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Research Article

**COMPARATIVE STUDY OF IMMUNOMODULATORY
ACTIVITY OF MARKETED AYURVEDIC FORMULATION****S.A Bagwan*, N.S Naikwade and J.Y Manure**Department of Pharmacology, Appasaheb Birnale College of Pharmacy,
Sangli-416416, India**Abstract:**

Introduction: The objective of the present study to compare immunomodulatory activity of Marketed Ayurvedic Formulation viz Formulation I and Formulation II.

Material and Method: In the present study the dose selection of Formulation I and Formulation II for the particular species (Rat) were selected on the basis of the method described by Paget and Barnes. The assessments of immunomodulatory activity were carried out by using Delayed Type Hypersensitivity Test, Carbon Clearance Test and Neutrophil adhesion test.

Result: Oral administration of Marketed Formulation I and Formulation II significantly ($P < 0.0001$) showed Immunomodulatory activity by increase in DTH response, phagocytic activity, and Neutrophil adhesion in Rat at experimental dose.

Conclusion: The study demonstrated that both the Formulation I and Formulation II shows significant immunomodulatory effect on both humoral as well as cell mediated immunity. While among two Formulation II showed more immunomodulatory activity as compare with Formulation I. Present study it was revealed that Formulation II showed more immunomodulatory activity than Formulation I due to presence of multiple immunomodulatory ingredient in its Formulation (Tinospora Cardifolia, Ocimum Sactum, Glycyrrhiza Glabra, Aconitum hetrophyllum, Cyperus rotundus, Pistacia integerrim), when compare with Formulation I it contain single ingredient (Tinospora Cardifolia). Ocimum Sactum which is present in Formulation II is a more potent immunomodulator than Tinospora Cardiofolia.

Keywords: Formulation I, Formulation II, Delayed Type Hypersensitivity test, Carbon Clearance Test, Neutrophil Adhesion test.

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INTRODUCTION:

Human system is under constant threat from a variety of pathogenic organisms (bacteria, viruses, fungi, protozoa etc.), parasite and tumour cells. Even nonliving substance such as organic, inorganic molecules, pollutants in the atmosphere poisons and toxins etc. are also cause a potential threat. The immune system is remarkably versatile system that has evolved to defense itself against this vast range of harmful agents. It is able to generate an enormous variety of cell and molecules capable of specifically recognizing and eliminating variety of foreign invaders. Immune activation is a protective as well as effective approach against emerging infectious disease. Immunostimulation constituent either an alternative to or an adjuvant for conventional chemotherapy and prophylaxis of infection, for tumor as well as autoimmune diseases, especially when the host immune system is impaired. [1] Immunomodulators are natural or synthetic substance that help to regulate or normalize the immune system. Immunomodulators correct immune systems that are out of balance. Immunomodulators are recommended for individuals with autoimmune diseases and they are widely used in chronic illness to restore immune system, and in individuals who have been on lengthy course of antibiotics or antiviral therapies. [2]

The suppression of the immune system associated with tuberculosis (TB), cancer, surgery or HIV infection is characterized by a reduction in the number and phagocytic function of neutrophils and macrophages as well as a reduction in the intracellular bactericidal capacity of these cells. This profound suppression of the individual elements of the system allows opportunistic pathogens to overwhelm the host so that secondary infection becomes the most common cause of the mortality in such individuals.

The Concept of Immunostimulation in Ayurveda has been used successfully in the treatment of immunocompromised condition like AIDS, Tuberculosis, cancer and hepatic disease. One of the main strategies in ayurvedic medication is to increase body's natural resistance to disease causing agent rather than directly neutralizing the agent itself. In practice this achieved by using extracts of various plant material called "Rasayanas", a group of non-toxic herbal drug preparation which are used to improve the general health by stimulation of body's immunity.[3,4,5.]

In the light of above we considered it appropriate to screen some immunomodulators Ayurvedic Formulation sold in the Indian market for animals

(preclinical) studies. Thus present work is undertaken to investigate and compare the immunomodulatory properties of two herbal Formulation viz. Formulation I and Formulation II.

MATERIALS AND METHODS:

Animals: All the experimental were carried out using male albino rat of Wistar strain. Weight around 150-200 gm. The animals are free access of food and water, and they were housed in a natural (12 h each) light -dark cycle. The animals were acclimatized for at least 5 day to the laboratory conditions before the experiment. The experimental protocol was approved by the institutional animal ethics committee (IAEC/ABCP/01/2016-17) and the care of laboratory animal was taken as per the guideline of CPCSEA.

