Review on Immunomodulatory Nutraceuticals

Venkatachalam Dhanapal
Sree Sastha Pharmacy College, Chembarambakkam, Chennai

A B S T R A C T

Objective: A large number of plants and their isolated constituents have been shown to have potential immunity. In this review focusing on the immunomodulatory activity of Ashwagandha (Withania somnifera) belonging to Solanaceae family and Neem (Azadirachta indica) belonging to Meliaceae family.

Methods: The Ashwagandha churna was evaluated immunomodulatory activity by using Albino wester rats and measured Delayed-type hypersensitivity response (DTH) (footpad swelling) and Percent neutrophil adhesion. The immuno-stimulatory effect of aswagandha root extract (WSE) evaluated by dexamethasone induced immunosuppressed mice in vitro model. The immunomodulatory effect of Neem leaves infusion was evaluated by using broiler chicks, measuring the antibody titer determination against Infectious Bursal Disease (IBD) using ELISA Kit techniques. The effects of Neem on some hematological parameters were measured the Estimation of Packed Cell Volume (PCV) and total leucocyte count (TLC).

Results: The results obtained from this study, Ashwagandha churna when orally administered the neutrophil adhesion (p<0.05) was significantly increased with the dose of 300 mg kg⁻¹ day⁻¹ when compared with untreated control indicating possible immune-stimulant effect, and the DTH response, which is a direct correlate of cell mediated immunity (CMI), was significantly increased at a doses of 200 and 300 mg kg⁻¹ day⁻¹. In vitro immuno-stimulatory activity of Ashwagandha root extract 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. The immunomodulatory action of Neem leaves infusion; the mean antibody titer against IBD was higher for group C than all other groups. The effects of neem on some Hematological parameters of packed cell volume of the control group was significantly lower than that of the treated groups at p<0.001, but with that of C2 at p<0.05.

Conclusions: The current review on Immunomodulatory herbs shows great potential of herbs to cure or prevent certain diseases, which are beyond the reach of allopathic medicines in certain cases.

Key words: Immunomodulatory activity, Ashwagandha, Neem, DTH, PCV, TLC

INTRODUCTION

Nutraceuticals which have also been called medical foods, designer foods, phytochemicals, functional foods and nutritional supplements, include such everyday products as “bio” yoghurts and fortified breakfast cereals, as well as vitamins, herbal remedies and even genetically modified foods and supplements. Nutraceuticals may be used to improve health, delay the aging process, prevent chronic diseases, increase life expectancy, or support the structure or function of the body. Immunomodulation is an alteration of the immune system and interfering with its functions; if it results in an enhancement of immune reactions it is named as an immuno-stimulation which primarily implies stimulation of...
non-specific system, that is, granulocytes, macrophages, complement, certain T-lymphocytes and different effector substances. Immunosuppression implies mainly to reduce resistance against infections, stress and may occur on account of environmental or chemotherapeutic factors. Immuno-stimulation and immunosuppression both need to be tackled in order to regulate the normal immunological functioning. Hence, both immune-stimulating agents and immuno-suppressing agents have their own standing and search for better agents exerting these activities is becoming the field of major interest all over the world.²

Nutraceuticals is defined as a food or any part of food that gives a health benefit above and beyond providing simple nutrition. This generally accepted definition establishes that health benefit may include not only the prevention or treatment of disease but also a simple improvement in the body’s immune system and performance. Further, it is also hypothesized that a combination of foods and/or multiple dietary agents may offer better therapeutic potential as compared to isolated compounds. Immuno-suppressive and immuno-stimulative agents. But there are a major limitation to the general use of these agents such as increased risk of infection and generalized effect throughout the immune system. Immunosuppression is a major drawback in the conventional therapy of cancer such as radiation and chemotherapy. Both this method have sever side effect such as nausea, vomiting, alopecia, mucosal ulceration etc. Modulation of immune responses to alleviate the diseases have been of interest for many years and the concept of ‘Rasayana’ in Ayurveda is based on related principles. Some of the herbs affecting immune system are Ashwagandha, Astragalus, Moringa, Phyllanthus, shilajit etc.³

In this review given detailed information about the immunomodulatory activity of Ashwagandha (Withania somnifera) belonging to Solanaceae family and Neem (Azadirachta indica) belonging to family Meliaceae.

