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Histological and Histometrical Examination in Assessment of Potential Immune Modulator Properties of *Glycyrrhizaglabra* Extract in Rats

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Abstracts: There is growing interest in the use of herbs to aid in the maintenance of health. Licorice root (*Glycyrrhiza*) has been employed in traditional Iranian medicine to treat infectious diseases. Thirty-two Sprague-Dawley male rats were randomly distributed into four groups. Experimental groups were injected intra-peritoneal with aqueous extract of *G. glabra* at 50, 100 and 200 mg/kg/day, respectively, for 30 consecutive days while control group was injected with distilled water without any add-on material. At the end of the experiments, blood samples were drawn and total as well as differential leucocytes count was performed. Also, the histological specimens from spleens, thymuses, adrenal glands and sub iliac lymph nodes were taken. The number of splenic megakaryocyte in the *G. glabra* treated animals decreased also the diameter of the splenic follicles showed a significant increase compared to those of the controls ($P<0.01$). The size of the adrenal glands of experimental animals showed significant increase in the medulla as well as in the reticular layer of cortex ($P<0.01$). In the *G. glabra* treated animals thymic cortex and medulla thickness were significantly higher than those of the controls ($P<0.01$). The diameter of the lymph nodes follicles of *G. glabra* given animals was higher than those of the controls ($P<0.01$). Total leucocytes count significantly increased in the *G. glabra* treated animals compared with control samples ($P<0.01$). Also, significant increases were also observed in the lymphocytes count in the peripheral white blood cells ratio of the *G. glabra* treated groups ($p<0.05$). In addition, significant decreases were also observed in the neutrophils and monocytes counts in the peripheral white blood cells ratio of the *G. glabra* treated groups ($p<0.05$). It is may be concluded that *G. glabra* extract had more beneficial impacts on histological and histometrical aspects of immune system.

Key words: Glycyrrhizaglabra · Histology · Potential Immune-Regulatory · Rat

INTRODUCTION

Glycyrrhizaglabra (Licorice) belonging to leguminosae family of the plants has been used for centuries as an herbal medicine for treatment of various diseases including chronic liver diseases, infections and inflammatory diseases [1].It 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]. 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 [2,3]and is also reported as an antiviral agent [4]. Liquorice decreases serum testosterone level in women.

Immune system is a very complex homeostatic system consisting of a network of interacting cells, tissues and organs. It allows the organism to exist within itself and maintains a surveillance mechanism to recognize certain components that are considered uyjhm. Immune modulators are substances, which modify the activity of the immune system. Immune modulators have biphasic effects some tend to stimulate immune system which are low while inhibit

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host defense parameters which are normal or already activated [5]. Some of the plants with known immunomodulatory activity are *Viscum album* [6], *Tinosporacordifolia* [7], *Withaniasomnifera* [8] and *Echinacea speczies* [9].

Since, liquorice extract is used in auto-immune conditions and has therapeutic benefit in immunodeficiency conditions like AIDS [10]; the present study was aimed at exploring potential immune-regulatory properties of *Glycyrrhizaglabra* extract histological and histometrical methods.

MATERIALS AND METHODS

Experimental Animals: The study was carried out for one month and for carrying out the experiments, thirty-two Sprague-Dawley male rats aged 10 to 12 weeks and initial weight of 298 ± 16.8 g were randomly distributed into four groups (n=8). The treatment groups were injected intra-peritoneal with aqueous extract of *G. glabra* at 50, 100 and 200 mg/kg/day, respectively, for 30 consecutive days. Control group was injected with distilled water without any add-on material. The doses were determined on the basis of a primary study. The animals of each group were housed in separate cages with sawdust bedding. Rats were housed in one stainless-steel cage under conventional conditions (temperature 22 ± 1 ºC; relative humidity 50 ± 10%; 12: 12 h light-dark natural cycle) and had ad-lib access to drinking water and food. The animals were allowed to be acclimatized to the laboratory environment at least 6 days before commencement of testing. All procedures that involved animals were approved by the Veterinary Ethics Committee of the Faculty of Para-Veterinary Medicine of Ilam University.

Plant Extraction: The *G. glabra* (Licorice) root was purchased from Emam-Reza medicinal plants market (Ilam, Iran) and botanical identification was confirmed at the herbarium of Ilam University (Exsiccate number: 132-4-91). For extraction preparation, the roots of plant was washed with sterile water, dried in shade at room temperature for 3 weeks and ground in an electric mill to obtain particles smaller than 4 mm. This material was extracted by maceration in 70% methanol solution at 50 °C for 2 hours. The extract was filtered through a ≠1 Wattman 1 paper and evaporated to dryness in a rotary evaporator under reduced pressure. The dried material was stored under refrigeration at 4-8 °C until its use.

