EVALUATION OF THE CHEMOPROTECTIVE EFFECT OF *Biophytum sensitivum* (L.) DC EXTRACT AGAINST CYCLOPHOSPHAMIDE INDUCED TOXICITY IN SWISS ALBINO MICE

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SUMMARY

An alcoholic extract of *Biophytum sensitivum* was studied against cyclophosphamide (CTX) induced toxicity in mice. Intraperitoneal administration of the extract with CTX significantly increased the total WBC count (3,356 ± 236 cells/cm^2), bone marrow cellularity (15.6 ± 0.42 cells/femur) and α-esterase positive cells (846 ± 30 cells) when compared to control mice treated with CTX alone. The relative organ weight of the spleen and thymus was also found to be increased after *B. sensitivum* administration when compared to the control mice. Reduction of GSH in liver (4.9 ± 0.22 nmol/mg protein) and in intestinal mucosa (10.6 ± 1.02 nmol/mg protein) of CTX treated controls was significantly reversed by *B. sensitivum* administration (liver: 6.5 ± 0.18 nmol/mg protein; intestinal mucosa: 16.5 ± 0.88 nmol/mg protein), with amelioration of changes in serum and liver ALP, GPT and lipid peroxidation. Histopathological analysis of the small intestine also suggests that *B. sensitivum* could reduce CTX induced intestinal damage. The level of the pro-inflammatory cytokine, TNF-α, which was elevated during CTX administration, was

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significantly reduced by the administration of *B. sensitivum* extract. The lowered levels of cytokines IFN-γ, IL-2 and GM-CSF after CTX treatment were also found to be increased by *B. sensitivum* extract administration.

KEY WORDS

Biophytum sensitivum, chemoprotection, cyclophosphamide, antioxidants, cytokines, mouse

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 /1/. Cyclophosphamide (CTX), a cytotoxic alkylating agent belonging to a class of oxazaphosphorines, is used in chemotherapeutic regimens of lymphoproliferative disorders, certain solid tumors, and non-neoplastic diseases such as nephritic syndrome, systemic lupus erythematosus, and rheumatoid arthritis /2/. It is inactive *in vitro* but is activated to intracellular alkylating metabolites, such as acrolin and phosphoramide mustard, by the hepatic cytochrome P450 monooxygenase system /3/. CTX administration causes nausea, vomiting, mucosal ulceration, intestinal pulmonary fibrosis, hepatic toxicity, lymphocytopenia and alopecia. Higher doses of CTX administration produce severe urotoxicity with haemorrhagic cystitis in the urinary bladder /4,5/. Cytotoxicity towards normal host tissue is the primary dose-limiting factor in CTX therapy that reduces the quality of life and restricts the treatment protocol. Hence there is a continued interest and need for the identification and development of non-toxic and effective chemopreventive compounds that can reduce the side effects of CTX.

CTX is now being used in combination with various detoxifying and protective agents with the purpose of reducing or eliminating its adverse toxic effects. Previous work in our laboratory has shown that several plant extracts, such as *Allium sativum* /6/, *Tinospora cordifolia* /7/, *Withania somnifera* /8/, *Phyllanthus amarus* /9/ and *Andrographis paniculata* /10/, have exhibited ameliorating effects on CTX toxicity.

Biophytum sensitivum (L.) DC (Syn. *Biophytum petersianum* Klotzch) is an important medicinal plant widely used in traditional medicine in Asia, Africa and the Pacific islands, especially in Indian medicine /11,12/. The reported beneficial effects of *B. sensitivum* include anti-inflammatory /13/ and antidiabetic /14/ effects. A polysaccharide isolated from *B. sensitivum* has been found to enhance complement fixation /12/. We have already proved that ethanolic extracts of *B. sensitivum* possess antioxidant /15/, anti-angiogenic /16/, immunomodulatory and anti-tumor /17/ activities. Since little is known about its chemoprotective activity, the current study focused on protection against CTX-induced toxicity in Swiss albino mice.

MATERIALS AND METHODS

Animals

Swiss albino mice (male; 20-25 g, 6-8 weeks old) were taken from the breeding section of Amala Cancer Research Centre. The animals were fed with normal mouse chow (Sai Durga Feeds, Bangalore, India) and water *ad libitum*. All animal experiments were performed according to the rules and regulations of the Animal Ethics Committee, Government of India.

