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Research Article

Anticancer Activity of *Clitoria ternatea* Linn. Against Dalton's Lymphoma

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ABSTRACT

The aim of the study was to evaluate the anticancer activity of *Clitoria ternatea* in Dalton's lymphoma(DLA) bearing mice. Tumour was induced in mice by the intraperitoneal injection of DLA cells. After 24 hours of tumour inoculation, methanol extract of *Clitoria ternatea(*MECT*)* was administered at doses of 100 and 200mg/kg body weight for 14 consecutive days. The effect of MECT was assessed using in vitro cytotoxicity, survival time, peritoneal cell count, hematological studies and antioxidant parameters. Treatment with MECT led to a decrease in tumour volume, packed cell volume and viable count. It also increased the non-viable cell count and mean survival time, thereby increasing the life span of EAC bearing mice. Hematological profile reverted to more or less normal levels in the treated group. The results suggest that MECT exhibit significant antitumour effects in DLA bearing mice.

key words: anticancer; antioxidant; Dalton's lymphoma; flavonoids; *clitoria ternatea.*

INTRODUCTION

Cancer is a major public health problem worldwide. It is the second most common cause of death in the developed world and a similar trend has emerged in the developing countries too[1]. According to the American Cancer Society, deaths arising from cancer constitute 2-3% of the annual deaths recorded worldwide.In India, it has been estimated that there is about 1.5 million cases of cancer in the country at any given point of time with about 0.5 million new cancer cases being added every year.

The search of natural products for cancer therapy represents an area of great interest in which plants had been the most important source. The World Health Organization estimates that approximately 80% of the world's inhabitants rely on traditional medicine for their primary health care[2] and that plants have long been used in the treatment of cancer[3]. Drugs obtained from natural sources are perceived to have fewer side effects while having same ability to cure disorders in much the same way as their synthetic counterparts. So it is anticipated that plants can provide potential bioactive compounds for the development of new leads to combat cancer.

Clitoria ternatea Linn (Fabaceae), known as Aparajitha in India is a persistent, herbaceous perennial legume. It is native to south-east Asia and widely distributed through out the world, mainly in tropical countries. The roots, seeds and leaves of this plant are of medicinal importance[4]. The plant is reputed for its folkloric uses in various diseases[5]. The roots are bitter, ophthalmic, laxative, intellect promoting, diuretic, depurative, aphrodisiac and is used as tonic. It is used in ophthalmology, helminthiasis, leprosy, leucoderma, elephantiasis, bronchitis, asthma, ascites, ulcers and fever. The seeds are cathartic and are useful in visceralgia. Leaves are useful in otalgia, hepatopathy and eruptions. The plant has been scientifically studied for various pharmacological activities including antioxidant,[6] anthelmintic,[7,8] analgesic,[9] anxiolytic, antidepressant, anticonvulsant, sedative,[10] hypoglycemic,[11] larvicidal[12] and anticancer[13]activities.It has been found to enhance acetylcholine content in rat hippocampus[14] and is also used as a local anaesthetic[15]. Finotin, a protein isolated from the seeds of the plant possess antimicrobial properties[16]. The present study was undertaken to evaluate antitumour activity of crude methanol extract of *Clitoria ternatea* against Dalton's lymphoma.

MATERIALS AND METHODS

Collection and extraction of plant material: *Clitoria ternatea* seeds were collected from various parts of Kottayam district, Kerala. The plant was identified and authenticated by Dr.V.T Antony, Dept.of Botany, S.B college, Changanassery with the help of herbarium sheets of sample species and a voucher specimen was deposited in the lab.

Preparation of plant extract

The seeds were dried under shade and pulverised.100gram of the seed powder was extracted with methanol by hot continuous extraction method using a soxhlet apparatus. The solvent was evaporated under reduced pressure at 50^{o}C and dried in vacuum. The yield of solvent free methanolic extract was 4.9% (w/w). It was stored in sterile amber coloured storage vials in refrigerator until used for experiment. The dried extract obtained was dissolved in isotonic saline solution and used for all the experiments.

