Asian Journal of Complementary and Alternative Medicine 02 (02); 2014; 06-.10

Asian Journal of Complementary and Alternative Medicine

ISSN: 2347-3894

RESEARCH ARTICLE

**Received on: 11-11-2013**
**Accepted on: 17-11-2013**
**Published on: 22-01-2014**

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Conflict of Interest: None Declared !

# Immunostimulatory activities of Withania somnifera root extract in dexamethasone induced immunocompromised mice and in vitro model.

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## ABSTRACT

The present study was designed to evaluate the immunostimulatory effect of Withania somnifera root extract (WSE) in in vitro and dexamethasone-induced immunosuppressed mice. WSE remarkably enhanced in vitro lymphocytes proliferation compared with the negative control. The optical densities at 540 nm for 2.5 µl and 5 µl of WSE were 2.24±0.14 and 2.67±0.20 respectively. While optical densities for Con A treated and untreated cells were 1.36±0.20 and 0.53±0.14, respectively. Administrations of WSE remarkable improved the primary and secondary antibodies titers in dexamethasone induced immunocompromised mice. Further, significantly enhanced CMI response in these mice was also revealed. Thus, in conclusion WSE have immunostimulatory activities in the both in vitro and in vivo modalities.

**Keywords:** *Dexamethasone; immunostimulatory; lymphocyte proliferation; mice; Withania somnifera*

**Cite this article as:**
Priyambada Kumari, Shanker K. Singh, Umesh Dimri, Meena Kataria, Sushma Ahlawat. Immunostimulatory activities of Withania somnifera root extract in dexamethasone induced immunocompromised mice and in vitro model.
Asian Journal of Complementary and Alternative Medicine 02 (02); 2014; 06-10.

## 1. INTRODUCTION

*Withania somnifera* (Ashwagandha) is used commonly in the management of various ailments in India (Khan et al., 2006). Roots ashwagandha constitute a range of rejuvenating compounds including alkaloids, withanolides, flavanoids and reducing sugars (Bandyopadhyay et al., 2007; Tong et al., 2011; Mir et al., 2012). Various preclinical and clinical studies have shown promising immuno protective and antioxidant and antibacterial activities of *W. somnifera* (Mirjalili et al., 2009; Singh et al., 2011a; Sehgal et al., 2012; Verma et al., 2012). It is widely utilized for therapeutic management of various clinical ailments including arthritis, rheumatism as well as to prevent disease in athletes, the elderly, and during pregnancy (Jain et al., 2012; Mir et al., 2012). Various preclinical and clinical studies demonstrated the *W. somnifera* as a multi-purpose remedial (Mir et al., 2012; Kaurav et al., 2012; Baitharu et al., 2013).

The immune system is an organization of the cells and molecules with very specialized roles in defending the host against infectious agents. Despite of the ability of innate immune system to eliminate some pathogens, evolutionary innovations have resulted in development of adaptive immune responses (Budhia et al., 2006). Differential and regulated expression of cytokines and their receptors are needed for the homeostasis of the immune system (Blanco et al., 2008; Sabat et al., 2010). Albeit, recombinant cytokines or cytokine antagonists has been reported to reinstate the altered immune homeostasis in various ailments (Stephens et al., 2002). However, their clinical efficacy has been limited and is associated with complications (Wieland et al., 2005).

Glucocorticoids are commonly recommended as anti-inflammatory and immunosuppressive remedial. Inhibition of almost all known cytokines as well as masking of the cell surface molecules needed for immunity are responsible for the immunosuppressive effect of the glucocorticoids (Rhen and Cidlowski, 2006; Barnes, 2006; Coutinho and Chapman, 2011; Palma et al., 2011).

Both the adaptive immune responses against specific antigens as well as natural immune reactions confer the capability to cope with diseases and thus, present study was designed to evaluate immunomodulatory effect of *W. somnifera* in *in vitro* and dexamethasone-induced immunosuppressed mice.

