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Full Length Research Paper

POTENTIAL MODULATORY ROLE OF *GLYCYRRHIZA GLABRA* AGAINST HEPATIC MORBIDITY IN CHRONIC MURINE SCHISTOSOMIASIS

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This work is a trial to elucidate the effect of liquorice root extract (*G. glabra*) on the course of chronic hepatic schistosomiasis mansoni. Sixty Swiss Albino mice were used in the experiment. Animals were divided into 6 groups. Group 1- chronically infected for 16 weeks. Group 2- chronically infected for 16 weeks and treated with both *G. glabra* (1st week post infection) and praziquantel (PZQ) (6th week post infection). Group 3- infected mice given *G. glabra* starting from the 1st till the 16th week P. I. Group 4- animals received PZQ on the 16th week P. I. for two consecutive days. Group 5- normal un infected non treated mice. Group 6- normal non infected mice given *G. glabra* for 16 weeks. All groups were sacrificed 16 weeks P. I. Histopathological studies of the liver were done for granuloma measurements. Serum Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST) and transforming Growth Factor Beta One (TGFβ1) were calculated. Mice given the combination regimen revealed complete eradication of worms 16 weeks post infection (P. I), with vanishing of both mature and immature ova in the oogram, and minimal hepatic granuloma diameter. Again, *G. glabra* given with praziquantel (PZQ) normalized the level of both serum liver enzymes, (ALT) and (AST), and (TGFβ1). The data recorded in this group (2), were significantly comparable to the infected control group. PZQ remarkably improved parasitological parameters. The promising impact on liver morbidity could be attributed to the protective role of liquorice against inflammation and oxidative stress. This study could be of value in endemic areas where schistosomiasis mansoni is a devastating public health insult to the community.

Keywords: Chronic schistosomiasis mansoni, hepatic granuloma, liquorice, praziquantel (PZQ), Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST) and Transforming Growth Factor beta one (TGFβ1).

INTRODUCTION

Natural compounds are used in the treatment of refractory diseases more and more frequently because of their efficacy and low toxicity. The root extract of the liquorice plant, *Glycyrrhiza glabra* (*G. glabra*) is known in the traditional Chinese medicine as Gancan. Compounds isolated from the root of this plant have immunomodulating (Kores *et al*, 1997) antioxidant (Bing *et al*, 2011) and free radical scavenging activity (Belink *et al*, 1998). Numerous studies have revealed many pharmacological activities of liquorice, such as antimicrobial (Cheever *et al*, 1998), antiviral (Du *et al*, 1999) antitumor (Sugihara *et al*, 1999), anti-inflammatory

[Jeong *et al*, 2005], and many other activities (Albanis *et al*, 2003). It is reported that liquorice contains nearly 300 flavonoids and more than 20 triterpenoids, among which glycyrrhetic acid (GA), a pentacyclic triterpene acid with numerous biological activities, has anti-inflammatory (Fenwick *et al*, 2003), antiviral (Valenzuela and Garrido 1994), antiallergic (Down *et al*, 1974), and antitumor proliferative effects (Mata-Santos *et al* 2010).

In vivo evidence of potential adverse effects involving (*G. glabra*) might cause herbal –drug interactions including its impact on blood pressure and hypokalemia. Hence, doses of (*G. glabra*) must be cautiously used to overcome such interactions (Qiao *et al*, 2015).

Schistosomiasis caused by *S. mansoni* continues to be an important cause of parasitic morbidity and mortality

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worldwide and is the most common fibrotic disease to arise due to inflammation and the deposition of scar tissue around parasite eggs trapped in the liver (Burke *et al* 2010). Less effective drugs are directed to reversing the existing hepatic fibrosis, especially at the chronic and advanced stages of schistosomiasis. Therefore, treatment targeting hepatic fibrosis of schistosomiasis remains a challenging proposition (Andrade, 2008).

