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Research Article

IMMUNOMODULATORY ACTIVITY OF ETHANOLIC EXTRACT OF *Wrightia tinctoria* LEAVES

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ABSTRACT

Wrightia tinctoria is belongs to Apocynaceae, it is generally called as Indigo plant. It is commonly used for skin diseases, jaundice etc…, In folk medicine it is used for some breast related disorders. Most of the herbal drugs are having good immune response and to prevent the diseases. In this study, the effect of the methanolic extract of the leaves of the plant *Wrightia tinctoria* on the primary and secondary antibody responses was evaluated by the humoral antibody response for a specific immune response. The effect of *Wrightia tinctoria* on the neutrophil activation was evaluated by the neutrophil adhesion test for a nonspecific immune response. The data was analyzed by one-way ANOVA followed by Duncan's multiple range test/unit. On oral administration, *Wrightia tinctoria* showed a significant increase in the primary and secondary humoral antibody responses, by increasing the hemagglutinating antibody titre at doses of 100 and 200mg/kg/bw. There was a significant increase in the percentage neutrophil adhesion at doses of 200mg/kg/bw. Also *Wrightia tinctoria*- possesses significant delayed hypersensitivity response in the increasing doses showing the greater activity in the dose of 200mg/kg/bw. The serum immunoglobulin test evoked a significant rise in the ethanolic extract of the leaves of the plant *Wrightia tinctoria* by increasing doses and shows maximum at 200mg/kg/bw.The present study reveals that the ethanolic extract of the *Wrightia tinctoria* holds a promise as an immunomodulatory agent, which acts probably by stimulating both the specific and nonspecific arms of immunity.

Keywords: *Wrightia tinctoria*, Immunomodulatory activity, Immune system, Immunostimulant, Antibody titre, neutrophil

INTRODUCTION

Herbal medicine is the oldest form of healthcare known to mankind.About three quarters of the world's population currently uses herbs and other forms of traditional medicines to treat the ailments. Thus the traditional systems of medicine are recognized increasingly as instruments for realizing the objective of providing holistic and cheaper health alternatives of primary health care.[1]

Traditional and folklore medicines play an important role in health services around the globe. India has an extensive forest cover, enriched with plant diversity. Several plants have been used in folklore medicine.[2]The rational design of novel drugs from traditional medicine offers new prospects in modern healthcare. Herbal drugs are known to possess immunomodulatory properties and generally act by stimulating both specific and nonspecific immunity.[3]Many plants used in traditional medicine have immunomodulating activities. Some of these stimulate both humoral and cell-mediated immunity, while others activate only the cellular components of the immune system. [4]i.e. the phagocytic function, without affecting the humoral or cell-mediated immunity. Some of these plants also suppress both humoral and cell-mediated immunity. [5-8]

Wrightia tinctoria of the family Apocynaceae, commonly known as 'Jaundice cure tree' has been used extensively in the Indian system of medicine for the treatment of various diseases like psoriasis, jaundice etc.[9]*Wrightia tinctoria* is a small, deciduous tree with a light gray, scaly smooth bark. Native to India and Burma, Wrightia is named after a Scottish physician and botanist William Wright From a distance, the white flowers may appear like snowflakes on a tree. The fruits pendulous, long paired follicles joined at their tips. The hairy seeds are released as the fruit dehisces. The leaves of this tree yield a blue dye called Pala Indigo.[10] Sweet Indrajao is called dhudi because of its preservative nature. The plant is distributed throughout tropical India, Ceylon, Malay Peninsula.

