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RESEARCH ARTICLE

Immunomodulatory Activity of *Pinus radiata* Extract against Coccidiosis in Broiler Chicken

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A B S T R A C T

Immunomodulatory activity of *Pinus radiata* extract (PRE) against experimental *Eimeria* infection in broiler chicks was evaluated. For *in-vivo* trials, 175 day-old broiler chicks were divided into five equal groups (A, B, C, D and E) containing 35 chicks in each group. At one week of age, the groups A, B and C were given orally with graded doses of PRE (100, 200 and 300 mg/kg of body weight, respectively) 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 infected orally with 60,000 sporulated oocysts of mixed *Eimeria* species. Cell mediated immune response was evaluated by Phytohemagglutinin-P, Concanavalin-A, Carbon clearance assay and Dinitrochlorobenzene tests. Humoral immune response was evaluated by microplate hemagglutination test using sheep red blood cells. Results of study demonstrated that a dose dependent immune response was observed in PRE treated groups. Cell meditated and humoral immune response of group treated with PRE @ 300 mg/kg of body weight was comparable to positive control group (Vitamin-E treated) ($P>0.05$). *Pinus radiata* extract treated groups showed significantly higher cell mediated and humoral response as compared to negative control (PBS) ($P<0.05$).

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INTRODUCTION

Coccidiosis is an infection of intestine caused by the protozoa, which belongs to subclass Coccidian, family *Eimeriidae* and genus *Eimeria.* There are seven major *Eimeria* species infecting chickens each having specific predilection site in digestive tract. These pathogens cause high morbidity and mortality due to extensive damage to digestive system of chicken (Abbas *et al.,* 2010). Among all species, *Eimeria tenella* (*E. tenella*) is the most pathogenic and causes heavy economic losses to commercial poultry farming all over the world including Pakistan (Chapman, 2014; Abbas *et al*., 2015; Bachaya *et al*., 2015).

The disease is clinically characterized by bloody diarrhea, dehydration, anemia and poor feed conversion ratio (Abbas *et al*., 2010). Infection to chickens occurs when it ingests a sporulated oocyst of *Eimeria* from litter or soil particles. Coccidiosis is mainly controlled by using anticoccidial drugs but, continuous and irrational use of these anticoccidial drugs has resulted in development of resistance to all *Eimeria* species across different countries such as Brazil (Kawazoe and Difabio, 1994), China (Lie *et al*., 2004), India (Yadav and Gupta, 2001) and Pakistan (Abbas *et al*., 2015). As of immunological control, different live vaccines have been developed against avian coccidiosis like coccivac and immunocox (Chapman, 2014). Though widely practiced, vaccination control of avian coccidiosis has been, by and large, reported to be ineffective (Chapman, 2014) unless more specific strains of *Eimeria* and sophisticated technologies are used in manufacturing such vaccines.

Due to widespread emergence of anticoccidial drug resistance, frequent failures of vaccines, residual and toxic effects of drugs have led to focus on alternative approaches to control coccidiosis. In this regard, different botanicals have been reported promising for their use as immunomodulatory or anticoccidial agents (Akhtar *et al*., 2012; Abbas *et al*., 2017; Idris *et al*., 2017). Moreover, botanical immunomodulators are considered as ideal approach due to abundance, inexpensive, easy processing, non-residual effect and high efficacy against different diseases including avian coccidiosis (Akhtar *et al.,* 2012).

Pinus radiata belongs to family *Pinaceae,* which is commonly known as Monterey pine or radiata pine, have phenolic compounds due to which it has been used as traditional medicinal plant in various countries including Pakistan (Anwar *et al.,* 2012; Yazaki, 2015). Pine bark extract is an excellent mixture of antioxidant compounds including polyphenolic compounds (Proanthocyanidins) and condensed tannins. Ahn *et al*. (2007) reported that pine bark extract (Pycnogenol) effectively reduced the number of pathogens and retarded the formation of thiobarbituric acid-related substances in raw beef.

Keeping in view the above mentioned importance and effective role of different botanicals and their antioxidant compounds against cocccidiosis, current study was designed to evaluate the immunomodulatory effects of aqueous methanolic extract of bark of *P. radiata* against experimentally induced mixed *Eimeria* infection in broiler chicks.

MATERIALS AND METHODS

Plant material: Bark of *Pinus radiata* was obtained from local market. The plant material was identified and authenticated by a botanist of University of Agriculture Faisalabad. The extract of *P. radiata* was obtained from powdered plant material in 70% methanol using Soxhlet's apparatus at 80°*C*. The aqueous methanolic extract of *Pinus radiata* (PRE) was evaporated in a rotary evaporator, under reduced pressure at 35°C. The extract was then freeze dried and stored at 4°Cuntil used.

