

Immunomodulatory Activity of *Atalantia monophylla* DC. roots

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ABSTRACT

Objective: The objective of the present study was to evaluate the immunomodulatory activity of the roots of *Atalantia monophylla*. In the present study Pet. Ether, Chloroform and Methanolic fractions of the ethanolic extract of the roots of *Atalantia monophylla*, were administered orally, in the doses of 10 mg/kg and 30 mg/kg, to evaluate the immunomodulatory activity. **Materials and Methods:** The Methanolic fraction showed most significant effect when compared with control group, in the dose of 30 mg/kg, in the *E.Coli* induced abdominal sepsis and Carbon Clearance Test as models for non-specific immune response. In the models of the Specific immune response, cell mediated immune response to SRBC - delayed type of hypersensitivity (DTH) the methanolic fraction in the dose 30 mg/kg, when compared with control group, showed most significant effect on decrease in footpad edema after treatment. **Results:** Humoral immune response was assessed by Sheep erythrocyte agglutination test, in which the Methanolic fraction of *Atalantia monophylla* in the dose of 30 mg/kg showed most significant ($p < 0.05$) increase in antibody titer after treatment when compared with control group. **Conclusion:** The present investigation reports that the Methanolic fraction of the ethanolic extract of the roots of *Atalantia monophylla*, in the dose of 30 mg/kg showed most significant immunomodulatory activity.

Key words: *Atalantia monophylla*; Immunomodulatory; Phagocytosis; Cell-mediated immune response; Humoral immune response.

INTRODUCTION

In coming years, use of indigenous drugs have increased to a very great extent as an alternative medicine. So, there is increasing need of undertaking studies for development of parameters of assessing these drugs scientifically. The main concepts of Rasayana drugs of Ayurvedic medicines are that these drugs increase the resistance of body as rejuvenating agents. Many plants under this class are reported for promoting and restoration of health. In many cases natural products are used as an alternative to the conventional chemotherapy against a variety of diseases. Immune system plays an important role in contributing to maintenance of homeostasis and establishment of body's

integrity. In the therapy immunomodulators are used to stimulate or suppress the immune response of the host. These are now recognized as an alternative to conventional chemotherapy in a variety of diseased conditions, especially when host's defense mechanisms have to be activated because of impaired immune response. A selective immunomodulators have to be induced in situations where it acts by stimulating specific and nonspecific responses and may be even useful for prevention and treatment of immunodeficiency related disorders allergic reactions, organ transplantation and AIDS.¹

The scientifically unexplored plants have tremendous potential for developing new pharmaceutical products containing patient friendly phytochemicals coupled with lesser chances of drug resistance. In this regard, the objective of the present study is to investigate Immunomodulatory activity of the Roots of *Atalantia monophylla* was undertaken.

Atalantia monophylla DC, is a large, thorny shrub or small tree. It is found in peninsular India, Orissa, Assam, Meghalaya and in the Andamans. In the local language it is called as 'Jungli Limbu' or 'Makad-limbu'. The roots possess anti-spasmodic, stimulant and antirheumatism property.^{2,3} The plant have been reported to have

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Antioxidant activity, Antifungal activity⁴ and Mosquitocidal activity.⁵ The drugs acting on rheumatoid arthritis are also proved to be immunomodulators.^{6,7} It is also reported to have anti-oxidant property, which also justified its role as an immunomodulator,⁸ that's why the roots of the plant *Atalantia monophylla* were selected to investigate its immunomodulatory activity.

MATERIALS AND METHODS

Plant material and its Authentication

The roots of the plant *Atalantia monophylla* DC were collected in the month of August from the local area of Tirupati, Dist. Chittoor (A.P.) India. It was then shade dried. The roots of the plant *Atalantia monophylla* DC. were authenticated from the Botanist and Taxonomist, Dr. K. Madhava Chetty, Asst. Prof., Dept. of Botany, Sri Venkateswara University, Tirupati-517502. (Reference no. 2008-09/135).

Preparation of Extracts & Fractions

The dried roots of *Atalantia monophylla* DC (AM) were extracted with ethanol as a solvent by using maceration technique. 2 kgs of the powdered crude drug was kept in a jar containing sufficient amount of ethanol for around three weeks. Vigorous intermittent shaking with the help of a mechanical shaker was done frequently. It was then filtered. Filtrate was then evaporated to dryness with the help of Rota evaporators and Vacuum oven. The dried ethanolic extract of AM was then processed for fractionation by using different solvents like Pet. Ether (40-60), Chloroform and Methanol. All the fractions were evaporated to dryness.

