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Immunomodulatory Activity of *n*-Butanol Extract of *Oroxylum indicum*

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Abstract

The present study evaluated the immunomodulatory activity and mechanism of action of the *n*-butanol extract [100 mg/kg body weight (b.w.), per oral (p.o.)] of *Oroxylum indicum* Vent. (Bignoniaceae) root bark against different experimental animal models, i.e., immune response to sheep red blood cells in stress-induced immunosuppression, carbon clearance assay and neutrophil adhesion test. In the immune response to sheep red blood cell model, the *n*-butanol extract of *Oroxylum indicum* treatment group showed a significant rise ($P < 0.05$) in circulating antibody-titer as compared to the stress-induced control group. This observation suggests that the drug treatment caused potentiation of humoral antibody immune response to antigen. Also, it significantly decreases the rise in malondialdehyde content along with an increase ($p < 0.05$) in superoxide dismutase, catalase and reduced glutathione levels as compared to the stress-induced control group and, thus, exhibits significant antioxidant potential. Pretreatment with the *n*-butanol extract of *Oroxylum indicum* showed a significant rise ($P < 0.05$) in phagocytic index indicating phagocytic activity. In the neutrophil adhesion model, a significant increase ($P < 0.05$) in percentage neutrophil adhesion was observed in the *n*-butanol extract treatment group as compared to the control group. From these findings, the *n*-butanol extract of *Oroxylum indicum* possesses immunomodulatory activity by enhancing specific immune response (humoral immunity) and non-specific immune response (phagocytosis) of the body as well as exhibiting antioxidant potential. Our results suggest that the *n*-butanol extract of *Oroxylum indicum* root bark possesses a significant immunostimulant activity.

Keywords: Immunomodulation, *n*-butanol extract, O*roxylum indicum,* oxidative stress.

Introduction

Immunity is the state of resistance or insusceptibility exhibited by the host to toxic molecules, microorganisms, and foreign cells. The function and efficiency of the immune system might be influenced by many exogenous factors such as food, pharmaceuticals, physical and psychological stress, hormones, etc., resulting in either immunostimulation or immunosuppression. The immune system is known to be involved in the etiology as well as pathophysiologic mechanism of many diseases. Modulation of immune responses to alleviate disease has been of interest for many years and is a concept of Rasayana in Ayurveda. Medicinal plants have been used in various traditional systems and have immune potential against various diseases (Dahanukar et al., 2000). Plant origin immunomodulatory agents act by altering both non-specific and specific immunity (Atal et al., 1986). *Oroxylum indicum* Vent. (Bignoniaceae), is reportedly used as an astringent, carminative, diuretic, stomachic, has aphrodiastic properties, and is valued for stimulating digestion, curing fevers, coughs, and other respiratory disorders (Warrier et al., 2001). This plant is also one of the important ingredients in most commonly used Ayurvedic formulations such as Dasamula, Brahma Rasayana, Amartarista, Dantyadyarista, Dhanawantara Ghrita, Narayana Taila, etc. (Anonymous, 1998). The plant is reported to possess anti-inflammatory, diuretic, antiarthritic, antifungal, and antibacterial activities (Warrier et al., 2001). However, the root bark of *Oroxylum indicum* has not been thoroughly evaluated for its immunomodulatory activity. From our earlier data (Khandhar et al., 2006) examining the antiulcer and hepatoprotective activity of *Oroxylum indicum*, *n*-butanol extract of root bark (provided at 100 mg/kg body weight, per os, single dose and once daily for 7 days for respective activities) was found to be most potent at reducing gastric lesions and hepatotoxicity. A

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preliminary study showed that the *n*-butanol extract of *Oroxylum indicum* provided at 100 mg/kg body weight, per os, single dose and once daily for 7 days, was the most effective in preventing *Escherichia coli*-induced peritonitis in treated host. Therefore, the present study evaluated the immunomodulatory activity of a *n*-butanol extract (100 mg/kg, b.w., p.o.) of O*roxylum indicum* root bark using different experimental animal models.

