Evaluation of the immunomodulatory activity of the methanol extract of *Ficus benghalensis* roots in rats

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**ABSTRACT**

Objective: To evaluate the immunomodulatory activity of the aerial roots of *Ficus benghalensis* (Family Moraceae).

Materials and Methods: Various extracts of the aerial roots of *Ficus benghalensis* were evaluated for potential immunomodulatory activity, using the *in vitro* polymorphonuclear leucocyte (human neutrophils) function test. The methanol extract was evaluated for immunomodulatory activity in *in vivo* studies, using rats as the animal model. The extracts were tested for hypersensitivity and hemagglutination reactions, using sheep red blood cells (SRBC) as the antigen. Distilled water served as a control in all the tests.

Results: The successive methanol and water extracts exhibited a significant increase in the percentage phagocytosis versus the control. In the *in vivo* studies, the successive methanol extract was found to exhibit a dose related increase in the hypersensitivity reaction, to the SRBC antigen, at concentrations of 100 and 200 mg/kg. It also resulted in a significant increase in the antibody titer value, to SRBC, at doses of 100 and 200 mg/kg in animal studies.

Conclusion: The successive methanol extract was found to stimulate cell mediated and antibody mediated immune responses in rats. It also enhanced the phagocytic function of the human neutrophils, *in vitro*.

KEY WORDS: Allergic reaction, hemagglutination, immunostimulant, moraceae

**Introduction**

Traditional and folklore medicines play an important role in health services around the globe. About three quarters of the world’s population relies on plants and plant extracts for healthcare. India has an extensive forest cover, enriched with plant diversity. Several plants have been used in folklore medicine.[1] The rational design of novel drugs from traditional medicine offers new prospects in modern healthcare. Ayurveda, the traditional medicinal system in India, describes certain plants which strengthen the host immune system. *Ficus benghalensis* (Family Moraceae) is a very large tree, 20-30 meters high, with wide-spreading branches bearing aerial roots.[2]

A decoction of the aerial roots of *Ficus benghalensis* has been used by ayurvedic practitioners, in rural Maharashtra, to boost the immune system to fight a number of diseases. However, no phytochemical and pharmacological investigations of the aerial roots have been conducted so far to substantiate this practice. The current study aimed at exploring the immunomodulatory potential of the aerial roots of *Ficus benghalensis*.

**Materials and Methods**

Preparation of extracts

The growing tips (fresh, light buff coloured) of the aerial roots of *Ficus benghalensis* were collected from Mumbai Park during the rainy season (June to September, 2004-05) and authenticated at Agarkar’s Research Institute, Pune, India. The roots were shade-dried, powdered and stored in airtight containers. The powder was subjected to successive Soxhlet extraction using solvents of varying polarity: petroleum ether (60°C-80°C), benzene, chloroform, acetone, methanol and water. The solvent was removed under reduced pressure to obtain a total of six extracts, i.e, petroleum ether, benzene, chloroform, acetone, methanol and water. The extracts were standardised with respect to their physico-chemical parameters such as consistency, pH and extractive value as prescribed in the Indian Pharmacopoeia. All the extracts were subjected to qualitative chemical tests to determine the nature of the phytoconstituents.[3]

All the extracts were evaluated for immunomodulatory activity, using the *in vitro* polymorphonuclear (PMN) function test.
An aqueous dispersion of the successive methanol extract (SME) was used for in vivo animal experiments. The vehicle (distilled water) served as the control.

High performance thin layer chromatography (HPTLC) study

HPTLC studies were carried out on the SME, using precoated silica gel G 60 F 254 TLC plates as the stationary phase, and 1-propanol: water (7:2) as the mobile phase. The plates were spotted using the Camag Linomat applicator IV and the developed plates were scanned under UV – 254 nm and UV – 366 nm, using the Camag Scanner III.

Animals

Random bred albino rats of both sexes [reared in the C.U. Shah College of Pharmacy] were used for acute toxicity and pharmacological studies. The animals were maintained at room temperature and fed with standard pellet diet (Lipton India Ltd.) and tap water, ad libitum.

The studies were approved by the Institutional Animals Ethical Committee [CUSEP/IAEC/11/2003-2004 and CUSEP/IAEC/12/2003-2004].

Antigen

Sheep red blood cells (SRBCs), collected in Alsevier’s solution, washed in large volumes of sterile normal saline thrice and adjusted to a concentration of 5 X 10^6 cells/ml, were used for immunisation and challenge.

Polymorphonuclear leucocytes (PMN cells)

PMN cells, collected from normal healthy volunteers (18-22 years) with no evidence of bacterial, fungal or viral infection, were used in the study. This test protocol was approved by the Institutional Human Ethics Committee (Department of Clinical Pharmacology, Nair Hospital).

