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"Evaluation of Immunomodulatory Activity of Ayurvedic Formulation by In-Vitro and In-Vivo Methods"

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ABSTRACT : Aim: Evaluation of immunomodulatory activity of ayurvedic formulation by in-vitro and in-vivo method.
Material and Methods: The assessment of immunomodulatory activity of effect of various doses(0.9 ml/kg b.w, p.o. twice a day) of Marketed Ayurvedic Formulation (i.e.) Jambhasava were done by Carbon clearance test and Neutrophil adhesion test for Non-specific immunity, Delayed Type Of Hypersensitivity Reaction for Cell mediated immune response, and T-Cell population test. In the present research work, the dose selection of Jambhasava Syrup for the particular species (Rat) were selected on the basis of the method described by Paget and Barnes.
Results: The present demonstrate that, jambhasava shows potent immunomodulatory effect on both humoral as well as cell mediated immunity. Further, on the basis of the results obtained in current study; it can be concluded that, as dose of jambhasava increases the immunomodulatory activity simultaneously increases and it may be used further as a potential therapeutic candidate in several immunosuppressed clinical conditions.
Keywords- Immunostimulant action, coronavirus disease, Ayurveda, Levamisole, etc

I. INTRODUCTION

In 21st century, the world is facing large number of increase in incidences of the diseases, variety of antibiotics & antiviral drugs are available in market which are effective to resist infection but; after prolonged use they seems to be ineffective due to development of antibiotic resistance by the bacteria & antiviral resistance by viruses.[1]

The coronavirus disease 2019 (COVID-19) pandemic, was caused by sever acute respiratory syndrome coronavirus two (SARS-CoV-2) continues to spread globally. More than 2-3 million people have infected & many of them have died. Today, no any treatment have been definitively shown to be effective, however the prevention strategies such as vaccination are ideal but these strategies are unlikely to be available in time to address current clinical needs. Many of drugs acts, at least in part, to directly limit viral replication. On other hand, the use of immune modulators might have benefits by controlling the pathological immune response to the virus. So, researchers are thinking towards the immunomodulation as a future of treatment.[2]

Immunology is one of the most rapidly developing area of biomedical research & has great scope with regard to prevention & treatment of broad range of immunological disorders. The immune system is involved in the etiology as well as pathophysiologic mechanism of various diseases.[3]The term immunomodulation relates to the alteration of immune response which may increase or decrease the immune responsiveness. An immunomodulators may defined as; substances, biological or synthetic, which can stimulate, suppress or modulate any of the components of immune system including both innate and adaptive arms of immune response. The very high manifestations of immunomodulatory action of biologically active substances are cause the immunosuppression & immunostimulation. The immunomodulating drugs are needed for the treatment of various disease statuses such as infections, organ transplantation, cancer, rheumatoid arthritis, systemic lupus erythematosus, down syndrome, crohn's and autoimmune diseases and the acquired immune deficiency syndrome.[4] Hence, it is becoming the field of major interest all over the world. Hence, the immune system has been called "Pacemaker" of life and changes occurring in this system have implicated in age related decrement of tissue vitality.[5]

Medicinal plants, since ancient times, have been virtually used in all traditions as a source of medicine for altering the immune system.[6]

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Many medicinal plants has been found for immunomodulatory potentials and they have proved to have useful effect on alteration of immune system by diverse mechanism in humans. The modulation of immune response through concept of rasayana in Ayurveda is popular, where plants with rejuvenating activity have been described. Several plants have been identified as rasayanas in the Indian Ayurveda and various other systems of medicine possessing various immune-pharmacological properties such as immunostimulant, immunoadjuvants, neurostimulant, antiaging, antirheumatic, anticancer, adaptogenic, antistress, etc. Immunomodulation by using various traditional medicinal plants can provide an another alternative to conventional chemotherapy for a variety of diseased conditions especially when host defence mechanism has to be activated under the conditions of physiologically impaired immune response or when a selective immunosuppression is desired in situations like autoimmune disorders or lupus.[7]

This traditional knowledge with its holistic and systemic approach supported by experimental evidences can serve as innovative and powerful discovery engine for newer, safer and affordable medicines. Thus in light of above, it is more appropriate to screen the immunomodulatory activity of Ayurvedic Formulation sold in Indian market with animal (preclinical) studies. Thus current work is undertaken to investigate immunomodulatory properties of marketed herbal formulation.

II. MATERIALS AND METHODS

Dose Selection of Drug:

In the present research work, the dose selection of Jambhasava Syrup for the particular species (Rat) were selected on the basis of the method described by Paget and Barnes. The method was based on body surface area of various species. In this 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 the rat dose on the basis of body's surface area ratio. (Conversion Factor = 0.018 for Rats)[8]

Conversion Factor For Rats Calculated As Below:

Human Dose X 0.018 = A gm/200gm of Rat.
A gm X 5 = Y gm/Kg of Rat.

Table 1: Dose Selection of Drug

Formulation	Human Dose(ml)	Calculated Dose For 200 gm rat (ml)
Jambhasava	20 ml twice a day	0.36 ml twice a day

Experimental Animal:

All the experiments were carried out by using male albino rat of wistar strain.
Weight:- Around 150 to 200 gm.

The animals have free access of food and water; they were housed in a natural (12 hrs each) light and dark cycle. The animals were acclimatized for at least 5 days to the laboratory conditions before the experiment. The experimental protocol was approved by the institutional animal ethics committee and the care of laboratory animals was taken as per guideline of CPCSEA, Ministry of Forests and Environment Government of India.

Drug Treatment:

In the present investigation, the rats were divided into five different groups consisting six rats in each group, for employing various immunomodulatory models.

Laboratory models for testing immunomodulatory activity were as follows;

- ❖ In-Vivo Methods
1) Delayed Type Hypersensitivity Reaction Test.
2) Carbon Clearance Test.
3) Neutrophil Adhesion Test.
- ❖ In-Vitro Methods
1) T-cell Population Test.

