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Original Article

Development of polyherbal formulation for immunomodulation

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ABSTRACT

A polyherbal formulation was 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 100 mg, 200 mg, 400 mg were screened on humoral and cellular immunity i.e. delayed type hypersensitivity on mice. The mice were also subjected to cyclophosphamide induced myelosupression. The present study demonstrated that the formulation 200 mg, 400 mg have synergistic immunostimulant activity in HA titre, DTH, on lymphoid organ weight and it also showed protection against cyclophosphamide induced myelosupression.

1. INTRODUCTION

Herbal preparations have been used as an immunomodulator in traditional medicine. 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. One of the main approaches in Ayurvedic medicine is to "increase the body's natural resistance to the disease/stress" known as "Rasayana" (Rejuvenation) [23]. 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 [1]. Many plants have been extensively used as 'Rasayana' drugs in Ayurveda for the management of neurodegenerative diseases, as rejuvenators, immunomodulators, aphosidiac and nutritional supplements [2]. Some polyherbal formulations such as Immunocin, Chyvangrans, Gerifort, RV08 [19], Immune – 21 [20] exhibits immunomodulatory activity and showed synergistic effects [17] but all these formulation contains more than six ingredients and this make standardization difficult.

Sphaeranthus indicus [14, 15] Linn. is a branched herb with purple flowers that grows abundantly in rice field and distributed throughout India. *Sphaeranthus indicus* showed immunomodulatory, antimicrobial, antibacterial, anxiolytic, wound healing action activities. Phytoconstituents isolated from *S. indicus* were eudesmanolides, isoflavonoids, 7-hydroxy eudesmanolides , sterol glycoside, essential oils.

Withania somnifera is the plant distributed throughout the dried subtropical regions of India. The roots contain seven withanolides with beta sitosterol glucoside and stigmasteroglucoside a, b [16]. The plant is used as rasayana, aphrodisiac, antioxidant, alterative, tonic and bronchitis.

Ocimum sanctum is a sacred plant distributed throughout India. It contains alkaloids, eugenol, glycosides, saponins and tannins. It is given as rasayana [23] and used as antidote in poison, anti-stress agent, carminative, anti-helmentic, tonic, aphrodisiac [22], laxative, expectorant and bronchitis.

Centella asiatica is indigenous to southeast Asia, India and Srilanka. It contains triterpenoid saponins i.e. asiaticoside, madecassoside, brahminoside, bramhoside, alkaloids and essential oils. It has been used as promoter of strength, nervine

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tonic, promotes longevity, and used in mental disorders. It is major intellect promoting rasayana.

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 [24]. It is used as anti-inflammatory, antidepressant, antispasmodic, antihypertensive, and antioxidant. It is mainly used as bioavailability enhancer in "Trikatu" formulation. Piperine is a major chemical constituent which shows immunomodulatory activity. All these drugs were previously studied for immunomodulatory activity [3, 4]. Thus, the present study was to evaluate immunomodulatory effect of this combination in normal and immune compromised 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* and *Centella asiatica* were obtained from medicinal garden of Dr. D. Y. Patil Institute of Pharmaceutical Sciences and Research, Pimpri, Pune and dried flower heads of *Sphaeranthus indicus* and dried fruits of *Piper nigrum* were obtained from local market of Pune. All plant materials were authenticated from Botanical Survey of India, Koregaon Road, Pune. The methanolic [21] 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 with 1% 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. 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.

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

The method described by Puri *et al* was adopted [5]. The animals were divided into five groups consisting of six animals each. All the treatment groups were treated with the 100mg, 200mg, and 400mg combination in sodium carboxy methyl cellulose daily for 7 days. The control group received 1.0% sodium carboxy methyl cellulose solution as a vehicle. The animals were immunized by injecting 0.1ml of 20% of fresh sheep red blood cells suspension intraperitoneally on day 0 [6]. Blood samples were collected from all the animals separately by retro orbital puncture on day +7 (before challenge) for primary antibody titre and on day +14 for secondary antibody titre. It is then centrifuged to obtain serum, normal saline as used as diluents and SRBC was adjusted to 20%. Each well of microtitre plate was filled initially with 25l saline. 25l of serum was mixed with 25l saline in the first well of microtitre plate. Two fold dilutions of the pooled serum samples were made in 25l of normal saline. 25l of SRBC (1%) were added to each of these dilutions and the plates were incubated at 37^0C for one hour and then observed for haemagglutination [7]. The reciprocal of the highest dilution of the test serum giving agglutination was taken as the antibody titre [8].

2.5 Delayed type hypersensitivity (DTH) response (Cellular immune response):

Foot pad edema in mice was used for detection of cellular immune response. On 7^{th} day, the thickness of right hind footpad was measured using digital vernier caliper and injection of 1% sheep RBC in the subplanatar region of right hind paw in the volume of 20l. Footpad thickness was again measured after 24 and 48 hrs. of this challenge. The difference between the pre and post challenge foot thickness express in mm was taken as a measure of delayed type hypersensitivity (DTH).

