IMMUNOMODULATORY ACTIVITY OF ETHANOLIC EXTRACT OF *DODONAEA VISCOSA* L.F.

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Summary

The immunomodulatory activity of an Indian medicinal plant i.e. ethanolic extract from *Dodonaea viscosa* L.F. namely DV was studied for there phagocytic activity, cell mediated and humoral immune system on rat/mouse. Immunomodulatory effect was assessed in carbon clearance test, delayed type of hypersensitivity (DTH), T-cell population test, and sheep erythrocyte agglutination test (SEAT) in animal treated with DV at doses of 200 and 400 mg/kg. In carbon clearance test, *D.viscosa* exhibited significantly high phagocytic index against control group, indicating stimulation of the reticulo-endothelial system. Significant decrease in mean difference, in the foot paw thickness in DTH indicates its anti-inflammatory activity. In SEAT *D. viscosa* treated groups at 200, 400 mg/kg doses

showed significant increase in antibody titer against control in normal immune status animals while In T-cell population test, showed significant increase in T-cell rosette formation against control. These results confirm the immunomodulatory activity of *D. viscosa* extract, which is a known immunomodulator in indigenous medicine.

Keywords: *Dodonaea viscosa* – immunomodulators – delayed type hypersensitivity

Introduction

In recent years there has been a renewed interest into the biological activity of traditional plant medicines and the role of natural products in drug discovery (1). Reasons for this include the great need for new molecular models, as leads into potential new drugs, and for authenticating traditional applications for use in current therapy (2).

Natural adjuvants, synthetic agents, antibody reagents are used as immunosuppressive and immunostimulative agents. But there are major limitation to the general use of these agents such as increased risk of infection and generalized effect throughout the immune system (3). Immunosuppression is a major drawback in conventional therapy of cancer such as radiation and chemotherapy (4). Both these method have sever side effect such as nausea, vomiting, alopecia, mucosal ulceration etc.

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. Immunostimulation in a drug-induced immuno-suppression model and immunosuppression in an experimental hyper-reactivity model by the same preparation can be said to be true immunomodulation (5). Apart from being specifically stimulatory or suppressive, certain agents have been shown to possess activity to normalize or modulate

pathophysiological processes and are hence called immunomodulatory agents (6).

A number of medicinal plants as rasayanas have been claimed to possess immunomodulatory activity. Some of the rasayana drugs known as immunomodulatory agents are *Withania somnifera, Tinospora cordifolia, Asparagus racemosus* and *Mangifera indica* (7, 8, 9, 10). A lot more are still to be explored and offer scope for further investigation.

Dodonaea viscosa a member of the family Sapindaceae popularly known as "vilayati mehandi" in India. It is an evergreen shrub or small tree abundantly available in Western Ghats of Tamilnadu and distributed throughout India. Plants were employed largely as analgesic, anti-inflammatory, antiviral, spasmolytic, laxative, antimicrobial and hypotensive agent. (11). In India the infusion of leaves were used to treat rheumatism, gout, hemorrhoids, fracture and snake BITES (12,13). The leaves were reported to possess local anesthetic, smooth muscle relaxant (Rojas et al, 1996), antibacterial (14,15) antifungal (16,17) anti-inflammatory (18,19) and anti-ulcerogenic activity (20). Aliarin, dodonic acid, viscosol, stigmosterol, isorhamnetin (21) penduletin, quercetin, doviscogenin (22) dodonosides A and B (23) have been isolated *D. viscosa*.

Present study attempts to extend the reported immunopotentiating activity of botanical immunomodulators for their possible applications in immunotherapeutic and immunochemical industry.

Materials and Methods

Male albino rats (wistar Strain, 125– 150 g) / mouse (Swiss albino, 25-30g) were obtained from Yash Farms, Pune used for the study. The animals were maintained at 25 ± 2 °C in the institute's animal house with food (Chakan Oil Mills, Pune, India) and water ad libitum. The study was approved by Institute's animal ethical committee and confirmed to national guidelines on the care and use of laboratory animals (CPCSEA/IAEC/PC10/07-2K8).