## 2. MATERIAL AND METHODS

### Plant extract

Roots of *Withania somnifera* were freshly harvested from two year old plants and duly authenticated from Central Herbarium (Botanical Survey of India, Government of India, Howrah-03) (voucher no. CHN/I (174)/2007/TechII/104). Thoroughly washed roots were dried in shade and powdered. The powder was subjected to methanol (70%) extraction under reflux and was concentrated. Finally, *W. somnifera* root extract (WSE) was suspended in normal saline and stored in refrigerator until the use.

### In vitro Immuno-stimulatory Assay

### Peripheral blood mononuclear cells isolation and counting

Peripheral blood mononuclear cells were isolated from heparinized blood samples by density gradient centrifugation. Briefly, 5 ml of heparinized blood samples were obtained aseptically from three clinically healthy dogs. Further, 3 ml of blood samples were carefully layered over 3 ml Histopaque-1077 (1.077 g/ml, Sigma, St Louis, MO, USA) and centrifuged at 700 *g* for 30 min at 25°C. Lymphocytes were collected from the Hisptopaque-1077 mid layer, washed twice in RPMI-1640 growth media (RPMI-GM), resuspended, and counted. Red blood cells were lysed using Tris buffer (0.16 mol/l $NH_4Cl$, 0.17 mol/l Tris). After final washing cells were resuspended in RPMI-GM supplemented with 10% (vol/vol) heat inactivated fetal calf serum, 100U/ml penicillin, 100mg/ml streptomycin and 25 mol/l HEPES buffer (Sigma). Cell viability was determined by trypan blue exclusion method (>95%). Cell concentration was adjusted to $2 \times 10^6$ cells/ml culture medium.

### Lymphocyte Proliferation Assay

Mitogen-induced lymphocyte proliferation was used as comparative indicator of cellular immune function. Briefly, 100 µl of lymphocytes ($2 \times 10^6$ cells/ml) obtained from each dog were placed into a sterile 96-well flat-bottom plate. Further, 2.5 µl and 5 µl of WSE (35 mg/ml) was added in triplicate for each dog's sample. As a positive control 10 µl Concanavalin-A (0.2 mg/ml) (Sigma) was also added in triplicate. As an untreated negative control 100 µl of lymphocytes were also kept in triplicate. Finally, the volume of each well was adjusted to 200 µl by adding RPMI-GM. The plates were incubated for 72 h at 37°C under 5% $CO_2$ in a humidified incubator. Twenty microliters of MTT [3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide; Sigma] were added to each well, and the plates were incubated for 4 h. Further, 150 µl dimethyl sulphoxide (DMSO) was added in each well, mixed thoroughly and then read using a microplate ELISA reader at a test wavelength of 540 nm with a reference wavelength of 650 nm. The results were expressed as lymphocytes proliferation index (LPI):

$$LPI = \frac{\text{Optical density of stimulated cells}}{\text{Optical density of nonstimulated cells}}$$

The mean LPI values of each dog was calculated and finally mean ± SD of three dogs was considered as final proliferation index for the tests and controls. Percentages of

proliferation, provided in the results section, were calculated by using the lymphocyte proliferation indexes as compared with untreated controls (that is, an LPI of 1.8 indicates 80% increase in proliferation)

**In Vivo Immunomodulatory Assay**

**Animals**

Fifty male Swiss albino mice (IVRI strain), weighing 28-32 g were obtained from the Laboratory Animal Resource Section, IVRI, Izatnagar. All mice were maintained under standard laboratory conditions (27 ± 1$^{o}$C temperature; 12:12 h light/dark and 50-60 % humidity) and quarantined for 7 days prior to the start of study. Standard rodent chow and tap water were provided *ad libitum* to the experimental animals. The experiment performed was in full compliance with Institutional Animal Ethics Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

**2.3.1. Experimental protocol:**

Fifty mice were randomly divided into five groups of 10 each, group I and II (controls), received 0.1 ml of normal saline by gavages for 2 weeks on alternate days, while groups III, IV and V were treated with methanolic *W. somnifera* root extract (WSE) dissolved in normal saline (NS) by gavages @ 100 mg, 250 mg and 500 mg/kg BW respectively for 2 weeks on alternate days. At day 14$^{th}$ dexamethasone (DEX) (Sigma) was administered @ 2 mg/kg BW, intraperitonially to all groups except group I. Further, Groups III, IV and V were treated with WSE as earlier for 18 days.

