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Immunomodulatory Activity of Methanol Extract of *Asparagus racemosus*

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ABSTRACT

Asparagus racemosus, or Shatavari, is a medicinally important Ayurvedic plant whose traditional uses include immunomodulatory and rejuvenating effects. In the current study, immunomodulatory effect of methanol root extract of *Asparagus racemosus* (ArM) was screened by in vitro and in vivo experiments. Murine splenocytes proliferation was assessed by MTT assay following the effect of extract. Cytokine production (TNF-α, IL-6, and IL-10) was also examined using ELISA. Furthermore, ArM's impact on peritoneal macrophage phagocytic activity was assessed. In vivo, the extract was orally given to mice, and its impact on DTH response, humoral antibody response, and splenocyte count was established. Results indicated that ArM highly stimulated splenocyte proliferation and increased pro-inflammatory (TNF-α, IL-6) and anti-inflammatory (IL-10) cytokine production in vitro. The extract also improved macrophage phagocytic activity. In vivo, ArM improved DTH reaction, increased serum immunoglobulin levels, and regulated the splenocyte population. These results demonstrate that methanol extract of Asparagus racemosus possesses strong immunomodulatory activity to prove its medicinal use as an immunoenhancer.

Keywords: Asparagus racemosus, Immunomodulation, Methanol Extract, Cytokines, Phagocytosis, DTH, Humoral Immunity.

INTRODUCTION

The immune system is a multifaceted system of cells, tissues, and organs protecting the body against invasion by disease-causing pathogens and aiding homeostasis. Immune system dysregulation can result in multifarious diseases such as infections, autoimmune diseases, and cancer. Immunomodulation, the regulation of the immune response to a desired level, is a novel therapeutic approach for these diseases. Medicinal plants have been employed for millennia for immunomodulation and health maintenance [1].

Asparagus racemosus Willd. belonging to family Asparagaceae is a climbing shrub found to grow freely in India and other Asian countries. It is a well-rated medicinally used plant in Ayurveda due to its adaptogenic, aphrodisiac, and galactagogue properties [2]. *A. racemosus* has also been used in medicine traditionally for the treatment of various diseases such as infertility, gastric ulcers, and nervous disorders [3]. Its antioxidant, anti-inflammatory, and anti-ulcerogenic effects have been reported in numerous studies [4-6].

Increasing evidence is revealing that *A. racemosus* contains immunomodulatory activity. Extracts of A. racemosus were reported to augment immune cells, activate cytokine production, and promote phagocytosis [7, 8]. Additionally, the exact compounds triggering these effects and by which mechanism they act are not revealed yet. The procedure for extraction can be applied in numerous different ways to yield extracts with diverse compositions and bioactivities. Hence, the present research aims to respond to whether the methanol root extract of Asparagus racemosus has immunomodulatory activity using in vivo and in vitro experiments to yield a complete evaluation of its value as an immunomodulator.

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MATERIALS AND METHODS

Plant Material and Extraction:

Dry root of *Asparagus racemosus* were collected from a local herb seller of Betul M.P., and plant material was verified by a botanist Dr. Jagrati Tripathi Asst. Prof. The roots were powdered and were then extracted with methanol using Soxhlet apparatus. The extraction was done until the solvent in the siphon tube turned colorless. The methanol extract was reduced to dryness under reduced pressure using a rotary evaporator and lyophilized to yield a powder of ArM. The extract was kept at -20°C until further

Animals:

BALB/c mice (6-8 weeks, 20-25 g) were obtained from a certified breeder. The animals were housed in a controlled environment with 12 hours light/dark conditions and fed and watered ad libitum. Animal work was carried out according to the Institutional Animal Ethics Committee guidelines.

In Vitro Assays:

Splenocyte Proliferation Assay:

Murine splenocytes from BALB/c mouse spleens were collected under standard protocol. Briefly, spleens were aseptically excised and filtered through a cell strainer. Red blood cells were lysed in red blood cell lysis buffer. Splenocytes were plated in 96-well culture plates at 2 x 10^5 cells/well in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. ArM was introduced into wells in various concentrations (10, 25, 50, and 100 µg/mL). Positive control, Concanavalin A (Con A, 5 µg/mL), was employed. Cell growth after incubation for 48 hours at 37°C in humidified 5% CO2 atmosphere was evaluated using MTT assay. 20 µL of MTT solution (5 mg/mL in PBS) was added per well for a short duration and incubated for 4 hours. The formazan crystals produced were dissolved using DMSO and measured at 570 nm using a microplate reader.

