RESEARCH COMMUNICATION

Ameliorating Effects of *Andrographis Paniculata* Extract Against Cyclophosphamide-Induced Toxicity in Mice

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Abstract

Major drawbacks of chemotherapeutic agents are their toxic side effects and lack of tumor specificity. Immunological and biochemical studies were here carried out to investigate protective effects of ethanolic extract of *Andrographis paniculata* against cyclophosphamide (CTX) induced toxicity *in vivo*. Intraperitoneal administration of the extract significantly increased the total WBC account (3256.5±196 cells/cm^2), bone marrow cellularity (17.1±10.4x10^6 cells/femur) and β–esterase positive cells (849±23.2 cells/4000 cells) in CTX treated animals, when compared to CTX alone treated control mice. Weights of lymphoid organs such as a spleen and thymus, reduced by CTX administration, were also increased by *A paniculata* treatment. Reduction of GSH in liver (4.8±0.21nmol/mg protein) and in intestinal mucosa (13±0.67 nmol/mg protein) of CTX-treated controls was significantly reversed by *A paniculata* administration (liver: 6.4±0.13, intestinal mucosa: 17.11±0.06), with amelioration of changes in serum and liver ALP, GPT, LPO (lipid peroxidation). Histopathological analysis of small intestine also suggests that extract could reduce the CTX induced intestinal damage. The level of proinflammatory cytokine TNF-α, which was elevated during CTX administration, was significantly reduced by the *A paniculata* extract administration. 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.

Key Words: Cyclophosphamide - toxicity - *Andrographis paniculata* - antioxidants - cytokines

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Introduction

Among the common therapeutic modalities of cancer, chemotherapy plays an important role. Most of the synthetic chemotherapeutic agents available today are immunosuppressant, cytotoxic and exert several side effects (Diwanay et al, 2004). Cyclophosphamide (CTX) is a cytotoxic alkylating drug with a high therapeutic index and broad spectrum of activity against a variety of cancers. (Haque, 2003). It is inactive in in vitro but is activated to intracellular alkylating metabolites, 4-hydroxy cyclophsosphamide and phosphoramide mustard by hepatic cytochrome p450 monoxygenase system (Colnvin et al, 1973). Major toxic side effects of CXT are hematopoietic depression, gastrointestinal toxicity and hemorrhagic cystitis (Slavin et al, 1975). Cytotoxicity towards normal host tissue is the primary dose-limiting factor in CTX therapy that reduces quality of life and restricts treatment protocol. Hence there is a continued interest and need for the identification and development of non-toxic and effective chemopreventive compound that can reduce the side effects of cyclophosphamide.

Protection against chemically induced toxicity and cancer using synthetic or natural compounds, constitute a promising means of disease control and prevention (Ma and Kineer, 2002). The use of plants or their active principle in the prevention or treatment of chronic disease is based on the experience of traditional system of medicine from ethnic societies, but their use in modern medicine suffer from lack of scientific evidences. Now-a-days, many medicinal plants have attracted the interest of scientists in this field and plant extracts used in traditional therapy are being reviewed for their chemopreventive activites. Earlier experimental studies in our laboratory have demonstrated that medicinal plant like *Withania somnifera* (Davis and Kuttan, 2003) and many naturally occurring sulphur compounds posses chemoprotective activities induced by CTX (Manesh and Kuttan, 2005).

Andrographis paniculata (Family: Acanthaceae), is one of the main ingredients of several Ayurvedic and herbal formulation. It is used as a hepatoprotective and heptatostimulative agent and has been reported to have protective effect against different hepatotoxins (Trivedi and Rawal, 2001). It also possesses anti-diabetic (Zhang and Tan,

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2000), antipyretic and anthelmintic properties (Gupta, 2004) and has role in delaying the hepatic tumorigenic condition. (Trivedi and Rawal, 1998). We have already proved that ethanolic extracts of *Andrographis paniculata* have antioxidant and anti-inflammatory acitivity (Sheeja et al, 2006). Since little is known about chemoprotective activity, the current study focused on protction against cyclophosphamide-induced toxicity in Swiss albino mice.

Materials and Methods

Preparation and administration of plant extract

Air-dried whole plants of *A.paniculata* were powdered and extracted (100g) twice with 100ml of 70% ethanol by stirring overnight at 40C. Supernatant was collected after centrifuging at 10,000 rpm at 40 C for 10 min. Ethanol was removed by evaporation and yield of the extract was 14 %. Preliminary phytochemical analysis showed presence of terpenoids and flavanoids.