Parasite: Chicken guts naturally infected with Eimeria were collected from different poultry sale points and outbreak cases in Faisalabad. Contents of guts were examined microscopically and Eimeria oocysts of different species were obtained and preserved in potassium dichromate solution (2.5%) by following the method as described by Ryley *et al*. (1976).

Birds and management: A total of 175 day-old broiler chicks were procured from local market in Faisalabad, Pakistan and were reared on floor pens under standard management practices according to guidelines provided by Zaman *et al.* (2012). Standard feed except anticoccidial additives was offered. Chicks were vaccinated for Newcastle Disease, Infectious Bronchitis and Infectious Bursal Disease following Zaman *et al.* (2012). 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), containing 35 chicks in each group. In each group, 20 and 15 chicks were used for evaluation of cell mediated and humoral immunity, respectively. Groups A, B and C were given orally graded doses of PRE (100, 200 and 300 mg/kg of body weight) for three consecutive days. Group D was provided orally with Vitamin E (served as positive control group) and group E served as negative control group (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. brunetii, E. mitis*).

Immunological evaluation

A-Evaluation of cell mediated immunity: Cell mediated immune response was analyzed through four different assays:

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 PRE, selected chicks were injected with Phytohemagglutinin-P (Sigma®, USA) intradermally (@100µg/100ml/chick) between interdigital spaces of the right foot. The left foot served as control (PBS). The thickness of the interdigital skin was measured at different hours post PHA-P injection with the help of screw gauge.

Concanavalin-A test: *In vitro* cell mediated response by chicken lymphocytes to Concanavalin-A (Con-A) was measured in both administered and control groups by following the method of Qureshi *et al.* (2000). The optical density value was accessed 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).

Dinitrochlorobenzene test: Cell mediated response to dinitrochlorobenzene (DNCB) was done by methods 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 cm^2 area on the skin of selected chicks followed by a secondary dose on day 21 of the age. Skin thickness (mm) was measured using Vernier caliper at pre and post 24 hours of application of DNCB.

B-Evaluation of humoral immunity: Humoral immunity was evaluated by collection of serum samples on post primary and secondary injection (I/M) of SRBCs (5%) using microplate hemagglutination test as described by Qureshi and Havenstein (1994).

Statistical analysis: Analysis of variance and Duncan's multiple range tests were used for the determination of statistical significance.

RESULTS

Cell mediated immunity: The higher cell mediated response (PHA-P) was observed in all treated groups ($P<0.05$) than negative control group (PBS). Among PRE treated groups, maximum cell mediated response was recorded in chicks treated with PRE @ 300 mg/kg of body weight followed in decreasing order in the groups treated with 200 and 100 mg/kg of body weight. Cell mediated response of groups treated with PRE @ 300 mg/kg of body weight and Vitamin E (positive control group) was comparable ($P>0.05$) (Fig. 1).

The cell mediated response (CON-A) was higher in all treated groups ($P<0.05$) than negative control group (PBS). Among PRE treated groups, maximum cell mediated response was recorded in chicks treated with PRE @ 300 mg/kg of body weight followed in

decreasing order in the groups treated with 200 and 100 mg/kg of body weight. Cell mediated response of groups treated with PRE @ 300 mg/kg of body weight and Vitamin E (positive control group) was comparable (P>0.05) (Fig. 2).

The carbon clearance index in all treated groups was lower (P<0.05) than negative control group (PBS). Among PRE treated groups, minimum carbon clearance index was recorded in chicks treated with PRE @ 300 mg/kg of body weight followed in increasing order by the groups treated with 200 and 100 mg/kg of body weight. Difference in carbon clearance index of groups treated with PRE @ 300 mg/kg of body weight and Vitamin E (positive control group) was comparable (P>0.05) (Fig. 3).

The higher cell mediated response (DNCB) was observed in all treated groups (P<0.05) than negative control group (PBS). Among PRE treated groups, maximum cell mediated response was recorded in chicks treated with PRE @ 300 mg/kg of body weight followed in decreasing order by the groups treated with 200 and 100 mg/kg of body weight. Cell mediated response of groups treated with PRE @ 300 mg/kg of body weight and Vitamin E (positive control group) was comparable (P>0.05) (Fig. 4).