Histological and Histometrical Assessment: At the end of the administration period; the animals were anaesthetized with chloroform vapor, quickly brought out of the jar and sacrificed. Heparinized blood samples were drawn by cardiac puncture. For tissue assessment; the specimens from spleens, thymuses, adrenal glands and sub iliac lymph nodes were immersion imprisoned overnight in 10% neutral buffered formalin to be fixed. Then the specimens were mounted to allow 5-µm sections. Sections were stained via Hematoxylin and Eosin (HandE) method and photographed directly using a stereo microscope in 400 high power fields with Microsoft system.

For exact description of the structural changes in the lymphatic tissues a histometrical analyze was performed. For this purpose, splenic megakaryocytes in unit area of (1.44×10^4 µm^2 tissue area) were determined by counting in 10 randomly selected areas in subcapsular white pulp regions [11] using Image Tool® 3.0 software (UTHSCSA, San Antonio, TX, USA). Also, in each animal from all of the groups 10 tissue sections (7µm) were taken at 21 µm intervals and splenic capsule thickness and also splenic follicular diameter were recorded. Furthermore, thymic capsule thickness, thymic cortex diameter as well as thymic medulla diameters were recorded. In addition, the thickness of the glomerular, fascicular, reticular and medullary layers of adrenal glands has been determined. Finally, the thickness of the lymph nodes capsule and the diameter of the lymph nodes follicles were determined.

Statistical Analysis: All statistical analyses were carried out using Statistical Package for Social Scientist (SPSS version 13, Chicago, IL, USA). Results were tested for normal distribution (Shapiro-Wilks) and homogeneity of variances (Bartlett test) and then expressed as standard error of the mean (SEM). The analysis of variance (ANOVA) was used to test the overall significance of differences among the means. Tukey-Kramer´s Multiple Comparison Test was applied for post hoc comparison. A probability level of less than 5% ($P < 0.05$) was considered as significant.

RESULTS

Spleen: The number of splenic megakaryocyte in the *G. glabra* treated animals have been decreased significantly in comparison with control animals ($P<0.01$) (Table 1) (Figure 1). The thickness of the spleen´s capsule was not affected by *G. glabra* administration

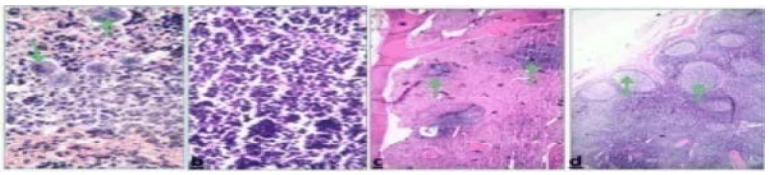

Fig. 1: Spleen cross sections of the control (a, c) and 100 mg/kg G. glabra treated (b, d) animals. The section shows the presence many of megakaryocytes (arrows) in the whole parenchyma of the spleen, but in the b part the number of splenic megakaryocyte in the G. glabra treated animals have been decreased. The c part shows the diameter of the splenic follicles (arrows) in the whole parenchyma of the spleen, but d part of figure indicates a significant increase in the diameter of splenic follicles (arrows). (H&E,)(a, b: × 400; c, d: × 200)

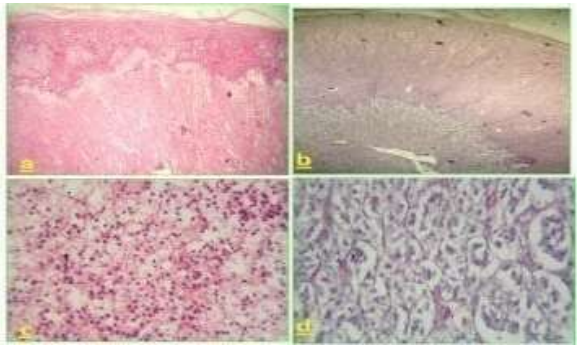

Fig. 2: Adrenal gland cross sections of the control (a, c) and 200 mg/kg G. glabra treated (b, d) animals. The figure shows significant increasing in the size of medulla as well as reticular layer of cortex in the G. glabra treated animals. (H&E,)(a, b: × 200; c, d: × 400)

Table 1: Summarized histometric changes in the spleen and adrenal glands of the rats administrated with different concentrations of G. glabra.

