.46 ±0.04[a]	38.8 ±2.16[a]
EIC 200	0.28 ± 0.01	27.3 ±1.02[a,d]	31.4 ± 2.67[a,d]	0.31 ± 0.01[e]	16.3±1.06 [c,d]
EIC 400	0.41±0.04 [e]	32.8 ± 1.63[b,d]	22.6 ± 1.24[d]	0.28 ± 0.03[e]	11.2 ± 0.98[d]

n=5; Data were expressed as mean ± SEM, [a]P<0.001; [b]P<0.01; [c]P<0.05 vs. Normal; [d]P<0.001; [e]P<0.01 vs. Tumour 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 Ethanol Extract of *Indigofera cordifolia* on Solid Tumour Volume of EAC Tumour Bearing Mice

Design of Treatment	Tumour Volume (cc.)			
	15th Day	20th Day	25th Day	30th Day
Tumour Control	1.12 ± 0.15	1.36 ± 0.28	1.84 ± 0.41	2.12 ± 0.31
EIC 200	0.9 ± 0.1	1.02 ± 0.08	1.24 ± 0.06	1.31 ± 0.09[b]
EIC 400	0.58 ± 0.04[a]	0.61 ± 0.03[b]	0.71 ± 0.04[a]	0.82 ± 0.02[a]

N=6; Values were expressed as mean ± SEM; [a]P<0.01; [b]P<0.05 vs Tumour Control. Data were analysed by One way ANOVA followed by Dunnett's Test.

DISCUSSION

Cancer is a disease of misguided cells that have high potential of excess proliferation without apparent relation to the physiological demand of the process. It is the second largest cause of death in the world. Plants belonging to the genus *Indigofera* and several of their constituents have shown potent anticancer properties in many models based on the studies conducted throughout the world. Based on these observations, in the present study, the EIC was evaluated for its *in vivo* antitumour properties.

The reliable criteria for judging the value of any anticancer drug is the prolongation of life span, the disappearance of leukemic cells from the blood and reduction of solid tumour volume [21, 22]. Transplantable tumour cells such as EAC are rapidly growing cancer cells with aggressive behavior. The tumour implantation includes a local inflammatory reaction, with increasing vascular permeability, which results in an intense ascetic fluid accumulation. The ascitic fluid is vital for tumour augmentation, since it constitutes a direct nutritional source for cancer cells [23, 24]. Our results show an increase in life span accompanied by a reduction in WBC count in EIC treated mice. The plant extract also inhibited the accumulation of ascitic fluid in the peritoneal cavity of the tumour bearing animals. These results clearly demonstrated the antitumour effect of EIC on EAC tumour cells.

The most common problems encountered in cancer chemotherapy are bone marrow suppression and anemia [22, 25]. Anemia is found frequently in cancer patients. Similar results were observed in the present study in animals of the EAC tumour 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. Treatment with EIC brought back the hemoglobin content, RBC and WBC counts near to normal. This indicates that the extract have a significant protective effect on the hemopoietic system.

Excessive production of the free radicals leads oxidative stress, which results in damage to macromolecules such as lipids, and can encourage lipid peroxidation *in vivo.* Malondialdehyde, the end product of lipid peroxidation has been reported to be higher in carcinomatous tissue than in non diseased organ [26]. Glutathione, a powerful inhibitor of neoplastic process, plays a vital role as an endogenous antioxidant system that is originate particularly in high concentrations in liver and is known to have key functions in the protective process. The free radical scavenging system, superoxide dismutase and catalase are present in all oxygen metabolising cells and their function is to provide a defense against the potentially damaging reactive of superoxide and hydrogen peroxide [27]. Decrease in SOD action in tumour bearing animals, which might be due to loss of Mn-SOD activity in cancer cells and loss of mitochondria, leading to a reduction in total SOD activity in the liver. Inhibition of SOD and catalase activities as a result of cancer growth was also reported [22, 28]. Treatment with EIC in different dose levels significantly increased the SOD and catalase levels in a dose dependent manner. Plant derived extracts containing antioxidant principles such as flavonoids, phenolic compounds and tannins showed cytotoxicity towards cancer cells and anticancer activity in experimental animals [22]. Anticancer activity of these antioxidants is either through induction of apoptosis or by inhibition of angiogenesis. The lowering of lipid peroxidation, GST, GPx and increase in levels of SOD and catalase in EIC treated group indicates its potential as an inhibitor of cancer induced intracellular oxidative stress.

In EAC tumour bearing animals, there was a regular and hurried increase in ascetic fluid volume. Ascitic fluid is the direct dietary source for tumour growth and it meets the nutritional requirements of tumour cells [24]. EIC treatment decreased the volume of solid tumour and increases the life span of the tumour bearing animals. Hence it may concluded that EIC, by a direct cytotoxic effect or by decreasing the nutritional fluid volume and arresting the tumour cell growth. The present study revealed that the extract was cytotoxic towards EAC cell lines and it was also found to be potent cytotoxic against human cancer cell lines. The cytotoxic potency of the extract was confirmed by the *in vitro* cytotoxic assay methods against animal cancer cells lines and human cancer cell lines. The extract exhibits potent cytotoxicity against all the tested cancer cell lines. At the same time, the IC_{50} for the normal cell line was found to be high when compared to cancer cells, which indicated that the extract is having cytotoxicity against the cancer cells, but it is safe for normal cells.

Preliminary phytochemical studies indicated the occurrence of flavonoids, saponins, tannins and phenols in EIC. Many such compounds are known to possess potent antitumour properties [4]. The extract of *Indigofera cordifolia* is rich in flavonoids and saponins. Flavonoids have been found to possess antiangiogenic, antimutagenic and antimalignant effect [26, 27]. Moreover, they have a chemo preventive role in cancer through their effects on signal transduction pathway in cell proliferation and inhibition of neovascularization [28, 29]. Antitumour and cytotoxic properties of the extract may be due to these phytochemical constituents.

CONCLUSION

In conclusion, the present study demonstrates the potent antitumour properties of ethanolic extract of *Indigofera cordifolia*. Further studies to characterize the active principles and to elucidate the mechanism of action are in progress.

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CONFLICTS OF INTEREST STATEMENT

The authors have no potential conflicts of interest.

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