



Full Length Article

Immunomodulatory Activity of *Glycyrrhiza glabra* Extract against Mixed *Eimeria* Infection in Chickens

Kashif Hussain^{1*}, Zafar Iqbal¹, Rao Zahid Abbas^{1*}, Muhammad Kasib Khan¹ and Muhammad Kashif Saleemi²

¹Department of Parasitology, University of Agriculture Faisalabad-38040, Pakistan

²Department of Pathology, University of Agriculture Faisalabad-38040, Pakistan

*For correspondence: raouaf@hotmail.com; kashifhussain1167@gmail.com

Abstract

This study was conducted to evaluate the immunomodulatory activity of aqueous methanolic extract (AME) of *Glycyrrhiza glabra* (roots) against mixed *Eimeria* species infection in broiler chickens. For *in vivo* trial, 175 day old broiler chicks were divided into five equal groups (A, B, C, D and E). At one week of age, groups A, B and C were given orally three graded doses of *G. glabra* AME (100, 200 and 300 mg/kg of body weight respectively) for three consecutive days. Group D served as positive (Vitamin E treated) and Group E as negative control (PBS treated). At 14th day of age, all groups were infected orally with 60,000 sporulated oocysts of mixed *Eimeria* species. Cell mediated immune response was evaluated by four tests (Phytohemagglutinin-P, Concanavalin-A, Carbon clearance assay and Dinitrochlorobenzene). Humoral immune response was evaluated by microplate hemagglutination test using sheep red blood cells. Results revealed a dose dependent immune response in *G. glabra* AME treated groups. Cell mediated and humoral immune response of group treated with *G. glabra* AME @ 300 mg/kg of body weight was almost similar ($P>0.05$) to the positive control group (Vitamin E treated). *G. glabra* AME treated groups showed significantly higher ($P<0.05$) cell mediated and humoral response as compared to negative control (PBS treated). © 2017 Friends Science Publishers

Keywords: Coccidiosis; *Glycyrrhiza glabra* extract; *Eimeria*; Immunity; Chicken

Introduction

Poultry industry is facing many threats of different viral, bacterial and parasitic diseases (Hafez, 2011; Chapman, 2014; Bachaya *et al.*, 2015; Naqvi *et al.*, 2017). Various chemical, biological and immunological agents have been used for the control of avian coccidiosis (Blake and Tomley, 2014; Chapman, 2014); however, owing to emergence of drug-resistant parasite strains and residual effects, use of chemicals tend to be restricted (Nogueira *et al.*, 2009). Use of live vaccines is the another tool for the control of coccidiosis but due to complexity of strains and life cycle stages and delayed immunity development in broilers, this practice may lead to development of clinical disease in broilers maintained under poor management (Chapman, 2005). Therefore, there is need for some new, effective and cheaper resources as alternatives for the control and eradication of coccidiosis. Among alternatives, botanicals rich in antioxidant compounds such as phenols, flavonoids, tannins and saponins has gained special importance and are being used as an alternative strategy to treat coccidiosis (Nweze and Obiwulu, 2009; Abbas *et al.*, 2012). During last decade, some botanicals have been reported promising for their use as anticoccidials (Abbas *et al.*, 2012; Zaman *et al.*, 2012; Idris *et al.*, 2017) and immunomodulators (Abbas *et al.*, 2015, 2017 a & b).

Glycyrrhiza glabra (local name 'Mulethi') is rich in phytochemicals including volatile oil, amino acids, amines (glucose and sucrose 5–15% sugars) starch, flavonoids, saponins, isoflavonoids, tannins, vitamin B2, B3, B6 and vitamin E (Pandit *et al.*, 2011). In view of reported antiviral, immunomodulatory and antioxidant properties (Pandit *et al.*, 2011; Omer *et al.*, 2014), *G. glabra* was tested for its possible role in the control of avian coccidiosis in broiler chickens as an alternate to the commercial chemical based anticoccidials.

