Anticancer activity of *Cyathula prostrata* (Linn) Blume against Dalton's lymphomae in mice model

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Abstract: *Cyathula prostrata* (Linn) Blume herbs are commonly used for the treatment of inflammatory and pain in Nigeria. The objective of the present study was to assess the antitumor and antioxidant activity of *Cyathula prostrata* (Linn) Blume in mice model. The treatment of Dalton's lymphoma ascites cells induced tumor by the methanolic extract of *Cyathula prostrata* was determined at concentration of 100 mg/ kg body weight given orally for 11 days, antitumor activity was assessed by monitoring the mean survival time, body weight, effect on hematological parameters, antioxidant enzyme levels and histopathological evidence. The results showed that the methanolic extract of *Cyathula prostrata* increased the survival period of animals, decreased the body weight and also altered many hematological markers and also restored the antioxidant enzymes when compared to the mice of the DLA control group. These findings indicate that the methanolic extract of *C. prostrata* has anti-tumor activity by preventing the lipid peroxidation and thereby promoting the antioxidant systems in Dalton's lymphoma ascites induced mice. So, these extract could be a natural anticancer agent for human health.

Keywords: Dalton's lymphoma ascites, antitumor, *Cyathula prostrata,* hematological, antioxidant.

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. Treatment at an early stage of carcinogenesis with chemo preventive drugs will be a better option than attempts to rule out fully developed tumors. The safety aspects of these chemo preventive agents are narrow due to drug resistance and limiting toxicity, which may damage the host cells. Natural products may play an important role in cancer treatment (Sohail, 2013). Sixty percent of anticancer agents which are used currently are sources of natural products including plants, which act as an effective anticancer agent. Nowadays, pharmaceutical industries focus on natural products and have started researching on the pharmacological evaluation of plants used in traditional system of medicine (Newman *et al.,* 2003).

Cyathula prostrata (Linn.) Blume, the young foliage is red coloured is an annual herbs, commonly disseminated in Africa, Asia, Australia and America. *C. prostrata* is a tidy of cultivated areas, waste land and forest margins. In the Ivory Coast, the debilitates is applied to aching, chancres, ear drops for otitis and headache, seeds used for paste with or without clay are used on sores, burns and fractures. Traditionally articular rheumatism, dysentery is treated with *C. prostrata* plant in Nigeria and Cameroon.

Similarly *C. prostrata* used for the treatment of eye troubles, wounds and urethral discharges in Gabon (Burkill, 1995). Hence based on the ethnomedical claims, the present study was investigated to explore the possible *in vivo* antitumor activity of methanolic extract of *C. prostrata* on DLA cells induced tumor in animal model.

MATERIALS AND METHODS

Collection and authentication of Cyathula prostrata
The fresh leaves of *Cyathula prostrata* (Linn.) Blume (Amaranthaceae) were collected from Kerala, India. Taxonomic authentication was done by Dr. V. S. Ramachandran, Taxonomist, Bharathiar University, Coimbatore, Tamilnadu, India.

Preparation of methanolic extract of C. prostrata leaf (MECP)
The fresh leaves were shade dried with occasional shifting and then powdered with a mechanical grinder, passing through sieve #40, and stored in a tight container. The powdered leaves (100 gm) were extracted with 1000ml of methanol using soxhlet apparatus 65°C for 24 hrs. The extract was filtered through filter paper to get clear filtrate. The filtrate obtained by repeated maceration was evaporated under reduced pressure at 40°C using rotary evaporator. A dark semisolid material obtained, was stored at -4°C, until use.

Chemicals
The chemicals and solvents used in the study were of highest purity and analytical reagents grade. The

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Chemicals were purchased from SD Fine Chem., Himedia and Qualigens, India.

Experimental animals
Seventy days old Swiss albino mice weighing 20±2g were used for the present study. The mice were procured from the Kerala Agricultural University, India. The mice were divided into five groups (six mice per group) and maintained in polyacrylic cages at a temperature of 25±2°C, suitable humidity, dark/ light cycle, with feed and water (*ad-libitum*). The mice were acclimatized to laboratory conditions for 10 days prior to the commencement of the experiment. The animal care and handling was done according to the regulations of Council Directive CPCSEA No: 659/02/a about good laboratory practice (GLP) on animal experimentation. All animal experiments were performed in the laboratory according to the ethical guidelines suggested by the international animal ethics committee (IAEC).