It flowers and fruits during April to August.The leaves are acrid, thermogenic, anodyne and hypotensive and are very useful in odontalgia, vitiated conditions of vata and hypertension. The seeds are bitter, astringent, acrid, carminative, constipating, depurative, anthelmintic and febrifuge.[11]Its pungent fresh leaves quickly relieve toothaches. White, close-grained wood looks like ivory and is much used for carving and wood-turning. They are used in piles, fever, diarrhoea, roundworm and colic diseases.[12]

The leaves are applied as a poultice for mumps and herpes and sometimes, they are also munched to relieve toothache. In folk medicine, the dried and powdered roots of Wrightia is mixed with milk and orally administered to women for improving fertility.[13]The bark and seeds are effective against psoriasis and non-specific dermatitis. It has anti-inflammatory and anti-dandruff properties and hence is used in hair oil preparations. [14].

However, no phytochemical and pharmacological investigations on leaves have been conducted so far to substantiate this practice. The current study aimed at exploring plants is believed to promote positive health and maintain organic resistance against infection by establishing body equilibrium. It is tempting to speculate that the restorative and rejuvenating power of these herbs may be due to their action on the immune system. The concept of immunomodulation relates to non-specific activation of the function and efficiency of macrophages, granulocytes, complement, natural killer cells, and lymphocytes, and also to the production of various other cells. [15-16]

Chemical constituents

9-hydroxy-cis-12-octadecenoic acid, 9- hydroxy stearic acid, triisoricinoleoyl glycerol,di and tri isoricinoleoyl glycerols, myristic acid,palmitic acid, β sitosterol, α-amyrin and its acetate, oleanolic acid urosolic acids, Lupeol and its benzoate, cycloartenone, cycloeucalenol.

MATERIALS AND METHODS

The leaves of the plant *Wrightia tinctoria* (Family: Apocyanaceae) was collected from vidrudhunagar district of Tamil nadu, India. The plant material was taxonomically identified by botanist. A voucher specimen has been preserved in our laboratory for future reference. The leaves were dried under shade and then powdered with a mechanical grinder and stored in airtight container. The dried powder material of the leaves was defatted with petroleum ether and the marc thus obtained was then extracted with methanol in a soxhelt apparatus. Ethanolic extract is used because the chemical constituents were easily extracted in ethanol. The solvent was completely removed under reduced pressure and a semisolid mass was obtained. The dried *Wrightia tinctoria* extract was suspended in normal saline and used for the present study.

The standardized coarse powder of plant material was subjected to soxhlet extraction using ethanol as the solvent. The dark green

filtrate obtained was concentrated. The crude ethanolic extract was used for the present studies.

The extract was subjected to preliminary phytochemical investigations to identify various phytoconstituents present in the leaves. Preliminary phytochemical screening of Wrightia tinctoria showed the presence of alkaloids, glycosides and tannins in ethanolic extract.

Sheep red blood cells SRBC were collected in the alsevers solution,washed three times in large quantities 30 ml of pyrogen free 0.9%normal saline and adjusted to a concentration of (0.5X 10^9 SRBC) and was used as Antigen for inducting inflammation in the study.

Healthy young adult male albino wistar rats (110-160 gm) were kept separately in individual polypropylene cages (38 cm × 23 cm × 10 cm) with stainless steel hopper. The temperature in the experimental animal room was 22ºC ± 3ºC. Although the relative humidity was 30 % and preferably not exceeding 70 % other than during room cleaning, the aim was 50-60 %. Lighting used artificially, the sequence being 12 hours light and 12 hours dark. The animals were housed individually. For feeding, conventional laboratory diets was used with an unlimited supply of drinking water. The animals were uniquely identified and kept in their cages for five days prior to dosing for acclimatized to the laboratory conditions. During acclimatization the animals were observed for ill health. Animals demonstrating signs of spontaneous disease or abnormality prior to the start of the study were eliminated from the study. The experiments are done according to the CPCSEA guidelines and approved by the Institutional Animal Ethical Committee. The test substance was dissolved in distilled water and the dose was selected at 100 and 200 mg/kg body weight. The test substance pretreated with animal for 14 days by oral gavage. Albino rats were divided into groups comprising of six animals each. Group I served as control and was administered vehicle only. Group II was administered 100 mg kg^{-1} bodyweight ethanolic extract. Group III received 200 mg kg^{-1} body weight of ethanolic extract. Each experiment was performed on fresh group of animals unless specified.