Reagents

The minimum essential medium (MEM) used for bioassay was procured from HiMedia Lab Pvt. Ltd. Ficoll Hypaque and bovine serum albumin were procured from Sigma Chemical Co. Candida albicans ATCC-10231, maintained on Sabourauds agar HiMedia, was used as the test microorganism in the bioassay. All the solvents, reagents and chemicals used were of analytical grade.

In vitro phagocytosis test

All the extracts were evaluated for immunomodulatory activity, using the PMN function test. Peripheral venous blood, 10 ml, was collected from volunteers in a sterile heparinised tube. Neutrophils were isolated by Ficoll Hypaque density gradient sedimentation. The RBC-PMN pellet was then subjected to dextran sedimentation. The supernatants, containing more than 90% of PMN cells, were collected and the cell density adjusted to 1X10^6 cells/ml using MEM.

Candida albicans (cell density adjusted to 1X10^6 cells/ml using MEM) was used as the test microorganism. The PMN cells (cell density adjusted to 1X10^6 cells/ml using MEM) were mixed with 1X10^6 cells/ml of Candida albicans and incubated at 37°C for one hour in 5% CO₂ atmosphere, in the presence of the test extracts. The control was the identical solution minus the test extracts. Cytosmears were prepared after incubation. The smear was fixed with methanol, stained with Giemsa and studied under 100 x ‘oil immersion objective’ to determine the phagocytic activity of PMN cells. Neutrophils (100 nos.) were scanned and the cells with ingested microorganisms were counted. The parameters evaluated were percentage phagocytosis (percentage of PMN cells involved in phagocytosis) and phagocytic index (ratio of number of Candida albicans engulfed to the total number of neutrophils).

In vivo tests

Acute toxicity study: The acute toxicity study for the SME was conducted in rats as per the prescribed guidelines. Three animals of either sex were used. Their weights were recorded before beginning the study. They were administered a single bolus dose of the SME (2000 mg/kg) per orally and observed over 14 days for mortality and physical/behavioural changes.

Hypersensitivity reaction which measures cellular immunity

Hypersensitivity reaction to SRBC was induced in rats, following the prescribed method. The SME (in doses of 50, 100 and 200 mg/kg, body weight) was administered to the animals (test group) orally for five days and the vehicle was administered to the control animals. Each group consisted of six rats – three male and three female. The SME was administered orally on each of the two days prior to the immunisation, on the day of the immunisation and on each of the two days after the immunisation (i.e., Days -2, -1, 0, +1, +2).

The rats were immunised by injecting 0.1 ml of SRBC subcutaneously into the right hind footpad on day 0. The animals were challenged seven days later by injecting the same amount of SRBC into the left hind footpad. The thickness of the left hind footpad was measured with a micrometer at 4 h and 24 h after the challenge.

Hemagglutination reaction which measures the humoral immunity

The SME (in doses of 50, 100 and 200 mg/kg, body weight) was administered to the animals (test group) orally for five days and the vehicle was administered to the control animals. Each group consisted of six rats – three male and three female. The SME was administered orally on each of the two days prior to the immunisation, on the day of the immunisation and on each of the two days after the immunisation (i.e., Days –2, -1, 0, +1, +2).

The rats were immunised by injecting 0.5 ml of SRBCs intraperitoneally (i.p.) on the day of the immunisation. Blood samples were collected by retro-orbital puncture on the tenth day after the immunisation. Antibody levels were determined by the hemagglutination technique. The antibody titer was determined by a two-fold serial dilution of one volume (100 µl) of serum and one volume (100 µl) of 0.1% bovine serum albumin (BSA) in saline. One volume (100 µl) of 0.1% SRBCs in BSA in saline was added and the tubes were mixed thoroughly. They were allowed to settle at room temperature for about 60-90 min until the control tube showed a negative pattern (a small button for pattern). The value of the highest serum dilution showing visible hemagglutination was taken as the antibody titer.

Statistical analysis

The data was analysed using one-way analysis of variance (ANOVA), followed by Dunnett’s test. P<0.05 was considered significant.
Results

Physico-chemical and phytochemical investigations

All the extracts were evaluated for physico-chemical parameters viz. consistency, colour, pH and extraction values. All the successive extracts had an acidic pH, except the water extract, which was alkaline. The water extract had the highest extractive value, indicating the presence of a high amount of water-soluble polar phytoconstituents in the aerial roots.