Cell line

Ehrlich ascites carcinoma (EAC) cells were procured from Adayar Cancer Institute, Chennai, India, and maintained as ascites tumor in the peritoneal cavity of Swiss albino mice.

Chemicals and ELISA kits

Cyclophosphamide (Ledoxan) was obtained from Dabur Pharma Ltd, New Delhi, India. Glutathione (GSH) and 5-5′-dithiobis (2-nitrobenzoic acid) (DTNB) were 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 from SPAN Diagnostics Ltd. All other chemicals used were of analytical reagent grade.

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

Plant extract

Authenticated *Biophytum sensitivum* obtained from Amala Ayurvedic Centre was dried at 45°C and powdered. Ten grams was stirred overnight in 70% methanol (100 ml), centrifuged at 10,000 rpm at 4°C for 10 min, and the supernatant was collected. Methanol was removed by evaporation, and the yield was 12% (w/w). Phytochemical analysis showed the presence of flavonoids.

Drug administration

For animal studies *B. sensitivum* extract was suspended in 1% gum acacia to the desired concentration (500 µg/dose/animal) and administered intraperitoneally simultaneously with CTX. The drug was continued for 10 consecutive days.

Haematological parameters

Three groups of animals (six mice/group) were used for this study. Group I animals received 10 doses of *B. sensitivum* extract. Group II and III animals received CTX at a dose of 25 mg/kg body weight i.p. for 10 consecutive days: Group II was the control group treated with CTX alone, while group III animals were treated simultaneously with *B. sensitivum*, beginning on the same day as CTX administration and continued for 10 consecutive days with the CTX. Blood was collected from the caudal vein, and total white blood cell (WBC) count (haemocytometer), differential count (Leishman's stain) and haemoglobin level (cyanmethaemoglobin) were recorded prior to the extract administration and continued every third day for 30 days after the administration of the extract.

Lymphoid organ weight, bone marrow cellularity and α-esterase activity

Swiss albino mice were divided into three groups (18 animals per group). Treatments were the same as in the previous experiment. Six

animals from each group were sacrificed at different time intervals (days 2, 7 and 11) by cervical decapitation. The body weight of each animal was recorded before sacrifice, and the weight of the lymphoid organs - liver, spleen and thymus - were recorded and expressed as relative organ weights.

Bone marrow cells from the above preparation were made into single cell suspensions and their number determined using a haemocytometer. Bone marrow cells from the above preparations were smeared on clean glass slides and stained with Harri's hematoxylin to determine non-specific α-esterase activity by the azodye coupling method /18/.

Biochemical assays

Swiss albino mice were divided into two groups (18 animals per group). Treatments were the same as in the previous experiment. Six animals from each group were sacrificed at different time intervals (days 2, 7 and 11) by cervical decapitation. Blood was collected from each animal by heart puncture immediately after sacrifice and serum was separated. The liver and intestine were also excised and washed thoroughly in ice-cold phosphate buffered saline, and samples were used to estimate various biochemical parameters. The intestinal mucosa was collected and used to estimate the levels of GSH by the method of Moron *et al.* /19/. A portion of the intestine (jejunum) was kept in formaldehyde for histopathological analysis. Liver homogenate was made in ice-cold Tris buffer (0.1 M, pH 7.4) and centrifuged at 4°C at 1,200 rpm for 10 min. The supernatant was used for the estimation of ALP /20/, GPT /21/ and lipid peroxidation (LPO) /22/. Serum was also used to estimate all the above parameters.

Cytokine assay

Swiss albino mice were divided into two groups (18 animals per group). Treatments were the same as in the previous experiment. Six animals from each group were sacrificed at different time intervals (days 2 and 11) by cervical decapitation. Blood was collected from each animal by heart puncture immediately after sacrifice and serum was separated. The level of cytokines IL-2, IFN-γ, GM-CSF and TNF-α were measured on the day of sacrifice using ELISA kits specific for murine cytokines according to the manufacturer's instructions.

Statistical analysis

Values are expressed as means ± SD. Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Dunnett's test. P values less than 0.05 were considered to be significant.

