



EVALUATION OF IMMUNOMODULATORY ACTIVITY OF AQUEOUS EXTRACT OF *FICUS BENGALENSIS* AERIAL ROOTS IN WISTAR RATS

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ABSTRACT

There is no effective drug for treatment of certain infections like AIDS, hepatitis, and other viral infections. For other infections the drug (mainly antibiotics) being used are becoming ineffective due to development of microbial resistance, necessitating the search for newer drugs. Any such new drug will be available only at an exorbitant cost due to the product patent norms under WTO agreement. This work is to prove the immunomodulatory property of *Ficus bengalensis* in wistar rats by studying the Delayed Type Hypersensitivity (DTH), Humoral Antibody titre (HA), Total Leukocyte Count (TLC), and Differential Leukocyte Count (DLC) In The Aqueous Extract of *Ficus bengalensis* aerial roots. The effect of this extract were comparable to the standards drug Levamisole all the data represents the Immunostimulatory activity of aqueous extract of *Ficus bengalensis* aerial roots.

Key words: Immunomodulation, *Ficus bengalensis*, DTH, HA, TLC, DLC, Levamisole.

INTRODUCTION

Immunology as a science probably began with the observations by Metchnikoff in 1882 that starfish when pierced by a foreign object (A rose thorn responded by coating it with cells (Latter identified as Phagocytes). Immunology – the study of the way in which the body defends itself against invading organisms or internal invaders (Tumors) has developed rapidly over the last 40 years, and particularly during the last 10 years with the advent of molecular techniques.² It is now a rapidly moving field that contributing critical tools for research and diagnosing, and therapeutics for treatment of a wide range of human disease. Thus, it is an integral part of college like science course and medical studies. *Ficus bengalensis*, a plant widely used in the traditional system of medicine in India, have been reported possess the anti viral, anti bacterial anti inflammatory activity.^{1,3} In the present study, the aqueous extract of *Ficus bengalensis* aerial roots has been investigated for its effect on cell mediated and humoral components of the immune system in rats. Administration of test extract produced increases in humoral antibody (HA) titre and delayed typed hypersensitivity (DTH) in rats. It is concluded that test extract is a promising drug with immuno stimulation properties. There is no effective drug for treatment of certain infections like AIDS, hepatitis, and other viral infections.

For other infections the drug (mainly antibiotics) being used are becoming ineffective due to development of microbial resistance, necessitating the search for newer drugs.

Any such new drug will be available only at an exorbitant cost due to the product patent norms under WTO agreement.¹ In Siddha, Ayurveda and other ancient systems of medicine, many plants and plant preparations are reported to be useful in the treatment of infections. When screened by modern scientific methods these preparations did not show any anti-microbial activity.

These drugs may not probably act directly upon the microbes. Instead may stimulate the body's defense mechanism (immune system) and there by help to cure the infection. Hence by screening herbal drugs and their extracts for their immunostimulant property it may be possible to get effective, cheaper new molecular entity for the treatment of various infections.

It may be hoped that such type of drugs will not produce microbial resistance, since they do not act on the microbes and will not have adverse side effects since they are from (natural) plant origin.⁴ This work is to prove the immunomodulatory property of *Ficus bengalensis* aerial roots in wistar rats by studying the Delayed Type Hypersensitivity (DTH), Humoral Antibody titre (HA), Total Leukocyte Count(TLC), and Differential Leukocyte Count (DLC) In The Aqueous Extract of *Ficus bengalensis* aerial roots.^{1,3,5}

METHODOLOGY

COLLECTION OF PLANT MATERIALS

The dried aerial roots of *Ficus bengalensis* belonging to the family Moraceae were taken powdered and the resultant powder was taken for extraction

PREPARATION OF AQUEOUS EXTRACT

The drug was extracted with sufficient quantity of distilled water; total 350 gm of drug was subjected to extraction. The drug and water was kept in the ratio of 1:5.^{1,2,6} Then it was filtered through a thin muslin cloth., the resultant extract was subjected to freeze drying at the Center of Advanced Studies (CAS) at the Marine Biology department, of Annamalai University at Parangipettai The yield was 9 gm.

EXPERIMENTAL ANIMALS

The experimental protocol has been approved by institutional animal ethics committee. Rajah Muthiah medical college, Annamalai University. Regd no:-160/1999/CPCSEA, Rats of wistar strain weighing between 150 to 250 gms were maintained under standard laboratory conditions. They were provided with a standard diet supplied by pranav agro industries ltd India and water ad libitum at central animal house.

