

RESEARCH COMMUNICATION

***Cardiospermum halicacabum* inhibits Cyclophosphamide Induced Immunosuppression and Oxidative Stress in Mice and also Regulates iNOS and COX-2 Gene Expression in LPS Stimulated Macrophages**

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Abstract

The effect of a methanolic extract of *Cardiospermum halicacabum* L was studied against cyclophosphamide (CTX)-induced toxicity in mice. Administration of CTX (25 mg/kg b.wt, i.p.) for 10 days produced significant myelosuppression as evidenced by a decreased WBC count and bone marrow cellularity. Co-treatment with *Cardiospermum* significantly increased the total WBC count, bone marrow cellularity and α -esterase positive cells, and the relative organ weights of spleen as well as thymus compared to the CTX alone treated group. *Cardiospermum* further reduced the enhanced levels of ALP, GPT, LPO, and proinflammatory cytokine TNF- α , and also significantly increased the glutathione (GSH) level in CTX treated animals. The lowered levels of other cytokines like IFN- γ , IL-2, GM-CSF, after CTX treatment were also found to be increased by extract administration. Histopathological analysis of small intestine also suggested reduction of CTX-induced intestinal damage. Moreover the extract down-regulated the inducible nitric oxide synthase (iNOS) and cyclo-oxygenase-2 (COX-2) mRNA expression in LPS stimulated macrophages. These studies indicate that *C. halicacabum* could reduce cyclophosphamide induced oxidative stress and immunosuppression through enhancing the antioxidant status and immunomodulation by stem cell proliferation.

Keywords: *Cardiospermum halicacabum* L - CTX - immunomodulation - oxidative stress - iNOS - COX-2

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Introduction

Conventional cancer treatments have many modalities, all directed at killing tumor cells or preventing their proliferation. The most common approach is chemotherapy, which is not selective toward tumor cells, but damaging to normal cells too. Immunosuppression is a major drawback of chemotherapy (Hersh and Freireich, 1968). Cyclophosphamide (CTX) is a cytotoxic alkylating drug with a high therapeutic index and broad spectrum of activity against a variety of cancers. CTX is one of the most widely used drugs for the treatment of chronic and acute leukemias, multiple myeloma, lymphomas, and rheumatic arthritis (Pass et al, 2005). However, use of CTX is often restricted because of its wide adverse side effects and toxicity that includes nausea, vomiting, alopecia, mucosal ulceration, pulmonary fibrosis, cardiac, hemopoietic suppression, nephrotoxicity, urotoxicity, cardiotoxicity and hepatic toxicity (Morandi et al., 2005; Papaldo et al., 2005; Schwartz et al., 2005; Amudha et al., 2007). Drugs that could reduce these side effects, as well as stimulate immunity, will be of great help in improving cancer treatment strategies.

Natural products have been shown to be an excellent and reliable source for the development of new drugs (Newman and Cragg, 2007). Since the immune system plays a fundamental role in host defense against pathogens as well as surveillance against tumours, the search for a safe and effective compound with immunomodulatory properties for clinical use has become a major goal of many research laboratories. Botanical based immunomodulators are often employed as supportive or adjuvant therapy to overcome the undesired effects of cytotoxic chemotherapeutic agents and to restore health to normal. Previously our laboratory has reported the chemoprotective activity of *Withania somnifera* (Davis and Kuttan, 1998) and *Andrographis Paniculata* (Sheeja and Kuttan, 2006).

Cardiospermum halicacabum L. of the family *Sapindaceae* has been used in Ayurveda and folk medicine for a long time in the treatment of rheumatism, lumbago, cough, hyperthermia, nervous diseases, as a demulcent in orchitis and in dropsy (Neuwinger, 2000). Various pharmacological actions of *Cardiospermum halicacabum* L. have been investigated in animal models. The anti-inflammatory, analgesic and vasodepressant activities

of this plant have been established (Gopalakrishnan et al., 1976; Sadique et al., 1987). The ethanol extract of the plant suppresses the production of TNF- α and nitric oxide in human peripheral blood mononuclear cells (Venkateshbabu, 2006). The ethanol extract of this plant has shown remarkable antipyretic and anti-ulcer activity in rats (Asha and Pushpangadan, 1999; Sheeba and Asha, 2006). Extract of this plant have been reported to contain different triterpenoids, glycosides, and a range of fatty acids (Ahmed et al., 1993; Ferrara et al., 1996; Srinivas et al., 1998). This study has been undertaken to investigate the effect of the methanolic extract of *Cardiospermum halicacabum* L. on cyclophosphamide induced immunosuppression and oxidative stress.