MATERIALS AND METHODS:

Method-1

Immunomodulatory Activity of the Ayurvedic Formulation “Ashwagandha churna”

Ashwagandha churna is an Ayurvedic formulation that is popular as a home remedy for several diseases and human requirements. It is also an official drug and is mentioned in the Indian Pharmacopoeia. Regular usage of the ashwagandha churna is reported to purify the body and increase the life force.

Albino Wistar rats of the either sex (180–200 g) were used for the current study. They were maintained under standard environmental conditions and were fed standard pellet diet and water ad libitum. Fresh sheep red blood cells (SRBCs) are used. SRBCs collected in Alsever’s solution were washed three times in large volumes of pyrogen-free 0.9% normal saline and adjusted to a concentration of 0.5*10⁹ cells/ml for immunization and challenge. The animals were divided into five groups consisting of six animals each. A group of six untreated rats were taken as control (group I). The Ashwagandha churna formulation was dissolved in water and fed orally for 14 days at a dose of 50 mg kg⁻¹ day⁻¹ (group II), 100 mg kg⁻¹ day⁻¹ (group III), 200 mg kg⁻¹ day⁻¹ (group IV), and 300 mg kg⁻¹ day⁻¹ (group V) for assessment of immunomodulatory effect.

Delayed-type hypersensitivity (DTH) response (footpad swelling):

Six animals per group (control and treated) were immunized on day 0 by i.p. administration of 0.5x10⁹ SRBCs/rat and challenged by a subcutaneous administration of 0.025 ×10⁹ SRBCs/ml into right hind footpad on day 14. The ashwagandha churna was administered orally from day 0 until day 13. DTH response was measured at 24 h after SRBC challenge on day 14 and expressed as mean percent increase in paw volume (plethysmometrically)

Neutrophil adhesion test:-

On the 14th day of drug treatment, blood samples were collected (before challenge) by puncturing the retro orbital plexus into heparinized vials and were analyzed for total leukocyte counts (TLC) and differential leukocyte counts (DLC) by fixing blood smears and staining with Field stain I and II–Leishman’s stain. After initial counts, blood samples were reincubated with 80mg/ml of nylon fibers for 15 min at 370C. The incubated blood samples were again analyzed for TLC and DLC. The product of TLC and % neutrophil gives neutrophil index (NI) of blood sample. Percent neutrophil adhesion was calculated as shown below:

Neutrophil adhesion (%) = \frac{N_{II} - N_{Iu}}{N_{II}} \times 100

Where, N_{II} is neutrophil index of untreated blood sample, N_{Iu} is neutrophil index of treated blood sample.

Method -2:

Immunostimulatory activities of Ashwagandha root extract in dexamethasone induced immunosuppressed mice (in-vitro)

The present study was designed to evaluate the immunostimulatory effect of Withania somnifera root extract (WSE) in dexamethasone induced immunosuppressed mice (in-vitro). Roots ashwagandha constitute a range of rejuvenating compounds including alkaloids, withanolides, and flavonoids and reducing sugars.

Plant extract:

Roots of Withania somnifera were freshly harvested from two year old plants and duly authenticated. 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 and centrifuged at 700 g for 30 min at 25°C. Lymphocytes were collected from the Histopaque-1077 mid layer, washed twice in RPMI-1640 growth media (RPMI-GM), re suspended, and counted. Red blood cells were lysed using Tris buffer (0.16 mol/l NH₄Cl, 0.17 mol/l Tris). After final washing cells were re suspended in RPMI-GM supplemented with 10% (v/v) heat inactivated fetal calf serum, 100U/ml penicillin, 100mg/ml streptomycin and 25mol/l HEPES buffer (Sigma). Cell viability was determined by trypan blue exclusion method (>95%). Cell concentration was adjusted to 2 × 106 cells/ml culture medium.