Induction of lymphoma
DLA cells were obtained from Amala Cancer Research Institute, Kerala, India. The cells were maintained *in vivo* in Swiss albino mice by intraperitoneal transplantation of $1x10^6$ cells/mouse. The DLA cells aspirated from the peritoneal cavity of the mice were washed with saline and given intraperitoneally to the experimental animals to develop ascitic tumors.

Experimental design
Thirty Swiss albino mice were divided into five groups (n = 6) and given food and water *ad libitum*. The vehicle and extract were administrated orally for 11 days. Group A served as control group; group B mice induced with DLA cells; group C mice induced with DLA and treatment with Methanolic extract of *Cyathula prostrata* at 100 mg/kg body weight from the next day of induction for 11 days; group D mice induced with DLA cells and treated with Methotrexate at 3.4mg/kg body weight from the next day of induction for 11 days; Group E mice orally administered with methanolic extract of *Cyathula prostrata* at 100 mg/kg body weight for 11 days.

Twenty-four hours from last dose and 18 hours of fasting, 6 animals of each group were sacrificed by cervical dislocation to measure antitumor activity. The blood was collected from the animals by incision in the jugular vein under slight anesthesia (diethyl ether). From the collected blood sample, the following hematological parameters such as RBC, WBC and hemoglobin content were estimated using a cell analyzer (Medonic CA 530, Boule Medical AB, Sweden). The differential counts of WBC were carried out in the blood smear.

Similarly, the serum was analyzed for the following parameters: aspartate amino transferase (AST) and alanine amino transferase (ALT) was measured by the Reitman and Frankel method (Reitman and Frankel, 1957), alkaline phosphatase (ALP) was measured according to the procedure proposed by King and Armstrong (1934), acid phosphatase (ACP) activity was done by the following method of King (1965), 5'-nucleotidase (5'N) level was analysed by the method of Campbell (1962), the quantitative measurement of lipid peroxidation (LPO) in tissue homogenate was determined according to the method described by Niehius and Samuelson (1968), superoxide dismutase (SOD) level was measured by the following the method of *Das et al.* (2000), catalase (CAT) activity was measured by following the method described by Sinha (1972), peroxidase (Px) was done by following the method of Addy and Goodman (1972), total reduced glutathione (GSH) content was measured by a colorimetric technique (Moron *et al.,* 1979), levels of vitamin C (vit C) and vitamin E (vit E) was analysed by the following methods of Omaye *et al.* (1979) and Rosenberg (1992) respectively.

Histopathological study
Histopathological studies were carried out by the technique of Dunn, 1974. Liver tissue was placed in 10% formal saline (10% formalin in 9% sodium chloride) for 1 hr to rectify shrinkage due to higher concentration of formalin. The tissue was dehydrated by ascending grades of isopropyl alcohol by immersing in 80% isopropanol overnight and 100% isopropyl alcohol for 1 hr. The dehydrated tissues were cleared in two changes of xylene, 1 hr each. Then the tissues were impregnated with histology grade paraffin wax (melting point 58- 60°C) at 60°C for one hour. The wax – impregnated tissues were embedded in paraffin blocks using the same grade wax. The paraffin blocks were mourned and cut with a rotary microtone at three micron thickness. The sections were floated on a tissue floatation bath at 40°C and taken on glass slides and smeared with equal parts of egg albumin and glycerol. The sections were then melted in an incubator at 60°C and after 5 mins the sections were allowed to cool. The liver and spleen sections were stained with Ehrlich's hematoxylin and were examined microscopically and photographed for the evaluation of histopathological changes.

STATISTICAL ANALYSIS

The results obtained were reported as mean ± SD. One way Analysis of Variance (ANOVA) was performed to analyze statistical significance of the data using Agres Statistical Package.

