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Effect of polyherbal formulation for immunomodulation in immunosuppressed mice

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Abstract
A Polyherbal Formulation is prepared by using Methanolic extract of *Sphaeranthus indicus* flowerheads, fruits of *Piper nigrum*, Hydroalcoholic extracts of *Withania somnifera*, *Ocimum sanctum*, *Centella asiatica* for immunomodulatory activity. The effects of 100mg, 200mg, 400mg were screened on humoral and cellular immunity i.e. HA titer and Delayed Type Hypersensitivity on immunosuppressed mice. The present study demonstrated that the formulation200mg, 400mg have synergistic immunostimulant activity in HA titer and DTH, and it also showed protection against cyclophosphamide induced immunoosupression.

Keywords: Immunomodulation, immunosuppression, *Sphaeranthus indicus*, *Withania somnifera*, *Piper nigrum*, Haemagglutionation

1. Introduction
Plants are complex mixtures of compounds and no single compound can provide the desired activity. Some compound potentiate a desired therapeutic action, while others reinforce the same and yet others interact to neutralize and counteract any possible side effect that may exist. Therefore several plants with the common desired activities are selected so that the final formulation will have a concentrated desired activity.

Herbal preparations have been used as an immunomodulator in traditional medicine8. Several herbal preparations that can enhance the body immune status are extensively used in the indigenous system of medicine. There is an upsurge in the clinical usage of indigenous drugs because they are associated with fewer side effects [1].

One of the main approaches in Ayurvedic medicine is to "increase the body's natural resistance to the disease/stress" known as "Rasayana" (Rejuvenation). Some of these are believed to promote positive health and maintain organic resistance against infection by re-establishing the body's equilibrium and conditioning the body tissues. Rasayanas are a group of non toxic herbal drug preparations which are used to improve the general health by stimulating body's immunity [2]. Many plants have been extensively used as 'Rasayana' drugs in Ayurveda for the management of neurodegenerative diseases, as rejuvenators, immunomodulators, aphosidiac and nutritional supplements [3]. Some polyherbal formulations such as Immunocin, Chyvangrans, Gerifort, RV0818, Immune - 2119 exhibits immunomodulatory activity and showed synergistic effects but all these formulation contains more than six ingredients and this make standardization difficult.

Sphaeranthus indicus Linn. is a branched herb with purple flowers that grows abundantly in rice field and distributed throughout India. *Sphaeranthus indicus* showed immunomodulatory [7], antimicrobial, antibacterial, anxiolytic, wound healing action activities. Phytoconstituents isolated from *S. indicus* were eudesmanolides, isoflavonoids, 7-hydroxy eudesmanolides [22], sterol glycoside, essential oils.

Withania somnifera is the plant distributed throughout the dried subtropical regions of India. The roots contains seven with anolides with beta sitosterol glucoside, stigmasterol glucoside a, b. The plant is used as rasayana, aphrodisiac, antioxidant, alterative, tonic, bronchitis, immunomodulator [10, 17].

Ocimum sanctum is a sacred plant distributed throughout India. It contains alkaloids, eugenol, glycosides, saponins, tannins. It is given as rasayana and used as antidote in poison,

antistress agent, carminative, antihelmentic, tonic, aphrodisiac, immunomodulator [20], laxative, expectorant, bronchitis [21].

Centella asiatica is indigenous to Southeast Asia, India, Srilanka. It contains triterpenoid saponins i.e. asiaticoside, madecassoside, brahminoside, bramhoside, alkaloids, essential oils. It has been used as promoter of strength, nervine tonic, promotes longevity, in mental disorders. It is major intellect promoting rasayana [13].

Piper nigrum is mostly cultivated in hot and moist parts of India. It contains essential oils, alkaloids such as piperine, pipericine, piperidine, glycosides, and flavones. It is used as anti- inflammatory, antidepressant, antispasmodic, antihypertensive, and antioxidant. It is mainly used as bioavailability enhancer in "Trikatu" formulation [14]. Piperine is a major chemical antistress agent, carminative, antihelmentic, tonic, aphrodisiac, immunomodulator [20], laxative, expectorant, bronchitis [21].