For animal administration the extract was dissolved in minimum quantity of ethanol, then resuspended in 1% gum accacia and given at a concentration of 10mg/animal/dose intraperitoneally.

Chemicals and ELISA kits

Cyclophsophamide (LEDOXAN) was obtained from Dabur Pharma Ltd, New Delhi, India. GSH, 5-5' dithiobis (2-nitrobenzoic acid) (DTNB) was purchased from SISCO research laboratory, Bombay, India. Pararosanline and naphthylacetate were obtained from Loba Chemie, Mumbai, India. Glutamate pyruvate transaminase (GPT) and alkaline phosphatase (ALP) analyzing kits were obtained form SPAN diagnostics Ltd. All other chemicals used in the present study were of analytical reagent grade.

Highly specific quantitative "sandwich " ELISA Kits for mouse IL-2,GM-CSF, IFN-γ and TNF-α were purchased from Pierce Biotechnology USA .

Animals

Inbred Swiss albino (6-8 weeks) mice, weighing 25-28 g, were taken from Amala cancer Research Centre, breeding section. They were kept in well-ventilated cages under standard conditions at room temperature, pressure and humidity. The animals were fed with normal mouse chow (Sai Durga Feeds and Foods, Bangalore, India.) and water ad libitum. All animal experiments were conducted according to the rules and regulations of Animal Ethics Committee, Government of India.

Cyclophosphamide administration

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

*Determination of the effect of **Andrographis paniculata** on hematological changes after CTX administration*

Three groups of animals (6mice/group) were used for this study. Group I animals received 10 doses of *A. paniculata extract.* Group II and III animals received 10 doses of CTX at a dose of 25mg /Kg b. wt (i.p) for 10 consecutive days of which group II was kept as CTX alone treated control while group III animals were treated with extract, 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 (cyanmethylhemoglobin method).

*Determination of the effect of **A. paniculata** on lymphoid organ weight, bone marrow cellularity and β-esterase activity after CTX administration*

Fifty four animals were randomly divided into 3 groups having 18 animals in each group. Treatments of CTX as well as Andrographis paniculata for each group were same as the previous experiment. Six animals from each group were sacrificed at different time intervals (2nd, 7th and 11thday) after the last dose of CTX and Andrographis paniculata administration by cervical dislocation. Body weight of each animal was taken before sacrifice, lymphoid organs such as thymus and spleen was excised, weighed and expressed as relative organ weight.

Bone marrow cells from above preparation was smeared on a clean slide and stained with p-rosaniline and Haris haematoxylin as per the method of Bancroft and Cook (1984) to determine the presence of β-esterase activity and expressed as number of positive cells/ 4000 bone marrow cells.

*Determination on the effect of **A. paniculata** on enzyme levels after CTX administration*

Thirty six animals were divided into 2 groups. All the animals received CTX at a concentration of 25mg/kg b.wt for 10 consecutive days. Group I was kept as CTX alone treated control while Group II received 10 doses of Andrographis paniculata (10mg/doses/animal, i.p) simultaneously with CTX. Six animals from each group were sacrificed at 2nd, 7th and 11th day after last dose of CTX administration, by cervical dislocation. Blood was collected from each animal by heart puncture immediately after sacrifice and serum was separated. Organs such as liver and intestine were also excised, and washed thoroughly in ice cold phosphate buffered saline and samples were used to estimate various biochemical parameters. Intestinal mucosa was also collected and used to estimate the levels of GSH by the method of Moron et al (1979). A portion of intestine (jejunum) was kept in formaldehyde for histopathological analysis. Liver homogenate was made in ice cold Tris buffer (0.1M PH7.4) and centrifuged at 40 C at 1200 rpm for 10 min. The supernatant was used for the estimation of alkaline phosphatase (ALP) (King and Amstrong, 1980), glutamate pyruvate transaminase (GPT) (Bermeyer and Bernt, 1980) and lipid peroxidation (Ohakawa, 1979). Serum was also used to estimate all the

above parameters.

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 4μm thickness were cut and stained with haematoxylin and eosin and histopathological analysis was carried out.