RESULTS

Effect of *B. sensitivum* on hematological parameters

CTX administration reduced the total WBC count in mice (Fig. 1). The animals in both group showed a reduction in the total WBC count. The animals treated with CTX alone showed a drastic reduction in total WBC count on day 12 to 2,016 ± 120, while total WBC count was 3,356 ± 236 in the animals treated with *B. sensitivum* plus CTX on the same day; furthermore, WBC increased and normalized on day

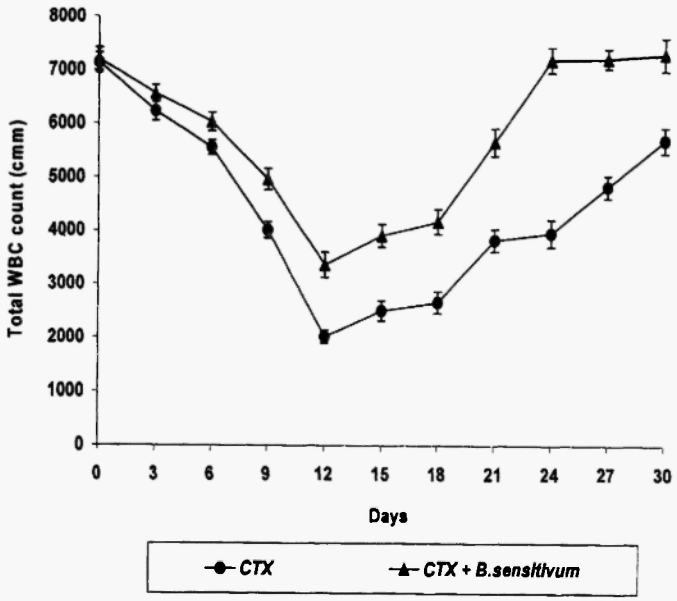

Fig. 1: Effect of *Biophytum sensitivum* on total white blood cell (WBC) count in cyclophosphamide (CTX)-treated mice.

24. The CTX alone group recovered normal WBC count only on day 36 after CTX administration (data not shown). Differential counts and haemoglobin content did not show any significant changes in either treated or untreated animals (data not shown).

Effect of *B. sensitivum* on organ weight, bone marrow cellularity and α-esterase activity

The CTX treated control animals showed a large reduction in the weight of the thymus and spleen, 0.07 ± 0.002 g/100 g b.wt. and 0.14 ± 0.020 g/100 g b.wt., respectively, on day 2 after CTX administration, and had not normalized even by day 11. Even though *B. sensitivum* treated mice showed a reduction in the weight of the thymus to 0.09 ± 0.002 g/100 g b.wt. and the spleen to 0.18 g/100 g b.wt. on day 2 in CTX treated animals, they gradually increased and normal weight was attained by day 11 (spleen: 0.33 ± 0.01, and thymus: 0.15 ± 0.003 g/100 g b.wt.). There was an increase in the weight of the spleen (0.45 ± 0.02 g/100 g b.wt.) and thymus (0.16 ± 0.01 g/100 g b.wt.) when normal animals received 10 doses of *B. sensitivum* extract.

Normal animals after treatment with *B. sensitivum* showed an increase in bone marrow cellularity (22.5 ± 2.0 x 10^6 cells/femur) and α-esterase positive cells (1,502.6 ± 42.5 cells/4,000 cells). The number of bone marrow cells and α-esterase positive cells were decreased drastically in the control animals treated with CTX alone, but this was reversed by administration of *B. sensitivum*. In the control animals, on day 2 after the last dose of CTX there was a drastic reduction in the number of bone marrow cells (5.6 ± 0.6 x 10^6 cells/femur) and α-esterase positive cells (235.6 ± 15.5 bone marrow cells/4,000 cells) compared to normal animals (bone marrow cellularity: 16.2 ± 0.56 cells/femur; α-esterase: 880 ± 30.2 bone marrow cells/4,000 cells). Even after day 11, the bone marrow cells (11.4 ± 2.0 x 10^6 cells/femur) and α-esterase positive cells (540.6 ± 20.2/4,000 cells) did not reach normal values. Treatment with *B. sensitivum* elevated bone marrow cellularity and the number of α-esterase positive cells, thus protecting the mice from the myelosuppressive effect of CTX. In the *B. sensitivum* treated group of animals, bone marrow cellularity and α-esterase positive cells were found to be 8.2 ± 0.6 x 10^6 cells/femur and 490 ± 30.6 cells/4,000 bone marrow cells, respectively, after 2 days,

and these were further enhanced to 15.6 ± 0.42 cells/femur and 846 ± 30 cells/4,000 bone marrow cells on day 11 after the last dose of CTX.

