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BARK EXTRACT OF *SHOREA ROBUSTA* ON MODULATION OF IMMUNE RESPONSE IN RATS

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ABSTRACT

Immune activation is an effective and protective approach against emerging infectious diseases. The immunomodulatory activities of *Shorea robusta* bark was evaluated by testing the various immunological parameters. Sheep red blood cells (5×10^9 cells/ml) were used for immunizing the animals that belongs to immunized groups. This study was performed with a set of immunomodulation such as the humoral antibody response (hemagglutination antibody titers, immunoglobulins), cell mediated immune response (delayed type hypersensitivity and phagocytosis), nitroblue tetrazolium (NBT) reduction test, total lymphocyte count (TLC) and DC. Ethanolic extract of *Shorea robusta* bark was administered p.o. (orally) to the mice at a dose of 100mg and 300mg/kg body weight per day for 14 days. In this study, *Shorea robusta* bark extract administrated rat models at 300mg/kg per day, i.p should significant important in stimulating immunomodulatory response, thus *Shorea robusta* bark is an effective natural health product for in modulating immune system.

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INTRODUCTION

Modulation of immune responses to alleviate the diseases has been of interest for many years and the concept of 'Rasayana' in Ayurveda is based on related principles (Sharma, 1983). Further, immunomodulation using medicinal plants can provide an alternative to conventional chemotherapy for a variety of diseases especially when host defense mechanism has to be acquired under the conditions of impaired immune responsiveness (Lily, et al., 2005). The use of plant products in the indigenous system of medicines as immunomodulators, indeed, can modulate the body's immune system, as a variety of plant derivatives such as polysaccharides, lectins, peptides, flavonoids and tannins have been reported to modulate the immune system in various *in vivo* models (Shivaprasad *et al.*, 2006). Therefore, the search for more effective and safer agents exerting immunomodulatory activity is becoming a field of major interest all over the world (Patwardhan, et al., 1990).

Shorea robusta belongs to family Dipterocarpaceae is reported to possess antimicrobial properties for its resin (Alluri *et al.*, 2005), and being used in ulcers (Narayan *et al.*, 2004) and found to show anti-aging and wound healing activity (Sharma *et al.*, 2009). The chemical constituents of *Shorea robusta* such as 2-(e-iminoacetic acid)-3-(2H)-benzofurnone, shoreaphenol, flavonoids, glucose of 4'hydroxychalcone, leuconathocyanidin, hopcaphenol, triterpenoids and a terpene alcohol, monomethylether and dimethylether of homocatechol, alkybenzene derivatives, pentosans, lignin, tannin, amino acids and fatty acids (Patra *et al.*, 1992; Prajapati *et al.*, 2006).

The bark traditionally used as astringent, acrid, cooling, antihelmintic, alexeteric, anodyne, constipating, urinary astringent, union promoter depurative and tonic. They are useful in vitiated conditions of *kapha* and *pitta*, ulcers, wounds otalga, bacterial infections diarrhea, dysentery, gonorrhoea, leucorrhoea, pruritus, leprosy, cough, and anaemia (Prajapati *et al.*, 2006). However no reports are available on immunomodulatory activity of *Shorea robusta* bark. Therefore, the present study has been undertaken to investigate the immunomodulatory activity of *Shorea robusta* bark on cell mediated and humoral immunity in the experimental animal models.

EXPERIMENTAL METHODS

Animals

Male Swiss albino mice weighing between 20 and 25g were used to evaluate immunomodulatory activity is against *Shorea robusta* bark. Four groups were maintained for the experiment. The animals were housed at standard conditions of temperature ($25 \pm 1^\circ\text{C}$), relative humidity ($55 \pm 10\%$), 12/12 h light/dark cycles in the institute's animal house with standard food and water *ad libitum*. Animals were divided into four groups (I–IV). Each group comprised of a minimum of six animals. Group I (control) received normal saline; Group II- Control Immunized; Group III- plant extract 100 mg/kg body weight; group III- plant extract 300 mg/kg. The treatment was started after the last dose of animals was immunized.

