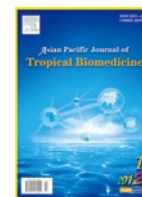




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Evaluation of immunomodulatory activity of *Glycyrrhiza glabra* L roots in combination with zing

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ABSTRACT

Objective: To determine the immunomodulatory properties of roots of *Glycyrrhiza glabra* (*G. glabra*) L. (Liquorice), and also to check whether significant potentiation in immunomodulation occurs or not with the combination of zinc. **Methods:** Immunomodulatory potential of aqueous liquorice extract (ALE) 0.75 and 1.5 g/kg BW and its combination with zinc (45 mg/kg BW) were determined by the effect on leukocyte count and spleen weight, *In vivo* phagocytosis (carbon clearance method), determination of cellular immune response, haemagglutination antibody titre and plaque forming cell (PFC) assay using sheep red blood cells (SRBC). In addition, the effect of the extract on Systemic Anaphylactic reaction was also measured. **Results:** Leukocyte count and phagocytic index (carbon clearance) was increased significantly with the treatment of ALE (1.5 g/kg) compared to control ($P < 0.05$). Zinc (45 mg/kg) in combination with ALE (0.75 g/kg) showed highly significant increase of leukocyte count and phagocytic index compared to control ($P < 0.01$). Same groups also showed positive significant action on spleen weight. In cellular immune response study, an enhancement in foot pad thickness was observed when compared to control group. Neither of the two doses of ALE showed significant increase in HA titre value and antibody secreting cells of mouse spleen. However, combination of zinc (45 mg/kg) and ALE (0.750 g/kg) showed significant increase ($P < 0.05$) in HA titre and antibody secreting cells of mouse spleen value compared to vehicle control. In systemic anaphylaxis reaction test, results indicate a positive effect on anaphylaxis with the treatment of ALE in both doses and in combination with zinc. **Conclusions:** The present study has shown the immunomodulatory activity of aqueous root extract of *G. glabra* L. at the dose 1.5 g/kg BW. *G. glabra* L. in combination with zinc has shown potentiation of immunomodulatory activity in all aspects of the study.

1. Introduction

The immune system is a remarkably versatile defense system that has evolved to protect animals from invading pathogenic microorganisms and to eliminate disease. It is able to generate an enormous variety of cells and molecules capable of specifically recognizing and eliminating an apparently limitless variety of foreign invaders[1]. Immunomodulation is required when host defense mechanism has to be activated under the conditions of impaired immune response or when a

selective immunosuppression is desired in situations like autoimmune disorders. Immunomodulatory medicinal plants are comparatively a recent concept in phytomedicine. Immunomodulation using medicinal plants can provide an alternative approach to conventional chemotherapy for a variety of diseases, especially when host defense mechanism has to be activated under the conditions of impaired immune response or when a selective immunosuppression is desired in situations like autoimmune disorders.

Glycyrrhiza glabra (*G. glabra*) L. (Liquorice) belongs to the family Fabaceae is a low growing, perennial shrub, member of pea family native to Europe (southeast and southwest), Mediterranean countries (North of Africa) and central Asia[2]. Liquorice root decoction is often used as an antitussive, respiratory balm and to relieve gastriculcers[3]. Glycyrrhizin a triterpenoid compound, accounts for the sweet taste of liquorice root, has been used for more than 60 years in the treatment of chronic hepatitis C[2,4] and is also

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reported as an antiviral agent[5]. Liquorice decreases serum testosterone level in women[2] and is beneficial in aplastic anemia and Addison's disease[4]. Since, liquorice extract is used in auto-immune conditions and has therapeutic benefit in immunodeficiency conditions like AIDS, the present study was aimed at exploring immunomodulatory principles, cellular and/or humoral, of an aqueous extract of roots of *G. glabra* L.

Zinc is one of the essential minerals that the body needs in order to function properly. Studies are showing that even moderately low levels of zinc may lead to depressed immune system function. More recently, zinc has been prescribed for immunodeficient hosts, to modulate the immune system so that to a certain extent it can combat against opportunistic pathogens[6]. Hence, the present study is aimed to check whether significant potentiation in immunomodulation occurs or not with the combination of zinc and liquorice, so that such combinations can be used later in suitable formulations.