IN-VIVO METHODS

[A] DELAYED TYPE OF HYPERSENSITIVITY RAECTION: (21 Days Model)

- **Purpose and Rationale:**

Delayed type of hypersensitivity reaction is reaction of cell mediated immunity and becomes visible only after 16 to 24 hrs.

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- **Procedure:**[9, 10, 11]

1. In this experimental model, animals were divided into five groups comprising of six animals in each group.
2. The Group-I was considered as a control group and received vehicle (water) only with a dose of 10 ml/kg b.w. p.o.
3. The Group-II was considered as a standard group and received the standard drug levamisole with a dose of 50 mg/kg b.w.p.o.
4. The Group-III was considered as a Test I group and received the Ayurvedic Formulation with a dose of 0.9 ml/kg b.w.p.o.
5. The Group-IV was considered as a Test II group and received the Ayurvedic Formulation with a dose of 1.8 ml/kg b.w.p.o.
6. The Group-V was considered as a Test III group and received the Ayurvedic Formulation with a dose of 3.6 ml/kg b.w.p.o.
7. Immunized the rat with 0.1 ml of 20% SRBCs in normal saline intraperitonially on 14th day of the study.
8. On 21st day, animals from all groups get challenged with 0.03 ml of 1% SRBCs in sub-plantar region of right hind paw.
9. Then the footpad reaction was assessed after 24 hrs. i.e. on 22nd day.
10. The Increase in the footpad oedema was measured with help of Vernier calliper.

Table 2: Grouping and Treatment Schedule for DTH Test

Sr. No.	Group	Test Substance	Dose
1	Group I	Control (Water)	10 ml/kg b.w, p.o.
2	Group II	Standard (Levamisole)	50 mg/kg b.w, p.o.
3	Group III	Ayurvedic Formulation (Test 1)	0.9 ml/kg b.w, p.o. twice a day
4	Group IV	Ayurvedic Formulation (Test 2)	1.8 ml/kg b.w, p.o. twice a day
5	Group V	Ayurvedic Formulation (Test 3)	3.6 ml/kg b.w, p.o. twice a day

- **Antigenic Material:**

Preparation of Sheep's RBCs:

The first step was to collect the Sheep's blood in sterile Alsever's solution in 1:1 proportion. In this the Alsever's solution used was freshly prepared at the time of it's use. Then the blood was kept in the refrigerator and processed for the preparation of SRBCs batch, by centrifugating it at 2000 rpm for 10 minutes and further washing it with physiological saline solution for 4 to 5 times. Then suspend it into buffered saline for further use.

Table No. 3: Composition of Alsever's Solution for DTH Test

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

- **Statistical Analysis:**

The result of the test was expressed as Mean Value ± SEM. The variation in a set of data was estimated by performing the one way analysis of variation (ANOVA) technique. The individual comparison of group mean value were done by Dunnet's Test.

P value < 0.05, were considered as statistically significant.

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[B] CARBON CLEARANCE TEST: (10 Days Model)

- **Purpose and Rationale:**

The phagocytic activity of reticulo-endothelial system will be assayed by carbon clearance test. The phagocytic index was calculated as the rate of carbon elimination by reticulo-endothelial system in carbon clearance test.

- **Procedure:**[12, 13, 14]

1. In this experimental model, animals were divided into five groups comprising of six animals in each group.
2. The Group-I was considered as a control group and received vehicle (water) only with a dose of 10 ml/kg b.w. p.o.
3. The Group-II was considered as a standard group and received the standard drug levamisole with a dose of 50 mg/kg b.w.p.o.
4. The Group-III was considered as a Test I group and received the Ayurvedic Formulation with a dose of 0.9 ml/kg b.w.p.o.
5. The Group-IV was considered as a Test II group and received the Ayurvedic Formulation with a dose of 1.8 ml/kg b.w.p.o.
6. The Group-V was considered as a Test III group and received the Ayurvedic Formulation with a dose of 3.6 ml/kg b.w.p.o.

Table 4: Grouping and Treatment Schedule for Carbon Clearance Test

Sr. No.	Group	Test Substance	Dose
1	Group I	Control (Water)	10 ml/kg b.w, p.o.
2	Group II	Standard (Levamisole)	50 mg/kg b.w, p.o.
3	Group III	Ayurvedic Formulation (Test 1)	0.9 ml/kg b.w, p.o. twice a day
4	Group IV	Ayurvedic Formulation (Test 2)	1.8 ml/kg b.w, p.o. twice a day
5	Group V	Ayurvedic Formulation (Test 3)	3.6 ml/kg b.w, p.o. twice a day

7. On 10th day, 3 hours after the last dose; all the animals of each group were given colloidal carbon suspension intravenously in a volume of 10 µl/ gm body weight of rat.
8. The blood sample (25 µl) were then withdrawn from the retro-orbital plexus under mild ether anaesthesia at 5 min and 15 min after injection of colloidal carbon ink lysed in 0.1% of sodium carbonate solution (3 ml).
9. Then the optical density was measured spectrophotometrically at 660 nm.
10. The Phagocytic activity was calculated using the following formula:

$$K = \frac{\text{Log } OD_1 - \text{Log } OD_2}{t_2 - t_1}$$

Where,

The OD_1 and OD_2 were the optical densities at time t_1 and t_2 respectively.

- **Preparation of carbon ink suspension:**

The Camlin Ink was diluted eight times with the saline solution and used for intravenous injection in carbon clearance test in a dose of 10 µl/gm body weight of the rat.[48]

- **Statistical Analysis:**

The result of the test was expressed as Mean Value ± SEM. The variation in a set of data was estimated by performing the one way analysis of variation (ANOVA) technique. The individual comparison of group mean value were done by Dunnet's Test.

P value < 0.05, were considered as statistically significant.

