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IMMUNOMODULATORY EFFECT OF ASHWAGANDHA AGAINST DOXORUBICIN TOXICITY

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ABSTRACT

Doxorubicin is a member of the Anthracyclin drug family and one of the most frequently used drug to treat many forms of cancer such as leukemia, lymphoma and solid tumors. Doxorubicin is an essential component of treatment for childhood solid tumors and aggressive lymphomas and shows activity in acute lymphoblastic or myeloblastic leukemias. The use of the drug induced cardio toxicity and affects the immune functions. This toxic side effect makes the problem during cancer chemotherapy causes myelosuppression, mucosal ulceration, alopecia and diarrhoea etc. The aim of the study was to evaluate therapeutic impact of Ashwagandha for ameliorating the toxic side effects being produced during doxorubicin administration. In the present investigation aqueous root extract of Ashwagandha @ 300 mg/kg b.w. was used against administration of doxorubicin @ 5 mg/kg b.w. in rats. White Blood Cell count, Platelets and Absolute Lymphocyte Count were observed for immune function. A marked reduction in total count of WBC, ALC and Platelets were observed on day 5th however slight reduction in the above counts were also observedday 21st .and elevated level of Lipid Peroxidaion was observed on day 21st. When Ashwagandha @ 300 mg/kg b.w. administered five days prior to Doxorubicin administration and continued for 21 days, then significant increase in total count of WBC, ALC and Platelets were observed through out the cycle. Level of lipid Peroxidaion was also declined towards normal during cycle. Thus findings of present investigation showed that therapeutic potency of Ashwagandha ameliorate the toxicity produced during cancer chemotherapy.

KEYWORDS: Ashwagandha, Doxorubicin, White Blood Cell Count, Absolute Lymphocyte Count, Platelets and LPO.

INTRODUCTION

Doxorubicin is a member of the Anthracyclin drug family and one of the most frequently used drug to treat many forms of cancer such as leukemia, lymphoma and solid tumors (Singal et al., 1995). Doxorubicin is one of the important antitumor agents having a variety of therapeutic potency against variety of human tumors including soft tissue sarcoma, breast cancer, small cell carcinoma of the lung and acute leukemias.Doxorubicin is one of the most popular chemotherapeutics (Tan et al., 2009).However, its clinical use is limited due to its side effects in high and repeated doses. The use of the drug induced cardiotoxicity and affects the immune functions (Santos et al., 2010). Reportedly, doxorubicin suppressed the production of IL-2, INF-gamma, lymphocyte proliferation and CD4+/CD8+ ratio in tumour-bearing mice (Zhang et al., 2005).

Since Doxorubicin is a very good anti-cancer drug which is used for the treatment of solid tumors, similarly it has toxic effect on various parts of the body especially on immune system and heart. Whenever this drug is used for chemotherapy purpose on cancer patients, its toxicity acts on immune system of the patient and suppresses the immunity. Thereafter receiving the doxorubicin as a chemotherapy cycle, patients looses their immunity so fast, which in turns patients unable to receive another cycle of doxorubicin.

Thus present investigation was aimed to evaluate therapeutic potency of Ashwagandha to ameliorate the toxicity produced during cancer chemotherapy so that the patient could easily get full cycle of chemotherapy.

The root extract of *Withania somnifera* has been shown to have health promoting effects such as anti-stress, anti-arthiritic, anti-inflammatory, analgesic, anti-pyretic, anti-

oxidant and immunomodulatory properties (Al-Hindawi *et al*., 1992; Panda and Kar 1996; Agarwal *et al*., 1996; Archana and Namasivayam 1999; Dhuley 2000; Davis and Kuttan 2002; Prakash *et al*., 2002; Gupta *et al*., 2003). Beside these properties it also has anti-ageing, aphrodisiac, thyroregulatory, antiperoxidative (Mishra *et al*., 2000), anti-inflammatory (Sudhir *et al*., 1986), antitumor (Sharada *et al*., 1996), antioxidant, hemopoietic and rejuvenating properties (Dhuley 2000). The root of *W. somnifera* contains several alkaloids, withanolides and a few flavanoids and reducing sugars (Umadevi 1996). More than 20 active constituents have been reported to date, including withaferin A, sitoindosides VII–X, withanosides I–VII, choline and beta-sitosterol (Ganzera *et al*., 2003).

MATERIALS AND METHODS

Animals

In the present investigation, experiments were performed on 16 -18 weeks old healthy charles foster rats. For the optimal growth and development, the rats were kept in ideal condition under a well regulated light and dark (12h:12h) schedule at 23±1°C in the animal house, Mahavir cancer Institute & Research centre, patna, India (CPCSEA Regd. No. 1129/bc/07/CPCSEA, dated 13/02/2008) and the experiment was duly approved by the IAEC. Animals were given food and water *ad libitum.*

Doxorubicin: Drug was procured from pharmacy of Mahavir cancer institute.