An improvement in the antioxidant capacity in alcohol-fed mice via recovery of the hepatic glutathione (GSH) pool could make liquorice valuable in the treatment of alcoholic liver disease (Jae-chul *et al* 2016)

Previous studies suggested that liquorice has anti-inflammatory activity in lipopoly saccharide stimulated microglial cells and anti-oxidative activity in oxidative liver damage (Shetty *et al*, 2002). In this study, the potential effect of liquorice on the course of chronic liver morbidity due to experimental schistosomiasis has been investigated.

MATERIAL AND METHODS

Animals

Sixty CD-1 Swiss male albino mice, weighing 15-18 g were provided by the Schistosome Biology Supply Center (SBSC) of the Theodor Bilharz Research Institute (TBRI), Giza, Egypt. The mice were maintained on a standard commercial pelleted diet (El-Kahira Company for oils and soap) in an air-conditioned animal house at 20-22°C. The animal experiments were conducted at the TBRI animal unit in accordance with international valid guidelines.

Infection of animal

Animals were infected with the Egyptian strain of *S. mansoni* (50 ± 5 cercariae/mouse) using subcutaneous injection of mice (Peters and Warren, 1969)

Preparation of root extract of *Glycyrrhiza glabra*

Dried roots of *G. glabra* were purchased from the local market and powdered and extracted with 70% methanol. The methanol extract was flash evaporated at 45°C–50°C under vacuum to give a powder (yield 0.8%) which was dissolved in water and used for the study.

Drugs and dosage

Glycyrrhiza glabra L was given orally in a dose of 100 mg/kg/day (Jae *et al*, 2016), 3days/week for 16 weeks in the form of aqueous suspension. Praziquantel® (Praziquantel-Sedico Pharmaceutical Co. 6th of October

City, Egypt) was given orally in a total dose of 1000 mg/kg divided equally on two consecutive days [Gonnert & Andrews 1977] in the form of suspension in 2% Cremophor EL.

Experimental design

In this study 60 animals were divided into 6 main groups where each group included 10 mice:-

- Group I: mice were chronically infected being maintained for 16 weeks.
 - Group II: chronically infected mice for 16 weeks treated with both *G. glabra* (starting on 1st week post infection) and Praziquantel (6th week PI)
 - Group III: including infected mice treated with *G. glabra* starting from 1st weeks and continued to the 16th week PI
 - Group IV: in this infected group, animals received PZQ in the 6th week PI, the drug was given for 2 consecutive days
 - Group V: A normal non infected, non –treated group of mice
 - Group VI: A normal non infected, non –treated group of mice given *G. glabra* for 16 weeks
- All groups were sacrificed at week 16 post-infection

Histopathological studies

For granuloma measurement, microtome sections were cut at a thickness of 5 µm and at a distance of 250 µm apart to avoid re-measurement. Sections were stained with hematoxylin–eosin and Masson's trichrome stain that showed the amount and pattern of collagen formation in the granuloma. The ocular micrometer was used for measurement of the diameter of the liver eggs granulomas (Von Lichtenberg 1962). The mean diameter of each granuloma was obtained by measuring perpendicular diameters using an ocular micrometer. The percent reduction in granuloma diameter relative to the infected control was calculated

Determination of aspartate and alanine transaminase activity in serum

Concentrations of aspartate transaminase (AST) and alanine transaminase (ALT) in the collected sera were estimated using the available commercial kits (Sentinel CH, Milan, Italy) by White *et al.* (1970) and Harold (1975).

Determination of TGF-β1 serum levels

Blood sample were collected from all animals and the serum was separated by centrifugation. The serum TGF-

Table 1 Effect of *G. glabra* with/without praziquantel on total worm burden, 16 weeks post infection of mice with *S.mansoni*

Animal group	Mean worm burden $\pm$ SE (liver and porto-mesenteric)			Total worm burden	% parasite reduction
	Male	Female	Couples		
I) Infected control	8 $\pm$ 1.56	5.11 $\pm$ 0.79	4.5 $\pm$ 0.67	22.11 $\pm$ 2.25	
II) Infected and treated <i>G. glabra</i> + pzq	0	0	0	0	100
III) Infected + treated <i>G. glabra</i>	1.83 $\pm$ 0.49	0.91 $\pm$ 0.24	0.80 $\pm$ 0.08	4.34 $\pm$ 0.65	80.37
IV) Infected treated with PZQ	0	1.02 $\pm$ 0.31	0	1.02 $\pm$ 0.31	95.39