**Lymphocyte Proliferation Assay:**

Mitogen-induced lymphocyte proliferation was used as comparative indicator of cellular immune function. Briefly, 100μl of lymphocytes (2× 106 cells/ml) obtained from each dog were placed into a sterile 96well flat-bottom plate. Further, 2.5μl and 5μl of WSE (35 mg/ml) was added in triplicate for each dog’s sample. As a positive control 10μl Concanavalin-A (0.2 mg/ml) (Sigma) was also added in triplicate. As an untreated negative control 100μl of lymphocytes were also kept in triplicate. Finally, the volume of each well was adjusted to 200μl by adding RPMI-GM. The plates were incubated for 72hr at 37°C under 5% CO₂ in a humidified incubator. Twenty microliters of MTT [3-(4, 5dimethylthiazol-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):

\[
\text{LPI} = \frac{\text{Optical density of stimulated cells}}{\text{Optical density of non – stimulated cells}}
\]

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:**

Fifty male Swiss albino mice (IVRI strain), weighing 28-32g were obtained. All mice were maintained under standard laboratory conditions (27 ± 10C 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).

**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 methanol extract of Aswagandha (WSE) dissolved in normal saline (NS) by gavages at 100 mg, 250 mg and 500 mg/kg BW respectively for 2 weeks on alternate days. At day 14th dexamethasone (DEX) (Sigma) was administered at 2 mg/kg BW, intra peritoneally to all groups except group I. Further, Groups III, IV and V were treated with WSE as earlier for 18 days.

**Preparation of sheep red blood cells (SRBCs) antigens:**

SRBC were collected aseptically from Jugular vein of sheep, stored in cold sterile Alsever’s solution for immunization and challenge, at required time schedule. Stored sheep blood cells were centrifuged and washed three times with pyrogen free sterile normal saline (0.85% NaCl w/v) and adjusted to a required concentration for immunization. Humoral antibody response (Hab) was analyzed using standard method. The mice were immunized by injecting 0.2 ml of 5×109 SRBC / ml i.p. and plant extracts were administered orally (100mg/Kg body wt.) for 5 consequent days after immunization. Two parallel controls were run simultaneously. One of them received only normal saline water, named ‘Normal Control’, while the other received Levamisole (2.5 mg / Kg body wt.) and Cyclophosphamide (250 mg/Kg body wt. post oral). The mean titre values of the drug treated groups were compared with the normal control. Delayed type hypersensitivity (DTH-CMI) method was employed to access SRBC induced DTH response in mice. Mice were immunized by injecting 20μl of 5×109 SRBC/ subcutaneously into the right hind footpad. The day of sensitization was designated as day 0. Seven days later the thickness of the left hind footpad was measured using a sphericomter (0.01mm pitch) and considered as control. Then the sensitized mice were challenged with the same amount of SRBC i/m into the left hind footpad. The test materials (doses= 100 mg/Kg body weight) were administered orally with a metal feeding cannula for 7 days from the day of immunization. The control animals were given an equal volume of 1% Gum acacia as vehicle. The challenging dose of 20μl of 5×109 SRBC/ml in mice were injected to assess the standard control response for DTH [4,5]. Swiss mice (n=6) bearing cancer were treated daily with *Withania somnifera* (L.) Dunal extract (100mg/kg) ip for 5 days. Blood samples were collected by puncturing the retro-orbital plexus. Total WBC and RBC count was determined using a hemocytometer. A normal control group received normal saline (5mg/kg/ip) and positive control group treated with 5-Fluouracil (5-FU), an anticancer drug.

