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Immunomostimulatory activity of aqueous extract of *Azadirachta indica* flowers on specific and non specific immune response

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Abstract

Neem (*Azadirachta indica* A. Juss) is one of the most useful traditional medicinal plants in India. Neem flowers are traditionally used as tonic and stomachic. Neem leaf, stem and seed oil has proved immunomodulatory activity along with the adverse effect on sperm count. Therefore, the aim of the present study was to evaluate immunomodulatory activity of aqueous extract of *Azadirachta indica* flowers on humoral and cell mediated immune response to ovalbumin, phagocytic activity by carbon clearance test and cyclophosphamide induced myelosuppression. The extract was also examined for the ability to induce secretory and cellular responses in murine peritoneal macrophages *in vitro*. The extract increased the production of super oxide (O_2-) and nitric oxide (NO) in murine peritoneal macrophages. These results indicate that reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNI) are likely major mediators of cytotoxic activity of extract treated macrophages. The extract showed significant increase in phagocytic index which indicates activation of reticuloendothelial system through release of the mediators. The extract also showed increase in antibody titer against the ovalbumin and protection towards the cyclophosphamide induced myelosuppression in dose dependent manner. Increase in cell mediated immunity by increasing paw edema against ovalbumin showed significant effect of the extract on stimulation of T cell at the dose 400 mg/kg. Present study, therefore, reveals that the extract holds promise as immunomodulatory agent, which acts by stimulating both specific (humoral and cell mediated immunity) and non specific immune response (cytotoxic and phagocytic activity of macrophages).

Keywords: Antibody titer, *Azadirachta indica*, Immunomostimulation, Myelosuppression, Nitric oxide, Ovalbumin

1. Introduction

Azadirachta indica, commonly known as neem, has attracted worldwide prominence in recent years, owing to its wide range of medicinal properties. The sanskrit name of the neem tree is 'Arishtha' meaning 'reliever of sickness'. Neem elaborates a vast array of biologically active compounds that are chemically diverse and structurally complex. More than 140 compounds have been isolated from different parts of neem [1]. All parts of the neem tree- leaves, flowers,

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seeds, fruits, roots and bark have been used traditionally for the treatment of inflammation, infections, fever, skin diseases and dental disorders. The medicinal utilities have been described especially for neem leaf. Neem leaf and its constituents have been demonstrated to exhibit immunomodulatory, anti-inflammatory, anti-hyperglycaemic, antiulcer, antimalarial, antifungal, antibacterial, antiviral, antioxidant, antimutagenic and anticarcinogenic properties [2-6].

The aqueous extract of neem bark possesses anticomplement activity, acting both on the alternative as well as the classical pathway of complement activation in human serum [7]. An aqueous extract of stem bark has been shown to enhance the immune response of Balb-c mice to sheep red blood cells *in vivo* [8]. In addition, the polysaccharides present in aqueous extracts of the stem bark have been shown to possess antitumor, interferon inducing and anti-inflammatory activities [9].

The aqueous extract of leaf also possesses potent immunostimulant activity as evidenced by both humoral and cell-mediated responses [10]. Oral administration of leaf extract showed higher levels of IgM and IgG along with increased titer of antiovalbumin antibody. Neem oil has been shown to possess immunostimulant activity by selectively activating the cell-mediated immune mechanisms to elicit an enhanced response to subsequent mitogenic or antigenic challenge [11].

The dry flowers are considered tonic and stomachic. Stem, leaves and seed oil has shown immunomodulatory activity with toxic effect on sperm count [12-14] that inspire to work on flower to study its potential for immunomodulation which has not yet exploited. Hence, the present study was designed to study action and mechanism of aqueous extract of *Azadirachta indica* flowers on specific and non specific immune response on experimental animals.

2 Materials and Methods

2.1 Plant material and extraction

The fresh flowers of *Azadirachta indica* were collected from the Ahmedabad, Gujarat, India in the month of May. Flowers were then shade dried at room temperature. Dry material was coarsely pulverized to powdered form. The dried powdered of *Azadirachta indica* flower were extracted with distilled water using maceration technique. The aqueous extract was dried at 40°C using a vacuum evaporator and then investigated for immunomodulatory activity. The yield of aqueous extract was found to be 21% w/w of dried flower powder.