Preparation of Drug Solution

All the powdered fractions of the ethanolic extract were accurately weighed, and then dispersed in distilled water using a suspending agent TWEEN 80. The appropriate stock suspensions of the drugs were prepared. The doses were administered orally by selecting the appropriate concentration of the stock solution. The plain suspension of the distilled water with TWEEN 80 served as control.

Animals

Swiss male albino mice (18-22 gm) and wistar rats of either sex (150-200 gm) were used. They were maintained at $25 \pm 2^\circ$ C and relative humidity of 45 to 55% and under standard environmental conditions (12 h light: 12 h dark cycle). The animals had free access to food (Amrut feed,

Chakan oil mills, India) and water *ad libitum* throughout study. Institutional Animal Ethical Committee approved the protocol (Proposal No. 03/IAECCOP/2009). All the experiments were carried out between 9:00-16:00 hour.

Preliminary Phytochemical Screening of the Fractions

All the fractions of the ethanolic extract were investigated for preliminary phytochemical analysis using various tests to determine the presence of different phytoconstituents in different fractions.⁹

Acute Toxicity Studies

In the Acute Toxicity Study, Oral administration of all the fractions of the ethanolic extract of crude drug at the doses of 175, 550 and 2000 mg/kg in mice showed no adverse effect or mortality was observed in wistar rats up to 2000 mg/kg, p.o of extracts during the 24 hours and 14 days observation. From pilot study data and review of literature, two different doses 10 mg/kg and 30 mg/kg were selected for further study.^{10,11}

Pharmacological Screening of Different Fractions

All the fractions of ethanolic extracts of drugs were screened for their immunomodulatory effect by using following pharmacological screening models.

Models for Non-specific Immune Response

E. coli Induced Abdominal Sepsis (Determination of Host Resistance)

Four groups each consisting of 06 animals. All animals were injected with 1.0 mg of E.coli serotype 0111:B4 LPS in a volume of 500 μ l of sterile solution. Immediately after this, group I to IV received isotonic NaCl solution, (10mg/kg), test drug dose I.

There after quantities of bacteria in the intra abdominal fluid (10ml of sterile saline was injected into the abdominal cavity and then sample of peritoneal lavage fluid was collected under anaesthesia and subjected to quantitative evaluation of the bacteria.) and rate of lethality was observed at every 24 hour for the period of 72 hours.¹²

Carbon Clearance Test (Phagocytic Response)

In this test three groups of animals were used with 6 rats in each group. Group I served as a control and received vehicle i.e. plain suspension of sterile water with TWEEN

80 (10ml/kg) only. On the other hand, animals of group II received doses D1 mg/kg of particular fraction of drug extract orally daily for the period of 05 days. Carbon ink suspension was injected via the tail vein to each rat 48 hours after the five-days treatment. Blood samples (25 µl) were then withdrawn from the retro-orbital plexus under mild ether anesthesia at 0 and 15 minutes after injection of colloidal carbon ink and lysed in 0.1% sodium carbonate solution (03 ml). The optical density was measured spectrophotometrically at 660 nm. The phagocytic index was calculated using the following formula;

$$K = \log OD 1 - \log OD 2 / t_2 - t_1$$

Where, OD1 and OD2 are the optical densities at time t_1 and t_2 .^{13,14}

Models for Specific Immune Response

Cell-mediated Immune Response to SRBC (DTH)

Cell mediated immune response was assessed by T - cell population test and delayed type hypersensitivity (DTH).

T - cell Population Test

In this test, three groups of rats were used with 6 rats in each group. Group I served as a control and received vehicle i. e. plain suspension of sterile water with TWEEN 80 (10 ml/Kg) only. Rats of group II received doses D1 mg/kg of particular fraction of drug extract orally daily for 10 days. On 11th day blood was collected from retro orbital plexus and anticoagulated with sodium citrate in separate test tubes. The test tube containing blood was placed in a left sloping position 45° at 37 °C for 01 hour. Supernatant fluid containing lymphocytes and leukocytes were removed using micropipette.^{15,16}

Delayed Type Hypersensitivity (DTH)

The mice were divided into 04 groups, each containing 06 animals. Group I served as normal control Group and received plain suspension of sterile water with TWEEN 80 orally (01 ml/Kg) for the period of 21 days. Group II served as negative control Group receives Cyclosporine 100 µg/mouse, i.p. on 14th day of study. Animal of group III were administered doses D1 mg/kg of particular fraction of drug extract orally daily for the period of 21 days. Mice were immunized with 0.1ml of 20% SRBC's in normal saline intraperitoneally on 14th day of study.