**Humoral immune response:**

All mice were antigenically challenged with SRBCs (50 x 106 cells/100 gm BW) intra-peritoneally. The first challenge was given at day 15th and 2nd challenge was given at day 22nd of the experiment. At day 22nd and 29th blood samples were withdrawn aseptically from retro-orbital plexus of all groups. 25 μl of serum was serially
diluted with 25 μl of PBS, further 25 x 106 SRBCs were added and incubated at 370 C for 1 hr. Antibody titers were measured using micro haemagglutination test. The rank of minimum dilution that exhibited haemagglutination was considered as antibody titer. The level of antibody titer on day 22nd of the experiment was considered as primary humoral immune response, whereas the one estimated on day 29th of the experiment was the secondary humoral immune response. The values were expressed after taking log10 of microhaemagglutination antibody titers.

Cellular immune response:
This was assayed by footpad reaction method in mice. On day 30th of the experiment, SRBCs (25 x 106 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.

STUDY ON IMMUNOMODULATORY ACTION ON NEEM:

METHOD -1: 

Immunomodulatory and growth promoting effects of Neem leaves infusion in broiler chicks:

Materials and Methods
Hundred and sixty (160) day-old broiler chicks were randomly divided into four groups A, B, C and D (each group representing four replicates and 10 chicks per replicate). All the chicks were reared in an open sided house, using separate places for each replicate and sand as bedding material. Strict sanitation practices were maintained in the house before and during the course of the experiment. Group A, B and C was given neem infusion @ 30, 40 and 50ml/liter of drinking water respectively, and group D was kept as control.

Preparation of 4% (w/v) Concentrated Neem Leaves Infusion
Fresh Neem leaves were collected from the local area and dried for 24 hours at 37*C in oven. Exposure to sunlight was avoided to prevent the loss of active components. Dry leaves were then ground and 40 g of dried ground leaves were taken in a non-metallic jar. One liter of hot boiled distilled water was poured on it and kept at room temperature for 5-8 hours to prepare an infusion.

Antibody Titer Determination
At the end of experiment, blood samples were collected form all birds in test tubes. Test tubes were kept in slanting position to separate serum. Serum was then taken in small size labeled plastic bottles and stored in iceboxes. The serum samples were then sent for antibody titer determination against Infectious Bursal Disease (IBD) using ELISA Kit techniques as described.

Method -2

The Effects of Neem on some Hematological parameters of Wistar Rats:

Extraction of Plant Material:
Water Extraction 1000g of powdered Neem leaves were dissolved in distilled water in a soxhlet apparatus; the liquid extract was further concentrated in a rotary evaporator to yield a solid aqueous extract weighing 30g. Methanol Extraction using Soxhlet extractor, 1000g of powdered Neem leaves were extracted in methanol to yield 20g of dry, concentrated extract with the aid of rotary evaporator.

Animal Treatment Procedure. Twenty-five male Wistar rats of proven fertility weighing between 200g-250g, were used. They were housed in a Perspex cage with stainless steelmesh tops, kept in the animal house. The rats had free access to food and water and were maintained in standard environmental conditions. The rats were fed growers mash and provided with water ad libitum. The rats were divided into 5 groups, A, B1, B2, C1 and C2. Group A is the control group while groups B to C were treatment groups.

Group A: this is the control group consisting of 5 male rats. This group received normal diet and water.

Group B: this group was treated orally with water extract of Neem leaves. 

B1- received 20% w/w equivalent of water extract of Neem leaves mixed with the feed; for 12 weeks while

B2.-received 30% w/w equivalent of the aqueous extract mixed with the feed for 12 weeks.

Group C: This group was treated orally with methanol extract of Neem leaves.

C1 group- received 20% w/w equivalent of methanol extract of Neem leaves mixed feed for 12 weeks.

