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IN-VITRO & IN-VIVO EVALUATION OF IMMUNOMODULATORY ACTIVITY OF FLAVANOIDAL CONSTITUENTS FROM BERBERIS VULGARIS ROOT

Pradeep Kumar.Challa¹, Narsimha Reddy. Yellu*²

¹Department of Pharmacology, Vaageswari College of Pharmacy, Near L.M.D colony, Karimnagar -505481, Andhrapradesh, India.

²Department of Pharmacy, University College of Pharmaceutical Sciences, Hanamkonda, Warangal. 506001.

Email: ynrku@yahoo.co.in

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Abstract

To study the immunomodulatory activity of aqueous and ethanol extracts of roots of *Berberis vulgaris* (Family: Berberidacea) on Albino rats. The ethanolic and aqueous extract of roots of *Berberis vulgaris* was administered orally at the dosage levels of 200 mg/kg/day and 400 mg/kg/day to Azathioprine induced rats. The assessment of immunomodulatory activity was studied by delayed type hypersensitivity, neutrophil adhesion test and evaluation of Immunoprophylactic effect in rats. *Berberis vulgaris* showed a significant ($p < 0.001$) increase in the neutrophil count, upon treatment with aqueous and ethanolic extracts of the root, compared to control. In response to sheep red blood cells (SRBCs). *Berberis vulgaris* showed significantly ($p < 0.05$) decreased delayed type hypersensitivity reaction which is due to decreased inflammatory response response to SRBCs in sensitized rats. Immunoprophylactic effect exhibited significant macrophage stimulation and 14 to 50% mortality as compared to control rats. The study demonstrates that *Berberis vulgaris* produced a significant immunostimulatory effect compared to controls. Hence, it holds promise for used as an immunostimulating agent.

Keywords: *Berberis vulgaris*, Humoral immunity, cell-mediated immunity, Hypersensitivity, Flavanoids.

1. Introduction

The ability of the body to defend itself against specific invading agents such as bacteria, toxins, viruses and foreign tissue is called specific resistance (or) immunity. Immunogenicity is the ability to response by stimulating the production of specific antibodies, the proliferation of specific Tcells. Antigens are the substances that have reactivity; substances with both immunogenicity and reactivity are considered complete antigens. The small parts of antigen molecules that initiate immune responses are called Epitopes (or) Antigen determinants. This induces production of antibodies (or) activates a specific Tcells. ^[1]

Determination of antigen nature is by Size, chemical nature, Foreignness, Antigenic specificity, Species specificity, Isospecificity, Auto specificity, Organ specificity, Heterogenetic (Heterophile) specificity. Antigens are classified in to two types T cell dependant (TD) and T cell independent (TI) antigens. Some antigens can directly stimulate antibody production by B cells, without the apparent participation of T cells; such antigens are called TI antigens. Others that require T cell participation to generate an immune response are called TD antigens. [2]

An antibody, also known as an immunoglobulin, an antibody (Ab) can combine specifically with the epitope on the antigen that triggered its production. Immunity is classified in to different types they are innate immunity and acquired immunity. Innate immunity or native immunity is the resistance to infections that an individual possesses by virtue of his or her genetic and constitutional make-up. It is not affected by prior contact with microorganisms or immunisation. It may be nonspecific, when it indicates a degree of resistance to infections in general, or specific where resistance to a particular pathogen is concerned. Innate immunity may be considered at the level of the species, race or individual. [2]

The resistance that an individual acquires during life is known as acquired immunity, as distinct from inborn innate immunity. Acquired immunity is of two types active and passive. Active immunity is the resistance developed by an individual as a result of an antigenic stimulus. Artificial active immunity is the resistance induced by vaccines.

Vaccines are preparations of live or killed microorganisms or their products used for immunization. Passive immunity is the resistance that is transmitted passively to a recipient in a 'readymade' form is known as passive immunity. Here the recipient's immune system plays no active role. [2]

Immunostimulants, also known as immunostimulators, are substances that stimulate the immune system by inducing activation or increasing activity of any of its components. (Example is the granulocyte macrophage colony-stimulating factor). In recent days, lot of medicine, chemicals as well as natural products have been introduced in order to stimulate the non-specific defense mechanism as well as specific immune responses if the treatment is followed by infection or vaccination these are termed as immunostimulants. Traditional Indian system of medicine like Siddha and Ayurveda has suggested means to increase the body's natural resistance to disease. [3] A number of plants used in the traditional medical system of remedies in India. They have been shown to possess immunostimulating activity acting at different levels of the immunesystem.

