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Immunomodulatory Effect of *Withania somnifera*, *Asparagus racemosus* and *Picrorhiza kurroa* Roots

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Abstract: *Withania somnifera* (commonly known as "Ashwagandha"), *Asparagus racemosus* (Shatavari) and *Picrorhiza kurroa* (Kutki) are conventional herbal drugs used in Indian traditional system of medicine. They have been claimed to possess aphrodisiac, sedative, rejuvenative and life prolonging properties. In the present study, immunomodulatory properties were studied using the three models; viz., delayed type hypersensitivity reaction, neutrophil count and humoral antibody response in mice. The dried roots of *W. somnifera*, *A. racemosus* and *P. kurroa* were powdered and the ethanolic extract was obtained and standardized by High Performances Thin Layer Chromatography (HPTLC) method. The dried extract was administered orally as 0.1% suspension of carboxymethylcellulose at a dose of 100 mg kg^{-1} of body weight. After 14 days of oral administration immunomodulatory activity was done in male Swiss albino mice with and without an immunosuppressive agent Cyclophosphamide. The ethanolic extract of these herbs exhibited significant foot pad thickness in Delayed Type Hypersensitivity (DTH) reaction when measured after 24 h of sheep RBC antigen challenge in mice. *W. somnifera* revealed enhanced neutrophil counts and humoral antibody response. All the three selected herbs have immunostimulatory effects in common however, the overall order of immunostimulatory activity was established as *W. somnifera*>*A. racemosus*>*P. kurroa*.

Key words: Immunomodulatory, *Withania somnifera*, cyclophosphamide, neutrophils, polyherbal, antibody

INTRODUCTION

Plants have been attracting medical attention for their effective and amazing cures for thousands of years and are the most widely used medicines in the world today (Sarwar *et al*., 2011). Indian medicinal plants have a remarkable reputation among the indigenous medical practitioners. *Withania somnifera* is a member of the plant family Solanaceae and is known by many names as "Ashwagandha", "Queen of Ayurveda", "Indian ginseng" and "winter cherry", has been an important herb in the Ayurvedic and indigenous medical systems for more than 3,000 years. Its roots have been used as herb remedy to treat a variety of ailments and to promote general wellness. It has received much attention in recent years due to the presence of a large number of alkaloids and steroidal lactones (Grover *et al*., 2010; Singh *et al*., 2011). Research has shown that *W. somnifera* possess anti-inflammatory, antioxidant, anti-tumour and immunomodulatory properties (Pretorius *et al*., 2009; Singh *et al*., 2011). Ashwagandha is also considered to be an adaptogen, facilitating the ability to withstand with stressors. Administration of a powdered root extract from Ashwagandha was found to enhance total white blood cell count and antiperoxidative effect (Rasool and Varalakshmi, 2008). Also, *W. somnifera* had shown protective effect against cyclophosphamide induced myelosuppression (Winters, 2006; Grover *et al*., 2010).

EuMil, a herbal preparation composed of *Withania somnifera*, *Ocimum sanctum*, *Asparagus racemosus* and *Embelica officinalis* revealed adaptogenic and antistress activity (Muruganandam *et al*., 2002). A polyherbal formulation comprising of *A. racemosus*, *W. somnifera*, *Tribulus terrestris* and shilajit was found to possess adaptogenic activities (Bhattacharya *et al*., 2000). Various authors have reported the immunomodulatory activity of *W. somnifera* in mice (Davis and Kuttan, 2000).

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Asparagus racemosus (In India, commonly known as Shatavari) roots, belongs to family Asparagaceae, has long been used in Ayurveda as a tonic remedy to promote fertility and reducing menopausal symptoms (Gaur and Kaushik, 2011). It is also used for dry coughs and gastric ulcers. *Asparagus racemosus* was found to be effective for phagocytic activity and killing capacity of macrophages (Rege and Dhanukar, 1993). It is also used successfully for nervous disorders, inflammation, liver diseases, certain infectious diseases, immune modulator, increases corticosteroid production, ischemia and promotes cell regeneration (Potduang *et al*., 2008, Nandagopal *et al*., 2011; Velavan and Begum, 2007). The juice of fresh root of *A. racemosus* has curative effect in patients with duodenal ulcers. Oral administration of decoction of powered root enhances the immuno-modulatory effect (Uma *et al*., 2009).

Picrorhiza kurroa (*P. kurroa*, in India commonly known as Kutki) from the Scrophulariaceae family is a small perennial herb. Although, it shows anti-oxidant, anti-inflammatory and immunomodulatory activities, it is most valued for its hepatoprotective effect (Banerjee *et al*., 2008).