Plant material and extract preparation

The leaves of *Dodonea viscosa* were collected from authentic sources, dried under shade and powdered in the laboratory. The identity of crude drug material was authenticated by matching drug characters with specimen herbarium from Botanical Survey of India, Pune (Voucher Specimen No: VSJAA1). Powdered dug was weight (1kg) and defatted in soxhlet apparatus with petroleum ether (40-60 ^{0}C) about 35-40 complete cycles. The defatted material was dried to remove petroleum ether and subjected to extraction using 1 lit of ethanol (95%) in a soxhlet apparatus for 24hrs for complete extraction. The solvent was evaporated under vaccum. The extract was kept in air tight containers for further studies.

Drugs

Accurately weighed quantities of the ethanol extract were prepared into water as vehicle using Tween80 as a suspending agent. Cyclosporine was used as a standard immunosuppressant. A Sheep Red Blood Cell (SRBC) was collected from local slaughter house in Alsevers solution. SRBC were used as an antigen at the concentration of 20% for immunization and 1% for challenge.

Statistical analysis

Data were expressed as mean ± S.E.M. and statistical analysis was carried out using unpaired Student's t-test. *$p < 0.05$ was regarded as statistically significant.

Healthy adult albino mice (18- 22g) were subjected to acute toxicity studies as per guidelines suggested by the OECD 425. The mice were observed for 2 h for behavioral, neurological & autonomic profiles & after24 & 72h for any lethality. (24).

All mice were free of any toxicity up to the dose of 3 gm/kg. From this data, two different doses 200, 400 mg/kg were selected for further study.

Methods

T- Cell Population Test

The T-cell has an affinity for and binds spontaneously to sheep erythrocytes (25). In this test three groups of rats were used. Ethanolic extract group II (200mg/kg) group III 400mg/kg were administered orally daily for 10 days. Group I was kept as a control and received vehicle only. On 11^{th} day blood was collect from retro orbital plexus and heparinized with 50 IU heparin in separate test tubes.
Place test tube containing blood in a left sloping position 45^0 at 37^0 C for 1hrs. Collect supernatant which contain lymphocytes and leucocytes were removed using micropipette. An amount of 0.25ml this lymphocyte suspension and 0.25ml 0.5% SRBC were mixed in a test tube and incubated for 5min at 37^0C. The mixed suspension was spuned at 200 rpm for 5min and kept at 4^0c for 2hrs in a refrigerator. The supernatant fluid was removed and place one drop of cell suspension on a glass slide, covered with cover slip and sealed. Lymphocytes were counted and a lymphocyte binding with three or more erythrocytes considered as a rosette. By counting the number of rosette forming and non rosette forming lymphocytes the percentage of rosette formation was determined.

Sheep Erythrocyte Agglutination Test (SEAT)

To study humoral antibody response sheep erythrocyte agglutination test was performed (26). Three groups of animal were divided into I to III, each having six albino rats. Group I was kept as a control and received vehicle only. Group II (200mg/kg) and group III 400mg/kg were administered DV orally for 10 days.

All the animals were injected with 0.25ml of 5 x 10^9 SRBC /ml on 6^{th}, 8^{th}, and 10^{th} days for achieving maximum titer of antibody. On day 11 blood as collected and serum was separated by centrifuging at 200 rpm for 15min.100 l of serum diluted serially with normal saline in separate test tubes. Dilutions were made i.e.20, 40, 60 ….up to 1280. To this 50 l of SRBC added and incubated at 37^0 C for 18hrs. All the tubes were subjected to examine agglutination visually and compared with control. The highest dilution

giving hemagglutination was taken as the antibaody titer. The antibaody titers were expressed in the graded manner, the minimum dilution (1/2) being ranked as 1, and mean ranks of different groups were compared for statistical significance (27).

Carbon clearance test

Phagocytic activity of reticuloendothelial systems (RES) was assayed by carbon clearance test (28). Phagocytic index was calculated as a rate of carbon elimination of reticuloendo-thelial systems by carbon clearance test. In this test three groups of animals were used. Group I was kept as a control and received vehicle only. Ethanolic extract group II (200mg/kg) group III 400mg/kg were administered orally daily for 10 days.

