Research Article

Immunomodulatory Activity of Herbal Formulation (CE1 and CE2) Containing *Sphaeranthus indicus, Curculigo orchioides* and *Piper nigrum*

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ABSTRACT

Objectives: To screen two combinations (CE1 and CE2) of methanol extracts of flower heads *Sphaeranthus indicus,* rhizomes of *Curculigo orchioides* and fruits of *Piper nigrum* for immunomodulatory activity. **Methods:** Effects of CE1 and CE2 on humoral (Haemagglutination Antibody titre) and cellular immunity (Delayed Type Hypersensitivity) were studied in mice. These were also subjected to screening for cyclophosphamide induced myelosuppression in mice. **Results:** CE1 and CE2 stimulated humoral immunity and delayed type hypersensitivity (DTH) response as evidenced by increased antibody production and increase in paw edema. Both the combinations resulted in statistically significant results. CE1 and CE2 were found active in normalizing total WBC levels in case of cyclophosphamide induced myelosuppression in mice and also showed significant change in weight of lymphoid organs. The results suggest that combination CE1 was more effective immunomodulator than CE2, which acts by stimulating both humoral and cellular immunity. **Conclusion:** The results of study showed that formulation has resulted in synergistic effect on immune system and acted through humoral and cellular immunity.

Keywords: *Sphaeranthus indicus, Curculigo orchioides, Piper nigrum,* Humoral immunity.

INTRODUCTION

In recent times, focus on plant research has been intensified all over the world and a large amount of evidence has been collected to show immense potential of medicinal plants used in various traditional systems. In Indian system of medicine, a large number of herbal drugs have been advocated for various types of diseases/stress related disorders. The term immunomodulatory means regulation of the immune system by suppression and stimulation of the cells and organs of the immune system. The modulation of immune responses to alleviate disease has been of interest for many years and the concept of 'rasayana' in Ayurveda is based on related principles. The immunomodulatory agents from plant origin which are claimed to induce para-immunity, the non-specific immunomodulation of essentially granulocytes, macrophages, and natural killer cells and complement functions. It is now being recognized that immunomodulatory therapy could provide an alternative to conventional chemotherapy to a variety of diseased conditions[1].

The beneficial effects of polyherbal formulation in immunomodulatory activity may be attributed to the presence of multiple ingredients with multiple modes of actions such as antioxidant, stimulating leukopoiesis, stimulation of the reticulo endothelial system, activating the both specific and non-specific host defense responses[2]. This is further supported by the fact that some drugs when given as whole is non-toxic while many of individual components are reported to be highly toxic[3]. A combination of herbs exhibits augmented therapeutic efficacy than a single herb[4]. Different polyherbal formulations such as *Indukantha Ghritha*, *Haridradi Ghrita*, *RV08* and *Immu-21* are used for immunomodulatory activity and showed synergistic effects but all these formulation contains more than four ingredients and this make standardization difficult[3, 5-7].

Sphaeranthus indicus Linn. (Compositae) is a herb found mostly in southern part of India. Flowers (flower heads) are highly esteemed as alternatives, depuratives, refrigerants and tonics, useful as blood purifiers in skin diseases. The drug is also useful in urethral

discharges and in jaundice[8]. Considering this, the methanol extract of flower heads of *S. indicus* has earlier been studied for its effect on immunomodulatory activity[9].

Curculigo orchioides Gaertn. (Family: Amaryllidaceae) is a small herb found in India in the subtropical Himalayas. Its tuberous roots are considered to be tonic, alterative, demulcent, diuretic and restorative. The drug is claimed as a medical cure for piles, asthma, jaundice, diarrhea, colic and gonorrhea[10]. In the ayurvedic texts, *C. orchioides* is classified as a drug having properties similar to rasayanas. The methanol extract of rhizomes of *C. orchioides* has earlier been studied for its effect on humoral and cellular immunity in normal animals[11,12].

Piper nigrum used as bioavailability enhancer in Trikatu formulation[13]. Piperine is major chemical constituent of the plant has also been screened for immunomodulatory activity[14]. Therefore the aim of present study was to assess immunomodulatory effect of different combination of *S. indicus, C. orchioides* and *P. nigrum.*

MATERIALS AND METHODS

Animals

Swiss albino mice of either sex, weighing 20-25 gm, household in standard conditions of temperature, humidity and light were used. They were fed with standard rodent diet and water. The experimental protocol was approved by IAEC (Institutional Animal Ethical Committee).