Table 2 Effect of *G. glabra* with/without praziquantel on oogram pattern in 16 weeks post infection of mice with *S.mansoni*

Animal group	% Immature ova	% Mature ova	% Dead ova
I) Infected control	43.3	30.7	26
II) Infected and treated <i>G. glabra</i> + pzq	0	0	100
III) Infected + treated <i>G. glabra</i>	24	28	48
IV) Infected treated with PZQ	0	11	89

Table 3 Effect of *G. glabra* with/without praziquantel on number of ova per gram tissue (liver and intestine) in mice infected with ± 60 *S. mansoni* cercariae, 16 weeks post infection.

Animal group	Number of ova/gm mean number $\pm$ standard error	
	Liver	Intestine
I) Infected control	12197.08 $\pm$ 371	14863.39 $\pm$ 485
II) Infected and treated <i>G. glabra</i> + pzq	1231.52 $\pm$ 324	1232.34 $\pm$ 165
III) Infected + treated <i>G. glabra</i>	1914.93 $\pm$ 288	2801.12 $\pm$ 384
IV) Infected treated with PZQ	1652 $\pm$ 373	1469 $\pm$ 264

$\beta 1$ level was quantified using the Ready-set-go human/mouse TGF- $\beta 1$ kit (eBioscience, San Diego, CA, USA) (Hoff and Rlagt 2000).

RESULTS

Data of group II (infected and treated with both therapeutic agents) revealed complete eradication of worm 16 weeks post infection. PZQ resulted in disappearance of male worms and couples (group IV) the percentage reduction in mean worm load as compared to infected control (group I) was 95.39%. *G. glabra* (group III) decreased the number of female worms significantly while the percentage reduction in the mean number of worms reached 80.37% Table (1).

As regards the Oogram pattern, there was complete disappearance of both mature and immature ova in intestinal wall in animals of group II. PZQ resulted in disappearance of immature ova, 16 week PI (group IV) and the percentages of dead ova increased to 89 % in (group III) with decreased percentages of both mature and immature ova Table (2).

The number of ova / gram tissue in group (II) receiving a combination of Liquorice extracts and (PZQ) decreased significantly and was more pronounced than group (IV) given (PZQ) alone. *Glycyrrhiza glabra* administration also reduced ova number in tissue significantly (group III).

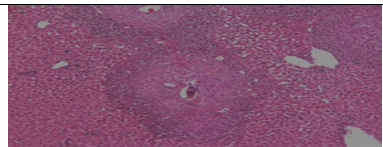
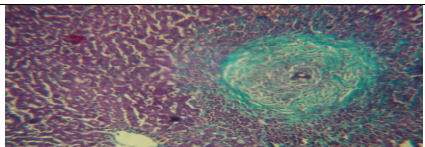
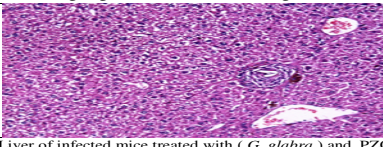
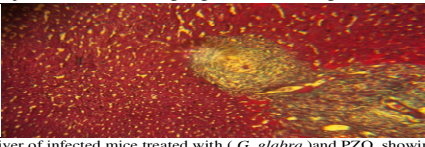
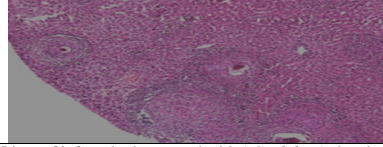
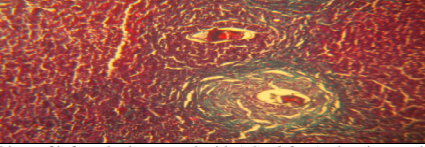
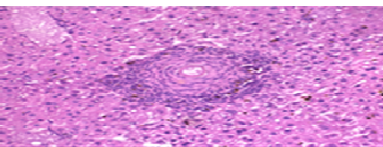
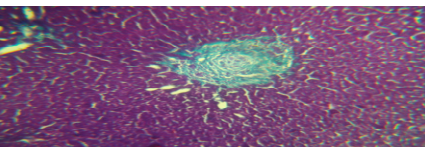
As regards pathological changes in liver of infected mice, the mean granuloma diameter decreased significantly (97.86%) in group (II) (combination regimen).