Neutrophil adhesion test[18,19] For14 days drug was given orally to each group except control.Control was given 1 ml distilled water. After 14 days of treatment of all the three groups, blood samples were collected by retro-orbital puncture in heparinized vials and subjected to total as well as differential leukocyte count. After initial counts the blood samples were incubated with 80 mg ml^{-1} of nylon fibers at 37°C for 15 min. The incubated samples were again analyzed for total and differential leukocyte count. The product of total leukocyte count and % neutrophil known as neutrophil index was determined for each of the respective groups (24). The % neutrophil adhesion for each of the test groups was determined as follows. Neutrophil index and neutrophil adhesion calculated.[20]

$$\text{Neutophil adhesion} = (NI_u - NI_t) / NI_u X\ 100$$

Where,

NIu: Neutrophil Index before incubation with nylon fibres

NIt: Neutrophil Index after incubation with nylon fibres

Haemagglutinating antibody **(**HA**)** titre [21] test was perfomed in rats. Rats of group II and III were pretreated with Wrightia tinctoria for 14 days. on 14 th day each rat was immunized with 0.5X10^9 SRBC/rat by intra peritoneal route, including control rats. The day of immunization was referred to as day 0. The animals were treated with Wrightia tinctoria extract for 14 more days. Blood was withdrawn from all animals on the fourteenth and twenty-first days, from the retro-orbital plexus, under mild ether anesthesia, and centrifuged to obtain the serum. The antibody titre was determined using microtitre plates. Each well of a microtitre plate was filled initially with 25µl of normal saline and 25µl of serum was mixed with 25µl of normal saline in the first well of the microtitre plate. Subsequently the 25µl diluted serum was removed from the first well and added to the next well to get twofold dilutions of the antibodies present in the serum. Further twofold dilutions of this diluted serum were carried out till the last well of the second row (twenty-first well), so that the antibody concentration of any of the dilutions is half of the previous dilution. Twenty-five microlitres of 1% SRBC was added to each well and the microtitre plates were incubated at 37°C for one hour and then observed for hemagglutination. The highest dilution giving hemagglutination was taken as the antibody titre. The antibody titre was expressed in a graded manner, the minimum dilution (1/2) being ranked as 1 and the mean ranks of different groups were compared for statistical significance. The antibody titre obtained on the fourteenth day after immunization and on the twenty-first day after challenge with SRBCs was considered as the primary and secondary humoral immune responses, respectively. The titre was determined by titrating serum dilutions with SRBC (0.025X10^9 cells).

Delayed Hypersensitivity Response Also Performed[22, 23]For the purpose of measuring the delayed hypersensitivity test, six animals per group were immunized on day 0 by intra peritoneal administration of 0.5X 10^9 SRBC per rat. Drug was administered orally for 14 days. (Control- 1ml, D. water, group I- 100 mg/kg,B.wt and group II 200 mg/kg, B.wt) On the 14 th day, After 24 hrs of last dose Subcutaneous administration of 0.5X 10^9 SRBC per ml by Sub plantar region to all Groups, in Right plantar region of rat's paw. When the antigen was induced the animal develops inflammation in the foot.the liberation of mediator's causes edema and thus paw volume is increased. The paw volume was measured in both Right and Left Hind Paw after 24 hrs of challenge Calculated % of inhibition. The values were noted for the delayed hypersensitivity response.

Serum Immunoglobulin test [24, 25]also did in the rats, Drug was administered orally for 21 days. Six hours after 21st day drug administration blood samples were collected by puncturing the retro orbital plexus of the wistar rats.For each serum sample a control tube containing 30 ml of distilled water and 30 ml of zinc sulphate were prepared. To each 0.1 ml of serum added from pippete.it was left to stand for I hour in plugged test tubes. Barium sulphate was developed which act as standard. Turbidity was developed and the obtained turbidity was compared with standard barium sulphate using nepheloturbidimeter.