Berberis vulgaris has ability to modulate humoral immune responses by acting at various levels in immune mechanism such as antibody production, release of mediators of hypersensitivity reactions and tissue responses to

these mediators in the target organs. Medicinal applications this plant are cognitive activities, hypotensive and antinephritic effect, immunostimulant effects, immunostimulant effects, antifungal activity, Antiallergic, anti-inflammatory effect, antibacterial. All parts of the tree are used medicinally, insomnia, it is mild sedative and it increases immune-system resistance. [5]

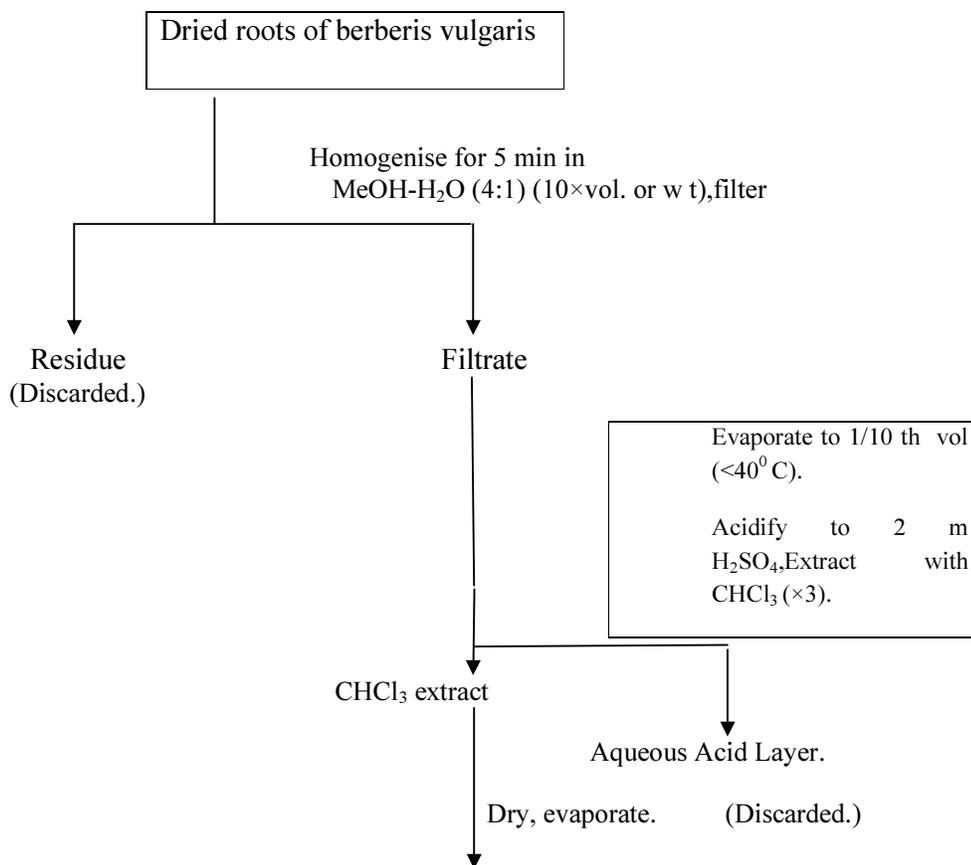
2. Materials and Methods

Chemicals: All the drugs and chemicals were of analytical grade while the other drugs were procured from Levamisole, Azathioprine (B&T Pharma, Hyderabad), *Escherichia coli* (IM Technology, Chandigarh, No.732).

Plant material: The dried roots of *berberis vulgaris* used in this study, collected at the fruiting stage (Month: January) from local area of Karimnagar, Andhrapradesh, India

Extraction

The dried roots of *berberis vulgaris* were cleaned, shade dried and coarsely powdered mechanically. The powdered material was soaked in 70% aqueous-methanol for 3 days with occasional shaking. It was filtered through a muslin cloth and then through a filter paper. This procedure was repeated thrice and the combined filtrate was evaporated on a rotary evaporator under reduced pressure to a thick, semi-solid mass of dark brown colour, i.e. the crude extract.



Flavanoidal content of dried roots of *Berberis vulgaris*

The roots were collected and dried on filter paper sheets under shade at room temperature. Roots were dried for 1 month. This powdered material was kept for ethanolic extraction in Soxhlet apparatus for 18 hours.