Table 4 Effect of *G. glabra* with /without praziquantel on hepatic granuloma size 16 weeks post-infection.

Animal group	number of granuloma in 10 successive power fields (10x10)	% reduction in number of granuloma	mean granuloma diameter in μm	% reduction of mean granuloma diameter	State of eggs (%)	
					Intact	degenerated
I) Infected control	18.33 $\pm$ 3.19		356.24 $\pm$ 25.83		85	15
II) Infected and treated <i>G. glabra</i> + pzq	1.14 $\pm$ 1.2	93.78	97.86 $\pm$ 23.25	72.53	8	92
III) Infected + treated <i>G. glabra</i>	7.78 $\pm$ 2.7	57.56	186.57 $\pm$ 37.34	47.63	54	46
IV) Infected treated with PZQ	5.24 $\pm$ 1.6	71.41	121.85 $\pm$ 43.27	65.79	17	83

Table 5 Effect of *G. glabra* with/without praziquantel on liver enzyme, TGF- β 1 and serum, 16 weeks post infection of mice with *S.mansoni*

Animal groups	ALT	AST	TGF - β 1 (pg./ml)
I) Infected control	102.67 $\pm$ 43.11	210 $\pm$ 61.34	627 $\pm$ 2.14
II) Infected and treated <i>G. glabra</i> + pzq	50 $\pm$ 12.5	48 $\pm$ 2.03	280.67 $\pm$ 0.67
II) Infected + treated <i>G. glabra</i>	82 $\pm$ 21.4	79 $\pm$ 2.14	521.6 $\pm$ 4.09
IV) Infected treated with PZQ	71 $\pm$ 23.52	123 $\pm$ 52.61	396.33 $\pm$ 1.24
V) Normal	66.43 $\pm$ 4.81	72.42 $\pm$ 3.47	123.61 $\pm$ 1.64
VI) Normal + <i>G. glabra</i>	54 $\pm$ 2.15	42 $\pm$ 2.21	488 $\pm$ 1.78

Liver stained with haematoxylin and eosin (x100).	liver stained with Masson's trichrome stain (x100).
	
Photograph of liver section of infected non treated control mice. Showing large number of fibrocellular granulomas	Photograph of liver sections of infected non treated control mice groups 16 weeks PI. Showing large of fibrocellular granuloma
	
Liver of infected mice treated with (<i>G. glabra</i>) and PZQ showing less number of fibrocellular granuloma,	Liver of infected mice treated with (<i>G. glabra</i>) and PZQ showing small fibrous granuloma with degenerated eggs,
	
Liver of infected mice treated with (<i>G. glabra</i>) showing decrease in size of granulomas,	Liver of infected mice treated with (<i>G. glabra</i>) showing small fibro-cellular granuloma ,
	
Liver of infected mice treated with (PZQ) showing small fibrous granuloma,	Liver of infected mice treated with (PZQ) showing small fibrous , granuloma surrounding degenerated eggs .

when compared to the other groups, the percentage of reduction in mean granuloma diameter reached 72.53%. A remarkable increase in degenerated eggs (92%) was observed. In both treated groups (III and IV) the use of *G. glabra* resulted in 47.63% reduction in mean diameter of granuloma and the percentage of degenerated eggs was 46%. In addition PZQ revealed 65.79% reduction in mean granuloma diameter and the percentage of degenerated eggs reached 83%. Table (4).

