Kurdish Studies
June 2024
Volume: 12, No: 5, pp. 178-184
ISSN: 2051-4883 (Print) | ISSN 2051-4891 (Online)
www.KurdishStudies.net

DOI: 10.53555/ks.v12i5.3197

Immunomodulatory And Protective Effects Of Oryza Sativa Derived Arabinoxylans (Axs)

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ABSTRACT
Immunomodulatory effects of rice bran (*Oryza sativa*)-derived arabinoxylans (AXs) were evaluated in chickens. Broiler chicks were randomly allocated into five equal groups of 25 chicks each. The humoral immune response was detected using sheep RBCs. Cellular immunity was assessed using phytohemagglutinin-P (PHA-P), concanavalin-A (Con-A), carbon clearance, and dinitrochlorobenzene (DNCB) assays. Following a 14-day course of AXs, all experimental and control groups were exposed to sporulated oocysts of mixed *Eimeria* species, and the anticoccidial potential was assessed based on the mortality rate, lesion score, oocysts per gram of stool (OPG), and weight of lymphoid organs on different days post infection. We observed that AXs-treated groups showed higher immunomodulatory effects. However, the group treated with AXs @ 300 mg/Kg of body weight showed higher antibodies, a higher cellular response to PHA-P and CON-A, and higher carbon clearance ($P<0.05$), while the group B chickens showed maximum skin thickness before and after 24 to 48 hours of DNCB dosage. This study could be useful in identifying an immunomodulatory agent for the control of chicken coccidiosis.

Keywords: Arabinoxylans, Immunity, Chicken, *Oryza Sativa*.

INTRODUCTION

Coccidiosis is caused by an important protozoan parasite of the genus *Eimeria*. *Eimeria* species that cause poultry coccidiosis include *E. tenella, E. necatrix, E. acervulina, E. maxima, and E. brunetii* (Abbas et al., 2017). These *Eimeria* species cause enteritis and bloody diarrhoea by destroying enterocytes, but the severity of the disease varies by species and may also cause immunosuppression in addition to other secondary infections. Coccidiosis is primarily treated with chemotherapeutic or anticoccidial agents. However, the failure of this approach has been attributed to anticoccidial drug resistance, the diversity of *Eimeria* species, and synthetic drug residual problems (Abu-Akkada and Awad, 2015).

Nowadays, the main focus is on botanical and herbal medications as an alternative to conventional chemotherapy drugs for the treatment and control of coccidiosis (Ahad et al., 2017). Due to recent events, alternate regimens utilizing both natural and artificial immunomodulators must be implemented (Akhtar et al., 2012). Botanical immunomodulators are regarded as suitable agents due to their availability, simplicity of processing, compelling effectiveness, and low residue levels in animal products (Ani et al., 2013). Cereals are the main suppliers of pharmacologically active compounds in this context, which has increased researchers' interest in the biological significance of these carbohydrates (Badr El-Din et al., 2008).

An annual plant in the Poaceae family called Oryza sativa, popularly known as Asian rice, has demonstrated immunostimulatory effects through enhanced lymphocyte proliferation, cellular responsiveness, and interleukins (Blake & Tomley, 2014; Corrier, 1990). Rice bran (Oryza sativa) and its derivatives have anti-diabetic, anti-inflammatory, and anti-aging properties. According to many studies, some rice bran derivatives can decrease inflammatory mediators and reactive oxygen species, which in turn can enhance osteogenesis (Forster et al., 2013; Ghatak & Panchal, 2012).

To the best of our knowledge, there is no information regarding the immunomodulatory and anticoccidial effects of arabinoxylans of Oryza sativa in chickens against coccidiosis in Pakistan. Therefore, the objective of this study was to evaluate the immunomodulatory and anticoccidial effects of arabinoxylans derived from Oryza sativa on chickens. We hypothesized that AXs derived from Oryza sativa might demonstrate strong immunostimulatory and therapeutic potentials against avian coccidiosis.