*Determination of the effect of **A. paniculata** on IL-2, IFN-γ, GM-CSF, and TNF-α production after CTX administration*

Swiss albino mice (25-28 g) were divided into 2 groups (12 animals/ group). Group I animals received 10 doses of Andrographis paniculata extract. Group II and Group III animals were treated with 10 doses of CTX (25mg/kg b. wt.). Group II was kept as control without any further treatment, while group III animals were also treated with Andrographis paniculata for 10 consecutive days simultaneously with CTX. Six animals from each group were sacrificed at 2nd and 11th day after the last dose of CTX administration and blood was collected by heart puncture. Serum was separated and the level of various cytokines such as IL-2, IFN-γ, GM-CSF and TNF-α were measured on the same day of sacrifice using sand witch ELISA Kit specific for the murine cytokines according to the manufacture's protocol.

Statistical analysis

Data are expressed as mean ±standard deviations (SD). Significance of differences was assessed at $p<0.05$.

Results

Effects of Andrographis paniculata on hematological parameters of CTX administered animals

CTX administration reduced the total WBC count in mice. The animals in both group showed a reduction in the Total WBC count. The CTX alone treated animals showed drastic reduction in total WBC count on 12th day to 1915±114, while Total WBC count was 3256±196 in the animals treated with Andrographis paniculata along with CTX on the same day, further it was increased and normalized by 24th day. The reduced level of total WBC was observed in control animals throughout the period of study. Differential counts and haemoglobin contents did not show any significant changes in both treated and untreated animals (data not shown).

Effect of Andrographia paniculata on organ weight, bone marrow cellularity and β-esterase activity after CTX administration

The CTX treated control animals showed high reduction in the weight of thymus and spleen, 0.06±0.002g/100g b.wt. and 0.13±0.004g/100g b.wt respectively on the 2nd day after CTX administration and did not normalize even after 11th day. The *Andrographis paniculata* treated mice showed a reduction in the weight of thymus to 0.08±0.002g/100g b.wt and spleen to 0.17±0.002 on 2nd day after the last dose of CTX administration, but it gradually increased and attained normal weight by day 11th day (spleen: 0.32±0.01and thymus 0.14±0.003 g/10gb.wt). There was an increase in the weight of spleen (0.47±0.02g/100g b.wt) and thymus (0.15±0.01g/100g b.wt) when normal animals received 10 doses of Andrographis paniculata extract.

Normal animals after treatment with *A paniculata* showed an increase in the bone marrow cellularity 21.3±1.8x10^6 cells /femur and β-esterase positive cells (1401.8±41.9 cells/4000 cells). The number of bone marrow cells as well as β-esterase positive cells was decreased drastically in CTX alone treated control animals, but this was reversed by administration of *A paniculata*. In control animals, on the 2nd day after last dose of CTX, there was a drastic reduction in the number of bone marrow cells (5.2±0.5 x10^6 cells/femur) and β-esterase positive cells (237.4±15.5 bone marrow cells/4000cells) compared to normal animals (bone marrow cellularity: 15.75±0.54 cells /femur, β-esterase positive cells: 867±29.5 cells /4000 cells). Even after 11th day, the bone marrow cells (11.4± 1.15 x10^6 cells/femur) and β-esterase positive cells (537.8±19.7/ 4000cells) did not reach back to normal value. Treatment with Andrographis paniculata could elevate the bone marrow cellularity and number of β-esterase positive cells, thus protect the mice from myelosuppressive effect of CTX. In treated group of animals bone marrow cellularity and β-esterase positive cells was found to be 7.38±0.58x10^6 and 487.4±30.9 cells /4000 bone marrow cells respectively, after 2 days and it was again enhanced to 17.14±1.04x10^6 cells/ femur and 849±23.2 cells /4000 BM cells on 11th day after last dose of CTX.

Effect of Andrographis paniculata on enzyme levels after CTX administration

CTX administration in mice was found to decrease the levels of GSH in liver as well as intestine (Table 1) but this

Table1. Effect of *Andrographis paniculata* Treatment on the Intestinal and Liver GSH levels in CTX Treated Animals

	Intestine GSH (nmol/mg protein)			Liver GSH (nmol/mg protein)		
Group	2nd day	7th day	11th day	2nd day	7th day	11th day
Normal	17.5 ± 0.27			6.5 ± 0.3		
CTX alone	5.4 ± 0.31	10.0 ± 1.06	13.8 ± 0.92	1.9 ± 0.04	2.4 ± 0.06	4.8 ± 0.21
CTX+*A paniculata*	9.7 ± 0.41 a	14.4 ± 0.41[1]	17.1 ± 0.6[1]	4.0 ± 0.21[1]	5.4 ± 0.08[1]	6.4 ± 0.13[1]

The treated animals received 10 doses of *Andrographis paniculata* (10mg/dose/animlas i.p) and 10 dose of CTX (25mg/kg/b.wt. i.p) simultaneously. Animals were sacrificed on 2nd, 7th day and 11th day after the last dose of CTX administration and GSH level in liver and intestinal mucosa were estimated. The data are mean±SD values. [1]$p<0.001$ compared to CTX alone.