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Table 1: *In vitro* cytotoxicity assay of MECT by trypan blue method

Conc. of MECT	25 µg	50 µg	75 µg	100 µg	200µg
DLA($1X10^6$ cells)	14	24	53	83	100
Lymphocytes	-	-	3	8	12

Animals: Studies were carried out using male Balb/c mice (20-25g) obtained from Veterinary College, Mannuthy, Trichur. They were housed in polypropylene cages in a controlled environment (temperature 25 ± 2^0C, humidity 60-70% and 12 h dark/light cycle). They were given standard pellet diet(M/s Hindustan Lever Ltd., Bombay, India) and water ad libitum.

Tumour cells: Daltons lymphoma ascites tumour(DLA) were obtained from Amala Cancer Research Institute, Trichur. The cells were maintained by the intraperitoneal inoculation of 10^6 cells/mouse every 14 days.

Determination of *in vitro* cytotoxicity

Short term cytotoxicity studies were conducted by incubating $1X10^6$ DLA cells in 1ml PBS containing various concentrations of the extract at 37^0C for 3hrs.The viable cell count was done using trypan blue exclusion method[17]. Normal lymphocytes served as control.

Antitumour activity of MECT

Male albino mice were divided in to four groups of twelve animals (n=12) each.

Group I Pair fed control

Group II Received 1×10^6 DLA cells(i.p)

Group III Received 1×10^6 DLA cells(i.p)+MECT at a dose of 100mg/kg body weight

Group IV Received 1×10^6 DLA cells(i.p)+MECT at a dose of 200mg/kg body weight

DLA cells collected from the donor mouse were suspended in sterile isotonic saline and the viable count was adjusted to 1×10^6 cells/ml. These were injected intraperitoneally (i.p.) on the first day of the experiment. Fourteen doses of MECT were injected intraperitoneally (i.p.) from the first day up to the 14th day at 24-hr intervals. Control animals received only vehicle (saline solution). On day 15, half of the animals (n = 6) in each cage were killed by decapitation. Ascitic fluid and blood were collected for the analysis of various parameters. The remaining animals were kept to observe for the life span of the hosts. The anti-tumor activity of the methanol extract of *Clitoria ternatea* (MECT) was measured in DLA animals with respect to the following parameters:

Effect of MECT on ascites: The antitumour activity of the methanolic extract was measured with respect to the following parameters:

Tumor volume: The volume of the ascitic fluid collected from the peritoneal cavity was measured by taking it in a graduated centrifuge tube and packed cell volume was determined by centrifuging at 1000 rpm for 5min.

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 number of cells in the 64 small squares was counted.

Viable/non-viable tumor cell count: 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 stain were nonviable. These viable and nonviable cells were counted.

$$\text{Cell count} = \frac{\text{No. of cells X dilution}}{\text{Area} \times \text{Thickness of liquid film}}$$

Percentage increase in life span (% ILS)

The effect of MECT on tumor growth was monitored by recording the mortality daily for a period of 6 weeks and percentage increase in life span (%ILS) was calculated.

$$\%\text{ILS} = \left(\frac{\text{Mean survival of treated group}}{\text{Mean survival of control group}} - 1 \right) \times 100$$

$$\text{Mean survival} = \left(\frac{\text{Day of } 1^{st} \text{ death + Day of last death}}{2} \right)$$

Hematological Parameters: Blood collected was used for the estimation of hemoglobin (Hb) content[18], red blood cell count (RBC) and white blood cell count[19].

Estimation of in vivo Antioxidants: After collecting the blood samples, the liver was excised, rinsed in ice-cold normal saline solution followed by cold 0.15 M Tris-HCl (pH 7.4), blotted dried and weighed. A 10% w/v homogenate was prepared in 0.15M Tris-HCl buffer and was used for the estimation of lipid peroxidation (LPO) and reduced glutathione(GSH).The assay of the antioxidant enzymes catalase and superoxide dismutase were also conducted.

Lipid peroxidation was measured in terms of thiobarbituric acid reactive substances, TBARS[20]. Changes in the antioxidant status were assessed by estimating the activities of GSH[21], catalase[22] and superoxide dismutase[23].

Phytochemical analysis: Preliminary phytochemical screening of the methanolic extract of *Clitoria ternatea* was carried out for the detection of phytochemical components using standard conventional protocols.

STATISTICAL ANALYSIS

The results are presented as mean±SEM.One-way analysis of variance (ANOVA) followed by Dunnet's test was applied for statistical analysis. p values <0.05 are considered significant.

