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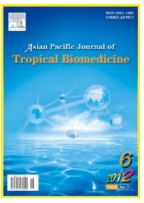

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Immunomodulatory activity of butanol fraction of *Gentiana olivieri* Griseb. on Balb/C mice

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ABSTRACT

Objective: To explore the immunomodulatory properties of 80% ethanol extract and butanol fraction of *Gentiana olivieri* (*G. olivieri*) Griseb on Balb/C mice. **Methods:** The study was performed with basic models of immunomodulation such as the humoral antibody response (hemoglutination antibody titres), cell mediated immune response (delayed type hypersensitivity and *in vivo* carbon clearance or phagocytosis). Ethanol (80%) extract of flowering aerial parts of *G. olivieri* and its butanol fraction were administered p.o. (orally) to the mice. Levamisole, 2.5 mg/kg was used as standard drug. **Results:** There was a potentiation of immune response to sheep red blood cells by cellular and humoral mediated mechanisms comparable to levamisole (2.5 mg/kg) by both 80% ethanol extract and the butanol fraction at doses of 50-200 mg/kg in male Balb/C mice. Both significantly ($P<0.01$) potentiated the humoral immune response in cyclophosphamide (250 mg/kg) immunosupressed mice at 100 and 200 mg/kg of each extract and fraction as compared to control. The potentiation of delayed type hypersensitivity response was statistically significant ($P<0.01$) at 200 mg/kg of ethanol extract and 100, 200 mg/kg of butanol fraction as compared to control. The phagocytosis was significant at 200 mg/kg with butanol fraction of *G. olivieri*. **Conclusions:** The results reveal the immunostimulant effects of plant *G. olivieri* in mice by acting through cellular and humoral immunity in experimental models of immunity in mice. Butanol fraction is the most effective at a dose level of 200 mg/kg.

1. Introduction

The use of medicinal plant products as immunomodulators as possible therapeutic measure is becoming a new subject of scientific investigations[1]. Traditionally, the plant *Gentiana olivieri* (*G. olivieri*) Grieseb is used for treatment of a variety of disorders. The plant is reported to be sudorific in Ayurveda[2], widely used in east and south-east Anatolia as bitter tonic, stomachic and to combat some mental disorders in the different regions of Turkey. Macerated dried flowering herb in water is used to lower the blood pressure in type-2 diabetic patients, while infusion (2%-3%) is used as appetizer and as antipyretic[3]. The plant is known to possess a number of alkaloids, triterpenoid acids, fats, bitter secoiridoids glycosides, flavonoids (iso-orientin and its derivatives) and xanthones[2-5]. The presence of these phytoconstituents was confirmed by different qualitative tests performed on different extracts and fraction of *G. olivieri* (not shown in this study).

The different active phytoconstituents of plant such as polysacchrides, lectins, peptides, flavonoids have been reported to modulate the immune system in different experimental models[6]. Therefore, the chemical profile indicates herb *G. olivieri* may be a good source of immunomodulatory agents. Further the plant is known to possess hepatoprotective, antidiabetic, antimicrobial and anti-inflammatory bioactivities. However, till date no scientific evaluations are conducted for its immunomodulatory activity. Thus, this study was designed to evaluate the immunomodulatory activity of 80% ethanol extract and butanol fraction of aerial part of *G. olivieri* Griseb. in different experimental models of cellular and humoral immunity in mice.

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2. Materials and methods

2.1. Plant material

G. olivieri Griseb. (flowering aerial part) was procured from Himalya Herbal Store, Saharanpur, UP (India). The sample was identified on the basis of exomorphic characters, chemical reaction and review of literature by Dr. Singh HB, Taxonomist, NISCAIR, CSIR New Delhi. The voucher specimen of the sample (NISCAIR/RHMD/Consult/2009-10/1255/1259) was deposited in the NISCAIR, RHM Division, Dr. KS Krishna Marg (Near Pusa Gate), New Delhi (India).