Materials and Methods

Cyclophosphamide (LEDOXAN) was obtained from Dabur Pharma Ltd, New Delhi, India. GSH 5-5' dithiobis (2-nitrobenzoic acid (DTNB) was purchased from SISCO Research Laboratory, Mumbai, India. Pararosaniline and naphthyl acetate were obtained from Loba Chemie, Mumbai, India. Glutamate Pyruvate Transaminase (GPT) and Alkaline Phosphatase (ALP) analyzing kits were obtained from SPAN Diagnostics Ltd. All other chemicals used in the present study were of analytical reagent grade.

ELISA Kit

Highly specific quantitative 'Sandwich' ELISA kits for mouse granulocyte monocyte - colony stimulating factor (GM-CSF), interferon-gamma (IFN- γ), interleukin-2 (IL-2) and tumor necrosis factor-alpha (TNF- α) were purchased from Pierce Biotechnology, USA.

Primers

Oligonucleotide primer sequences of genes for reverse transcription-polymerase chain reaction (RT-PCR) were purchased from Maxim Biotech Inc. (San Francisco, California, USA). Mouse primers of iNOS (Forward 5'-CTTCAACACCAAGGTTGTCTGCAT-3' and Reverse 3'-ATGTCATGAGCAAAGGCCGAGAAC-5'; 231bp) and COX-2 (Forward 5'-GTGGAAAA CCTCGTCCAGA-3' and Reverse 3'-ATGGTTTTGTCCGGTGGTAGT-5'; 256bp) genes were amplified against GAPDH (Forward 5'-CGTCCCCTAGACAAAATGGT-3' and Reverse 3'-TTGAAACCGTAACACCTTCC-5'; 557bp) standard. Cells to cDNA II kit purchased from Ambion Inc. (Austin, Texas, USA) was used for cDNA synthesis.

Animals

Inbred Balb/c mice (6-8 weeks) weighing 25-28g was taken from Amala Cancer Research Centre, breeding section, and were kept in well ventilated cages under standard conditions of room temperature, pressure and humidity. They were fed with normal mouse chow (Sai Durga Feeds and Foods, Bangalore, India) and water ad libitum. All the animal experiments were carried out with the prior approval of the Institutional Animal Ethics Committee (IAEC) and were conducted strictly adhering to the guidelines of Committee for the purpose of Control and Supervision of Experiments on Animals

(CPCSEA) constituted by the Animal Welfare Division of Government of India.

Cyclophosphamide administration

Cyclophosphamide (CTX) was administered, intraperitoneally at a concentration of 25 mg/Kg body weight for 10 consecutive days.

Extract preparation

Cardiospermum halicacabum was collected locally and a voucher specimen was deposited at the herbarium of Amala Ayurveda Research Centre, Kerala, India after authentication. The whole plant was dried at 45°C and powdered. It was then extracted with 70% methanol in soxhlet apparatus and the extract was evaporated to dryness. The yield of the extract was 19%. The dried extract was stored in an amber glass bottle at -20°C and used as and when required. The extract was resuspended in PBS containing 1% gum acacia for *in vivo* experiments.

Toxicological evaluation

Balb/c mice were divided into 5 groups (N=10). The control group received 1% gum acacia in PBS while the experimental groups received 10 doses of *C.halicacabum* extract (25, 50, 100 and 500 mg/kg B.Wt.) intraperitoneally for 10 days. Animals were observed for the mortality, behavior changes and change in body weight. All the animals were sacrificed by cervical dislocation after 14 days of drug administration. Selected organs such as liver, spleen, thymus, kidney, and lungs were dissected out and weights were recorded. Blood was collected by heart puncture immediately after sacrifice; serum separated and was used for the analysis of hepatic and renal functions. Liver function markers, such as alkaline phosphatase (ALP) (King, 1965), glutamate pyruvate transaminase (GPT) (Bergmeyer and Bernt, 1980) and kidney function markers such as creatinine (Toro and Ackermann, 1975) and blood urea nitrogen (BUN) (Murray, 1984), were determined.

