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Chemo Protective Action of *Asparagus racemosus* against Cyclophosphamide Induced toxicity

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ABSTRACT

Plants and their extracts have been the basis of treatment of human diseases since time immemorial. Use of plants as a source of immunomodulators is under extensive use and significances. In the present study, the aqueous extract of *Asparagus racemosus* significantly enhanced the total WBC count, hemoglobin content and bone marrow cellularity in animals treated with Cyclophosphamide. It also enhanced the weight of lymphoid organs such as thymus and spleen, which indicates its effect on cellular immune response. The number of α-esterase positive cells, which were decreased by CTX administration, was also found to be increased by *Asparagus racemosus* treatment indicating its effect on stem cell differentiation .These observation indicated that the *Asparagus racemosus* could overcome the immunosuppression induced by Cyclophosphamide. Thus it can be concluded that the *Asparagus racemosus* can be used as an adjuvant along with cancer chemotherapy.
Keywords: immunosuppressant, immunomodulators, Cyclophosphamide, *Asparagus racemosus*

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INTRODUCTION

One of the current most devastating afflictions faced by mankind is cancer. Cancer is caused by the progressive growth of the progeny of a single transformed cell. Most cancers are clonal in origin and in tumor in progression. Carcinogens are agents, which are associated with or responsible for the process of neoplasia. Carcinogens include all exogenous chemicals and other stimuli that are associated with an increase in cancer incidence. A wide variety of chemicals and chemicals classes can cause cancer in animals and humans [1]. The N-nitroso compounds are environmentally important chemical carcinogens [2], which alkylate different cellular macromolecules, the most critical target being DNA [3] in addition to organic compounds, a number of inorganic elements and their compounds such as Beryllium, Iron, cobalt, Zinc, Lead and Platinum are carcinogenic in both animals and humans [4]. Carcinogenic polycyclic hydrocarbons are metabolized by cellular enzymes to epoxide intermediates, which cause the neoplastic transformation [5]. The alkyl ting agents such as nitrogen mustard, ethylene mine etc. are direct carcinogens (Marcel, 1986. Apart from chemical agents physical agents, can induce cancer- ionizing radiations, UV radiation and mineral fibers.

Cancer formation can be induced by radiation, but certain types of cancer predominate in radiation such as; leukemia [61] thyroid cancer [7], breast cancer, lung cancer and bone cancer [8]. The mechanism of radiation- induced cancer includes DNA Injury [9], virus activation [10], DNA repair [11] and immunosuppression [12]. Asbestos is a naturally occurring fiber, which is a potent carcinogen [13].

Besides physical and chemical agents, some of the DNA viruses can induce cancer. Viral infections account for many of the human cancers worldwide. Papilloma virus, Hepatitis B Virus, SV40, Polyoma Virus, Epstein- Barr virus are some of the DNA viruses that induce cancer. Human T-Lymphotrophic Virus -1 is the only RNA virus currently known to be associated with a human cancer, an endemic adult T- Cell leukemia [14]. In addition to all these external carcinogens certain endogenous alteration such as the activation of oncogenes and in activation to turnover suppressor genes also results in the development of cancer [15].

Curing of cancer requires removal or destruction of all malignant cells without killing the patient. Surgical therapy, Chemotherapy, Immunotherapy and Radiotherapy are the commonly used therapies for cancer. Cancer chemotherapy involves the use of various anticancer drugs. A wide spectrum of human malignancies can be affected by the administration of antineoplastic drugs. Cyclophosphamide, Ifosfamide, Chlorambucil, Busulfan, Cisplatin, Carboplatin etc. are some of the anticancer drugs [16].

Immunotherapy involves the use of agents that stimulate host resistance to cancer and these agents are called Biological Response Modifiers (BRMs). Interferon's, Human haematopoietic colony stimulating factors, IL -1, IL- 2, and Tumor Necrosis factors are some of the commonly used BRMs [17].

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Radiotherapy is a clinical medical therapy in which ionizing radiation is used to treat patients with cancers. Most radiation therapy is carried out with beams of X- rays or γ- rays. Isotopes such as ^{313}Cs, ^{192}Ir, ^{60}Co are used in the radiation treatment.