Materials and Methods

Procurement of plant material and extraction procedure

The fresh root bark of *Oroxylum indicum* was collected from Van-Aaushadhi Ektrikaran Udyan, Ahwa, Dang Forest, Gujarat, India, in January 2002. The authentification of this plant was established by the taxonomist of Gujarat Ayurved University, Jamnagar, India, and a voucher specimen (404) was deposited in the Department of Pharmacognosy and Phytochemistry, L.M. College of Pharmacy, Ahmedabad, India (Khandhar et al., 2006). The root bark was dried and powdered to 60 (mesh). The powder of root bark was first defatted using petroleum ether (remaining post-extraction material constituted 0.32% w/w of the original solute (i.e., root bark powder that underwent the extraction)). The residual unextracted material was then air-dried; this defatted powder was then moistened with NH_3 solution and extracted with chloroform (post-extraction remaining material constituted 0.78% (w/w) of pre-extracted material). After drying, the remaining un-extracted material was then extracted with ethyl acetate (post-extraction remaining material = 1.52% w/w). Finally, extracted powder was extracted with *n*-butanol (post-extraction remaining material = 1.68% w/w). The solvent-specific eluents were recovered in each extraction regimen then air-dried and their corresponding powder extract was stored in air-tight borosilicate glass containers until usage.

Phytochemical analysis

Phytochemical analysis of the *n*-butanol extract was performed using standard methods. Specifically, the extract was analyzed for the presence of alkaloids (Sim, 1969), flavonoids (Geissman, 1955), saponins (Fischer, 1952), tannins (Robinson, 1964), and carbohydrates. Thin-layer chromatography was employed to check for the presence of baicalein. Quantification of aglucone was performed using reverse phase HPLC (Khandhar et al., 2006) and extrapolation against a standard curve generated using purified baicalein.

Drugs and chemicals

All different organic solvents were used for extraction under study were obtained from S.D. Chem. Pvt. Ltd. (Mumbai, India), Analytical Research (A.R.) grade. Fresh drug solution was prepared in 1% carboxymethyl cellulose (CMC) and administered orally. Hydrogen peroxide and Ciocalteau phenol reagents were obtained from S.D. Chem. Pvt. Ltd. Trichloroacetic acid, thiobarbituric acid, phosphate buffer, 5,5′-dithio*bis*-2-nitrobenzoic acid (DTNB), epinephrine, and dexamethasone were obtained from Sigma-Aldrich (St. Louis, MO). A pure sample of baicalein was purchased from Sigma-Aldrich Chemical Co. Spectrochem HPLC grade methanol, acetonitrile, orthophosphoric acid and water were used.

Animals

Wistar albino rats (Zydus Cadila Ltd., Ahmedabad, India) of either sex weighing 150–250 g as well as male albino mice weighing 30–40 g were selected for the study. Animals were provided a standard chow diet (certified Amrut brand rodent feed, Pranav Agro Industries, Pune, India) and filtered tap water, ad libitum. The rats were maintained under standard condition of a 12 h dark-light cycle, 60 ± 10% humidity and a temperature of 21.5 ± 1°C. Caprophagy was prevented by keeping the animals in cages with gratings on the floors. These experiments complied with the University guidelines for animal experimentation. The animals were killed with an overdose of ether anesthesia after the completion of the experiments.

Stress-induced immunosuppression

Stress-induced immunosuppression was induced as per method described previously with a slight modification according to Sen et al. (1992). Wistar albino rats of either sex 150–250 g were selected for the study. The animals were randomly divided into two groups of 6 animals each.

Group I. Control group: The animals received only aqueous suspension of 1% CMC as vehicle without any treatment once daily, for 5 days.

Group II. Drug treated animals: Animals received *n*-butanol extract (100 mg/kg, p.o.) once daily, for 5 days.

Animals were immunized with SRBC (0.5×10^9 cells/ml/100 g b.w.) on day 0. On day 7 they received the same dose of antigen that is SRBC. Immediately after the booster dose, the rats were food deprived (but not water) for 18 h and then subjected to restraint stress (RS) at room temperature (22 ± 2°C) in Plexiglas restrainers. After 24 h of restraint stress, rats were lightly anesthetized with ether. The blood was collected from the retro-orbital plexus using the microcapillary technique and following estimations were performed:

1. Antibody titer (Joharapukar et al., 2003);
2. Oxidative stress parameters, i.e., lipid peroxidation, superoxide dismutase, catalase and reduced glutathione.

The effect of drug administration on antioxidant enzymes (i.e., reflected by antioxidant enzymes and lipid peroxide level) was assessed in the blood of the rats, which was withdrawn from the retro-orbital plexus on day 22 of the treatment regimen. The effect of administration of *n*-butanol extract of *Oroxylum indicum* root bark on antioxidant enzymes, i.e., superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) was assayed by the method of Mishra and Frodvich (1973), Aebi et al. (1974), and Beutler et al. (1963), respectively. The level of melondialdehyde (MDA) was estimated (μmoles/mg of protein) in terms of thiobarbituric acid reactive substances (TBARS) at 535 nm (Shimandzu UV Spectrophotometer) as described by Kiso et al. (1984).