Centella asiatica is indigenous to Southeast Asia, India, Srilanka. It contains triterpenoid saponins i.e. asiaticoside, madecassoside, brahminoside, bramhoside, alkaloids, essential oils. It has been used as promoter of strength, nervine tonic, promotes longevity, in mental disorders. It is major intellect promoting rasayana [13].

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Thus, the present study was to evaluate immunomodulatory effect of this combination in immunocompromised mice.

2. Materials and Methods

2.1 Animals

Swiss Albion mice of either sex weighing between 18 to 25 gm were used. Animals were housed under standard conditions of temperature, 12hr/12hr, light/dark cycle and fed with standard pellet diet and tap water. All the experiments were approved and conducted as per guidelines of Institutional Animal Ethical Committee (IAEC).

2.2 Plant Material and extract preparation

The plant *Ocimum sanctum, Centella asiatica* was obtained from medicinal garden of Dr. D.

Y. Patil Institute of Pharmaceutical Sciences and Research, Pimpri, Pune and Dried flower heads of *Sphaeranthus indicus*, dried fruits of *Piper nigrum* was obtained from local market of Pune. All plant materials were authenticated from Botanical Survey of India, Koregaon Road, Pune.

The methanolic and hydroalcoholic extracts were prepared by Soxhlet extraction and were subjected to Qualitative chemical analysis such as TLC, HPTLC.

The extracts were combined as parts of extracts

S. indicus: W. somnifera: O. sanctum: C. Asiatica: P. nigrum (1:1:1:1:0.25)

The extracts were mixed with1% sodium carboxy methyl cellulose and different doses were prepared as 100mg, 200mg, and 400mg/kg. The control animals were given an equivalent volume of Sodium carboxy methyl cellulose as a vehicle. Group II is a Cyclophospahamide treated control. Cyclophosphamide was used as a standard immunosuppressant.

2.3 Antigen

Fresh blood was collected from sheep sacrificed in local slaughterhouse. Sheep red blood cells (SRBC's) were washed three times in normal saline and adjusted to a concentration 20% for immunization and 1% for challenge [11].

2.3.1 Antigen challenge

On '0' day of the study, mice from all the groups (i.e. group I to V) were immunized with 0.1ml sheep RBC's (20%) intraperitoneally [16]

3. Methods

3.1 Humoral Antibody (H.A.) titer: (Humoral immune response)

The method described by Bafna and Mishra, 2006 was adopted [16]. The animals were divided into five groups consisting of six animals each. All the treatment groups were treated with the respective treatment as shown in Table [1]. Group I (Control group) and Group II (Cyclophosphamide treated Control) received 1.0% sodium carboxy methyl cellulose solution as a vehicle for a period of 7 days. Group III – V were given the formulation daily for 7 days. The animals of Group II – V were treated with Cyclophosphamide (50mg/ kg, p.o.) on 4th, 5th and 6th day, 1hour after the administration of respective treatment.

The animals were immunized by injecting 0.1ml of 20% of fresh sheep red blood cells suspension intraperitoneally on day 0. Blood samples were collected from all the animals separately by retro orbital puncture on day +7 and serum was seperated. Antibody levels were determined by haemagglutination technique. Briefly, equal volumes pf individual serum samples of each group were pooled. Two fold dilutions of the pooled serum samples were made in 25 l of normal saline in microtitration plate and to that 25 l of SRBC (1%) were added to each of these dilutions and the plates were incubated at room temperature for one hour and then observed for haemagglutination under microscope. The reciprocal of the highest dilution of the test serum giving agglutination was taken as the antibody titer [15].

Table 1: Protocol for Antibody titer in immunosuppressed mice

Sr. No.	Groups	Treatment and Dose/Day	Treatment Days
1	Control	1% NaCMC	1-7
2	Cyclophosphamide	50mg/kg (p.o.)	4th, 5th, 6th
3	Formulation 100 + CP	100mg/kg (p.o.)	1-7 On 4th, 5th, 6th
4	Formulation 200 + CP	200mg/kg (p.o.)	1-7 On 4th, 5th, 6th
5	Formulation 400 + CP	400mg/kg (p.o.)	1-7 On 4th, 5th, 6th

