

Bioactivity Guided Fractionation of the Aqueous Extract of the Indian Banyan and Evaluation of Immunomodulatory Activity - Validating the Traditional Use

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Rec date: May 2, 2014, Acc date: June 15, 2014, Pub date: June 17, 2014

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Abstract

The Indian Banyan is a large tree, bearing many aerial roots. An aqueous decoction of the fresh aerial roots of this tree has been used by traditional ayurvedic medical practitioners to boost the immune system in various diseases. In AIDS, death occurs eventually due to the failing immune system hence new therapies directed towards augmenting the functional capacity of the immune system are the need of the hour. Studies carried out in our laboratory have indicated the immunostimulant activity of the methanol and aqueous extracts of the aerial roots. The aqueous extract was found to enhance the cell mediated and antibody mediated immune responses in rats. The aqueous extract was subjected to fractionation using solvents of varying polarity. This process generated four fractions namely chloroform fraction, ethyl acetate fraction, n-butanol fraction and the aqueous fraction. These fractions were screened for immunomodulatory activity using the *in vitro* lymphocyte proliferation assay in an effort to identify the most bioactive fraction. The results of this assay indicated that the aqueous fraction exhibited maximum immunostimulatory activity (80% stimulation of lymphocytes at 1 µg/ml) while the other three fractions did not exhibit stimulation of lymphocytes at the concentrations tested (0.001- 10 µg/ml). Hence the aqueous fraction was taken up for evaluation of *in vivo* immunomodulatory activity. The aqueous fraction was evaluated at doses ranging from 25 mg/kg to 200 mg/kg body weight in rats using the hypersensitivity reaction assay and the hemagglutination reaction assay. The maximum response was observed at 50 mg/kg. The aqueous fraction of the aqueous extract exhibited significant stimulation of cell mediated and antibody mediated immunity and could prove beneficial in diseases involving suppression of the immune system like AIDS.

Keywords: *Ficus benghalensis*; Aerial roots; Immunomodulation; Phenolics; Hypersensitivity reaction assay; Hemagglutination reaction assay; Lymphocyte proliferation assay

Introduction

Right from the time mankind first displayed susceptibility to disease and injury, the discovery of medicinal agents from renewable resources has been an essential and intrinsic aspect of all evolving creatures. Terrestrial plants are an especially viable opportunity for the discovery of biologically active natural products which may serve as commercially significant entities in their own right or which may provide lead frameworks for the investigation of modified derivatives possessing enhanced activity and/or reduced toxicity. Plants continue to provide key leading structures and therapeutic agents for a myriad of debilitating diseases [1]. Indian medicinal plants are a rich source of substances that are claimed to induce immunity [2]. Ayurveda, the traditional Indian system of medicine, offers certain plant products termed as Rasayana, which can strengthen the tissue resistance to disease [3]. Ayurvedic pharmacotherapy lays emphasis on drugs of plant origin and Rasayana therapy. Rasayanas are claimed to improve physical and mental health, increase the resistance of the body to infection and other external factors tending to perturb the homeostasis of the human system, promote revival of physiological functions after debilitating diseases and to augment intellect [4].

Ayurvedic concept of Rasayanas and the modern concept of new class of plant derived drugs, the "adaptogens", appear to induce a state of nonspecific increase in resistance of an organism to diverse aversive assaults that threaten the internal homeostasis [5]. Thus leads derived from traditional medicines through scientific studies appear to be influential for the discovery of new immunomodulatory compounds and novel mechanisms of action [6].

Natural product derived immunomodulators can be classified as low and high molecular weight compounds. A more specific classification reveals that active compounds belong to the following classes: carbohydrates, terpenes, steroids, phenolics, coumarins, aminoacids, peptides/proteins, glycoproteins, alkaloids and other nitrogen containing organic compounds [7].

Ficus benghalensis was selected based on ethnomedical use. A decoction of aerial roots of *Ficus benghalensis* has been used by Ayurvedic practitioners in the rural areas (Dhule region) of Maharashtra to boost the immune system in a variety of diseases that are accompanied by impairment of the immune system. However no phytochemical and pharmacological investigations of aerial roots have been reported so far. Our initial studies indicated a significant immunomodulatory activity of the methanol and aqueous extracts of the aerial roots of *Ficus benghalensis* [8,9]. This paper reports the bioactivity-guided fractionation of the active aqueous extract and the subsequent evaluation of its fractions for immunomodulatory activity using *in vitro* and *in vivo* studies.