C 2 group-received 30% w/w equivalent of methanol extract of Neem leaves mixed with feed for 12 weeks (Each group consisted of 6 wistar rats. )

Estimation of Packed Cell Volume (PCV) and White blood Cell (WBC):

PCV was determined by the micro-hematocrit method. In this method blood samples collected by means of tail nipping were collected in heparinized capillary tubes and sealed off by rotating the blood- free ends in a flame avoiding charring the blood or bending the tubes. The sealed tubes were then spun in a centrifuge for about 15 minutes at 3,000 rp.m. The hematocrit then read off directly using the microhematocrit reader provided. WBC counts were estimated using the new improved Neubauer counter haemocytometer as described.

RESULT AND DISCUSSION:

Immunomodulatory action of Ashwagandha:
Ashwagandha churna Ayurvedic formulation showed significant increase in neutrophil adhesion (p < 0.05) at a dose of 300mg kg-1 day-1 in rats. The DTH response to SRBCs, which corresponds with cell-mediated immunity, showed a dose-dependent increase due to the treatment with ashwagandha churna. 

In the current study, ashwagandha churna when orally administered significantly increased the adhesion of neutrophils to nylon fibers, which correlates with the process of margination of cells in blood vessels. The neutrophil adhesion was significantly increased with the dose of 300mg kg -1day-1 when compared with untreated control indicating possible immunostimulant effect. The DTH response, which is a direct correlate of cell mediated immunity (CMI), was significantly increased at a doses of 200 and 300mg kg-1 day-1 of the ashwagandha churna Ayurvedic formulation.

Method 2: 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 administered 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 ≤ 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 ≤ 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 ≤ 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 ≤ 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 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 ≤ 0.01). However, significant reduction (P ≤ 0.01) in cellular immune response was recorded DEX alone administered mice as compared to healthy controls.

Immunomodulatory action of Neem:
Method 1:

<table>
<thead>
<tr>
<th>Groups</th>
<th>Neem leaves infusion litter*of water (ml)</th>
<th>Mean antibody titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>30</td>
<td>5940</td>
</tr>
<tr>
<td>B</td>
<td>40</td>
<td>5147</td>
</tr>
<tr>
<td>C</td>
<td>50</td>
<td>6768</td>
</tr>
<tr>
<td>D</td>
<td>Control</td>
<td>6558</td>
</tr>
</tbody>
</table>

Table 2: Mean Antibody Titer against IBD in broilers given Neem leaves extract in drinking water

DTH, delayed-type hypersensitivity; SRBCs, sheep red blood cells.

The values are mean ± SD of six rats in each group. One-way ANOVA followed by Student’s t-test; *p < 0.05

<table>
<thead>
<tr>
<th>Group</th>
<th>Neutrophil index</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Utrearted blood</td>
</tr>
<tr>
<td>B</td>
<td>Fiber treated blood</td>
</tr>
<tr>
<td>C</td>
<td>Neutrophil adhesion (%)</td>
</tr>
<tr>
<td>D</td>
<td>DTH Response</td>
</tr>
</tbody>
</table>

Table 1: Effect of ashwagandha churna on neutrophil adhesion and DTH response to antigen challenge by SRBC s in rats
Nutraceuticals for diverse uses.

**Method 2:**

**Haematological Parameters**

Packed Cell Volume (PCV): PCV of the control group was significantly lower than that of the treated groups at p<0.001, but with that of C2 at p<0.05.

Total Leucocytes Count (TLC): There was no significant variation between the mean TLC of the control and the neem treated groups at P > 0.05. This study was designed to ascertain whether water and methanol extracts of Neem leaves had effects on the haematological indices of male Wistar rats.

**CONCLUSION**

The present study was undertaken to evaluate the effect of two selected medicinal plants on immunomodulatory activity. Ashwagandha is a highly valued medicinal plant with diverse therapeutic uses in the traditional Indian systems of medicines such as Ayurveda, Unani and Siddha. It shows great potential as a safe and effective in vivo model. Asian Journal of Complementary and Alternative Medicine. 2014; 02(02):06-10

**REFERENCES**