RESULTS

Table 1 showed that the activity of methanolic extract of *Cyathula prostrata* increased the average life span of DLA cells bearing mice from 20 to 54 days when

compared to that of DLA cells induced group mice, methotrexate (standard drug) also significantly increased the life span to 55 days at 3.4mg/kg body weight. These results supported by that the methanolic extract of *Cyathula prostrata* having the property of percentage increase in body weight in dose dependent manner when compared to that of DLA cells induced mice with MECP treatment mice (fig. 1).
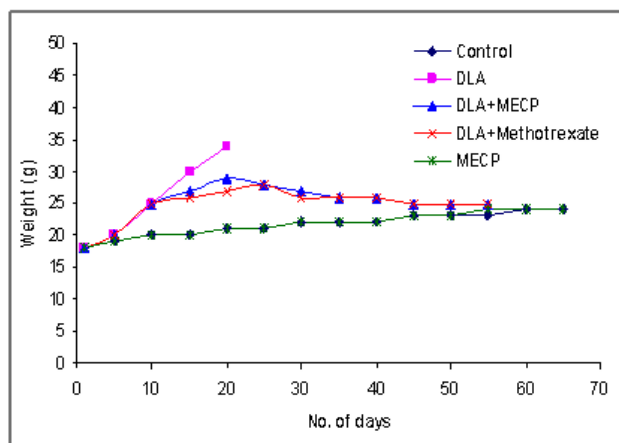

Fig. 1: Survival period verses body weight of control and dalton's lymphoma ascites induced mice

DLA cells induced mice showed (table 2) the significant increased level of total WBC (10.88±0.44), neutrophils and a significant reduced levels of RBC (2.10±0.24), hemoglobin, platelet count, eosinophils and lymphocytes (7.50±0.57; 132.59±1.00; 4.03±0.06 and 33.65±0.62) when compared to the control mice (P<0.05). The treatment of *Cyathula prostrata* extract and also standard drug reversed these parameters towards normal, also treatment of MECP showed a significant decreased levels of WBC, neutrophils and increased levels of RBC and lymphocytes when compared with DLA control animals was observed in dose dependent manner.

Table 3 shows the activities of marker enzymes in control and lymphoma bearing animals respectively. In lymphoma bearing animals, the activities of the AST and ALT were significantly (69.40±2.48; 82.87±4.98) increased, whereas ALP, LDH and 5'NT were found to be significantly (56.51±3.81; 40.02±1.28; 8.97±1.33) increased when compared to the control mice (P<0.05). All the marker enzymes were found to be significantly increased in the serum of tumor bearing animals when compared to normal control mice. On MECP treatment, the activities of all these enzymes were significantly brought back to near normal levels.

In the present study a significant increase in lipid peroxidation (fig 2) was observed in DLA induced mice (Group B). DLA cells induced mice showed a significant (P<0.05) decreased levels of SOD (2.04±0.09), CAT (15.63±1.40) and POD (20.13±1.85). The treatment of

MECP at 100 mg/kg body weight showed a significant increased levels of SOD (6.15±0.36), CAT (22.21±1.60) and POD (33.92±1.20) when compared to the normal mice group (P<0.05). The levels of non-enzymic antioxidant were significantly depleted in mice induced with DLA. Treatment of MECP significantly increased the levels of non-enzymic antioxidants in the DLA induced animals (table 4).
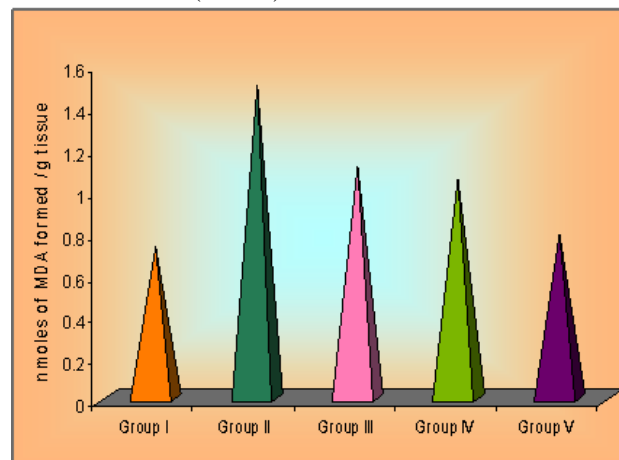

Fig. 2: Lipid peroxidation of control and Dalton's lymphoma ascites induced mice

Histoarchitecture of the liver tissue under a light microscope was done to observe the efficacy of MECP on the morphological structure of the cells. The normal mice liver tissue showed the normal histoarchitecture under a light microscope (fig. 3a). DLA cells induced mice liver tissue showed slightly enlargement in the hepatocytes, dilated sinusoidal, and collections of lymphocytes (fig. 3b). The treatment of MECP at 100mg/kg body weight showed normal architecture of livers tissue no lymphocytes, indicating its efficacy of hepatoprotective activity when compared to (methotrexate) standard drug treatment (fig. 3c). Methotrexate (at 3.4 mg/kg body weight) treated mice liver tissue exhibited normal histopathological appearance, with a few lymphocytic collections in the portal area (fig. 3d) and the animal treated with MECP (extract alone) at 100mg/kg body weight also showed normal architecture of liver tissue (fig. 3e).