**2.3.1. Preparation of sheep red blood cells (SRBCs) antigens**

Sheep blood was obtainer aseptically from jugular vein in a disposable syringe containing sterilized Alsever's solution (1:1). After thorough mixing, it was incubated over night at 4$^{o}$C and centrifuged at 3000 rpm for 5 min. The sediment washed thrice with sterilized phosphate buffer saline (PBS, pH 7.2), further SRBCs suspension was prepared in PBS. Erythrocytes were counted as per Jain (1986) and concentration adjusted to 25 x10$^{6}$/ml with PBS.

*Humoral immune response*

All mice were antigenically challenged with SRBCs (50 x 10$^{6}$ cells/100 gm BW) intraperitoneally. The first challenge was given at day 15$^{th}$ and 2$^{nd}$ challenge was given at day 22$^{nd}$ of the experiment. At day 22$^{rd}$ and 29$^{th}$ blood samples ware withdrawn aseptically from retro-orbital plexus of all groups. 25 µl of serum was serially diluted with 25 µl of PBS, further 25 x 10$^{6}$ SRBCs were added and incubated at 37$^{o}$C for 1 hr. Antibody titers were measured using microhaemagglutination test (Moudgil and Singh, 1997). The rank of minimum dilution that exhibited haemagglutination was considered as antibody titer. The level of antibody titer on day 22$^{nd}$ of the experiment was considered as primary humoral immune response, whereas the one estimated on day 29$^{th}$ of the experiment was the secondary humoral immune response. The values were expressed after taking $\log_{10}$ of microhaemagglutination antibody titers.

**Cellular immune response**

This was assayed by footpad reaction method in mice. On day 30$^{th}$ of the experiment, SRBCs (25 x 10$^{6}$ cells) were injected in the sub-planter region of right hind paw of all groups. An equal volume of PBS was injected in sub-planter region of the left hind paw of all the mice, to compare as control. The reaction was assessed by measuring the increase in paw volume with help of a Vernier's calipers at zero hr and 48 hrs after challenge. The mean percentage increase in paw volume was considered as delayed type of hypersensitivity reaction and considered as an index of cell mediated immunity.

**Statistical analysis**

Comparisons between the groups were performed using the MANOVA and Post Hoc Tukey's tests. The level of statistical significance for all the comparisons made was established at $P \leq 0.05$.

## 3. RESULTS

**In vitro immuno-stimulatory activity**

WSE remarkably enhanced lymphocytes proliferation compared with the negative control (untreated cells). Both 2.5 µl and 5 µl of WSE (35 mg/ml) revealed significant ($P < 0.001$) stimulation of lymphocytes proliferation by approximately 322% and 403% respectively compared with the untreated cells. Concurrently the positive controls, 10 µl of Con A (0.2 mg/ml) also revealed significant ($P < 0.001$) stimulation of lymphocytes proliferation by approximately 156% in comparison with the untreated cells. The mean ± SD of optical densities at 540 nm for 2.5 µl and 5 µl of WSE were 2.24±0.14 and 2.67±0.20 respectively. While, optical densities at 540 nm for Con A treated and untreated cells were 1.36±0.20 and 0.53±0.14, respectively.

**Body weight and mortality assessment**

After giving dexamethasone initially there was decrease in body weight in all treated groups as compared to control group, but later the body weights of the groups III, IV and V, treated with WSE were found to be increasing, while body weight of dexamethasone treated group II, was found to be decreasing continuously throughout the study period. Increase in body weight was revealed in WSE administered mice in a dose dependent manner. Thirty percent mortality was observed in DEX alone administered group, while 10% mortality was found in DEX + WSE administrated at the dose rate of 100 mg/kg group (Group III). There was no mortality and morbidity found in other groups (I, IV and V).

