IMMUNOMODULATORY EFFECTS OF *CAMELLIA SINENSIS* AGAINST COCCIDIOsis IN CHICKENs

A. Abbas¹, Z. Iqbal¹, R. Z. Abbas¹, M. K. Khan¹, J. A. Khan², K. Hussain¹, M. S. Mahmood³ and H. M. Rizwan¹

¹Department of Parasitology, Faculty of Veterinary Science, University of Agriculture, Faisalabad, Pakistan
²Institute of Pharmacy, Pharmacology and Physiology, University of Agriculture, Faisalabad, Pakistan
³Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan

Corresponding author: abbasasghar255@gmail.com

ABSTRACT

Purpose of current study was to evaluate the immunomodulatory potential of *Camellia sinensis* in broiler chickens artificially infected with mixed *Eimeria* species. A total of 108 chickens were equally divided in six groups. *Camellia sinensis* in powder form was administered in feed in three graded doses (4, 5 and 6%) containing three control groups (positive control, negative control and normal control). Cell mediated immunity was evaluated using Phytohemagglutinin-P (PHA-P) through classical toe-web assay. Humoral response was determined by micro-hemagglutination test using sheep red blood cells. Results of study demonstrated that *C. sinensis* treatment enhanced cellular and humoral immunity against coccidiosis in dose dependent manner by increasing lymhoproliferative immune response and increased immunoglobulin levels (Total immunoglobulins, IgG and IgM) in broiler chickens. It was concluded from the present study that *C. sinensis* has a potential to be used as immunomodulatory agent against coccidiosis in broiler chickens.

Key words: *Camellia sinensis*, Coccidiosis, *Eimeria*, Immunomodulation

INTRODUCTION

Coccidiosis is recognized as major diseases of poultry which is caused by single-celled protozoon belonging to genus *Eimeria* having different species (Abbas et al., 2012; Chapman, 2014). The main species responsible for coccidiosis in poultry are *E. acervulina*, *E. tenella*, *E. praecox*, *E. mitis*, *E. brunetti*, *E. maxima* and *E. necatrix* (Chapman, 2014). Major noticeable characteristics are bloody diarrhea, poor feed conversion ratio, low productivity, and increase in morbidity and mortality of affected chickens (Masood et al., 2013). Coccidiosis causes heavy economic losses to commercial poultry farming and is thought to be the one of the most expensive infectious diseases of poultry all over the world including Pakistan (Abbas et al., 2012; Blake and Tomley, 2014; Bachaya et al., 2015). According to an estimate, coccidiosis causes about $127 million losses to US poultry industry annually and likewise similar losses may occur worldwide (Chapman, 2014).

Anticoccidial drugs have been effectively administrated for the control of avian coccidiosis (Tewari and Maharana, 2011) but, their frequent and irrational use caused development of anticoccidial drug resistance to different *Eimeria* species. With this reason, the alternative methods are required to overcome this threat. Another effective way to control coccidiosis is the use of vaccines available in both live as well as attenuated forms. Vaccination is not appealing approach because of some constraints like geographical screening before their use, high cost of production and outbreak of diseases in poorly managed poultry production systems. Therefore, there is need to find out the safe alternatives to prevent avian coccidiosis (Zaman et al., 2012, 2015; Abbas et al., 2015).

In this context, many plants and herbal products have been found to have chemotherapeutic effect against coccidiosis in poultry and are being commercialized after a series of experimental trials for their validation (Abbas et al., 2012). Among herbal anticoccidials, *Camellia sinensis* (green tea) has received a great attention especially due to its polyphenolic contents having strong antioxidant properties (Izziqreen and Mohd-Fadzelly, 2013). *Camellia sinensis* is an important plant which contains natural flavonoids. These flavonoids have antioxidant properties due to which they have excellent anticoccidial properties (Jang et al., 2007). It has been used as anticarcinogenic and anti-inflammatory agent. Dried leaves of *C. sinensis* contain 30% flavonoids by its dry weight (Perva-Uzunalic et al., 2006). Plants, rich in antioxidant compounds, having the justification to be explored for their anticoccidial potential because *Eimeria spp.* result in high oxidative stress during coccidiosis disease. Therefore, keeping in view the above mentioned valuable antioxidant properties and positive effects of *C. sinensis*, current study was planned to evaluate immunomodulatory potential of *C. sinensis* in broiler chickens.
MATERIALS AND METHODS

Plant materials: Leaves of *Camellia sinensis* were purchased from local market in Faisalabad (Pakistan). The plant material was identified and authenticated by a botanist in the Department of Botany, University of Agriculture, Faisalabad, Pakistan. Powdered plant material was prepared following method described by Abbas et al. (2010). Briefly, plant material was dried under shade and ground finely to powder in an electric mill in the department of Parasitology, University of Agriculture, Faisalabad.