2. Materials and methods

2.1. Plant material

Roots of *G. glabra* L. were collected from Herbal store, local market of Ranchi, Jharkhand. The roots were identified and authenticated by Dr. Kaushal Kumar, Senior Scientist and Head, Patanjali Herbal Garden & Agro Research Department, Haridwar. The plant specimen (Herbarium) was also submitted in Dept. of Pharmaceutical Sciences, BIT, Mesra, Ranchi.

2.2. Extraction

The roots were carefully dried in shade for 15 d. After drying roots were subjected to size reduction. The crushed mass of root was then ready for extraction. About 500 g of dried *G. glabra* root (crushed) mass was simply extracted with 2 500 mL distilled water [water/root mass ratio 5:1] at 70–80 °C for 5 h. The aqueous extract obtained after vacuum filtration was evaporated to dryness, dried at 50 °C and stored in cool and dark place in tight pack container for further use.

2.3. Animals

Swiss male albino mice weighing 25–30 g of either sex were used in the study. Animals were procured from Laboratory Animal House of Birla Institute of Technology, Mesra (Reg. no.: 621/02/ac/CPCSEA). All animal experiments strictly complied with the approval of institutional animal ethics committee. The animals were kept in polyacrylic cages and maintained under standard housing conditions of temperature (24–27 °C) and humidity (60%–65%) with 12:12

light:dark cycles. They were acclimatized for seven days. Food was provided in the form of dry pellets and water ad libitum.

2.4. Preliminary phytochemical screening

Preliminary tests were carried out for the presence or absence of phytoconstituents like glycosides, flavonoids, saponins, alkaloids, carbohydrates, sterols, proteins, phenolic compounds and reducing compounds[7].

2.5. Procurement of sheep red blood cells (SRBC)

10 mL blood was withdrawn from the jugular vein of a healthy sheep from the Veterinary College, Birsa Agricultural University, Ranchi, Jharkhand, and collected in 10ml of autoclaved Alsever's solution (2.05% w/v glucose, 0.42% w/v sodium chloride (NaCl), 0.8% w/v trisodiumcitrate, 0.055% w/v citric acid). Then it was centrifuged at 500 g for 15 min at 4 °C in cooling centrifugation apparatus to separate the red blood cells (RBCs). Then these sheep RBCs were washed thrice with sterile normal saline and adjusted to concentration of 0.1 mL containing 1×10^8 cells (10% SRBC) using Alsever's solution for immunization and stored in refrigerator at 4 °C.

2.6. Selection of doses

From the acute toxicity studies there was no mortality till the dose level of >7.5 g/kg BW of *G. glabra* root extract in mice by oral route. Thus, the two different doses selected for test groups were 1/10th (0.75 g/kg BW) and 1/5th (1.5 g/kg BW) of the safe dose. As zinc has showed good immunomodulatory activity at the dose 45 mg/kg BW in mice, the same dose was selected for study.

2.7. Treatment protocol

The animals were numbered, weighed and then divided into 4 groups with 6 animals in each as follows:

Group I: Served as vehicle control and received PBS (pH 7.4) 1 mL/100 g BW, p.o.

Group II: Received 0.750 g/kg BW, aqueous extract of liquorice root, p.o.

Group III: Received 1.5 g/kg BW, aqueous extract of liquorice root, p.o.

Group IV: Received 0.750 g/kg BW, p.o. of aqueous extract of liquorice root + zinc solution 45 mg/kg BW, p.o.

2.8. Effect on leukocyte count and spleen weight

Mice were treated as stated above (treatment protocol) for 5 consecutive days. On day 6, blood was collected from caudal vein for white blood cells (WBC) count. The results of analyses compared with control groups. The animals were sacrificed by cervical dislocation and their spleens were

harvested for weighing. The results of this analysis were compared with that of vehicle control[8].