Determination of the effect of *Cardiospermum halicacabum* extract on hematological changes after CTX administration

Two groups of animals (8 mice/group) were used for this study. All the animals received 10 doses of CTX at a dose of 25 mg /Kg b. wt (i.p) for 10 consecutive days of which group I was kept as CTX alone treated control while group II animals received 10 doses of *C.halicacabum* extract (20 mg /Kg b. wt, i.p.), beginning on the same day as CTX administration. Blood was collected from all the animals by tail vein bleeding prior to CTX administration and every third day thereafter, and following parameters were determined: a) Total WBC count; b) Differential count; c) Haemoglobin content (cyanmethyhemoglobin method).

Determination of the effect of *Cardiospermum halicacabum* extract on lymphoid organ weight, bone marrow cellularity and α -esterase activity during and after CTX administration

Forty eight animals were used in this study and they

were divided into 2 groups (24 animals/group). Group 1 animals were treated intraperitoneally with CTX alone (25 mg/Kg body weight) for 10 days. Group 2 animals were treated with CTX along with *C.halicacabum* extract (10 doses). Eight animals from each group were sacrificed by cervical dislocation at different time points (48th h, 7th day and 11th day). Body weight of animals were taken before sacrifice, lymphoid organs such as thymus and spleen were excised, weighed and expressed as relative organ weight. Bone marrow cells were collected from both femurs and the cell number were determined according to Sredni et al. 1992 using haemocytometer and expressed as total live cells ($\times 10^6$ cells / femur). Bone marrow cells from above preparation were smeared on a glass slide and stained as per the method of Bancroft and Cook, 1984 to determine the presence of α -esterase activity, which was expressed as number of positive cells/4000 bone marrow cells.

Determination of the effect of Cardiospermum halicacabum extract on enzyme levels during and after CTX administration

From each animals grouped above, blood was collected by heart puncture immediately after sacrifice and serum was separated. Intestinal mucosa was collected and used to estimate the levels of GSH by the method of Moron et al., 1979. Liver homogenate was made in ice cold Tris buffer (0.1 M, pH-7.4) and centrifuged at 4°C and 1200 rpm for 30 minutes. The supernatant was used for the estimation of ALP (King, 1965), glutamate pyruvate transaminase (GPT) (Bergmeyer and Bernt, 1980) and LPO (Okhawa, 1979). Serum was also used to estimate all the above parameters.

Determination of effect of the Cardiospermum halicacabum extract on IL-2, IFN- γ , GM-CSF and TNF- α production after CTX administration

Serum from the above experiment was also used on the same day of sacrifice to determine the level of various cytokines, such as IL-2, IFN- γ , GM-CSF and TNF- α using sandwich ELISA kit according to the manufactures protocol.

Determination of the effect of Cardiospermum halicacabum extract on iNOS and Cox-2 gene expression in LPS activated macrophages

Macrophages were elicited by injecting 5% sodium caseinate intraperitoneally in BALB/c mice. Macrophages were washed with PBS and resuspended in RPMI-1640 with 10% FCS. The cells were plated in 96 well culture plates and incubated for 2 hrs at 37°C, in a 5% CO₂ atmosphere. After incubation, non adherent cells were removed and the adherent macrophages were incubated (2×10^6 cells/well) in complete medium (RPMI-1640, 10% FCS, 100 μ g/mL streptomycin and penicillin, 2mM glutamine). Macrophages were cultured with LPS (5 μ g/ml) in the presence and absence of *C. halicacabum* extract (5 μ g/ml) and incubated for 4hrs at 37°C, in a 5% CO₂ atmosphere. cDNA was synthesized using the Cells - cDNA kitTM (Ambion Inc., Austin, Texas, USA). In short macrophages were washed with PBS and heated in cell lysis II buffer (provided in the kit) to release the RNA

into the solution. This was followed by a heating step to inactivate endogenous RNase. The genomic DNA was further degraded by treating with DNase, followed by the inactivation of DNase by heating at 70°C. Reverse transcription was performed at 42°C for 50 min using Moloney murine leukemia virus RT (supplied along with the kit). Gene expression studies were performed by PCR analysis. The mouse iNOS and COX-2 genes were amplified against GAPDH standard. The cycling conditions were as follows: 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C for 40 cycles, followed by a 10-min extension at 72°C. Amplified PCR products were electrophoresed on a 1.8% agarose gel and stained with ethidium bromide and photographed under ultraviolet light.