		Groups				
Parameters		Control	50 mg/kg/day G. glabra	100 mg/kg/day G. glabra	200 mg/kg/day G. glabra	Significance
Spleen	SCT(µm)	4.3 ±0.01	4.2 ±0.03	3.8 ±0.02	4.7 ±0.09	-
	GCD (µm)	84.5 ±0.6	134.1 ±6.9	173.4 ±7.5	136.6 ±8. 9	**
	MC/UA	4.1 ±0.04	1.6 ±0.07	0.4 ±0.01	0.3 ±0.05	**
Adrenal	GLT(µm)	74.6 ±0.4	74.2 ±0.5	73.8 ±0.8	76.2 ±0.4	-
gland	FLT(µm)	94.3 ±0.4	95.5 ±0.6	98.4 ±0.7	96.5 ±0. 3	-
	RLT(µm)	12.1 ±0.04	33.4 ±0.06	29.9 ±0.3	24.6 ±0.8	**
	MT(µm)	14.4 ±0.6	74.7 ±0.5	67.4 ±0.7	56.1 ±0.2	**

-: not significant,* : P<0.05, ** : P<0.01

SCT: Splenic capsule-thickness, GCD: The diameter of germinal center of the lymphoid follicles, MC/UA: Megakaryocyte count/unit (1.44×104µm2) tissue area, GLT: Glomerular layer thickness of adrenal, FLT: Fascicular layer thickness of adrenal, RLT: Reticular layer thickness of adrenal, MT: Medullary layer thickness of adrenal.

Table 2: Summarized histometric changes in the thymus and sub iliac lymph nodes of the rats administrated with different concentrations of G. glabra.

		Groups				
Parameters		Control	50 mg/kg/day G. glabra	100 mg/kg/day G. glabra	200 mg/kg/day G. glabra	Significance
Thymus	TCT(µm)	5.6 ±0.03	5.2 ±0.04	5.3 ±0.09	5.4 ±0. 03	-
	TMD(µm)	24.5 ±0.6	35.7 ±0.3	42.5 ±0.6	38.9 ±0.3	*
	TCD(µm)	17.3 ±0.7	61.2 ±0.9	35.9 ±0.8	65.1 ±0.4	**
Lymph node	LCT(µm)	7.3 ±0.06	7.1 ±0.09	6.4 ±0.02	6.8 ±0. 08	-
	LFD(µm)	27.3 ±0.8	49.5 ±0.2	49.7 ±0.9	58.5 ±0. 3	**

-: not significant, * : P<0.05, ** : P<0.01

TCT: Thymic capsule thickness, TMD: Thymic medulla diameter, TCD: Thymic cortex diameter, LCT: Lymph node capsule thickness, LFD: Lymph node follicular diameter.

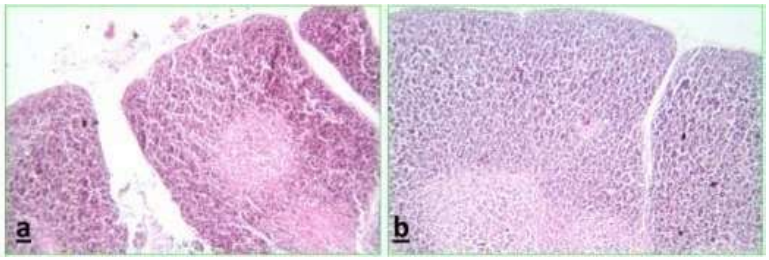

Fig. 3: Thymus cross sections of the control (a) and 200 mg/kg *G. glabra* treated (b) animals. The figure shows significant high thickness of thymic cortex and also medulla in the *G. glabra* treated animals. (HandE,) (a: × 200; b: × 400)

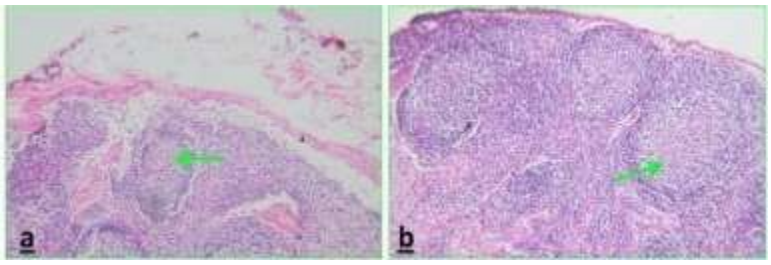

Fig. 4: Transverse sections of sub iliac lymph nodes in the control (a) and 100 mg/kg *G. glabra* treated (b) animals. The figure shows significant high diameter of the lymph nodes follicles in the *G. glabra* given animals. (HandE,) (a: × 200; b: × 400)

Table 3: Mean ± standard deviation and range of the leukocyte differential count values of the control and mice administrated with different concentrations of G. glabra.