The powdered material was weighed about 50 mg it was dissolved in 150 ml of ethanol (1:3 ratio) and ethanolic extraction was done in Soxhlet apparatus. The extract which was formed in round bottom flask, this ethanolic extract were evaporated under reduced pressure at room temperature (30°C) to dryness to yield yellowish brown color extract of *berberis vulgaris* stored in an airtight container for further experimental studies. [6]

The dilution of aqueous extract of roots of *Berberis vulgaris* was prepared in distilled water and the aqueous suspension of ethanolic extract of roots of *Berberis vulgaris* was prepared in distilled water prior to oral administration to animals. [7]

Animals

Albino rats of either sex weighing between 120-180 g purchased from Mahaveer Enterprise, Hyderabad was used. Institutional Animal Ethics Committee approved the experimental protocol; The animals were maintained under standard conditions of temperature (23 ± 2°C), humidity (35-60%), and 12:12 light and dark cycle throughout the period of experimental study approved by Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA). The animals were given pellet food and water *ad libitum*.

Acute toxicity study: Acute toxicity studies were performed using male Wistar rats. The animals were fasted overnight prior to the experiment and maintained under standard laboratory conditions.) Zizyphus jujube extract was administered orally using various doses up to 2000 mg/kg and observed for the mortality and behavioural changes (OECD, 2000).

Experimentation on the animals were conducted as ethical guidelines and been approved by institutional ethical committee by no. IAEC.VCP/2011/10/1/9

Test compound formulations: The dilution of aqueous extract of roots of *Berberis vulgaris* (AEZJ) was prepared in distilled water and the aqueous suspension of Methanolic extract of roots of *Berberis vulgaris* (MEZJ) was prepared in distilled water prior to oral administration to animals. It was used within 7 days and stored at 8°C while for further use, freshly prepared solution was used. The vehicle alone served as control. [6]

Fresh culture preparation: Nutrient broth was prepared by 1.3 gms of agar was dissolved in 100 ml of distilled water. Then this solution was sterilized under autoclave for 2 hours and cooled. Culture of *Escherichia coli* was transferred in to this sterilized medium under aseptic conditions in laminar air flow chamber. It was taken in to

rotary shaker for 18hours to increase the growth of organisms in that sterilized medium. This will show “log phase” and rapid growth of micro organisms in the culture medium. 100ml of distilled water was taken it was kept in autoclave for 1hour at 15lb pressure. Then 0.5gms of phosphate buffer solution (PBS) was transferred in to sterilized water under aseptic conditions in laminar air flow chamber by this 0.5% of phosphate buffer solution was prepared. In this solution 0.5ml of *Escherichia coli* was added a dose of 0.5ml/100gm body weight was used. [8]

Antigenic material

Preparation of Sheep RBCs (SRBCs): Induction

Sheep blood was collected in sterile Alsever’s solution in 1:2 proportion of Alsever’s solution (freshly prepared). Blood was kept in the refrigerator and processed, for the preparation of Sheep RBCs batch, by centrifugating at 2000 rpm for 10 minutes and washing with physiological saline 4-5 times and then suspending into buffered saline for further use. [9]

Preparation of Alsever’s solution

Composition:

Dextrose: 2.05 gm

Sodium citrate: 0.8 gm

Sodium chloride: 0.4 gm

Citric acid: 0.05 gm

All the ingredients were weighed and dissolved in 100ml of distilled water. Alsever’s solution was used in the proportion of 1:2 (Sheep blood: Alsever’s solution) for washing sheep blood. [10]

3. Experimental Design

Induction of Immunomodulatory

Azathioprine is an immune suppressive agent. 1mg/ml of azathioprine solution was administered to azathioprine control for 1 day and for and treatment for 22 days and then *Berberis vulgaris* extract administered and on the same day blood samples were collected for estimate of haematological and serological tests (Hesham A et al., 2010).

Albino wister rats weighing 140-200g were maintained in animal house and they were divided in to 5 groups of 6 animals in each. The weight range of the animals was equally distributed throughout the groups. They were acclimatized to housing conditions for at least one week prior to use. The animals of

Group 1: Rats served as normal control group.

Group2: Azathioprine intoxicated rats received I.P with a single dose of Azathioprine.

Group 3: Rats treated with *Berberis vulgaris* plant extract at a dose 100mg/kg (low dose).

Group 4: Rats treated with *Berberis vulgaris* plant extract at a dose of 200mg/kg (high dose).

Group 5: Rats treated with Levamisole (standard drug) at 100mg/kg p.o at day 1-22 respectively.