Materials and Methods

Plant Material

Roots of *G. glabra* were obtained from local market and got authenticated by a botanist. Plant material was dried under shade and ground finely to powder in an electric mill. One kilogram of plant material in powder form was soaked in 10.5 L of aqueous methanol (70%) and extracted using soxhlet apparatus. The suspension was evaporated in rotary evaporator at temperature not exceeding than 50°C till aqueous methanolic extract (AME) in the form of a paste weighing about 500 g was achieved. The AME was further dried by using freeze drier at -40°C and stored at 4°C for different biological assays.

Preparation of *Eimeria* Infection

Caeca of naturally infected chicken with *Eimeria* were collected from different poultry sale points and outbreak cases in Faisalabad. Contents of caeca were examined as described by Ryley *et al.* (1976). Sporulation of oocysts was done in 2.5% potassium dichromate solution by incubating at 25–29°C for 48 h and maintaining 60–80% humidity (Ryley *et al.*, 1976). The infection dose (60,000 sporulated oocysts/mL) was maintained in Phosphate buffered saline using McMaster technique (MAFF, 1986).

Management of Birds

A total of 175 days-old broiler chicks were procured from local market in Faisalabad and reared on floor pens under standard management practices (Zaman *et al.*, 2012). All chicks were offered broiler starter ration for first two weeks followed by broiler finisher ration. Standard feed except anticoccidial additives was offered. Feed and water were provided *ad libitum*. Chicks were vaccinated for Newcastle Disease, Infectious Bronchitis and Infectious Bursal Disease following Zaman *et al.* (2012). Temperature during the first week of age was maintained at 85–90°F; however, it was reduced on weekly basis by 5°F. Light was provided for 24 h throughout the experimental period. Experiment was continued for 42 days.

Experimental Design

For *in vivo* trial, broiler chicks (n=175), at one week of age, were divided into five equal groups (A, B, C, D and E). Of the total 35 infected chicks in each group, 20 and 15 chicks were used for investigations on cell mediated and humoral immunity, respectively. Groups A, B and C were given orally three graded doses of *G. glabra* AME (100, 200 and 300 mg/kg of body weight) for three consecutive days. Group D served as positive control (Vitamin E treated) and Group E served as negative control (PBS treated). At 14th day of age, all groups were administered orally with 60,000 sporulated oocysts of mixed *Eimeria* species (*E. tenella*, *E. necatrix*, *E. brunetti*, *E. mitis*). Cell mediated immune response was evaluated by Phytohemagglutinin (PHA-P), Concanavalin-A antigens (Con-A), Carbon clearance assay (CCA) and Dinitrochlorobenzene (DNCB) tests. Humoral immunity was evaluated by microplate hemagglutination test using sheep red blood cells (SRBCs).

Evaluation of Immunological Parameters

A-Evaluation of Cell Mediated Immune Response

Phytohemagglutinin-P test: Cell mediated response to Phytohemagglutinin-P (PHA-P) was evaluated by using classical toe web assay method as described by Corrier (1990). At 14th day post-administration of *G. glabra* AME, selected chicks were injected with Phytohemagglutinin-P

(Sigma®, USA) intradermally (100 µg/100 mL/chick) between interdigital spaces of the right foot. The left foot served as control (PBS). The thickness of the interdigital skin was measured at 24, 48 and 72 h post PHA-P injection with the help of screw gauge. Lympho-proliferative response to PHA-P was calculated by using the formula:

$$\text{Lympho-proliferative response} = (\text{PHA-P response, right foot}) - (\text{PBS response, left foot})$$

Concanavalin-A test: *In vitro* cell mediated response by chicken lymphocytes to Concanavalin-A (Con-A) was measured in both administered and control groups following Qureshi and Saif (2000). Briefly, the serum was collected from selected chicks of each group at day 7 and 14 post administration of *G. glabra* AME, Vitamin-E and PBS. The optical density (OD) value was determined by using ELISA reader.