2.2. Extraction and fractionation-phytochemicals

Aerial flowering herb (1 kg) was macerated with 80% ethanol (1:5 ratio) for 70 h. The solvent was filtered, marc was drained and the procedure was repeated thrice for the complete extraction of phytochemical. The combined extracts were reduced to one eighth of their original volume under rotavapour (Heidolph Hei Vap Advantage, MLIG3) at 50 ℃ and lyophilized to get a yield of 37 g 80% ethanol extract of *G. olivieri* Griseb. This lyophilized extract was thoroughly treated with butanol to get 9.8 g butanol fraction.

2.3. Animals

Male Balb/C mice (*Mus musculus*) 8-10 weeks old and weighing 18-22 g, in groups of six each were used for the study. The animals were housed under standard laboratory conditions with a temperature of (23±1) ℃, relative humidity of (55±10)%, 12/12 h light-dark cycles and fed with a standard pellet diet (Lipton India Ltd.) and water was given *ad libitum*. None of the animals were sacrificed throughout the study. Drugs for oral administration were freshly prepared as a homogenized suspension of 80% ethanol extract and butanol fraction of *G. olivieri* at doses of 50, 100, 200 mg/g each in gum acacia and administered orally, once daily for the duration of the experiment to Balb/C mice. Levamisole at the dose of 2.5 mg/kg (p.o.), was used as a standard immunostimulant drug. Cyclophosphamide and cyclosporine-A were used as the standard immunosuppressive agents at 250 and 5 mg/kg (p.o.).

2.4. Chemicals

Bovine albumin saline (BSA) was purchased from Himedia Mumbai. Ethylene diamine tetra acetic acid (EDTA), cyclophosphamide, cyclosporin-A and levamisole were purchased from Sigma Aldrich, New Delhi. All other reagents used were of analytical grade.

Fresh blood was collected from a healthy sheep from a local farmer. Sheep red blood cells (SRBCs) were washed thrice with normal saline adjusted to a concencentration of 0.1 mL containing 5×10^9 cells for immunisation and challenge.

2.5. Experimental protocols

All experimental protocols and the number of animals used for the experimental work were duly approved by the Institutional Animals Ethics Committee (IAEC); vide approval No. ASCB/IAEC/02/10/014, dated June 05,2010.

2.5.1. Humoral antibody response

The mice were divided into 10 groups, each consisting of 6 animals. Mice in group I (control) were given 0.1% bovine serum albumin (BSA saline) 0.3 mL/mouse for 7 days. Mice in group II (sensitized control) were given SRBCs on day 0. Mice in group III-VIII were given cyclophosphamide 250 mg/kg on day 0 and 80% ethanol extract and butanol fraction of *G. olivieri* at doses of 50, 100, 200 mg/kg bw (orally) for seven days. Mice in group IX and X were given levamisol 2.5 mg/kg and cyclophosphamide 250 mg/kg, respectively on day 0. The animals were immunized by injecting 200 μL of 5×10^9 SRBCs/mL intraperitonially (i.p.) on day 0. Blood samples were collected in microlitre tubes from individual animals of all the groups by retroorbital vein puncture on day 8. The blood samples were centrifuged and the serum was separated. Then, haemoglutination primary and secondary titres were performed[7,8].

2.5.2. Delayed type hypersensitivity

A new area of research is the discovery or/and development of immunomodulatory agents that are free from any toxic side effects and can be used for a long duration, resulting in continuous immuno-activation[9]. Animals were divided into ten groups of 6 each. Group I and II served as control and sensitized control, respectively as in humoral antibody response titre. Mice in group III-VIII were administered both extract and fraction of *G. olivieri* after SRBCs sensitization and once daily for seven days. Levamisole (2.5 mg/kg) and cyclosporine-A (5 mg/kg) were administered as standard immunostimulant (group IX) and T-cell suppressor (group X), respectively. The mice were then challenged by injecting the same amount of SRBCs intradermally into the right hind footpad, whereas left hind footpad served as control[10,11].

The footpad thickness was measured with sphaeromicrometer (pitch 0.01 mm) at 0, 24 and 48 h of SRBCs challenge.

