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IMMUNOSUPPRESSIVE EFFECT OF *SWERTIA CHIRATA* BUCH HAM. ON SWISS ALBINO MICE

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ABSTRACT: *Swertia chirata* Buch Ham (Gentianaceae) commonly known as chirata is a perennial herb. The present study was undertaken to evaluate the immunomodulatory effect of methanolic extract of *S. chirata* aerial part on Swiss albino mice. Haemagglutination Test (Humoral antibody response) and Delayed-type hypersensitivity (DTH) response test and were done to investigate the effect of methanolic extract of *S. chirata* on humoral and cell mediated immunity responses and flow cytometric studies for the estimation of T lymphocytes (CD3 and CD19) and Th1 cytokines (IL-2, IFN-γ and IL-4). Levamisole and Cyclophosphamide were used as immunostimulatory and immunosuppressive standard drugs. The methanolic extract of *S. chirata* showed dose related decrease in primary and secondary antibody response and Delayed type hypersensitivity (DTH) response with the maximum decrease at 200 mg kg^{-1} b wt (primary antibody response- 5.41±0.24, secondary antibody response- 5.39±0.19 and DTH response- 0.62±0.05). It also showed dose dependent decrease in the production of T lymphocytes (CD3 and CD19) and Th 1 cytokines (IL-2, IFN-γ and IL-4). The maximum decrease in the production of CD3, CD19, IL-2, IFN-γ and IL-4 was 33.66, 19.70, 31.12, 35.09 and 14.63% at 200 mg kg^{-1} b wt. The results revealed that *S. chirata* showed significant decrease in the production of CD3 and IFN-γ. Phytochemical screening showed the presence of phenolic compound such as flavonoids, tannins and alkaloids which may be responsible for the activity. The above results suggest that *S. chirata* can be further explored for the development of potent immunosuppressive drug.

INTRODUCTION: The functional and efficiency of immune system may be influenced by many exogenous and endogenous factors.

Apart from the natural mechanism, there are compounds that are capable of interacting with immune system, to up regulate or down regulate specific aspects of the host response can be classified as immunomodulators and the mechanism involve with these is known as immunomodulation.

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The basic strategy underlying immunomodulation is to identify aspects of the host response that can be enhanced or suppressed in such a way as to augment or complement a desired immune response.

An immunomodulator may be defined as a substance, biological or synthetic, which can stimulate, suppress or modulate any of the components of the immune system including both innate and adaptive arms of the immune response [1].

Immunomodulators may be Immunostimulants and Immunosuppressant. Immunosuppressant used for control of pathological immune response in autoimmune diseases, graft rejection, graft versus host disease, hypersensitivity immune reaction (immediate or delayed type), and immune pathology associated with infections.

Out of the list the maximum use of these agents has been for prevention of graft rejection and treatment of autoimmune diseases [1]. These immuno-modulators may be synthetic drugs or may be of herbal origin. Due to side effect of synthetic drugs, herbal drugs gaining popularity in the world market.

Swertia chirata is an herb commonly known as chirata and belongs to family Gentianaceae. The plant is a native of temperate Himalayas, found at an altitude of 1200–3000 m (4000 to 10,000 ft), from Kashmir to Bhutan, and in the Khasi hills at 1200–1500 m (4000 to 5000 ft). It can be grown in sub-temperate regions between 1500 and 2100 m altitudes[5]. It is a perennial herb, it has an erect, about 2–3 ft long stem, the middle portion is round, while the upper is four-angled, with a prominent decurrent line at each angle.

The stems are orange brown or purplish in colour, and contain large continuous yellowish pith. The root is simple, tapering and stout, short, almost 7 cm long and usually half an inch thick [5]. Plant is reported to contain a yellow bitter acid, ophelic acid, two bitter glucosides, chiratin and amarogentin, gentiopicrin, two yellow crystalline phenols, a neutral, yellow crystalline compound, and a new xanthone, swerchirin [5].