The rats were divided into four groups of six animals each. The control group I orally received 1ml of 5% gum acacia, while animals of treatment group II and III were administered DV at doses of 200, 400mg/kg/day, p.o., respectively for five days. Carbon ink suspension was injected via the tail vein to each rat 48 hours after the five-day treatment. Blood samples (25µl) were then withdrawn from the retro-orbital plexus under mild ether anesthesia at 0 and 15 minutes after injection of colloidal carbon ink and lysed in 0.1% sodium carbonate solution (3ml). The optical density was measured spectrophotometrically at 660nm. The phagocytic index was calculated using the following formula

$K = \log OD1 - \log OD2 / t_2 - t_1$

Where OD1 and OD2 are the optical densities at time t_1 and t_2, respectively.

Delayed Type Hypersensitivity (DTH)

The method described by Joharpurkar *et al.,* 2003 (29) and Bafna and Mishra 2004 (30), was adopted. The mice were

divided into 4 groups, each containing six animals. Normal control Group I was given distilled water orally for 21 days. Negative control Group II receives cyclosporine 100 µg/mouse, *i.p*. on 14th day of study. Animal in the extract treated group III, IV were administered D *viscosa* at a dose of 200 and 400 mg/kg/day, orally respectively for 21 days. Immunized mice with 0.1ml of 20% SRBC's in normal saline intraperitonially on 14th day of study. On day 21st, animal from all group get challenged with 0.03ml of 1% SRBC's in subplantar region of right hind paw. Footpad reaction was assessed after 24hrs i.e. on 22nd day. Increase in foot paw edema was measured with the help of Digital Plethysmometer – LE7500 (Panlab, USA)

Results

T- Cell Population Test

In this test percentage increase in rosette formation was found to be 14.51 ± 4.09 % and 26.20 ± 5.62 % when animals were administered with group II (200 mg/kg) and group III (400 mg/kg) respectively. Both group shows significant activity ($P<0.05$) when compared with normal control group.

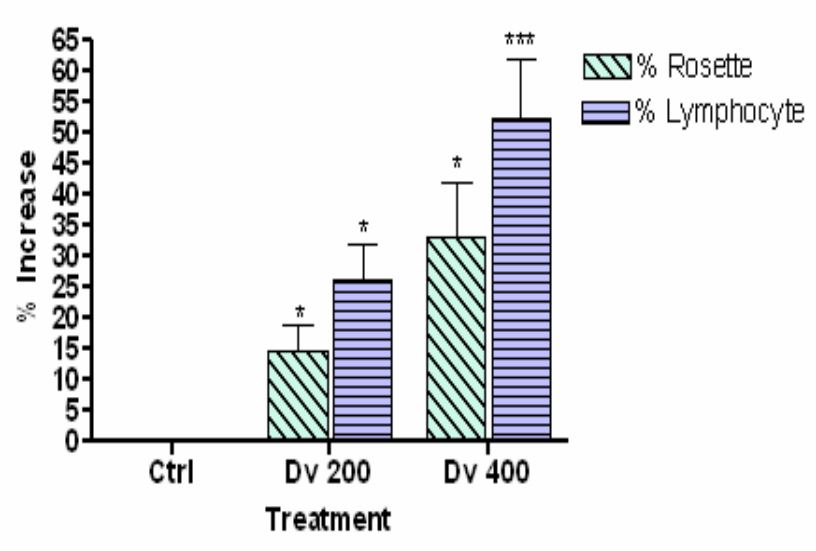

Fig.1 Effect of Ethanolic Extract of DV on T- Cell Population Test

Results are expressed as mean ± SEM. (n = 6).Data were analysed by one way analysis of variance (ANOVA) followed by *Dunnett's* test. *p<0.05 and **p<0.01.

(DV 200, Ethanolic extract of *D. viscosa* 200 mg/kg; DV 400: Ethanolic extract of *D. viscosa* 200 mg/kg).

Lymphocyte formation were compared with control group I, significant increase in lymphocyte formation was found to be 52.12 ± 9.66 % (P<0.001) in group III when animals were administered with ethanolic extract 400 mg/kg body weight. Group II (200 mg/kg) also shown significant increase in lymphocytes formation as 32.78 ± 9.22 % (P < 0.05).