EXPERIMENTAL PROTOCOL 24 rats were divided into four groups of six animals each.

Group-I : Control

Group-II : *Ficus bengalensis*.Linn aqueous extract was administered at a dose of 200mg/kg/day by oral route for 14 days

Group-III : *Ficus bengalensis*.Linn aqueous extract was administered at a dose of 400mg/kg/day by oral route for 14 days

Group-IV : Standard - Levamisole was administered at a dose of 50mg/kg/day by oral route for 14 days

EXPERIMENTAL SETUP

The animal model is required to study the following

- Delayed type hypersensitivity (DTH) response
- Humoral antibody (HA) titer
- Total leukocyte count
- Differential leukocyte count

DETERMINATION OF DELAYED TYPE HYPERSENSITIVITY RESPONSE (DTH)

The animals were immunized by injecting 0.1 ml of SRBCs suspension, containing 1×10^8 cells, intraperitoneally, on day 0. On Day 8, after immunisation the thickness of the right hind footprint was measured using a Vernier calliper.

The rats were then challenged by injection of 1×10^8 SRBCs in the left hind footpad. The footpad thickness was measured again after 24 h of challenge. The difference between the pre- and post challenge footpad thickness, expressed in mm was taken as a measure of the DTH response. The following formula to be used to measure the DTH response.^{1,6,8,10}

$$\frac{(\text{Left foot pad challenged with antigen} - \text{Right foot pad control}) \times 100}{\text{Left foot pad challenged with antigen}}$$

HUMORAL ANTIBODY TITRE:

The animals were immunized by injecting 0.1 ml of SRBCs suspension, containing 1×10^8 cells, intraperitoneally, on day 0. Blood samples were collected in micro centrifuge tubes from individual animals of all the groups by retro orbital vein puncture on day 10. The blood samples were centrifuged and the serum separated. Antibody levels were determined by the hemagglutination technique.^{1,7}

Method for Serial dilution

This was performed by using 96 wells (12x8) U bottomed titre plate. The wells were marked from I to XII. In the first (I) and last well (XII) 25 microlitre of serum collected from treated animals was added and inactivated at 56 degree celcius for 30 minutes. Afterwards to all the wells except well number XII, 25 microlitre of PBS was added. 25 microlitre was taken from first well and added to 2nd well again 25 microlitre from second well was taken and added to third well and continued the same procedure up to well number XI. After this 25 microlitre of sample from well number XI was discarded. Finally 25 microlitre of 1% SRBC was added to all the wells and was kept at room temperature for two hours.

Observation:

The button formation was observed. The well which is previous to the well showing button formation is considered as Antibody titer.

Well no	Dilution (antibody titer)
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1024

TOTAL LEUKOCYTE COUNT

W.B.C diluting pipette:

It has got three graduations. Two graduations 0.5 and 1 are present on the stem of the pipette and the third mark 11 is placed just above the bulb. Blood is drawn up to mark 0.5 and the rest of the bulb is filled by sucking up diluting solution up to the mark 11, the bulb of the pipette is so constructed that it holds exactly 20 times the volume of fluid contained in the stem of the pipette up to mark 1. Although fluid is drawn up to 11, the dilution of the blood will be 20 because the last part of the fluid remains locked up in the stem and is not available for dilution.⁹

The counting chamber:

The ruling area consists of 9 square millimeters. The central of the smallest squares are separated by triple lines in which RBC will be counted. The side of each square for counting WBC is $\frac{1}{4}$ mm.

Diluting fluid for WBC (Turks fluid)

Commonly the fluid is made up as follows:

Glacial acetic acid -1.5ml

1% solution of gentian violet in water -1ml
Distilled water 98ml
The glacial acetic acid haemolysis the red cells, while the gentian violet stains the nucleus of leukocytes

Method of counting W.B.C.

The white cells are counted in four corners of 1 square millimeter ruled area on both sides. The white cells are recognized by the retractile appearance and by the slight colour given to them by the stain contained in the diluting fluid. The cells touching the left side and upper side of boundary line are not counted.