2.9. *In vivo phagocytosis (carbon clearance method)*

The aqueous extract of *G. glabra* (in doses stated above) was administered to the animals (test group) orally for 5 consecutive days. Control group received PBS (pH 7.4). After 48 h of last dose mice were injected via the tail vein with colloidal carbon (Indian ink), which was diluted with PBS (pH 7.4) to eight times before use ($10 \mu\text{L/g BW}$). Blood samples were drawn from the caudal vein at 0 and 15 min. The blood ($25 \mu\text{L}$) was dissolved in 0.1% sodium carbonate (2 mL) and the absorbance was measured at 660 nm[8,9]. The phagocytic index, K, was calculated by equation:

$$K = \ln OD_1 - \ln OD_2 / t_2 - t_1$$

where, OD1 and OD2 depict the optical densities at times t_1 and t_2 , respectively.

2.10. *Cellular immune response*

The aqueous extract of *G. glabra* (in doses stated above) was administered to the animals (test group) orally on -3, -2, -1, 0, +1, +2, +3, +4, +5, +6 days. Control group received PBS (pH 7.4). Mice were sensitized with 0.1 mL of SRBC suspension containing 1×10^8 cells intraperitoneally on day 0 and challenged on day 7 with same volume of SRBC, intradermally into the left footpad of each mouse. The increase in footpad thickness (FPT) was measured 24 h after SRBC challenge, using plethysmometer. The degree of DTH (delayed type hypersensitivity) reaction was expressed as the percentage increase in FPT over the control values[10].

2.11. *Haemagglutination antibody titre*

The aqueous extract of *G. glabra* (in doses stated above) was administered to the animals (test group) orally on -4, -3, -2, -1, 0, +1, +2, +3, +4 days (0 day : the day of immunization with SRBC, -4, -3, -2, -1 : the days before immunization, +1, +2, +3, +4 : the days after immunization). Control group received PBS (pH 7.4). Mice were intraperitoneally immunized with 1×10^8 SRBC in the volume of 0.1 mL on day 0. Blood samples were collected from caudal vein in microcentrifuge tubes to separate the sera.

Antibody titre was determined following the procedure reported by Nelson and Mildenhall[11]. To two-fold serial dilutions of serum samples made in $25 \mu\text{L}$ volumes of normal saline containing 0.1% bovine serum albumin (BSA saline) in V bottom heamagglutination plates (96 well microtitre plates) were added $25 \mu\text{L}$ of 0.1% suspension of SRBC in BSA saline. After thorough mixing SRBC were allowed to settle at room temperature for 90 min until control wells showed small button of cells (negative pattern). The values of the highest serum dilution causing visible heamagglutination

was considered as the antibody titre[12].

2.12. *Plaque forming cell (PFC) assay*

The assay was performed according to the method reported by Jerne and Nordin. After immunizing the animals with SRBC on day 0, they were treated as stated above for 5 consecutive days. Briefly, the spleen cells of SRBC immunized extract treated mice were separated in RPMI-1640 medium, washed twice and suspended in same medium. Glass petridishes were layered with 1.2% agarose in 0.15 M Sodium chloride to form bottom layer.

A mixture of 2 mL agarose (0.6%) in RPMI-1640 medium (42°C), 0.1 mL suspension of 20% SRBC and 1×10^6 spleen cells in a volume of 0.2 mL was poured over the bottom layer of agarose followed by an incubation period of 90 min at 37°C . A 2 mL quantity of 1:9 diluted fresh rabbit serum was added to petridish and plates were reincubated for 40 min to allow the formation of plaques. The numbers of plaques were counted immediately and values were expressed as counts per 10^6 spleen cells[13].

2.13. *Systemic anaphylaxis reaction*

Animals were sensitized by 1mg bovine serum albumin (BSA) subcutaneously in 0.2 mL PBS (pH 7.4) at day 0 and were shocked by intravenous injection of 1mg BSA in PBS (pH 7.4) on day 14. Immunomodulatory activity was examined by injecting different doses as stated in treatment protocol on alternative days from day 0 to day 14 for seven times before shocking injection. The systemic anaphylactic reaction was observed within 30 min following the shocking injection and rated in following fashion: positive reaction, mortality or animal rendered still for at least 1 min; negative reaction with no change or normal movement[8].