Histopathological analysis of intestine of experimental mice

A small portion of small intestine was taken and fixed in 10 % formaldehyde. After several treatments for dehydration in alcohol, sections having 4 μ m thickness were cut and stained with haematoxylin and eosin and histopathological analysis was carried out.

Data analysis

All data are expressed as mean \pm SD. CTX alone treated group was compared with *Cardiospermum halicacabum* extract along with CTX treated group by one way ANOVA followed by post hoc Dunnet's test.

Results

Toxicological evaluation

Short-term administration of *C. halicacabum* extract at doses 25 and 50mg/kg b.wt. for 10 days did not produce any mortality, change in behavior, body weight, relative organ weight, hepatic and renal functions compared to normal untreated animals. *C. halicacabum* at 100 mg/Kg showed a mild toxicity whereas 500mg/kg exhibited an observable toxicity showing behavior changes, mortality, weight loss, and decrease in the organ weight and also alterations in both hepatic and renal functions.

Effects of Cardiospermum halicacabum on hematological parameters of CTX administered animals

CTX administration reduced the total WBC count in both the groups of animals (Figure 1). The CTX alone treated animals showed drastic reduction in total WBC count on 12th day to 2156 ± 255 cells/mm³, while Total WBC count was 3785 ± 251 cells/mm³ in the animals also treated with *Cardiospermum halicacabum*. Differential counts and haemoglobin contents did not show any significant changes in both treated and control animals (data not shown).

Effect of Cardiospermum halicacabum on organ weight, bone marrow cellularity and α -esterase positive cells after CTX administration

Relative organ weights of thymus and spleen were drastically reduced in CTX treated control animals.

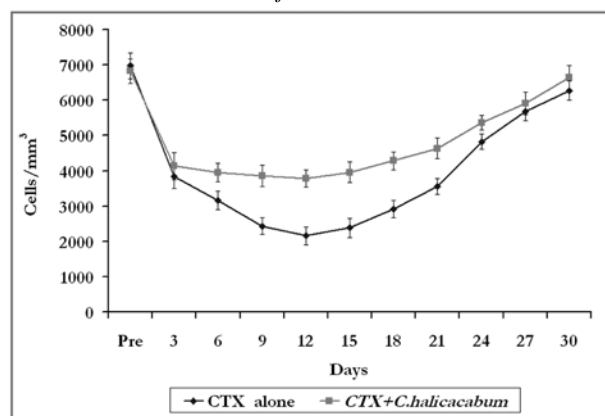


Figure 1. Effect of *C. halicacabum* Extract on Total White Blood Cell Counts

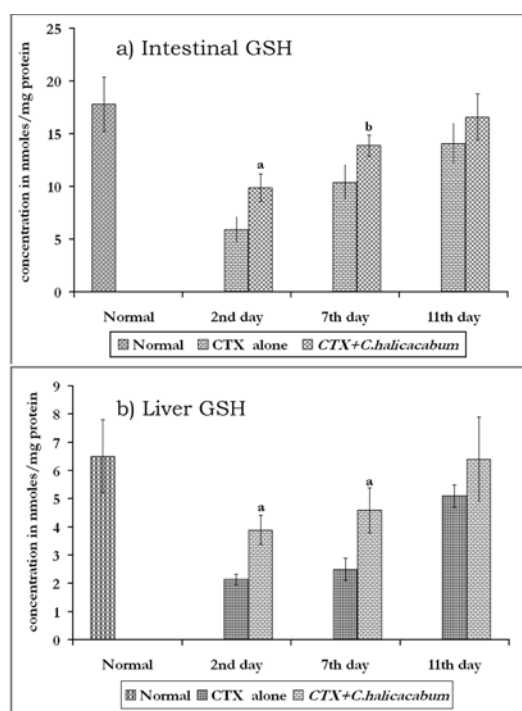


Figure 2. Effect of *C. halicacabum* Extract on GSH Level. Values are the mean \pm S.D.; ^a $p < 0.001$ compared to CTX alone treated control; ^b $p < 0.01$ compared to CTX alone treated control

Administration of *Cardiospermum halicacabum* extract significantly increased both organ weights in CTX treated animals.