Elevation of ALT & AST and TGF- β 1 levels following infection at week 16 was recorded in group I (infected control). Treatment with a combination of *G. glabra* and PZQ, normalized the level of both enzymes. Administration of *G. glabra* alone groups (III), significantly ameliorated the level of both liver enzymes in serum, PZQ also, improved their levels in serum, yet the combination regimen yielded the best outcome. (Table 5)

DISCUSSION

The root extract of liquorice plant, *Glycyrrhiza glabra* (*G. glabra*) is to be a common herbal medicine in many countries, researchers have shown that the main active substances include polysaccharides, triterpenoid, saponin and flavonoids compounds (Zeng *et al*, 2015). Significant improvement of immunologic function and growth performance of mice has been reported (Chen *et al*, 2016) in addition to its anti-oxidant activity (Shetty *et al*, 2002).

Since alcohol – induced changes in liver of mice were effectively inhibited by liquorice treatment (Jae *et al*, 2016) the present study investigated the possible hepato- protective properties of the root extracts of this plant in murine schistosomiasis *mansoni*.

Among all infected groups, (*G. glabra*) was given to animals (group III) from 1st week of infection till the 16th week. (*G. glabra*) resulted in significant reduction of the mean total worm burden as compared to infected control group (1). In addition; the number of ova /gm tissues was remarkably reduced. The percentage of dead ova in the oogram pattern increased, yet both mature and immature ova were still seen with lower percentages as compared to infected control. The histopathological examination of liver sections revealed a high reduction in both the mean granuloma diameter and number with an increase in degenerated eggs. This promising impact on liver morbidity could be attributed to the protective role of liquorice against inflammation and oxidative stress (Jae *et al*, 2016).

It was also stated that liquorice was a promising candidate to alleviate alcoholic fatty liver (Jae *et al*, 2016). Excessive ingestion of liquorice induces a syndrome of hypokalemia and hypertension that reflects increased activation of renal mineralocorticoid receptors by cortisol (Walker and Edwards 1994). In this study a relatively low dose of liquorice was used 100mg/kg/day

(Jae *et al* 2016) to overcome these side effects, the safe dose was accordingly combined with PZQ to overcome possible adverse effects. These undesirable effects could be attributed to inhibitory role of liquorice on cytochrome enzyme (CYP3A4). (Qiao *et al*, 2015).

In this work biochemical analysis, of serum ALT and AST activities corresponded to the histopathologic findings where, both serum enzymes were significantly decreased. It was suggested that the hepatoprotective effect of liquorice could be associated with an augmentation of antioxidant defense and anti-inflammatory response (Jae *et al*, 2016).

In the evolution of the granulomatous response to the *S. mansoni* eggs, the production of TGF- β 1 may modulate inflammation and regulate fibro genesis. Several investigators indicated that TGF- β 1 is a regulatory cytokine that is mainly produced by regulatory T cells which provides an effective mechanism of control of the progression of fibrosis in association with IL-10 (Kitani *et al*. 2003).

Kanzler *et al*. (1999) detected the important role of TGF- β 1 in stellate cell activation and liver fibrogenesis. Moreover, Alves-Oliveira *et al*. (2006) found that high levels of TGF β 1 appeared to be associated with protection against fibrosis. Compared to the infected untreated group in this study, treatment with silymarin resulted in a significant reduction in serum TGF- β 1 at 10th and 18th weeks PI. Moreover, the administration of PZQ in combination with silymarin caused normalization with significant reduction of serum TGF- β 1 at 10th and 18th weeks post infection, when compared to the corresponding PZQ treated group.

TGF- β 1 has the ability to induce synthesis of collagen type I (Trappoliere *et al* 2005). This could be due to the activated hepatic stellate cell (HSCs) during the acute stage of fibrosis (El- Lalkany *et al* 2012), the authors added that TGF- β 1 levels continued to rise reaching the highest level at the 18th week post infection in *S.mansoni* infected mice.

The evident rise in collagen expression in the liver, seven weeks post infection was associated with the early development of the granulomatous response (Singh *et al*, 2004).