CALCULATIONS

The area of the smallest square = $\frac{1}{16}$ mm²
Volume of smallest square = $\frac{1}{160}$ mm³
Total number of square counted = $16 \times 4 = 64$
Total number of cells counted = X
64/160 mm³ of diluted blood contains = X cells
So, 1 mm³ of diluted blood contains = $\frac{160}{64} \times X$ cells
1 mm³ of undiluted blood contains = $\frac{160}{64} \times 20 \times X$ cells

DIFFERENTIAL LEUKOCYTE COUNTS

A thin blood film was made on a clean, dry, glass slide. It was dried fixed and stained to differentiate the different types of leukocytes. Hundred leukocytes were counted and percentage of different leukocytes was calculated.⁹

Composition of leishman's stain

It contains a mixture of methylene blue and eosin dissolved in acetone free methanol.

PROCEDURE

A thin blood film was made on a clean dried glass slide. It was dried and stained with leishman's stain solution. The drop of leishman's stain was counted & 2 minutes was allowed to fix the blood film. Fixation means nucleus and various cellular organs will be fixed without any damage to the cells or cellular organs.

After 2 minutes double the quantity of distilled water was added over the slide and waited for 7 minutes. In the mean time the stain will initiate the chemical reaction. The acidic dye eosin will initiate various acidophilus structures and some neutrophilic granules and basic dye will stain structure like nucleus, basophilic granules, and cytoplasm of the lymphocyte and monocytes.

After 7 minutes the slide was washed in a slow stream of water later it was dried in air. One drop of cedar wood oil was placed over the film. The cells were identified and entered into 100 squares. This gives the % of different types of leukocytes present in rat blood.⁹

RESULTS

Effects of Test Extracts and Standard Drug on DTH Response in Rats Using Sheep's RBCs as Antigen.

Table -1: Delayed type hyper sensitivity

Group	Treatment	Dose	DTH response in (mm) mean paw edema \pm SEM (n=3)
1	Control		2.23 \pm 0.033
2	Test extract-I of <i>Ficus bengalensis</i>	200mg/kg	2.23 \pm 0.033
3	Test extract II of <i>Ficus bengalensis</i>	400mg/kg	2.23 \pm 0.033
4	Standard levamisole	50 mg/kg	3.56 \pm 0.066**

DUNNETT t test and p values as significant * if $p < 0.05$, highly significant ** if $p < 0.01$, as compared to control.

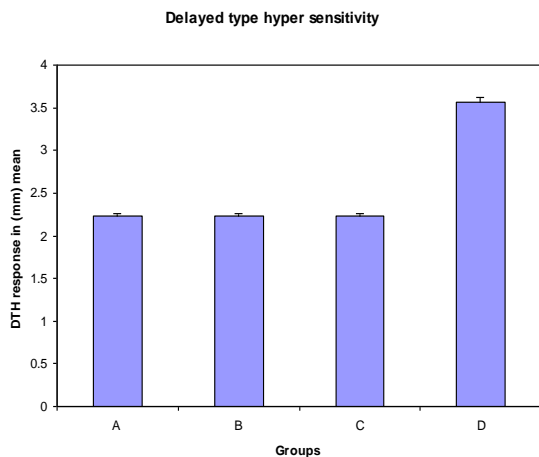


Figure -1: Delayed Type Hypersensitivity Reaction (A- Control, B- *Ficus bengalensis* 200mg/Kg, C- *Ficus bengalensis* 400mg/Kg, D- Levamisole 50mg/Kg)

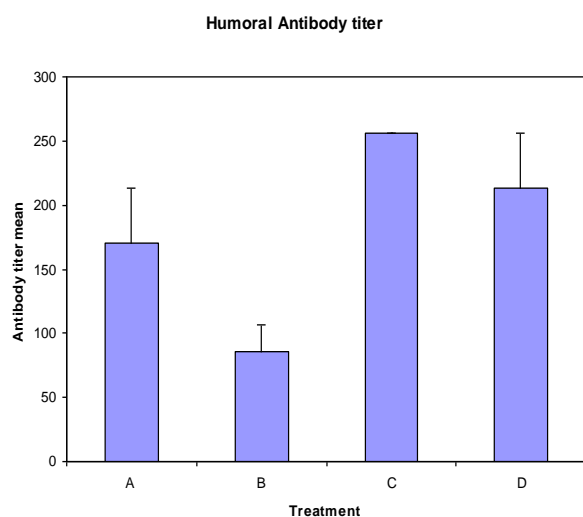


Figure -2: Humoral Antibody Titre (A- Control, B- *Ficus bengalensis* 200mg/Kg, C- *Ficus bengalensis* 400mg/Kg, D- Levamisole 50mg/Kg)