Table 2. Effect of *Andrographis paniculata* Treatment on the Serum and Liver ALP Levels in CTX Treated Animals

	Serum ALP (KA units)			Liver ALP (KA units)		
Group	2nd day	7th day	11th day	2nd day	7th day	11th day
Normal	13.0 ± 0.4			14.0 ± 0.26		
CTX alone	22.7 ± 0.53	20.8 ± 1.4	16.0 ± 0.45	23.4 ± 0.36	19.3 ± 0.65	17.5 ± 0.53
CTX+ *A paniculata*	14.4 ± 0.34[1]	13.8 ± 0.1[1]	12.8 ± 0.14[1]	14.5 ± 0.3[1]	13.9 ± 0.33[1]	13.8 ± 0.25[1]

The data are mean±SD values. [1]p<0.001 compared to CTX alone.

Table 3. Effect of *Andrographis paniculata* Treatment on the Serum and Liver GPT Levels in CTX Treated Animals

	Serum GPT (U/ml)			Liver GPT (U/ml)		
Group	2nd day	7th day	11th day	2nd day	7th day	11th day
Normal	14.0 ± 0.74			50.3 ± 0.4		
CTX alone	37.1 ± 0.8	31.3 ± 0.4	29.8 ± 0.82	81.2 ± 1.7	75.9±1.14	62.4 ± 1.39
CTX+ *A paniculata*	25.9 ± 1.04[1]	18.7 ± 0.92[1]	14.8 ± 0.89[1]	77.0 ± 1.0[1]	63.1±0.94[1]	53.1 ± 1.13[1]

The data are mean±SD values. [1]p<0.001 compared to CTX alone.

Table 4. Effect of *Andrographis paniculata* Treatment on the Serum and Liver LPO Levels in CTX Treated Animals

	Serum LPO (nmol/ml)			Liver LPO (nmol/ml)		
Group	2nd day	7th day	11th day	2nd day	7th day	11th day
Normal	1.42 ± 0.24			1.26 ± 0.42		
CTX alone	3.10 ± 0.14	2.35±0.04 a	1.84±0.08 a	4.80 ± 0.24	3.53 ± 0.12	2.05 ± 0.16
CTX+ *A paniculata*	2.08 ± 0.11[2]	1.68 ± 0.08[2]	1.46 ± 0.05[2]	3.50 ± 0.18[2]	1.85 ± 0.1[1]	1.26 ± 0.11[1]

The data are mean±SD values. [1,2]p<0.01, p<0.001 compared to CTX alone.

was significantly reversed by the *Andrographis paniculata* treatment.

Tables 2-4 show the effects of *Andrographis paniculata* extract on serum and liver ALP, GPTand LPO levels in CTX treated mice. In all cases increase due to the toxin was reversed to a significant extent by the *Andrographis paniculata* treatment.

Histopathological analysis

Histopathological analysis of jejunam portion of the CTX alone treated control animals showed severe damage to the intestinal villii when compared to normal. The lengths of the villi were markedly reduced and the crypt architecture was largely destroyed. This was inhibited by the *Andrographis paniculata* extract.

Effects of Andrographis paniculata on cytokine production during CTX treatment

Table 5 shows the serum IL-2 and IFN-γ profiles while Table 6 summarizes data for GM-CSF and TNF-α. CTX caused significant decrease in IL-2, IFN-γ and GM-CS, while markedly elevating TNF-α. All of these changes were reversed by *Andrographis paniculata*.

Discussion

CTX administration induces acute and transient myelosuppression, primarily through damage to rapidly proliferating hematpoietic progenitors and their mature progeny leading to decline in the number of peripheral blood cells. (Fishman et al., 2001). Administration of ethanolic

Table 5. Effect of *Andrographis paniculata* Treatment on Serum IFN-γ and IL-2 Levels in CTX Treated Animals

	IFN-γ (pg/ml)		IL-2 (pg/ml)	
Group	2nd day	11th day	2nd day	11th day
Normal	1980 ± 34.9		11.1 ± 0.47	
A paniculata	2856 ± 8.6[1]		29.1 ± 0.54[1]	
CTX alone	550 ± 20.8	720 ± 19.6	5.2 ± 0.34	10.6 ± 0.23
CTX+ *A paniculata*	1476 ± 26.3[2]	2161 ± 74.8[2]	21.8 ± 0.34[2]	39.7 ± 0.69[2]

The data are mean±SD values. [1,2]p<0.001 compared to normal and CTX alone, respectively.