On day 21st, animal from all group get challenged with 0.03

ml of 1% SRBC's in sub plantar region of right hind paw. Footpad reaction was assessed after 24 hours i.e. on 22nd day. Increase in foot paw edema was measured using digital plethysmometer – LE7500 (Panlab, USA).¹⁵

Humoral Immune Response (Haemagglutination Antibody Titer)

Humoral immune response was assessed by Sheep erythrocyte agglutination test.

Sheep Erythrocyte Agglutination Test

Rats were divided into three groups with 06 rats in each group. Group I served as a control and received vehicle i.e. plain suspension of sterile water with TWEEN 80 (10 ml/Kg) only. Rats in groups II were administered two doses D1 mg/kg of particular fraction of drug extract orally daily for 10 days.¹⁵

Preparation of Sheep Red Blood Cells (SRBC's)

Sheep blood was collected from local slaughter house in sterile Alsever's solution in 1:1 proportion of Alsever's solution (freshly prepared). RBC's were counted microscopically (5 x 10⁹/ml). Blood was kept in the refrigerator at 4 °C and processed, for the preparation of Sheep RBC (SRBC's) batch, by centrifugating (3000rpm for 05minutes) and washing with physiological saline 4-5 times followed by suspending into buffered saline for further use.¹³

Formula of Alsever's Solution

Sodium chloride	:	0.42 gms/100 ml
Sodium citrate	:	0.80 gms/100 ml
Glucose	:	2.05 gms/100 ml

All the rats were injected with 0.25ml of 5 x 10⁹ SRBC/ml on 6th, 8th, and 10th days for achieving maximum titer of antibody. On day 11, blood was collected and serum was separated by centrifuging at 200 rpm for the period of 15 minutes. 100 l of serum diluted serially with normal saline in separate test tubes. Dilutions were made i.e.20, 40, 60 up to 1280. To this, 50 l of SRBC added and incubated at 37 °C for 18 hours. All the tubes were then subjected to examine under microscope for agglutination and compared with control. The highest dilution giving hemagglutination was taken as the antibody titer. The antibody titers were expressed in the graded manner, the minimum dilution (01/02) being ranked as 01, and mean ranks of different groups were compared for statistical significance.¹⁷

Data Analysis

Data obtained were subjected to statistical analysis using one way ANOVA followed by Dunnetts 't' test using graphpad software.

RESULTS AND DISCUSSION

Plant Extraction & Fractionation

The dried roots of *Atalantia monophylla* DC were extracted with the help of ethanol by maceration method. The yield was 5.78%.

The ethanolic extracts of the roots of *Atalantia monophylla* DC was fractionated by using different solvents like Pet. Ether (40-60), Chloroform and Methanol with increasing polarity. The yields of the fractions were calculated and have been shown in the Table 1.

Preliminary Phytochemical Screening of the Fractions

The results of the Preliminary phytochemical screening of the Pet. Ether, Chloroform and Methanol fractions of the ethanolic extract of the roots of *Atalantia monophylla* R.Br. have been shown in Table 2.

Models for Non- specific Immune Response

E. coli Induced Abdominal Sepsis (Determination of Host

Table 1: Yield of the fractions of the ethanolic extract of crude drug

Crude Drug	Pet. Ether Fraction % Yield	Chloroform Fraction % Yield	Methanol Fraction % Yield
<i>Atalantia monophylla</i> DC	16.75	21.15	18.50

Table 2: Preliminary phytochemical screening of the fractions of the ethanolic extract of *Atalantia monophylla* DC

Phyto constituents	Pet. Ether fraction	Chloroform fraction	Methanolic fraction
Carbohydrates	-	-	+
Glycosides	-	-	-
Saponins	-	-	-
Flavonoids	-	-	+
Alkaloids	-	+	-
Tannins	-	-	+
Steroids	+	-	-
Amino Acids	-	-	-
Proteins	-	-	-

Resistance)

The Methanolic fraction of AM 30 mg/kg showed most significant ($p<0.05$) effect when compared with control group for 24 hrs, 48 hrs and 72 hrs. Other doses of AM fractions do not showed any significant effect. The results have been shown graphically in the Figure 1.

Carbon Clearance Test (Phagocytic Response)

The Methanolic fraction of AM 30 mg/kg showed most significant ($p<0.05$) and ($p<0.01$) dose dependent increase in carbon clearance when compared with control. The Methanolic fraction of AM 10mg/kg showed significant ($p<0.05$) increase in carbon clearance when compared with control. AMC10 mg/kg, AMC 30mg/kg, AMPE 10mg/kg and AMPE 30mg/kg also showed some significant activity by increase in carbon clearance when compared with control. The results have been shown graphically in the Figure 2.