Carbon Clearance Assay

The phagocytic ability of chick's blood cells was determined by carbon particle clearance assay by following the steps as described by Zhang *et al.* (2004). At 14th day post administration of *G. glabra* AME, Indian ink was centrifuged to get supernatant (0.05 mL/10 g/b.wt) and then injected into wing vein of selected chicks and blood samples were collected from other wing vein at 15 min post injection of Indian ink. The samples of blood were placed in tubes containing 2 mL of 0.1% sodium carbonate and OD values were measured at 460 nm in ELISA reader.

Dinitrochlorobenzene Test

Cell mediated response to dinitrochlorobenzene (DNCB) was assessed as described by Blumink *et al.* (1974). Briefly, on day 14 of age, a primary dose (0.1 mL) of 2% DNCB in acetone was applied on 4 cm² area on the skin of each of the five chicks followed by a secondary dose on day 21 of the age (7 days post primary dose). Skin thickness (mm) was measured using a vernier caliper at pre and 24 h post-application of DNCB both after the primary (day 14) and secondary (day 21) exposure to DNCB.

B- Evaluation of Humoral Immune Response

Humoral immunity was evaluated by using microplate hemagglutination test using sheep red blood cells (SRBCs) as described by Qureshi and Havenstein (1994). At 14th day post-administration of *G. glabra* AME, selected chicks were injected with 1 mL of SRBCs (5%) by I/M route. At day 7th post primary injection, a booster injection of 1 ml of SRBCs (5%) by I/M route was given. Serum was obtained from the blood samples at one and two weeks of post primary and secondary injection. Anti-SRBCs antibody titers were calculated by hemagglutination test, which was carried out in 96 well round bottom micro-titration plates (Flow Lab. UK).

Statistical Analysis

Analysis of variance and Duncan's multiple range tests were used for the determination of statistical significance using SAS statistical analysis software.

Results

Cell Mediated Immune Response

The cell mediated immune response to PHA-P in all treated groups was higher ($P < 0.05$) than negative control group (PBS treated group). Among *G. glabra* AME treated groups, maximum cell mediated immune response to PHA-P was recorded in chickens treated with *G. glabra* AME @ 300 mg/kg of body weight followed in decreasing order by groups treated with *G. glabra* AME @ 200 and 100 mg/kg of body weight. Cell mediated immune response, to PHA-P, of groups treated with *G. glabra* AME @ 300 mg/kg of body weight and Vitamin E treated group was comparable ($P > 0.05$; Fig. 1).

The carbon clearance index in all treated groups was lower ($P < 0.05$) than negative control group (PBS treated). Among *G. glabra* AME treated groups, minimum carbon clearance index was recorded in chickens treated with *G. glabra* AME @ 300 mg/kg of body weight followed in increasing order by groups treated with *G. glabra* AME @ 200 and 100 mg/kg of body weight. Carbon clearance index of group treated with *G. glabra* AME @ 300 mg/kg of body weight was comparable ($P > 0.05$) with Vitamin E treated group (Fig. 2).

The cell mediated immune response to DNCB in all treated groups was higher ($P < 0.05$) than negative control group (PBS treated). Among *G. glabra* AME treated groups, maximum cell mediated immune response to DNCB was recorded in chickens treated with *G. glabra* AME @ 300 mg/kg of body weight followed in decreasing order by groups treated with *G. glabra* AME @ 200 and 100 mg/kg of body weight. Cell mediated immune response, to DNCB, of groups treated with *G. glabra* AME @ 300 mg/kg of body weight was statistically not different ($P > 0.05$) from that of Vitamin E treated group (Fig. 3).

The cell mediated immune response to CON-A in all treated groups was higher ($P < 0.05$) than negative control group (PBS treated). Among *G. glabra* AME treated groups, maximum cell mediated immune response to CON-A was recorded in chickens treated with *G. glabra* AME @ 300 mg/kg of body weight followed in decreasing order by groups treated with *G. glabra* AME @ 200 and 100 mg/kg of body weight. The almost same cell mediated immune response was observed in Vitamin E treated group (Fig. 4).