Table 6. Effect of *Andrographis paniculata* Treatment on Serum GM-CSF and TNF-α Levels in CTX Treated Animals

	GM-CSF (pg/ml)		TNF-α (pg/ml)	
Group	2nd day	11th day	2nd day	11th day
Normal	33.1 ± 0.46		20.2 ± 0.79	
A paniculata	39.1 ± 0.56		19.9 ± 0.87	
CTX alone	18.4 ± 0.05	24.27 ± 0.65	279.4 ± 7.94	264.7 ± 5.41
CTX+ *A paniculata*	27.4 ± 0.22 a	38.33 ± 1.05 a	141.4 ± 0.93 a	112.6 ± 1.16 a

The data are mean±SD values. [1]p<0.001 compared to CTX alone.

extract of *Andrographis paniculata* in CTX treated mice, found to enhance the total WBC count, bone marrow cellularity and β-esterase positive cells, which were drastically reduced in the CTX alone treated control animals suggest that CTX induced myelosuppression was reversed or inhibited by extract administration possibly through its immunomodulatory activity. 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.

Alkaline phosphatases (ALPs) are group of enzymes found primarily in the liver and bone. The cells lining the intestine and kidney also produce a small amount of ALPs. The primary importance of measuring ALP is to check the pathological condition of the animal especially, liver injury. 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. Thus serum alkaline phosphatase level is also a measure of the hepatobiliary system. Administration of Andrographis paniculata in CTX treated mice reduced the ALP level which was elevated during CTX administration indicate the protective effect from liver injury due CTX. Andrographis paniculata was also found to decrease the activity of serum and liver GPT in the CTX treated animals support the ameliorating effect of Andrographis paniculata on CTX induced liver damage. The process of lipid peroxdation (LPO) is one of the oxidative conversions of polyunsaturated fatty acid to a product known as malondialdehyde (MDA), or lipid peroxides (Samir, 1999). MDA, owing to high cytotoxicity and inhibitory action on protective enzymes, is suggested to act as a tumor promoter (Seven, 1999). The oxidative product of CTX is responsible for the induction of LPO, which has devastating effect on the cell membrane (Haque, 2003). Lipid peroxidation was found to be decreased in along Andrographis paniculata with CTX treated mice compared to CTX alone treated animals indicate that one of the mechanisms by which *Andrographis paniculata* exerts its chemoprotective action may be by inhibiting lipid peroxidation.

Reduced glutathione (GSH) which is one of the major cellular non- enzymatic antioxidants protects cell against reactive oxygen species (ROS) generated by CTX. (Haque, 2003). It is also very important constituent in the detoxification pathways. Metabolism of CTX in the body produces ROS, which produce devastating effect on cell membranes. The depleted GSH level in CTX alone treated animals is probably due to enhanced utilization of GSH to detoxify the ROS. Administration of Andrographis paniculata along with CTX elevated the GSH level in both liver and intestine thereby increases its ability to cope with the free radical produced during CTX administration. This also suggests the protective effect of the extract against CTX induced toxicity.

The present histopathological analysis demonstrated intestinal villi of CTX treated mice to be ruptured and this damage could be reduced or reversed by *Andrographis paniculata* treatment. Since the epithelial cells of villi play an important role in mucosal immunity (Kumar et al, 2004) its protection during chemotherapy is very important.

Cytokines are low molecular weight proteins that are secreted principally by activated lymphocytes and macrophages and are responsible for the preservation and restoration of homeostasis through coordination of lymphoid cell, inflammatory cells and hematipoietic cells (Dunlop and Campbell, 2000). Tumor necrosis factor-α, is a mediator of number of inflammatory toxic responses to chemicals and therefore represents a promising target for the prevention of chemical induced inflammatory toxicity (Ma and Kinneer, 2002), as indicated by our present results. 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). Granulocyte Monocyte Colony Stimulating Factor (GM-CSF), a hemopoietic growth factor plays a pivotal role in regulation of bone marrow progenitor cells proliferation (Griffin, 1988). The fact that lowering of levels of IFN-γ, IL-2 and GM-CSF after CTX treatment was reversed by administration of *Andrographis paniculata* suggests chemoprotective effects by stimulating the immune cells of the animals and protecting them from toxic side effect of CTX.

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

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