3.2 Cellular mediated immune response

3.2.1 Delayed Type Hypersensitivity (DTH) response

Foot pad edema in mice was used for detection of cellular immune response. The animal were divided into five groups of six animals each. All the groups were treated with their respective treatment as described in Table 2. On 0 day, all the groups I to V were immunized with SRBC (20%, s.c.) in normal saline. The animals of Group II to V were ttreated with cyclophosphamide (50mg/kg, p.o.) on 4th, 5th, 6th day, and 1hr. after the administration of the respective treatment. On day 7th all animal from all the groups were challenged with injection of 1% sheep RBC in the subplanatar region of right hind paw in the volume of 20 l. footpad reaction was assessed after 24 hr. and 48hr. i.e. on day 8thand 9th, in terms of increase in the thickness of footpad due to edema caused as a result of hypersensitivity reaction. The difference between the pre and post challenge foot thickness express in mm was taken as a measure of delayed type hypersensitivity (DTH) [16].

Table 2: Protocol for delayed type hypersensitivity (DTH) in immunosuppressed mice

Sr. No.	Groups	Treatment and Dose/Day	Treatment Days
1	Control	1% NaCMC	1-7
2	Cyclophosphamide	50mg/kg (p.o.)	4th, 5th, 6th
3	Formulation 100 + CP	100mg/kg (p.o.)	1-7 On 4th, 5th, 6th
4	Formulation 200 + CP	200mg/kg (p.o.)	1-7 On 4th, 5th, 6th
5	Formulation 400 + CP	400mg/kg (p.o.)	1-7 On 4th, 5th, 6th

4. Statistical Analysis

Test drug treated groups were compared with Cyclophosphamide group. Statistically analysed by one way ANOVA followed by Tukey – Kramar multiple comparisons test. Values are expressed as Mean ± S.M.E. with the level of significance set at $p<0.05$.

5. Results and Discussion

5.1 Effect of formulation on Humoral Antibody Titer in immunosuppressed mice

Humoral response to SRBC's was measured as primary antibody titer as show in Table 3. Primary antibody titre in control group was 90.666 ± 17.364. In cyclophosphamide group it was 34.666 ± 6.422. On 7th day in the group of mice with normal immune status did not showed significant rise in antibody titer except formulation 400mg/kg administration produced a significant rise in HA titer ($p<0.01$). Formulation 200mg/kg shoed rise in antibody titer but it is statistically not significant. All groups were compared with cyclophosphamide group.

Table 3: Effect of formulation on Antibody titer in immunosuppressed mice

Sr. No.	Groups	Dose/Day	Antibody Titre Mean ± S.E.M.
1	Control	1% NaCMC	90.666 ± 17.364
2	Cyclophosphamide	50mg/kg	34.666 ± 6.422##
3	Formulation 100	100mg/kg	96 ± 35.054
4	Formulation 200	200mg/kg	122.666 ± 31.372
5	Formulation 400	400mg/kg	170.666 ± 26.985**

Values are expressed as Mean ± SME, n = 6, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Test drug treated groups were compared with cyclophosphamide Group (Statistically analyzed by one way ANOVA followed by Tukey – Karmar multiple comparisons test).

5.2 Effect of formulation on Delayed Type Hypersensitivity

Footpad edema in DTH response was performed by injecting SRBCs into subplanatar region of hind paw of mice, the degree of footpad thickness was measured using digital vernier caliper. The result shown in the Table 2 indicates that the formulation 200mg/kg, 400mg/kg treated groups has shown significant increase ($p<0.01$) in the mean difference in the paw thickness after 24 hrs and 48 hours. Formulation 100mg/kg showed increase in footpad thickness ($p<0.05$).

Table 4: Effect of formulation on Delayed type hypersensitivity response in immunosuppressed mice

Sr. No.	Groups	Dose/Day	Footpad Thickness after 24hrs Mean + S.E.M.	Footpad Thickness after 48hrs Mean + S.E.M.
1	Control	1% NaCMC	0.4016 ± 0.00477	0.49 ± 0.00365
2	Cyclophosphamide	50mg/kg	0.2066 ± 0.01308 # #	0.255 ± 0.00619 # #
3	Formulation 100	100mg/kg	0.705 ± 0.03063	0.9066 ± 0.02155
4	Formulation 200	200mg/kg	0.8316 ± 0.01167*	1.185 ± 0.1105
5	Formulation 400	400mg/kg	1.3223 ± 0.00760**	1.695 ± 0.01204

Values are expressed as Mean ± SME, n = 6, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Test drug treated groups were compared with cyclophosphamide Group (Statistically analyzed by one-way ANOVA followed by Tukey – Karmar multiple comparisons test).