Humoral Immunity

Overall, a higher ($P < 0.05$) total antibody titer (GMT) was observed in all treated groups than negative control group (PBS treated). Among *G. glabra* AME treated groups,

maximum GMT was recorded in chickens treated with *G. glabra* AME @ 300 mg/kg of body weight followed in decreasing order by groups treated with *G. glabra* AME @ 200 and 100 mg/kg of body weight. However, total antibody titer (GMT) was higher ($P < 0.05$) in Vitamin E treated group but it was comparable ($P > 0.05$) with *G. glabra* AME @ 300 mg/kg of body weight (Fig. 5).

Discussion

A large number of research reports have shown that botanicals and their antioxidant compounds have excellent anticoccidial potential and proven to be best alternative to synthetic anticoccidial drugs (Abbas *et al.*, 2015; Alzahrani *et al.*, 2016; Fall *et al.*, 2016; Gotep *et al.*, 2016). Plants, rich in antioxidant compounds, have also shown remarkable immunomodulatory effects (Abbas *et al.*, 2017a & b).

The results of cell mediated and humoral responses to different antigens (PHA-P, DNCB, CON-A, Carbon and SRBCs) showed that there was no significant difference ($P > 0.05$) between the both cell mediated and humoral responses in groups treated with respective plant extract @ 300 mg/kg of body weight and Vitamin E treated group. Higher antibody titers and cell mediated response in groups treated with plant extract indicated that the plant ingredients may increase animal's ability to resist physiological and infectious insults. The plant extracts may stimulate the cellular immune responses by the activation of host defense potentiators, through maturation, proliferation or differentiation of the immune cells. The similar enhanced immune response in various animals, by plant extracts, was reported in previous studies (Akhtar *et al.*, 2012; Nandini *et al.*, 2016). Likewise, different plant extracts have been reported to increase splenocytes proliferation, delayed type of hypersensitivity reaction and phagocytic activity of macrophages in experimental animals (Cao *et al.*, 2010). Similarly, *G. glabra* (Glycyrrhizin) extract is used in auto-immune conditions and has therapeutic benefit in immunodeficiency conditions like AIDS (Mazumder *et al.*, 2012).

Moreover, aqueous and ethanolic extracts from *Aloe vera* have shown immunomodulatory potential against coccidiosis in broiler chickens by improving humoral and cellular immune response of chickens infected with mixed *Eimeria* species (Akhtar *et al.*, 2012). Later on, some other researchers have also reported similar types of dose dependent trends on evaluation of immunomodulatory potential of different herbal extracts (Laxmi *et al.*, 2015; Singh *et al.*, 2015). Similar positive findings were described by Akhtar *et al.* (2012) with increased production of interleukin-6, which is a potent stimulant of B-cells. Likewise, Awais *et al.* (2011) evaluated the immunomodulatory potential of *Saccharum officinarum* extract against avian coccidiosis. *Saccharum officinarum* extract produced cellular and humoral immunity against *E. tenella* infection and also showed therapeutic effects by

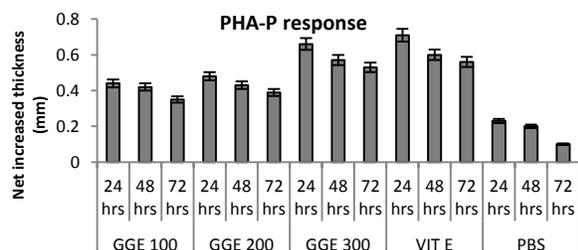


Fig. 1: Cell mediated response (PHA-P) of *Glycyrrhiza glabra* extract (n=4)
GGE: *Glycyrrhiza glabra* extract; Vit E: Vitamin E treated group served as positive control group; PBS: Phosphate buffered saline treated group served as negative control group