Materials and Methods

All the solvents and chemicals used in the fractionation, isolation processes, chemical tests and bioassays were of analytical grade.

Plant material

The fresh growing tips of the aerial roots of *Ficus benghalensis* were collected from Mumbai during the rainy season (June–September 2012-13). The plant was authenticated by Dr. SunitaShailajan at RamnarainRuia College, Mumbai. A voucher specimen was deposited at the same institute. The roots were air dried, powdered in a hammer mill and stored in airtight plastic containers.

Preparation of the plant extract

The crude drug powder (50 g) of the aerial roots of *Ficus benghalensis* was subjected to Soxhlet extraction using distilled water (1.5 L) to give the aqueous extract (12.185 g, % extractive = 24.37). The water was removed in vacuum under reduced pressure using a rotary evaporator (This extract of *Ficus benghalensis* was used for further fractionation using solvents of varying polarity).

Fractionation of the aqueous extract

The aqueous extract was extracted using a separating funnel with chloroform as the solvent. The chloroform layer was collected and the solvent removed on a rotary evaporator to give the chloroform fraction of the aqueous extract. Similarly the aqueous extract was subjected to sequential extraction with ethyl acetate, n-butanol to give the ethyl acetate and n-butanol fractions. The remaining fraction was termed as n-butanol insoluble or aqueous fraction of the aqueous extract [10]. The fractionation scheme is given in Figure 1.

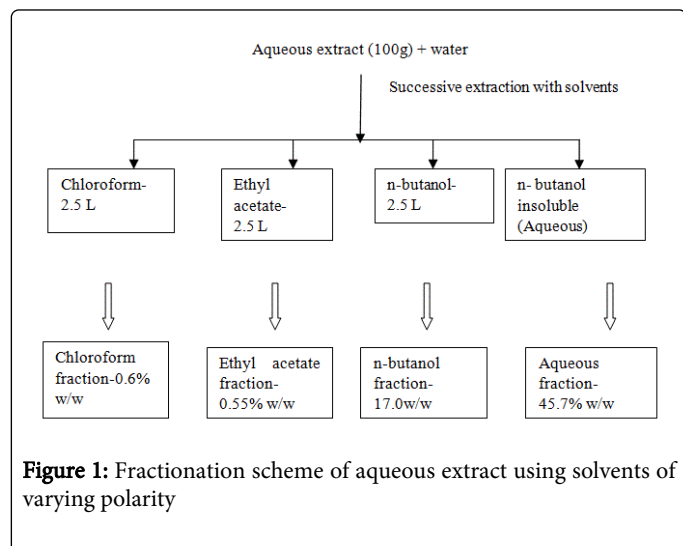


Figure 1: Fractionation scheme of aqueous extract using solvents of varying polarity

Phytochemical screening of fractions

The four fractions obtained from solvent fractionation were subjected to chemical tests to identify the phytoconstituents present in them.

HPTLC studies on the fractions

Preliminary HPTLC studies were conducted for the four fractions of the aqueous extract of the aerial roots of *Ficus benghalensis* using precoated silica gel 60 F₂₅₄ plates (Merck India Ltd.) in an effort to develop a suitable mobile phase. An entire range of single solvents (hexane, toluene, dichloromethane, acetone, ethyl acetate, n-propanol, ethanol, methanol and water) and solvent combinations were explored to achieve optimum separation of components. The test samples were applied on the HPTLC plates using Linomat CAMAG LINOMAT IV applicator. The plates after development were sprayed with 5% alcoholic ferric chloride solution, ASR, alcoholic potassium hydroxide solution and FolinCiocalteu reagent in an effort to identify the class of phytoconstituents present in the fractions. The plates were scanned using CAMAG Scanner 3 and the densitograms recorded using CATS 4 software.