Drugs and Chemicals: All the drugs and Chemical were of analytical grade while the other drugs were procured - Levamisole (Johnson & Johnson Ltd.), colloidal carbon (Indian Ink, camel India Pvt. Ltd.) Formulation I & Formulation II (Indian Market)

Selection of doses: In the present study the dose selection of Formulation I and Formulation II for the particular species (Rat) were selected on the basis of the method described by Paget and Barnes. The method is based on body surface area of various species and a dose for one strain can be calculated with the help of another strain whose dose is previously known. The doses of the drugs were calculated by extrapolating the therapeutic dose to rat dose on the basis of body surface area ratio (conversion factor 0.018 for rats) [6]

Conversion factor for rat:

Humans dose x 0.018 = X g/200g. Of rat.

$X \times 5 = Y$ g/kg of rat.

PHARMACOLOGICAL SCREENING

The immunomodulatory activity is carried out using the following in-vivo immunomodulatory models.

1. Delayed Type Hypersensitivity Reaction.
2. Carbon Clearance Test. (Test for Phagocytosis).
3. Neutrophil Adhesion Test.

1. DELAYED TYPE HYPERSENSITIVITY REACTION: (21 Days Model)**Purpose and Rationale:**

Delayed hypersensitivity reaction is a reaction of cell mediated immunity and become visible only after 16-24 hrs.

Procedure:

1. In this test animals were divided into four group comprising 6 animals in each.
2. Group I was kept as a control and received vehicle only (water) 10 ml/kg.
3. Group II was kept as a standard and received standard drug levamisole (50 mg/kg).

4. Group III was kept as test I and received the ayurvedic Formulation I.
5. Group IV was kept as test II and received the ayurvedic Formulation II.

Table 1: Grouping and Treatment Schedule for DTH Test

Sr. No	Group	Test Substance	Dose
1	Group I	Control (water)	10 ml/kg
2	Group II	Std (Levamisole)	50 mg/kg
3	Group III	Formulation I	0.54 ml twice a day
4	Group IV	Formulation II	0.54 ml twice a day

6. Immunized Rat with 0.1ml of 20% SRBCs in normal saline intraperitoneally on 14th day of the study. On day 21st, animals from all groups get challenge with 0.03ml of 1% SRBCs in sub plantar region of right hind paw. Foot pad reaction was assessed after 24hrs i.e. on 22nd day. Increase in foot pad edema was measured with the help of vernier caliper or by using Plethysmometer. [7,8]

Antigenic material:

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

Composition of Alsever's Solution

Table 2: Composition of Alsever's Solution

Chemicals	Quantity(g/L)
Sodium Chloride	4.2
Sodium Citrate	8.0
Citric acid anhydrous	0.55
Glucose	20.5
Distilled water q.s.	1000ml

Statistical analysis: Result was expressed as mean value \pm SEM. The variation in a set of data has been estimated by performing one way analysis of variation (ANOVA). Individual comparison of group

mean value were done using Dunnett's test. P value < 0.05, were considered statistically significant.

2. CARBON CLEARANCE TEST: (10 Days Model)

Purpose and Rationale: Phagocytic activity of reticuloendothelial system was assay by carbon clearance test; phagocytic index was calculated as a rate of carbon elimination of reticuloendothelial system by carbon clearance test.

Procedure:

1. In this test Animals were divided into four group comprising 6 animals in each.
2. Group I was kept as a control and received vehicle only (water) 10 ml/kg.
3. Group II was kept as a standard and received standard drug Levamisole (50 mg/kg).
4. Group III was kept as test I and received the ayurvedic Formulation I.
5. Group IV was kept as test II and received the ayurvedic Formulation II.

Table 3: Grouping and Treatment Schedule for Carbon Clearance Test

Sr. No	Group	Test Substance	Dose
1	Group I	Control (water)	10 ml/kg
2	Group II	Std (Levamisole)	50 mg/kg
3	Group III	Formulation I	0.54 ml twice a day
4	Group IV	Formulation II	0.54 ml twice a day

6. Carbon ink suspension was injected via tail vein to each rat 48 hours after the five day treatment
7. Blood sample (25 μ l) were then withdrawn from the retro-orbital plexus under mild ether anesthesia at 5 and 15 min after injection of colloidal carbon ink lysed in 0.1% sodium carbonate solution (3 ml).
8. The optical density was measured spectrophotometrically at 660 nm.
9. The phagocytic activity was calculated using the following formula. [7,8]

$$K = \frac{\text{Log OD1} - \text{Log OD2}}{t_2 - t_1}$$

Where ODI and OD2 are the optical densities at time t1 and t2, respectively.