**Humoral immune response**

Comparative study of various dose treatments on the humoral immune response is depicted in Table 1. In the

WSE administered group V, significantly ($P \leq 0.01$) higher haemagglutination antibody titers were recorded as compared to DEX alone administered group II at day 7th post-sensitization. While significant decrease ($P \leq 0.01$) in antibody titers were recorded in DEX alone administered mice in comparison with the healthy controls. At day 14th post-sensitization, the secondary antibody titer was also found significantly higher ($P \leq 0.01$) in groups III, IV and V as compared to the DEX alone administered group. While, antibody titer of secondary immune response was significantly decreased ($P \leq 0.01$) in DEX alone administered mice as compared to the healthy controls. The humoral immune response of various doses of WSE was found to be in dose dependent manner. WSE at the dose rate of 500 mg/kg BW was found to be the most protective.

**Cellular immune response**

The study with regard to assessment of the cell mediated immune responses at various doses of WSE (Table) indicates WSE modulates cellular immune response in a dose dependent manner. Significant cellular immune response was observed in all administered WSE groups compared with DEX alone administered mice ($P \leq 0.01$). However, significant reduction ($P \leq 0.01$) in cellular immune response was recorded DEX alone administered mice as compared to healthy controls.

## 4. DISCUSSION

In the present study, the potential to stimulate lymphocyte proliferation by WSE implies the immuno-stimulating activities of *W. somnifera*. Similarly concanavalin A, the standars polyclonal phytomitogens stimulates the T cells proliferation (Gajewski et al., 1989). Moreover, mitogens induced remarkable proliferation of lymphocytes, bone marrow cells and thymocytes of *W. somnifera* administered mice has been reported (Davis and Kuttan, 2002). *W. somnifera* confers significant increase in the stress-induced depleted T-cell population and increase in the expression of Th1 cytokines in chronically stressed mice (Khan et al., 2006). Thus, the enhanced lymphocytes proliferation by the WSE suggests its possible mitogen imitating activities.

The immunological responsiveness of an organism can be enhanced or reduced by the immunomodulatory agent through interfering with the regulatory mechanisms. These may be antigen independent and may directly induce production of mediator and effecter molecules by the immunocompetent cells. This type of antigen independent immunity is thus distinct from the one achieved by conventional immunization or by passive immunization using antibodies. The macrophages thought to be the primary target of the immunomodulatory agents, play a key role in the generation of an immune response. A number of herbal medicines and products are claimed to modify or boost immunity without scientific support. Previous scientific studies have demonstrated the immunostimulatory activities of W. *somnifera* root extracts in BALB/c mice by enhancing the total WBC (Davis and Kuttan, 2000a; Kumar et al., 2011).

The results of the present study clearly demonstrate the immunorestorative activities of *W. somnifera* root extract, as remarkable improvement in primary and secondary antibodies titers by the dexamethasone induced immunocompromised mice have been revealed. Further, enhanced CMI response in these mice also validates the immuno-stimulatory potential of *W. somnifera* root extract. In accordance to results of the present study, administration of *W. somnifera* extract revealed enhancements in the circulating antibody titer, plaque forming cells in the spleen and phagocytic activity of peritoneal macrophages in mice (Davis and Kuttan, 2000b; Kumar et al., 2011). Previously, we have also demonstrated the immunorestoratve and antioxidant activities of *W. somnifera* root extract in immoncompromised clinical demodicosed dogs (Singh and Dimri, 2010; Singh et al., 2011b).

## 5. CONCLUSION

Thus, in conclusion methanolic *W. somnifera* root extract have immunostimulatory activities in the both *in vitro* and *in vivo* modalities. It can be recommended for the salvage immune homeostasis in immnocompromised patients. Further, large scale preclinical and clinical studies are required to validate its therapeutic potential.

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