2.2 Experimental animals

Albino mice (20-22 gm) were used for immunomodulatory activity. The animals were housed under good hygienic conditions in the departmental animal house. Animals were housed under standard conditions of temperature (25ºC±5ºC), 12h/12h light and dark cycle, fed with standard pellet diet (Amrut Ind Ltd. Pune) and had access to water, *ad libitum*. All the experiments were performed in accordance with the Institutional Animal Ethics Committee (IAEC) constituted as per directions of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), under the Ministry of animal welfare division, Government of India, New Delhi.

2.3 Chemicals

Ovalbumin, Freund's complete adjuvant and Tetra methyl benzidiene/Hydrogen peroxide (TMB/H_2O_2) were procured from Bangalore Genei, India. Streptomycin, penicillin and HEPES buffer were procured from Himedia pvt ltd, India. Fetal bovine serum and (PHA-M) Phytohemagglutinin were procured from sigma Aldrich, USA. All the other chemicals were procured from standard local source.

2.4 Isolation of peritoneal macrophage and culture conditions

Peritoneal macrophages were obtained from mice that had been injected intraperitoneally 3 days previously with 2 ml of 3% thioglycolate broth (Himedia, India). Three days later, the peritoneal exudates cells (PEC) were collected in Roswell Park Memorial Institute (RPMI-1640) containing 10% fetal bovine serum (FBS), 20 µM 2-mercaptoethanol, 100U/ml penicillin, 100 µg/ml streptomycin and 25 mM HEPES buffer. The exudates were centrifuged at 1000 rpm, 25°C for 20min and erythrocytes were lysed by hypotonic lysis. The mixture was centrifuged and the cell pellets were washed twice and resuspended in RPMI-1640 medium. The cell numbers were determined by a hematocytometer and cell viability was tested by trypan-blue dye exclusion technique. The collected cells were then adjusted to required cell counts per ml, and seeded into a 96-well plate with RPMI-1640. The cells were cultured at 37°C for 2 h under a humidified atmosphere of 95% air and 5% CO_2. The growth medium was replaced to a sample dissolved in the medium containing 0.1% DMSO, and then it was maintained for 24 h under the same condition.

2.5 Nitric oxide assay

NO production was determined by assaying culture supernatants for nitrite using Griess reagent by the method of Keller *et al* [15]. PEC at 5 x 10^5 cells/well was incubated with different concentration of extract and PHA for 24 h at 37°C in 5% CO_2 atmosphere. Cell-free supernatant (75 µl) was mixed with 75 µl of Griess reagent (sulfanilamide 1%, phosphoric acid 5%, napthylethylenediamine 0.1%) and incubated at room temperature for 10 min. Cells incubated with PHA (100 µg/ml) were used as a positive control. After incubation, the absorbance of the wells was determined by using ELISA reader (Biotek, USA) equipped with a 540 nm filter. Nitrite concentration was determined using dilutions of sodium nitrite in culture medium as standards.

2.6 Superoxide assay: NBT (Nitro blue tetrazolium dye) reduction

The reduction of NBT to insoluble blue formazan was used as a probe for superoxide generation, although it is not entirely specific for O_2- [16]. 50 µl of 0.3% NBT solution in the medium were distributed to the wells. Following incubation for 2 h, the supernatants were removed and the macrophages were fixed by the addition of 200 µl of absolute methanol, washed twice with 70% methanol and then dried. The formazan-deposits were solubilized in 120 µl 2 M KOH and 140 µl DMSO. After homogenization of the contents in the wells, the extinction was read at 630 nm.

2.7 Phagocytic activity

Phagocytic index was determined as per the method reported by Gonda *et al* [17]. Mice were divided into 3 groups, of six animals each. The control group received distilled water only as vehicle; while animals of the treatment groups were given test extract (200 and 400 mg/kg, p.o.) in distilled water daily for 20 days. Carbon ink suspension diluted with saline (1:8) was injected via tail vein to each mouse 48 hours after 20 days treatment. Blood samples were drawn from retro orbital plexus under ether anesthesia at 0 and 15 min. Blood (25 µl) was mixed with 0.1 % sodium carbonate (2 ml) and subjected for determination of optical densities at 660 nm. The phagocytic index K, was calculated by using following equation: $K= (\ln OD_1 - \ln OD_2) / (t_2 - t_1)$ where OD_1 and OD_2 are the optical densities at times t_1 and t_2, respectively.