2.5.3. In vivo carbon clearance test

The mice were divide into 8 groups. Each group consists of 6 animals. Group I (control) was given 1% sodium carboxy methyl cellulose in water (0.3 mL/mouse, orally) for 5 days, Mice in group II-VIII were given different concentrations of ethanol extract and butanol fraction of *G. olivieri* at doses of 50, 100, 200 mg/g, p.o., and standard drug (levimasole 2.5 mg/kg, p.o.) for 5 days. At the end of 5 days, after the gap of 48 h, the mice were injected, *via* the tail vein, with carbon ink suspension (10 μL/g bw). Blood samples were drawn (in EDTA solution 5 μL), from the retroorbital vein, at interval of 0 and 15 min. A 25 μL sample was mixed with 0.1% sodium

carbonate solution (2 mL) and absorbance was measured at 660 nm. The carbon clearance was calculated using the following equation: (Log_e OD_1–Log_e OD_2)/15, where, OD_1 and OD_2 are optical densities at 0 and 15 min, respectively[12].

2.6. Statistical analysis

Data were expressed as mean±standard error of the means (SEM) and statistical analysis was carried out employing the ANOVA followed by Dunnett test, which compares the test groups and standard drug group with the control group.

3. Results

3.1. Humoral antibody titre

Ethanol extract and butanol fraction of *G. olivieri* at all doses selected (50, 100 and 200 mg/kg) produced dose dependently increase in the primary and secondary antibody formation comparable to levamisole in immunosupressed (cyclophosphamide treated) mice (Table 1). The increase

Table 1

Effect of 80% ethanol and butanol fraction of *G. olivieri* at doses of (50, 100 and 200 mg/kg) on haemagglutination titre in mice (mean±SEM) (n=6).

S.No.	Treatments	Doses (mg/kg)	Primary HA titre	% Change	Secondary HA titre	% Change
1.	Control	–	5.50±0.22	–	5.60±0.21	–
2.	Sensitized control	–	6.50±0.40	–	7.20±0.40	–
3.	Butanol fraction of *G. olivieri* + cyclophosphamide	50 + 250	6.00±0.40	9.09 ↑	6.80±0.40*	21.43 ↑
		100	7.00±0.30**	27.27 ↑	7.80±0.23**	39.28 ↑
		200	7.60±0.56**	38.18 ↑	8.40±0.64**	50.00 ↑
4.	Ethanol extract of *G. olivieri* + cyclophosphamide	50 + 250	5.80±0.40	5.45 ↑	6.40±0.51*	14.28 ↑
		100	6.80±0.51*	23.63 ↑	7.50±0.63**	33.93 ↑
		200	7.40±0.54**	34.54 ↑	8.00±0.40**	42.86 ↑
5.	Levamisole	2.5	8.00±0.40**	45.45 ↑	9.33±0.60**	66.07 ↑
6.	Cyclophosphamide	250	3.00±0.22**	45.45 ↓	4.00±0.40**	28.57 ↓

Statistical analysis was carried out employing the ANOVA followed by Dunnett test. HA: humoral antibody; *: P<0.05, **: P<0.01 comparing with the control; ↑: Potentiation; ↓: Suppression.

Table 2

Effect of 80% ethanol and butanol fraction of *G. olivieri* at doses of (50, 100 and 200 mg/kg) on delayed type hypersansitivitey response in mice (mean±SEM) (n=6).

S.No.	Treatments	Doses (mg/kg)	Mean of right foot pad thickness (mm)			
			24 h	% Change	48 h	% Change
1.	Control	–	0.80±0.00	–	0.71±0.00	–
2.	Sensitized control	–	1.37±0.03	–	1.28±0.07	–
3.	Butanol fraction of *G. olivieri*	50	1.15±0.05*	43.75 ↑	1.09±0.08*	53.52 ↑
		100	1.25±0.07**	56.25 ↑	1.17±0.04**	64.79 ↑
		200	1.31±0.02**	63.75 ↑	1.27±0.03**	78.87 ↑
4.	Ethanol extract of *G. olivieri*	50	1.10±0.06*	37.50 ↑	1.06±0.08*	49.29 ↑
		100	1.14±0.06*	42.50 ↑	1.09±0.06*	53.52 ↑
		200	1.24±0.08**	55.00 ↑	1.17±0.03**	64.79 ↑
5.	Levamisole	2.5	1.47±0.04**	83.75 ↑	1.44±0.05**	102.82 ↑
6.	Cyclosporine-A	5.0	0.52±0.01**	35.00 ↓	0.64±0.04**	9.86 ↓