Collection of coccidian oocysts: Chicken guts susceptible to coccidian infection were collected from outbreak cases of poultry farms and different poultry sale points of Faisalabad. Guts were opened and contents thus collected from intestines were examined microscopically. The contents were placed in separate desiccators containing 25% laboratory grade sodium hypochlorite @ 4:1 for 25 minutes to discard debris. To remove the chemical, about four times more water was added to the desiccators and sediment was obtained. Coccidian oocysts were extracted following the method described by Ryley et al. (1976).

Sporulation of oocysts: Positive samples were placed for sporulation in 2.5% potassium dichromate solution in petri dishes and sporulation of oocysts was done by using method of Ryley et al. (1976). Examination of coccidian oocysts was done by making slide and examining under light microscope at 40X to confirm sporulation of oocysts. The oocysts with 4 sporocysts were considered sporulated regardless the shape and size of the sporocysts. The oocysts were slightly flattened under the pressure of a cover slip to better illustrate morphology (Molan et al., 2009).

Isolation of the sporulated oocysts: The sporulated oocysts were separated by Zinc sulphate floatation technique (Ryley et al., 1976). The counting of washed sporulated oocysts was done by McMaster technique (MAFF, 1986).

Experimental design: The experimental design used in the present study was authenticated and approved by department of parasitology in accordance with approved published research ethics guidelines. Research trial was conducted at experimental station of department of parasitology, University of Agriculture Faisalabad. For *in vivo* trial, 108 (day-old) broiler chicks were procured from local market. Chicks were reared under standard management practices. All the chicks were kept on mash feed ration. At 15th day of age, the chickens were randomly divided into six groups and at the same day first three groups were offered with graded doses of plant material (Table 1) till end of experiment (42 days). Half chickens (n=9) from each group were reserved for evaluation of cell mediated and half (n=9) were reserved for evaluation of humoral immunity. At 18th day of age, the chickens of all groups except group VI were inoculated orally with sporulated oocysts (60,000/chick) with mixed *Eimeria* species.

Table 1. Experimental plan for *in vivo* trial (n=108).

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Treatment groups</th>
<th>Number of birds</th>
<th>Oocyst challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Camellia sinensis</em> 4%</td>
<td>18</td>
<td>60,000</td>
</tr>
<tr>
<td>2</td>
<td><em>Camellia sinensis</em> 5%</td>
<td>18</td>
<td>60,000</td>
</tr>
<tr>
<td>3</td>
<td><em>Camellia sinensis</em> 6%</td>
<td>18</td>
<td>60,000</td>
</tr>
<tr>
<td>4</td>
<td>IM</td>
<td>18</td>
<td>60,000</td>
</tr>
<tr>
<td>5</td>
<td>INM</td>
<td>18</td>
<td>60,000</td>
</tr>
<tr>
<td>6</td>
<td>NN</td>
<td>18</td>
<td>60,000</td>
</tr>
</tbody>
</table>

IM: Infected medicated group (treated with Toltrazuril), served as positive control
INM: Infected non-medicated group served as negative control
NN: Non-infected, non-medicatd group

Immunological evaluation

(a) Cell mediated immunity: Classical Toe-web assay by Phytomagglutinin-P (PHA-P) was used to assess the cell-mediated immunity or lymphoblastogenic response in infected chickens following Corrier (1990). For this purpose, on day 14th post-administration of plant material (29th day of experiment), experimental and control chickens were injected PHA-P (Sigma®, USA) (100µg/500µl/ chicken) intradermally between the third and fourth digits of the right foot. The left foot injected with PBS (100µl) served as negative control. The thickness of the interdigital skin was measured with a pressure sensitive micrometer screw gauge at 24, 48 and 72 hours post injection. Lymphoproliferative response to PHA-P was calculated by the formula:

\[
\text{Lymphoproliferative response} = (\text{PHA-P response, right foot}) - (\text{PBS response, left foot})
\]

(b) Humoral immunity: Sheep red blood cells (SRBCs) were used to demonstrate antibody titers which were detected by using micro plate haemagglutination test (Qureshi and Havenstein, 1994). For this purpose, on day 7th post oral administration of *C. sinensis*, chickens were injected SRBCs (5%) via intramuscular route (1 ml/chicken) followed by a booster at day 7th post primary injection. Blood was collected at day 7 and 14 post primary and secondary injections to separate the serum. Antibody titers were calculated by following procedure.