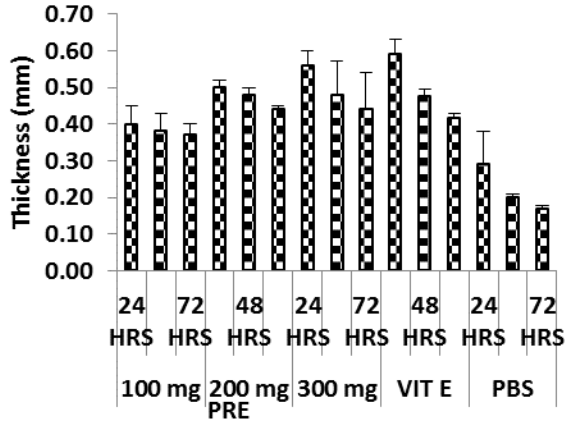

Fig. 1: Cell mediated response (PHA-P) of *Pinus radiata* extract (n=4); PRE: *Pinus radiata* extract**;** Vit E: Vitamin E served as positive control group**;** PBS: Phosphate buffer saline served as negative control group.

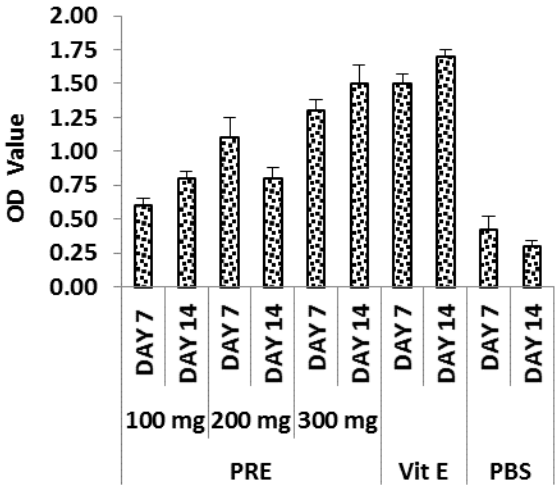

Fig. 2: Cell mediated response (CON-A) of *Pinus radiata* extract (n=3); PRE: *Pinus radiata* extract**;** Vit E: Vitamin E served as positive control group**;** PBS: Phosphate buffer saline served as negative control group.

Humoral immunity: The total antibody titers in all treated groups were higher (P<0.05) than negative control group (PBS). Among PRE treated groups, maximum antibody titers were recorded in chickens treated with PRE at 300 mg/kg of body weight followed in decreasing order by the groups treated with 200 and 100 mg/kg of body weight. Antibody titers of groups treated with PRE @ 300 mg/kg of body weight and Vitamin E (positive control group) were comparable (P>0.05) (Figure 5).

DISCUSSION

A large number of experiments have shown remarkable effects of different plants and their compounds against avian coccidiosis (Abbas *et al.*, 2010; Abbas *et al.*, 2015; Zaman *et al*., 2015; Alzahrani *et al*., 2016). Results of present study demonstrated that dose dependent cellular and humoral immune response was observed in infected chicks treated with PRE @ 100, 200, 300 mg/kg of body weight. Immune response shown by group treated with PRE @ 300 mg/kg of body weight was comparable to Vitamin-E treated group (P>0.05). The similar, enhanced immune response in various animal models by different plant extracts have been reported in previous studies (Akhtar *et al.*, 2012; Mahmood *et al.*, 2016; Nandini *et al*., 2016).

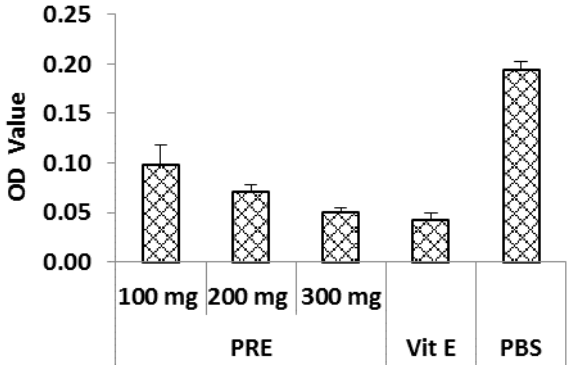

Fig. 3: Carbon clearance index of *Pinus radiata* extract (n=4); PRE: *Pinus radiata* extract; Vit E: Vitamin-E served as positive control group A; PBS: Phosphate buffer saline served as negative control group.

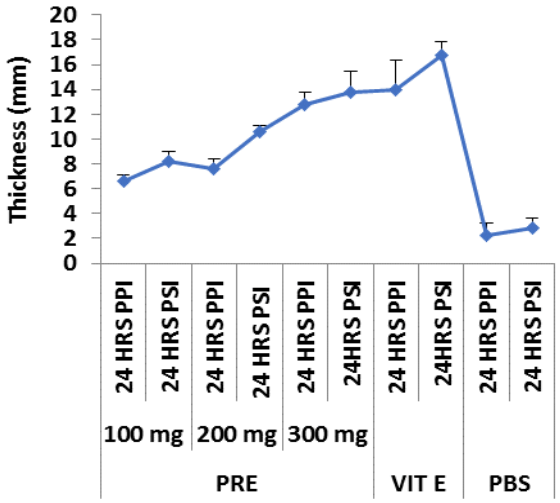

Fig. 4: Cell mediated response (DNCB) of *Pinus radiata* extract (n=5); PRE: *Pinus radiata* extract**;** Vit E: Vitamin E served as positive control group**;** PBS: Phosphate buffer saline served as negative control group; PPI: Post primary injection; PSI: Post-secondary injection.