2.14. *Statistical analysis*

The values were calculated as mean \pm S.D. The significance of the difference of the mean value with respect to control group was analyzed by one way ANOVA followed by Dunnet's *t*-test using statistica 8.0. $P < 0.05$ or above was considered to be significant.

3. Results

3.1. *Preliminary phytochemical screening*

The preliminary phytochemical analysis of the aqueous root extract of *G. glabra* L. showed the presence of different class of constituents such as, glycosides, flavonoids, saponins, carbohydrates, sterols, proteins, phenolic compounds and reducing compounds.

Table 1.

Effect of aqueous liquorice extract (ALE), and its combination with zinc on leukocyte count, spleen weight and carbon clearance index.

Group	Treatment	Leukocyte count/mm ³	Spleen weight (g/100 g BW)	Carbon clearance index
Group I	PBS (pH 7.4)	3 540.00±82.76	0.360±0.025	0.034 8±0.003 0
Group II	ALE (0.75 g/kg)	3 850.00±106.07	0.418±0.020	0.040 4±0.004 0
Group III	ALE (1.50 g/kg)	4 040.00±106.54*	0.508±0.041*	0.068 0±0.010 0*
Group IV	ALE (0.75 g/kg) +Zn (45 mg/kg)	4 450.00±155.72**	0.523±0.045*	0.083 6±0.010 0**

Values are expressed as mean±S.E.M. **P*<0.05 (significant compared to control) ***P*<0.01 (highly significant compared to control).**Table 2.**

Effect of aqueous liquorice extract (ALE), and its combination with zinc on footpad thickness (paw volume).

Group	Treatment	Percentage increase in paw volume
Group I	PBS (pH 7.4)	16.67±10.54
Group II	ALE (0.75 g/kg)	33.34±16.67
Group III	ALE (1.50 g/kg)	58.34±15.37
Group IV	ALE (0.75 g/kg) +Zn (45 mg/kg)	66.67±30.73

Table 3.Effect of aqueous liquorice extract (ALE), and its combination with zinc on HA titre and plaque forming cells/10⁶ cells.

Group	Treatment	HA titre	Plaque forming cells/10 ⁶ cells
Group I	PBS (pH 7.4)	76.80±12.80	298.00±5.83
Group II	ALE (0.75 g/kg)	115.20±12.80*	308.00±5.83*
Group III	ALE (1.50 g/kg)	153.60±25.60*	312.00±3.74*
Group IV	ALE (0.75 g/kg) +Zn (45 mg/kg)	204.80±31.35**	410.00±8.94**

Values are expressed as mean±S.E.M. **P*<0.05 (insignificant compared to control) ***P*<0.05 (significant compared to control).**Table 4.**

Effect of aqueous liquorice extract (ALE), and its combination with zinc on systemic anaphylaxis reaction.

Sensitizing injection 0.1 mL	Shocking injection 0.1 mL	Treatment	Number of mice		
			AS	D	T
BSA (<i>s.c.</i>)	BSA (<i>i.v.</i>)	PBS (pH 7.4)	6	0	6
BSA (<i>s.c.</i>)	BSA (<i>i.v.</i>)	ALE (0.75 g/kg)	4	0	6
BSA (<i>s.c.</i>)	BSA (<i>i.v.</i>)	ALE (1.50 g/kg)	3	0	6
BSA (<i>s.c.</i>)	BSA (<i>i.v.</i>)	ALE (0.75 g/kg) +Zn (45 mg/kg)	2	0	6

BSA: bovine serum albumin; PBS: phosphate buffer saline; ALE: aqueous liquorice extract; AS: anaphylactic symptoms; D: death; T: total number of mice.

3.2. Effect on leukocyte count and spleen weight

The effect of aqueous liquorice extract administration as such and that in combination with zinc on leukocyte count and spleen weight is shown in Table 1. Leukocyte count was increased significantly with the treatment of ALE (1.5 g/kg) compared to control (*P*<0.05). Zinc (45 mg/kg) in combination with ALE (0.750 g/kg) showed highly significant increase of leukocyte count compared to control (*P*<0.01). Significant positive effect was observed in Groups III & IV, compared to vehicle control (*P*<0.05). ALE (0.750 g/kg) did not show significant positive effect on spleen weight.