Plant material and extract preparation

Dried flower heads of *S. indicus,* rhizomes of *C. orchioides,* and fruits of *P. nigrum* were collected from the local market of Pune and were authenticated in Regional Research Institute (Ay), Kothrud. Maceration of air-dried, powdered flower heads of *S. indicus* and rhizomes of *C. orchioides* afforded 6.65% methanol extract (w/w) whereas extraction of dried powdered fruits of *P. nigrum* afforded 7.85% methanol extract (w/w). The extracts showed the presence of alkaloids, phenolics, tannins, saponins, and steroids when subjected to qualitative chemical tests.

Drugs

The methanol extracts were combined as (Parts of extracts)-

CE1: *S. indicus: C. orchioides: P. nigrum* (2:1:0.25)

CE2: *S. indicus: C. orchioides: P. nigrum* (1:2:0.25)

CE1 and CE2 were suspended in 1% sodium carboxy methylcellulose to prepare different doses from 50, 100 and 200 mg/kg. The control animals were given an equivalent volume of sodium carboxy methylcellulose vehicle. Cyclophosphamide was used as a standard immunosuppressant.

Antigen

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

METHODS

Humoral antibody (HA) and delayed type hypersensitivity (DTH) response

The method described by Puri et al. was adopted[15]. Animals were divided into seven groups of six animals each. The control group received 1.0 % sodium carboxy methylcellulose solution only as vehicle; while animals in the treatment groups were given the CE1 and CE2 (50, 100 and 200 mg/kg, p.o.) in 1.0 % sodium carboxy methyl cellulose daily for 7 days. The animals were immunized by injecting 0.1 ml of 20% of fresh sheep red blood cells suspension intraperitoneally on 0 day. Blood samples were collected in micro centrifuge tubes from individual animal by retro-orbital plexus on 7th day to obtain serum (before challenge) for primary antibody titre and on day 14th for secondary antibody titre. Antibody levels were determined by haemagglutination technique. Briefly, equal volumes of individual serum samples of each group were pooled. Two fold dilutions of pooled serum samples were made in 25 µl volumes of normal saline in microtitration plate and to it added 25 µl of 1% suspension of sheep red blood cells in saline. After mixing, the plates were incubated at room temperature for 1 hr. and examined for haemagglutination under microscope. The reciprocal of the highest dilution of the test serum giving agglutination was taken as the antibody titre.

On 7th day, the thickness of the right hind footpad was measured using digital vernier calipers. The mice were then challenged by injection of 20µl of 1% SRBCs in right hind footpad. Foot thickness was again measured after 24 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).

Cyclophosphamide induced Myelosuppression

Cyclophosphamide induced myelosuppression was studied according to the method described by Manjarekar et al.[16]. Animals were divided into eight groups of six animals each. The control group and cyclophosphamide group received 1.0 % sodium carboxy methylcellulose solution only as vehicle daily for 13 days while animals in treatment groups were given CE1 and CE2 (50, 100 and 200 mg/kg, p.o.) in 1.0 % sodium carboxy methyl cellulose daily for 13 days. On days 11, 12, 13 all the animals except in the control group were injected with cyclophosphamide (30 mg/kg, i.p.) 1 hour after administration of the extracts. Blood samples were collected on day 14 and total white blood cell (WBC) count was determined.

Effect on lymphoid organs

The animals were divided into seven groups consisting six animals each. The control group received 1.0 % sodium carboxy methylcellulose solution only as vehicle; while animals in the treatment groups were given CE1 and CE2 (50, 100 and 200 mg/kg, p.o.) in 1.0 % sodium carboxy methyl cellulose daily for 7 days. On 7th day, all the mice were sacrificed to remove lymphoid organs (Spleen, Liver, Kidney, Thymus gland). All lymphoid organs were kept in phosphate buffer of physiological P^{H} and weighed separately[17, 18].

Statistical analysis

Data were expressed as mean ± S.E.M. and difference between the groups was statistically determined by analysis of variance followed by Tukey–Kramer Multiple Comparisons test, with the level of significance set at $p < 0.05$.