[C] NEUTROPHIL ADHESION TEST: (16 Days Model)

- **Purpose and Rationale:**

Increase the recruitment of neutrophil adhesion to nylon fibres was correlates with the process of Margination of cell in the blood vessels.

- **Procedure:**[12, 14, 16]

1. In this experimental model, animals were divided into five groups comprising of six animals in each group.
2. The Group-I was considered as a control group and received vehicle (water) only with a dose of 10 ml/kg b.w. p.o.

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3. The Group-II was considered as a standard group and received the standard drug levamisole with a dose of 50 mg/kg b.w.p.o.
4. The Group-III was considered as a Test I group and received the Ayurvedic Formulation with a dose of 0.9 ml/kg b.w.p.o.
5. The Group-IV was considered as a Test II group and received the Ayurvedic Formulation with a dose of 1.8 ml/kg b.w.p.o.
6. The Group-V was considered as a Test III group and received the Ayurvedic Formulation with a dose of 3.6 ml/kg b.w.p.o.
7. On 16th day of treatment, blood sample from all the groups were collected by puncturing the retro-orbital plexus under the mild anaesthesia.
8. The blood was collected in vials which are pre-treated with disodium EDTA and analysed for total leukocyte count (TLC) and differential leukocyte count (DLC).
9. After the initial blood count was over; the blood sample were collected with nylon fibre (80 mg/ml, previously sterilized by 95% of alcohol) for 15 minutes at 37^0C.
10. The incubated blood sample were further analysed for TLC and DLC.
11. The product of TLC and % Neutrophil was considered as Neutrophil Index and % Neutrophil Adhesion was calculated.

Table 5: Grouping and Treatment Schedule for Neutrophil Adhesion Test

Sr. No.	Group	Test Substance	Dose
1	Group I	Control (Water)	10 ml/kg b.w, p.o.
2	Group II	Standard (Levamisole)	50 mg/kg b.w, p.o.
3	Group III	Ayurvedic Formulation (Test 1)	0.9 ml/kg b.w, p.o. twice a day
4	Group IV	Ayurvedic Formulation (Test 2)	1.8 ml/kg b.w, p.o. twice a day
5	Group V	Ayurvedic Formulation (Test 3)	3.6 ml/kg b.w, p.o. twice a day

12. The % Neutrophil Adhesion was calculated as follows:

$$\% \text{ Neutrophil Adhesion} = \frac{NI_U - NI_T}{NI_U}$$

Where,

NI_U = Neutrophil Index of Untreated Blood Sample.

NI_T = Neutrophil Index of Nylon fibre Treated Blood Sample.

- **Statistical Analysis:**

The result of the test was expressed as Mean Value ± SEM. The variation in a set of data was estimated by performing the one way analysis of variation (ANOVA) technique. The individual comparison of group mean value were done by Dunnet's Test.

P value < 0.05, were considered as statistically significant.

IN-VITRO METHODS

[A] T-CELL POPULATION TEST: (11 DAYS MODEL)

- **Purpose and Rationale:**

The increase in number of lymphocyte formation and rosette formation in T cell Population test indicates its effect on cell mediated immunity and T cell activity.

- **Procedure:**[17, 18]

1. In this experimental model animals were divided into five groups comprising of six animals in each group.
2. The Group-I was considered as a control group and received vehicle (water) only with a dose of 10 ml/kg b.w. p.o.
3. The Group-II was considered as a standard group and received the standard drug levamisole with a dose of 50 mg/kg b.w.p.o.
4. The Group-III was considered as a Test I group and received the Ayurvedic Formulation with a dose of 0.9 ml/kg b.w.p.o.
5. The Group-IV was considered as a Test II group and received the Ayurvedic Formulation with a dose of 1.8 ml/kg b.w.p.o.
6. The Group-V was considered as a Test III group and received the Ayurvedic Formulation with a dose of 3.6 ml/kg b.w.p.o.

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Table 6: Grouping and Treatment Schedule for T cell Population Test

Sr. No.	Group	Test Substance	Dose
1	Group I	Control (Water)	10 ml/kg b.w, p.o.
2	Group II	Standard (Levamisole)	50 mg/kg b.w, p.o.
3	Group III	Ayurvedic Formulation (Test 1)	0.9 ml/kg b.w, p.o. twice a day
4	Group IV	Ayurvedic Formulation (Test 2)	1.8 ml/kg b.w, p.o. twice a day
5	Group V	Ayurvedic Formulation (Test 3)	3.6 ml/kg b.w, p.o. twice a day

7. Antigen Challenge: On 0^{th} day of treatment, all groups were sensitized with 0.1 ml of SRBCs containing 1 x 10^8 cells, i.p.
8. On 11^{th}dayof treatment, blood was collected from the retro-orbital plexus and anticoagulated with Alsever's Solution in separate test tubes.
9. Then the test tubes containing blood were kept in sloping position (45^0) at 37^0C for 1 hour. The RCBs are allowed to settle at bottom and supernatant was collected from each test tube by using micropipette which contains lymphocytes.
10. Then 50 µl of blood lymphocyte suspension and 50 µl of SRBCs were mixed in test tube and incubated.
11. The resultant suspension was centrifuged at 200 rpm for 5 minutes and kept in refrigerator at 4^0C for 2 hours.
12. The supernatant fluid was removed and one drop of cell suspension was placed on a glass slide. Total lymphocyte were counted and a lymphocyte binding with three or more erythrocytes i.e. RBCs was considered as rosette and number of rosettes was calculated.

- **Antigenic Material:**

Preparation of Sheep's RBCs:

The first step was to collect the Sheep's blood in sterile Alsever's Solution in 1:1 proportion. In this the Alsever's Solution used was freshly prepared at the time of its use. Then the blood was kept in the refrigerator and processed for the preparation of SRBCs batch, by centrifugating it at 2000 rpm for 10 minutes and further washing it with physiological saline solution for 4 to 5 times. Then suspend it into buffered saline for further use.