Plant material and preparation of extract

The plant bark of *Shorea robusta* was collected from Sengipatti, Thanjavur District in TN, India. The collected

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Shorea robusta bark was cut into small pieces and shade dried at room temperature. The *Shorea robusta* bark was soaked with ethanol (70%) for 48 hours. A semi solid hydro-alcoholic extract was obtained after complete elimination of alcohol under reduced pressure. The extract contained both polar and non-polar phytocomponents of the plant material used. The extract was stored in refrigerator until used. The *Shorea robusta* bark extract was dissolved in distilled water just before oral administration.

Dosage

The plant extract was suspended in normal saline and was administered orally for 14 days at dose of 100 mg and 300mg/kg body weight. The dose volume was 0.2 ml. Control animals received the same volume of normal saline.

Immunization:

The sheep red blood cells (SRBC) were used to immunize the animals, which were collected in a sterile Alsever's solution and washed thrice with pyrogen free normal saline and adjusted to 5×10^9 cells per ml. The animals were immunized, by injecting 20% (1 ml) SRBC intraperitoneally (i.p). The day of immunization was considered as day 0. On the 14th day, the blood samples were collected to carry out the immunological parameters.

ASSESSMENT OF IMMUNOMODULATORY ACTIVITY

Total Leukocyte Count (TLC): Blood sample was drawn and diluted with Turk's fluid in WBC pipette, in which red cells were lysed without affecting the leukocyte population. Leukocyte count was carried out by using an improved Neubauer's counting chamber (Srikumar et al., 2005).

Differential Leukocyte Count (DLC): Blood smear was made on a clean glass slide and stained with Leishman's stain. Among the hundred leukocytes counted, different population of leukocytes were differentiated and identified based on the cell size, presence of granules, size and colour of granules and shape of the nucleus under an oil immersion preparation (Srikumar et al., 2005).

Candida Phagocytosis:

The phagocytic ability of neutrophil was assessed by separating the buffy coat from the blood sample. To this, the incubating medium (0.1 ml of minimum essential medium (MEM), 0.1 ml of inactivated fetal calf serum and 0.1 ml of heat killed *Candida albicans* 2×10^8 cell/ml) was added and incubated at 37°C for 15 min, followed by centrifugation. From the sediment, thin smears were made and stained with Leishman's stain. The number of neutrophils positive for *candida* in 100 cells gives phagocytic index (PI). (Srikumar et al., 2005).

Nitroblue Tetrazolium (NBT) Reduction Test: The killing ability of the neutrophils was assessed by nitroblue tetrazolium reduction test (NBT). Briefly, the blood sample was incubated at 37°C for 30 min in a clean glass slide. After incubation, the slide was gently washed with cold saline to remove other cell populations. To this NBT medium (0.2 ml of 0.34% sucrose solution), (0.2 ml of 0.28% NBT) and 0.2 ml

of inactivated fetal calf serum) was added and incubated at 37°C for 30 min. After incubation, slide was washed with cold saline and stained with safranin. When NBT was phagosomes by the cells, intracellular dye is converted to an insoluble blue crystalline form (Formazon crystals). One hundred cells were observed and the positive cells with the formazon granules were counted (Srikumar et al., 2005).

Footpad thickness

Delayed-type hypersensitivity reactions, an in-vivo indicator of specific cell mediated immune responsiveness by T-cells are measured as footpad thickness in rats (Tewari et al., 1982). The day of immunization of the rats using 20% sheep red blood cells (SRBC) was considered as '0' day. Four days later the left hind footpad was challenged by an injection of 0.1ml of 50% SRBC suspended in normal saline. The right hind footpad was injected with 0.1 ml of saline. Twenty-four hours later (5th day) footpad was measured using a vernier caliper. The thickness of the saline injected footpad (Y) was subtracted from that of SRBC injected footpad (X) to determine DTH reaction ($DTH = (X - Y) / Y \times 100$). The value was reported as mean % of swelling.