Sheep Erythrocyte Agglutination Test

In this test agglutination titer to SRBC were compared with control Group I. Group II treated with ethanolic extract of DV 200 mg/kg shown agglutination titer in X: 80, X: 160 dilutions while in group III shown agglutination titer up to X: 320, X: 640 serum dilutions indicates significant increase in agglutination titer (p<0.01**).**

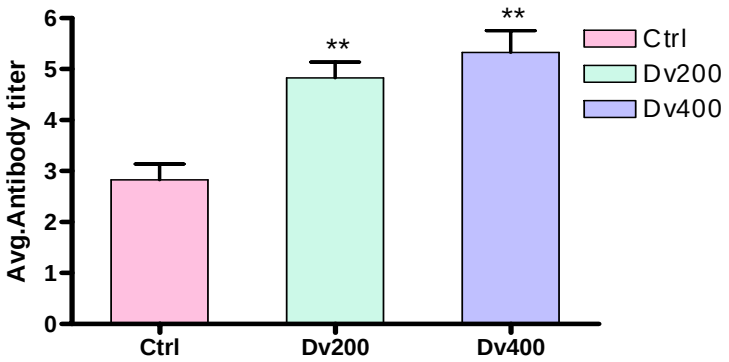

Fig.2 Effect of Ethanolic Extract of DV on Humoral Immune Response in Rat Using Heamagglutination Titer
Results are expressed as mean ± SEM. (n = 6).Data were analysed by one way analysis of variance (ANOVA) followed by *Dunnett's* test. *p<0.05 and **p<0.01.
(DV 200, Ethanolic extract of *D. viscosa* 200 mg/kg; DV 400: Ethanolic extract of *D. viscosa* 200 mg/kg).

Delayed Type Hypersensitivity (DTH) Response
The result obtained in Fig. 2 indicates that there was significant decrease in mean difference, in the foot paw thickness at doses of 200, 400 mg/kg in ethanolic extract of *D. viscosa* administered group when compared against normal control. Negative control group having treatment cyclosporine (100 µg/mouse) showed significant decrease, ($p<0.01$) in the mean difference, in the foot paw thickness as compared to control group. Group treated with *D. viscosa* at dose 200 mg/kg showed significant decrease ($p<0.05$) in DTH response whilst group treated with *D. viscosa* at dose 400 mg/kg showed significant decreases ($p<0.01$) DTH response in terms of mean difference, in the foot paw thickness, when compared against control. The drug influences cell mediated immune response in dose dependent manner.

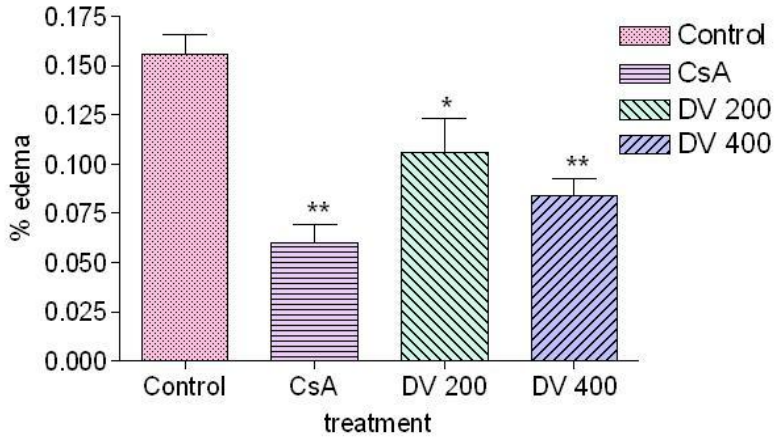

Fig.3 Effect of *D. viscosa* on Mean Difference, in Foot Paw Thickness in Mice as Assessed by Delayed Type of Hypersensitivity.

Results are expressed as mean ± SEM. (n = 6).Data were analysed by one way analysis of variance (ANOVA) followed by *Dunnett's* test. *p<0.05 and **p<0.01. DV 200: Ethanolic extract of *Dodonaea viscosa* 200 mg/kg, DV 400: Ethanolic extract of *Dodonaea viscosa* 200 mg/kg, CsA: Cyclosporine.