Phytochemical screening (chemical tests) of all the successive extracts was conducted to determine the presence of various phytoconstituents. These investigations revealed the presence of steroids and flavonoids in the petroleum ether, benzene and chloroform extracts. The acetone, methanol and water extracts were found to contain flavonoids, phenolics, steroids, glycosides, carbohydrates and proteins. [Table 1]

HPTLC studies

The results of the HPTLC studies conducted on the successive methanol extract indicated the presence of fluorescent phenolic compounds. The HPTLC fingerprint at 254 nm showed the presence of eight components, while the HPTLC chromatogram at 365 nm showed the presence of seven components.

Spraying the HPTLC plates with anisaldehyde-sulfuric acid reagent resulted in the formation of three green bands and one bluish violet band, indicating the presence of steroidal and triterpenoidal saponins in this extract.

Spraying the plates with Folin Ciocalteau reagent resulted in the formation of two blue bands, indicating the presence of phenolics with hydroquinone/ catechol nucleus. As the pH of the SME is around 5.0, these results indicate the presence of phenolic acids.

Pharmacological investigations

Acute toxicity study: The results of the acute toxicity study indicated that the LD₅₀ of the SME of Ficus benghalensis was more than 2000 mg/Kg body weight.

In vitro phagocytosis test: All the extracts were evaluated at concentrations of 0.5, 1.0 and 2.0 mg/ml. The petroleum ether, benzene, chloroform and acetone extracts did not show any significant increase in the percentage phagocytosis versus
Hypersensitivity reaction

The first line of host defense by PMN formation test and cytokine studies are currently available. Indian J Med Res 2000;1

PMN function test showed a significant increase in percentage phagocytosis. The SME showed significant activity at concentrations of 0.5 mg/ml (53%), 1.0 mg/ml (49%) and 2.0 mg/ml (46%) as compared to 31% in the control.

The water extract also exhibited a 55% phagocytosis at a concentration of 1.0 mg/ml as compared to 32% in the control. [Table 2] As neutrophils form the first line of host defense by virtue of their ability to phagocytose invading microorganisms, they have a major role in modulating the immune function. The stimulation of neutrophils results in an increase in the immediate cellular immune response.

Hypersensitivity reaction: Per oral administration of the SME (50, 100 and 200 mg/kg) for five days produced a dose related increase in early (4 h) and delayed (24 h) hypersensitivity reaction in rats. The 4 hour-reaction was found to be of higher magnitude than the 24 hour-reaction. [Table 3] These results indicate that the extract has a greater effect on the early hypersensitivity reaction and a less pronounced effect on the delayed hypersensitivity reaction.

Hemagglutination reaction: The antigen antibody reaction results in agglutination. The relative strength of an antibody titer is defined as the reciprocal of the highest dilution which is still capable of causing visible agglutination. The antibody titer is useful to measure the changes in the amount of the antibody in the course of an immune response.

Per oral administration of the SME (50, 100 and 200 mg/kg) for five days produced a dose related increase in the antibody titer in rats. [Table 3]

Discussion

The present study demonstrates, for the first time, the immunostimulant potential of the SME of Ficus benghalensis. The results of the in vitro PMN function test showed a significant increase in the percentage phagocytosis and phagocytic index for successive methanol and water extracts. This indicates that these extracts enhance the phagocytic efficacy of the PMN cells by causing more engulfment of the Candida cells versus control, thereby stimulating a non-specific immune response.

As the SME showed promising immunostimulant activity in the in vitro test, it was taken up for in vivo animal studies.

The results of in vivo animal studies showed an increase in the early and delayed hypersensitivity reaction to SRBC at doses of 100 mg/kg and 200 mg/kg. This indicated the stimulatory effect of SME on chemotaxis dependent leukocyte migration. In the early hypersensitivity reaction, the antigen antibody formed immune complexes, which are known to induce local inflammation with increased vascular permeability, edema and infiltration of PMN leukocytes. The early increase in vascular permeability as well as neutrophil influx has been ascribed to the complement C3a fragment which is activated by this immune complex.

Antibody molecules which are secreted by plasma cells mediate the humoral immune response. The SME showed an increase in the hemagglutination titer at doses of 100 mg/kg and 200 mg/kg in animal studies. This augmentation of the humoral response to SRBC indicated an enhanced responsiveness of the macrophages and T and B lymphocyte subsets involved in antibody synthesis.

The SME probably stimulates lymphocyte proliferation, which in turn leads to production of cytokines that activate other immune cells such as B cells, antigen-presenting cells and other T cells. Studies such as the lymphocyte transformation test and cytokine studies are currently underway to understand the exact mechanism for the observed immunostimulation.

The SME of Ficus benghalensis was found to have a significant immunostimulant activity on both the specific and non-specific immune mechanisms. These results are encouraging enough to pursue bioactivity-guided fractionation of this extract and structure elucidation of the active phytoconstituents.

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References