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IMMUNOMODULATORY ACTIVITY OF ETHANOLIC EXTRACT OF AERIAL PARTS OF *ARTEMISIA PALLENS*.

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Abstract: Background: *Artemisia pallens* belonging to the family Asteraceae commonly known as Davana in Tamil is a south Indian annual herb erect in habit, up to 60cm tall. The plant finds use in traditional systems of medicine viz., anthelmintic, tonic and antipyretic properties. It is reputed for its medicinal use as antihyperglycemic. Davana improves the hormonal balance. **Objective:** To study the immunomodulatory activity of ethanolic extract of aerial parts of *artemisia pallens*. **Material and methods:** Ethanolic extract of *Artemisia pallens* was administered orally at doses of 100 and 200mg/kg/day to healthy mice (Albino) divided into three groups consisting of six animals each. The evaluation of immunomodulatory activity was carried out by testing humoral (antibody titre) and cellular (foot pad swelling) immune responses to the antigenic challenge by sheep RBC's and Neutrophil adhesion test. **Results:** On oral administration of ethanolic extract of *Artemisia pallens* (APE) showed a significant decrease in delayed type hypersensitivity (DTH) responses and the humoral responses to Sheep RBCs and also showed non-significant reduction in primary antibody titre and at the doses of 100 and 200mg/kg/day shows significant reduction in secondary antibody and foot pad thickness. When orally administered, significantly decreases the adhesion of neutrophils to nylon fibers. The decrease was found to be significant at a dose of 100 mg/kg/day and highly significant at 200 mg/kg/day when compared to untreated control. **Conclusion:** The study demonstrated that ethanolic extract of aerial parts of *Artemisia pallens* shows immunosuppressant effect by cellular and humoral immunity.

Keywords: Artemisia, Cellular immunity, DTH response, Humoral immunity, Immunosuppressant activity,



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INTRODUCTION

Plant and animals product have been the basis of treatment of human diseases since time immemorial. Every country in the world has lists of herbal remedies for the treatment of diseases in humans¹. Ayurvedic medicines are largely based upon herbal and herbomineral preparations and have specific diagnostic and therapeutic principles². Ayurveda gives emphasis on promotion of health a concept of prevention of diseases and strengthening of both physical and mental health. Genus *Artemisia* (Asteraceae) popularly known as "Sage Brush" or "Worm wood" is bitter aromatics. This genus is named in honour of Artemisia the Greek goddess of chastity. Some of them are sources of volatile oils. Sesquiterpene lactones are known to be present in almost all species³ *Artemisia pallens* commonly known as "Davana" has been traditionally used in Indian folk medicine for the treatment of diabetes mellitus, wound healing, anthelmintic, antipyretic, antibacterial, antifungal and stimulant⁴. The literature survey revealed that the *Artemisia pallens* found to have antioxidant, antimicrobial, and immunomodulatory activity but not scientifically evaluated for phytochemical investigations and pharmacological potentials. Therefore in the present work *Artemisia pallens* is evaluated for its immunomodulatory effects.

MATERIAL AND METHODS

Plant material and sample extracts

The aerial parts of *Artemisia pellens* were collected from dindugal, Tamil Nadu, India. The plant species were identified by Dr. Suresh Baburaj, Survey of Medicinal Plants and Collection Unit, Ootacamund, Tamilnadu, India. The voucher specimens were preserved in the Department of Pharmacognosy, J.S.S. College of Pharmacy, Ootacamund, for further reference. After authentication, the plant materials were dried under shade, coarsely powdered and stored in well closed container till further use. The coarsely powdered aerial parts of *Artemisia pellens* were extracted with 95% ethanol by cold maceration and the marc was again extracted with ethanol. The process was repeated four times and the filtrates were combined, distilled and evaporated.

Preliminary phytochemical analysis

A systematic and complete study of crude drugs should include a complete investigation of both primary and secondary metabolites derived from plant metabolism. The different qualitative chemical tests are to be performed for establishing profiles of the given extracts for their nature of chemical composition. The ethanolic extracts of the selected plants were subjected to the qualitative chemical tests for the identification of various phytoconstituents⁽⁵⁾.

Acute toxicity study:

Toxicity studies were conducted as per internationally accepted protocol drawn under OECD guidelines in Swiss Albino mice at a dose level upto 3000 mg/kg^(6,7).