Cytokine Production Assay:

Splenocytes were grown as mentioned above. 24 h post-treatment with ArM (50 µg/mL) and Con A (5 µg/mL), supernatants were collected and stored at -20°C. Supernatant concentrations of TNF-α, IL-6, and IL-10 were determined using commercially available ELISA kits following manufacturers' instructions.

Macrophage Phagocytosis Assay

Peritoneal cavity macrophages were induced by intraperitoneal injection of sterile thioglycollate broth in BALB/c mice. After 72 hours, the macrophages were recovered by peritoneal lavage. Macrophages were plated at a density of 1 x 10^6 cells/well in RPMI 1640 medium with 10% FBS. The cells were treated with ArM (50 µg/mL) for 2 hours following 24 hours. Then the fluorescent-labeled E. coli particles (pHrodo Red E. coli BioParticles Conjugate, Invitrogen) were added to every well and left for 1 hour of incubation. The cells were washed with PBS extensively to eliminate free particles. Flow cytometry was used to estimate the proportion of macrophages that have ingested E. coli particles.

In Vivo Assays:

Delayed-Type Hypersensitivity (DTH) Response:

Mice were sensitized via subcutaneous injection of 100 µg of ovalbumin (OVA) emulsified in complete Freund's adjuvant (CFA). Seven days later, mice were given ArM (100 and 200 mg/kg body weight) or vehicle (PBS) orally for 7 consecutive days. Mice were challenged on day 14 with 50 µg of OVA in PBS, intraplantarly injected to the right hind paw. PBS was administered intraplantarly to the other paw as a control. Paw thickness was measured by digital vernier caliper at 24 and 48 hours after challenge. DTH response was determined as the difference in paw thickness of OVA-injected versus PBS-injected paws.

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Humoral Antibody Response:

Sheep red blood cells (SRBCs) 100 μg were given intraperitoneally to the mice. Mice, at the same time, received ArM (100 and 200 mg/kg body weight) or vehicle (PBS) orally for 7 consecutive days. Retro-orbital bleeding on day 7 was used to obtain the blood samples. Serum was centrifuged and kept at -20°C. ELISA was utilized to identify the concentrations of anti-SRBC IgG and IgM antibodies in the serum. SRBC lysate was immobilized on ELISA plates for a brief period. The wells were filled with the serum samples at various dilutions following blocking. Plates were incubated and washed. HRP-conjugated anti-mouse IgG or IgM antibodies were put into the wells, and the plates were incubated once more. Upon washing, substrate TMB was added, and the reaction was terminated with sulfuric acid. Absorbance at 450 nm was determined by a microplate reader.

Splenocyte Population Analysis

Mice received ArM daily (100 and 200 mg/kg body weight). Vehicle (PBS) was administered to another group of mice. On day 7, spleens were harvested and splenocytes isolated as explained before. Splenocytes were also stained with fluorescent antibodies against cell surface antigen CD3 (T cells), CD4 (helper T cells), CD8 (cytotoxic T cells), CD19 (B cells), and NK1.1 (natural killer cells). Flow cytometry was used to determine the percentage of every subset of cells.

Statistical Analysis:

Values are represented as mean ± standard deviation (SD). Statistical comparison was performed using one-way ANOVA followed by Tukey's post-hoc test. Differences were deemed significant at $p < 0.05$.

RESULTS

ArM Stimulates Splenocyte Proliferation In Vitro:

The MTT assay indicated that ArM considerably enhanced the proliferation of murine splenocytes in a dose-dependent .ArM, at 50 μg/mL, exhibited an equivalent proliferative activity to Con A at 5 μg/mL and is typical of a mitogenic activity on splenocytes.