Neutrophil adhesion test ^[11]

The rats were divided into four groups consisting of six animals each. Rats in group I received control only for 14 days. Rats in treatment group II and III were given aqueous extract of roots of *Berberis vulgaris*(400 mg/kg/day/p.o) and ethanolic extract of roots of *Berberis vulgaris*(200 mg/kg/day/per oral) daily for 14 days respectively. Group IV received Levamisole (50 mg/kg/per oral) for 14 days respectively. On the 14th day of the treatment, blood samples from all the groups were collected by puncturing retro-orbital plexus under mild ether anesthesia. Blood was collected and analyzed for total leukocyte count (TLC) and differential leukocyte count (DLC) by fixing blood smears and staining with Leishman's stain. After initial counts, blood samples were incubated with nylon fiber (80 mg/ml of blood sample) for 15 min at 37⁰C. The incubated blood samples were again analyzed for TLC and DLC. The product of TLC and % neutrophil gives neutrophil index (NI) of blood sample.

Percentage of neutrophil adhesion was calculated as follows,

$$\text{Neutrophil adhesion} = \frac{\text{NI}_U - \text{NI}_T}{\text{NI}_U} \times 100$$

NI_U

Where,

NI_U: Neutrophil Index before incubation with nylon fiber.

NI_T : Neutrophil Index after incubation with nylon fiber.

Delayed type hypersensitivity (DTH) response ^[12]

The rats were divided into four groups consisting of six animals each. Rats in group I received control only for 21 days. Rats in treatment group II and III were given aqueous extract of roots of *Berberis vulgaris*(400 mg/kg/day/per oral) and ethanolic extract of roots of *Berberis vulgaris*(200 mg/kg/day/per oral) daily for 21 days respectively. Group IV received Levamisole (50 mg/kg/per oral) for 21 days respectively. On 14th day of the study, all the groups of I, IV were immunized with Sheep RBCs (0.1ml of 20% Sheep RBCs intra peritoneal) in normal saline. On day 21st all animals from all the groups were challenged with 0.03 ml of 20% Sheep RBCs in sub plantar region of right hind paw foot pad edema in rat was used for detection of cellular immune response.

On 21st day, injection of 0.1ml of 20% SRBCs in the sub plantar region of right hind paw in the volume of 0.03 ml and normal saline in left hind paw in same volume. Foot pad reaction was assessed after 24hours on 22nd day, in terms of increase in the thickness of footpad as a result of hypersensitivity reaction due to edema; the thickness of the right hind footpad was measured using Digital plethysmometer. The footpad reaction was expressed as the difference in the thickness (millimeter) between the right foot pad injected with Sheep RBCs and the left footpad injected with normal saline.

Evaluation of immunoprophylactic effect

The rats were divided into three groups consisting of six animals each. Rats in group I received control only for 15 days. Rats in treatment group II and III were given aqueous extract of roots of *Berberis vulgaris*(400 mg/kg/day/p.o) and ethanolic extract of roots of *Berberis vulgaris*(200 mg/kg/day/per oral) daily for 15 days respectively. On 15th day, 3hr. after the last dose of aqueous extract and ethanolic extract *Escherichia coli* (0.5ml/100gm body weight, intra peritoneal) was injected to control, aqueous and ethanolic groups of rats and percentage of mortality was observed after 24hours. [8]

Statistical Analysis

All the results were expressed as Mean±Standard deviation (SD). Data were analyzed using one-way Analysis of Variance (ANOVA) followed by Tukey-Kramer multiple comparison test. P-values <0.01 were considered as statistically significant.

3. Results

Neutrophil adhesion test

Effect of aqueous extract of roots of *Berberis vulgaris* and ethanolic extract of roots of *Berberis vulgaris* on neutrophil activation by the neutrophil adhesion test is shown in [Table1]. Cytokines are secreted by activated immune cells for margination and extravasation of the phagocytes mainly polymorphonuclear neutrophils. The percentage neutrophil adhesion was significantly ($p < 0.001$) increased by aqueous extract of roots of *Berberis vulgaris*(400 mg/kg/per oral) and ethanolic extract of roots of *Berberis vulgaris*(200 mg/kg/per oral) when compared with the control group, showed possible immunostimulant effect. *Berberis vulgaris* significantly evoked increase in the adhesion of neutrophils to nylon fibers which correlates to the process of margination of cells in blood vessels.

Table-1: Effect of *Berberis vulgaris* treatment on neutrophils activation by neutrophil adhesion test.