Table 7: Composition of Alsever's Solution for T cell Population Test

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

- **Statistical Analysis:**

The result of the test was expressed as Mean Value ± SEM. The variation in a set of data was estimated by performing the one way analysis of variation (ANOVA) technique. The individual comparison of group mean value were done by Dunnet's Test.

P value < 0.05, were considered as statistically significant.

III. RESULTS AND DISCUSSION

IN-VIVO METHODS

DELAYED TYPE OF HYPERSENSITIVITY TEST

The effect of various doses of Marketed Ayurvedic Formulation (i.e.) Jambhasava on cell mediated immune response by DTH induced Foot Pad Oedema is shown in below table [Table No. 8]. The Ayurvedic formulation treated groups

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significantly showed increase in footpad oedema (P<0.0001) when compared with control group.

Table 8:- Result of DTH

Sr. No.	Group	Treatments	Dose and Route of Administration	Mean Difference in Paw Oedema (mm) [Mean ± SEM]
1	I	Control (Water)	10 ml/kg (P.O.)	2.677 ± 0.01 (100%)
2	II	Standard (Levamisole)	50 mg/kg (P.O.)	4.443 ± 0.02**** (165.96%)
3	III	Marketed Ayurvedic Formulation (0.9 ml/kg b.w.)	0.9 ml/kg twice a day (P.O.)	2.715 ± 0.02ns (101.42%)
4	IV	Marketed Ayurvedic Formulation (1.8 ml/kg b.w.)	1.8 ml/kg twice a day (P.O.)	3.903 ± 0.008**** (145.79%)
5	V	Marketed Ayurvedic Formulation (3.6 ml/kg b.w.)	3.6 ml/kg twice a day (P.O.)	4.328 ± 0.02**** (161.67%)

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

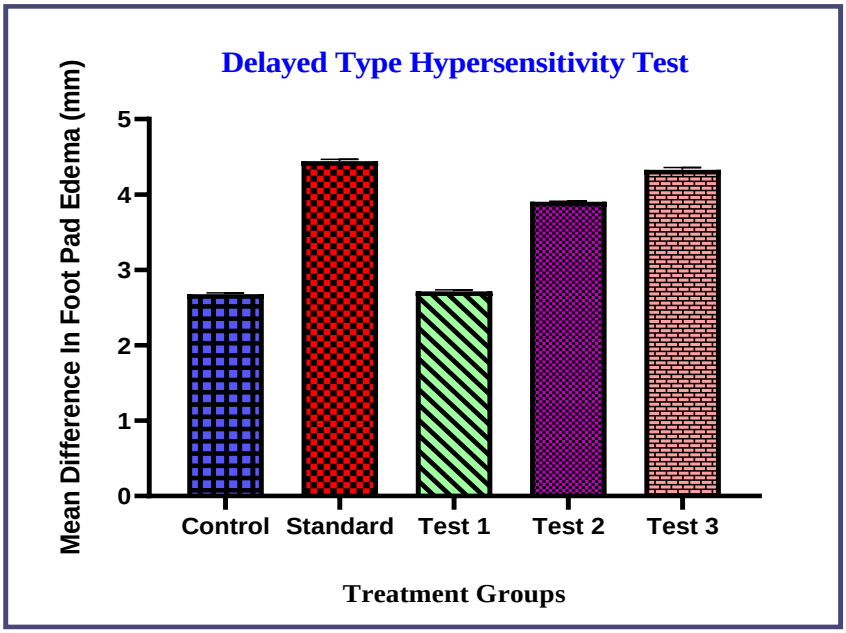

Fig. No. 1:- Graphical Representation of DTH

Where,
Control:- Water (10 ml/kg)
Standard:-Levamisole (50 mg/ml)
Test 1:- Marketed Ayurvedic Formulation (0.9 ml/kg b.w.)
Test 2:- Marketed Ayurvedic Formulation (1.8 ml/kg b.w.)
Test 3:- Marketed Ayurvedic Formulation (3.6 ml/kg b.w.)

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CARBON CLEARANCE TEST:

The effect of various doses of Marketed Ayurvedic Formulation (i.e.) Jambhasava on phagocytic activity by Carbon Clearance Test is shown in below table [Table No. 9]. The phagocytic activity of reticulo-endothelial system is generally measured by the rate of removal of carbon particle from the blood stream. The Ayurvedic Formulation treated groups significantly showed increase in footpad oedema (P<0.0001) when compared with control group. The Test 1 group i.e. Ayurvedic Formulation treated group with dose of 0.9 ml/kg b.w. showed increase in phagocytic index (0.03678 ± 0.0015) which indicates to the stimulation of reticulo-endothelial system to 117.62% when compared with control group. The Test 2 group i.e. Ayurvedic Formulation treated group with dose of 1.8 ml/kg b.w. also showed increase in phagocytic index (0.0472 ± 0.0012) which indicates to the stimulation of reticulo-endothelial system to 150.94% when compared with control group. The Test 3 group i.e. Ayurvedic Formulation treated group with dose of 3.6 ml/kg b.w. showed the maximum increase in phagocytic index (0.05513 ± 0.0013) which indicates to the stimulation of reticulo-endothelial system to 176.30% when compared with control group.