The selection of these herbs was made as these herbs are available in abundance in the local vicinity of Western U.P. (Bareilly district), India and are well known for different curative properties in addition to their immunomodulatory effects in common. They are regularly used by local Indian traditional medicine system practitioners and many other benefits of these herbs are also recognized internationally.

Therefore, the aim of the present study was (1) to find out the immunomodulatory effects in the presence and absence of an immunosuppressant, Cyclophosphamide (CP) and (2) to find out the comparative overall order of immunomodulatory properties of *W. somnifera*, *A. racemosus* and *P. kurroa* in mice utilizing three models; viz., delayed type hypersensitivity (DTH) reaction, neutrophil count and humoral antibody (HA) response. The reports that published the benefits of herbal combinations are scanty, so it will be appropriate to conduct further researches in this area in order to develop them into a medicinal formulation to support the immune system.

MATERIALS AND METHODS

Plant materials and chemicals: The air dried roots of *W. somnifera* (Ashwagandha), *P. kurroa* (Kutki) and *A. racemosus* (Shatavari) were purchased from Natural Drugs and Botanicals, Sahibabad, Ghaziabad (U.P.) India. The herbal materials were identified by a taxonomist Prof. M.P. Sharma, Department of Botany, Faculty of Science, Jamia Hamdard, New Delhi, India and the voucher specimens of all the three drugs have been deposited at Phytochemistry Research Laboratory, Faculty of Pharmacy, Jamia Hamdard, New Delhi, India.

Cyclophosphamide (Ledoxan) was procured from Dabur India Limited, Ghaziabad (U.P.) India and used as immunosuppressant agent. For dosing purpose, the drug was dissolved in water for injection I.P. Carboxymethylcellulose (CMC) and sodium chloride were purchased from S.D. Fine Chemicals Private Limited, Mumbai, India.

Extract preparation: The air dried roots were coarsely powdered and exhaustively extracted in a Soxhlet apparatus with ethanol (95%) for 72 h. The yield of the extract was found to be 32.4%. The extract was concentrated and dried on rotary flash evaporator to get a dark brown mass. The dried extracts from three herbs were standardized by HPTLC method before the use for the present study.

Animals: Male Swiss albino mice weighing 20-22 g were housed in standard conditions of temperature, humidity, 12/12 h light/dark cycles and free access to food with standard pellet diet (Hindustan Lever Pellets, Bangaluru, India) and tap water ad libitum. The animals were obtained from the Laboratory Animal Resource Section, Division of Animal Genetics, Indian Veterinary Research Institute, Izat Nagar, Bareilly, (U.P.) India. The animal experimentation was approved by the ethical committee for the purpose of control and supervision of experiments on animals (CPCSEA), vide approval number 453/01/a/CPCSEA. All efforts were made to minimize both the suffering and number of animals used. Vehicle-injected mice were used as controls.

Antigen: Sheep red blood cells were obtained from Division of Pathology, Indian Veterinary Research Institute, Izat Nagar, Bareilly, India, collected in Alsever's solution and washed three times in large volumes of pyrogen-free, sterile saline and adjusted to a concentration of 0.5×10^{8} cells mL^{-1} for immunization and challenge.

Methods: Mice were divided into eight groups of six each. Separate groups were utilized for the individual activity in mice. Group 1 was administered 0.1% Carboxymethylcellulose (CMC) orally and served as control group for non cyclophosphamide treated groups. In the group 2, 3 and 4 the 95% ethanolic extracts of roots of *P. kurroa, A. racemosus* and *W. somnifera*,

respectively was administered as 0.1% suspension of Carboxymethylcellulose (CMC) orally at a dose of 100 mg kg^{-1} b.wt. day^{-1}, from day 1 to day 14.

Cyclophosphamide (CP) was dissolved in water for injection i.p. to prepare a dose of 100 mg kg^{-1} b.wt. and was injected intraperitoneally (i.p.) on day 12 to the animals of group 6, 7 and 8 in addition to the ethanolic extract of the *P. kurroa*, *A. racemosus* and *W. somnifera* treatment, respectively. However, group 5 was administered 0.1% Carboxymethylcellulose (CMC) orally plus cyclophosphamide 100 mg kg^{-1} i.p. and served as control group for cyclophosphamide treated groups.

Sheep Red Blood Cells (SRBC) were adjusted to a concentration of 0.5×10^8 SRBC/mice administered intraperitoneally on day 7 as sensitizing dose and challenged by a subcutaneous administration of 0.25×10^8 SRBC mL^{-1} into right hind foot paw on day 14.