Models for Specific Immune Response

Cell-mediated Immune Response to SRBC (DTH)

Cell mediated immune response was assessed by T - cell population test and delayed type hypersensitivity (DTH).

T - cell Population Test

None of the doses of fractions of the crude drug showed any significant change. As no significant changes were observed during the T-cell Population test so the data have not been Presented.

Delayed Type Hypersensitivity (DTH)

The Methanolic fraction of AM 30 mg/kg showed most significant effect ($p<0.01$) decrease in footpad edema after treatment when compared with control group. The Methanolic fraction of AM 10 mg/kg also showed significant effect ($p<0.01$). The other fractions and doses of AM did not show any significant activity. The results have been shown graphically in the Figure 3.

Humoral Immune Response (Haemagglutination Antibody Titer)

Humoral immune response was assessed by Sheep erythrocyte agglutination test.

Sheep Erythrocyte Agglutination Test

The Methanolic fraction of AM 30mg/kg showed most

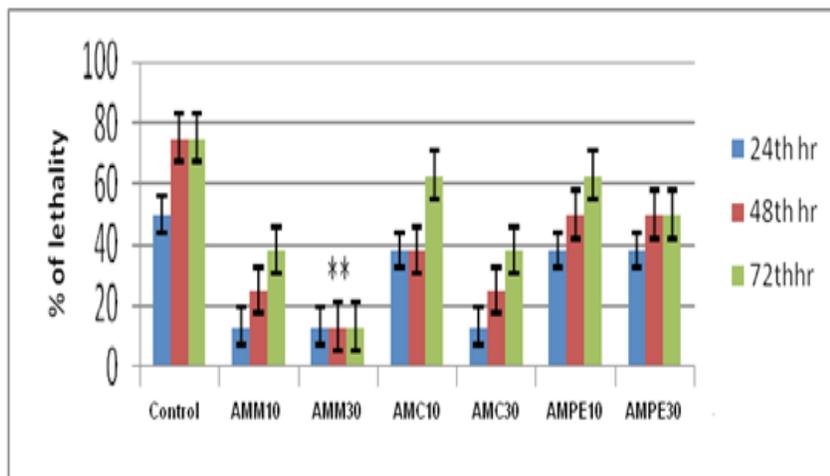


Figure 1: Effect of *Atalantia monophylla* fractions treatment on *E. coli* induced abdominal sepsis

Results are expressed as Mean \pm SEM (n=6). The data was analysed using One-way Analysis of Variance (ANOVA) followed by Dunnett's t test. *P<0.05, **P<0.01

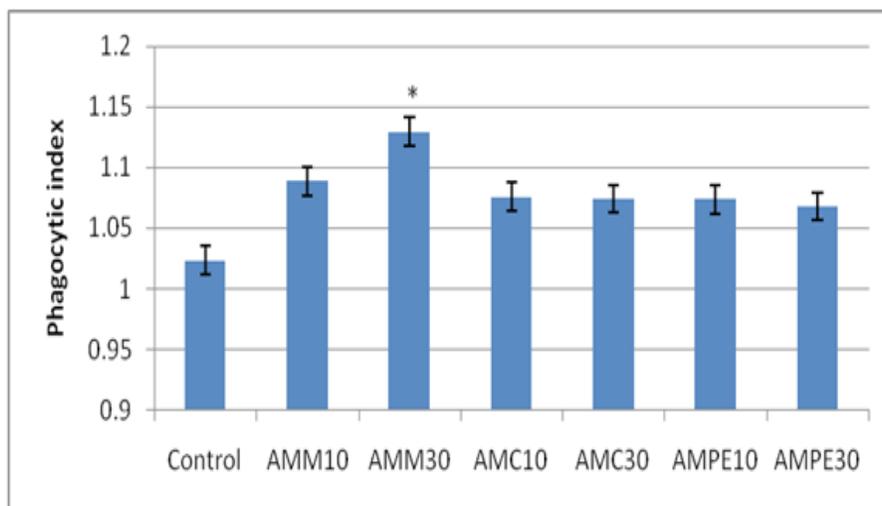


Figure 2: Effect of *Atalantia monophylla* fractions treatment on carbon clearance test

Results are expressed as Mean \pm SEM (n=6). The data was analysed using One-way Analysis of Variance (ANOVA) followed by Dunnett's t test. *P<0.05, **P<0.01

significant ($p < 0.05$) increase in antibody titer after treatment when compared with control group. The other fractions AMM 10mg/kg, AMC 10mg/kg, AMC 30mg/kg, AMPE 10mg/kg, AMPE 30mg/kg did not show any significant increase in antibody titer after treatment when compared with control group. The results have been shown graphically in the Figure 4.