The major drawback of cancer chemotherapy and radiotherapy is suppression of the immune system [18]. Drugs that could alleviate these side effects will be highly useful in cancer therapy. The main objective of cancer treatment is eradication of the tumor. For this different therapies such as Surgery, Chemotherapy, Immunotherapy and Radiotherapy can be applied either singly or in combination, such that all the malignant cells are destroyed without killing the patient.

Surgery is the mode of treatment when the tumor is localized and confined to a specific site. Surgery is not possible if the tumor is adhering to vital structure like the aorta or may be very difficult if the tumor is not in an accessible area [19].

Immunomodulators are materials, which can modify body's defense mechanism. Many plant extracts are found to have immunomodulatory effects, but the use of plants as sources of immunomodulators is under trial. Some of the plants and their compounds with known immunomodulatory activity are *Viscum album Tinospora cordifolia*, [20] *Withania somnifera* [21, 22], *Asparagus racemosus* [23], *Panax ginseng* [24], *Rasayanas* [25, 26].

Asparagus racemosus is a member of the plant family Liliaceae and Genus racemosus contains substantial quantities of isothiocyanates, some of which are very potent inducers of phase 2 detoxification enzymes of xenobiotic metabolism [27] and thereby protect cells against mutagenesis and neoplasia.

The present study is an analysis of the chemo protective effects of *Asparagus racemosus* on Cyclophosphamide treated mice with the following.

OBJECTIVES

1. Determination of hematological parameters,
2. Determination of organ weight, bone marrow cellularity and
3. Determination of the effect on X- Esterase positive cells.

METHODOLOGY

Test compound

Cyclophosphamide I.P., manufactured by Getwell Pharmaceuticals, was prepared by dissolving it in distilled water at a concentration of 25mg/Kg body weight and administered intraperitonial.

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Animals

Swiss albino mice (4-6 week old of 20-25 body weight) were used. The animals were made to acclimatize with the lab conditions for a period of 15 days in a healthy condition prior to study.

A. Determination of the effect of an aqueous extract of *Asparagus racemosus* on the Haematological parameters of cyclophosphamide induced mice.

Swiss albino mice were divided into 3 groups (8 mice/ group).

Group I - Treated with a doses (500 μg /dose/animal) of aqueous extract of *Asparagus racemosus* on consecutive days.
Group 2 - Treated with 10 doses of CTX (25mg/kg body weight) on consecutive days.
Group 3-Treated with 10 doses each of CTX and aqueous extract of *Asparagus racemosus*. on consecutive days.

Blood was collected from caudal vein and parameters such as total WBC count (Haemocytometer), Differential count (Leishman's stain) and Haemoglobin content (Cyanmethemoglobin method) and the body weights were recorded prior to the drug treatment and every third day for one month.

i. Differential Count of Leukocytes - using Haemocytometer

ii. Determination of Haemoglobin Content [28]

B. Determination of the Effect of Aqueous Extract of Asparagus on Organ weight.

Swiss albino mice were grouped into 4 groups.

Group 1- consists of 4 animal kept as normal (control)
Group 2 and 3- (8mice/group) were treated with 10 doses (500μg//animal) of aqueous extract of *Asparagus racemosus* on consecutive days.
Group 3 and 4- (8mice/group) were treated with 10 doses (25mg/kg body weight) of CTX on consecutive days. The extract and CTX were administered intraperitonial.

Group 1 animals were sacrificed and lymphoid organs such as spleen and thymus were collected, weighed and expressed as relative organ weight.
Group 2, 3 & 4 – animals (4) from -were sacrificed on 11^{th} and 15^{th} day after the 1^{st} day or CTX. Lymphoid organs such as spleen and thymus were collected, weighed and expressed as relative organ weight. Body weights of the animals were recorded prior to the sacrifice.

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C. Determination of the Effect of Aqueous Extract of *Asparagus racemosus* on Bone Marrow Cellularity.

Bone marrow cellularity was determined by the method of [29]

Swiss albino mice were grouped into 4 groups.