		Groups				
Parameters		Control	50 mg/kg/dayG. glabra	100 mg/kg/dayG. glabra	200 mg/kg/dayG. glabra	Significance
Total leucocytes count(×103 /ul)	12.2 ±1.76	23.89 ±2.03	34.8 ±1.72	41.7 ±2.49	**	
Neutrophils	(%)	33.4 ±0.5	27.8 ±0.6	21.8 ±1.9	24.2 ±0.4	*
	Range	24.8 -41.3	13.5 -29.8	20.3 -22.7	17.4 -32.5	-
Eosinophils	(%)	0.4 ±0.01	0.9 ±0.06	0.3 ±0.09	0.5 ±0.03	-
	Range	0.1 -0.9	0. 9 -1.9	0.1 -0.6	0.1 -1.1	-
Basophils	(%)	0.1 ±0.08	0.4 ±0.06	0.5 ±0.02	0.0 ±0.08	-
	Range	0.0 -0.2	0.3 -0.6	0.3 -0.7	0.0 -0.1	-
Lymphocytes	(%)	55.4 ±0.5	76.2 ±1.1	75.2 ±2.8	73.2 ±1.6	**
	Range	44.8 -61.3	68.5 -84.1	55.3 -94.5	57.8 -86.9	-
Monocytes	(%)	10.7 ±0.08	0.5 ±0.02	2.1 ±0.01	2.1 ±0.01	**
	Range	5.9 -12.9	0.5 -0.7	0.9 -3.7	1.4 -4.0	-

(- : not significant, *: P<0.05, **: P<0.01 in comparison with control).

(Table 1) also the diameter of the splenic follicles showed a significant increase compared to those of the controls (P<0.01) (Table 1) (Figure 1).

Adrenal Glands: The size of the adrenal glands of experimental animals have been show significant increasing in the medulla as well as in the reticular layer of cortex (P<0.01) (Figure 2) and (Table 1).

Thymus: The thickness of the thymic cortex and medulla in the *G. glabra* treated animals were greater than those of the controls (P<0.01) (Table 2) (Figure 3).

Lymph Node: The diameter of the lymph nodes follicles of G. glabra given animals was higher than those of the controls (P<0.01). In addition, the thickness of the lymph nodes capsule was not affected by *G. glabra* treatment (Table 2).

Peripheral Blood Cells: Total leucocytes count significantly increased in the *G. glabra* treated animals (P<0.01) (Table 3) compared with control samples. Also, significant increases were also observed in the lymphocytes count in the peripheral white blood cells ratio of the *G. glabra* treated groups (p<0.05) (Table 3).

In addition, significant decreases were also observed in the neutrophils and monocytes counts in the peripheral white blood cells ratio of the *G. glabra* treated groups ($p<0.05$) (Table 3).

DISCUSSION

Aqueous extract of the root is reported to inhibit the spinach mosaic virus [12].However; the concept of immune boosting is vague. Immune boosting may involve an activation of different cell types of the immune system, an increase in cell number, or it may involve another process such as a shift from one cytokine pattern to another [13]. It should be noted, that the total leucocytes count was increased in this study that probably fortified immune system. The immune effects of *Glycyrrhiza*are not as well studied. *Glycyrrhiza* activated peritoneal macrophages in a mouse model [14]. Recent *in vitro* studies showed its antiviral effects for viruses causing severe acute respiratory syndrome (SARS) [15], human immunodeficiency virus (HIV) [16] and Kaposi sarcoma-associated herpes virus (KSHV) [17] through the inhibition of viral replication by an unclear mechanism. These effects of *Glycyrrhiza* may partially be in related with total number of leucocytes. An increase in immune cell numbers suggests that one effect of these herbal tinctures was to stimulate immune cell proliferation within 24 h of use. Using the flow cytometry data, the number of each immune cell type was examined at each time point. A comparison of immune cell counts at 24 h shows a trend towards an increase [18] with the use of *Glycyrrhiza* tincture, the total numbers of immune cells increased [18]. *Glycyrrhiza* herbal tinctures ingested by human subjects stimulate/activation and proliferation of various immune cells [18].

High intake of liquorice can cause disabling the sodium-potassium ATPase, edema, increased blood pressure and depression of the renin-angiotensin-aldosterone system. As a consequence, a number of other clinical symptoms have also been observed. Glycyrrhizic acid is hydrolysed in the intestine to the pharmacologically active compound glycyrrhetic acid, which inhibits the enzyme 11β-hydroxysteroid dehydrogenase (in the direction of cortisol to cortisone) as well as some other enzymes involved in the metabolism of corticosteroids. Inhibition of 11β-hydroxysteroid dehydrogenase leads to increased cortisol levels in body [18].our study indicated significant increasing in the medulla as well as in the reticular layer of cortex that may be suggest *Glycyrrhiza* directly is effective in this respect showing level of cotisol released. In the *G. glabra* treated animals thymic cortex and medulla thickness were significantly higher than those of the controls. The diameter of the lymph nodes follicles of *G. glabra* given animals was higher than those of the controls, all of these findings support the immune fortification influence of *G. glabra* extract.

In present work, it is may be concluded that *G. glabra* extract had more beneficial impacts on histological and histometrical aspects of immune system

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