DISCUSSION

Many of chemical compounds or substances from natural resources used for cancer treatment also as lead compounds for further research. Many plant extracts are used for cancer treatment in the Indian traditional system of medicine, but most of these plants have not been technically evaluated for cancer treatment (Pushpangadan *et al.*, 1995). Therefore, a great possibility exists for identifying new potent anticancer compounds from plants. Based on ethnomedical claims, the present study was to evaluate the methanolic extract of *C. prostrata* (MECP) for *in vivo* antitumor properties.

Table 1: Effect of methanolic extract of *C. prostrata* on survival period of control and experimental mice

Groups	Survival in days	Life span (%)	Increase in life span (%)
Control	> 60	-	-
DLA	20.0 ± 0.82	100	-
DLA + MECP	54.5 ± 0.58	179.49	79.49
DLA +Methotrexate	55.25±0.50	184.60	84.60

% - Percentage; DLA - dalton's lymphoma ascites; MECP – methanolic extract of *C. prostrata*
Values are expressed by mean ± SD of four animals in each group

Table 2: Effect of methanolic extract of *C. prostrata* on hematological parameters of experimental mice

Parameters	Group A	Group B	Group C	Group D	Group E
Hb (g/dl)	12.99±0.70	7.50±0.57a	9.17±0.46b	10.17±0.99b	7.08±0.33b
RBC ($1x10^6$/mm^3)	5.20±0.46	2.10±0.24 a	2.90±0.16b	3.34±0.38b	5.06±0.25b
WBC ($1x10^3$/mm^3)	5.25±0.45	10.88±0.44a	8.15±0.30b	6.77±0.42b	5.23±0.51b
PLT (10^3/mm^3)	168.91±2.14	132.59±1.00a	156.00±1.81b	161.90±0.94b	172.90±1.06b
WBC Differential count (%)					
Lymphocytes	67.08±1.06	33.65±0.62a	54.62±0.62b	61.95±0.92b	67.55±1.18b
Neutrophils	33.17±0.72	68.35±0.68a	44.98±0.78b	36.59±0.48b	33.52±0.56b
Eosinophils	5.76±0.09	4.03±0.06a	4.86 ± 0.07b	4.98±0.06b	5.65±0.16b

Results were expressed as Mean ± SD (n=6). [a]$P<0.05$ compared with normal group of mice. [b]$P<0.05$ compared with DLA-induced group of mice

Table 3: Effect of methanolic extract of *C. prostrata* on the activities of tissue marker enzymes in liver of control and Dalton's lymphoma ascites induced mice

Parameters	Group A	Group B	Group C	Group D	Group E
AST	39.68±1.67	69.40±2.48a	50.15±2.66b	49.68 ± 1.40b	40.25±1.22b
ALT	69.31±3.23	82.87±4.98a	75.85±3.91b	74.07 ± 3.59b	70.13±3.07b
ACP	4.93 ± 1.13	15.42±1.46a	9.12 ± 0.82b	8.09 ± 0.78b	5.10 ± 0.42b
ALP	32.67±1.82	56.51±3.81a	41.19±2.57b	40.84 ± 1.36b	33.28±1.66b
LDH	21.07±0.82	40.02±1.28a	26.26±0.69b	25.34 ± 0.63b	21.87±0.48b
5'NT	5.28 ± 0.89	8.97 ± 1.33a	6.09 ± 0.20b	5.46 ± 0.73b	5.47 ± 0.64b

Results were expressed as Mean ± SD (n=6). [a]$P<0.05$ compared with normal group of mice. [b]$P<0.05$ compared with DLA-induced group of mice. AST, ALT & LDH – nmoles of pyruvate liberated /min / mg protein; ACP & ALP - nmoles of phenol liberated /min / mg protein; 5'NT-nmoles of phosphate liberated /min / mg protein.

