Immunomodulatory Activities of *Wrightia Tinctoria* (Roxb.) R. Br Bark Extracts

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Summary

Wrightia tinctoria (Roxb.) R.Br. (WT) has been reported to exhibit number of therapeutic uses such as astringent, stomachic, febrifuge, skin diseases and tonic in India. The objective of the present study was to investigate the immunomodulatory activity of the bark extracts of WT using delayed type hypersensitivity reaction and carbon clearance assay. Powdered dried barks of WT were extracted with petroleum ether 60 - 80°C (PEWT), alcohol (ALWT) and aqueous alcohol (AQWT) (60% water + 40% ethanol) successively. PEWT and ALWT extracts (200,400mg/kg, p.o) produced a significant increase in delayed type hypersensitivity in response to sheep red blood cells (SRBC). PEWT showed better activity than ALWT in delayed type hypersensitivity response. ALWT (200 and 400mg/kg, p.o) in dose dependent manner have shown significantly increase in the phagocytic activity. The above results reveal that ALWT possesses immunostimulant activity in carbon clearance assay whereas PEWT and ALWT showed immunomodulatory activity in delayed type hypersensitivity model.

***Keywords*:** *Wrightia tinctoria;* Delayed-type hypersensitivity; Carbon clearance assay.

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Introduction

Wrightia tinctoria (Roxb.) R.Br. (Apocynaceae), commonly known as Pala indigo plant or 'Sweet Indrajao' in English and 'Dudhi' in Hindi. It is a small deciduous tree with a light gray, scaly smooth bark and native to India and Burma **(1).** It is very popular as medicinal agent in ethnic medicine; the plant was mentioned in ancient Ayurvedic text like "Rajanighantu" and "Sva". It is used in Ayurveda, Unani and Siddha medicines. Traditional system of medicine claim usefulness of *Wrightia tinctoria* for the treatment of stomachic, febrifuge, skin diseases, abdominal pain and used as tonic **(2)**. The leaves are useful in psoriasis, non-specific dermatitis, anthelmentic, aphrodisiac, haemorrhoids dipsia and dropsy. The bark contains β-sitosterol, β-amyrin and its acetate and lupeol benzoate and seeds yield up to 40% fixed oil **(1).** Previous studies have reported that *Wrightia tinctoria* possesses the anti-bacteria, anti-fungal, antinociceptive and wound healing properties **(3, 4, 5, 6).** Phytochemical screening of the plant has shown the presence of indole, flavonoid, sterols, fixed oil and triterpenoid compounds **(7, 8, 9, 10).** Since, the drug is used as a general tonic and the other medicinal properties in traditional systems of medicine, it may think worthwhile that its effect may be due to the action on immune system, as a therapeutic measure.

Due to lack of scientific studies, as well as to establish its potential pharmacological properties, this present study aimed to investigate the immunomodulatory activity of the various extracts of the bark of *Wrightia tinctoria* (Roxb.) in animal models.

Materials and methods

Plant material and preparation of plant extract

The bark of *Wrightia tinctoria* was obtained from Tiruchengodu, Tamilnadu in the month of November 2007 and authenticated by Dr. K. P. Sreenath, Reader and Taxonomist, Department of Botany, Bangalore University, Bangalore. A voucher specimen (collector no-1) has been deposited in herbarium of P E S College of Pharmacy, Bangalore, India.

The powdered bark (80g) was subjected for successive solvent extraction using petroleum ether 60 - 80°C (PEWT), alcohol (ALWT) and aqueous alcohol (AQWT) (60% water + 40% ethanol) successively for 18 h. Each obtained extracts were concentrated by distillation of the solvent and evaporating them to dryness at low temperature (40^{o}C). They were then weighed and their yields were 4.81%, 11.45% and 2.82% respectively (calculated in terms of air dried weight of the plant material).