Effect of *B. sensitivum* on biochemical parameters after CTX administration

CTX administration in mice was found to decrease the levels of GSH in liver as well as intestine but this was reversed by the treatment with *B.sensitivum* (Table 1).

Tables 2-4 show the effects of *B. sensitivum* extract on serum and liver ALP, GPT, and LPO levels in CTX treated mice. In all cases the adverse effects caused by the toxin were reversed to a significant extent by *B. sensitivum* treatment.

Histopathological analysis

Histopathological analysis of the jejunum of the control animals treated by CTX alone showed severe damage to the intestinal villi when compared to normal. The lengths of the villi were markedly reduced and the crypt architecture was largely destroyed. This was inhibited by the *B. sensitivum* extract (Fig. 2).

Effect of *Biophytum sensitivum* on cytokine production during CTX treatment

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

DISCUSSION

The two fundamental cancer treatments, chemotherapy and radiotherapy, have long been known to have a magnitude of short- and long-term adverse effects. Many drugs are used as chemotherapeutic agents against various forms of cancer. However, most of the agents have cell toxicity and can induce genotoxic, carcinogenic and teratogenic effects in non-tumor cells that can give rise to secondary tumors. Cyclophosphamide is an anticancer prodrug that is dependent on cytochrome P450 metabolism for its therapeutic efficacy. CTX is

TABLE 1

Effect of *Biophytum sensitivum* treatment on intestinal and liver glutathione (GSH) levels in cyclophosphamide (CTX) treated mice

Group	Intestine GSH (nmol/mg protein)			Liver GSH (nmol/mg protein)		
Day	2	7	11	2	7	11
Normal	18.2 ± 0.88	–	–	7.2 ± 0.8	–	–
CTX alone	6.5 ± 0.62	9.5 ± 2.02	10.6 ± 1.02	2.2 ± 0.08	2.5 ± 0.08	4.9 ± 0.22
CTX +*B. sensitivum*	12.2 ± 1.2*	15.4 ± 1.05	16.5 ± 0.88	4.8 ± 1.02	5.8 ± 1.0	6.5 ± 0.18

The treated animals received 10 doses of *B. sensitivum* (500 µg/dose/animal, i.p.) and 10 doses of CTX (25 g/kg/b. wt., i.p.) simultaneously. Animals were sacrificed on day 2, 7 and 11 after the last dose of CTX administration, and the GSH levels in the liver and intestinal mucosa were estimated. Values are means ± SD.

* $p < 0.05$ compared to CTX alone.

TABLE 2

Effect of *Biophytum sensitivum* treatment on serum and liver alkaline phosphatase (ALP) levels in cyclophosphamide (CTX) treated mice

Group	Serum ALP (KA units)			Liver ALP (KA units)		
Day	2	7	11	2	7	11
Normal	13.5 ± 0.45	–	–	14.2 ± 0.26	–	–
CTX alone	25.5 ± 2.36	22.4 ± 1.08	21.0 ± 1.0	4.6 ± 0.24	8.8 ± 0.85	11.2 ± 0.8
CTX +*B. sensitivum*	19.2 ± 1.6*	16.4 ± 0.25*	14.5 ± 1.08*	9.5 ± 0.86*	13.4 ± 1.0*	14.4 ± 0.18

Values are means ± SD.

* $p < 0.05$ compared to CTX alone.