Table 9:- Result of Carbon Clearance Test

Sr. No.	Group	Treatments	Dose and Route of Administration	Absorbance		Phagocytic Index Mean [Mean ± SEM]
				5 min	15 min	
1	I	Control	10 ml/kg (P.O.)	0.173	0.083	0.03127 ± 0.0017 (100%)
2	II	Standard (Levamisole)	50 mg/kg (P.O.)	0.161	0.037	0.06337 ± 0.0016**** (202.65%)
3	III	Marketed Ayurvedic Formulation (0.9 ml/kg b.w.)	0.9 ml/kg twice a day (P.O.)	0.177	0.077	0.03678 ± 0.0015* (117.62%)
4	IV	Marketed Ayurvedic Formulation (1.8 ml/kg b.w.)	1.8 ml/kg twice a day (P.O.)	0.168	0.057	0.0472 ± 0.0012**** (150.94%)
5	V	Marketed Ayurvedic Formulation (3.6 ml/kg b.w.)	3.6 ml/kg twice a day (P.O.)	0.168	0.047	0.05513 ± 0.0013**** (176.30%)

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

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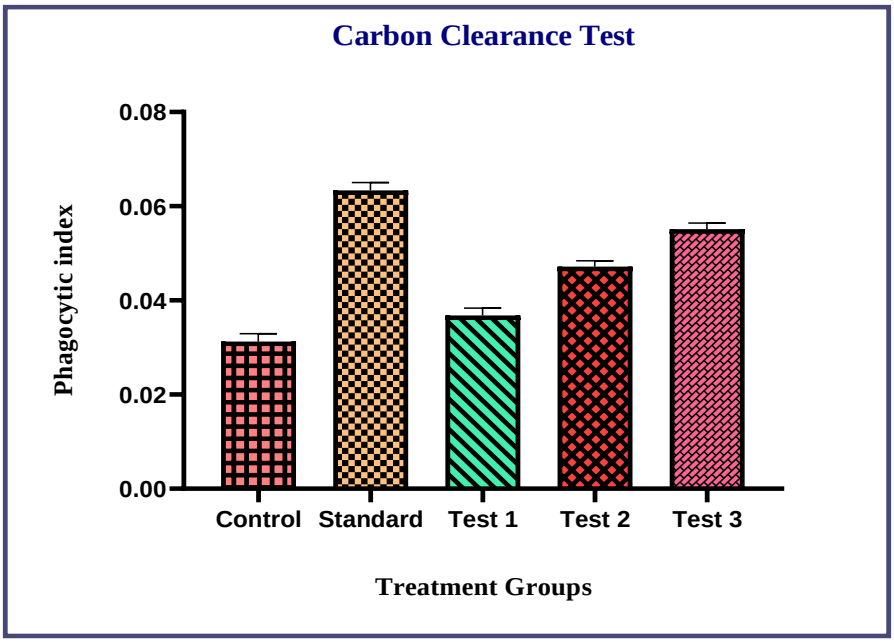

Fig. No. 2:- Graphical Representation of Carbon Clearance Test

Where,
Control:- Water (10 ml/kg)
Standard:-Levamisole (50 mg/ml)
Test 1:- Marketed Ayurvedic Formulation (0.9 ml/kg b.w.)
Test 2:- Marketed Ayurvedic Formulation (1.8 ml/kg b.w.)
Test 3:- Marketed Ayurvedic Formulation (3.6 ml/kg b.w.)

NEUTROPHIL ADHESION TEST

The effect of various doses of Marketed Ayurvedic Formulation (i.e.) Jambhasava on neutrophil activation by Neutrophil Adhesion Test is shown in above table [Table No. 10]. Cytokines are secreted by activated immune cell for margination and extravasation of the phagocytes mainly in Polymorphonuclear neutrophils.The percentage neutrophils adhesion was significantly ($P<0.0001$) increases by Ayurvedic Formulation treated groups when they were compared with the control group. The Test 1 group i.e. Ayurvedic Formulation treated group with dose of 0.9 ml/kg b.w. showed increase in % Neutrophil Adhesion (51.3 ± 0.6421) which indicates to the stimulation Neutrophil Adhesion to nylon thread to 188.18% when compared with control group. The Test 2 group i.e. Ayurvedic Formulation treated group with dose of 1.8 ml/kg b.w. also showed increase in % Neutrophil Adhesion (54.54 ± 0.8068)which indicates to the stimulation Neutrophil Adhesion to nylon thread to 200.07% when compared with control group. The Test 3 group i.e. Ayurvedic Formulation treated group with dose of 3.6 ml/kg b.w. showed the maximum increase in % Neutrophil Adhesion (61.16 ± 0.7795) which indicates to the stimulation Neutrophil Adhesion to nylon thread to 224.35% when compared with control group.

Table 10:- Result of Neutrophil Adhesion Test

Sr. No.	Group	Treatments	Dose and Route of Administration	% Neutrophil Adhesion Mean [Mean ± SEM]
1	I	Control (Water)	10 ml/kg (P.O.)	27.26 ± 1.58 (100%)
2	II	Standard (Levamisole)	50 mg/kg (P.O.)	66.86 ± 1.993**** (245.26%)

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3	III	Marketed Ayurvedic Formulation (0.9 ml/kg b.w.)	0.9 ml/kg twice a day (P.O.)	51.3 ± 0.6421**** (188.18%)
4	IV	Marketed Ayurvedic Formulation (1.8 ml/kg b.w.)	1.8 ml/kg twice a day (P.O.)	54.54 ± 0.8068**** (200.07%)
5	V	Marketed Ayurvedic Formulation (3.6 ml/kg b.w.)	3.6 ml/kg twice a day (P.O.)	61.16 ± 0.7795**** (224.35%)