All the data were statistically analyzed by ONE WAY ANOVA following Duncans multiple range test. The values are expressed as mean ± SD for 6 rats in each group. Values are not sharing a common superscript. Significance at P where compared with control.

RESULT

Neutrophil Adhesion Test: This test is an indicative of the marginalization of phagocytic cells in the blood vessels, an indication of immunostimulation. The % neutrophil adhesion in control group animals was 42.5 1±048 in Group2 (100 mg /kg) ethanolic extract-treated group it was 50 1±89whilst for Group3(200 mg/kg)it was 52.2±85. As is evident from the results of neutrophil adhesion test, nearly 27.7 % ($P < 0.05$) increase in neutrophil adhesion is observed after administration of Group2 ethanolic extract, whilst a 30% and significant($P < 0.05$) increase in neutrophil adhesion is observed in ethanolic extract Group3 (Table 2).

Ha titre showed significant increase when wrightia tinctoria was orally administered which was given in Table 3.This was due to the agglutination reaction between antigen and antibody and this was due to the humoral mediated response. Thus it can be concluded that the plant *Wrightia tinctoria* possess significant humoral mediated response in rat.

The serum immunoglobulin test[26] evoked a significant rise in the results which are given in Table 3. The serum when added showed the turbidity and can be concluded that immunoglobulin g is present and can thus confirm the immunomodulatory activity.

Delayed hypersensitivity response was performed, In the control group animals, after +48 and +72 h of challenge the DTH response was either equal or slightly more than the 0 h response; therefore, the peak edema after +24 h of challenge was the evaluating parameter. Methanolic extract (200 mg kg^{-1} per orally) was most effective ($P < 0.05$) compared to group 1 (100 mg kg^{-1} per orally) treatment in increasing the delayed-type hypersensitivity response (Table 4).[27]

Table 1: Effect of *Wrightia tinctoria* on the neutrophil adhesion in rats

Group	TLC(10^3/mm)		Neutrophil %	
	UTB	FTB	UTB	FTB
Group I	6.5±0.1210	5.9 ±0.22	42.5 ±0.48	37.1±0.94
Group II	7.28±0.45**	6.41±0.168**	50.1±0.89**	41.3.±0.6**
Group III	7.78±0.558**	6.61±0.223**	52.2±0.85**	42.8±3.125**

Table 2: The effect of *Wrightia tinctoria* on the neutrophil adhesion in rats

Group	Neutrophil Index	Neutrophil Index	Neutrophil Adhesion
	UB	FB	%
Group I	216 ± 11.463	218.3 ± 10.4	20.9%
Group II	364 ± 33**	262.81 ± 23**	27.7%
Group III	404.5 ± 37.6**	282.9 ± 29.8**	30.0%

Note: The values are expressed as mean ± SD for 6 rats in each group. Values are not sharing a common superscript. Significance at P ≤ 0.05 ONE WAY ANOVA following Duncans multiple range test/unit.

Table 3: The effect of *Wrightia tinctoria* on HA titre and serum immunoglobulin test

S. No.	Groups	Mean Value of HA TITRE	ZST Nephelo Units
1	Group I	6.33±1.038	48.6 ± 0.194
2	Group II	22.83±1.602**	66.8±0.983**
3	Group III	47.66±1.366**	97.7.12***

Note: The values are expressed as mean ± SD for 6 rats in each group. Values are not sharing a common superscript. Significance at P ** ≤ 0.05, P *** ≤0.001 when compared to control. ONE WAY ANOVA following Duncans multiple range test

Table 4: The effect of *Wrightia tinctoria* on DTH response to antigenic challenge by sheep RBCS in rats.