Haemagglutination test: Serum obtained from the blood samples at 7th and 14th days post first and booster injections to detect the anti-SRBCs antibody titers by haemagglutination test, carried out in 96 well round bottom micro-titration plates (Flow Lab. UK).
Test Procedure: A 50 µl phosphate buffer saline was added in each well of the microtitration plate. A 50 µl of test sera was added in first well and after proper mixing again 50 µl was taken out from 1st well and transferred to the next one and so on up to 11th well to make the two fold serial dilutions whereas the last well was kept as control. Then, 50 µl suspensions of 5% sheep red blood cells was added in each well of the microtitration plate and mixed gently. The plates were incubated at 25°C for half an hour and results were recorded. The titer of the well containing 50% agglutination and 50% reticulum settling (clumping) was considered as the total anti-SRBC antibody titer of the test sera. Results were expressed in terms of geometric mean titers (Burg, 1978).

Determination of IgG Levels: Same protocol was adopted for IgG titers but in the first step 50 µl of 0.01M 2-mercaptoethanol (Riedel-de Haen, Germany) was added in phosphate buffered saline in each well that destroyed the IgM immunoglobulins and the remaining titer was of IgG.

Determination of IgM Levels: Total IgM titer was calculated by subtracting the IgG titer from total antibody titer of the respective samples or by using following formula:

\[ \text{Total IgM titers} = \text{Total antibody titer} - \text{IgG titer} \]

Statistical Analysis: One way analysis of variance (ANOVA) and Duncan’s multiple range tests were used for the determination of statistical significance using SAS statistical analysis software version 2004 (SAS, 2004). Difference were considered statistically significant at (P>0.05).

RESULTS

There was no significant difference in pre-PHAP cellular response in all groups at 24, 48 and 72hrs. *** and ** show that there is no significant difference (P 0.05) in all groups. The results are the means and standard error of means.

Figure 1: Lympho-Proliferative response to PHAP-Pre infection in C. sinensis 4%, 5% and 6% treated broiler chickens artificially infected with mixed Eimeria species.

IM: Infected medicated group
INM: Infected non-medicated group
NN: Non-infected non-medicated group

Figure 2: Lympho-Proliferative response to PHAP-Post infection in C. sinensis 4%, 5% and 6% treated broiler chickens artificially infected with mixed Eimeria species.

There was significant difference (P 0.05) in post PHA-P cellular response of C. sinensis 4, 5 and 6% treated groups as compared to INM group. *** and ** show significant difference (P<0.05) among each other. C. sinensis treated chickens showed significant increase in the cellular response as compared to INM group. The results are the means and standard error of means.

Figure 3: Total anti-SRBCs (sheep red blood cells) antibody titer in C. sinensis 4, 5 and 6% treated broiler chickens artificially infected with Eimeria species.
Total anti-body titers at 7 days of PPI were non significantly different (P > 0.05) in all groups. At 7 and 14 days of PSI these titers were non significantly different (P > 0.05) in C. sinensis treated chickens but, were significantly different (P < 0.05) to INM group as shown in fig. 3.

IgM titers at 7 days PPI were non significantly different (P > 0.05) in all groups. While IgM titers at 7 and 14 days PSI were non significantly different (P > 0.05) in C. sinensis treated chickens but, were significantly different (P < 0.05) to INM group as shown in fig 5.

DISCUSSION

Recently, a significant number of scientific publications have demonstrated the potential benefit of different chemicals of plant origin against avian coccidiosis (Jang et al., 2007; Gandi et al., 2016; Idris et al., 2017). Use of botanicals and natural products has been proved safe, effective and economically cheap alternatives to anticoccidial drugs for the control of coccidiosis in poultry. Several experiments have confirmed that supplementation of natural herbal preparations in animal feed enhances immune response and causes reduction in severity of various kinds of infections (Jang et al., 2007; Awais et al., 2011; Aslam et al., 2016; Awaad et al., 2016). Natural plants contain several useful compounds such as tannins, flavonoids, natural polyphenols and essential oils which show remarkable biological, physiological and therapeutic properties (Ishaq et al., 2015; Javed et al., 2015; Yue et al., 2016; Rehman et al., 2016; Chen et al., 2016; Liaqat et al., 2016).