Pharmacological screening of fractions

The following pharmacological assays were conducted to evaluate the immunomodulatory activity of fractions of the aqueous extract:

In vitro lymphocyte proliferation assay [11]

Separation of peripheral blood mononuclear cells: Peripheral blood mononuclear cells (PBMC) were separated from the blood sample of K3 ethylene diaminetetraacetate containing bulb by using the Ficoll Hypaque density gradient separation. Whole blood (5 ml) was overlaid on 5 ml of Ficoll Hypaque–1077 gradient (Sigma chemical Co.). This was then centrifuged at 1500 rpm for 15 minutes. The buffy coat layer of PBMC was isolated and washed once with sterile RPMI-1640 medium. The cells were then cryopreserved at a density of 2x10⁶ cells/ml using RPMI-1640 medium, 10% fetal calf serum and 10% dimethylsulfoxide (DMSO) till further use.

Peripheral blood lymphocyte (PBL) was obtained from the buffy coat residues by the Ficoll Hypaque method. Under sterile conditions, 50 µl of PBL suspension (5x10⁶ cells/ml), 50 µl sample dilutions and 50 µl of Phytohemagglutinin (PHA) (5 µg/ml) were mixed in sterile 96 well flat-bottomed microtitre plates. A solution of *Ashwagandha* capsules in DMSO was used as a positive control (0.25 mg/ml) in this assay. The plates were incubated at 37°C in a 5% CO₂ incubator for 48–72 hours. After incubation cell growth was quantitated using MTT. For this 25 µl of MTT (1 mg/ml) was added to each well after which the plates were incubated at 37°C for 4 hours. Next 50 µl of acidified propanol (0.04 M hydrochloric acid in isopropanol) was added and the contents of each well mixed thoroughly. Plates were read on an automated ELISA reader at 540 nm. Controls consisted of PBL with PHA (100% activity), PBL with RPMI-1640 medium (0% activity) and sample with PHA (background). The four fractions were screened at concentrations ranging from 0.001- 10 µg/ml.

Test wells

PBMC (50 µl) + PHA (50 µl) + sample (50 µl)

Background wells

PHA (50 l) + sample (50 µl)

Control wells

Activity (100%): PBMC (50 µl) + PHA (50 µl)

Activity (0%): PBMC (50 µl) + RPMI-1640 medium (50 µl)

The absorbance values of test fractions and control were recorded and percentage

proliferation of lymphocytes was calculated using the formula given below.

$$\% \text{ proliferation} = \frac{\text{Absorbancecontrol} - \text{Absorbance test}}{\text{Absorbancecontrol}} \times 100$$

***In vitro* cytotoxicity assay [12]**

Cell Counting Kit-8 (CCK-8) from SIGMA ALDRICH - 96992 was used for the determination of number of viable cells. It is based on the reduction of the yellow colored 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) by mitochondrial dehydrogenases of metabolically active cells, to a blue formazan which is measured spectrophotometrically. The amount of formazan generated is directly proportional to the number of living cells. This method was used to measure the cytotoxicity of all the fractions of the aqueous extract using human PBMC. The four fractions (at a concentration of 10 µg/ml each) were prepared using DMSO as the solvent which also served as the vehicle control. The estimation was conducted in triplicate and the results were reported as percentage cytotoxicity after 48 hours sample exposure.

***In vivo* hypersensitivity reaction assay [13]**

The *in vivo* immunomodulatory activity of the aqueous fraction was evaluated using the hypersensitivity reaction assay with albino rats (Wistar strain) as the animal model. This assay was used to study the effect of the aqueous fraction on cell mediated immunity. The animals were inbred and given a standard pellet diet and tap water ad libitum. Sheep erythrocytes (SRBC-concentration 5x10⁹ cells/ml) were used as the antigen in this study for immunization and challenge. The aqueous fraction was administered for five days at doses ranging from 25-200 mg/kg body weight. The control group received only the vehicle (0.5% sodium carboxymethyl cellulose in water). Ashwagandha capsules (Himalaya Drug House, Mumbai) were used as the reference standard. A suspension of the capsule powder in 0.5% sodium carboxymethyl cellulose in water was prepared and administered per orally to the animals at a dose of 100 mg/kg. Animals were immunized by injecting 0.1 ml of SRBC subcutaneously into the right hind footpad at day 0. The animals were challenged seven days after the immunization by a subplantar injection of the same amount of antigen into the left hind footpad. Footpad thickness was measured using a micrometer before and after giving the antigen. The test protocol was approved by Institutional Animal Ethics Committee (CPCSEA/IAEC/BNCP/P-02/2012).