3.3. In vivo phagocytosis (carbon clearance method)

The effect of aqueous liquorice extract administration as such and that in combination with zinc on carbon clearance (phagocytosis) is shown in Table 1. Phagocytic index was significantly increased after the administration of ALE (1.5 g/kg) compared to vehicle control (*P*<0.05).

ALE (0.750 g/kg) in combination with zinc (45 mg/kg) showed highly significant increase in phagocytic index compared to vehicle control (*P*<0.01). ALE in lower dose did not show

significant effect on phagocytosis.

3.4. Cellular immune response

The effect of aqueous liquorice extract administration as such and that in combination with zinc on T-cell mediated DTH reaction is shown in Table 2. An enhancement in foot pad thickness was observed when compared to control group.

3.5. Haemagglutination antibody titre

The effect of aqueous liquorice extract administration as such and that in combination with zinc on HA titre is shown in Table 3. Haemagglutination antibody titer was determined to establish the humoral response against SRBC. At neutral pH, red blood cells possess negative ion cloud that makes the cells repel from one another.

This repulsive force is referred to as zeta potential. Because of its size and pentameric nature, IgM can overcome the electric barrier and get cross-link red blood cells, leading to subsequent agglutination. Neither of the two doses of ALE showed significant increase in HA titre value. However,

combination of zinc (45 mg/kg) and ALE (0.750 g/kg) showed significant increase ($P<0.05$) in HA titre value compared to vehicle control.

3.6. Plaque forming cell (PFC) assay

The effect of aqueous liquorice extract administration as such and that in combination with zinc on number of antibody producing cells in spleen is shown in Table 3. ALE in both doses did not show significant effect on antibody secreting cells of mouse spleen. But, ALE (0.750 g/kg) in combination with zinc (45 mg/kg) produced increase in antibody secreting cells in mouse spleen. Effect was significant ($P<0.01$) compared to control.

3.7. Systemic anaphylaxis reaction

Effect of aqueous liquorice extract administration as such and that in combination with zinc on active systemic anaphylaxis is shown in Table 4. Results indicate a positive effect on anaphylaxis with the treatment of ALE in both doses and with the treatment of ALE in combination with zinc, suggesting a reduction in number of animals presenting anaphylactic symptoms. In the case of vehicle control group, all mice exhibited anaphylactic symptoms.

4. Discussion

Initially, effects on blood leukocyte count and spleen weight were determined to investigate immunomodulatory effect of ALE and that in combination with zinc. Among different organs of immune system, spleen represents a major secondary lymphoid organ involved in elicitation of immune response. Unlike lymph nodes, which are specialized to trap-localized antigen from regional tissue spaces, the spleen is adapted to filtering blood and trapping blood-borne antigens and thus can respond to systemic infections[8].

Results from the present study revealed a significant increase in the blood leukocytes count ($P<0.05$) and weight of spleen ($P<0.05$) compared to vehicle control group, suggesting an enhancement of immune status in groups III & IV.

Anaphylactic reaction is mediated by immunoglobulin E (IgE) class of antibodies. IgE has high affinity to Fc receptors on the surface of tissue mast cells and blood basophils and thus binds readily to these cells. Such IgE coated mast cells and basophils are said to be 'sensitized.' A later exposure to the same allergen cross-links the membrane bound IgE on sensitized mast cells and basophils causing degranulation of these cells.

The pharmacologically active mediators released from the granules exert biological effects like vasodilatation and smooth muscle contraction that may be either systemic or

localized depending on the extent of mediator released[14]. In the present study, the ALE in higher dose(1.5 g/kg BW) and combination of ALE (0.750 g/kg BW) and zinc (45 mg/kg BW) showed a decrease in total number of animals showing anaphylactic symptoms. Thus we can conclude that through several probable mechanisms liquorice extract and zinc could have prevented anaphylactic symptoms, when sensitized and subsequently challenged by BSA.