Group 1 consists of 4 animal kept as normal (control)
Group 2 and 3 (8 mice/group) were treated with 10 doses of (500μg/dose/animal) of aqueous extract of *Asparagus racemosus* on consecutive days.
Group 3 and 4 (8 animals/group) were treated with 10 doses of CTX (25mg/kg body weight) on consecutive days. The extract and CTX were administered intraperitonally in all animals.

Group 1 animals were sacrificed by cervical dislocation. Bone marrow was collected from the femur by flushing out of the medullary cavity using a jet of PBS containing serum, with needle and syringe. Bone marrow was made into a single cell suspension and the cell number was determined using Haemocytometer and expresses as total number of live cells as femur Haemocytometer and expressed as total number of live cells per femur. Four (4) animals from group 2, 3 &4 were sacrificed on 11th and 15th days after 1st dose of CTX and bone marrow cellularity was determined as above.

D. Determination of a-esterase positive cells - Azo dye coupling [30]

The cells were stained with α Naphthyl acetate and Pararosaniline hydrochloride (45 minutes) and counter stained with Hematoxy1ine (1 minute). The number of a-esterase positive cells was expressed out of 4000 cells.

RESULTS

Administration of CTX significantly lowered the total WBC count in mice (figure 1) Myelosuppression as can be seen from the WBC count, was observed throughout the period of CTX administration. The count was drastically reduced to 2743.75±152.96Cell/mm3 compared to normal (6850±152.96Cell/mm3) on the 9th day in animals treated with CTX alone. For animal treated with CTX along with *A.racemosus*, it was reduced to only 5331.25±152.96Cell/mm3 on the same day.

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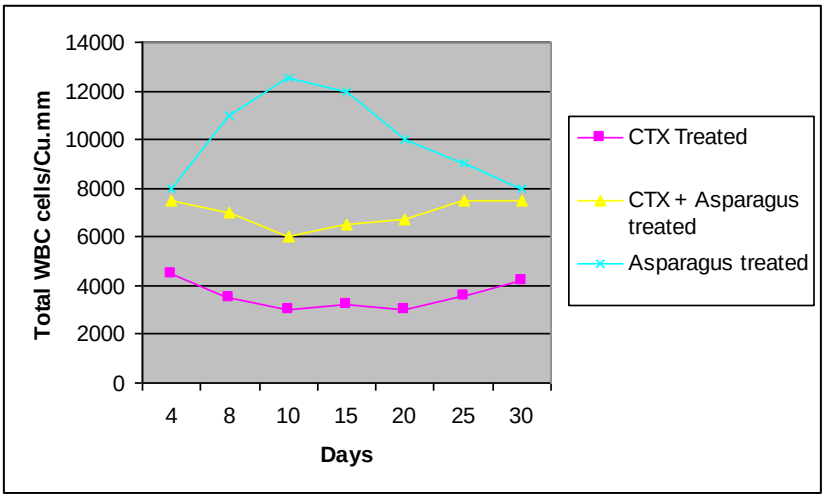

Figure 1 – Effect of *A.racemosus* extract on Total WBC Count among CTX treated mice

Day 12 onwards the animals showed an increase in their WBC counts. In CTX alone treated animals, this increase was rather slow compared to animals treated with CTX along with *A.racemosus* treated animals the total WBC counts reached normal (6583.7±84.54Cell/mm3) by the 18th day itself, but for animals treated with CTX alone, it took 30 days to reach the normal (6462.5±136.4Cell/mm3). This indicates that *A.racemosus* extract significantly elevated the level of WBC.