Wheat bran derived polysaccharides which are named as arabinoxylans were tested against mixed Eimeria infections in chickens. It was concluded that organ body weights and production of immune cells by the thymus elevated in groups treated with wheat bran derived polysaccharides which ultimately showed immunostimulatory response against Eimeria infection. So, it was confirmed that Wheat (Triticum aestivum) bran derived polysaccharides arabinoxylans have excellent ability to enhance immunity level against poultry coccidiosis (Akhtar et al., 2012a). Awais et al. (2011) reported that aqueous and ethanolic extracts of Saccharum officinarum which is commonly known as sugar cane have immunotherapeutic effects against the coccidiosis in broiler chickens. The extracts of Saccharum officinarum induced the immune response, improved body weight and reduced the oocyst shedding in chickens infected with E. tenella. Moreover, the ethanolic extracts showed higher anticoccidial index as compared to aqueous extracts of Saccharum officinarum. Akhtar et al. (2012b) conducted a study to evaluate the anticoccidial and immunomodulatory effects of Aloe vera extracts against coccidiosis in broiler birds and reported excellent results of Aloe vera in terms of improved immune response and increased weight gain in birds against mixed Eimeria infection. So, it can also be used as an immunomodulatory agent against avian coccidiosis.

Dalloul et al. (2006) evaluated the immunostimulatory and anticoccidial effect of a lectin obtained from the
mushroom Fomitella fraxinea. Both the in vivo and in vitro activity of F. fraxinea lectins (FFrL) was determined. FFrL was injected into embryos of 18 day old chicken embryos and mixed Eimeria infection was given orally after hatch. Results showed that FFrL treatment resulted in favorable results in terms of weight gain and reduced expulsion of oocyst in feces. Experiment results demonstrated that FFrL extract can be used as an excellent agent for promotion of growth and immunity in broiler birds against coccidiosis.

Recently, similar type of dose dependant immunomodulatory efficacy of Carica papaya aqueous extract was observed in rats (Ramesh et al., 2016). In another study, Carthamus tinctorius (sunflower) leaves have also shown immunomodulatory effects against avian coccidiosis in dose dependent manner (Lee et al., 2009). In another study, methanolic extract of Beta vulgaris caused significant inhibition of growth of tumor cells and showed immunomodulatory effects against cancer (Tripathy and Pradhan, 2013).

Neystani et al. (2007) evaluated antimicrobiological effects of tea extract against Escherichia coli by in vitro method. Results showed that it has antimicrobial effect in dose dependent manner. At high concentration, it caused more antimicrobial activity. Engelhardt (2010) reported that C. sinensis also contains some important compounds including alkaloids, carotenoids, minerals, amino acids, carbohydrates, lipids and volatile compounds. Plants containing such type of antioxidant compounds can be used as an effective anticoccidial agent in birds as similar immunomodulatory results are reported in current study.

Previously, Jang et al. (2007) have also reported the anticoccidial effects of C. sinensis against Eimeria maxima infection in chickens. Immunomodulatory or anticoccidial potential of C. sinensis might be attributed due to action of its antioxidant compounds like catechins and their derivatives such as epicatechin, epigallocatechin, epicatechin gallate and epigallocatechin gallate (Jang et al., 2007). Epigallocatechin gallate was used as an antioxidative (Fraga et al., 1987), anti-inflammatory (Lin and Lin, 1997), antiproliferative (Shammas et al., 2006), antibacterial (Mabe et al., 1999), antiviral (Williamson et al., 2006; Mahmood et al., 2016), trypanocidal agent (Paveto et al., 2004) and also showed inhibitory effect against ovine Babesia (Abou-Laila et al., 2010). Furthermore, C. sinensis has also been reported very effective in terms of its in vitro anthelmintic activity against Teladorsagia circumcincta and Trichostrongylus colubriformis (Ryu, 1982). Most of the above mentioned studies regarding the antiparasitic and especially anticoccidial activity of C. sinensis suggest its activity because of having antioxidant compounds; however, the exact mode of action is still unknown.

Conclusion: Present study confirmed the immunomodulatory effect of Camellia sinensis in dose dependent manner against coccidiosis in chickens. This work is helpful for making an herbal immunomodulator to prevent coccidiosis in poultry.

Acknowledgements: All authors acknowledge the financial grant for this work from Punjab Agricultural Research Board-Lahore, Pakistan.

REFERENCES


Neyestani, T.R., K. Niloufar and G. Azam (2007). Black and green teas may have selective synergistic or antagonistic effects on certain antibiotics against...