CONCLUSION

The roots of *Atalantia monophylla* DC. were selected to investigate the immunomodulatory activity. Traditionally the crude drug was used in the treatment of chronic rheumatoid arthritis^{2,3} and crude drug was scientifically reported for having anti-oxidant property.⁴ As the anti-oxidant property and anti-rheumatism drugs can be used as

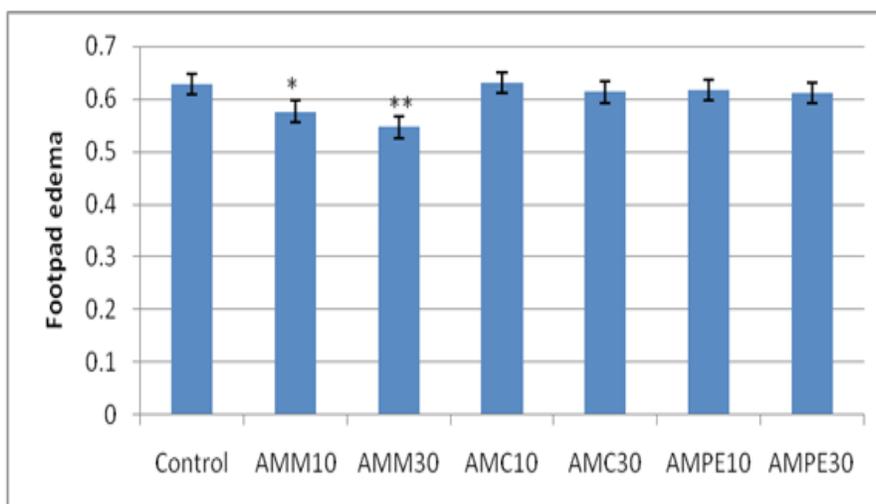


Figure 3: Effect of *Atalantia monophylla* fractions treatment on foot pad edema
 Results are expressed as Mean ± SEM (n=6). The data was analysed using One-way Analysis of Variance (ANOVA) followed by Dunnett's t test. *P<0.05, **P<0.01

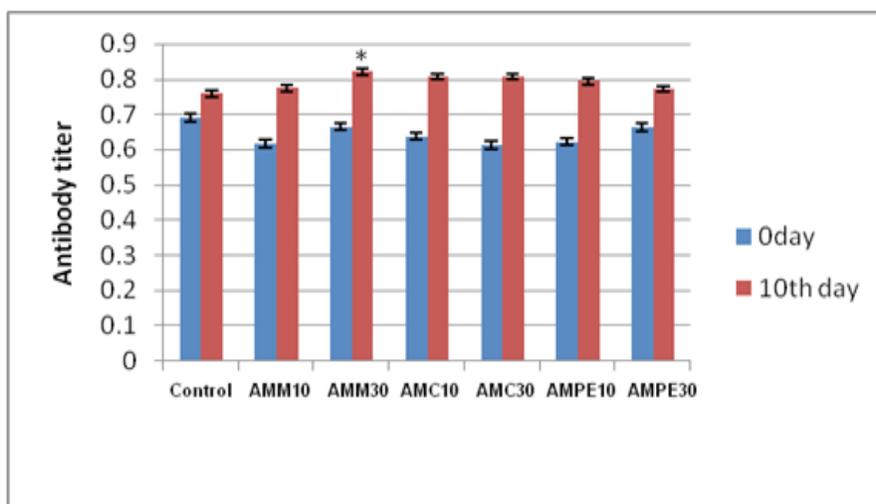


Figure 4: Effect of *Atalantia monophylla* fractions treatment on sheep RBC
 Results are expressed as Mean ± SEM (n=6). The data was analysed using One-way Analysis of Variance (ANOVA) followed by Dunnett's- test. *P<0.05, **P<0.01

immunomodulators.⁶⁻⁸ So the selection of the crude drug is justified.

The present investigation indicates that the methanolic fraction of the ethanolic extract of the roots of *Atalantia monophylla* DC., exert a significant immunomodulatory activity by enhancing Specific Immune response as well as Non-specific immune response. The Preliminary phytochemical screening of the methanolic fraction of the crude drugs revealed the presence of flavonoids

which may be the phytoconstituents responsible for immunomodulatory activity.¹⁸ Thus the crude drug showed significant immunostimulant property, and which may be due to the flavonoids present in it. Further investigation is underway to find out the phytoconstituents present in the fraction which is responsible for this action.

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