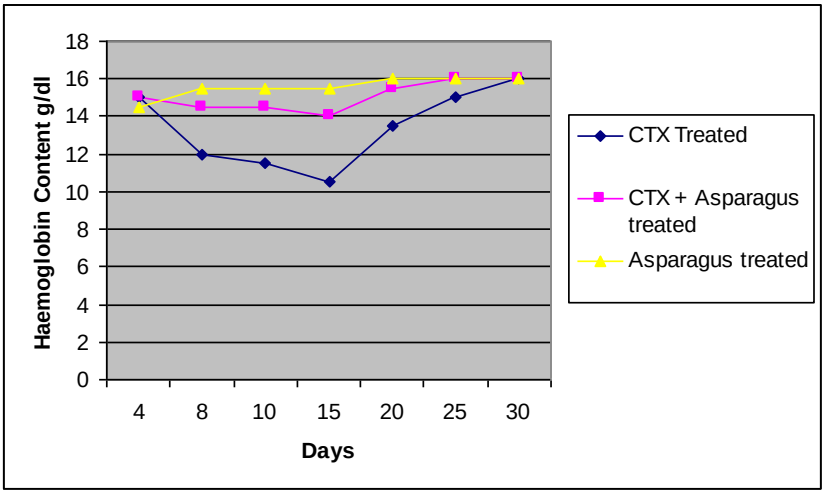

Figure 2 – Effect of *A.racemosus* extract on Hb content among CTX treated mice

Differential count did not show any significant verification Haemoglobin levels also increased in *A.racemosus* treated animals compared to that of CTX treated animals (figure 2). On 9th day the Haemoglobin level of CTX alone treated animals was only 10.69±0.23g/dl compared to normal 15.44±0.19 g/dl. In the animals treated with CTX alone with *A.racemosus*, the Haemoglobin level was 14.06±0.19 g/dl. This indicates that *A.racemosus* was effective in maintaining the Haemoglobin content, throughout the CTX treatment. The Haemoglobin levels

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reached normal (15.21±0.26 g/dl) on 15th day itself in CTX along with *A.racemosus* treated animals.

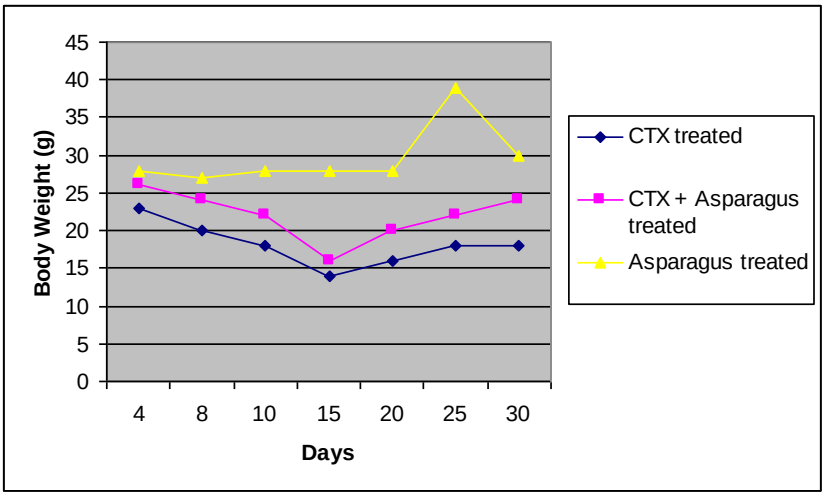

Figure 3 – Effect of A.racemosus extract on body weight among CTX treated mice

There was also a significant decrease in body weight of animals treated with CTX (Figure.3). The decrease in body weight of animals treated with CTS with *A.racemosus* extract was less compared with CTS alone treated animals. This can be seen from the observation that on 9th day for CTX alone treated animals the body weight was lowered from 25.58±0.368g to 17.06±0.368g, while the body weight of CTX alone with *A.racemosus* treated animals was reduced to only 20.03±1.22g from the normal on the same day. This clearly indicates the effect of *A.racemosus* on body weight.

	11th day		15th day	
	Relative organ weight (g / 100 g body weight)			
Treatment	Thymus	spleen	Thymus	spleen
Control	0.15±0.002	0.41+0.02	0.14±0.01	0.38±0.01
CTX treated	0.08±0.01	0.30±0.01	0.10±0.002	0.32±0.005
CTX + Asparagus treated	0.11±0.008	0.36±0.02	0.12±0.001	0.39±0.006
Asparagus treated	0.17±0.006	0.73±0.01	0.20±0.002	0.75±0.004

Table 1: Determination of the Effect of Aqueous Extract of Asparagus on organ weight (Results are the mean of replicates – g/ 100g)

CTX treatment has significantly reduced the relative organ weigh of lymphoid organs such as thymus and spleen (Table 1) on 11th day after CTX treatment, the relative, organ weight of thymus and spleen of CTX alone treated animals were only (0.06 ±0.001) g/100g body weight and (0.31 ±0.011) g/100g body weight respectively, compared to the relative organ weight of thymus and spleen of normal (0.31 ±0.011) g/100g body weight respectively compared to normal. This indicates its protective effect on theses organs.