Estimation of immunoglobulins

Immunoglobulins-IgA, IgG and IgM in serum were determined by single radial immunodiffusion (Mancini et al., 1965). Soluble immune complex was estimated by the method of Seth and Srinivas (Seth and Srinivas., 1981). This turbidometric assay was based on the precipitation of immune complex by low concentration of polyethylene glycol (PEG). Values were expressed as PEG index.

Hemagglutination assay

Antibody titer was estimated by the hemagglutination technique according to the method described by Puri et al. (1994). Blood samples were collected from individual animals by retro-orbital puncture. Two-fold dilutions of sera were performed in 0.15 mol/l Phosphate buffered saline (PBS) and were aliquoted into 96 well V-bottomed microtiter plates. Fresh 1% SRBC suspension in PBS was dispensed into each well and mixed thoroughly. The plates were incubated at room temperature for 2 h and examined visually for agglutination. The value of the highest dilution of the test serum giving agglutination was expressed as the hemagglutination titer.

Statistical analysis

One way analysis of variance (ANOVA) was used to compare the interaction between various experimental groups. Values were significant at $p < 0.001$; 0.01; 0.05. All the analysis was carried out using SPSS statistical package. Values are expressed as mean \pm SD for 6 rats in each group.

RESULTS

The current study revealed a alteration in immunological parameters in control immunized and evaluated the beneficial role of *Shorea robusta* bark in enhancing these vital functions (Table 1).

Phagocytic function is the primary mechanism through which the immune system eliminates most of the extracellular pathogens. Analysis of intracellular reduction capacity by NBT reduction test indicates an increase in NBT positive cells

Table 1 Immunomodulatory activity of *Shorea robusta* bark in experimental rats

Parameters	Control	Control Immunized	Plant (100/mg)	Plant (300/mg)
NBT-reduction (no. of positive cells/100 cells)	11.1±0.35	12.2±0.62	13.63±0.71***	14.7±0.81*
Phagocytic index (no. of positive cells/100 cells)	70±1.32	71.1±1.40	73.78±1.61	74.2±1.75***
DTH (% of swelling)	28.3±1.52	29.4±1.57	32.2±1.62***	33.3±1.73*
Immunoglobulin G (mg/dl)	237.5±2.74	240.2±3.14	247±3.68***	252±4.58*
Immunoglobulin A (mg/dl)	20.3±1.2	20.8±1.15	22.4±1.16	23.7±1.17**
Immunoglobulin M (mg/dl)	84.2±1.11	86±1.13	91.6±1.6***	94.3±1.72*
Hemagglutination assay (antibody titer)	74.7±1.5	74.9±1.67	85.3±1.79*	85.3±2.02*

Values are expressed as mean ± SD; one-way analysis of variance, ANOVA followed by Tukey– Multiple Comparisons Test (n = 6), Values are compared with control immunized animals. *P < 0.001; **P < 0.01; ***P < 0.05;

Table 2 Total and Differential count of leucocytes on *Shorea robusta* bark in experimental rats

Groups	Total count (TLC) (Cu.mm/ml)	Differential count (DLC) (%)				
		Neutrophil	Eosinophil	Monocyte	Basophil	Lymphocyte
Control	4628.33 ± 80.37	41.33 ± 2.04	1.43 ± 0.21	1.31 ± 0.32	1.12 ± 0.19	50.63 ± 1.82
Control Immunized	5313.30 ± 101.54	42.66 ± 0.80	0.93 ± 0.16	1.42 ± 0.41	1.76 ± 0.21	52.66 ± 2.15
Plant (100 mg)	5796.63 ± 137.60*	38.33 ± 2.92	1.76 ± 0.21	0.93 ± 0.34	0.60 ± 0.22**	58.83 ± 2.10***
Plant (300 mg)	5926.66 ± 182.56*	33.83±1.07**	2.43 ± 0.33*	0.90 ± 0.31	0.93 ± 0.16***	60.66 ± 0.49**