Chemicals;

Dipotassium EDTA, disodium EDTA, dextrose anhydrous purified was obtained from Merck (India) Pvt. Ltd, Mumbai, India. Sodium chloride was obtained from Rankam laboratories. Glucose, sodium citrate, citric acid were purchased from Ranbaxy laboratories Ltd., Mohali. All other chemicals used in the experiment were of analytical grade.

Animals

Healthy adult Albino mice of Swiss strain weighing between 18-22 g, were procured from J.S.S College of Pharmacy animal house, Ootacamund, India. The animals were fed with rat pellet feed supplied by M/S Hindustan Lever Ltd., Bangalore, India and water *ad libitum* (Aquaguard® filter water). The study was approved from the IAEC, proposal number JSSCP/IAEC/Ph.D/01/2006-07.

Suspension of test extracts:

Suspension (1% w/v) of the dried test extract was prepared by using 5% gum acacia.

Dose administered and dose volume

The test materials were administered orally to the animals through an oral gavage stainless steel needle. The dose volume for mice was 0.1 ml/ 10 g.

Blood withdrawal and processing

The mice were lightly anaesthetized with anesthetic ether. A fine capillary was gently inserted into the lower angle of eye at 45° and the blood was withdrawn from retro-orbital plexus. The blood was collected in eppendorf's tube containing a pinch of dipotassium EDTA and fed to the cell analyzer (CA 13 Medonics, Germany). Blood was collected in a tube containing 0.1ml of 11% sodium citrate for 1ml blood and centrifuged (Remi centrifuge, BNLC-1159) for 10 min at 3500 rpm and the supernatant was collected. The blood serum was collected in a clean eppendorf's tube with no anticoagulant and allowed to clot and then centrifuged as described above. The separated plasma or the serum was stored at 8°C till tests were carried out.

Antigenic material

The antigenic material used was sheep RBCs (SRBCs). The blood was withdrawn from the external jugular vein of sheep. It was mixed with Alsever's solution in 1:1 proportion and stored at 4°C in refrigerator.

Immunomodulatory activity

Treatment

Ethanollic extract of *Artemisia abrotanum* was used for this study. A group of six untreated mice were taken as control (Group-1). The ethanollic extract was fed orally for 14 days at a dose 100 mg/kg/day (Group-II), 200mg/kg/day (Group-III) for the assessment of immunomodulatory effect.

Neutrophil adhesion test⁽⁸⁾

On day 14 of drug treatment, blood samples were collected (before challenge) by puncturing the retro-orbital plexus into heparanized vials and analyzed for total leukocyte count (TLC) and differential leukocyte count (DLC) by fixing blood smears and staining with Field stain, 1 & 11 leishmans stain. After initial count, 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 % neutrophils gives the Neutrophil index (NI) of blood samples. Percent neutrophil adhesion was calculated as shown below (Wilkinson, 1978):

$$NI_u - NI_t$$

$$\text{Neutrophil adhesion (\%)} = \frac{\quad}{NI_u} \times 100$$

$$NI_u$$

Where, NI_u = Neutrophil index of untreated blood sample

NI_t = Neutrophil index of treated blood sample.

Haemagglutinating antibody (HA) titer⁽⁹⁾

On day 7, mice were immunized with 1×10^8 SRBC (i.p). On day 14, one hour after the administration of the test drug, the blood was withdrawn and serum was separated. Two fold dilutions (25µl) of sera were prepared in 0.05M phosphate buffer of pH 7.2 containing 0.1M NaCl administered in V shaped well microtiter plates. To each well 25

μl of 1% SRBC was added. The plates were incubated at 37°C for 1 hour and the observed for the formation of agglutination.

Delayed type hypersensitivity (DTH) response (foot pad swelling)⁽¹⁰⁾

For 14 days albino mice (Swiss) were orally administered once daily with various doses of test extracts. On day 7, mice were immunized with 1×10^8 SRBC (i.p). On day 14, one hour after the administration of the test drugs, the mice were challenged by infecting 50 μl of 1×10^8 SRBC in the planter region of the right hind paw. The paw volume was measured before and after 24 hour the challenge by using water plethysmometer (Ugo Basil, cat.No.7140). The mean increase in paw volume (ml) and percentage of increase in inflammation was calculated for each group.

% of inflammation= [(mean change in paw volume of treated group - mean change of paw volume in control group) / mean change of paw volume in control group] x 100.