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

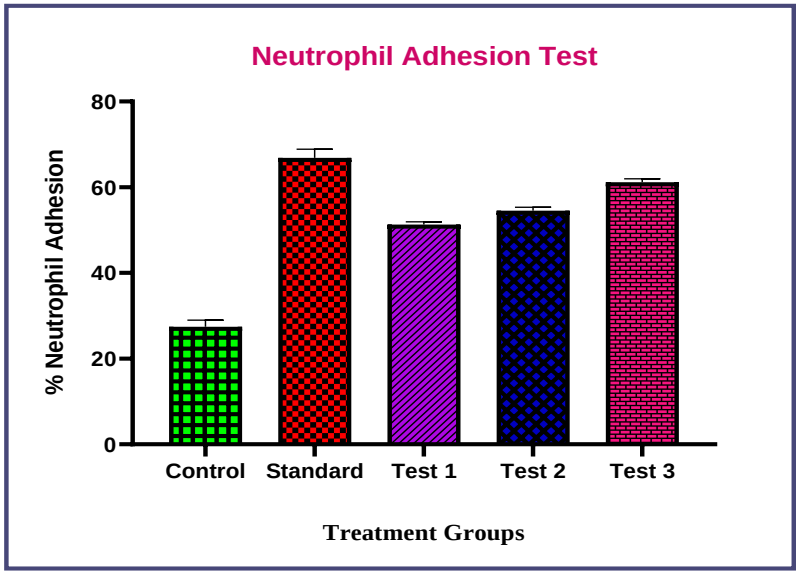

Fig. No. 3:- Graphical Representation of Neutrophil Adhesion Test

Where,
Control:- Water (10 ml/kg)
Standard:-Levamisole (50 mg/ml)
Test 1:- Marketed Ayurvedic Formulation (0.9 ml/kg b.w.)
Test 2:- Marketed Ayurvedic Formulation (1.8 ml/kg b.w.)
Test 3:- Marketed Ayurvedic Formulation (3.6 ml/kg b.w.)

IN-VITRO METHODS
T CELL POPULATION TEST

In present study, the effect of various doses of Marketed Ayurvedic Formulation (i.e.) Jambhasava on Lymphocyte formation by T Cell Population Test is shown in below table [Table No.11]. The total lymphocyte countwas significantly (P<0.0001) increases by Ayurvedic Formulation treated groups when they were compared with the control group. The Test 1 group i.e. Ayurvedic Formulation treated group with dose of 0.9 ml/kg b.w. showed increase in total lymphocyte count (1067 ± 104.7) which indicates to the stimulation of lymphocyte formation to 153.52% when compared with control group. The Test 2 group i.e. Ayurvedic Formulation treated group with dose of 1.8 ml/kg b.w. also showed increase in total lymphocyte count (1657 ± 83.47) which indicates to the stimulation of lymphocyte formation to 238.41% when compared with control group. The Test 3 group i.e. Ayurvedic Formulation treated group with dose of 3.6 ml/kg b.w. showed the maximum increase in total lymphocyte count (2041 ± 157.1) which indicates

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to the stimulation of lymphocyte formation to 293.66% when compared with control group.

Table 11:- Result of Total Lymphocyte Count for T Cell Population Test

Sr. No.	Group	Treatments	Dose and Route of Administration	Total Lymphocyte Count Mean (per mm^3) [Mean ± SEM]
1	I	Control (Water)	10 ml/kg (P.O.)	695 ± 93.52 (100%)
2	II	Standard (Levamisole)	50 mg/kg (P.O.)	2022 ± 126.4 **** (290.93%)
3	III	Marketed Ayurvedic Formulation (0.9 ml/kg b.w.)	0.9 ml/kg twice a day (P.O.)	1067 ± 104.7 ^{ns} (153.52%)
4	IV	Marketed Ayurvedic Formulation (1.8 ml/kg b.w.)	1.8 ml/kg twice a day (P.O.)	1657 ± 83.47 **** (238.41%)
5	V	Marketed Ayurvedic Formulation (3.6 ml/kg b.w.)	3.6 ml/kg twice a day (P.O.)	2041 ± 157.1 **** (293.66%)

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

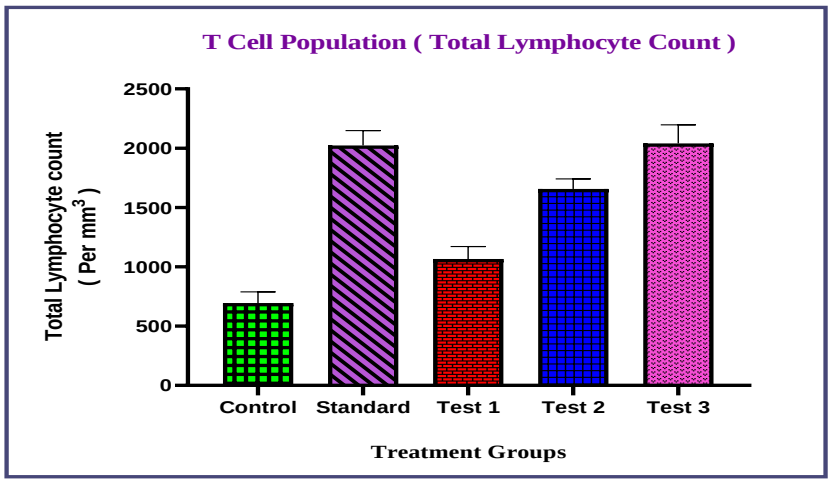

Fig. No. 4:- Graphical Representation of Total Lymphocyte Count for T Cell Population Test

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Where,
Control:- Water (10 ml/kg)
Standard:-Levamisole (50 mg/ml)
Test 1:- Marketed Ayurvedic Formulation (0.9 ml/kg b.w.)
Test 2:- Marketed Ayurvedic Formulation (1.8 ml/kg b.w.)
Test 3:- Marketed Ayurvedic Formulation (3.6 ml/kg b.w.)

1) Observation Table For Rosettes Count:-

In present study, the effect of various doses of Marketed Ayurvedic Formulation (i.e.) Jambhasava on rosette formation by T Cell Population Test is shown in below table [Table No. 12]. The rosette count was significantly (P<0.0001) increases by Ayurvedic Formulation treated groups when they were compared with the control group.