Table 4: Effect of methanolic extract of *C. prostrata* on the levels of enzymic antioxidants in liver of control and Dalton's lymphoma ascites induced mice

Groups	SOD	CAT	POD
Group A	8.01±0.22	28.55±1.64	36.00±1.13
Group B	2.04±0.09[a]	15.63±1.40[a]	20.13±1.85[a]
Group C	6.15±0.36[b]	22.21±1.60[b]	33.92±1.20[b]
Group D	6.30 ± 0.19[b]	23.29 ± 1.62 [b]	33.87 ± 1.88[b]
Group E	8.04±0.44 [b]	28.29±1.48 [b]	36.09±1.29[b]

Results were expressed as Mean ± SD (n=6). [a]$P<0.05$ compared with normal group of mice. [b]$P<0.05$ compared with DLA-induced group of mice. SOD–50% inhibition of nitrate / min / mg protein; CAT – µmoles of H_2O_2 decomposed / min / mg protein; POD – µmoles of H_2O_2 reduced/min/mg protein,

Ascites 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. The reliable criteria for judging the value of an anticancer drug is the prolongation of the life span of animals. 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 due to hemolytic or myelopathic conditions (Gupta *et al.,* 2004).

Tumor growth is generally associated with marked changes in hematopoiesis and immune response, myelosuppression and anemia (Kumarappan and Mandal, 2007). Anemia has been found to not only reduce the quality of life, but also to reduce survival rates. The red blood cell flux is greater in tumors than in normal tissues, and the tumor cells have the capacity to lyse erythrocytes through a hemolytic factor. Lymphocytopenia was also observed in animals with cancer, and this condition is not clearly understood. Impaired lymphocyte activation is a common feature in patients with cancer (Taveira *et al*., 2008). The present findings are similar to Natesan *et al.,* (2007), who reported the decreased level of RBC and hemoglobin with simultaneous increased level of WBC in DLA bearing mice and were reverted to near normal on treatment with methanolic extract of *Careya arborea* bark against lymphoma bearing mice.

Biochemical markers are used as an adjunct for diagnosis and prognosis of disease. Malignancy can be explained on the basis of the biochemical changes in the tissue. Extensive biochemical studies have been carried out on tumor tissue and peripheral blood to explore the etiology of cancer and to establish tumor markers (Jackson *et al.,* 2008). Liver damage induced by tumor cells generally reflects disturbances in liver cell metabolism, which can lead to characteristic changes in serum enzyme activities.

It was reported that the presence of tumors in the human body or in experimental animals is known to affect many functions of the vital organs especially the liver, even when the site of the tumor does not interfere directly with organ functions (Dongre *et al.,* 2008). The increased levels of ACP, ALP, LDH (P<0.05) in serum and liver tissue, of DLA induced mice showed that the liver damage and loss of functional morphology of cell membranes, and elevated levels of AST, ALT in the liver tissues of the DLA induced tumor control mice group. Elevated levels of AST and ALT in the liver tissue due to the damage of the hepatocellular, which are released from liver into the blood and also ALP activity, increase the ALP activity is due to increased biliary pressure. Restoration of these marker enzymes level in liver tissues, is an indicate stability of plasma membrane and also repairment of tissue damage caused by DLA cells induction. As a consequence, decreased levels of microsomal enzymes activities showed in the tissue due to lipid peroxidation. Nevertheless, the standard drug (methotrexate) treatment showed a significant reverted of these alterations when compared to the DLA induced mice group. These distorted parameters were restored towards the normal levels by MECP treatment, indicating its hepatoprotective nature (Natesan *et al.,* 2007). Lipid peroxidation was noticed that tumor cells produced more peroxides when they proliferate actively after induction of tumor. This, increased levels of peroxides indicate that the production of oxygen free radicals. The end product of
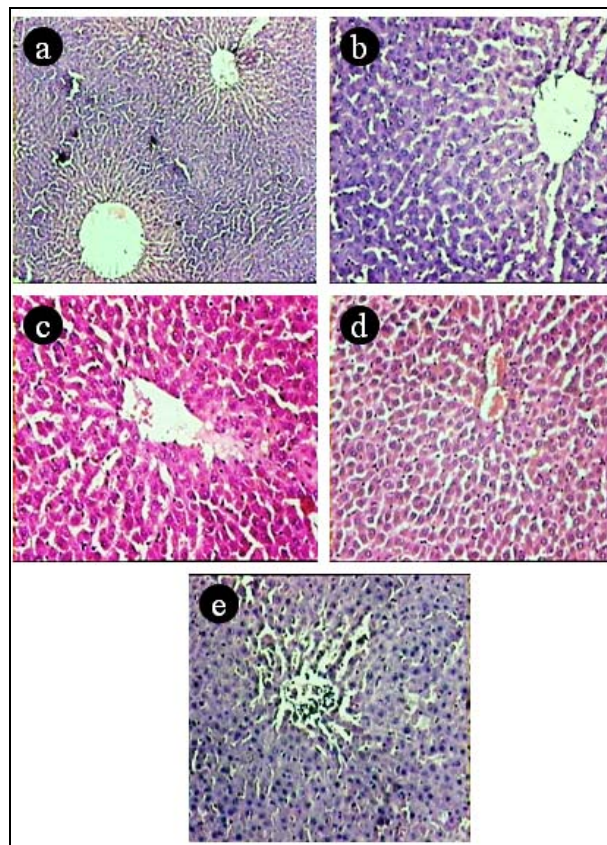