RESULTS

Humoral antibody and delayed type hypersensitivity response

Humoral response to SRBCs was measured as primary and secondary antibody titre. Primary antibody titre in control group was 40.00 ± 8.00 and secondary 77.33 ± 17.73. Administration of 50, 100 and 200 mg/kg of CE1 raised the levels of primary antibody titre to 5461.30 ± 863.51, 6144.00 ± 915.89, 10240.00 ± 2048.00 and secondary antibody titre to 8874.70 **±** 1644.10, 10923.00 **±** 1727.00 and 15019.00 **±** 3909.70 respectively. CE2 raised levels of primary antibody titre to 1280.00 **±** 256.00, 6144.00 **±** 915.89, 7509.3.30 **±** 1954.90 and secondary antibody titre to 2048.00 **±** 457.90, 10240.00 **±** 2048.00 and 11605.00 **±** 2222.60 at 50, 100, 200 mg/kg doses, respectively. However statistically significant results were obtained by CE1 and CE2 at dose of 100 and 200 mg/kg (Table 1).

DTH response was checked by increased footpad thickness using digital vernier calipers. Administration of CE1 and CE2 extracts produced increase in thickness of footpad of mice as a measure of DTH response. DTH response of control group was 0.26 **±** 0.04. CE1 at doses of 50, 100, 200 mg/kg resulted in DTH response as 0.47 **±** 0.03, 0.57 **±** 0.04, 0.85 **±** 0.01 respectively, whereas CE2 showed DTH response as 0.43 **±** 0.02, 0.46 **±** 0.05 and 0.49 **±** 0.04 at doses 50, 100 and 200 mg/kg respectively. The results were statistically significant in both the cases at all doses administered (Table 1).

Cyclophosphamide induced myelosuppression

Administration of cyclophosphamide at the dose of 30 mg/kg, i.p. has significantly lowered the levels of total WBC 3701.70 **±** 41.26 ($p< 0.05$) as compared to control group. CE1 when administered in doses 50, 100 and 200 mg/kg resulted in WBC levels as 4145.00 **±** 24.05 ($p< 0.05$), 4391.70 **±** 109.10 ($p< 0.001$) and 5566.70 **±** 110.81 ($p< 0.001$) as compared to cyclophosphamide group. CE2 also showed raised levels of WBC (Table 1).

Effect on lymphoid organ

Effect of CE1 and CE2 extracts on lymphoid organ to assess immune response. Weight of lymphoid organ such as Spleen, Thymus, Kidney, Liver increases with CE1 and CE2 extracts. Statistically significant ($p<0.001$) increase in weight of lymphoid organ were observed in CE1 and CE2 at dose of 200 mg/kg (Table 2).

DISCUSSION

Immunomodulatory activity of combinations (CE1 and CE2) of extracts of flower heads of *S. indicus*, rhizomes of *C. orchioides* and fruits of *P. nigrum* was explored by evaluating effects on antibody titre, DTH response, lymphoid organs and cyclophosphamide induced myelosuppression in mice.

The haemagglutination test was performed to confirm effect of CE1 and CE2 on the humoral arm of the immune system. The humoral immunity involves the interaction of B cells with antigen and their subsequent proliferation and differentiation into antibody-secreting plasma cells. During the primary response, IgM is secreted initially, often followed by switch to an increasing proportion of the IgG. The magnitude of secondary antibody response to the same antigen is amplified in

terms of antibody production[19]. Methanol extracts of *S. indicus* and *C. orchioides* have been showed to possess stimulatory effect on antibody titre and piperine showed increase in number of plaques forming cells [12,14,20]. Comparison of results clearly showed synergistic effects exist. CE1 proved to be more potent than CE2.

DTH is antigen specific and causes erythema and induction at the site of antigen infection in immunized animals. The histology of DTH can be different for different species, but the general characteristics are an influx of immune cells at the site of injection, macrophages and basophils in mice and induction becomes apparent within 24-72 hours. DTH directly correlates with cell mediated immunity[1].

In present study combination of *S. indicus* and *C. orchioides* with bioavailability enhancer *P. nigrum* increased the level of antibody titre and footpad thickness significantly indicating the enhanced responsiveness of B and T lymphocytes involved in antibody production [21].

A high degree of cell proliferation renders the bone marrow a sensitive target particularly to cytotoxic drugs. In fact, bone marrow is the organ most affected during any immunosuppression therapy with this class of drugs. Loss of stem cells and inability of the bone marrow to regenerate new blood cells results in thrombocytopenia and leucopenia[22.] Administration of the CE1 and CE2 extract was found to increase the total WBC count, which was lowered by cyclophosphamide, a cytotoxic drug.