Ashwagandha: Dry root of *W. somnifera* (Ashwagandha) were purchased from Haridwar Medicinal Store, Haridwar, Uttrakahand, India. The identity of the medicinal plant was confirmed by Prof. Ashok Kumar Ghosh (Botanist), Department of Environmental Science, A. N. College, Patna.

Preparation of aqueous root extract : 10g of root powder was dissolved in 100ml of distilled water in a conical flask and boiled at 100·C in water bath for 6 hrs and then filtered through Whatmann no.1 filter paper .The filtrate was then stored at room temperature for further study.

METHODOLOGY

Study Design: Eighteen rats were used in the study and were grouped into three groups. **Group A:** 6 untreated rats kept as control and served with equal volume of distilled water by gavage method. **Group B:** rats treated with Doxorubicin @ 5 mg/kg b.w. **Group C:** Ashwagandha (300mg/kg b.w.) administered five days prior to Doxorubicin administration and continued for 21 days (21 days was counted from the first exposure of Doxorubicin). Blood extracted from control (Gr. A), Doxorubicin treated group (Gr. B) and (Gr. C) Doxorubicin (5 mg/kg b.w) along with Ashwagandha (300mg/kg b.w.) of rats on day 5^{th} and day 21^{st} for total count of WBC, ALC, Platelets and level of lipid Peroxidaion (LPO).

Collection of Blood

The blood from the control and treated rats were obtained from heart puncture. Rats were anaesthetized for this purpose. Collection of blood from heart puncture is one of the most effective methods, which causes least stress to the animal. The blood was collected in EDTA vaccutainer tube for haematological (WBC, ALC, Platelets) study & LPO.

White blood cell count (WBC) - A 1:20 dilution of blood was made by adding 10 µl of blood to 200 µl of wbc diluting fluid in a plastic tube. After tightly corking the tube the suspension was well mixed by rotation. The improved Neubauer counting chamber was loaded with the diluted blood by means of pasteur pipette. The loaded counting chamber was allowed for two minutes for cells to settle, after which the preparation was viewed under the microscope 10 mm objective. The cells were counted in the 4 large corner squares of the counting chamber. The calculation of total white blood cells was made using the formula N x 2.5 x 20.

Platelets Count – Thin film of blood smear was made and stained by Leishmann's stain. Observation was made at 100 x magnification. Number of thrombocytes observed at five fields and after averaging of five fields, calculated value was multiplied by 20,000. (N x 20,000)

Absolute Lymphocyte Count – Absolute lymphocyte count was made by multiplying the total number of WBC with percentage of lymphocyte. (ALC = Total no. of WBC X % of lymphocyte).

Assessment of MDA for Lipid Peroxidation

MDA, as a marker for LPO, was determined by the double heating method of Draper and Hadley (1990). The principle of the method was spectrophotometric measurement of the colour produced during the reaction of thiobarbituric acid (TBA) with MDA. For this purpose, 2.5 ml of 100 g/l triochloroacetic acid (TCA) solution was added to 0.5 ml erythrocytes in a centrifuge tube and placed in a boiling water-bath for 15 min. After cooling in tap water, the mixture was centrifuged at 1000 rpm for 10 min, and 2 ml of the supernatant was added to 1ml 0.67% TBA (w/v) solution in a test-tube and placed in a boiling water-bath for 15 min. The solution was then cooled and the absorbance was measured using a spectrophotometer at 532 nm. The concentration of MDA was calculated by the absorbance coefficient of MDA-TBA complex $1.56x10^5$ $cm^{-1}M^{-1}$ and expressed in nmol/ml blood.

Statistical analysis

Data were analyzed with statistical software (Graphpad Prism 5) and values were expressed as Mean ± SEM. And differences between the groups were statistically

analyzed by one-way analysis of variance (ANOVA) using the Dunnett's test.

Table – 1:

Parameter	Control (n = 6) Gr. I	Doxo Day 5th (n = 6) Gr. II	Doxo Day 21st Gr. II	Ashwagandha + Doxorubicin Day 5th (n = 6) Gr. III	Ashwagandha + Doxorubicin Day 21st Gr. III
WBC (cumm)	8217 ± 289.2	4350 ± 170.8	5067 ± 88.19	4733 ± 185.6	7750 ± 183.9
ALC (lymphs/mm3)	4617 ± 74.91	1917 ± 87.24	2833 ± 202.8	2287 ± 33.71	4050 ± 114.7
PLT (cumm)	3,10,833 ± 8604	1,86,833 ± 5510	2,20,000± 1155	1,95,333± 6360	2,78,333 ± 3018
Level of MDA (nmol/TBARS/ml blood)	1.802 ±0.050	2.418 ± 0.144	5.064 ± 0.222	3.055 ± 0.271	1.673 ±.0952

Values are expressed as Mean ± SEM, one way ANOVA followed by Dunnet's Test, Treated groups are compared with control group. WBC = White Blood Cells, ALC = Absolute Lymphocyte Count , PLT = Platelets and MDA marker of LPO.