RESULTS

In vitro cytotoxicity assay: The results of the *in vitro* cytotoxicity test by the trypan blue method is given in Table 1. 53% percentage cell death occured at a concentration of 75 µg, 83% at 100 µg and 100% at a concentration of 200 µg, whereas the effect of the extract on normal lymphocytes was negligible.

Table 2: Effect of MECT on tumor volume, packed cell volume, cell count and mean survival time in DLA bearing mice

Treatment	Dose	Tumour volume (ml)	Packed cell volume(ml)	Tumour cell count		Median survival time(days)	%ILS
				Viable	Non-viable		
DLA	_	4.2	2.1	8.8	0.8	18	-
	100mg/kg	1.3	0.7	3.2	1.2	34	70
	200mg/kg	2.4	1.1	4.6	0.8	29	45

Table3: Effect of MECT on hematological parameters

Treatment	Hb (gm%)	RBC (million/mm^3)	WBC(10^3 cells/mm^3)	Lymphocyte (%)	Neutrophils (%)	Monocytes (%)
GroupI Normal control	13.5±0.4987	5.8±0.1932	6.3±0.2499	69±4.091	30±1.932	2±0.0775
GroupII DLAcontrol	10.6±0.7339**	3.6± 0.2978**	15.4±0.3661**	31±2.033**	68±2.781**	0±0**
GroupIII DLA+MECT (200mg/kg)	11.8± 0.3759ns	5.4± 0.3396**	7.5±0.3615	54±3.077*	46±2.266 **	1.5±0.057**
GroupIV DLA+MECT (100mg/kg)	11.2±0.7398*	5.1±0.2955*	9.3±0.2887**	46±3.838**	51±2.646 **	1±0.093**

All the values are mean± SEM

p<0.05, p<0.001***

Antitumour activity of MECT: Antitumor activity of MECT against DLA tumor bearing mice was assessed by the parameters such as tumor volume, packed cell volume, cell count (viable and non-viable), mean survival time and percentage increase of life span. The results are shown in Table 2. The tumor volume, packed cell volume and viable cell count were found to be significantly increased and nonviable cell count was significantly low in DLA control animals when compared with normal control animals. Administration of MECT at the dose of 100 and 200 mg/kg significantly decreased the tumor volume, packed cell volume and viable cell count. Non-viable cell count was higher in MECT treated animals when compared with DLA control animals. Furthermore, the median survival time was increased to 34 (%ILS = 70) and 29(%ILS = 45) on administration of MECT at 100 and 200 mg/kg body weight respectively. All these results clearly indicate that the MECT has a remarkable capacity to inhibit the growth of solid tumor induced by DLA cell line in experimental animals.

Hematological Parameters: Hematological parameters (Table 3) of tumor bearing mice were found to be significantly altered compared to the normal group. The total number of RBC showed a notable change, with a reduction of Hb content. The total WBC count was found to be increased in the tumour bearing group. In differential count of WBC, the percent of neutrophils increased while the lymphocyte count decreased. Administration of MECT at a concentration of 100mg/kg treatment restored all the altered hematological parameters to almost near normal. MECT at the concentration 200mg/kg treatment also brought back these altered parameters towards normal, though MECT 100mg treatment was found to be more effective.

Antitumor Effect of MECT on TBARS, GSH, catalase and superoxide dismutase levels: Table 4 illustrates the levels of TBARS, GSH, catalase and superoxide dismutase in tumour bearing and experimental groups. ROS formed in cancer tissues results in lipid peroxidation and subsequently to increase in malondialdehyde(MDA) level. In the present study, the levels of MDA were significantly increased in DLA control animals when compared with normal control animals. The treatment with MECT at 100 mg and 200 mg/kg significantly reduced the MDA levels when compared with DLA control animals. The levels of reduced GSH were significantly decreased in DLA control group when compared with normal control group. The levels of reduced GSH were found to be increased on administration of MECT at 100mg and 200mg/kg when compared with DLA control group.

The levels of catalase and superoxide dismutase in tumour bearing and experimental groups is shown in table 4.The concentration of these antioxidant enzymes decreased in DLA bearing mice. Administration of MECT at 100 mg and 200 mg/kg significantly increased the levels of the enzymes when compared with DLA control animals.