Data for effects of *Cardiospermum halicacabum* extract on bone marrow cellularity and α -esterase positive cells is shown in Tables 1 and 2, respectively. CTX administration drastically decreased bone marrow cellularity and α -esterase positive cell number in control mice.

Administration of *Cardiospermum halicacabum* significantly enhanced both the parameters in CTX treated animals.

Effect of Cardiospermum halicacabum on enzyme levels after CTX administration

CTX administration in mice was found to decrease the levels of GSH in intestine (Figure 2a) as well as liver (Figure 2b) but this was significantly reversed by the

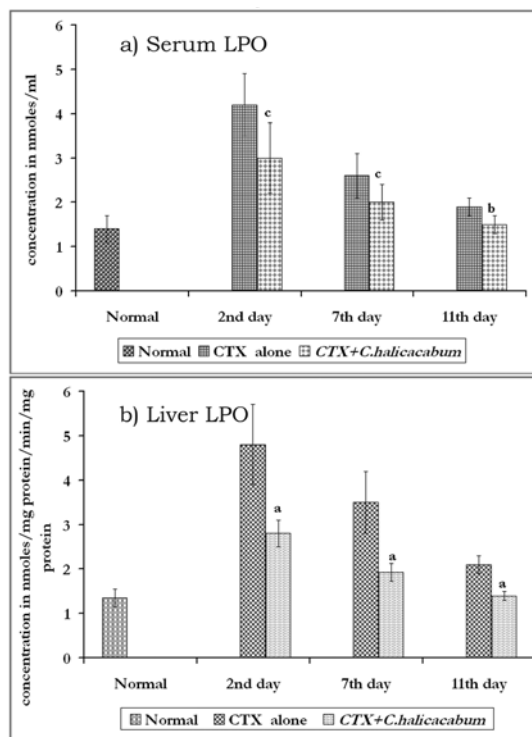


Figure 3. Effect of *C. halicacabum* Extract on LPO Levels. Values are the mean \pm S.D.; ^a $p < 0.001$ compared to CTX alone treated control; ^b $p < 0.01$ compared to CTX alone treated control; ^c $p < 0.05$ compared to CTX alone treated control

Cardiospermum halicacabum treatment.

Figure 3-5 shows the effects of *Cardiospermum halicacabum* extract on serum and liver LPO, ALP and GPT levels in CTX treated mice. In all cases the enzyme levels were elevated due to the toxicity, was reversed to a significant extent by the *Cardiospermum halicacabum* treatment.

Effects of Cardiospermum halicacabum extract on cytokine production during CTX treatment

Figure 6 shows the serum IFN- γ and TNF- α profile, while Figure 7 summarizes data for GM-CSF and IL-2. CTX caused significant decrease in IFN- γ , IL-2 and GM-CSF, while markedly elevating TNF- α .

All these changes were reversed by the treatment of

Table 1. Bone Marrow Cellularity

Group	Bone marrow cellularity $\approx X 10^6$		
	48 th h.	7 th day	11 th day
Normal	15.2 \pm 1.2		
CTX alone	4.3 \pm 0.3	6.6 \pm 0.5	11.8 \pm 0.8
CTX + C.halicacabum	7.5 \pm 0.6 ^a	9.5 \pm 0.8 ^a	14.2 \pm 1.3 ^a

Values are the mean \pm S.D.; ^a $p < 0.001$ compared to CTX alone treated control

Table 2. α -Esterase Activity (Number/ 4000 cells)

Group	α - esterase activity		
	48 th h.	7 th day	11 th day
Normal	975 \pm 60		
CTX alone	121 \pm 8.5	6.6 \pm 0.5	11.8 \pm 0.8
CTX + C.halicacabum	382 \pm 34 ^a	651 \pm 53 ^a	894 \pm 72 ^a