2.6 Cyclophosphamide induced myelosupression:

The method by Manjarekar *et al,* Bafna and Mishra was adopted [18, 19]. The animals were divided into five groups consisting six animals each. All the treatment groups were treated with the 100mg, 200mg, and 400mg combination in sodium carboxy methyl cellulose daily for 13 days. On 11^{th}, 12^{th}, 13^{th} day, all the animals of each group except control were given cyclophosphamide (30mg/kg, i.p.), one hour after administration of extract. On 14^{th} day blood samples were withdrawn from retro orbital plexus lysed in sodium carbonate solution from all groups and total white blood cell (WBC) count was determined [6,7].

2.7 Effect on lymphoid organ weight

The method by Kuttan, Sharififar was adopted. The animals were divided into four groups consisting 6 animals each. All the groups except control group were treated with 100mg, 200mg and 400mg/kg and control group was treated with 1% sodium CMC for 5 days. On 6^{th} day all the mice were anesthetized by anesthetic ether and about 1 to 2 cm midline incision was made through the abdominal wall, lymphoid organs (spleen, liver, kidney, thymus gland) were identified and carried out in phosphate buffer of physiological pH solution and each organ were weighed [9].

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2.8 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 $p < 0.05$.

3. RESULTS

3.1 Humoral antibody titre

Humoral response to SRBC's was measured as primary and secondary antibody titre. Primary antibody titre in control group was 90.666 ± 17.364 and secondary 149.33 ± 35.697. In cyclophosphamide group it was 34.666 ± 6.422 and 64 ± 14.311. When formulation with 100mg, 200mg and 400mg/kg was administered, it increased the levels of primary antibody titre to 112 ± 32.79, 224 ± 65.58, 298.666 ± 71.395 and secondary antibody titre was raised to 192. ± 28.622, 362.666 ± 69.456, 448 ± 131.16 respectively. Statistically significant results were obtained by doses 200 and 400mg/kg (Table 1).

Table 1. Effect of formulation on HA titre

Sr. No.	Groups	Dose/ Day	Primary HA titre (Mean ± SME)	Secondary HA titre (Mean ± SME)
1	Control	1% Sodium CMC	90.666 ± 17.364	149.33 ± 35.697
2	Cyclophos-phamide	50mg/kg	34.666 ± 6.422	64 ± 14.31
3	Formula-tion 100	100mg/kg	112 ± 32.79	192 ± 28.622
4	Formula-tion 200	200mg/kg	224 ± 65.58*	362.666 ± 69.456*
5	Formula-tion 400	400mg/kg	298.666± 71.395**	448 ± 131.16**

Values are expressed as Mean ± SME, n = 6, * p < 0.05, **p < 0.01, *** p < 0.001 (Statistically analysed by One Way ANOVA followed by Tukey – Kramar Multiple Comparison test). All treated groups were compared with Control group and Cyclophosphamide group for HA titre,

3.2 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 2. Effect of formulation on DTH response

Sr. No.	Groups	Dose/ Day	Footpad Thickness (Mean ± SME) 24 hrs.	Footpad Thickness (Mean ± SME) 48 hrs.
1	Control	1% Sodium CMC	0.40 ± 0.00683	0.49 ± 0.00683
2	Cyclophos-phamide	50 mg/kg	0.2133 ± 0.01202	0.2566 ± 0.00558
3	Formulation 100	100 mg/kg	0.7583 ± 0.00946*	0.9383 ± 0.007923*
4	Formulation 200	200 mg/kg	0.985 ± 0.00922**	1.265 ± 0.007638**
5	Formulation 400	400 mg/kg	1.5133 ± 0.01022**	1.9733 ± 0.01054**

Values are expressed as Mean ± SME, n = 6, * p < 0.05, **p < 0.01, *** p < 0.001 (Statistically analysed by One Way ANOVA followed by Tukey – Kramar Multiple Comparison test). All treated groups were compared with control group for DTH response.

3.3 Cyclophosphamide induced myelosupression

The blood samples were withdrawn from retro – orbital plexus on 14th day from all the groups and total WBC count was determined. The result shown in Table 3 indicates that cyclophosphamide group has shown significant decrease ($p < 0.01$) in the mean WBC count as compared to control group. Formulation 400mg/kg administration was found protecting Cyclophosphamide induced myelosupression significantly ($p < 0.01$). Formulation 200mg/kg has shown significant results ($p < 0.05$).

Table 3. Effect of formulation on Cyclophosphamide Induced Myelosupression

Sr. No.	Groups	Dose/ Day	Total WBC count (Mean ± SME)
1	Control	1%Sodium CMC	5816.666 ± 27.889
2	Cyclophosphamide	30mg/kg	2575 ± 21.889
3	Formulation 100	100mg/kg	3141.6666 ± 20.069
4	Formulation 200	200mg/kg	4066.666 ± 24.721*
5	Formulation 400	400mg/kg	5066.666 ± 24.721 **

Values are expressed as Mean ± SME, n = 6, * p < 0.05, **p < 0.01, *** p < 0.001 (Statistically analysed by One Way ANOVA followed by Tukey – Kramar Multiple Comparison test). All treated groups were compared with cyclophosphamide group.