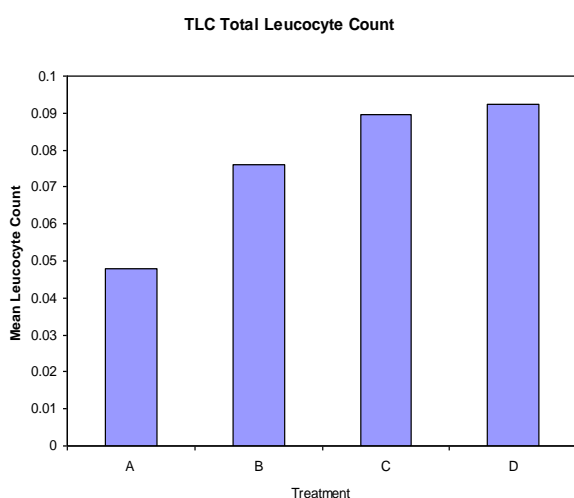


Figure -3: Total Leukocyte Count (A- Control, B- *Ficus bengalensis* 200mg/Kg, C- *Ficus bengalensis* 400mg/Kg, D- Levamisole 50mg/Kg)

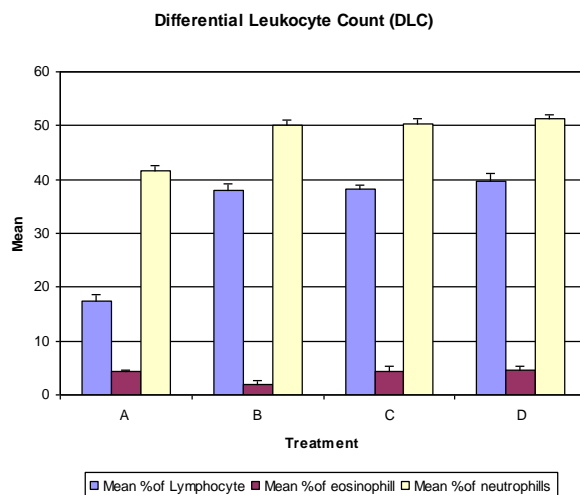


Figure -4: Differential Leukocyte Count (A- Control, B- *Ficus bengalensis* 200mg/Kg, C- *Ficus bengalensis* 400mg/Kg, D- Levamisole 50mg/Kg)

Table -2: Humoral Antibody Titer

Group	Treatment	Dose	Antibody titer mean± SEM (n=3)
1	Control		170.67±42.67
2	Test extract-I of <i>Ficus bengalensis</i>	200mg/kg	85.33±21.33
3	Test extract II of <i>Ficus bengalensis</i>	400mg/kg	256.00±0.00
4	Standard levamisole	50 mg/kg	213.33±42.67

DUNNETT t test and p values as significant* if p<0.05, highly significant** if p<0.01, and extremely highly significant*** if p<0.001 as compared to control

Table 3: Total Leukocyte Count

Group	Treatment	Dose	Mean Leukocyte Count (n=3)
1	Control		4.78x10 ³ cells / cumm ± 0.44
2	Test extract-I of <i>Ficus bengalensis</i>	200mg/kg	7.76 x 10 ³ cells/ cumm ± 0.13**
3	Test extract II of <i>Ficus bengalensis</i>	400mg/kg	8.95 x 10 ³ cells/ cumm ± 0.28**
4	Standard levamisole	50 mg/kg	9.23 x 10 ³ cells/ cumm ± 0.44**

DUNNETT t test and p values as significant* if p<0.05, highly significant** if p<0.01, and extremely highly significant*** if p<0.001 as compared to control

DISCUSSION

Two doses of the aqueous extract were used for the pharmacological investigation. The doses were administered in the form of oral solution.

DELAYED TYPE HYPERSENSITIVITY RESPONSE

Oral administration of *Ficus bengalensis* extracts (200-400 mg/kg p.o) for 14 days caused the following DTH reactivity in rats. The

Table -4: Differential Leukocyte Count

Group	Treatment	Dose	Mean % of Lymphocyte	Mean % of eosinophill	Mean % of neutrophills
1	Control		17.33±1.20	4.33±0.33	41.67±0.88
2	Test extract-I of <i>Ficus bengalensis</i>	200mg/kg	38.00±1.15**	2.00±0.58	50.00±1.15*
3	Test extract II of <i>Ficus bengalensis</i>	400mg/kg	38.33±0.67**	4.33±0.88	50.33±0.88*
4	Standard levamisole	50 mg/kg	39.67±1.45**	4.67±0.67	51.33±0.67*

DUNNETT t test and p values as significant* if p<0.05, highly significant** if p<0.01, and extremely highly significant*** if p<0.001 as compared to control

results obtained indicate that the control animals did not show any characteristics increase in paw edema.