***In vivo* hemagglutination reaction assay [14]**

The hemagglutination reaction assay was used to study the effect of the aqueous fraction on antibody mediated immunity in rats (Wistar strain). The aqueous fraction was administered orally to the test animals for five days at doses ranging from 25-200 mg/kg body weight. The control group received only the vehicle (0.5% sodium carboxymethyl cellulose in water). Ashwagandha capsules (Himalaya Drug House, Mumbai) were used as the reference standard. A suspension of the capsule powder in 0.5% sodium carboxymethyl cellulose in water was prepared and administered per orally to the animals at a dose of 100 mg/kg. Animals were immunized on day 3 by injecting SRBC (0.5 ml) intraperitoneally. Blood samples were collected retro-orbitally on day 14 of the study. The antibody titer was

determined by two fold serial dilution of one volume of serum and one volume of 0.1% bovine serum albumin (BSA) in saline. SRBC suspension (0.1%) in BSA in saline was added to each of the dilutions and mixed thoroughly. Erythrocytes were allowed to settle down at room temperature for 60-90 minutes till control tubes showed an unequivocal negative pattern (a small button). The highest serum dilution showing visible hemagglutination was taken as the antibody titer. The test protocol was approved by Institutional Animal Ethics Committee (CPCSEA/IAEC/BNCP/P-02/2012).

Results

Phytochemical screening of the fractions of aqueous extract

Phytochemical screening of chloroform, ethyl acetate and n-butanol fractions indicated the presence of flavonoids, phenolics and coumarins. The n-butanol insoluble fraction (aqueous fraction) was found to contain flavonoids, phenolic compounds and saponins.

Pharmacological screening of the fractions of aqueous extract

***In vitro* lymphocyte proliferation assay:** The chloroform, ethyl acetate, n-butanol and aqueous fractions obtained were subjected to *in vitro* lymphocyte proliferation assay. The fractions were evaluated for their effect on the mitogen induced proliferation of lymphocytes using the MTT assay. The chloroform and ethyl acetate fraction did not exhibit a significant stimulation of lymphocytes. The n-butanol fraction exhibited a maximum stimulation of 18.31% at 1.0 µg/ml. The aqueous fraction was found to stimulate the proliferation of lymphocytes by 86.39% at a concentration of 1 µg/ml (Table 1). There was a decrease in response above these concentrations.

Concentration µg/ml	Percentage proliferation			
	Chloroform fraction	Ethyl acetate fraction	n-butanol fraction	Aqueous fraction
0.001	0.26 ± 0.45	0.85 ± 0.23	1.25 ± 0.65	29.69 ± 0.75
0.01	0.67 ± 0.50	1.02 ± 0.41	5.14 ± 0.34	32.81 ± 0.84
0.1	1.07 ± 0.31	1.58 ± 0.76	13.17 ± 0.55	54.49 ± 0.18
0.5	1.35 ± 0.52	1.94 ± 0.28	17.5 ± 0.39	60.49 ± 0.25
1.0	1.59 ± 0.56	2.02 ± 0.31	18.31 ± 0.58	86.39 ± 0.63
10	1.75 ± 0.87	2.59 ± 0.45	17.19 ± 0.92	49.33 ± 0.86
Ashwagandha (AGD-0.25 mg/ml)	72.5 ± 0.35			

Table 1: *In vitro* lymphocyte proliferation assay of the fractions of aqueous extract Values are mean ± SEM (n=3); *P<0.05, **P<0.01 versus control; Dunnett's test

***In vitro* cytotoxicity assay:** The chloroform fraction was found to be cytotoxic at 10 µg/ml while the other three fractions did not exhibit cytotoxicity at 10 µg/ml.

***In vivo* hypersensitivity reaction assay:** The aqueous fraction was found to be the most bioactive fraction in the *in vitro* lymphocyte proliferation assay, hence was taken up for *in vivo* bioassays in rats.