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Treatment	11th day	15th day
Control	19.6±0.002x10^6	19.4x10^6
CTX treated	4.97±0.01 x10^6	9.6x10^6
CTX + Asparagus treated	10±0.006 x10^6	16x10^6
Asparagus treated	22±0.39 x10^6	26x10^6

Table 2 :Determination of the Effect of Aqueous Extract of *Asparagus* on bone marrow cellularity (cells of femur) (Results are the mean of replicates – g/ 100g)

Animal treated with CTX alone had a significantly lowered bone marrow cellularity (5.25 ±0.55 x 10^6Cells / femur) on 11^{th} day after CTX administration compared to normal (17.25 ±0.56 x 10^6Cells / femur) (Table 2). This was increased with CTX along with *A.racemosus,* the above marrow cellularity on 11^{th} day (7.37 ±0.4 x 10^6Cells / femur) has significantly increased to 14 ±0.35 x 10^6Cells / femur) on 15^{th} day compared to normal. This clearly indicates the effect of *Asparagus racemosus* extract on bone marrow cell proliferation.

Treatment	11^{th} day	15^{th} day
Control	778±8.4	772±8.4
CTX treated	543±9.4	409±7.3
CTX + Asparagus treated	705±5.6	756±6.9
Asparagus treated	1254±9.3	1312±4.7

Table 3: Effect of *A.racemosus* on α- esterase positive cells of cyclophosphamide treated mice (Results are the mean of replicates – g/ 100g)

Levels of μ- esterase positive cells were significantly decreased after CTX administration (Table 3). *Asparagus racemosus* treatment elevated the number of α- esterase positive cells in animals treated with CTX along with *A.racemosus*. on 11^{th} day, after CTX administration, the number of α- esterase positive cells treated with CTX along was only 477.5±7.63 Cells/4000 cell compared to that of normal 863.±22.29 cells / 4000 cells. In the case of animals treated with CTX along with *A.racemosus* extract, the number of α- esterase positive cells was 722.5.±5.68 cells / 4000 cells on 11^{th} day compared with normal. This was significantly increased to 833.25.±6.76 cells / 4000cells on 15^{th} day but the number of α- esterase positive cells in the case of animals treated with CTX alone was increased only up to 683.25.±6.98 cells / 4000cells on 15^{th} day. This indicates the effect of *A.racemosus* on stem cell proliferation and differentiation.

DISCUSSION

Immunotherapy have also been associated with certain side effect such as fever, chills, malaise, nausea, vomiting, acute respiratory complications, inflammation and ulceration at sites of administration of microbial products [30]. There are several complications associated with

radiation therapy. Bone marrow toxicity, immunosuppression [31, 32], skeletal complications [33] such as osteonecrosis, sarcoma [34] hepatic injury [35] are some of them. Carcinogenesis may be a late consequence of radiotherapy [36].

Drugs used in cancer chemotherapy can be classified into

1. Alkylating agents
2. Antibiotics
3. Plant derivatives
4. Antimetabolites
5. Hormones and hormone antagonists and
6. Miscellaneous agents

There are 4 ways chemotherapy is generally used

1. As an induction treatment for advance disease.
2. As an adjunct to local methods of treatment.
3. As the primary treatment for patients who present with localized cancer and,
4. By direct installation into sanctuaries or by site- directed perfusion of specific region of the body most affected by the cancer [13].