MATERIALS AND METHODS Processing of rice bran

Commonly accessible rice (Oryza sativa) bran was purchased and prepared as directed by Ani et al. (2013). Rice bran was passed through a 200-mm mesh after already being immersed in distilled water for 60 minutes at 4 °C. It was rinsed three times in water of 5–6 volumes (w/v) to eliminate the starch. The rinsed bran mixture was heated for approximately 12 hours at 50 °C minimum to remove up to 10% of the water. Bran was air dried before being crushed into a fine powder and samples were filtered through a 60-mm filter. The resulting fraction was de-starched and preserved at 4 °C for future usage.

Extraction of arabinoxylans (AXs)

Following directions given by Ani et al. (2013), the de-starched bran was processed to extract the polysaccharide. In a water bath at 80 °C for 90 min, 100 g of powdered rice bran was dissolved in 1.5 L of 0.15N sodium hydroxide (having 0.5 % water, volume by volume). After being chilled to 26 °C, the mixture was centrifuged at 5000 g for 30 minutes. After being centrifuged (5000 g for 30 min), the resultant supernatant was neutralized with HCL, which has a 0.2 normality at 4.5 pH, to reduce the volume under reduced pressure to as little as 1/4th of the initial volume. Centrifugation (5000 g/5 minutes at 4°C) and ethanol (65%) were applied to precipitate the concentrate. The ethanol was used to separate the supernatant up to 65 percent. Then the sediments were mixed in water and centrifuged at 5000 g for about 30 min at 4 °C. The freeze-dried mixture was used to make alkaline extracts of arabinoxylans.

Parasite

The present study used local isolates of mixed *Eimeria* species from coccidiosis outbreaks (Ghoneum & Jewett, 2000). As described earlier (Hajtó et al., 2013), the infected gut contents were sporulated, then washed three times with phosphatebuffered saline. The McMaster counting method was then used to quantify the amount of sporulated oocysts per milliliter of suspension (Khaliq et al., 2016). After diluting 4.2 × 104 oocysts/ml of PBS to 6.0 x 104 oocysts/ml of PBS, the solution was stored at 4 °C in a sterilized screw-capped bottle. According to a recent study, prediation site, shape, and size were used to distinguish sporulated oocysts of different species of Eimeria. Four distinct Eimeria species, like E. mitis, E. tenella, E. brunetii, and E. necatrix, were isolated from the caeca of infected chickens in this research.

Experimental Design

A total of 125 one-day-old broilers were purchased from a neighboring hatchery and raised at the Experimental Station of the Department of Parasitology, University of Agriculture Faisalabad. Following normal vaccinations, the chicks were fed ad libitum and given water. On the seventh day, the chicks were separated into five equal groups of 25, according to chance. Each set of 15 birds was evaluated for their humoral and cell-mediated immunity. Mixed Eimeria species were introduced to the ten remaining birds in each group. Group A received AXs @ 100 mg/kg of BW, Group B received AXs @ 200 mg/kg of BW, and Group C received AXs @ 300 mg/kg of BW orally dissolved in 5 mL of PBS. Group E acted as a negative control, receiving phosphate-buffered solution @ 1 mL per bird for three days, while Group D was named as a positive control group, and it received Vitamin E @ 87 mg/kg of BW.

Evaluation of Immune Response

Experimental and control chickens were analyzed using sheep red blood cells (SRBCs) following the method described by Lee et al. (2009). A booster dose of 5 percent SRBCs was given intramuscularly on the 14th day after AXs supplementation. On days 7 and 14 after the first and second injections, blood samples were collected, and the sera were separated. A microplate agglutination test was used to measure the anti-SRBC antibody titers of total immunoglobulins (Igs), IgM, and IgG. Cellmediated immunity was assessed in both treatment and control groups using the traditional MTT and toe-web tests using Phytohemagglutinin-P (PHA-P) and Concanavalin-A (Con-A) (Liao et al., 2006; Maeda et al., 2004). Phytohemagglutinin-P (Sigma-Aldrich, St. Louis, MO 63178) was injected into the dermis (100 g/100 mL per chicken) into the toe-web of the chicken on day 14 following arabinoxylans therapy. At 24, 48, and 72 hours after injection, the interdigital skin's thickness was measured by a pressure-sensitive micrometer screw gauge. The MTT assay was performed on chicken leukocytes from the treatment and control groups in a 96-well plate with Con-A at a dose of 25 g (2 x 106 cells) to determine the in vitro proliferation response to Con-A.