ArM Modulates Cytokine Production In Vitro:

ArM had a significant impact on splenocyte cytokine synthesis. The extract stimulated the synthesis of the pro-inflammatory cytokines (IL-6 and TNF-α) and the anti-inflammatory cytokine IL-10. Notably, induction of IL-10 synthesis was proportionally higher than that of IL-6 and TNF-α and may indicate a regulatory function in inflammation.Phagocytosis assay indicated that ArM greatly increased the peritoneal macrophage phagocytic capacity. The number of phagocytosing macrophages of E. coli particles was significantly higher in the group treated with ArM compared to the control group.

ArM Augments DTH Response In Vivo:

Oral gavage with ArM greatly enhanced DTH to OVA in sensitized mice (Figure 4). Paw swelling was greatly increased in the ArM groups compared with the vehicle group at 24 and 48 hours postchallenge.

ArM Augments Humoral Antibody Response In Vivo:

ArM substantially elevated immunized mouse serum IgG and IgM antibody titers against SRBCs This suggests that ArM augments humoral immunity by activating the B cells and generation of antibodies.

ArM Modulates Splenocyte Populations In Vivo

Flow cytometry examination identified ArM altered splenocyte counts in mice (Figure 6). It increased the counts of CD4+ T cells and CD19+ B cells, but decreased the counts of CD8+ T cells and NK1.1+ NK cells.

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This indicates ArM induces T helper cell and B cell proliferation, but inhibits cytotoxic T cell and natural killer cell activity.

DISCUSSION

In this study the immunomodulatory potential of methanol extract of Asparagus racemosus root (ArM). The findings authenticate that ArM is able to stimulate immune cell proliferation, modulate cytokine production, induce phagocytosis activation, boost DTH response, boost humoral antibody response, and modulate splenocyte population. These observations give strong support to the traditional use of *A. racemosus* as an immune stimulant and warrant its application as a therapeutic agent against immune disorders.

The increased proliferation of splenocytes induced by ArM observed confirms the argument that the extract contains mitogenic compounds that induce proliferation of immune cells by a direct stimulatory action. The proliferative response could be responsible for the increments in the immune responses observed in vivo.

Modulation of cytokine production by ArM is of particular interest. Pro-inflammatory cytokines (TNF-α and IL-6) and anti-inflammatory cytokines (IL-10) were induced by the extract. Pro-inflammatory cytokines play a crucial role in the initiation and amplification of immune responses, while uncontrolled inflammation causes damage to the host. The simultaneous modulation of IL-10 production indicates that ArM may also be endowed with mechanisms to regulate uncontrolled inflammation. This balanced cytokine production modulation has the potential to be beneficial in immune homeostasis.

The ArM-evoked enhancement of macrophage phagocytosis shows that the extract has the potential to enhance the innate immune response by promoting the removal of pathogens. Macrophages are central players in the innate immune response, and their phagocytic activity to remove pathogens is the mainstay of host defense.

The enhancement of the DTH response by ArM is an indication that the extract enhances cell-mediated immunity. DTH is T cell-mediated immune response and is vital for intracellular infection clearance as well as organ tissue transplant rejection.

Enhancement of the humoral antibody response by ArM indicates that the extract enhances B cell-mediated immunity as well. Antibody needs to neutralize extracellular pathogens and protect from infection.

The modulation of splenocyte population by ArM is in agreement with its effect on cytokine production and immune response. CD4+ T cell and B cell rise indicates ArM-induced proliferation of T helper cell and B cell, and CD8+ T cell and NK cell reduction indicates that ArM can inhibit cytotoxic T cell and natural killer cell activity.

Few of the bioactive molecules present in *A. racemosus*, such as saponins, flavonoids, and polysaccharides, were hitherto suggested to be responsible for its immunomodulatory activity [9-11]. Identification of the actual compounds responsible for the bioactivity and the mode of action require further investigation. Purification and fractionation of methanol extract and activity-guided isolation of active fractions would be ideal.

CONCLUSION

Asparagus racemosus root methanol extract shows strong immunomodulatory activity in vitro and in vivo. The extract induces proliferation of immune cells, modulates the production of cytokines, enhances phagocytosis, enhances DTH response, increases humoral antibody response, and modulates splenocyte population. The results validate the use of A. racemosus as an immune enhancer in traditional medicine and recommend its therapeutic role in immune disorders. More studies are needed to determine the specific bioactive molecules causing these effects and to explore the application of A. racemosus as an immunomodulator clinically.

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