DISCUSSION

The present investigation was carried out to evaluate the antitumor activity and antioxidant status of methanolic extract of *Clitoria ternatea (*MECT) in DLA tumor bearing mice. The MECT treated animals at the doses of

TABLE 4. Antitumor Effect of MECT on TBARS, GSH, catalase and superoxide dismutase(SOD) levels in kidney

	TBARS(mM/ 100g tissue)	GSH (mg/g tissue)	SOD(U/mg protein)	Catalase (U/mg protein)
Group I Normal control	1.42±0.2221	3.01±0.2781	4.37±0.2978	13.24±0.3474
Group II DLAcontrol	2.45±0.118**	1.583±0.2098**	1.32±0.1335**	7.488±0.3044**
Group III DLA+MECT (200mg/kg)	1.79±0.228^{ns}	2.647±0.188**	3.68±0.2449^{ns}	10.46±0.4211**
Group IV DLA+MECT (100mg/kg)	1.40±0.1390^{ns}	2.116±0.1211*	2.15±0.1476**	8.032±0.3464**

*All the values are mean± SEM . p<0.001*** , ns-nonsignificant*

100 and 200 mg/kg significantly inhibited the tumor volume, packed cell volume, tumor (viable) cell count, and brought back the hematological parameters to more or less normal levels. The extract also reduced the hepatic lipid peroxidation and increased free radical scavenging GSH as well as antioxidant enzymes SOD and CAT in tumor-bearing mice to near normal levels.

In the tumor bearing mice, a rapid increase in ascitic tumor volume was observed . It was reported that the 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[24]. Treatment with MECT inhibited the tumor volume, viable tumor cell count and increased the life span of the tumor bearing mice. As the reliable criteria for judging the value of any anticancer drug are the prolongation of the life span of animals[25], it may be concluded that MECT by decreasing the nutritional fluid volume and arresting the tumor growth increases the lifespan of EAC-bearing mice. Thus, MECT has antitumor activity against DLA bearing mice.

Usually, in cancer chemotherapy the major problems that are being encountered are of myelosuppression and anemia[26, 27]. 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 due to hemolytic or myelopathic conditions[28]. Treatment with MECT brought back the hemoglobin (Hb) content, RBC and WBC count more or less to normal levels. This clearly indicates that MECT possess protective action on the hemopoietic system.

Excessive production of free radicals resulted in oxidative stress, which leads to damage to macromolecules, for example, lipid peroxidation *in vivo*[29]. Malondialdehyde formed during oxidative degeneration [30] is accepted as an indicator of lipid peroxidation[31]. MDA level was also reported to be higher in cancerous tissues than in normal tissues[32]. Table 4 indicate that TBARS levels in the tumour bearing mice are higher than those in normal mice. It was found to be significantly lowered in the MECT treated experimental group.

Glutathione, a potent inhibitor of the neoplastic process plays an important role in the endogenous antioxidant system. It is found in particularly high concentration in the liver and is known to have a key function in the protective process In the present study, GSH levels decreased in the control group, whereas in experimental mice GSH was found to be significantly elevated when compared to the DLA bearing mice.

SOD and CAT are involved in the clearance of superoxide and hydrogen peroxide. The inhibition of SOD and CAT activities as a result of tumor growth were reported [33].A decrease in SOD activity in DLA bearing mice may be due to loss of Mn^{2+} containing SOD activity in DLA cells and the loss of mitochondria, leading to a decrease in total SOD activity in the tissues [34]. A reduced amount of catalase in tumor cells was also reported [34]. Similar findings were observed in the present study in DLA bearing mice. The administration of MECT at two different doses significantly increased the SOD and CAT levels.

Preliminary phytochemical screening indicated the presence of alkaloids, saponins and flavonoids in MECT. Flavonoids have received considerable attention in recent years due to their diverse pharmacological properties including antioxidant and antitumor activity [35, 36].Moreover, flavonoids have a chemopreventive role in cancer through their effects on signal transduction in cell proliferation[37] and angiogenesis[38]. The cytotoxic and antitumor properties of the extract may be due to these compounds. The present study points to the potential anticancer activity of *Clitoria ternatea*.

In conclusion, the present study demonstrates that the methanol extract of *Clitoria ternatea (MECT*) increased the life span of EAC tumor bearing mice and decreased lipid peroxidation and thereby augmented the endogenous antioxidant enzymes in the liver. All these parameters suggest that the methanol extract of *Clitoria ternatea (MECT*) seeds exhibits potential antitumor and antioxidant activities.

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