Prophylactic treatment of ALE and that in combination with zinc enhanced the rate of carbon clearance from the blood when compared to control group. Phagocytosis represents an important innate defense mechanism against ingested particulates including whole pathogenic microorganisms. The specialized cells that are capable of phagocytosis include blood monocytes, neutrophils and tissue macrophages. Once the particulate material is ingested into phagosomes, the phagosomes fuse with lysosomes and the ingested material is then digested[1]. Enhanced uptake of particulate matter with the treatment of ALE (1.5 g/kg BW) and combination of ALE (0.750 g/kg BW) with zinc (45 mg/kg BW) is evident from carbon clearance test.

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 a more rapid clearance of foreign particulate matter from the blood. ALE and zinc were found to stimulate the phagocytic activity of the macrophages as evidenced by an increase in the rate of carbon clearance. However, a dose proportionate response was not observed, since immune response is not always directly related with the immunomodulator concentration.

Number of antibody secreting cells from spleen was determined using plaque forming cell assay. Since spleen contributes immensely to the humoral as well as cellular arms of immune system, its role in generation of antibody secreting cells was studied. There were no effects of ALE in the anti-sheep red blood cell plaque forming assay as compared to vehicle control animals. This result indicate that short term administration of liquorice extract at concentration upto 1.5 g/kg BW has no effect on antibody producing cells of spleen. However, ALE (0.750 g/kg BW) in combination with zinc (45 mg/kg BW) produced significant enhancement in number of cells secreting antibodies against SRBC, which served as specific antigen.

Heamagglutination antibody titer was determined to establish the humoral response against SRBC. At neutral pH, red blood cells possess negative ion cloud that makes the cells repel from one another. This repulsive force is referred to as zeta potential. Because of its size and pentameric nature, IgM can overcome the electric barrier and get cross-link red blood cells, leading to subsequent agglutination. The smaller size and bivalency of IgG, however, makes them less capable to overcome the electric

barrier.

This characteristic may be accounted for, IgM being more effective than IgG in agglutinating red blood cells. ALE in both doses did not show significant increase of hemagglutination antibody titer value as compared to control group reflecting that the ALE has no effect on humoral immune response on short term treatment. However ALE (0.750 g/kg BW) in combination with zinc produced significant enhancement in antibody titer value ($P < 0.05$) compared to vehicle control group.

Delayed type hypersensitivity reaction is characterized by large influxes of nonspecific inflammatory cells, in which the macrophage is a major participant. It is a type IV hypersensitivity reaction that develops when antigen activates sensitized T_H1 cells[14].

These cells generally appear to be a TH1 subpopulation although sometimes TC cells are also involved. Activation of T_H1 cells by antigen presented through appropriate antigen presenting cells results in the secretion of various cytokines including interleukin-2, interferon- γ , macrophage migration inhibition factor and tumor necrosis factor- β .

The overall effects of these cytokines are to recruit macrophages into the area and activate them, promoting increased phagocytic activity vis-a-vis increased concentration of lytic enzymes for more effective killing[8,14]. Several lines of evidence suggest that DTH reaction is important in host defense against parasites and bacteria that can live and proliferate intracellularly. Treatment with liquorice extract in higher dose and liquorice extract in lower dose with zinc enhanced DTH reaction, which is reflected from the increased footpad thickness compared to control group suggesting heightened infiltration of macrophages to the inflammatory site. This study may be supporting the possible role of ALE in assisting cell-mediated immune response.

The present study has shown the immunomodulatory activity of aqueous root extract of *G. glabra* L. in cellular arm response (cellular immunity), phagocytic response and anaphylactic reaction at the dose 1.5 g/kg BW. However *G. glabra* L. did not show significant effect on humoral immunity and number of antibody producing cells of spleen, reflecting *G. glabra* L. has no effect on such responses on short term treatment. *G. glabra* L. in combination with zinc has shown potentiation of immunomodulatory activity in both humoral as well as cellular arms of the immune system, suggesting its therapeutic usefulness in immune compromised patients on long term basis.

Conflict of interest statement

We declare that we have no conflict of interest.

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