Similarly, the results showed that pre-treatment of rats with liquorice extract effectively protected the animals against CCL4- induced hepatic destruction as evidenced by decreased serum ALT, AST and ALP activities (Huo *et al*, 2011).

The histological examination of liver of animals pre-treated with liquorice extract, suppressed the acute hepatic damage against CCl₄, and was consistent with improvement of the serum biological parameters for hepato- toxicity (Huo *et al*, 2011).

It was also reported that, supplementation of the alcohol diet with liquorice, in mice, for the same period significantly reversed the changes in liver injury markers and effectively abrogated fat accumulation (Chen *et al*

2016).

They added that Liquorice was a promising candidate to alleviate alcoholic fatty liver via attenuating the effects of ethanol on triglyceride accumulation in the liver and ALT and AST activities in serum (Chen *et al*, 2016).

Praziquantel (PZQ), is Known to eliminate mature worm effectively, thus preventing the accumulation of *S.mansoni* eggs in tissues the drug control group given PZQ (group IV) yielded a high significant reduction in all parasitological parameters studied .These data were accompanied by significant reduction in both ALT and AST serum levels with improvement of schistosoma induced pathology .The main explanation of these results is presumed to be due to removal of schistosoma worms with subsequent reduction of egg deposition (Berhe *et al*, 2008).

Administration of (*G. glabra*) in addition to PZQ (group II) resulted in complete eradication of all worms with complete disappearance of both mature and immature ova. Although worms were completely eradicated still ova were counted in times. This could be due to dead or degenerated ova that were layed prior to PZQ treatment. (*G. glabra*) is not considered an anti-parasitic agent as compared to PZQ but still the parasitological data of (group III) are interesting.

Both therapeutic agents normalized ALT and AST serum enzymes .PZQ alone (group IV) or in combination with (*G. glabra*) (group II), significantly reduced serum TGF- β 1 to reach normal levels. The remarkable improvement in histopathology of liver during chronic Schistosomal infection (16 week) was manifested by a high significant reduction in the mean granuloma diameter and marked decrease in fibrous tissue surrounding degenerated eggs as shown in liver sections stained with masson tichrome of group II. In this regard, PZQ alone seemed to be effective in reducing hepatic fibrosis after parasitological cure as previously reported (Chen, 2005).Combined treatment using PZQ and *G. glabra* extracts in chronically infected mice ameliorated histopathological findings, normalized liver enzymes levels. Subsequent to parasite eradication, disappearance of mature and immature ova with removal of their toxins and alleviation of liver inflammation supervened. Previous in vitro studies showed that *G. glabra* extracts can protect liver microsomal membraness by reduction of lipid peroxidation (Shetty *et al*, 2002). Further studies showed that *G. glabra* inhibited proliferation of the HT-29 cell line which implied an ability of *G. glabra* to induce apoptosis revealing its anti –cancer poverty(Nourazarian *et al* ,2014).Therefore, *G. glabra* can significantly improve the growth performance and immunologic function of mice (Chen *et al* ,2016).

Last but not the least, treatment with PZQ, was used in murine schistosomiasis, where specific eradication of the parasite was necessary to enhance the effect of *G. glabra*. The combination of both drugs could reduce hepatic fibrosis by their action on the production of pro-

inflammatory cytokines.

CONCLUSION

Liquorice could be a promising agent to prevent the progression of liver injury .This may be mediated through enhancing anti-oxidative and anti-inflammatory capacity.

Glycyrrhiza glabra had partial toxic effects on worms and eggs. Furthermore, the anti-fibrotic and anti-inflammatory effects of either *G. glabra* /PZQ alone and /or in combination were observed in the chronic fibro genesis induced by *S. mansoni* infection. This was evident by drop in serum levels and hepatic expression of TGF- β 1. These effects were more obvious by most of the studied parameters; accordingly, data of this study point to *G. glabra* is convenient and promoting agent in the treatment of schistosomal liver fibrosis. Further studies on mechanisms of action of *G. glabra* and praziquantel during schistosomal liver fibrosis or other chronic liver diseases may shed some light on developing therapeutic methods in clinical practice.

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