The plant has been reported to possess hypoglycemic activity [6], antiulcerogenic activity [7], anti-inflammatory activity [8], hepatoprotective activity [9], wound healing activity [10], anthelmintic activity [11] as well as antibacterial and antifungal activity [12, 13] on selected microbial strains and most important, the antimalarial activity [14].

On the basis of above background, the present study was undertaken for the evaluation of immunomodulatory activity of *S. chirata* methanolic extract.

MATERIALS AND METHODS:

Plant material: The dry aerial parts of *S. chirata* were obtained from Natural Remedies, Bangalore (Karnataka) and authenticated at the Department of Botany, Dr. H. S. Gour University, Sagar. The identification of the plant materials was done by Taxonomist and Herbarium incharge, Department of Botany, Dr. H.S. Gour University, Sagar (M.P.). The Voucher specimen number is Bot/Her/B/3116.

Extract preparation: Dried and powdered plant material (60 g) was defatted with petroleum ether (500 ml) and then successively extracted with methanol (500 ml at 40˚C) using soxhlet apparatus. The extract was cooled at room temperature, filtered and evaporated to complete dryness. The percentage yield of the extract was calculated and it was found to be 12.58%.

Phytochemical analysis: The methanolic extract was subjected to various chemical tests to detect the presence of various phytochemicals such as tannins, flavonoids, alkaloids etc. using standard procedure [15, 16].

Immunomodulatory activity:

- **Animals:** Swiss albino mice (weight- 20-25 g) were used for the experiment. After obtaining the clearance from Institutional Animals Ethics Committee, all the animals were kept in standard cages and maintained under standard laboratory conditions (temperature 22 ± 2˚C with 12 h light/ 12 h dark cycle) with free access to pellet food (Lipton India Ltd) and water *ad libitum* throughout the study.

  All the experimental procedures were carried out in strict accordance with the guidelines laid down by the Committee for the Purpose of Control and Supervision on Experimentation on Animals (CPCSEA) and experimental protocols were approved by the Institutional animal Ethics Committee (Reg. No.- 379/01/ab/CPCSEA).

- **Chemicals:** FACS lysing solution, FACS permeabilizing solution, Golgi plug, FITC (Fluorescein isothiocynate) labeled anti-CD3 monoclonal antibodies, PE (Pycoerytherin) labeled anti-CD19, IL-2, IFN-gamma and IL-4 monoclonal antibodies were purchased from B. D. Biosciences. All other reagents used were of analytical grade.

- **Antigen:** Sheep red blood cells (SRBC) suspension was collected in Alsever's solution and were washed three times with pyrogen free sterile normal saline (0.9% NaCl, w/v). After adjusting the cells count to 5×10^9 cells/ml, SRBC was used for sensitization and challenge at required time schedule.

- **Haemagglutination Test (Humoral antibody response):** The SRBC agglutination test was performed to study the humoral antibody response against antigens. Mice (n = 6) were immunized by injecting 200 µl of 5×10^9 SRBC/ml intraperitoneally (i.p.) on day 0. Test drugs were administered to the mice in graded doses (50, 100 and 200 mg kg^{-1} b wt) for seven days. The blood samples were collected from individual animals of all the groups by retro orbital bleeding 7^{th} day (before challenge) for Primary Antibody titre and on 14^{th} day (7 days after challenge with 5×10^9 SRBC/ml, i.p.) for secondary Antibody titre. Serum was separated from blood. Haemagglutination Antibody titres were determined by following the Haemagglutination technique, which is performed by using 96 wells (12×8) bottomed titre plate. The data obtained were subjected to statistical analysis [17].