2.8 Humoral antibody (HA) titer and Delayed type hypersensitivity reaction:

Animals were divided into 3 groups of six animals each. The control group received distilled water only as vehicle; while animals in

the treatment groups were given the test extract (200 and 400 mg/kg, p.o.) in distilled water daily for 20 days. On day 21 the animals were immunized (subcutaneously) with 3 mg of ovalbumin dissolved in normal saline emulsified with equal volume of Freund's complete adjuvant (Bangalore Genei).

The blood was collected by retro orbital plexus under ether anesthesia after 7 days of immunization. The serum was separated. Quantification of serum IgG against ovalbumin were carried out and the serum antibody titer was estimated by Enzyme Linked Immunosorbent Assay (ELISA) [18,19]. Flat bottom polystyrene plates were coated with 12.5µg of ovalbumin dissolved in 100µl of sodium carbonate buffer (pH 9.6) at 4°C for 12 h. The coated plates were washed three times with phosphate buffer saline (0.15M, pH 7.2) containing 0.05% Tween-20 (Tween-PBS). The wells were incubated with 100µl of 1% (BSA) Bovine serum albumin in sodium carbonate buffer at 37°C for 1 h. Serial dilutions of serum in Tween-PBS were prepared and 100µl was incubated with coated wells for 1 h at 37°C. After washing, diluted (1:2000) Anti-mouse IgG conjugated with peroxidase (100 µl) was added and the plates were incubated at 37°C for 1 h. The enzyme activity was determined by addition of Tetra methyl benzidiene (TMB). The enzyme reaction was stopped by addition of 50 µl, 8N sulphuric acid and the absorbance was measured at 450nm. The antibody titer was expressed as $\log_2$ of the reciprocal of the highest dilution of the test serum showing three times more absorbance as compared with normal serum.

For determination of the delayed type hypersensitivity (DTH) reaction, the mice were challenged (s.c.) with 50 µg ovalbumin in 50µl saline in the left hind footpad 14 days after the immunization. The increase in footpad thickness was measured 24 h after the challenge with the help of a dial caliper (Mitutoyo, Japan). The right hind footpad was injected with 50µl vehicle and this served as the control. The degree of DTH reaction was expressed as the percentage increase in footpad thickness (L-R) over the control value [10].

2.9 Cyclophosphamide induced Myelosuppression

Cyclophosphamide induced myelosuppression was studied according to the method described by Manjrekar *et al* [20]. Animals were divided into 4 groups of six animals each. The control groups (vehicle and negative) received distilled water only as vehicle; while animals in treatment groups were given the test extract (200 and 400 mg/kg, p.o.) in distilled water daily for 16 days. On days 17, 18, 19 all the animals except in the vehicle control group were injected with cyclophosphamide (30mg/kg, i.p.) 1 h after administration of the extract or vehicle. Blood samples were collected on day 20 and total white blood cell (WBC) count was determined.

2.10 Statistical Analysis

Results expressed as Mean ± SD. Data were analyzed by one way ANOVA followed by Dunnet's test for multiple comparisons with the level of significance chosen at $p<0.05$.

3. Results

3.1 Nitric oxide and superoxide production

Increase in the nitrite and superoxide production has a significant effect on the macrophages killing ability. It increases the cytotoxic activity of the macrophages. Effects of different concentrations of the plant extract on superoxide generation in terms of the reduction of NBT dye and nitrite release in culture supernatant were demonstrated (Fig. 1). The extract gave phagocytic modulation, in dose dependent manner. The aqueous extract at 832 µg/ml gave the most effective NBT dye reduction which was approximately 56% more than that of control.

For nitrite release in culture supernatants, the aqueous extract at 416 µg/ml and 832 µg/ml significantly increases nitrite release from peritoneal macrophages with 47% and 117% respectively as compare to control. The extract at 832 µg/ml showed similar effect to that of positive standard PHA at 100µg/ml.