Carbon clearance assay

The capacity of phagocytosis of the blood cells of chicks was assessed in both the control and experimental groups using the clearance assay of carbon particles (Zhang 2007). In a nutshell, the supernatant from vortexing India ink was given intravenously to (n=5) birds at 0.05 ml/10 grams of BW. At 0, 3, and 15 minutes following the injection of the India ink, blood samples from different wing veins were drawn. The blood samples were then put in the 2 mL sodium carbonatecontaining tubes, and the values of optical density were calculated in an ELISA reader at 600 nm.

Dinitrochlorobenzene (DNCB) test

The Blumink et al. (1974) approach was used to assess delayed-type hypersensitivity reactions in experimental and control group chickens. Before DNCB treatment, skin thickness (in mm) was assessed using Vernier calipers. Twelve days following the main treatment, a second dose was given to a different site and after 24 and 48 hours, the increased skin thickness (measured in millimeters) was measured.

Statistical analysis

A complete randomized design (CRD) was used to statistically analyze the data. Comparisons between groups were made using a contrast table. For analysis, Statistics Analysis Software with 95% confidence intervals was used.

RESULTS Immunological evaluation

Using a microplate HA test, the immunological response of all chickens was evaluated. Post-primary inoculation and postsecondary inoculation of SRBCs were injected, and results were obtained on days seventh and fourteenth. In Group B, the chicken's immunological reaction to sheep RBCs on the 7th and 14th days was measured, and the PSI and PPI results show a high level of IgG and IgM (**Table 1**). Group C chickens fed 300 mg/kg arabinoxylans had a significantly higher antibody response to SRBCs than the other experimental and control groups ($P<0.05$). Traditional in vitro toe-web assays against Phytohemagglutinin-P and in vitro MTT assays for Concanavalin-A were used to measure the lymphoproliferative immune response in all groups of chickens. A pressure-sensitive micrometer screw gauge was used to determine the interdigital skin thickness of chickens at 24, 48, and 72 hours before and after PHA-P injection. Chickens in group C had the highest lymphoproliferative response ($P<0.05$) on the first, second, and third days after Phytohemagglutinin-P exposure (**Table 2**). At 7 and 14 days after administration, the MTT assay for chickens in group B showed that they had a higher lymphoproliferative response ($P<0.05$) to Concanavalin-A than the other groups (**Table 3**).

The DNCB test and carbon assay test are used to evaluate the phagocytic capacity of macrophages among all chickens. Among all groups, group C shows a higher optical density clearance index ($P<0.05$) than other groups (**Table 4**). After 3–15 minutes of administration of Indian ink, the blood samples' optical density was expressed as a clearance index. The group B chickens show maximum skin thickness before and after 24 to 48 hours of DNCB dosage (**Table 5**).

DISCUSSION

Bloody diarrhea, a low FCR, immunosuppression, weight loss, and a high death rate are clinical signs of coccidiosis. Due to the resistance of chemotherapeutic agents, both herbal and botanical agents are used to control and treat coccidiosis these days (Mehala and Moorthy, 2008; Reig and Toldra, 2008). Due to minimal residue, easy availability, easy processing, and high efficacy, botanical immunomodulators are considered the best for coccidiosis treatment (Patwardhan and Gautam, 2005). For an effective pharmacological response, cereals are a good source (Wills et al., 2000). Arabinoxylans are effective against digestive problems and even for tumors, and they are a major ingredient in wheat, oats, rice, and barley (Akhtar et al., 2012). In mammals and mice, Oryza sativa has immunostimulant potential (Din et al., 2008; Park et al., 2013). In various animal and human models, O. sativa has the potential to modify the biological response (Sato et al., 2012; Ani et al., 2013; Forster et al., 2013; Muhammad et al., 2013). AXs derived from O. sativa have immunomodulatory activities against murine neuroblastoma, hematoma, and metastasis (Hajto et al., 2013; Martinez et al., 2015).