Fig. 3: Histopathological observation of control and dalton's lymphoma ascites induced mice: The liver of normal animals showed normal histological appearance (fig. 3a). The tumor control animal liver showed slight enlargement of hepatocytes, dilated sinusoidal spaces containing lymphocytes, and portal triads showing collections of lymphocytes (fig. 3b). The animals treated with methanolic extract of *C. prostrata* at 100 mg/kg body weight also showed normal histology with no lymphocytes in the portal area, indicating its potent hepatoprotective action when compared to standard methotrexate treatment (fig. 3c). The animals treated with standard methotrexate at 3.4 mg/kg body weight exhibited almost normal histological appearance of liver cells, except for a few lymphocytic collections in the portal area (fig. 3d) and the animal treated with methanolic extract of *C. prostrata* only at 100mg/kg body weight also showed normal architecture (fig. 3e).

lipid peroxidation, MDA levels was higher in cancer tissue than in normal tissues. After the treatment of MECP showed a significant reduced level of TBARS liver tissue. This indicates the scavenging of radicals, restoration of cell membrane morphology and also MDA production (Natesan *et al*., 2007). Our results are in agreement with Manoharan *et al.,* (2005) and Rajneesh *et al.,* (2008). Endogenous enzymatic antioxidants offer protective defenses in the body to limit the levels of reactive

oxidants and the damage they inflict. In addition, consumption of dietary antioxidants appears to be associated with a lowered risk of degenerative diseases (Vijayakumar *et al.,* 2010). Superoxide dismutase is a major intracellular enzyme, which protects against oxygen free radicals by catalyzing the dismutation of superoxide free radical and anions to hydrogen peroxide and oxygen (Rumbey and Paterson, 1998). Besides catalase, peroxidase controls the actual level of peroxide in cells by reduction of H_2O_2 to H_2O at the expense of various phenols which acts as electron donor (Nakamura *et al.,* 1996). A decreased level of SOD, CAT and POD (P<0.05) activities as markers of malignant transformation. Sharma *et al.* (1994) results showed that numerous medicinal plants showed their chemo preventive activity by scavenging free radicals and improvement of antioxidant defense mechanism. Endogenous enzymatic antioxidants offer protective defenses in the body to limit the levels of reactive oxidants and the damage they inflict. In addition, consumption of dietary antioxidants appears to be associated with a lowered risk of degenerative diseases (Omisore *et al.,* 2005).

The chemo preventive effect of MECP is probably due to the presence of several bioactive chemo preventive principles and their synergistic effects. Several authors have suggested that the chemo preventive properties of plant anti-carcinogens are either due to anti-lipid peroxidative action (Scavenging excess ROS), modulating carcinogens detoxification (elevation of carcinogen detoxifying enzymes) or improving the antioxidant defence mechanism (Chandra Mohan *et al.,* 2003).

CONCLUSION

In summary, the methanol extract of *C. prostrata* leaves was effective in inhibiting the tumor growth in ascitic tumor models. The biochemical and histological studies supported its antioxidant and hepatoprotective properties. The present study has also opened avenues for further research especially with reference to the development of potent phytomedicine for cancer from *C. prostrata* leaves.

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