Carbon Clearance Test

Increase in phagocytic activity was observed in the present study when treated groups were compared with control. The rate of carbon clearance which was determined in terms of phagocytic index was 0.0694 ± 0.00062 and 0.0737 ± 0.00145 found in ethanolic extract treated group II and group III respectively. The mean phagocytic index of control (group I) was found to be 0.028 ± 0.00097 which clearly indicates that the amount of residual foreign particles in extract treated rats blood was significantly less (p<0.01)

Table No.1 Carbon Clearance Test of Ethanolic Extract of D *viscosa* L. F.

Sr. no	Treatment/ Time	Mean absorbance ± SEM	Phagocytic Index

		5 minutes	**15 minutes**	
1	**Control**	0.0769 ± 0.00058	0.0292± 0.00079	0.028± 0.00097
2	**DV 200**	0.1002 ± 0.00141	0.0108± 0.00015	0.0694± 0.00062 **
3	**DV 400**	0.1054 ± 0.00104	0.0096± 0.00036	0.0737± 0.00145 **

Results are expressed as mean ± SEM. (n = 6).Data were analysed by one way analysis of variance (ANOVA) followed by *Dunnett's* test. *p<0.05 and **p<0.01.
(DV 200, Ethanolic extract of *D. viscosa* 200 mg/kg; DV 400: Ethanolic extract of *D. viscosa* 200 mg/kg).

Discussion

Antibody production to T-dependent antigen SRBC requires co-operation of T- and B-lymphocytes and macrophages (31). The high values of haemagglutinating antibody titre obtained in case of Dodonea *viscosa* L.F. have indicated that immunostimulation was achieved through humoral immunity. Immunomodulators may activate cytotoxic effector cells, such as cytotoxic T lymphocytes, natural killer (NK) lymphocytes, macrophages, and activated neutrophils (32).

In the present study the drug may be capable to influence the role of immunoglobulins results activation of pre B cells and or dendritic cells results in activation of antibodies which give the higher agglutination titer against SRBC's antigens (33).

Increase in rosette formation and lymphocyte formation in T-cell population test indicates effect of ethanolic extract of D *viscosa* on cell mediated immunity. It shows dose dependant activity profile of the drug. The dug may activate the CD4 and CD8 cells which influence T-cell mechanism results increase in T-cell immune response significantly (34).

The reticuloendothelial system (R.E.S.) consist of the spleen, thymus and other lymphoid tissues, together with cells lining the sinuses of the spleen, bone marrow, and lymph nodes and capillary endothelium of the liver (Kupffers cells), and of the adrenal and pituitary glands. These comprise the sessile or fixed macrophages. In addition, free macrophages, such as the blood monocytes and other leucocytes and the tissue macrophages, are transported by the body fluids or wander through the tissues. The R.E.S. is the best defined functionally by its ability to scavenge debris or other foreign matter and forms first line of defense. The rate of removal of carbon particles, by the sessile intravascular phagocytes in the liver and spleen, from the bloodstream is a measure of reticuloendothelial phagocytic activity (35). In carbon clearance test; *D. viscosa* treated groups, exhibited significantly high phagocytic index. This indicates stimulation of the reticulo-endothelial system by drug treatment. It may be possible that the extract influence the mechanism of phagocytosis, largely distributed monocytes - macrophages or R.E.S. which result in significant increase in the phagocytic index with cabon clearance test (36).

Cyclosporine is used as an immunosuppressive drug in organ transplant recipients. It binds to cyclophilin, a cytoplasmic protein, thereby interfering with calcium-dependent events including secretion of interleukin-2 (IL-2) by T lymphocytes. Since IL-2 is necessary for T cell replication, this drug is a potent inhibitor of T cell proliferation and thereby inhibits T cell-mediated immune responses.

DTH reaction is antigen specific and causes erythema and induration at the site of antigen injection in immunized animals when encountered with activated Th1 cells by certain antigens, viz SRBC. DTH comprises of two phases, an initial sensitisation phase and effector phase. In initial sensitization phase Th1 cells are activated and clonally expanded by APC with class II MHC molecule. In effector phase subsequent exposure to the SRBC antigen induces DTH response, where Th1 cells secrete a variety of cytokines and other non specific inflammatory mediators (37, 38).

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