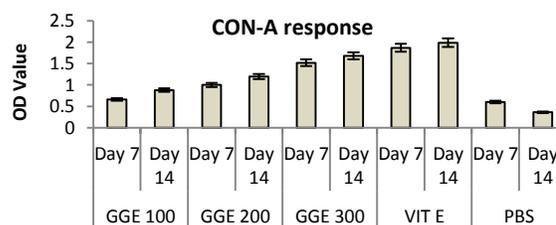


Fig. 4: Cell mediated response (CON-A) of *Glycyrrhiza glabra* extract (n=3)
GGE: *Glycyrrhiza glabra* extract; Vit E: Vitamin E treated group served as positive control group; PBS: Phosphate buffered saline treated group served as negative control group

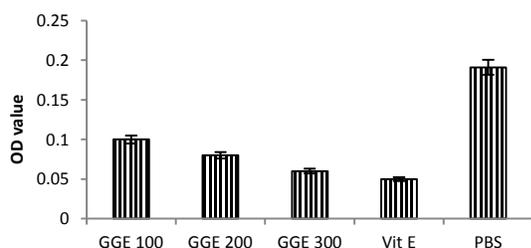


Fig. 2: Carbon clearance index of *Glycyrrhiza glabra* extract (n=4)
GGE: *Glycyrrhiza glabra* extract; Vit E: Vitamin-E treated group served as positive control group A; PBS: Phosphate buffered saline treated group served as negative control group

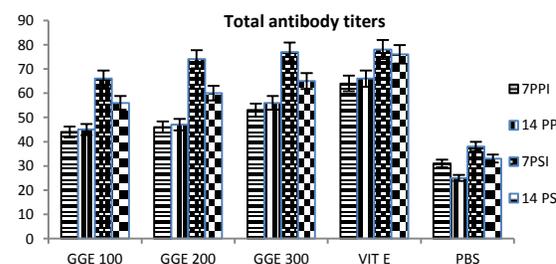


Fig. 5: Humoral immune response (total antibody titers) of *Glycyrrhiza glabra* extract (n=13)
GGE: *Glycyrrhiza glabra* extract; Vit E: Vitamin E treated group served as positive control group; PBS: Phosphate buffered saline treated group served as negative control group PPI: Seven days post primary injection; PSI: Seven days post-secondary injection

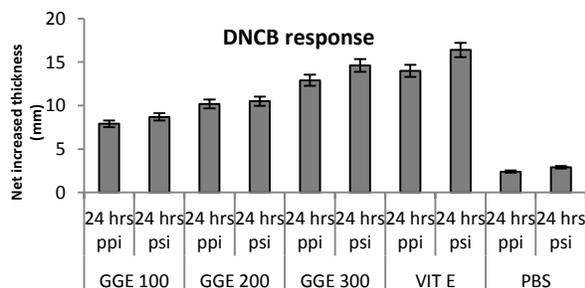


Fig. 3: Cell mediated response (DNCB) of *Glycyrrhiza glabra* extract (n=5)
GGE: *Glycyrrhiza glabra* extract; Vit E: Vitamin E treated group served as positive control group; PBS: Phosphate buffered saline treated group served as negative control group; PPI: Post primary injection; PSI: Post-secondary injection

increasing weight gain and reducing oocyst excretion in infected birds.

In the present study, *G. glabra* showed the considerable immunomodulatory activity against mixed *Eimeria* species which might be due to abundance of antioxidant compounds in this plant inducing the immune response as evident by increased leukocyte counts and spleen weight in chickens. Among different organs of immune system, spleen represents a major secondary

lymphoid organ involved in elicitation of immune response (Mazumder *et al.*, 2012). Similar, immunomodulatory results of different plants were also reported in previous studies (Awais *et al.*, 2011; Akhtar *et al.*, 2012; Laxmi *et al.*, 2015; Singh *et al.*, 2015).

In conclusion, present study showed that *G. glabra* aqueous methanolic extract has protective and immunomodulatory effects against coccidiosis in chickens. *G. glabra* showed immunomodulatory effects in dose dependant manner by increasing cellular and humoral immune response in infected chickens. However, further studies are needed to characterize the active compounds of *G. glabra* involved in enhancing the immunomodulatory potential against coccidiosis.

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