Delayed Type Hypersensitivity (DTH) reaction: The method described by Doherty was used in the present study (Doherty, 1981). Male albino mice of 20-22 g b. wt. were divided into eight groups of six each. Six groups (two sets of 3 groups for each testing material) were administered a dose of 100 mg kg^{-1} b. wt. day^{-1}, orally with ethanolic extracts of test drugs. Two groups were administered with 0.1% Carboxymethylcellulose (CMC) and served as control groups. The mice in all the groups were primed with 0.5×10^8 SRBC/mice, i.p., on day 7 and challenged on day 14 with 0.25×10^8 SRBC mL^{-1} in the right hind foot pad. The contra lateral paw received equal volume of saline. On day 12 one control group (group 5) and one set of group for each testing herb (group 6, 7 and 8, respectively) were administered with cyclophosphamide (100 mg kg^{-1} b. wt., i.p.). On day 14 the thickness of foot pad for both set of groups i.e., those which received cyclophosphamide (i.e., group 5, 6, 7 and 8) and those which did not received cyclophosphamide treatment (i.e., group 1, 2, 3 and 4) was measured at 24 and 48 h after CP challenging. The thickness of foot pad was measured by digital plethysmometer (Ugo Basil, Italy). The difference in the thickness of the right hind paw with respective left hind paw was used as a measure of delayed type hypersensitivity reaction in mL.

Neutrophil count: The method adopted for neutrophil count was described by Ziauddin *et al.* (1996). Male albino mice of 20-22 g b.wt. were divided into eight groups of six each. The treatment schedule was similar to that of SRBC-induced DTH reaction. After sensitization with SRBC on day 7, blood samples were collected from the retro-orbital plexus of individual animals on day 10 and day 14 in all the groups. The differential leukocyte count was performed by fixing the blood smears and staining with Field Stains A and B and percent neutrophil in each sample was determined.

Humoral Antibody (HA) response: Male albino mice of 20-22 g b. wt. were divided into eight groups of six each. The treatment schedule was similar to that of SRBC-induced DTH reaction. After sensitization with SRBC on day 7, blood samples were collected from the retro-orbital plexus of individual animals on day 10 and then on day 14 in all the groups. The antibody titres were determined using the method described by Puri *et al.* (1994). Briefly, an aliquot (25 μL) of two fold diluted sera in saline was challenged with 25 μL of 0.1% v/v SRBC suspension in microtitre plates (Laxbro). The plates were incubated at 37°C for 1 h and then observed for haemagglutination. The highest dilution giving haemagglutination was taken as the antibody titre. The antibody titres were expressed in a graded manner, the minimum dilution may be represented as ½. The mean dilutions of different groups were statistically compared.

Statistical analysis: Data were expressed as the Mean±Standard Error of the Mean (SEM) and statistical analysis was carried out by employing one way Analysis of Variance (ANOVA) followed by Dunnett's test. $p<0.05$ was considered to be statistically significant.

RESULTS

As evident from Table 1 extracts of *W. somnifera, A. racemosus* and *P. kurroa* showed increased foot pad thickness when measured after 24 h of sheep RBC challenge. Extract of *W. somnifera* roots produced the most significant increase on DTH ($p<0.001$), *A. racemosus* and *P. kurroa* also exhibited delayed type of hypersensitivity reaction ($p<0.001$, $p<0.05$, respectively). The foot pad thickness normalized within 48 h in all the groups. Administration of cyclophosphamide in animals caused the slight fall in the foot pad thickness in control (group 5) as compared to control (group 1) which is without cyclophosphamide administration (Table 1).

The neutrophil counts on day 14 was found to be significantly increased by *W. somnifera* ($p<0.001$) and *P. kurroa* ($p<0.005$) as compared to control group 1 (Fig. 1). Pre-treatment with *W. somnifera* and *A. racemosus* could elevate neutrophil count even in the presence of cyclophosphamide challenge ($p<0.001$). *P. kurroa* failed to augment neutrophil count in presence of cyclophosphamide treatment (Fig. 1).