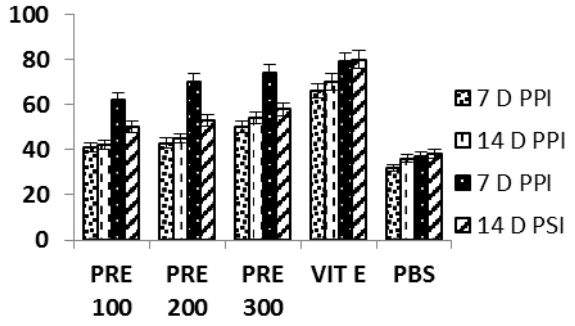

Fig. 5: Humoral immune response (total antibody titers) of *Pinus radiata* extract (n=13); PRE: *Pinus radiata* extract**;** Vit E: Vitamin E served as positive control group**;** PBS: Phosphate buffer saline served as negative control group; 7 DPPI: Seven days post primary injection; 7 DPSI: Seven days post-secondary injection.

Pine bark is rich source of polyphenolic compounds known as Proanthocyanidins. Proanthocyanidins are potent antibacterial agents, free radical scavengers and effective enzyme inhibitors (Yazaki, 2015). They also exhibited anti-inflammatory, vasodilatory, cardioprotective antiviral and immunostimulating activities. It has been reported that pine bark extract helps in treatment of diabetes due to its hypoglycaemic property (Molan *et al.*, 2009).

Positive effect of *P. radiata* extract on cellular and humoral immune response against coccidiosis may be due to its proanthocyanidins compounds which can play important role in increasing immune response by activation of immune cells (lymphocytes, phagocytes) and increasing antibody or cytokines in infected chicks. Moreover, different plant extracts have also been reported to enhance cellular and humoral immunity by increasing lymphocyte cell proliferation, phagocytic activity of macrophages and immunoglobulins production in experimental animals (Cao *et al*., 2010).

Molan *et al*. (2009) investigated that plants of genus *Pinus* had antioxidant activity as well as anticoccidial potential. Aqueous extract of *P. radiata* (500 µg per mL) resulted in 28–84% sporulation inhibition of *Eimeria* oocysts. Additionally, administration of 1000 µg per ml of pine bark extract changed external and internal morphology of *Eimeria* oocysts.

Present study demonstrated immunomodulatory potential of *P. radiata* extract in dose dependent manner. Recently, similar type of dose dependent immunomodulatory activity of *Carica papaya* aqueous extract was observed in rats (Ramesh *et al*., 2016). 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 showed therapeautic effects by increasing weight gain and reducing oocyst excretion in infected birds.

Nidaullah *et al*. (2010) studied the immunostimulatory and anticoccidial effect of herbal complex comprising of *Allium sativum*, *Azdirachta indica* and *Berberis lyceum* which was added in feed of broiler chickens for 35 days. Herbal complex enhanced the cellular and humoral immune response of infected chickens. Moreover, a significant reduction in oocyst excretion, lesion score and mortality was observed. In another study, extracted juice of whole plants of *Echinacea purpurea* increased cellular and humoral immunity in mice by increasing count of peripheral lymphocytes and monocytes. Additionally, it also activated the immune system by increasing total immunoglobulins (IgM, IgG) levels (Mishima *et al*., 2004). Moreover, aqueous and ethanolic extracts from *Aloe vera* have shown immunomodulatory potential by improving humoral and cellular immunityof broiler chickens infected with mixed *Eimeria* species (Akhtar *et al*., 2012).

Conclusions: Current study showed that *P. radiata* aqueous methanolic extract has positive effect on immune response in broiler chicks infected with mixed *Eimeria* species. *P. radiata* extract showed immunomodulatory potential in dose dependent manner against coccidiosis. It suggests that *P. radiata* can be used as a prophylactic remedy to prevent coccidiosis in chickens. However, further studies are needed to formulate and standardize herbal product from *P. radiata.*

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Authors contribution: RZA and ZI planed, designed and supervised the experiment. AA and MKK performed the complete experimental trial and analyzed the data. JAK helped in the extraction of plant material.

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