Macrophage phagocytosis by carbon clearance method⁽¹¹⁾

The plant extract at doses of 50, 100 and 200 mg/kg, p.o was administered 15 days prior to injection of carbon particles. On day 16, mice are injected with 0.1 ml of carbon suspension (Pelikan tuschea Ink, Germany) intravenously, through tail vein. Blood samples were collected from the retro-orbital plexuses immediately before and at 3, 6, 9 and 12 minutes after the injection of carbon suspension. An aliquot of 25 μl of blood sample was lysed with 2 ml of 0.1% acetic acid and the absorbance was measured spectrometrically at 675 nm (Biozzi *et al.*, 1953).

RESULTS AND DISCUSSION:

Plant material and extraction

The yield of the ethanolic extracts of *Artemisia pallens* was found to be 5.5 % w/w.

Physicochemical Constants

The physicochemical constants like ash value and extractive value were determined. The dried aerial parts show total ash (13.24 % w/w), acid insoluble ash (1.23% w/w), water soluble ash (6.63 % w/w), sulphated ash (17.37 w/w), alcohol soluble extractive (12.20 % w/w), and water soluble extractive (13.49% w/w). The results are shown in Table 1.

Preliminary phytochemical studies

Preliminary Phytochemical studies of *Artemisia pallens* reveal the presence of steroids, terpenoids, and flavonoids. The results obtained are shown in Table 2.

Neutrophil adhesion

The ethanolic extracts of AP, when orally administered significantly decreases the adhesion of neutrophils to nylon fibers which correlates the process of margination of cell in blood vessels. The decrease was found to be significant at a dose of 100 mg/kg/day and highly significant at 200 mg/kg/day when compared to untreated control. A decrease in neutrophils adhesion may represent a possible immunosuppressant effect (Table 3).

Humoral antibody titer

The ethanolic extract of AP was evaluated at the doses of 100 and 200 mg/kg of body weight for humoral antibody titer. The extract showed no significant reduction in primary antibody titer. However, the ethanolic extract of AP at a dose of 100 and 200 mg/kg/day showed a significant reduction in secondary antibody titer (4.91 ± 0.64 , 4.55 ± 0.76) when compared to untreated control group (9.66 ± 0.47), the above results indicate a dose dependent immunosuppressant effect on humoral immune response. The results are shown in (Table 4). Haemagglutination antibody titer has been shown to establish the humoral response against sheep red blood cell (SRBC). At neutral pH, red blood cells possess a negative ion that makes the cells repel from one another. This repulsive force is referred to as zeta potential. Because of its size and pentameric nature, IgM can overcome the electric barrier and cross-link red blood cells, leading to subsequent agglutination. The smaller size and bivalency of IgG, however, makes them less capable to overcome the electric barrier. This characteristic may account for, IgM being more effective than IgG in agglutinating red blood cells⁽¹²⁾.

Delayed type hypersensitivity (DTH) response

In order to assess the effect of the ethanolic extract on DTH response, a group of 18 mice were divided into three groups of six each. The ethanolic extracts at the dose levels of 100 and 200 mg/kg, p. o. showed significant decrease in the foot pad thickness when compared to untreated control group ($p < 0.05$) (Table 5). These results indicate a dose dependent activity on delayed type hypersensitivity (DTH)

Delayed type hypersensitivity is a part of the graft rejection, tumor immunity, and most important, immunity to many intracellular infectious microorganisms, especially those causing chronic diseases such as tuberculosis⁽¹³⁾. DTH requires the specific recognition of a given antigen by activated T-Lymphocytes, which subsequently proliferate and release cytokines. These in turn increase vascular permeability, induce vasodilatation, macrophage accumulation⁽¹⁴⁾ and activation, promoting increased phagocytic activity and increased

concentrations of lytic enzymes of more effective killing⁽¹⁵⁾. The reactions of DTH are classified into several kinds, and the presence of the CD₄T cells are necessary for this process. The use of DTH method has shown that the best time for the assessment of the responses in mice is 48 hours after the injection. There was a significant decrease in the percentage of DTH response at this time compared with other times ($p < 0.05$). Statistical studies indicate a significant decrease ($p < 0.05$) among the three groups that received the ethanolic extract of AP and the control group. Also, they show that immunosuppressive effect of the extracts more than that of the control. Our previous studies showed the presence of artemisinin⁽¹⁶⁾ and flavonoids⁽¹⁷⁾ in the extract and it is probable that their presence in extract is responsible for a decrease in IL-2 and its receptors and probably it blockades the IL-2 activity. AP extract shows a significant effect on delayed hypersensitivity reaction (48 h). This might be due to the inhibitory effect of the extracts on T-lymphocytes which mediates the cell mediated immune response i.e. foot pad thickness.