Values are the mean \pm S.D.; ^a $p < 0.001$ compared to CTX alone treated control

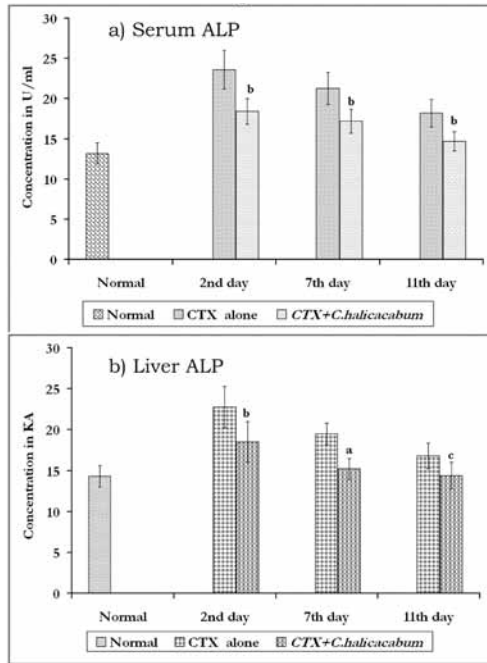


Figure 4. Effect of *C. halicacabum* Extract on ALP Levels. Values are the mean ± S.D; ^ap< 0.001 compared to CTX alone treated control; ^bp< 0.01 compared to CTX alone treated control; ^cp<0.05 compared to CTX alone treated control

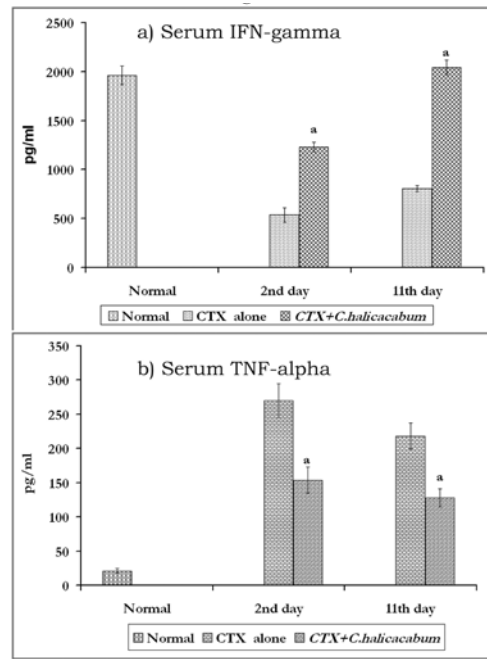


Figure 6. Effect of *C. halicacabum* Extract on IFN-γ and TNF-α. Values are the mean ± S.D; ^ap< 0.001 compared to CTX alone treated control

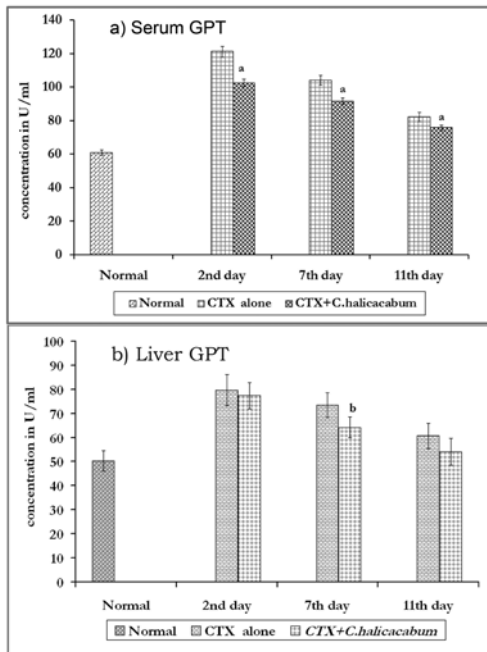


Figure 5. Effect of *C. halicacabum* Extract on GPT Levels. Values are the mean ± S.D; ^ap< 0.001 compared to CTX alone treated control; ^bp< 0.01 compared to CTX alone treated control

C. halicacabum extract.

Effect of Cardiospermum halicacabum extract on iNOS and COX-2 Gene Expression

RT-PCR analysis showed that *Cardiospermum halicacabum* (5 μg/mL) downregulated the iNOS and COX-2 mRNA expression in LPS stimulated cells (Figure 8).