3.4 Effect on lymphoid organs

Generation of lymphocytes in lymphoid organs is followed by their migration into the peripheral secondary tissues, which comprises of well organized encapsulated organs, the spleen which traps antigens from blood and lymph nodes, collectively referred to as systemic organs. Activated lymphocytes undergo

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clonal proliferation and differentiation into effector cells. The result shown in Table 4 indicates that administration of formulation 400mg/kg, 200mg/kg showed significant increase in weight of lymphoid organ Kidney and Thymus ($p<0.01$). Whereas formulation 400mg/kg showed extremely significant increase in weights of lymphoid organs Liver and Spleen ($p < 0.001$). All groups were compared with control group.

Table 4. Effect of formulation on lymphoid organs weight

Sr. No.	Groups	Liver (Mean ± SME)	Kidney (Mean ± SME)	Spleen (Mean ± SME)	Thymus (Mean ± SME)
1	Control (1% Sodium CMC)	4.0718 ± 0.00070	1.488 ± 0.0082	0.3611 ± 0.00048	0.2151 ± 0.00048
2	Formulation 100mg/kg	4.117 ± 0.00048	1.554 ± 0.001	0.4128 ± 0.0006	0.224 ± 0.00037
3	Formulation 200mg/kg	4.712 ± 0.00042**	1.6571 ± 0.00048**	0.5175 ± 0.00062**	0.2515 ± 0.00022**
4	Formulation 400mg/kg	4.997 ± 0.00031***	1.6971 ± 0.00031**	0.6263 ± 0.00067***	0.275 ± 0.00073**

Values are expressed as Mean ± SME, $n = 6$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Statistically analysed by One Way ANOVA followed by Tukey – Kramar Multiple Comparison test). All treated groups were compared with control group for Effect of formulation on lymphoid organs.

4. DISCUSSION

Immunomodulatory activity of formulation of extracts of flowerheads of *S. indicus*, roots of *W. somnifera*, Leaves of *C. asiatica*, *O. sanctum*, fruits of *P. nigrum* was explored by evaluating effects on antibody titre, DTH response, lymphoid organs and cyclophosphamide induced myelosuppression in 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. The magnitude of secondary antibody response to the same antigen is amplified in terms of antibody production [8]. In the present study assessment of humoral immunity was carried out using haemagglutination titre. The anti SRBC antibody titre was raised in the formulation treated groups in doses of 200mg/kg and 400mg/kg when compared with cyclophosphamide group but was found to be extremely significant at doses 400mg/kg in both primary and secondary antibody titre response.

DTH is directly related to cell mediated immunity (CMI). DTH is a part of graft rejection, tumor immunity. In DTH, circulating T cells sensitized to antigens from prior contact react with antigens and induce specific immune response which includes mitosis and the release of soluble mediators. This process causes attraction of more scavenger cells at the site of reaction. The infiltrating cells are thus immobilized to promote inflammatory reaction. Increase in DTH response indicates that drug has stimulatory effect on lymphocytes. In the model of DTH, formulation 200mg/kg, 400mg/kg treated groups has shown significant increase in mean difference in the paw thickness after 24hr and 48hrs. Formulation 100mg/kg also showed increase in footpad thickness but not significant.

Cyclophosphamide is an alkaylating agent and probably the most potent immunosuppressive drug. Administration of the formulation 200mg/kg, 400mg/kg was found to increase the total WBC count, which was lowered by cyclophosphamide, cytotoxic drug. The result indicates that the formulation can stimulate the bone marrow activity. This might be due to synergistic effect of flavones glycosides 5,6 – tetramethoxy flavones – D – diglucoside [25], sesquiterpene lactones present in flowerheads of *S. indicus* [6], piperine from *P. nigrum*, steroidal lactone withanolides present in *W. somnifera*, triterpenoidal saponins, asiaticoside present in *C. asiatica* [10]. But the other groups fail to restore the effect of cyclophosphamide induced myelosupression.

Administration of formulation 400mg/kg, 200mg/kg showed significant increase in weights of lymphoid organs kidney and thymus ($p<0.01$). Whereas formulation 400mg/kg showed extremely significant increase in weights of lymphoid organs liver and spleen ($p<0.001$). All groups were compared with control group. This shows that formulation may stimulate spleen and liver cells that kidney and thymus cells, thus enhance the immune system. This may be due to synergistic effect of sesquiterpene glycoside sphaeranthanolide [11], piperine from *P. nigrum* [12], steroidal lactone withanolides present in *W. somnifera*, teriterpenoidal saponins, asiaticoside present in *C. asiatica* [10], ursolic acid present in *O. sanctum* [13] 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. 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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