After sensitization with 0.5×10^8 SRBC/mice on day 7, the blood samples were taken from retro-orbital plexus

Table 1: Delayed Type Hypersensitivity (DTH) reaction in *W. somnifera*, *A. racemosus* and *P. kurroa* roots extract with and without cyclophosphamide

		DTH activity mean foot pad thickness (mL)	
Groups	Treatment	24 h (Mean±SE)	48 h (Mean±SE)
1	Control (0.1% CMC)	0.280±0.007	0.200±0.006
2	*P. kurroa*	0.310±0.005*	0.200±0.007
3	*A. racemosus*	0.330±0.007**	0.192±0.005
4	*W. somnifera*	0.390±0.006***	0.180±0.006
5	Control (0.1% CMC+CP)	0.160±0.006	0.245±0.131
6	*P. kurroa*+CP	0.200±0.006**	0.120±0.006
7	*A. racemosus*+CP	0.190±0.005**	0.130±0.006
8	*W. somnifera*+CP	0.260±0.005***	0.120±0.004

*p<0.05, **p<0.01, ***p<0.001, n: 6 mice per group, Tabular value represents Mean±SE, Dose: 100 mg kg^{-1}, p.o. for all the testing substances

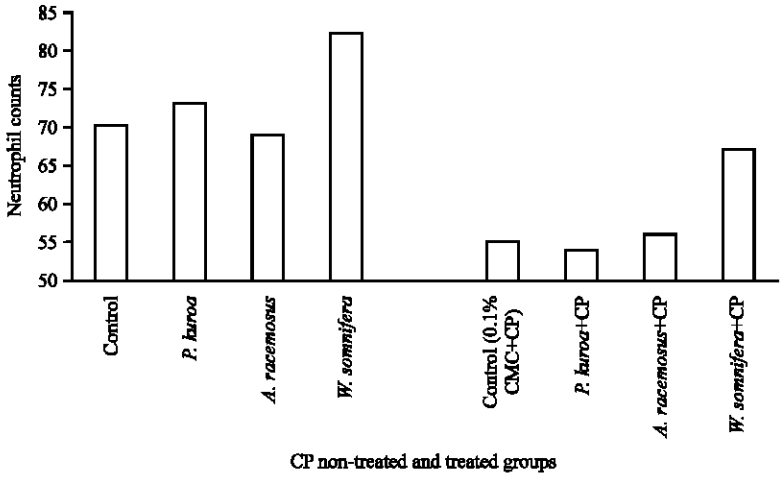

Fig. 1: Neutrophil count in ethanolic extract of *W. somnifera*, *A. racemosus* and *P. kurroa* roots

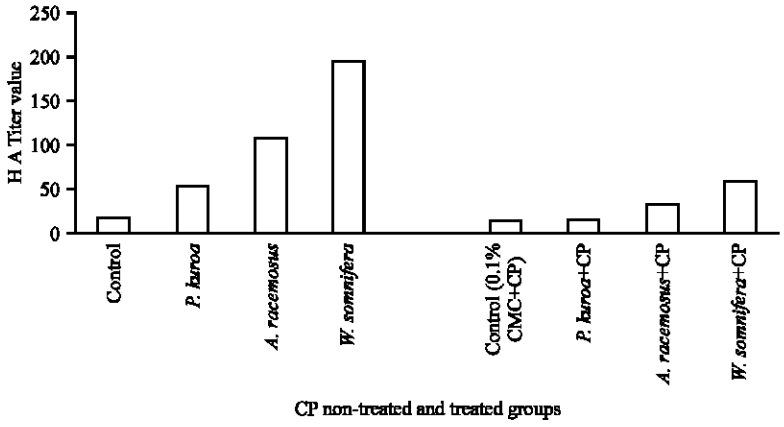

Fig. 2: Humoral antibody (HA) response in ethanolic extract of *W. somnifera*, *A. racemosus* and *P. kurroa* roots

from each animal. After 14 days of pretreatment with *W. somnifera*, *A. racemosus* and *P. kurroa* increased HA titre values (Fig. 2) as compared to control group ($p<0.01$. $p<0.05$, respectively). In Fig. 2, the myelosuppressant effect of cyclophosphamide treatment has been observed with a slight fall in HA titre value in control group (Group 5) as compared to control group without CP (Group 1). The HA titre value in cyclophosphamide treated groups reveals that *W. somnifera*, *A. racemosus*, and *P. kurroa*, (Fig. 2) could combat the

myelosuppressant effect of cyclophosphamide ($p<0.01$) as they showed increased HA titre value as compared to control group (group 5).