Group	Mean ±SD	% Inhibition in the PAW Volume
Group I	0.61 ± 0.4082	0
Group II	0.5±0.051**	18.3%
Group III	0.46±0.051**	24.5%

Note: The values are expressed as mean ± SD for 6 rats in each group. Values are not sharing a common superscript. Significance at P ≤ 0.05 when compared to control ONE WAY ANOVA following Duncan's multiple range test.

DISCUSSION

Immunomodulatory agents of the plant and animal origin enhance the immune responsiveness of an organism against a pathogen by activating the immune system. Ethanolic extract of *Wrightia tinctoria* administered orally showed significant invivo, immunomodulatory activity and possibly exerts its effect through diverse mechanisms that may involve cellular pathways. Further pharmacodynamic investigations are required to understand the precise mechanism of *in-vivo* immunomodulation by ethanolic extract.

In the present investigation, ethanolic extract of *Wrightia tinctoria*-was studied for its Immunomodulatory activity using acute animal model. In the present investigation, *Wrightia tinctoria* significantly increased the adhesion of neutrophils to nylon fibres which interrelates to the process of margination of cells in blood vessels. It was found to be highly significant when compared to control.

The ethanolic extract of *Wrightia tinctoria* evoked an increasing response to the haemagglutination test which was shown in the table. Thus it was confirmed that there is an antigen antibody reaction taking place and thus there is a humoral mediated immunity, produced by the B cells.

When SRBC added to the serum dilution, antibody is formed. This antibody bind to antigen and this will neutralize it or facilitate its elimination by cross linking to form latex that is more readily ingested by phagocytic cells. When the test animals pretreated, value of circulating antibody increases.The test involve double dilution of serum sample and addition of SRBC.If the serum antibody react with SRBC then agglutination occurs because of formation of antibody which bridges with neighbouring erythrocytes and these settle at the bottom as latex. Unagglutinted RBC as a button in cell bottom.If haemaglutination was detected in the serum wells a not in control wells then result is recorded as titre. RBC at neutral pH possess negative ions that cloud which repel one another.

In the present investigation, ethanolic extract of *Wrightia tinctoria*-was studied for its Immunomodulatory activity using acute animal model. SRBC induced inflammation is a biphasic phenomenon the first phase of edema is attributed to release of histamine and 5-Hydroxytryptamine. Plateau phase is maintained by kinin like substances and second accelerating phase of swelling is attributed to prostaglandin like substances. The knowledge of these mediators involved in different phases is important for interpreting mode of drug action.

Thus it can be concluded that the plant *Wrightia tinctoria*- possess significant anti-inflammatory activity in rats. Further studies involving the isolation of the active chemical constituents from the plant and the investigations in the biochemical pathways may result in the development of a potent immunomodulatory activity with less toxicity and better therapeutic index.

An immunoglobulin test measures the level of certain immunoglobulins, or antibodies, in the blood. Antibodies are proteins made by the immune system to fight antigens, such as bacteria, viruses, and toxins. Blood contains three globulins-alpha, beta, and gamma globulins based on their electrophoretic migration rate. Estimation of serum immunoglobulin is determined by using Zinc Sulphate Turbidity test. Immunoglobulin **G** (IgG), the most abundant type of antibody, is found in all body fluids and protects against bacterial and viral infections. Amount of IgG, present in the serum is determined by this particular test. Small amount of serum

is added to the Zinc Sulphate solution and it is incubated for 1 hour. This causes the precipitation of IgG which makes the solution cloudy instead of clear. This test is specific for immunoglobulin G.

CONCLUSION

Immunomodulatory agents of the plant and animal origin enhance the immune responsiveness of an organism against a pathogen by activating the immune system. ethanolic extract of *Wrightia tinctoria* administered orally showed significant invivo, immunomodulatory activity and possibly exerts its effect through diverse mechanisms that may involve cellular pathways. Further pharmacodynamic investigations are required to understand the precise mechanism of invivo immunomodulation by ethanolic extract.

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