TABLE 3

Effect of *Biophytum sensitivum* treatment on serum and liver glutamate pyruvate transaminase (GPT) levels in cyclophosphamide (CTX) treated mice

Group	Serum GPT (U/ml)			Liver GPT (μ moles of pyruvate formed/mg protein)		
Day	2	7	11	2	7	11
Normal	14.0 ± 0.74	–	–	2.51 ± 0.20	–	–
CTX alone	37.1 ± 0.8	31.3 ± 0.4	29.8 ± 0.82	0.88 ± 0.38	1.18 ± 0.12	2.0 ± 0.39
CTX +*B. sensitivum*	21.6 ± 1.06***	19.7 ± 0.95**	15.0 ± 0.95**	1.42 ± 0.24*	1.88 ± 0.15	2.48 ± 0.30

Values are means ± SD.
* p <0.05, ** p <0.01, *** p <0.001 compared to CTX alone

TABLE 4

Effect of *Biophytum sensitivum* treatment on serum and liver lipid peroxidation (LPO) levels in cyclophosphamide (CTX) treated mice

Group	Serum LPO (nmol/ml)			Liver LPO (nmoles MDA formed/mg protein)		
	Day 2	7	11	2	7	11
Normal	1.42 ± 0.24	–	–	1.4 ± 0 03	–	–
CTX alone	7.40 ± 0.14	6.98 ± 0.04	5.84 ± 0.08	4.80 ± 0.24	3.53 ± 0.12	2.05 ± 0.16
CTX +*B. sensitivum*	2.16 ± 0.14*	1.76 ± 0.08*	1.48 ± 0.05	2.80 ± 0.16*	1.65 ± 0.12	1.38 ± 0.11

Values are means ± SD.

* $p < 0.05$ compared to CTX alone.

Fig. 2: Histopathological analysis of the jejunum of mice treated by CTX alone (b) showed severe damage to the intestinal villi when compared to normal mice (a); the lengths of the villi were markedly reduced and the crypt architecture was largely destroyed. This was partly inhibited by the co-administration of *Biophytum sensitivum* extract (c).

TABLE 5

Effect of *Biophytum sensitivum* treatment on serum interferon (IFN)-γ and interleukin (IL)-2 levels in cyclophosphamide (CTX) treated mice

Group	IFN-γ (pg/ml)		IL-2 (pg/ml)	
Day	2	11	2	11
Normal	1,980 ± 34.9	–	11.1 ± 0.47	–
B. sensitivum alone	2158 ± 18.8	2775 ± 40.5	14.2 ± 1.82	16.5 ± 2.2
CTX alone	550 ± 20.8	720 ± 19.6	4.6 ± 0.34	5.5 ± 0.23
CTX +*B. sensitivum*	1,222 ± 33.5***	2,020 ± 25.6***	6.8 ± 1.26	11.0 ± 1.22*

Values are means ± SD.
* $p < 0.05$, *** $p < 0.001$ compared to CTX alone.

TABLE 6

Effect of *Biophytum sensitivum* treatment on serum granulocyte monocyte colony stimulating factor (GM-CSF) and tumor necrosis factor (TNF)-α level in cyclophosphamide (CTX) treated mice

Group	GM-CSF (pg/ml)		TNF-α (pg/ml)	
Day	2	11	2	11
Normal	33.1 ± 0.46	–	20.2 ± 0.79	–
***B. sensitivum* alone**	38 2 ± 1.2	40.5 ± 5.5	18.2 ± 0.65	17 2 ± 0.87
CTX alone	18 4 ± 0 05	24.27 ± 0.65	279.4 ± 7.94	264.7 ± 5.41
CTX +*B. sensitivum*	25.0 ± 2.46*	32.6 ± 1.08**	112.6 ± 2.93***	92.6 ± 3.36***

Values are means ± SD.
* p <0 05, ** p <0.01, *** p <0.001 compared to CTX alone

metabolized by hepatic cytochrome P450s via two major pathways. The first involves 4-hydroxylation to the active metabolite, 4-hydroxy-CTX. 4-Hydroxy-CTX exists in equilibrium with aldophosphamide, which breaks down to form the DNA cross-linking agent, phosphoramide mustard, and the toxic metabolite, acrolein /23-25/. The alternative pathway involves *N*-dechloroethylation of CTX to form the inactive metabolite, 3-dechloroethylcyclophosphamide, and the toxic by-product, chloroacetaldehyde /23,24,26/. Many of these metabolites are known to cause myelosuppression, primarily through damage to rapidly proliferating haematopoietic progenitors and their mature progeny, leading to decline in the number of peripheral blood cells /27/. Administration of an ethanolic extract of *B. sensitivum* in CTX treated mice was found to enhance the total WBC count, bone marrow cellularity and α-esterase positive cells, which were drastically reduced in the CTX alone treated control animals, suggesting that CTX induced myelosuppression was reversed or inhibited by the plant extract administration, possibly through its immunomodulatory activity. The weight of the lymphoid organs, especially the spleen and thymus, was also increased in CTX treated animals by plant extract administration, providing supportive evidence for its immunostimulative potential during CTX therapy.