Preparation of carbon ink suspension: Camlin ink was diluted eight times with saline and used for carbon clearance test in a dose of 10 µl/gm body weight of rat^[9]

Statistical analysis: Result was expressed as mean value ± SEM. The variation in a set of data has been estimated by performing one way analysis of variation (ANOVA). Individual comparison of group mean value were done using Dunnett's test. P value < 0.05, were considered statistically significant.

3. NEUTROPHILE ADHESION TEST (16 days Model)

Purpose and Rationale: Increase the recruitment of neutrophils adhesion to nylon fibers which correlates to the process of margination of cells in blood vessels.

Procedure:

1. In this test animals were divided into four group comprising 6 animals in each.
2. Group I was kept as a control and received vehicle only (water) 10 ml/kg.
3. Group II was kept as a standard and received standard drug Levamisole (50 mg/kg).
4. Group III was kept as test I and received the ayurvedic Formulation I.
5. Group IV was kept as test II and received the ayurvedic Formulation II

Table 4: Grouping and Treatment Schedule for Neutrophil adhesion Test

S. No	Group	Test Substance	Dose
1	Group I	Control (water)	10 ml/kg
2	Group II	Std (Levamisole)	50 mg/kg
3	Group III	Formulation I	0.54 ml twice a day
4	Group IV	Formulation II	0.54 ml twice a day

6. On 16th day of the treatment, blood sample from all the group were collected by puncturing retro-orbital plexus under mild ether anesthesia.

7. Blood was collected in vials pre-treated by disodium EDTA and analyzed for total leukocyte count (TLC) and differential leukocyte count (DLC).

8. After initial count blood sample were collected with nylon fiber (80 mg/ml, previously sterilized by 95% alcohol) for 15min at 37°C. the incubated drug sample were analyzed for TLC and DLC.

9. The product of TLC and % neutrophils adhesion was calculated as follows.[7,8]

$$\text{Neutrophil adhesion (\%)} = \frac{NI_U}{NI_U - NI_T} \times 100$$

Where,

NI_U: Neutrophil index before incubation with nylon fibers.

NI_T: Neutrophil index after incubation with nylon fibers.

Statistical analysis: Result was expressed as mean value ± SEM. The variation in a set of data has been estimated by performing one way analysis of variation (ANOVA). Individual comparison of group mean value were done using Dunnett's test. P value < 0.05, were considered statistically significant.

RESULT:

Delayed Type of Hypersensitivity Reaction: Effects of Formulation I and Formulation II on cell mediated immune response by DTH induce footpad edema is shown in [Table:5] Formulation I and Formulation II treated group significantly showed increase in footpad edema (P < 0.0001) when compare with control group. Formulation I showed increase in footpad edema 3.893 ± 0.01 which potentiate DTH response to 144.07 % when compare with control. Similarly Formulation II showed increase in footpad edema 4.495 ± 0.01 which indicate increase in DTH response up to (166.3 %) when compare with control.

Table 5: Result of DTH

Sr. No	Group	Treatments and route of Administration	Mean difference in paw edema in (mm) (Mean ± SEM)
1	I	Control (P.O.)	2.702 ± 0.01 (100 %)
2	II	Standard (Levamisole) (P.O.)	4.192 ± 0.01**** (↑155.1 %)
3	III	Formulation I (P.O.)	3.893 ± 0.01**** (↑144.07 %)
4	IV	Formulation II (P.O.)	4.495 ± 0.01**** (↑166.3 %)

Values are expressed as (Mean ± S.E.M). n=6 ****P < 0.0001 statistically significant when compared with control group by ANOVA followed by Dunnett test.