3.2 Carbon clearance test

Aqueous extract of *Azadirachta indica* flowers possess macrophage stimulatory activity as evidenced by increased phagocytic index in carbon clearance test. The phagocytic activity of reticuloendothelial is generally measured by the rate of removal of carbon particles from blood stream. The phagocytic index for control group was found to be 0.096 whereas the extract at the dose of 400 mg/kg increased it significantly to 0.147. (Table 1).

3.3 Humoral antibody (HA) titer

Humoral response to ovalbumin was studied by ELISA antibody titer. Animals treated with different doses of the aqueous extract of *Azadirachta indica* flowers showed an increase in the humoral antibody titer in a dose dependent manner. The antibody titer value in control group was found to be 16.64. Administration of the extract produced increase in humoral antibody titre to 19.30 and 20.14 at the dose of 200 mg/kg and 400 mg/kg respectively. (Table 1)

3.4 Delayed-type hypersensitivity (DTH) reactions

The cell-mediated immune response was assessed by DTH reaction, i.e. foot pad reaction. Aqueous extract of *Azadirachta indica* flowers produced a significant increase in DTH reactivity in mice in dose dependent manner. The extract showed 26% and 44% increase in paw edema at 200 mg/kg and 400 mg/kg dose respectively as compared to 20% increase in paw edema of control. Increase in DTH reaction in mice in response to T cell dependent antigen revealed the stimulatory effect of aqueous extract of *Azadirachta indica* flowers on T cells (Table 1).

3.5 Cyclophosphamide induced myelosuppression

A significant reduction in white blood cell count was observed in animals treated with cyclophosphamide alone (Negative control group) as compared to the control group. The aqueous extract of *Azadirachta indica* flowers increased the levels of WBCs in a dose dependent manner with significant effects at the dose levels of 200 mg/kg and 400 mg/kg as compared to the negative control group (Table 1).

Table 1. Effect of aqueous extract of *Azadirachta indica* flowers on phagocytic index, antibody titre, DTH reaction and cyclophosphamide induced myelosuppression.

Groups	Phagocytic index	Antibody % increase titer	DTH reaction in paw edema	Total WBC count per cu. mm.
Negative Control	-	-	-	4483 ± 386
Control	0.096 ± 0.01	16.64 ± 0.89	20.35 ± 3.68	9180 ± 2120*
AIA (200 mg/kg)	0.103 ± 0.02	19.30 ± 1.03*	26.16 ± 5.50	6183 ± 1585*
AIA (400 mg/kg)	0.147 ± 0.02*	20.14 ± 1.05*	43.84 ± 4.13*	6366 ± 592*

AIA = Aqueous extract of *Azadirachta indica* flowers, Values are expressed as Mean ± SD. (n = 6), *: Significantly different from Vehicle control/Negative control group ($p < 0.05$)

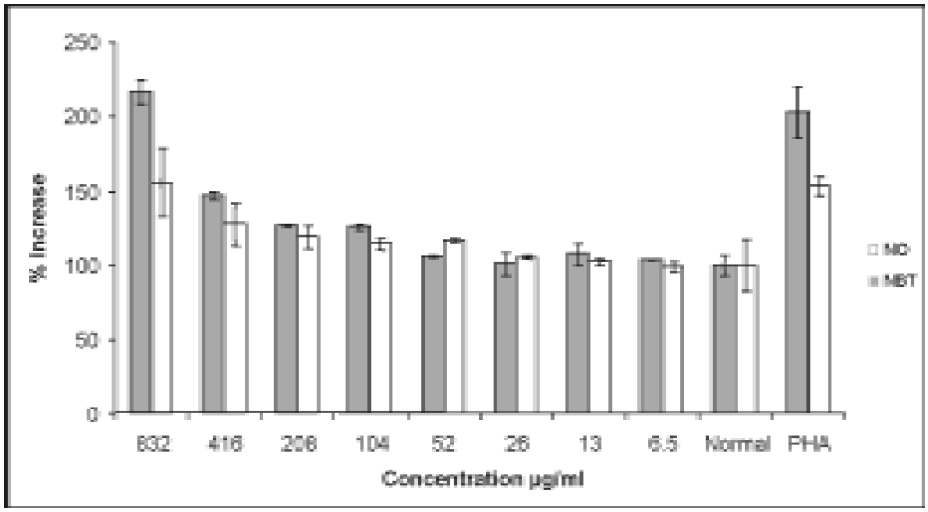

Fig. 1: Effect of aqueous extract of Azadirachta indica flowers on the nitrite and superoxide production from peritoneal macrophages. Data shown are Mean ± SD (n = 3), expressed as a percentage of normal control.