As per our observations, chickens receiving AXs on the 7th and 14th days after receiving both the primary and secondary injections of sheep RBCs at a dose of 200 mg/kg had the greatest total anti-Sheep RBC titer, including both IgG and IgM, among all the concentrations and controls. Akhtar et al. (2012) discovered comparable outcomes, in which the highest anti-SRBC titers were discovered at concentrations of 200 and 300 mg/kg on days 7 and 14, respectively. At 24, 48, and 72 hours after treatment, chickens in both groups had their cellular immune responses to PHA-P assessed. AXs supplied to chickens at the dose of 200 mg/kg at 24, 48, and 72 hours produced significantly higher in vivo lymphoblastogenesis than the treated and control groups, respectively. Specifically because of localized production of lymphocytes (Cheema et al., 2003), after PHA-P treatment, Khaliq et al. (2016) detected comparable in vivo T-cell lymphoproliferation in broiler chickens at 24, 48, and 72 hours. On days 7 and 14 after treatment, chickens from both the treated and control groups had an in vitro cellular immune reaction to Con-A as measured by measures of lymphoproliferation. Higher lymphoblastogenesis is seen in group B than in other groups. Similar to this, increased in vitro T-lymphoblastogenesis of cultured chicken WBCs was seen on days 7 and 14 following Con-A therapy by Qureshi et al. (2004). This increase may have been caused by the activation of IL-1 and its receptors (Yamanaka et al., 2001).

Through the use of a carbon particle clearance assay, the reticuloendothelial system's phagocytic capability was discovered in the current research. The clearance index of group B chickens was found to be higher. Ismail and Asad (2009) found that oral administration of Acacia catechu rats at doses of 5 mg/kg and 50 mg/kg resulted in a high phagocytic index, specifically due to stimulation of the mononuclear phagocytic system. Macrophages and neutrophils are involved in the phagocytic activity to engulf foreign particles (Patel and Asdaq, 2010).

On the fourteenth day, after 24 and 48 hours of AXs administration, a delayed hypersensitivity reaction was observed in chickens by using the DNCB test. At 24 and 48 hours after DNCB injection groups, B and C chickens have a high lymphoproliferative response. In contrast to these findings, Sajid et al. (2007) showed increased skin thickness in rabbits treated with ivermectin at higher doses (600 g/kg and 200 g/kg) and the same intervals after treatment. Furthermore, Munir et al. (2009) findings concurred with those of Sajid et al. (2007) in that salinomycin-treated chickens had a greater cellular response than those treated with levamisole, cyclosporine, and cyclophosphamide against ND and HPS. After 6 weeks of exposure to the challenge, weight gain was monitored on a regular basis, and Groups B and C had high body weight gain after the challenge. Ten days post-challenge, the outcomes observed were that the chicks being supplemented with egg yolk had the highest live weight gains and antibody IgY levels at rates of 0.02 percent and 0.05 percent, respectively (Lee et al., 2009). These results were similarly comparable to broiler chickens' having better live weight when given a herbal complex supplement for five weeks (Elkhair et al., 2014).

Within a six-week challenge, chickens from the experimental and control groups were slaughtered, and their relative organ-to-weight ratio of lymphoid organs was measured. Following the challenge, chickens from groups A and B caused increased

organ-to-weight ratios in the bursa, spleen, and thymus, respectively. Five weeks post-challenge, these findings were similar to the thymus, bursa, and spleen development of chickens treated with herbal complexes (Elkhair et al., 2014).
Treated and control groups were evaluated by OPG for the efficacy of therapeutics, six weeks after the challenge. B and C have fewer lesions and higher OPG scores after the challenge groups. These findings also demonstrated a correlation between reduced intestinal lesions and fecal oocysts in broiler chicks treated with hyperimmune egg yolk containing 0.5 percent IgY (Lee et al., 2009). The survival rates of broiler chickens treated with tannins from *Emblica officinalis* at 0.75 and 1 g/kg 12 days after a coccidian challenge were similar to these results (Kaleem et al., 2014).
Oryza sativa-derived AXs showed immunostimulatory potentials against avian coccidiosis.