Carbon clearance assay

Carbon clearance assay was done as per the method described previously by Sugiura et al. (1998). Male albino mice of 30–40 g were selected for the study. The animals were randomly divided into two groups of 6 animals each.

Group I. Control group: The animals received only aqueous suspension of 1% CMC as vehicle without any treatment once daily for 5 days.
Group II. Drug treated animals: Animals received *n*-butanol extract (100 mg/kg, p.o.) once daily for 5 days.

On day 6 each group was injected (intravenously) with 0.1 ml/10 g body weight of colloidal carbon. The blood was collected (20 μl) from the retro-orbital plexus at 5 and 10 min after the injection. Immediately after collection the blood was mixed with 1% acetic acid solution. Optical density of the mixture was measured at 660 nm with a spectrophotometer. The phagocytic index was calculated according to the following equation:

$$\text{Phagocytic index} = (\log\ \text{ODt5} - \log \text{ODt10})/(\text{t10} - \text{t5})$$

where ODt5 = Optical density at 5 min after the injection of carbon solution, ODt10 = Optical density at 10 min after the injection of carbon solution, t5 = 5 min, t10 = 10 min.

Neutrophil adhesion test

Neutrophil adhesion test was performed as per method described previously by Wilkonson (1978). Wistar albino rats of either sex weighing 150–250 g were selected for the study. The animals were randomly divided into two groups of 6 animals each.

Group I. Control group: The animals received only aqueous suspension of 1% CMC as vehicle without any treatment once daily, for 14 days.
Group II. Drug treated animals: Animals received *n*-butanol extract (100 mg/kg, p.o.) once daily, for 14 days.

On day 14 of drug treatment the blood was collected by puncturing the retro-orbital plexus into EDTA vials and it was analyzed for total leucocyte counts (TLC) and differential leucocyte counts (DLC) by Sysmax 3000, Japan. After initial counts, blood samples were incubated with 80 mg/ml of nylon fibers for 15 min at 37°C. The incubated blood samples were again analyzed for TLC and DLC. The product of TLC and % neutrophil gives neutrophil index (NI) of blood sample, NI = (TLC × % neutrophil).

Percentage neutrophil adhesion was calculated as shown below:

$$\text{Neutrophil adhesion}\,(\%) = \frac{(\text{NI}_\text{u} - \text{NI}_\text{t})}{\text{NI}_\text{u}} \times 100$$

where NI_u = NI of untreated blood sample (control), NI_t = NI of treated blood sample (*n*-butanol; 100mg/kg, p.o.).

Statistical analysis

The results were expressed as Mean ± SEM. The significance of difference between mean values for the various treatments was tested using Student's *t*-test. One way analysis of variance (ANOVA) followed by Tukey's multiple range test (Bolton, 1997) was used wherever applicable to assess statistical significance of difference between groups. P value less than 0.05 ($P < 0.05$) was considered as statistically significant.

Results

Phytochemical analysis

On preliminary phytochemical screening, the *n*-butanol extract of *Oroxylum indicum* showed the presence of alkaloids, tannins, flavonoids, and anthraquinones. Thin-layer chromatography was specifically employed to check the presence of a single flavonoid, baicalein. Reverse-phase HPLC analysis was used to quantify the baicalein present in the extract and the result showed that the *n*-butanol extract contained 11.56% w/v baicalein (Khandhar et al., 2006).

Stress induced immunosuppression

In this model, in rats exposed to restraint stress (RS) the antibody titer was significantly lower than that of non-restraint stress group. Restraint stress prevented the antibody response to the booster SRBC injection. Pretreatment with *n*-butanol extract clearly attenuated this effect of RS on the secondary antibody response (Table 1).

Antioxidant activity

In the present study, pretreatment with *n*-butanol extract significantly reversed the rise in MDA content along with significant rise in SOD, CAT, and reduced GSH levels as

Table 1. Effect of *n*-butanol fraction (100 mg/kg; p.o.) of *Oroxylum indicum* root bark on antibody formation in SRBC sensitized rats on stress-induced immunomodulatory activity.