*: Significantly different from vehicle control (Normal) group ($p < 0.05$)

4. Discussions

Modulation of the immune response through stimulation or suppression may help in maintaining a disease-free state. Agents that activate host defense mechanisms in the presence of an impaired immune responsiveness can provide supportive therapy to conventional chemotherapy [21]. The results obtained in the present study indicate immunostimulating property of aqueous extract of *Azadirachta indica* flowers by stimulating both the specific and non-specific immune mechanisms.

Macrophages incubated with the extract at concentrations of 6.5-832 µg/ml for 24 h, showed a significant activation of macrophages by modulate the secretion of mediators *in vitro* responsible for the cytotoxicity. Once activated, macrophages produce a large number of cytotoxic molecules, such as superoxide (O_2-) and nitric oxide (NO). NO is synthesized by NO synthase (NOS) [22] and mediates diverse functions, including vasodilatation, neurotransmission and inflammation [23]. NO has been shown to be the principal effector molecule produced by macrophages for cytotoxic activity and can be used as a quantitative index of macrophage activation [24]. The increase in carbon clearance index reflects the enhancement of phagocytic function of mononuclear macrophage *in vivo* and thus non-specific immunity. Phagocytosis by macrophages is important against the smaller parasites and its effectiveness is markedly enhanced by opsonisation of parasite with antibodies and complement C3b leading to more rapid clearance of parasite from blood [25]. The above results led to the assumption that the extract has the macrophage stimulatory abilities through release of chemical mediators in the lysis of foreign materials in terms of the engulfment by phagocytosis.

Antibody production to T-cell dependent antigen ovalbumin requires co-operation of T- and B-lymphocytes and macrophages [26]. The significant increased values of antibody titer in case of *Azadirachta indica* flowers indicate the immunostimulant activity of the extract through humoral immunity.

DTH is antigen specific and causes erythema and induction at the site of antigen infection in immunized animals. The general characteristics of DTH are an influx of immune cells at the site of injection, macrophages and basophils in mice and induction becomes apparent within 24-72 hours. T-cells are required to initiate the reaction [27, 28]. The significant increase in DTH reaction at the dose of 400 mg/kg in response to ovalbumin, T cell dependent antigen revealed the stimulatory effect of aqueous extract of *Azadirachta indica* flowers on lymphocytes and on other necessary cell types required for the expression of the reaction (Table 1).

A high degree of cell proliferation renders the bone marrow a sensitive target particularly to cytotoxic drugs. In fact, bone marrow is the organ most affected during any immuno-suppressant therapy especially with cyclopho-sphamide. Loss of stem cells and inability of the bone marrow to regenerate new blood cells results in thrombocytopenia and leucopenia [29]. Administration of the aqueous extract of flowers of *Azadirachta indica* was found to increase the total WBCs count, which was lowered by cyclophosphamide, a cytotoxic drug. The results of the present study indicate protective effect of the extract towards myelosuppression or/and stimulatory effect on bone marrow activity. Increased in humoral and cell mediated response indicate the stimulation of B and T-lymphocytes to the immunized antigen (ovalbumin). Extract also increased macrophages phagocytic and cytotoxic activity through release of superoxide and nitric oxide and also reveal protection towards myelosuppression.

5. Conclusion

The present investigation therefore suggests that aqueous extract of *Azadirachta indica* flowers may stimulate both specific and non specific immune responses by stimulating macrophages, humoral and cell mediated response. It can therefore be concluded that aqueous extract of *Azadirachta indica* flowers is a potential immunostimulant against cytotoxic drugs and can be used as a complimentary therapeutic agent.

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