DISCUSSION

In the present study, we evaluated the immunostimulatory effects of *W. somnifera*, *A. racemosus*, and *P. kurroa* in mice using three models. Delayed type hypersensitivity reaction is characterized by large influxes of non specific inflammatory cells in which macrophages are the major participants. It is a type IV hypersensitivity reaction that develops when antigen activates sensitized T_{DTH} cells. Activation of T_{DTH} cells by antigen through appropriate antigen reaction results in secretion of various cytokines including interlukin-2, interferon-γ, macrophage migration inhibition factor and tumor necrosis factor-β (Askenase and van Loverent, 1983; Meera *et al*., 2008; Hashemi and Davoodi, 2012). Secreted cytokines recruit macrophages into the area and activate them, promotes enhanced phagocytic activity and vis-à-vis augment concentration of lytic enzymes for more effective killing. Thus DTH reaction is important in host defense against parasite and bacteria that can live and proliferate within the cells. In the DTH model, SRBC sensitized animals when challenged with the same allergen, produced a significant increase in paw edema when compared with contra lateral paw receiving normal saline establishing the validity of the model (Hashemi and Davoodi, 2012; Oladunmoye, 2007).

Our results showed that *W. somnifera, A. racemosus* and *P. kurroa* significantly enhanced the foot pad thickness when measured after 24 h of sheep RBC challenge. As evident from the Table 1, *W. somnifera* produced the most significant effect on foot pad thickness ($p<0.001$), *A. racemosus* and *P. kurroa* also exhibited delayed type of hypersensitivity reaction ($p<0.001$, $p<0.05$, respectively). The foot pad thickness normalized within 48 h. Administration of cyclophosphamide in animals caused the fall of mean foot pad thickness in control group as compared to control group without cyclophosphamide exposure. In Table 1, all the three drugs showed significant enhancement in foot pad thickness and could combat the immunosuppressant effect of cyclophosphamide. This reveals that the test materials have effect on T-cells and may play a role in providing immunity against parasite or bacterial intracellular proliferation.

Phagocytosis represents an important innate defense mechanism against ingested particulates including whole pathogenic micro organisms. Neutrophils are capable of ingestion and their enhancement may provide immunity through phagocytosis, therefore neutrophil counts were also determined. The neutrophil count on day 14 was found to be significantly increased by *W. somnifera* ($p<0.001$) and *P. kurroa* ($p<0.005$) as compared to control. *W. somnifera* and *A. racemosus* (but not *P. kurroa*) could elevate neutrophil count even in the presence of cyclophosphamide challenge ($p<0.001$) (Fig. 1).

After sensitization with 0.5×10^8 SRBCs on day 7, the blood samples were taken from retro-orbital plexus from individual animal on day 14 and these samples exhibited significant humoral antibody response when antibody titre was determined for all the groups. Among all the treated groups *W. somnifera* (Fig. 2) exhibited maximum haemagglutination titre value ($p<0.001$). The immunomodulatory activity of *W. somnifera* has also been reported by Diwanay *et al*., (2004). *A. racemosus* and *P. kurroa* extract (Fig. 2) also enhanced the humoral response against sheep RBCs by significantly augmenting HA titre values as compared to control ($p<0.01$).

The pretreatment with *W. somnifera* and *A. racemosus* could combat the immunosuppressant effect of cyclophosphamide (Fig. 2) as the haemagglutination antibody response was significantly higher as compared to control in group 5 ($p<0.01$). *P. kurroa* failed to augment the humoral response against sheep RBCs in presence of cyclophosphamide treatment. *P. kurroa* revealed no significant effect on HA titre values (Fig. 2) as compared to control group 5 ($p<0.01$). Therefore, the extract of *P. kurroa* did not have myelostimulant effect which could combat the myelosuppressant effect of cyclophoshphamide.

Thus, the results revealed that *W. somnifera* is an impressive and excellent immunostimulatory agent as it influenced T-cells production, enhanced neutrophil counts and produced significant humoral response against sheep RBCs in absence as well as in presence of cyclophosphamide. The immunostimulant activities of *W. somnifera* might be attributed to two primary withanolides: Withaferin A (WA) and Withanolide D (WD). The principal withanolide in the Indian variety of the plant is WA (Grover *et al*., 2010).

A. racemosus comes next to *W. somnifera* as an immunostimulating agent as it could enhance immunity by T-cells and have good humoral response in presence as well as in absence of cyclophosphamide but had no effect on neutrophil count in absence of CP. Steroidal sapogenins and steroidal saponins are major secondary metabolites present in *Asparagus*

racemosus and a new steroidal sapogenin molecule having unique characteristics might be attributed to show the immunostimulatory effects of *A. racemosus* (Sharma *et al*., 2010).

In this study, the overall order of immunostimulatory activity was established as-*W. somnifera*>*A. racemosus* >*P. kurroa*. Utilizing their remarkable properties and appropriate dosage combination a polyherbal immunostimulant formulation can be discovered and formulated to support the immune system.

Further in depth study on the parameters investigated in the present experimentation need to be designed and explored for immunomodulatory activity.

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