- **Delayed-type hypersensitivity (DTH) response:** Test drugs (50, 100 and 200 mg kg^{-1} b wt) p.o. was administered after injecting 200 µl of 5×10^9 SRBC/ml i.p on day 0 to mice and once daily on consecutive days. Six days later, the thickness of the left hind foot was measured with a spheromicrometer (pitch, 0.01 mm) and this thickness of left hind foot was considered as a control. The mice were then challenged by injecting the 20 µl of 5×10^9 SRBC/ml intra-dermally into the left hind footpad. The foot thickness was measured again after 24 h. The difference between the pre and post challenge foot pad thickness expressed in mm was taken as a measure of delayed type hypersensitivity (DTH) and the mean value obtained for treatment groups were compared with that of control group [18].

- **Flowcytometric studies:** For Flow cytometric studies, experimental animals were sensitized by injecting 5×10^9 SRBC/ml intraperitoneally (i.p) on day 0. Drug administration was carried out till next 6 consecutive days. On day 7 animals were challenged by injecting same concentration of SRBC and on day 8, blood was collected from retro-orbital plexus of animals under ether anesthesia in EDTA coated tubes for lymphocyte immunophenotyping and intracellular cytokines estimation.

- **Lymphocyte immunophenotyping:** Immuno-phenotyping focuses on lymphocyte populations involved in acquired immunity and specific molecules present on the cell surface that defines characteristics of lymphocytes such as state of activation or functional capabilities. Lymphocyte subsets were measured by immunofluorescent antibody staining of whole blood and subsequently analyzed using two color flow cytometry (Becton & Dickinson, UK).

  Murine monoclonal antibodies conjugated to a fluorochrome and directed against receptors CD3 and CD19 were used for the study. FITC-labeled anti-mouse CD3 monoclonal antibody and PE-labeled anti-mouse CD19 monoclonal antibody were added directly to 100 µl of whole blood, which was then lysed using whole blood lysing reagent (BD Biosciences). Following the final centrifugation, samples were resuspended in phosphate-buffered saline (pH, 7.4) and analyzed directly on the flow cytometer (LSR, BD Biosciences) using Cell Quest Pro Software (BD Biosciences) [19].

- **Intracellular cytokines estimation:** 100 µl of whole blood was taken in falcon tubes and red blood cells were lysed by adding whole blood lysing reagent (BD Biosciences). After washing in Phosphate Buffer Saline (PBS), cells were permeabilized using permeabilizing solution and incubated with anti-mouse IL-2, anti-mouse IFN-gamma and anti-mouse IL-4 for the duration of 30 min in dark. After incubation, cells were given three washes of PBS and after final washing; cells were acquired directly on Flowcytometry [20].

Calculation of percentage activity:

$$\%\text{Activity} = \frac{\text{Mean Values of Control} - \text{Mean Values of Treated}}{\text{Mean Values of Control}} \times 100$$

Statistical analysis: The results were subjected to statistical analysis using ANOVA with post Bonferroni test and expressed as the Mean ± S.E. ***$p<0.001$; **$p<0.01$; *$p<0.05$.

RESULTS:

Haemagglutination Test (Humoral antibody response): In the present study, humoral antibody titre was found lowered by methanol extract of *S. chirata*. Methanol extract at different doses (50, 100 and 200 mg kg^{-1} b wt) showed primary agglutination titre as 6.00 ± 0.19, 5.77 ± 0.23 and 5.41 ± 0.24 and secondary agglutination titre as 5.90 ± 0.27, 5.69 ± 0.25 and 5.39 ± 0.19 respectively while, Cyclophosphamide (100 mg kg^{-1} b wt) showed primary agglutination titre and secondary agglutination titre as 2.91 ± 0.22 and 3.16 ± 0.19 respectively (**Table 1**).

The present studies indicate that humoral antibody titre is lowered by methanol extract of *S. chirata* at all doses level, i.e. 50- 200 mg kg^{-1} b wt.

Delayed type Hypersensitivity: In case of methanol extract of *S. chirata*, DTH response was found to be decreased in dose dependent manner. In this, footpad thickness at 50, 100 and 200 mg kg^{-1} b wt was measured as 0.78 ± 0.06, 0.71 ± 0.04 and 0.62 ± 0.05 respectively but showed significant decrease at 200 mg kg^{-1} b wt. Levamisole (2.5 mg kg^{-1} b wt) showed thickness 1.38 ± 0.07 and Cyclophosphamide (100 mg kg^{-1} b wt) showed 0.49 ± 0.03 (Table 1). The results were compared to standard drugs and control.