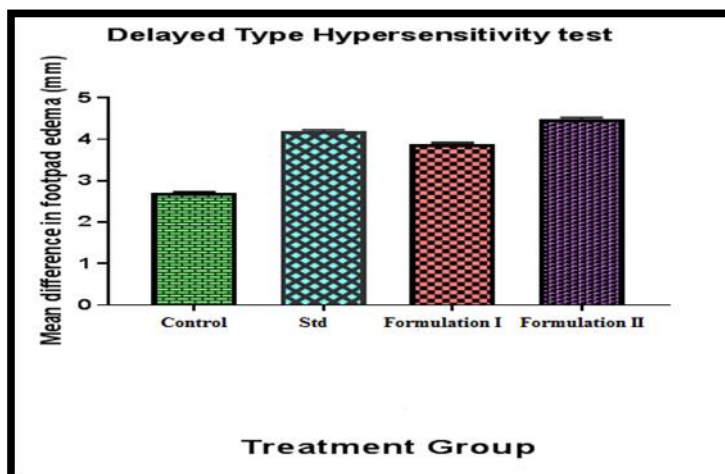


Fig.1: Graphical representation of DTH

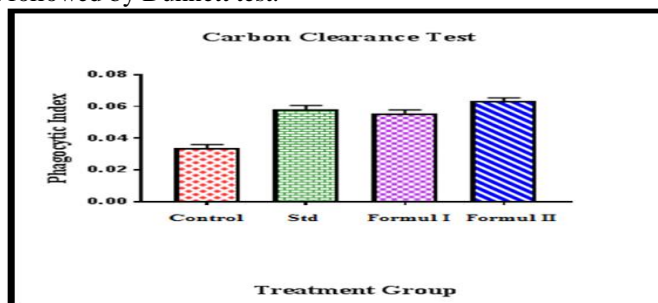
Carbon Clearance Test: Effect of Formulation I and Formulation II on the phagocytic activity by carbon clearance test is shown in [Table: 6]. The phagocytic activity of reticuloendothelial system is generally measured by the rate of removal of carbon particle from the blood stream. In carbon clearance test Formulation I & Formulation II treated all group exhibited significantly high phagocytic index ($P < 0.0001$) when compare with control group.

Formulation I treated group showed phagocytic index 0.05562 ± 0.0009 which indicated stimulation of reticuloendothelial system to 164.70 % when compare with control. Similarly Formulation II treated group showed phagocytic index 0.06335 ± 0.0008 which potentiate reticuloendothelial system to 187.59 % when compare with control group.

Table 6: Result of Carbon Clearance Test.

Sr. No	Group	Treatments	Dose and route of administration	Absorbance		Phagocytic index (Mean±SEM)
				5 min	15 min	
1	I	Control	10 ml/kg (P.O.)	0.169	0.078	0.03377±0.0009 (100 %)
2	II	Standard (Levamisole)	50 mg/kg (P.O.)	0.154	0.041	0.05815±0.0001**** (↑172.55 %)
3	III	Formulation I	0.54 ml twice a day (P.O.)	0.162	0.045	0.05562±0.0009**** (↑164.70 %)
4	IV	Formulation II	0.54 ml twice a day (P.O.)	0.152	0.035	0.06335±0.0008**** (↑187.59%)

Value are expressed as (Mean ±SEM), n=6 ****P<0.0001 statistically significant when compared with control group by ANOVA followed by Dunnett test.

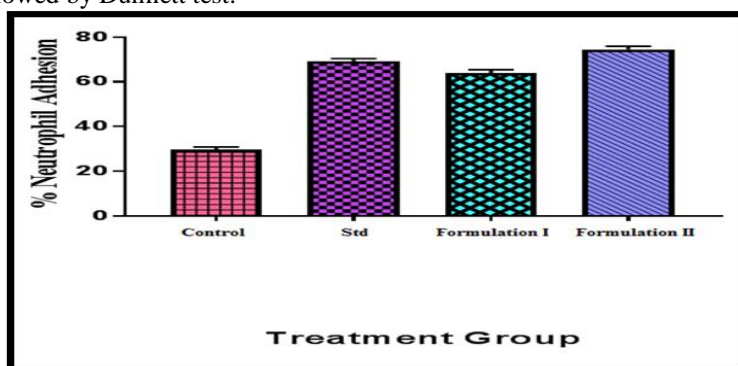


Graph: 2. Graphical representation of Carbon Clearance Test

Table: 7. Result of Neutrophil Adhesion test.

Sr. no	Group	%Neutrophil Adhesion
1	Control	29.55±0.6919
2	Standard	69.24±0.5614****
3	Formulation I	63.83±0.8031****
4	Formulation II	74.47±0.7064****

Values are expressed as (Mean ±S.E.M),n=4 ****P<0.0001 Statistically significant when compared with control group by ANOVA followed by Dunnett test.

**Fig: 3. Graphical representation of Neutrophil Adhesion Test.**

Result Of Neutrophil Adhesion Test: Effect of Formulation I and Formulation II on neutrophils activation by the neutrophils adhesion test is shown in [Table: 7] Cytokines are secreted by activated immune cell for margination and extravasations of the phagocytes mainly Polymorphonuclear neutrophils. The percentage neutrophils adhesion was significantly ($P<0.0001$) increase by Formulation I (63.83 %) and Formulation II (74.47 %) when compare with control group showed possible Immunostimulant effect. Formulation I and Formulation II significantly evoked increase in the adhesion of neutrophils to nylon fibers which correlates to the process of margination of cell in blood vessels.