Cyclophosphamide (CTX) is a chemical derivative of mecholrethamine synthesized in Germany in 1958 [37]. It is one of the most widely used alkylating agent and antineoplastic drug for the treatment of breast cancer, lymphomas, childhood tumors and many solid tumors [38] CTX is extensively metabolized in vivo to active and inactive metabolites [39] – Phosphoramide mustard and acrolein being cytotoxic. Minimizing the damage to normal tissues caused by chemotherapy has been instigated the development of methods to improve the therapeutic index [40]. Several agents like WR – 2771 [41], N- acetly1 Cysteine, mercaptioethane sulfonate (MESNA) [42] and mercaptoproponal glycine have been used as chemo protecting agents, but toxicity produced after repeated administration limits their clinical significance.

Recently there is an increase in interest in the search of potential drugs of plant origin that are capable of minimizing the toxicity induced by chemotherapy to normal cells without compromising its anti-neoplastic activity. Traditional system of Indian medicine extensively uses plant derived compounds and formulations to modulate the immune system of host. These herbal formulations were found to be less toxic or non – toxic. Extracts of garlic [43], *Viscum album* [21] and isolated compounds like curcumin [44] and multiherabal formulation like septilin [45], Brahmarasayana [46, 47] were found to have chemo protective activity in experimental animals as well as in patients receiving chemotherapy.

Asparagus racemosus

Asparagus racemosus belongs to the family Liliaceae and has more than one hundred glucosinolate (GS) derivatives Brassica. The primary product of hydrolysis of GS, catalyzed by

mycrosinae, is an unstable aglycone, which then decomposes to a range of products of which the most common is isothiocyanates. These isothiocyanates possess a range of antifungal [48] antibacterial and antimicrobial activities, which are probably the basis of the use in folk medicine.

Studies have shown that feeding Brassica vegetables to animals causes an increase in the activities of biotransformation enzymes in various tissues [49]. The enzyme inducing activity of Brassica vegetables is due to the hydrolysis products of glucosinloates [50] The phase 1 oxidative enzymes are mainly induced by the uptake of Brassica vegetables [51].

Specific Glucosinolate metabolites in certain sub- species of *Asparagus racemosus* and inhibit the growth of cancer cells. Benzyl isothiocyanates have a blocking effect against, 7,-12 diethyl 1 benazanthracence (DMBA) induced carcinogenesis and also suppress the formation of mammary tumors [52, 53].

Epidemiological data indicate that consumption of cruciferous vegetables is associated with a decreased incidence of cancer in human population [54, 55]. A decreased incidence of various cancer including prostate cancer [56] and lung cancer [57] in humans consuming large quantities of cruciferous vegetables. In cancer chemotherapy, alkylating agents stands first. Cyclophosphamide is one of the most commonly used alkylating agent in vivo to cytotoxic metabolites such as acrolein and phosphoramide mustard [58, 59]. Phosphoramidc mustard is known to cause myelosuppression.

Development of effective and non-toxic protectors of chemotherapy is essential for reducing the injury caused to normal cells. Many chemo protectors have been reported which include endogenous antioxidants and immunomodulators. Materials of plant sources like lectins [60] induce chemo protection.

In the present study, the chemo protective effect of *Asparagus racemosus* on Cyclophosphamide induced immunosuppression was analyzed. Animals treated with CTX showed a drastic reduction in their total WBC counts. Administration of an aqueous extract of *A.racemosus* increased the total WEC count, indicating its stimulatory effect on haemopoietic system. There was no change in differential count indicating that the extract did not alter the ratio of different WBC types. CTX administration decreased the hemoglobin content also but treatment with *A.racemosus* extract almost maintained the hemoglobin content, showing its protective effect.

Relative organ weight of Thymus and Spleen, which are the two major lymphoid organs, were also reduced significantly by CTX administration. Treatment with *A.racemosus* extract considerably increased the relative organ weight of these organs, indicating its potentiating effect on the immune system.

Bone marrow, which is exclusive for all stem cells, was also analyzed. Bone marrow serves as the major source of all blood cells including lymphocytes. Administration of CTX

reduced bone marrow cellularity. But Treatment with *A.racemosus* extract significantly increased the bone marrow Cellularity indicating its effect on stem cell proliferation. There was also a reduction in "the number of a-esterase positive cells by the administration of CTX. This was also increased by the treatment with *A.racemosus* showing its effect on stem cell differentiation.

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