The animals treated with lower dose 200 mg/kg showed no significant increase in paw edema as compared with the control. The animals treated with higher doses 400mg/kg showed no significant increase in paw edema when compared with the control.

The wistar rats treated with standard drug levamisole 50mg/kg showed highly significant increase in paw edema as compared to control. It is based on the stimulatory effect of the test drug and standard drug on chemotaxis dependent leukocyte migration, the antigen antibody formed immune complexes, which are known to induce local inflammation with increased vascular permeability, edema and infiltration of PMN leucocytes,

HUMORAL ANTIBODY TITER

Oral administration of *extract Ficus bengalensis* (200-400 mg/kg) for 14 days showed the following reaction in rats. The control animals did not show any characteristic humoral antibody titer.

The results obtained indicate that animals treated with lower dose (200 mg/kg) as well as higher dose (400 mg/kg) of *Ficus bengalensis* and standard drug Levamisole (50 mg/kg) showed no significant increase humoral antibody titer when compared with the control group.

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

TOTAL LEUKOCYTE COUNT

Oral administration of *Ficus bengalensis* for 14 days showed the following changes The results obtained indicates that there was not a significant increase in mean total leukocyte count in the control animals. The animals treated with lower dose of aqueous extract of *Ficus bengalensis* (200mg/kg) showed highly significant increase in mean total leukocyte when compared to control.

The animal treated with higher dose of aqueous extract of *Ficus bengalensis* (400mg/kg) also showed highly significant increase in mean total leukocyte count as compared to control. The standard drug levamisole (50mg/kg) showed a highly significant increase in the mean total leukocyte count as compared to control.

DIFFERENTIAL LEUKOCYTE COUNT

Oral administration of *Ficus bengalensis* for 14 days showed the following count in rats. The results obtained indicates that the animals treated with lower dose aqueous extract of *Ficus bengalensis* (200mg/kg) showed highly significant increase in mean percentage of lymphocytes and significant increase in mean % of neutrophils respectively as compared to control.

The animals treated with higher dose of aqueous extract of *Ficus bengalensis*. (400mg/kg) showed a highly significant increase in mean percentage of lymphocytes & neutrophils respectively as compared to control.

The standard drug levamisole (50mg/kg) also showed a highly significant increase in mean percentage of differential leukocyte count.

SUMMARY & CONCLUSION

The study was undertaken to carry out the Immunomodulatory activity of aqueous extract of *Ficus bengalensis*. For the experimental work the dried leaves was powdered and were extracted with distilled water and was freeze dried.

The aqueous extract of *Ficus bengalensis* in two different doses 200 mg/kg and 400 mg/kg was tested for their Immunomodulatory action, out of which the higher dose of 400 mg/kg showed statistically significant Immunomodulatory activity. This was evident from the different parameters that were measured.

DELAYED TYPE HYPERSENSITIVITY RESPONSE

In to this parameter the lower dose of the test showed no significant result. The higher dose also showed no significant increase in paw edema when compared with control. The standard drug Levamisole showed the maximum increase in paw volume.

HUMORAL ANTIBODY TITER

In this parameter both the dose of 200 mg/kg and 400 mg/kg of *Ficus bengalensis* produced no significant result, standard drug Levamisole at a dose of 50 mg/kg also produced no significant increase in the titre value.

TOTAL LEUKOCYTE COUNT

In this parameter the lower dose showed highly significant increase and higher dose of the aqueous extract of *Ficus bengalensis* showed a highly significant increase in the mean total leukocyte count, as compared to control. The results were highly significant for the standard drug levamisole.

DIFFERENTIAL LEUKOCYTE COUNT

For the differential leukocyte count the results revealed for lower dose was follows. The mean percentage of lymphocytes and neutrophils showed a highly significant increase in values as compared to control.

The results obtained from the animals that received higher dose of aqueous extract, revealed the fact there was a highly significant increase in the mean percentage of lymphocytes and significant increase in the mean percentage of neutrophils respectively when compared to control.

The effect of this extract were comparable to the standards drug levamisole all the data represents the Immunostimulatory activity of aqueous extract of *Ficus bengalensis*.

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