Combination of extracts showed significant stimulation of humoral immunity and cellular immunity as compared to individual plant extracts. This may be due to the synergistic effect resulted by combination of phytoconstituents like sesquiterpene glycoside sphaeranthanolide[23], eudesmanolides[24] present in flower heads of *S. indicus*, phenolic glycosides from *C. orchioides*[11] and piperine from *piper nigrum*[13,14].

CONCLUSION

From the results of study, it is then concluded that the combination of *Sphaeranthus indicus, Curculigo orchioides,* and *Piper nigrum* resulted in stimulated effect on humoral immunity and cell mediated immunity. This combination resulted in protection against cyclophosphamide induced myelosuppression. It was clearly evident the combination has effected humoral immunity in comparison with individual drug and hence further detail investigation are warranted in this direction.

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Table 1: Effect of CE1 and CE2 on HA titre and DTH response, cyclophosphamide induced myelosuppression

S.No.	Groups	Primary HA titre (Mean ± SEM)	Secondary HA titre (Mean ± SEM)	DTH (Mean ± SEM) 24 hrs	Total WBC count (Mean ± SEM)
1.	Control	40.00 ± 8.00	77.33 ± 17.72	0.26± 0.04	5848.30± 131.72
2.	Cyclophosphamide (30 mg/kg)	–	–	–	3701.70 ±41.265 [b]
3.	CE1 (50 mg/kg)	5461.30 ± 863.51 [a]	8874.70 ± 1644.10 [a]	0.47 ± 0.03 [c]	4145.00 ± 24.05 [b]
4.	CE1 (100 mg/kg)	6144.00 ± 915.89 [b]	10923.00 ± 1727.00 [b]	0.57 ± 0.04 [d]	4391.70 ± 109.10 [d]
5.	CE1 (200 mg/kg)	10240.00 ± 2048.00 [d]	15019.00 ± 3909.70 [d]	0.85 ± 0.01 [d]	5566.70 ± 110.81 [d]
6.	CE2 (50 mg/kg)	1280.00 ± 256.00 [a]	2048.00 ± 457.90 [a]	0.43 ± 0.02 [b]	4086.70 ± 21.70 [a]
7.	CE2 (100 mg/kg)	6144.00 ± 915.80 [b]	10240.00 ± 2048.00 [b]	0.46 ± 0.05 [c]	4055.00 ± 52.45 [a]
8.	CE2 (200 mg/kg)	7509.30 ± 1954.90 [c]	11605.00 ± 2222.60 [c]	0.49 ± 0.04 [c]	4923.30 ± 137.28 [d]

Values are expressed as mean ± S.E.M. n=6; [a] p>0.05, [b] p<0.05, [c] p<0.01 and [d] p<0.001. All treated groups are compared with control for HA titre, DTH response and whereas with cyclophosphamide group in case of cyclophosphamide induced myelosuppression.

Table 2: Effect of combination of extracts on lymphoid organs

S. No.	Groups	Kidney (Mean ± SEM)	Liver (Mean ± SEM)	Spleen (Mean ± SEM)	Thymus (Mean ± SEM)
1.	Control	1.498 ± 0.012	4.072 ± 0.055	0.361 ± 0.010	0.215 ± 0.005
2.	CE1 (50 mg/kg)	1.565 ± 0.009 [b]	4.208 ± 0.018 [a]	0.396 ± 0.005 [a]	0.231 ± 0.003 [a]
3.	CE1 (100 mg/kg)	1.583 ± 0.017 [c]	4.292 ± 0.062 [b]	0.410 ± 0.010 [b]	0.245 ± 0.003 [b]
4.	CE1 (200 mg/kg)	1.610 ± 0.016 [d]	4.483 ± 0.017 [d]	0.433 ± 0.014 [d]	0.255 ± 0.009 [d]
5.	CE2 (50 mg/kg)	1.538 ± 0.011 [a]	4.202 ± 0.046 [a]	0.386 ± 0.008 [a]	0.230 ± 0.003 [a]
6.	CE2 (100 mg/kg)	1.562 ± 0.016 [b]	4.288 ± 0.011 [b]	0.408 ± 0.008 [b]	0.241 ± 0.004 [b]
7.	CE2 (200 mg/kg)	1.600 ± 0.007 [d]	4.345 ± 0.073 [c]	0.421 ± 0.007 [c]	0.248 ± 0.007 [c]

Values are expressed as mean ± S.E.M. n=6; [a] p>0.05, [b] p<0.05, [c] p<0.01 and [d] p<0.001
All treated groups are compared with control.

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