Group	Antibody titer
Control	3.01 ± 0.34
n-Butanol	5.08 ± 0.49*

All values represent mean ± SEM, n = 6 in each group. $^{*}P < 0.05$, when compared with control group (unpaired Student's *t*-test).

Table 3. Effect of *n*-butanol fraction (100 mg/kg, p.o.) of *Oroxylum indicum* root bark on phagocytic activity as determined by carbon clearance method.

Group	Phagocytic index
Control	0.03 ± 0.01
n-Butanol	0.12 ± 0.03*

All values represent mean ± SEM, n = 6 in each group. $^{*}P < 0.05$, when compared with control group (unpaired Student's *t*-test).

compared to the stress-induced control group. Thus, the antioxidant potential of *n*-butanol extract of *Oroxylum indicum* was confirmed, which prevented the changes of stress-induced oxidative stress. Thus, the *n*-butanol extract of *Oroxylum indicum* attenuated the stress-induced immunosuppression through antioxidant mechanism (Table 2).

Carbon clearance assay

In the present study the rate of carbon clearance is a measure of phagocytic activity which was determined from the phagocytic index parameter. Pretreatment with *n*-butanol extract of *Oroxylum indicum* showed significant ($p < 0.05$) rise in phagocytic index (Table 3).

Neutrophil adhesion test

The result of the neutrophil adhesion test is shown in Table 4. Pretreatment with *n*-butanol extract (100 mg/kg) showed significant increase in % NA ($p < 0.05$) when compared with the control group.

Discussion

Phytochemical analysis of the *n*-butanol extract of *Oroxylum indicum* root bark has previously revealed the presence of alkaloids, tannins, flavonoids, and anthraquinones. In the present study, preliminary screening using thin-layer chromatographic technique revealed the presence of the flavonoid baicalein, an agent reported to possess immunomodulatory activity (Lien et al., 2003), in a *n*-butanol extract prepared from the root bark of *Oroxylum indicum.* Furthermore, reverse-phase HPLC analysis was performed to develop a complete chemo profile and to quantify the baicalein in the active fraction; the result indicated a significant quantity of baicalein in *n*-butanol extract. Therefore, any results in the current study that demonstrate broad immunomodulatory activities of this *n*-butanol fraction might be attributed to the presence of baicalein in the *Oroxylum indicum* root bark.

The immune system is known to be involved in the etiology as well as pathophysiological mechanisms of many diseases. Immunological defense is complicated by interplay between non-specific and specific, cellular and humoral immune responses, stimulation, or suppression of immunocompetent cells, and influence of endocrine and other mechanisms upon the immune system. Different agents of plant origin and their polyherbal formulations were reported to interact with the immune system in a complex way and modulated the pathophysiological processes (Atal et al., 1986).

In the present study, the stress-induced immunosuppression model has been used to investigate the effects of *n*-butanol extract (100 mg/kg; p.o.) of *Oroxylum indicum* root bark. Stress is known to break down the adaptive/coping mechanisms by disturbing the physiological homeostasis of the organism and these result in several autoimmune, endocrinal, and visceral disorders. Immunological responsiveness to antigens is known to be modified during restrain-stress (RS). During RS, plasma corticosteroids and norepinephrine levels are elevated (Borodin et al., 1985; Armario et al., 1986; Roth et al., 1988). Few reports reveal that the central depletion of catecholamine inhibits the humoral antibody response only to T-dependent antigen SRBC (Cross & Rozman, 1988). In rats immunized with SRBC, a booster injection of SRBC causes marked higher anti-SRBC antibody response, than seen in primary antibody response. However, in rats exposed to RS, the anti-SRBC antibody titer is significantly lower than the non-RS group, which means RS prevents the antibody response to the booster SRBC injection. In the present study, *n*-butanol

Table 2. Effect of *n*-butanol fraction (100 mg/kg; p.o.) of *Oroxylum indicum* root bark on lipid peroxidation and antioxidant enzymes on stress-induced immunomodulatory activity.

Group	MDA content (μmole/mg protein)	SOD (munits/mg protein)	CAT (units/min/mg protein)	GSH (μmole/mg protein)
Stress-control	1.060 ± 0.082	0.046 ± 0.001	0.280 ± 0.039	0.014 ± 0.001
n-Butanol	0.650 ± 0.045*	0.068 ± 0.002*	0.623 ± 0.006*	0.043 ± 0.011*

All values represent mean ± SEM, n = 6 in each group. $^{*}P < 0.05$, when compared with the control group (unpaired Student's *t*-test).