6. Discussion

Immunomodulatory activity of formulation of extracts of flowerheads of *S. indicus*, roots of *Somnifera*, Leaves of *C. asiatica*, *O. sanctum*, fruits of *P. nigrum* was explored by evaluating effects on antibody titer, DTH response in immunosuppressed mice.

When mice are sensitized with SRBCs, an antigen gets diffused in the extra vascular space and enters the lymph node via lymphatics. Particulate antigens are taken up by macrophages lining the sinuses or disperse in the lymphoid tissues and are processed. Small highly antigenic peptides are combined with MHC class II molecules. B cells with receptors for antigens bind and internalize it into an endosomal compartment and process and present it on MHC class II molecules to TH2 cells. These B cells are triggered to proliferate, giving rise to clones of large number of daughter cells. Some of the cells of these expanding clones serve as memory cells, other differentiates and become plasma cells that make and secrete large quantities of specific antibodies. During primary response IgM secreted initially, often followed by switch to an increasing proportion of IgG 9. In the present study assessment of humoral immunity was carried out using haemagglutination titer.

Immunostimulation in a drug – induced immunosuppression and immunosuppression in an experimental hyper reactivity model by the same preparation can be said to be true Immunomodulation. Immunosuppression is a major drawback in radiotherapy. Both methods have side effects such as nausea, vomiting, and mucosal ulceration, alopecia, and pulmonary fibrosis, cardiac and hyper toxicity. Drugs that could reduce these side effects as well as stimulate immunity will be of great help in improving cancer treatment strategies. Herbal drugs and many polyherbal formulation are known for their immunostimulatory actions.

Antibody production to T – dependent antigen SRBC requires co-operation of T and B – lymphocytes and macrophages. Cyclophosphamide has a particularly intense effect on short lived lymphocytes known to include a great proportion of B – cells.

In the present study the effect of formulation was explore by using H.A. titer method in immunosuppressed mice. The high values of haemagglutinating antibody titer obtained in case of formulation 400mg/kg have indicated that immunostimulation was achieved through humoral immunity. Other doses showed increase in H.A. titer but results were statistically not significant.

After administration of cyclophosphamide on day 4, 5 and there was elevation in DTH response. It has been established that the mechanism behind this potentiation of DTH by cyclophosphamide is the elimination of population of suppressor cells. Dosing with cyclophosphamide during period closer to elicitation of DTH is reported to have profound suppressive effect on all forms of DTH and cell mediated immunity. Formulation 400mg/kg treated group has shown significant increase in the mean difference in the paw thickness after 24 and 48 hours. Formulation100mg/kg, 200mg/kg showed increase in footpad thickness but statistically not significant. Increase in DTH response indicates that formulation possesses stimulatory effect on lymphocytes and on other necessary cell types required for the expression of the reaction in immunosuppressed mice.

In both HA titer and DTH model, it may be due to synergistic effect showed by sesquiterpene glycosides sphaeranthanolide12, Eudesmanolides 23, alkaloid sphaeranthine, sesquiterpene lactones6 present in flower heads of *S. indicus*, piperine from *P. nigrum*, flavonoid glycoside 3 – glucosylquarcetin and 3 – glucosylkaempferol. Sugars named arabinogalactan, pectin present in *C. asiatica*, steroidal lactone withanolides, alkaloids, glycowithanoloids sitoindosides present in roots of *W. somnifera*, glycosides, triterpenoid acid, ursolic acid, flavonoids, phenolics present in *O. sanctum*.

The present study demonstrates that the formulation of extracts possesses stimulant effect on Humoral and Cell mediated immunity. The formulation showed synergistic immunomodulatory activity against cyclophosphamide induced myelosuppression also. This has resulted in protection against cyclophosphamide induced myelosuppression and has shown effect on lymphatic cells. However, mechanism of action could be unfolded only after detail investigations and analysis on immunomodulatory activity of these naturally occurring compounds has to be carried out.

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