There was significant statistical difference ($p < 0.001$) was observed in the WBC of Doxorubicin treated group with compare to control. A significant increase however was seen in the *Ashwagandha* treated group of WBC. There was also significant statistical difference ($p < 0.001$) were observed in the Absolute Lymphocyte Count (ALC), & Platelets (PLT), with compare to control. A marked significant statistical increase ($p < 0.001$) was observed in the ALC & PLT during the period of the study in the *Ashwagandha* treated group. There was significant statistical difference ($p < 0.001$) in the level of MDA was seen in all groups with compare to control during the period of the study.

Cancer chemotherapeutic agent which is available today are immunosuppressant's, cytotoxic, and causes side effects like myelosuppression, mucosal ulceration, alopecia and diarrhoea etc. Medicinal plants based immunomodulators are often employed as supportive or adjuvant therapy to overcome the undesired effects of cytotoxic chemotherapeutic agents and to restore normal health.

Thus in the present study the role of *Withania somnifera* as immunomodulator has been studied against Doxorubicin toxicity.

In the present investigation ashwagandha showed marked increases in the WBC, ALC and platelets count after bone marrow suppression induced by Doxorubicin. This increase in the WBC, ALC and platelets were observed through out in one cycle of 21 days. Similar study was also observed Davis et al (2000) which is in support of my work that Treatment with five doses of WS was found to enhance the total WBC count on 10th day. Bone marrow cellularity as well as alpha-esterase positive cell number also increased

RESULTS AND DISCUSSION

Analysis of Haematological Parameters and Lipid Peroxidation.

significantly. Treatment with WS along with the antigen (SRBC) produced an enhancement in the circulating antibody titre and the number of plaque forming cells (PFC) in the spleen. Maximum number of PFC (985 PFC/10(6) spleen cells) was obtained on the fourth day. WS inhibited delayed type hypersensitivity reaction in mice (Mantoux test). Administration of WS also showed an enhancement in phagocytic activity of peritoneal macrophages when compared to control in mice. These results confirm the immunomodulatory activity of WS extract in indigenous medicine

Root extract of WS was tested for immunomodulatory effects in three myelosuppression models in mice: cyclophosphamide, azathioprin, or prednisolone (Ziauddin et al 1996). Significant increases in hemoglobin concentration, red blood cell count, white blood cell count, platelet count, and body weight were observed in WS-treated mice compared to untreated control mice. The authors also reported significant increases in hemolytic antibody responses toward human erythrocytes which indicated immunostimulatory activity which supports the present investigation of ashwagandha as immunomodulator against Doxorubicin toxicity.

The actions of WS on the immune system are subtler than simply suppressing the immune/ inflammatory response. WS modulates the immune response, increasing the expression of T-helper 1 (Th1) cytokines, as well as CD4 and CD8 counts, and natural killer (NK) cell activity (Bani et al 2006, Davis et al 2002, Khan et al 2006). In my investigation significant increase in ALC (Absolute Lymphocyte Count) count after ashwagandha treatment supports above study as ALC is the predictor of CD4 and CD8 count.

Lipid peroxidation (LPO) is a potential marker to asses the level toxicity in the body. In the present study there was significant increase in TBARS value (level of MDA) which is marker of lipid peroxidation (LPO) indicates that lipid peroxide produced in heart and liver tissues as

a result of Doxorubicin administration. On the other hand groups those received *Withania somnifera* along with Doxorubicin showed significant reduction in lipid peroxidation. El-Gawad *et. al.*, 2001 also showed that the intervention of *Withania somnifera* with Doxorubicin decreased the level of LPO and minimized the toxicity in rats which is in support of my work.

CONCLUSION

During chemotherapy, patients suffers a lot from other ailments, in such situation management of cancer chemotherapy can play an important role to decrease the incidence of serious side effects of anticancer drugs with preservation of their chemotherapeutic efficacy through plant extracts.

- Doxorubicin is a good anti cancer drug and being used in variety cancer cases but its toxicity causes myelosuppresion mucosal ulceration and alopecia etc. The present investigation was aimed to combat the toxicity of Doxorubicin through aqueous extract of immunomodulator plant like Ashwagandha as a adjuvant therapy. Ashwagandha (300mg/kg b.w.) administered five days prior to Doxorubicin administration and continued for one cycle of 21 days then significant increase in total count of WBC, ALC and Platelets were observed through out the cycle after treatment. Where as the administration of *Withania somnifera* reduced TBARS level in serum of experimental rats which was the reversal of doxorubicin toxic effects.

Thus findings of present investigation showed that therapeutic potency of Ashwagandha ameliorate the toxicity produced during cancer chemotherapy by mitigating the bone marrow depression and the dose of *Withania somnifera* (300 mg/kg) was observed most effective against doxorubicin induced immunosuppression and toxicity.

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