Table 4. Effect of *n*-butanol fraction (100 mg/kg, p.o.) of *Oroxylum indicum* root bark on neutrophil adhesion.

Group	% NA
Control	24.22 ± 3.03
n-Butanol	46.20 ± 1.33*

All values represent mean ± SEM, n = 6 in each group. *$P < 0.05$, when compared with control group (unpaired Student's *t*-test).

extract of *Oroxylum indicum* under the study attenuated the stress-induced suppression of antibody response to booster SRBC injection and caused rise in anti-SRBC antibody titer.

During stress, large amount of free radicals are generated which cause oxidative stress and immunosuppression. Free radicals induce lipid peroxidation and cause a rise in MDA content. The MDA content is a representative marker of oxidative stress and correlates with stress-induced immunosuppression. Pretreatment with *n*-butanol extract of *Oroxylum indicum* Vent. significantly reduced the stress-induced rise in MDA content, and at the same time increased ($P < 0.05$) the level of SOD, CAT and GSH. Thus, antioxidant potential of *Oroxylum indicum* is confirmed, and this prevented the changes of stress-induced oxidative stress parameters. From these observations it is suggested the *n*-butanol extract of *Oroxylum indicum* attenuated the stress-induced immunosuppression through antioxidant mechanism.

Various *in vitro* and *in vivo* models of phagocytosis have been used to screen the immune response. The carbon clearance assay reveals the effect of phagocytosis with *in vivo* experiments. In this assay, the enhanced phagocytic activity of macrophages is evaluated in terms of the rate of elimination of exogenously administered antigens such as carbon particles. The rate of carbon clearance is a measure of phagocytic activity, and it is determined from the phagocytic index parameter. The phagocytosis of pathogens by macrophages initiates the innate immune response (Aderem & Underhill, 1988). It is believed that the primary target of most of the immunomodulatory agents is macrophages. In the present study, the *n*-butanol extract of *Oroxylum indicum* caused a significant rise in the phagocytic index which in turn showed a significant rise in carbon clearance. This revealed that the *n*-butanol extract of *Oroxylum indicum* activated the reticuloendothelial system (RES) and enhanced phagocytic activity of monocytes and macrophages. This might give the host defense ability to counter the infectious stress.

Immunomodulatory agents of plant origin exert beneficial effects in the diseased state by enhancing non-specific and specific status of immune system. During non-specific defense status, the chemotactic chemicals cause vasodilatation and increase vascular permeability of walls of small blood vessels. This causes exudation of the fluid from the blood which gets accumulated in the interstitial space of connective tissues. Exudation of fluid increases blood viscosity and slows the rate of blood-flow. The chemotactic chemicals also attract the leukocytes, and the neutrophils are the first to arrive at the site of inflammation, whereas, leukocytes stick to the walls of small blood vessels. Due to increased permeability of capillaries, neutrophils and monocytes are able to squeeze through the tiny gaps between adjacent endothelial cells in the capillary wall and enter the interstitial space of connective tissues by amoeboid movement. This whole process is called diapedesis. The adhesion of neutrophils to nylon fibers represents the process of diapedesis. Neutrophil adhesion to nylon fibers was significantly increased in *n*-butanol extract-treated animals. This suggests the *n*-butanol extract of *Oroxylum indicum* stimulates migration of neutrophils in the blood vessels and thereby exerts immunomodulatory activity.

In summary, the results of these studies have demonstrated the potential effects of the *n*-butanol extract of *Oroxylum indicum* root bark on immune regulation. These findings confirm earlier ones regarding the general immunopotentiating role of this plant extract. These studies have also revealed the possible mechanisms underlying the immunomodulating activity of this active fraction. Specifically, the effects of *n*-butanol extract of *Oroxylum indicum* can be attributed to enhancement of specific immune responses, i.e., humoral immunity, antioxidant defense mechanism via attenuating stress-induced immunosuppression and stimulation of non-specific immune response, i.e., phagocytosis. Based on the phytochemical analyses, the effect of *n*-butanol extract of *Oroxylum indicum* on immune regulation might be associated with the presence of baicalein, a major flavonoid found in the *n*-butanol extract of *Oroxylum indicum*. Hence, the present study highlights the effect of *Oroxylum indicum* on immunocyte function not only in immunocompromised conditions but also during basal condition. Follow-up studies using the flavonoid alone or extracts that will selectively contain this agent, will help further to define precisely how and at what dose level this active agent exerts its effects in treated hosts.

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