Animals

Albino Swiss mice (weighing 18-25g, n = 6 per group) were purchased from Bioneeds, Nelamangala, Tumkur, India. The animals were housed under standard conditions of temperature (25 ± 1^{0}C), relative humidity (55±10%), and 12/12 h light / dark cycles and fed with standard pellet diet (Pranav Agro Industries Ltd, Bangalore) and portal water *ad libitum* under strict hygienic conditions. The animal studies were conducted after obtaining clearance from Institutional Animal Ethics Committee and the experiments were conducted in strict compliance according to ethical principles and guidelines provided by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Drugs

The test extracts (PEWT, ALWT and AQWT) were prepared in 0.5% v/v of Tween-80. The vehicle alone served as control. Cyclophosphamide (Cadila Health Care Ltd., Goa) was used as a standard immunosuppressant agent.

Antigens

Sheep red blood cells (SRBC) were collected from a healthy adult sheep sacrificed in the local slaughter house. SRBC collected in Alsever's solution, were washed thrice with Phosphate buffer saline (PBS). The number of SRBC was then adjusted to a concentration of 0.1ml containing $1X10^8$ cells after the RBC count. This RBC suspension was used for immunization and challenge.

Delayed type hypersensitivity (DTH) reaction using SRBC as an antigen

Method described by Doherty was used **(11)**. Mice of either sex were divided into eight groups of six each. The test extracts (PEWT, ALWT and AQWT at 200 and 400 mg/kg, p.o.) were administered on day 0 to day 7. Cyclophosphamide (50 mg /kg, i.p) were administered on 4^{th}, 5^{th} and 6^{th} day. The mice were primed with 0.1ml of SRBC suspension containing $1X10^8$ cells, i.p., on day 0 and challenged on day 7 with 0.05ml of 2×10^8 SRBC on the right hind paw. The contralateral paw received equal volume saline. The thickness of the foot pad was measured at 24 h after the challenge using screw gauge. The difference in the thickness of the right hind foot paw was used as a measure of delayed hypersensitivity (DTH) reaction.

Macrophage phagocytosis by carbon clearance assay

Mice of either sex were divided into eight groups of six each. The test extracts (PEWT, ALWT and AQWT at 200 and 400 mg/kg, p.o) were administered 7 days prior to the injection of carbon particles (0.1ml/10mg). Cyclophosphamide (50 mg /kg, i.p) was administered on 4, 5 and 6 day. On 7^{th} day all the animals received an intravenous injection of Indian ink suspensions **(12).** As soon as mouse eyes turned black 50 µl of blood samples were collected on the slide from each animal by retro orbital bleeding by using glass capillaries at an interval of 2 min (t_1) and 10 min (t_2) after the injection of ink suspension lysed with 4 ml of 0.1 % sodium carbonate solution. Absorbance of these samples was measured at 675 nm using spectrophotometer. Then the liver and spleen of individual mice were culled and weighed.

Rate of carbon clearance (K) and Phagocytic index (α) are calculated as follows:

$(K) = log\ A_1 - log\ A_2 / (t_1 - t_2)$, $(\alpha) = K^{1/3}$ X body weight / Liver wt. + spleen wt.

Rate of carbon clearance and phagocytic index of treated group animals was compared with the control group animals **(13).**

Statistical analysis

The results are presented as mean ± SEM from 6 animals. Statistical analysis between the groups was analysed by using One-Way ANOVA followed by Dunnett post-hoc test. $P<0.05$ was considered to be statistically significant.

Results

Delayed type hypersensitivity (DTH) reaction using SRBC as an antigen

The oedema achieved a peak at 24 hour. The control group (10ml/kg body weight, p.o) was found to be 0.671±0.014 mm. PEWT and ALWT (200 and 400mg/kg, p.o.) produced a significant dose-related increase in DTH reactivity in mice. Cyclophosphamide also showed significant increase in paw oedema as compared to control. AQWT did not significantly alter the paw oedema as compared to controls (Table 1).

Table 1: Effect of test extracts on delayed type hypersensitivity (DTH) response using SRBC's as an antigen in mice -7 days pre-treatment.

Group	Treatment	Dose (mg/kg, p.o)	DTH response (mm) mean paw edema ± SEM
I	Vehicle control	10ml/kg	0.671 ± 0.014
II	Cyclophosphamide	50	1.241 ± 0.027[a]
III	PEWT	200	1.010 ± 0.030 [a]
IV	PEWT	400	1.083 ± 0.022 [a]
V	ALWT	200	0.901 ± 0.036 [a]
VI	ALWT	400	0.946 ± 0.018 [a]
VII	AQWT	200	0.748 ± 0.041
VIII	AQWT	400	0.766 ± 0.037

Mean ± SEM, (n=6)
[a] $P<0.01$, statistically significant as compared to control.