ACKNOWLEDGEMENT
The project was funded by Pakistan Science Foundation, Islamabad under Research Project No.
PSF/NSLP/P-AU (185).

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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Table 1: Antibody response in experimental and control chickens

-----Total anti-SRBCs antibody titer-----

Group	Day 7PPI	Day 14PPI	Day 7PSI		Day 14PSI
A	44.00^{b}	41.00^{b}	50.00^{b}	54.00^{b}	
B	49.00^{b}	42.00^{b}	52.00^{b}	56.00^{b}	
C	56.00^{c}	49.00^{c}	60.00^{b}	60.00^{c}	
D	58.00^{c}	48.00^{c}	58.00^{b}	62.00^{c}	
E	32.00^{a}	36.00^{a}	40.00^{a}	40.00^{a}	

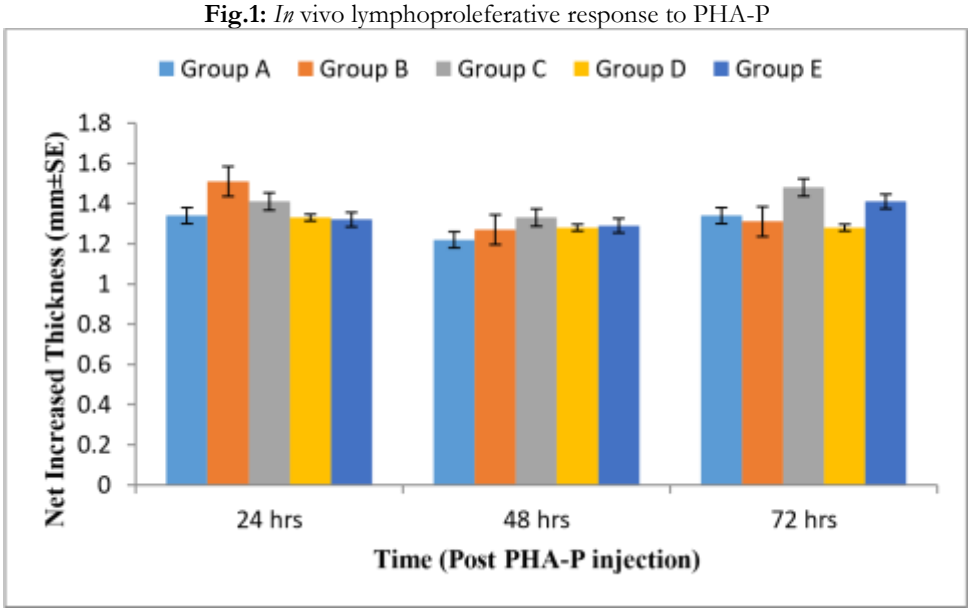
-----Immunoglobulins M and G-----

Group	Day 7PPI		Day 14PPI		Day 7PSI		Day 14PSI			
	IgM				**IgM**					
IgG		**IgM**		**IgG**		**IgG**		**IgM**	**IgG**	
A	32.00^{b}	12.00^{b}	27.00^{b}	14.00^{b}	26.00^{b}	24.00^{b}	26.00^{b}	28.00^{b}		
B	35.00^{b}	14.00^{b}	28.00^{b}	14.00^{b}	28.00^{b}	24.00^{b}	27.00^{b}	27.00^{b}		
C	38.00^{c}	18.00^{c}	31.00^{c}	18.00^{c}	30.00^{c}	30.00^{c}	30.00^{c}	30.00^{c}		
D	42.00^{c}	16.00^{c}	32.00^{c}	16.00^{c}	29.00^{c}	29.00^{c}	32.00^{c}	30.00^{c}		

E	22.00[a]	8.00[a]	26.00[a]	10.00[a]	23.00[a]	17.00[a]	20.00[a]	20.00[a]