Statistical analysis was carried out employing the ANOVA followed by Dunnett test. *: P<0.05, **: P<0.01 comparing with the control; ↑: Potentiation; ↓: Suppression.

Table 3

Effect of 80% ethanol and butanol fraction of *G. olivieri* at doses of (50, 100 and 200 mg/kg) on *in vivo* carbon clearance test in mice (mean±SEM) (n=6).

S.No.	Treatments	Doses (mg/kg)	Phagocytic index (k)	% Change
1.	Control	–	0.06±0.02	–
2.	Butanol fraction of *G. olivieri*	50	0.07±0.02	16.39 ↑
		100	0.08±0.01*	29.51 ↑
		200	0.08±0.01**	37.70 ↑
3.	Ethanol extract of *G. olivieri*	50	0.06±0.01	3.29 ↑
		100	0.07±0.02	9.84 ↑
		200	0.07±0.02	14.75 ↑
4.	Levamisole	2.5	0.09±0.02	50.81 ↑

Statistical analysis was carried out employing the ANOVA followed by Dunnett test. *: P<0.05, **: P<0.01 comparing with the control; ↑: Potentiation.

in primary and secondary antibody titre was higher with butanol fraction as compared to alcohol extract. Butanol fraction at a dose of 200 mg/kg produced the maximum increase of 38.18% and 50.00% primary and secondary antibody formation, respectively which were comparable to levamisole 2.5 mg/kg used as a standard drug inducing 45.45% and 66.07% increase in primary and secondary titres, thus indicating ethanol extract and butanol fraction of *G. olivieri* significantly ($P<0.01$) potentiate antibody formation. The production of secondary antibodies was more pronounced as compared to the primary antibodies. Cyclophosphamide 250 mg/kg, a standard immunosuppressor drug showed 45.45% and 28.57% decrease in primary and secondary antibody formation, respectively.

3.2. Delayed type hypersensitivity response

G. olivieri alcohol extract and fraction produced dose related 37.50% to 78.87% increase in delayed type hypersensitivity response at the selected range of doses *i.e.* 50-200 mg/kg. As with humoral antibody titre, the most significant ($P<0.01$) result of 63.75% and 78.87% was observed in 24 and 48 h, respectively with 200 mg/kg butanol fraction of *G. olivieri* in mice (Table 2). The ethanol extract at 200 mg/kg also sinificantly ($P<0.01$) potentiated the delayed type hypersensitivity response to 64.79% in 48 h. Levamisole produced 83.75% and 102.82% delayed type hypersensitivity response in 24 and 48 h, respectively. The results also indicated more potentiation of delayed hypersensitive response in 48 h as compared to that (early) in 24 h.

3.3. In vivo carbon clearance

Butanol fraction of *G. olivieri* exhibited significant increase in carbon clearnce at 100 and 200 mg/kg in mice. It exhibited the most significant results of 37.70% at the dose of 200 mg/kg as compared to 50.81% for levamisole 2.5 mg/kg. The butanol fraction at 100 mg/kg also significantly ($P<0.05$) increased the phagocytic index (Table 3). The alcohol extract although dose dependently increased the carbon clearance but no significant results were obtained at selected doses of 50-200 mg/kg in mice.