Histopathological analysis

Histopathological analysis of jejunam portion of the

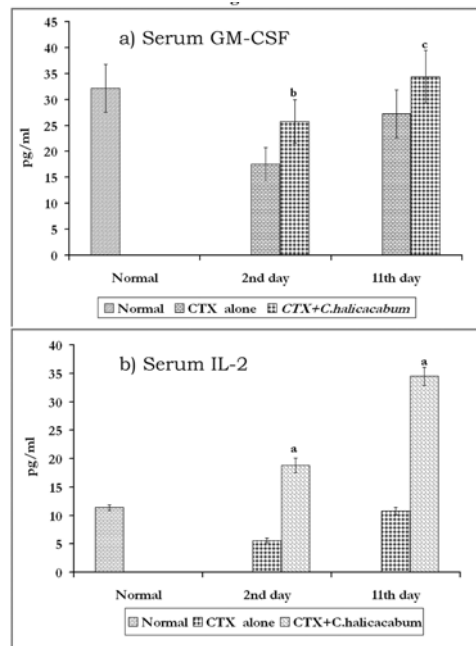


Figure 7. Effect of *C. halicacabum* Extract on GM-CSF and IL-2. Values are the mean ± S.D; ^ap< 0.001 compared to CTX alone treated control; ^bp< 0.01 compared to CTX alone treated control; ^cp< 0.05 compared to CTX alone treated control

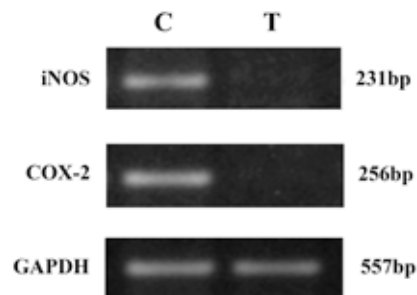


Figure 8. Expression of iNOS and COX-2 Genes

CTX alone treated control animals showed severe damage to the intestinal villi when compared to normal. The height of the villi was markedly reduced and the crypt architecture was largely destroyed. This was inhibited by the *C. halicacabum* extract.

Discussion

Most of the chemotherapeutic agents available today are immunosuppressants, cytotoxic and exert a variety of side effects that are particularly evident in cancer chemotherapy. Cyclophosphamide is an alkylating agent and a powerful immunosuppressant acting as it cross links DNA in actively multiplying cells (Gonsette, 1986). Modulation of the immune response through stimulation may help in maintaining a disease-free state. Agents that activate host defense mechanisms during an impaired immune responsive condition can provide supportive therapy to conventional chemotherapy (Wagner, 1984). Plant extracts used in traditional therapy are being reviewed for their chemoprotective and immunomodulatory activities.

It has been shown that immunomodulatory compounds used with chemotherapy may reduce myelosuppression and enhance the immune response (Praveen et al., 1996). In the present study we analysed the effect of *Cardiospermum halicacabum* extract on hematopoiesis. Administration of plant extract showed an increase in the total WBC count, bone marrow cellularity and α -esterase activity in CTX treated animals compared to CTX alone treated control mice showing their potential on stem cell proliferation and differentiation. Weight of lymphoid organs especially spleen and thymus was also increased in CTX treated animals by the extract administration, providing supportive evidence for its immunostimulative potential during CTX therapy.

The glutathione antioxidant system plays a fundamental role in cellular defense against reactive free radicals and other oxidant species (Meister and Andersen 1983). GSH plays a vital role in the protection of cells against oxidative stress and acts as an important water-phase non enzymatic antioxidant and an essential cofactor for antioxidant enzymes taking part in cellular redox reactions. Its high electron-donating capacity (sulphydryl group) endows GSH with great reducing power, which is used to regulate a complex thiol-exchange system (Dickinson, 2002). Depletion of GSH results in enhanced lipid peroxidation (Anderstam et al, 1992). Excessive lipid peroxidation can cause increased GSH consumption (Comporti, 1987). CTX toxicity reduced the GSH level in control animals. Treatment with *Cardiospermum halicacabum* extract increased the GSH production.