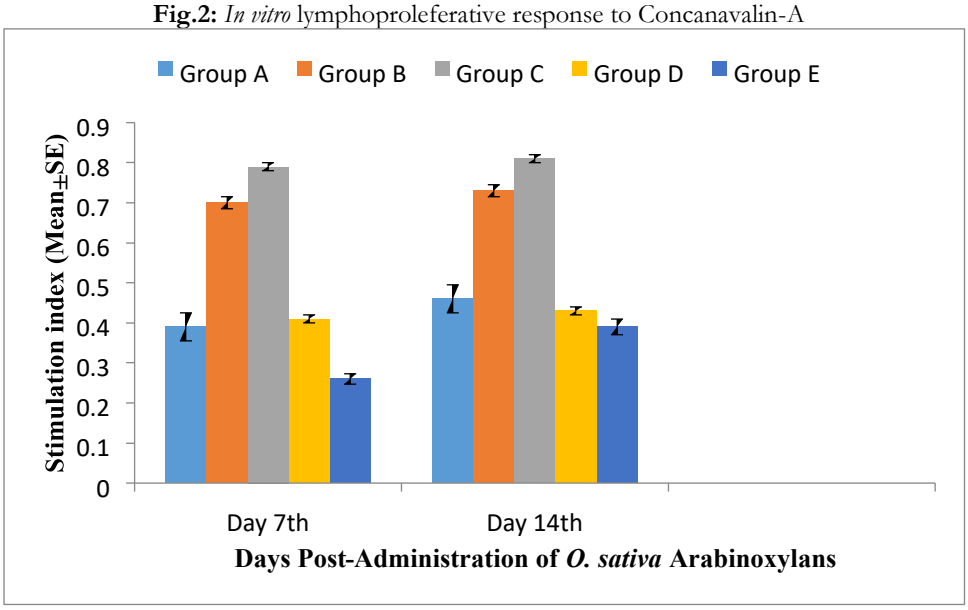
AXs : arabinoxylans ; A= AXS @ 100 mg/kg ; B= AXs @ 200 mg/kg; C= AXs @ 300 mg /kg; D= Vit-E@ 87mg/kg; E= PBS

Fig.1: *In* vivo lymphoproleferative response to PHA-P



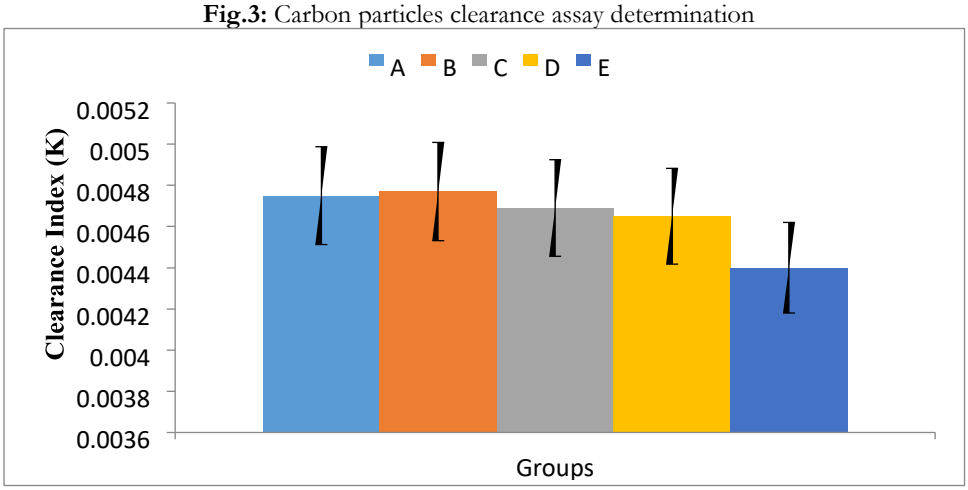
A= 100 mg/kg (AXs); B= 200 mg/kg (AXs); C= 300 mg/kg (AXs); D= +ve Control (Vit-E=87mg/kg); E= -ve Control

Fig.2: *In vitro* lymphoproleferative response to Concanavalin-A



A= 100 mg/kg (AXs); B= 200 mg/kg (AXs); C= 300 mg/kg (AXs); D= +ve Control (Vit-E=87mg/kg); E= -ve Control

Fig.3: Carbon particles clearance assay determination



A= 100 mg/kg (AXs); B= 200 mg/kg (AXs); C= 300 mg/kg (AXs); D= +ve Control (Vit-E=87mg/kg); E= -ve Control