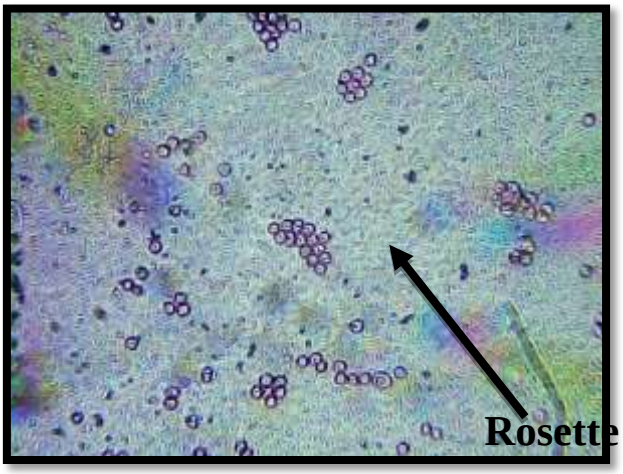

Fig. No. 5:- Rosette in T Cell Population Test

The Test 1 group i.e. Ayurvedic Formulation treated group with dose of 0.9 ml/kg b.w. showed increase in Rosette count (13 ± 1.778) which indicates to the stimulation of Rosette Formation to 118.18% when compared with control group. The Test 2 group i.e. Ayurvedic Formulation treated group with dose of 1.8 ml/kg b.w. also showed increase in Rosette count (16 ± 1.145) which indicates to the stimulation of Rosette formation to 145.45% when compared with control group. The Test 3 group i.e. Ayurvedic Formulation treated group with dose of 3.6 ml/kg b.w. showed the maximum increase in Rosette count (23 ± 1.544) which indicates to the stimulation of Rosette formation to 209.09% when compared with control group.

Table 12:- Result of Rosettes Count for T Cell Population Test

Sr. No.	Group	Treatments	Dose and Route of Administration	Rosettes Count Mean [Mean ± SEM]
1	I	Control (Water)	10 ml/kg (P.O.)	11 ± 1.145 (100%)
2	II	Standard (Levamisole)	50 mg/kg (P.O.)	$27 \pm 1.453^{****}$ (245.45%)
3	III	Marketed Ayurvedic Formulation (0.9 ml/kg b.w.)	0.9 ml/kg twice a day (P.O.)	13 ± 1.778^{ns} (118.18%)
4	IV	Marketed Ayurvedic Formulation	1.8 ml/kg twice a day (P.O.)	16 ± 1.145^{ns} (145.45%)

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		(1.8 ml/kg b.w.)		
5	V	Marketed Ayurvedic Formulation (3.6 ml/kg b.w.)	3.6 ml/kg twice a day (P.O.)	23 ± 1.544**** (209.09%)

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

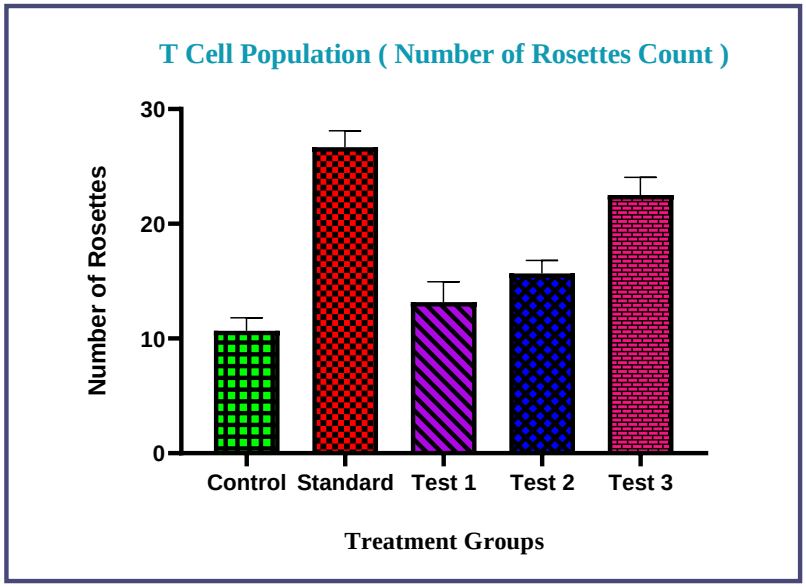

Fig. No. 6:- Graphical Representation of Rosette Count for T Cell Population Test

Where,
Control:- Water (10 ml/kg)
Standard:-Levamisole (50 mg/ml)
Test 1:- Marketed Ayurvedic Formulation (0.9 ml/kg b.w.)
Test 2:- Marketed Ayurvedic Formulation (1.8 ml/kg b.w.)
Test 3:- Marketed Ayurvedic Formulation (3.6 ml/kg b.w.)

IV. DISCUSSION

In present scenario, herbal therapeutics are gaining momentum in pharmacological applications and as a molecular targets in the drug development. The emerging trend in rising incident of diseases and associated complications with commercial medications poses a serious threat to mankind. The naturopathic treatment via. herbal medicaments offer respite from such problems as well as they are comparatively safe with less side effects. These medicinal herbs were served as a basic platform for ancient Ayurvedic system of medicine. They contains valuable source of number of bioactive compounds; which helps to cure diseases by activating on body's natural function.[19]

The immunomodulatory agents of plant and animal origin enhances or suppress the immune responsiveness of an organism against a pathogen or auto-immune diseases by acting on immune system. The modulation of immune responses to alleviate the diseases has been interest for many years. So, immunostimulation and immunosuppressant both needed to be tackle in order to regulate the normal immunological functions. As plants individually contain various phytochemical constituents which may effective in different pathophysiological conditions. In the market number of herbal drugs and formulations are available which where claimed for its specific functions but; they may be also effective in different other conditions. Hence, it becomes very

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important to screen such a formulation with pharmacological significance for investigation of unexploited potential in the formulation which may leads to a base for the development of more effective therapeutics. Thus, the present study was designed to explore the possible immunomodulatory effects of Ayurvedic formulation Jambhasava.

In present study, Delayed Type of Hypersensitivity Test, Carbon Clearance Test, Neutrophil Adhesion Test and T cell Population Test were selected for evaluation of the immunomodulatory effect of Ayurvedic formulation Jambhasava.