TABLE 1: EFFECT OF *S. CHIRATA* EXTRACT ON HUMORAL ANTIBODY (HA) TITRE AND DTH RESPONSE

Group	Dose (mg kg^{-1} b wt)	Primary HA titre		Secondary HA titre		DTH response	
		Mean±S.E.	% activity against control	Mean±S.E.	% activity against control	Mean±S.E.	% Activity against control
Control	-	6.26±0.35		6.12±0.31		0.93±0.06	
SCM	50	6.00±0.19(ns)	4.15%↓	5.90±0.27(ns)	3.59%↓	0.78±0.06(ns)	16.12%↓
SCM	100	5.77±0.23(ns)	7.82%↓	5.69±0.25(ns)	7.12%↓	0.71±0.04(ns)	23.65%↓
SCM	200	5.41±0.24(ns)	13.57%↓	5.39±0.19(ns)	11.92%↓	0.62±0.05(**)	33.33%↓
Levamisole	2.5	9.43±0.79(***)	50.63%↑	9.08±0.44(***)	48.36%↑	1.38±0.07(***)	48.38%↑
Cyclophosphamide	100	2.91±0.22(***)	53.51%↓	3.16±0.19(***)	48.36%↓	0.49±0.03(***)	47.31%↓

ns- non-significant; ↓-% suppression; ↑-% stimulation. Values are expressed as the Mean ± S.E. ***p<0.001; **p<0.01; *p<0.05 using ANOVA with post Bonferroni test. SCM: *Swertia chirata* methanolic extract.

Lymphocyte Immunophenotyping: *S. chirata* showed dose related decrease in the production of CD3 and CD19, but the maximum decrease was seen at 200 mg kg^{-1} b wt which was 33.66% (27.27 ± 1.90) and 19.70% (39.11 ± 2.12) for CD3 and CD19 respectively when compared against sensitized control (**Table 2**). In case of standard drugs Cyclophosphamide showed 43.59 and 46.39% decrease in the production of CD3 and CD19 respectively.

Intracellular cytokines estimation: In the assay of estimation of intracellular cytokines, methanol extract of *S. chirata* dose dependently inhibited the production of IL-2, IFN- γ and IL-4 (Table 2). The maximum decrease in the production was seen at 200 mg kg^{-1} b wt which was 31.12% (3.01 ± 0.10), 35.09% (2.46 ± 0.15) and 14.63% (2.80 ± 0.14) for IL-2, IFN-γ and IL-4 respectively when compared against sensitized control. In case of standard drugs, Cyclophosphamide showed 45.99, 45.38 and 42.37% decrease in the production of IL-2, IFN-γ and IL-4 respectively.

DISCUSSION: A wide range of immunosuppressive drugs have now been adopted to control unwanted immune responses, particularly those giving autoimmune disease and transplant rejection. The clinical application of immunosuppressants has significantly improved patient survival with first-year survival up to 90% for renal transplant [17]. But unfortunately immuno-suppressants are suffers from a number of serious adverse effects among which nephrotoxicity, hepatotoxicity, induction of diabetes, induction of hypertension and neurotoxicity are most notorious for cyclosporine and tacrolimus [21].

As a consequence, there continues to be a high demand for new immunosuppressants. The immunosuppressants without any side effects are still a challenge to the medical system. Suppression of immune response by medicinal plant products as a possible therapeutic measure has become a subject of scientific investigation recently [22]. The presence of immunostimulant compounds in higher plants has been extensively reviewed but only a limited amount of immunosuppressive products of

plant origin have been such products, if well tolerated by the patient, may be developed into alternative coadjuvants in the treatment of disorders caused by an exaggerated or unwanted immune response, such as in autoimmune diseases, allergies, glomerulonephritis, chronic hepatitis etc [23].