4. Discussion

Immunodulation may be specific *i.e.* limited to antigen/agent or non-specific, with a general effect on immune response. Potentiationation of the immune response is desired for certain cases such as for immunocompromised patients, whereas suppression of the immune response is needed for others, such as in organ transplantation, allergic and inflammatory diseased patients. Some plants modulate both humoral and cell-mediated immunity, while others activate only the cellular components of the immune system. The evaluation of plants and/or products that either promote or inhibit immunocyte proliferation is crucial to the study of immunomodulation and drug discovery[13-15]. In this study we found 80% ethanol extract and butanol fraction of flowering aerial part of *G. olivieri* Griseb possess immunostimulant activity in experimental models of cellular and humoral immunity. The effect of ethanol extract of *G. olivieri* and specifically butanol fraction of *G. olivieri* was found to be the most effective at doses of 50, 100, 200 mg/kg each in gum acacia when administered orally. The study was carried out by different methods; each provides information about effect on different components of immune system. As per the literature reviews this is the first ever study conducted to establish the immunostimulatory properties of plant *G. olivieri* Griseb.

Levamisole is the only known oral allopathic salt used as immunostimulant, which restores suppressed immune function of B cells, T cells, monocytes and macrophages whereas cyclophosphamide and cyclosporine-A are used as standard immunosuppressor suppressing B cells and T cells, respectively. Hence, comparative study of these standard drugs and 80% ethanol extract and butanol fraction of aerial part of *G. olivieri* Griseb was planned where effect on B cells, T cells and macrophages was studied *in vivo* in SRBCs immunized BALB/c mice. Both ethanol extract and butanol fraction of *G. olivieri* at doses of 50-200 mg/kg significantly dose dependently increased primary and secondary humoral antibody titre with the highest increase at 200 mg/kg of butanol fraction, as compared to the controls. Antibody molecules, consisting of B lymphocytes and plasma cells, are the central to humoral immune responses. The major immunoglobins, IgG and IgM are involved in immune processes in the form of complement activation, opsonization, neutralization of toxins, *etc*. The potentiation of haemagglutinating antibody titres indicated, that *G. olivieri* extracts induced immunostimulation through humoral immunity. Cell mediated responses involving T lymphocytes and lymphokines are critical to delayed type hypersensitivity reactions, tumor immunity and infection against foreign microorganisms. The delayed type hypersensitivity response directly correlates with cell mediated immunity which was dose dependently significantly increased by both extracts of *G. olivieri* at all doses and was tested with the highest at 200 mg/kg of butanol fraction. There is an evidence to suggest that delayed type hypersensitivity reaction is important in host defense against parasites and bacteria that can live and proliferate intracellularly. An increase in delayed type hypersensitivity response indicates that plant extract and fraction have a stimulatory effect on lymphocytes and accessory cell types required for the expression of reaction response and B cell activation[13,14,16].

Phagocytosis represents an important immune defence mechanism in which leukocytes ingest pathgenic microorganisms, malignant cells, tissue debris and inorganic particles (carbon ink). The *in vitro* phagocytosis test was done to evaluate the effect of extracts on the reticuloendothelial system (RES). It is a diffuse system containing phagocytic cells. When the colloidal carbon particles are injected directly into the systemic circulation,

it is cleared by RES involving phagocytes. Butanol fraction of *G. olivieri* showed remarkable augmentation at 200 mg/kg in the phagocytic index. The result is owing to a mechanism related to phagocytosis by macrophages. The process of phagocytosis by macrophages includes opsonisation of the foreign particulate matter with antibodies and complement C3b leading to more rapid clearance of foreign particulate matter from the blood[17-21]. Alcohol extract although produced dose dependently increase in phagocytic index but no significant value was obtained with selected doses. However, higher doses could have significantly potentiated the phagocytic index.

In conclusion, both the 80% ethanolic extract particularly its butanol fraction most significantly stimulated the immune system by acting through cellular and humoral immunity in animals. This is the first ever immunostimulatory property reported on this plant. The study justifies antidiabetic and hepatoprotective properties of the plant. The plant can be explored for its medical utilization in treatment of immunodeficiency diseases, cancer and as combinational therapy with antibiotics. The results are found to be encouraging enough to isolate the bioactive compound and trace the exact mechanism of action.

Conflict of interest statement

We declare that we have no conflict of interest.

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