The Delayed Type Hypersensitivity (DTH) test determines the activity of the cellular immune response, especially T lymphocytes and macrophages. The DTH is a part of the process of graft rejection, tumour immunity and most important, immunity against many intracellular infectious microorganisms, especially those causing chronic diseases such as tuberculosis. The DTH response requires special recognition of the antigen administered T lymphocytes which then proliferate to form lymphoblasts and release lymphokines and other cytokines, attracting more scavenger cells to the site of reaction. The release of cytokines from activated T lymphocytes will increase macrophage activity and enzyme concentration to accelerate the elimination process. In present study SRBCs, served as a sensitizer substance which in combination with skin protein produce antigenicity and generate hypersensitivity reaction in rat. These SRBCs antigen induces response in which Th1 cells secrete a number of cytokines that; activate the macrophages and other nonspecific inflammatory mediators. The delay in response time reflects the time required for cytokines to induce macrophage activation.[20, 21] In present research work, it was found that the Jambhasava at various doses i.e. 0.9 ml/kg b.w. [2.715 ± 0.02](101.42%), 1.8 ml/kg b.w. [3.903 ± 0.008] (145.79%), 3.6 ml/kg b.w.[4.328 ± 0.02] (161.67%) etc. and Levamisole [4.443 ± 0.02] (165.96%) causes the increase in footpad oedema after 24 hrs of the exposure to antigenic material i.e. SRBCs, when compared with control [2.677 ± 0.01] (100%). This indicated stimulation of cell mediated immunity and the increase in response occurs as dose increases.

The carbon clearance test was done to evaluate the effect of drugs on the reticulo endothelial system. The reticulo-endothelial system (RES) is a diffuse system consisting of phagocytic cells. Cells of the RES play a vital role in the clearance of particles from the bloodstream. When colloidal carbon particles in the form of ink were injected directly into the systemic circulation, the rate of clearance of carbon from the bloodstream by macrophage is governed and phagocytic index was calculated.[22] In present research work, phagocytic index of Ayurvedic formulation Jambhasava at various doses i.e0.9 ml/kg b.w. [0.03678 ± 0.0015] (117.62%), 1.8 ml/kg b.w. [0.0472 ± 0.0012] (150.94%), 3.6 ml/kg b.w. [0.05513 ± 0.0013] (176.30%) etc. and levamisole [0.06337 ± 0.0016] (202.65%) causes increase in phagocytic index, when compared with [0.03127 ± 0.0017] (100%). This increase in phagocytic index indicates that there was stimulation of reticulo-endothelial system and the response increases as dose of formulation increases.

Neutrophils are related to the cell-mediated immunity and help in the clearance of foreign particles by recognition and movement toward the particle, phagocytosis, and abolishing the foreign particle. The movement of neutrophils towards the foreign body is the first and most important step in phagocytosis process. Cytokines are secreted by activated immune cells for Margination and extravasation of phagocytes mainly polymorphonuclear neutrophils.[23] Here, experimentally the activation of neutrophils were studied by neutrophil adhesion test. Our results showed that jambhasava at various doses and levamisole were found to be stimulate neutrophil chemotaxis and increase in % neutrophil adhesion when compare with the control group. Further observed that, the jambhasava at dose of 3.6 ml/kg b.w. [61.16 ± 0.7795] (224.35%) shows highest % of neutrophil adhesion than other doses at 1.8 ml/kg b.w. [54.54 ± 0.8068] (200.07%) and 0.9 ml/kg b.w. [51.3 ± 0.6421] (188.18%) when it compared with vehicle control [27.26 ± 1.58] (100%).

The increase in rosette formation and lymphocyte formation in T cell population Test indicates the effect of Ayurvedic Formulation Jambhasava at various doses, on cell mediated immunity.[24] It shows dose dependant activity profile of the formulation. Further observed that,thejambhasava at dose of 3.6 ml/kg b.w. [2041 ± 157.1] (293.66%) shows maximum mean lymphocyte count than other doses at 1.8 ml/kg b.w. [1657 ± 83.47] (238.41%), 0.9 ml/kg b.w. [1067 ± 104.7] (153.52%) and levamisole [2022 ± 126.4] (290.93%) when it compared with control group [695 ± 93.52] (100%).

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The formulation may activate the CD4 and CD8 cells which influence T-cell mechanism results in T-cell immune response significantly.

In this study, the overall order of immunomodulatory activity was established as:

Levamisole>Jambhasava (3.6 ml/kg b.w.) >Jambhasava (1.8 ml/kg b.w.) >Jambhasava (0.9 ml/kg b.w.) > Control (water)

In the present study, it was revealed that the immunomodulatory activity of Jambhasava is due to the presence of multiple immunomodulatory ingredients in its formulation (Syzygium cumini, Azadirachta indica, Trigonella Foenum, Gymnema sylvestre, Terminalia bellirica, Picrorhiza kurrooa, Tribulus terrestris, Asparagus racemosus, Zingiber officinale, Withania somnifera, Cinnamomum verum, Boerhavia diffusa), when compared with control it gives a dose dependent effect profile and the dose at 3.6 ml/kg b.w. gives a more potent immunomodulatory effect.

V. CONCLUSION

The present demonstrate that, jambhasava at dose of 0.9 ml/kg b.w, 1.8 ml/kg b.w. and 3.6 ml/kg b.w. shows potent immunomodulatory effect on both humoral as well as cell mediated immunity which is due to;

- It enhances the capacity of monocytes macrophages system.
- By the activation of reticulo-endothelial system.
- Increase in the Polymorphonuclear neutrophils and their activation leading to margination in the blood vessels.
- Activation and proliferation of T lymphocytic cells.

Further, on the basis of the results obtained in current study; it can be concluded that, as dose of jambhasava increases the immunomodulatory activity simultaneously increases and it may be used further as a potential therapeutic candidate in several immunosuppressed clinical conditions. The overall order of immunomodulatory activity is;

Levamisole >Jambhasava (3.6 ml/kg b.w.) >Jambhasava (1.8 ml/kg b.w.) >Jambhasava (0.9 ml/kg b.w.) > Control (water)

However, more exhaustive work needs to be performed to substantiate the claim.

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