Values are expressed as mean ± SD; one-way analysis of variance, ANOVA followed by Tukey–Multiple Comparisons Test (n = 6), values are compared with control immunized animals. *P < 0.001; **P < 0.01; ***P < 0.05;

in control immunized animals than that of Group I rats. NBT positive cells markedly enhanced in *Shorea robusta* bark (100mg and 300mg respectively) treated animals when compared to the respective controls. A non-significant increase in phagocytic index was observed in Group II animals when compared to Group I animals. Supplementation of *Shorea robusta* bark markedly improved phagocytic index in 14 days treatment group when compared to immunized control. A non-significant increase in delayed-type response as assessed by footpad swelling was observed in Group II animals when compared to Group I animals. Administration of *Shorea robusta* bark improved delayed-type response in 14 days treated animals.

A non-significant increase in the concentrations of immunoglobulins IgG, IgA and IgM were observed in Group II animals on comparison with Group I animals. *Shorea robusta* bark (100mg and 300mg respectively) treatment increased the concentration of IgG in Group II animals. Administration of *Shorea robusta* bark for 14 days increased the concentration of IgA and IgM. Group II animals showed an increase in plaque forming cells and antibody titer when compared to control animals.

TLC and DLC

TLC count was significantly increased in a dose-dependent (100mg and 300mg respectively) manner. The lymphocyte count also increased but the results are more significant for group IV. From this study it was observed that the neutrophil and basophil populations were decreased for the extracts whereas the population of monocyte was not significantly changed in comparison with control (Table 2).

DISCUSSION

Recently phytopharmaceutical research received much attention in developing safe and effective lead compounds with potential immunomodulatory activities. Constant increase in antibiotic resistant strains of microorganisms has prompted scientists to look for herbal immunomodulators to treat various infections. The study of the immunomodulatory effects of medicinal plants on both cell mediated and humoral immune response is a matter of interest for many researchers. Several studies have previously demonstrated the immunomodulating effects of medicinal plants on lymphocyte proliferation in the presence of mitogen, allogenic cells and specific antigens (Summerfield and Saalmuller, 1998). Modulation of immune response through stimulation or suppression may help in maintaining a disease free state (Bafna and Mishra, 2005). In this present study, the immunomodulatory activity of ethanolic extract of *Shorea robusta* bark was investigated.

Many of the disorders today are based on the imbalances of immunological processes like DTH (cell mediated) reactions and humoral responses (Kanjiwani et al., 2008). DTH is a part of the process of graft rejection, tumour immunity and most important immunity to many intracellular infectious microorganisms, especially those causing chronic diseases viz tuberculosis (Elgert, 1996). Further, DTH requires the specific recognitions of a given antigen by activated T lymphocytes which subsequently proliferate and release cytokines. These in turn, increase vascular permeability, induce vasodilatation, macrophage accumulation (Descotes, 1999) and activations, promoting increased phagocytic activity and increased concentrations of lytic enzymes for more effective killing (Kuby, 1997). In the present study reaction was measured by foot-pad thickness. After 24h of antigenic challenge and

subsequent immunization with SRBC, the animal showed significant increase in volume of paw edema due to production of antibodies in response to the antigen. This potentiation of DTH response indicates that *Shorea robusta* bark has stimulatory effect on lymphocytes and accessory cell types required for the expression of the reaction and thus increases cell mediated immunity.