DISCUSSION:

Immunomodulation is a process which can alter immune system, specifically stimulation or suppression and thus immunomodulators are immune stimulant and immune suppressant. Modulation of immune responses to alleviate the diseases has been interest for many years, and immunostimulation and immunosuppressant both needed to be tackle in order to regulate the normal immunological function. [7] A number of medicinal plants of rasayanas have been claimed to possess immunomodulatory activity and many Formulation of these plant products are available to enhance the immune system. Hence present study was carried out to estimate the immunomodulatory activity of marketed Formulation viz Formulation I and Formulation II. Thus present

study was design to explore and compare the possible immunomodulatory activity of Formulation I and Formulation II.

In the present study carbon clearance test, Delayed type of hypersensitivity test, Neutrophils adhesion test and chronic administration of test Formulation were selected for evaluation of immunomodulatory effect of Ayurvedic Formulation I and Formulation II.

According to Sites DP et al. Delayed hypersensitivity test is part of the process of the graft rejection, tumor immunity, and most important, immunity against many intracellular infectious microorganisms, specially those causing chronic disease such as tuberculosis. Delayed hypersensitivity test required the specific recognition of antigen to activate T lymphocytes which substantially proliferate and release the cytokines, which in turn increase vascular permeability; induce vasodilatation, macrophage accumulation and activation promoting increase phagocytic activity and increase concentration of lytic enzyme for more effective killing. In the present study SRBCs, served as a sensitizer substance which in combination with skin protein produces antigenicity, and generate hypersensitivity reaction in rat. [10] In present research work it was found that Formulation I, Formulation II and levamisole causes increase in the footpad edema after 24 hrs of the exposure to SRBC $3.893\pm 0.01\text{mm}$ (144.07 %) , $4.495\pm 0.01\text{mm}$ (166.3 %) and 4.192 ± 0.01 (155.1 %)

respectively. when compared with control $2.070 \pm 0.01mm$ (100 %). This indicated stimulation of cell mediated immunity.

According to Smriti Tripathi et al. Phagocytic activity of reticuloendothelial system was assayed by carbon clearance test. Phagocytic index was calculated as rate of carbon elimination of reticuloendothelial system by carbon clearance test. [11] In the present work, phagocytic index of Formulation I was 0.05562 ± 0.0009 (164.70 %) Formulation II showed 0.06335 ± 0.0008 (187.59 %) and Levamisole 0.05815 ± 0.0001 (172.55 %) when compare with vehicle control 0.03377 ± 0.0009 . This revealed that Formulation II have highest phagocytic index than Formulation I and Levamisole. Thus increase in phagocytic activity indicated that there was stimulation of reticuloendothelial system.

According to Roitt I et al. Movement of neutrophils towards the foreign body is the first and most important step in phagocytosis process. Cytokines are secreted by activated immune cell for margination and extravasations of phagocytes mainly Polymorphonuclear neutrophils. Experimentally activation of the neutrophils can be study by neutrophils adhesion test. Our result showed that Formulation I, Formulation II and Levamisole were found to be stimulate neutrophils chemotaxis and increase % of neutrophils adhesion when compare with the control group. Further it was observed that Formulation II showed highest % of neutrophils adhesion ($74.47 \pm 0.7064\%$) than Formulation I ($63.83 \pm 0.8031\%$) and Levamisole ($69.24 \pm 0.5614\%$) when compared with vehicle control ($29.55 \pm 0.6911\%$).

In the present study it was revealed that Formulation II showed more immunomodulatory activity than Formulation I due to presence of multiple immunomodulatory ingredient in its Formulation (Tinospora Cardifolia, Ocimum Sactum, Glycyrrhiza Glabra, Aconitum hetrophyllum, Cyperus rotundus, Pistacia integerring), when compare with Formulation I it contain single ingredient (Tinospora Cardifolia). Ocimum Sactum which is present in Formulation II is a more potent immunomodulator than Tinospora Cardifolia. In this study, the overall order of immunomodulatory activity was established as Formulation II > levamisole > Formulation I

CONCLUSION:

The present study demonstrates that Formulation I and Formulation II shows significant

Immunomodulatory effect on both humoral as well as cell mediated immunity which is due to

- Increase the Polymorphonuclear neutrophils and their activation leading to margination in the blood vessels.
- By the activation of reticuloendothelial system.
- Enhance capacity of monocytes Macrophages system.

Further among the two Formulation, Formulation II shows more immunomodulatory activity than Formulation I and, the overall order of immunomodulatory activity is Formulation II > levamisole > Formulation I.

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