The aqueous fraction was evaluated at doses of 25, 50, 100 and 200 mg/kg. It produced a dose related increase in the early and delayed hypersensitivity reaction thereby indicating a significant stimulation of cellular immune responses at doses ranging from 25-200 mg/kg. The maximum response was observed at 50 mg/kg (Table 2). This indicates that phenolic compounds, flavonoids, triterpenoids, saponins, carbohydrates present in the aqueous fraction could be responsible for the immunostimulant activity.

Aqueous fraction (Oral dose in mg/kg)	Inflammation after challenge Difference in rat paw thickness (in mm) before and after giving the antigen	
	4 hrs	24 hrs
Control	0.55 ± 0.20	0.27 ± 0.18
25	1.09 ± 0.33	0.82 ± 0.22
50	1.52** ± 0.53	0.85 ± 0.31
100	1.36** ± 0.41	0.60 ± 0.25
200	1.25 ± 0.51	0.52 ± 0.15
<i>Ashwagandha</i> – 100 (AGD-100)	0.99 ± 0.15	0.72 ± 0.12

Table 2: Hypersensitivity reaction assay of the aqueous fraction. Values are mean ± SEM (n=6); *P< 0.05, **P<0.01 versus control; Dunnett's test.

Hemagglutination reaction assay: The aqueous fraction was evaluated for its effect on humoral immunity at doses ranging between 25-200 mg/kg body weight. The aqueous fraction produced a dose related increase in the antibody titer in rats. The aqueous fraction exhibited a maximum response at a dose of 50 mg/kg (Table 3).

Aqueous fraction (Oral dose in mg/kg)	Hemagglutination antibody titer	
	Range	Mean Antibody tier
Control	128-256	125.20 ± 24.02
25	1024-4096	2052.20** ± 112.17
50	4096-8192	6020** ± 112.17
100	4096-8192	5324.50** ± 183.17
200	1024-2048	2160.72 ± 183.17
<i>Ashwagandha</i> – 100 (AGD-100)	1024-2048	1638 ± 112.17

Table 3: Hemagglutination reaction assay of the aqueous fraction. Values are mean ± SEM (n=6); *P< 0.05, **P<0.01 versus control; Dunnett's test.

In this assay, the aqueous fraction exhibited a better immunostimulatory response than the reference standard. This augmentation of the humoral response to sheep red blood cells as evidenced by the increase in the hemagglutination antibody titers in rats indicated the enhanced responsiveness of B-lymphocyte subset, involved in antibody synthesis.

Conclusion

This research work is an effort to discover something useful for the therapeutic management of AIDS, a disease that has taken millions of lives and still continues to be a killer disease affecting men, women and children. The prime objective of this research project was to conduct fractionation of the bioactive aqueous extract and investigate the immunomodulatory activity of the fractions generated and explore its potential as a supporting therapy in diseases like AIDS.

There are no previous reports on the bioactivity guided fractionation of the aqueous extract of the aerial roots of *Ficus benghalensis*. The activation and proliferation of T lymphocytes and cytokine production post stimulation with antigens play important roles against bacterial and viral infection. Immune stimulation is important in many disease conditions where there is a suppression of normal immune responses viz. AIDS. In AIDS, the HIV infects the T-helper cells that are the central controllers of immune responses to antigens. When these cells become infected with HIV, many other cell types fail to function normally and this results in a severe depression of the immune system. The body becomes highly susceptible to infections with opportunistic pathogens eventually leading to death. Hence immune restoration becomes imperative in the overall management of AIDS because death eventually is a result of failed immune system rather than the virus itself. Thus improving immune health is an important part of a long-term strategy for managing AIDS. The bioactive aqueous fraction of *Ficus benghalensis* has a strong potential to be explored further as an immune based therapy along with anti-HIV drugs in the overall management of AIDS. The present study establishes the *in vitro* and *in vivo* cellular and humoral immunomodulatory activity of the aqueous fraction of the aqueous extract of the aerial roots of *Ficus benghalensis*.

Acknowledgement

The authors acknowledge the funding from AICTE, India to conduct this research project.

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