The index of humoral immune response is the increase in antibody titre value due to increase in immune response (Sehar, et al., 2008). The humoral immunity involves interaction of B-cells with the antigen and their subsequent proliferations and differentiations into antibody secreting plasma cells (Gokhale et al., 2003). Further, antibody functions as the effector of the humoral response by binding to antigen by neutralizing it or facilitating its eliminations by cross-linking to form clusters that are more readily ingested by phagocytic cells. To evaluate the effect of *Shorea robusta* bark on humoral response, its influence was tested on SRBC specific haemagglutination antibody titre in rats. *Shorea robusta* bark showed an enhanced production of circulating antibody titre. This augmentation of the humoral response to SRBC antigen by increase in haemagglutination antibody titre indicated the enhanced responsiveness of macrophages and T and B lymphocyte subsets involved in antibody synthesis (Benacerraf, 1978).

Phagocytic index generally increases whenever there is an increase in immune response and its effects are associated with varied pathologic conditions in humans (White & Gallin, 1986). Besides, phagocytosis represents an important innate defense mechanism against ingested particulates including whole pathogenic microorganisms (Atal, et al, 1986). The specialized cells that are capable of phagocytosis include blood monocytes, neutrophils and tissue macrophages. In a view of the pivotal role played by the macrophages in coordinating the processing and presentation of antigen to B-cells, *Shorea robusta* bark was evaluated for its effect on macrophage phagocytic activity. In this study, the increase in phagocytic index after treatment with *Shorea robusta* bark advocate effect of extract on macrophages and therefore on the subsequent stimulation of the immune system.

NBT reduction test relies on the generation of bactericidal enzymes (like NADPH-oxidase) in neutrophils during intracellular killing. These enzymes are necessary for normal intracellular killing against foreign antigens. During intracellular killing, the cellular oxygen consumption increases and glucose metabolism reduces the colorless NBT to blue formazan. *Shorea robusta* bark administrated group immunized with SRBC, showed increase in NBT reduction dose dependent manner. This may be due to increase in the bactericidal enzymes within the neutrophils (Srikumar et al., 2005).

Among the leukocytes, only antigen specific lymphocytes possess the diversity, specificity, memory and self-reorganization indicating an adaptive immune response (Goldsby, 2001). It was observed that *Shorea robusta* bark caused significant increase in TLC and lymphocyte population indicating the presence of immunological effects of *Shorea robusta* bark.

Humoral immunity involves interaction of B cells with antigens and their subsequent proliferation and differentiation into antibody-secreting plasma cells. Antibodies function as effectors of the humoral response by binding to antigens and neutralizing them or facilitating their elimination by cross-linking to form clusters that are more readily ingested by phagocytic cells (Gokhale et al., 2003). The estimation of serum immunoglobulin level is a direct measure to detect the humoral immunity. Serum immunoglobulin refers to a group of serum molecules produced by B-lymphocytes, they are soluble and secreted form of B-cell receptors and are produced to a maximum level to counter the invasion by an antigen, hence, they are also called as antibodies. Blood contains three types of globulins-alpha, beta and gamma, based on their electrophoretic migration rate. The concentrations of immunoglobulins IgG, IgA and IgM and serum soluble immune complexes were increased significantly in *Shorea robusta* bark treated animals when compared to controls. The increase in immunoglobulins observed after *Shorea robusta* bark supplementation in our study could be attributed to enhanced the antibody secreting cells by *Shorea robusta* bark. The reason of stimulation of antibody production by this drug may involve its effects on cytokines and cytokine producing cells like macrophages and T & B lymphocyte which are essential part of the humoral immune response activation.

In conclusion, this result provides primary evidence that hydro-alcoholic extracts of whole plant of *Shorea robusta* bark altered the total and differential WBCs count, potentiated the effect on cellular and humoral response and phagocytosis. Among the two extracts, 300mg/kg b.wt. possess potential immunomodulatory activity. The potential immunostimulating activity on the plant due to the presence of flavonoids, polyphenol and terpenoids in *Shorea robusta* bark. Thus the extract showed stimulation of defense system by modulating the immunological parameters and holds the promising therapeutic benefits of the plant parts on immunomodulation.

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