ALP plays an important role in carbohydrate metabolism and oxidative phosphorylation. ALP is present in tissues, especially in the cell membrane, and serves as a membrane marker in particular during liver injury. CTX administration elevated the level of serum ALP, and *B. sensitivum* co-administration significantly inhibited its production, indicating a protective effect against liver injury due to CTX. *B. sensitivum* was also found to decrease the activity of serum GPT in the CTX treated animals, supporting the ameliorating effect of *B. sensitivum* on CTX-induced liver damage. The process of lipid peroxidation is the oxidative conversion of polyunsaturated fatty acids to a product known as malondialdehyde (MDA), or lipid peroxides /28/. MDA, owing to high cytotoxicity and inhibitory action on protective enzymes, is suggested to act as a tumor promoter /29/. The oxidative product of CTX is responsible for the induction of LPO, which has a devastating effect on the cell membrane /30/. Lipid peroxidation was found to be decreased in mice treated with *B. sensitivum* plus CTX compared to CTX alone, indicating that one of

the mechanisms by which *B. sensitivum* exerts its chemoprotective action may be by inhibiting lipid peroxidation.

One of the major cellular non-enzymic antioxidants is GSH, which is involved in the detoxification of toxic electrophilic xenobiotics, hydrogen peroxide and oxygen free radicals. The metabolism of CTX in the body produces highly reactive electrophiles and the decreased amount of GSH in the CTX treated group is probably due to the electrophilic burden on the cells and also due to the formation of acrolein, which is known to deplete GSH content and DNA alkylation /31/. Co-administration of *B. sensitivum* with CTX elevated the GSH level in both liver and intestine, thereby increasing its ability to cope with the free radicals produced during CTX administration. This also suggests the protective effect of the extract against CTX induced toxicity.

The present histopathological analysis demonstrated the intestinal villi of CTX treated mice to be ruptured, and this damage was reduced or reversed by *B. sensitivum* treatment. Since the epithelial cells of the villi play an important role in mucosal immunity /32/, its protection during chemotherapy is very important.

Cytokines are peptides and low-molecular weight proteins, which affect cell functions and condition their interactions. They are produced by cells that possess regulatory properties and are found very close to or in direct contact with target cells. By binding to specific cell surface receptors, they affect cell proliferation, differentiation and functioning. These proteins mediate and regulate homeostasis through coordination of lymphoid cells, inflammatory cells and haematopoietic cells /33/. TNF-α is a mediator of a number of inflammatory toxic responses to chemicals and therefore represents a promising target for the prevention of chemical induced inflammatory toxicity /34/, as indicated by our present results. The lymphokine, IL-2, which was identified as T cell growth factor /35/, plays a central role in the maturation and development of lymphocytes and monocytes /36/, whereas IFN-γ stimulates phagocytic activity of macrophages and differentiation of T cells and cytotoxic effects /37/. GM-CSF, a haemopoietic growth factor, plays a pivotal role in the regulation of bone marrow progenitor cell proliferation /38/. The fact that the reduction in the levels of IFN-γ, IL-2 and GM-CSF after CTX administration was reversed by the co-administration of *B. sensitivum*

suggests chemoprotective effects by stimulating the immune cells of the animals and protecting them from the toxic side effects of CTX.

In conclusion, the results of the above experiments strongly suggest a chemoprotective effect of *B. sensitivum* extract, and this may be due to stimulation of the antioxidant as well as immune system. *B. sensitivum* reportedly contains flavonoids, including iuteolin 7-methyl ester, isoorientin, and others; biflavones, such as cupressuflavone and amentoflavone; as well as two acids, 4-caffeoylquinic acid and 5-caffeoyl quinic acid /39/, and phenolic compounds /40/. At present, we do not know which compounds are responsible for the chemoprotective effect produced by this extract. Further studies must be conducted to fully elucidate the ameliorative effect of *B. sensitivum* as well as its isolated compounds without inhibiting the anticancer activity of cyclophosphamide before exploiting this plant for clinical trials.

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