Ethanolic extract of *Cleome gynandra* also exhibited significant immunosuppression effect in dose dependent manner [24]. In another case, Aqueous extract of fruit of *Lagenaria siceraria* shows preferential suppression of the components of cell-mediated immunity and shows no effect on the humoral immunity [25].

TABLE 2: LYMPHOCYTE IMMUNO-PHENOTYPING AND INTRACELLULAR CYTOKINES ESTIMATION BY FLOWCYTOMETRY

Group	Dose (mg kg^{-1} b wt)	CD3	CD19	IL-2	IFN-γ	IL-4
		Mean±S.E	Mean±S.E	Mean±S.E	Mean±S.E	Mean±S.E
Normal Control	-	29.24±0.79	36.41±1.00	2.76±0.08	2.19±0.15	1.97±0.11
Sensitized control		41.11±1.82	48.71±1.87	4.37±0.10	3.79±0.13	3.28±0.12
SCM	50	34.17±1.79(ns) 16.88%↓	44.90±1.82(ns) 7.82%↓	3.94±0.12(ns) 9.83%↓	3.28±0.16(ns) 13.45%↓	3.00±0.13(ns) 8.53%↓
SCM	100	30.44±1.83(**) 25.95%↓	41.00±1.80 (ns) 15.82%↓	3.44±0.09(*) 21.28%↓	2.88±0.17(*) 24.01%↓	2.91±0.09(ns) 11.28%↓
SCM	200	27.27±1.90(***) 33.66%↓	39.11±2.12 (ns) 19.70%↓	3.01±0.10(***) 31.12%↓	2.46±0.15(**) 35.09%↓	2.80±0.14(ns) 14.63%↓
Levamisole	2.5	60.09±2.56(***) 46.16%↑	70.16±2.66 (***) 44.03%↑	6.69±0.43(***) 53.08%↑	5.82±0.33(***) 53.56%↑	4.94±0.27(***) 50.60%↑
Cyclophosphamide	100	23.19±1.34(***) 43.59%↓	26.11±1.27 (***) 46.39%↓	2.36±0.09(***) 45.99%	2.07±0.07(***) 45.38%↓	1.89±0.05(***) 42.37%↓

ns- non-significant, ↓-% suppression, ↑-% stimulation. Values are expressed as the Mean ± S.E. ***p<0.001; **p<0.01; *p<0.05 using ANOVA with post Bonferroni test. SCM: *Swertia chirata* methanolic extract.

Our research work focused on effect of methanol extract of *S. chirata* on immune response. And the results revealed that *S. chirata* methanol extract showed immunosuppression at humoral antibody and cell mediated responses, similarly it inhibited the production of lymphocytes (CD3 and CD19) and intracellular cytokines (IL-2, IFN-γ and IL-4). It showed the immunosuppressive efficacy of *S. chirata*. So we can say that these plants could be a source of drug having effective immuno-suppression properties. It was also investigated alkaloids have immunosuppressive activity [26]. The phytochemical analysis showed the presence of tannins, flavonoids and alkaloids (**Table 3**) which may be responsible for the immunosuppressive activity of the *S. chirata*.

TABLE 3: PHYTOCHEMICAL ANALYSIS OF METHANOL EXTRACT OF *S. CHIRATA*

Phytochemical	Methanol Extract (SCM)
Steroids	-
Terpenoids	-
Tannins	+
Alkaloids	+
Glycosides	+
Flavonoids	+
Saponins	-

CONCLUSION: In the above study, methanolic extract of *S. chirata* significantly decreased delayed type hypersensitivity and also significantly inhibited the production of lymphocytes (CD3) intracellular cytokines (IL-2 and IFN-γ). So, further studies can be performed to explore the *S. chirata* to develop an immunosuppressive drug which will be effective and having no side effect.

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