Lipid peroxidation is one of the main manifestations of oxidative damage initiated by ROS and it has been linked with impairment of membrane functioning, decreased fluidity, inactivation of membrane-bound receptors and enzymes, and increased non-specific permeability to ions (Sikka 2004). In the present study, the *Cardiospermum halicacabum* extract treated mice showing significant reduction in the LPO level both in serum and liver. This indicates the antioxidant activity of *C. halicacabum* and

ability for reducing CTX elevated ROS formation.

GPT and ALP are clear markers of cell proliferation. Alkaline phosphates activity decreases in cyclophosphamide-treated mice. Alkaline phosphatase plays an important role in maintenance of cellular permeability and acts on monophosphoesters. Damage to cell membrane caused by cyclophosphamide may be the reason for declined activity of alkaline phosphatase. CTX administration elevated the level of liver ALP due to impairment of tissues; indicate the pathological condition of the animal, lead to liver dysfunction. When the liver is not functioning properly, this enzyme is not excreted through bile but is released into blood stream. Administration of *Cardiospermum halicacabum* extract in CTX treated mice reduced the ALP and GPT level which were elevated during CTX administration indicate the protective effect from liver injury due to CTX.

Cytokines are soluble polypeptides that are produced by a wide variety of cell types either constitutively or after induction. They are involved in virtually every aspect of immunity and inflammation, including development and functioning of the immune system, cell proliferation and differentiation, cellular recruitment and activation, and regulation of cellular interactions with extracellular matrix proteins. Cytokines mediate the communication both locally between cells and tissues and distantly between organs (Nathan, 1991; Borish, 2003). TNF- α is a proinflammatory cytokine expressed by macrophages. It represents a key mediator in pathological situations, such as rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis (Dinarello, 2000). TNF- α level was drastically elevated after CTX treatment which was significantly reduced by the administration of *C. halicacabum* extract. The lymphokine, IL-2, which was identified as T cell growth factor (Ehrhacat et al, 1997) plays a central role in the maturation and development of lymphocytes and monocytes (Theze, 1996), whereas IFN- γ stimulates phagocytic activity of macrophage and differentiation of T cells and cytotoxic effects (Borish, 1996). GM-CSF is one of the major hematopoietic growth factors belonging to a family of glycoproteins that stimulate proliferation and function of a wide variety of myeloid progenitor cells. It is mainly produced by T lymphocytes or nonhematopoietic cells (Gasson, 1991). CTX drastically reduced the levels of IL-2, IFN- γ and GM-CSF, whereas treatment of *C. halicacabum* extract could enhanced the production of these cytokines and thereby stimulated the immune system even after CTX administration.

Cyclooxygenase-2 (COX-2), an enzyme that catalyses the transformation of arachidonic acid into prostaglandin, which is secreted by several cell populations such as fibroblasts, endothelial cells, and monocytes/macrophages by proinflammatory stimuli such as LPS, TNF- α , IL-1 β , and IL-6 (Payvandi et al., 2004). Macrophages are known to play a major role in the innate immune defense system against pathogens and tumor cells and represent one of the main cellular sources of COX-2 expression upon exposure to different stimuli (Lee et al., 1992). Overexpression of COX-2 has been demonstrated in different animal models of inflammation and tumors (Wang et al., 2001;

Petrangolini et al., 2003). Furthermore, the COX-2 expression has been shown to promote angiogenesis, tumor invasion and metastasis (Tsuji et al., 1998; Sengupta et al., 2003). Nitric oxide (NO), a free-radical gas, is synthesized by a nitric oxide synthase (iNOS) that mediates diverse functions, including vasodilatation, neurotransmission, inhibition of platelet aggregation, immune responses, and inflammation (Lowenstein and Snyder, 1992; Xie et al, 1992). Furthermore, recent studies have demonstrated the production of NO by murine macrophages stimulated with lipopolysaccharide (LPS) or certain Proinflammatory cytokines (Asai et al., 1996). *C. halicacabum* extract down regulated the COX-2 and iNOS mRNA expression in LPS stimulated macrophages, showing its anti-inflammatory potential.

In conclusion, the results of the above experiments strongly suggest the chemoprotective effect of the *C. halicacabum* extract and this may be due to the stimulation of the antioxidant as well as immune system.

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