Macrophage phagocytosis by carbon clearance assay

ALWT (200 and 400mg/kg, p.o) significantly, showed a dose related increased in phagocytic index as compared to control. The control group (10ml/kg body weight, p.o) was found to be 0.167×10^{-04}. Cyclophosphamide (50 mg/kg i.p.) showed a significant decrease in phagocytic index as compared to control. Both PEWT & AQWT (200 and 400mg/kg, p.o) showed dose related increase in the phagocytic index as compared to control but statistically not significant (Table 2).

Table 2: Effect of test extracts on macrophage phagocytic activity in mice -7 days pre-treatment.

Group	Treatment	Dose (mg/kg, p.o)	Rate of carbon Clearance (K)(10^{-02})	Phagocytic index (α) (10^{-04})
I	Vehicle control	10ml/kg	0.081± 0.013	0.167 ± 0.065
II	Cyclophosphamide	50	0.049 ± 0.007[a]	0.035 ± 0.012[a]
III	PEWT	200	0.095 ± 0.008	0.197 ± 0.040
IV	PEWT	400	1.154 ± 0.010	0.375 ± 0.082
V	ALWT	200	1.779 ± 0.011 [a]	1.335 ± 0.226 [a]
VI	ALWT	400	2.118 ± 0.010 [a]	1.783 ± 0.305 [a]
VII	AQWT	200	0.080 ± 0.019	0.199 ± 0.024
VIII	AQWT	400	1.135 ± 0.009	0.304 ± 0.087

Mean ± SEM, (n=6); [a] P<0.01, statistically significant as compared to control.

Discussion and conclusion

Use of herbs for improving the overall resistance of body against common infections and pathogens has been a guiding principle of Ayurveda **(14).** *Wrightia tinctoria* has been used as tonic traditionally. The present study has showed that the test extract of *Wrightia tinctoria* ALWT does not only potentiate non-specific immune response, but is also effective in improving cell mediated immunity.

In the present investigation 7 days pre-treatment in mice were selected in both the models. PEWT and ALWT (200,400mg/kg, p.o) produced a significant dose-related increase in DTH reactivity in mice. In DTH, circulating T cells sensitized to the antigen from prior contact with the antigen and induces specific immune response, which subsequently proliferates and release cytokines **(15).** The increase in response indicated that PEWT and ALWT have stimulating effect on lymphocytes. Dosing with cyclophosphamide during period closer to elicitation of DTH is reported to have profound suppressive effect on all forms of DTH and cell mediated immunity **(16).**

The carbon clearance test was carried out to evaluate the effect of drugs on the reticuloendothelial system (RES). It is generally considered that phagocytosis and elimination of foreign agents are typical functions in the RES **(17).** The event of phagocytosis is primarily the removal of microorganisms and foreign bodies, besides imparting the property of the elimination of dead or injured cells **(18).** Phagocytosis of the RES is performed by the Kupffer cells in the liver **(19).**

Hence, the carbon clearance was evaluated by the phagocytic index. The effects of ALWT (200,400mg/kg) significantly, shown a dose related increased in phagocytic index as compared to control, which indicates the capacity of macrophages system in removal of foreign particle, thereby an indicator of enhanced immunological response against foreign particles or antigens. Both PEWT and AQWT (200,400mg/kg, p.o) showed dose related increase in the phagocytic index as compared to control but statistically not significant.

In conclusion, the enhancement of DTH response, phagocytic index provides the first line of evidence about the immunomodulatory property of *W. tinctoria* bark extracts. The present investigation suggests that significant increased in the immunomodulatory activity of *W. tinctoria* bark extract could be attributed to the presence of phytosterols, fixed oils and fats, carbohydrates, glycosides, alkaloids, gum and mucilage, phenolics compounds, tannins, and saponins present in the drug. Further studies needed to evaluate the isolated compounds from *W. tinctoria* liable for exact immunostimulatory mechanism.

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