Phagocytic activity

Carbon clearance test was carried out to establish phagocytic activity of reticuloendothelial system after treatment with the ethanolic extract (Table 6). Phagocytic index significantly decreased after the administration AP at the dose levels of 100 and 200 mg/kg when compared to the control group ($p < 0.01$).

The role of phagocytosis is primarily the removal of microorganisms and foreign bodies, but also the elimination of dead or injured cells. Phagocytic defects are associated with varied pathological conditions in humans⁽¹⁸⁾. In view of the pivotal role played by the macrophages in coordinating the processing the presentation of antigen to B-cells, AP was evaluated for their effect on macrophage phagocytic activity. When the carbon particles are injected intravenously, the rate of clearance of carbon from blood by macrophage is governed by an exponential equation. This seems to be the general way in which inert particulate matter is not cleared from the blood. In the present study, phagocytic index was significantly decreases after the administration the plant extracts, when compared to the control group.

CONCLUSION

From the above study it was concluded that *Artemisia pallens* showed immunosuppressant effect on humoral as well as cellular immunity based on its effects on HA titer and DTH response.

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Declaration of Interest

This study was supported financially by AICTE, New Delhi (India).

“Table 1: Physicochemical constants of the aerial parts of AP”

Name of the species	Total Ash (%w/w)	Acid Insoluble Ash (% w/w)	Water Soluble Ash (%w/w)	Sulphated Ash (%w/w)	Alcohol Soluble Extractive value (%w/w)	Water Soluble Extractive value (%w/w)
<i>Artemisia pallens</i>	13.24	01.23	06.63	17.37	12.20	13.49

“Table 2: Qualitative Phytochemical analysis of AP”.

Phytochemical constituents	<i>Ethanollic extract of AP</i>
Steroids/Terpinoids	+
Alkaloids	-
Saponins	-
Glycosides	-
Tannins	-
Flavonoids	+
Sugars	-
Amino acids	-
Proteins	-

Table 3. The effects of ethanolic extracts of on neutrophil adhesion in mice.

Groups	Neutrophil Adhesion		Neutrophil
	UB	FTB	Adhesion (%)
Untreated	315.36±0.30	233.47±0.37	25.97±0.35
AP 100	310.37±0.48	245.73±0.25	20.83±0.29*
AP 200	312.40±0.55	275.47±0.52	11.82±0.58**

Values expressed in mean ± SEM; Mean difference between the groups were analysed using ANOVA followed by Dunnet's t-test comparison as post hoc;

* $P < 0.05$ and ** $P < 0.01$.

UB indicates untreated blood and FTB indicates fiber treated blood.

“Table 4: Effect of ethanolic extracts of AP on primary and secondary antibody titers to antigenically challenged mice”.

Sl. No	Treatment mg/kg	N	Mean hemagglutination antibody titre	
			Primary antibody titres	Secondary antibody titres
1.	Control	6	7.4 ± 0.5	9.66 ± 0.47
4.	AP100	6	7.0 ± 0.57	4.91 ± 0.64**
5.	AP200	6	6.83±0.89	4.55 ± 0.76**

Values expressed in mean ± SEM; n=6; Mean difference between the groups were analysed using ANOVA followed by Dunnet's t test comparison as post hoc;

* $p < 0.05$, significant, ** $p < 0.01$, highly significant (compared with respective control)

“Table 5. The effects of ethanolic extract of AP on the mean of DTH responses on antigenically challenged mice”.

Group	Extract (mg/kg/day)	Foot pad thickness i.e. mean % edema	
		24 h (after challenge)	48 h (after challenge)
I	Control	40.25± 6.50	31.54 ± 5.28
II	AP 100	13.75 ± 2.85**	12.45 ± 2.72**
III	AP 200	11.77 ± 1.68**	09.82 ± 3.18**

Values expressed in mean \pm SEM; Mean difference between the groups were analysed using ANOVA followed by Dunnet's t-test comparison as post hoc; ** $P < 0.01$.

, ** $p < 0.01$, highly significant (compared with respective control)

"Table 6. The effects of ethanolic extracts on Phagocytic response".

Groups	Phagocytic index
Untreated	34.48 \pm 0.44
AP 100	25.80 \pm 0.17*
AP200	10.71 \pm 0.34**

Values expressed in mean \pm SEM; Mean difference between the groups were analysed using ANOVA followed by Dunnet's t-test comparison as post hoc;

* $P < 0.05$ and ** $P < 0.01$

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