Treatment	% Neutrophil Adhesion
Control	45.72±9.81***
Aqueous extract of roots of <i>Berberis vulgaris</i>	65.98±6.21***
Ethanollic extract of roots of <i>Berberis vulgaris</i>	62.68±6.03***
Levamisole standard	67.57±7.41***

Values are expressed as (Mean ± SD), n= 6, ***p<0.001. All groups were compared with control group. Statistically analyzed by one- way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison tests. [6]

Delayed Type Hypersensitivity

Effect of aqueous extract of roots of *Berberis vulgaris*(400 mg/kg/per oral) and ethanolic extract of roots of *Berberis vulgaris*(200 mg/kg/per oral) on cell mediated immune response by delayed type hypersensitivity decreased footpad edema is shown in [Table 2]. On twenty first day all treated groups showed significant (p<0.05) decrease in the mean difference of paw thickness when compared to control group.

Table-2: Effect of *Berberis vulgaris* treatment on cell mediated immune response by delayed type hypersensitivity induced footpad edema.

Treatment	Mean diff.of paw edema in (cm)
Control	0.7±0.187*
Aqueous extract of roots of <i>Berberis vulgaris</i>	0.46±0.103*
Ethanollic extract of roots of <i>Berberis vulgaris</i>	0.43±0.121*
Levamisole standard	0.48±0.116*

Values are expressed as (Mean \pm SD), n= 6, *p<0.05. All groups were compared with control group. Statistically analyzed by one- way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison tests. [6]

Evaluation of immunoprophylactic effect

Table-3: Effect of aqueous and ethanolic extract of *berberis vulgaris* on *Escherichia coli* induced in albino rats.

S.NO	Total number of animals died	Protection
Control	6/6	—
Aqueous extract of roots of <i>Berberis vulgaris</i>	1/6	84%
Ethanolic extract of roots of <i>Berberis vulgaris</i>	3/6	50%

Immunoprophylactic effect was significantly enhanced in animals treated with the aqueous and ethanolic extracts before administering *Escherichia coli*. Animals treated with the *Berberis vulgaris* aqueous and ethanolic extracts in rats, showed only 14 to 50% mortality in comparison to 100% mortality in control rats. [8]

4. Discussion

Immunomodulation is a procedure which can alter the immune system of an organism by interfering with its functions, if it results in an enhancement of immune reactions it is named as an immunostimulative drug which primarily implies stimulation of specific and non-specific system, i.e. granulocytes, macrophages, T-lymphocytes. [6]

Generally, Cytokines are secreted by activated immune cells in order for the margination and extravasation of the phagocytes mainly polymorphonuclear neutrophils. A Significant increase in the adhesion of neutrophils to nylon fibers correlates to the process of margination of cells in blood vessels. In this present study, *Berberis vulgaris* showed a significant increase in the neutrophil count, upon treatment with aqueous and ethanolic extracts of the root, compared to control. This may be helpful in increasing immunity of body against microbial infections. [12]

In general cell mediated immunity involves antigen entry and this antigen engulfed by macrophages. Proteins in macrophages combines with antigen, antigen with macrophage proteins presents on macrophage surface then T_H cells recognizes antigen present on macrophage and binds to it, and gets activated. Activated T_H cells release

cytokines (gives to macrophages), and gets itself multiply to T_K cells, macrophages with those chemicals activates cytotoxic T cells (kill T cells) cytotoxic cells attacks on target cells this leads to cell lyses. [13]

Cytokines in turn increase vascular permeability, induce vasodilatation, macrophage accumulation, and activation, promoting increased phagocytic activity and increased concentrations of lytic enzymes for more effective killing. When activated T_{HI} cells encounter certain antigens, with Sheep RBCs. They secrete cytokines that induce a localized inflammatory reaction called delayed type hypersensitivity. [13]

From the present study the results showed that there was a decreased paw edema which is due to decreased inflammatory response up on treatment with aqueous, ethanolic extract of *Berberis vulgaris* compared to standard drug Levamisole.

The total aqueous and ethanolic extract of roots showed a significant immunostimulatory activity *Escherichia coli* treatment caused 100% mortality of the animals, due to abdominal peritonitis in untreated mice and extract treated mice. However, treatment with *Berberis vulgaris* reduced the mortality to 14 to 50% showing *Berberis vulgaris* has immunostimulatory activity. [8]

5. Conclusion

The results of the present study, it can be concluded that, *Berberis vulgaris* produced a significant immunostimulatory effect compared to controls. Hence, it holds promise for used as an immunostimulating agent. A further study has to be conducted as there a need for isolation of individual constituents from the extracts to get more precision over studies.

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Corresponding Author:

Narsimha Reddy, Yellu*

Email: naraasimhareddy@gmail.com