

Ameliorating effect of *Withania somnifera* root extract in *Escherichia coli*-infected broilers

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ABSTRACT The present study was undertaken to investigate the effect of aqueous *Withania somnifera* root (WSR) extract in broiler chicks experimentally infected with *Escherichia coli* O78 @ 10⁷ CFU/0.5 ml intraperitoneally. Clinical signs and mortality due to colibacillosis observed in infected chicks were mild and lasted for short duration in WSR extract supplemented group as compared with the nonsupplemented group. A significant increase in serum alanine transaminase, aspartate transaminase, lactate dehydrogenase, and creatine phosphokinase activities and a decrease in total protein and albumin concentrations were observed in the infected groups, though these changes were of lower magnitude in WSR extract supplemented group. A significantly higher activity of oxidative blood parameters such as superoxide dismutase, catalase, glutathione reductase, and glutathione-S-transferase enzymes were

noticed in WSR extract supplemented group. The WSR extract supplemented group revealed significantly higher *E. coli*-specific antibody titer and enhanced lymphocyte proliferation response as compared with the non-supplemented group. The gross and histopathological lesions of colibacillosis were mild in the WSR extract-supplemented infected group as compared with the nonsupplemented infected group. *Withania somnifera* root extract supplementation produced 31.48 and 34.38% protection in the gross and histopathological lesions in *E. coli* infected chicks, respectively. It is concluded that supplementation of 20% WSR extract @ 20 ml/L of water caused a reduction in the severity, mortality, and recovery period of *E. coli* infection and enhanced the humoral and cellular immune responses suggesting its protective effect on limiting the pathology of *E. coli* infection in broiler chickens.

Key words: *Withania somnifera*, *Escherichia coli*, chicken, immune response, lesions

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INTRODUCTION

Avian colibacillosis caused by *Escherichia coli* (*E. coli*) is of great concern as it burdens the poultry farmers with heavy economic losses. The acute form of the disease is characterized by septicemia and the subacute form by airsacculitis and fibrinous polyserositis (e.g., pericarditis, perihepatitis, and peritonitis). Consequently, it decreases egg production and increases condemnations, mortality,

and with increased costs of vaccination and chemotherapy results in economic losses (Kabir, 2010; Lau et al., 2010).

The *E. coli* strains are found to be resistant to various antimicrobials such as cephradine, tetracyclines, chloramphenicol, sulfonamides, β -lactam antibiotics, aminoglycosides, and fluoroquinolones (Blanco et al., 1997; Bass et al., 1999; Li et al., 2007; Roth et al., 2019). Attributed to the extensive use of antibiotics and lack of new drugs and vaccines, multidrug resistance is now a worldwide problem. A remedy can be found in the use of ethnoveterinary medicines. One of the approaches in developing successful drugs from medicinal plants is a phytotherapeutic approach wherein standardized crude drug preparations (extracts or active fractions) can be used as drugs with modern standards of safety and efficacy.

The prime objective of Ayurveda, the ancient Indian system of medicine, is the prevention of the disease process. *Withania somnifera* is an important medicinal plant, a small, woody shrub 60 to 200 cm high, in the

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Solanaceae family, which is described under many common names such as ginseng and ashwagandha. It is widely distributed in eastern Asia, Africa, and Australia. The roots are the main portions of the plant used therapeutically (Girdhari and Rana, 2007). The major biochemical constituents of ashwagandha roots are steroidal alkaloids and steroidal lactones in a class of constituents called withanolides (Oberholzer et al., 2008; Varma et al., 2011). Withaferin A and Withanolide D are the 2 main withanolides that contribute to most of the biological activity of *W. somnifera* (Harikrishnan et al., 2008; Mirjalilli et al., 2009). Recently, the plant was investigated to be effective in the treatment of some bacterial infections and was tested for antibacterial properties (Arora et al., 2004; Owais et al., 2005; Teixeira et al., 2006). Besides its use as an antibacterial product, several reports have demonstrated immunomodulator and tumor inhibiting activity of its root extract (Ziauddin et al., 1996; Dhuley, 1997; Davis and Kuttan, 2000). *W. somnifera* has been used as an antioxidant, adaptogen, aphrodisiac, liver tonic, antiinflammatory agent, and astringent agent (Rastogi and Mehrotra, 1998). Ashwagandha root powder (@ 0.5%) supplementation in feed has shown a reduction in the severity of *S. gallinarum* infection in chicks (Kumari et al., 2015a).

However, the literature on in vivo studies regarding the effect of this medicinal plant on pathological lesions and immunological response against *E. coli* infection in poultry is sparse. Keeping in view the above facts, the present study was undertaken to study clinical, biochemical, immunological indices, and pathological changes in *E. coli*-infected broiler chicks supplemented with *W. somnifera* root (WSR) extract.

MATERIALS AND METHODS

This study was performed after the approval from the Institutional Animal Ethics Committee of the university.

Experimental Birds, Housing, and Feeding

One hundred and twenty, 1-day-old, healthy and unvaccinated Cobb broiler chicks were procured from a local commercial hatchery and reared under cage system under strict hygienic conditions in a temperature controlled and ventilated animal house of the department. The housing, lighting, ambient temperature, relative humidity, and other physical conditions of the indoor facility were maintained in accordance to standard guidelines (Aviagen, 2014). These birds were fed *ad libitum* on commercial broiler feed (Godrej Agrovvet, Pvt. Ltd., Mumbai, India) in square-type bottom feeders attached outside the cages, and fresh water was provided by good quality plastic fountain-type drinkers.

Preparation of Aqueous Extract of Withania somnifera Roots *W. somnifera* roots were procured from the Medicinal, Aromatic, and Potential Crops Section, Department of Genetics and Plant Breeding, CCS

Haryana Agriculture University, Hisar, India and were chopped, washed, shade-dried, and powdered. Twenty percent aqueous root extract of *W. somnifera* was prepared and filtered through Whatmann (No. 1) filter paper (Pujari and Gandhi, 2012), which was given to chickens through oral route in drinking water @ 20 ml per liter. The dose used in the present experimental study was calculated on the basis of in vitro antibacterial effect of WSR extract on *E. coli* (Kumari and Gupta, 2015). Results of other workers revealed that total alkaloids content of WSR collected from the same source as that of present study varied between 0.26 and 0.31%, and tannins content varied between 0.66 and 0.84 mg/g (Gulati et al., 2017).

Experimental Design and Sampling The 1-day-old chicks were divided into 2 groups viz. Group A and B containing 60 birds each. The birds of group B were supplemented with 20% aqueous WSR root extract through oral route in the drinking water @ 20 ml per liter of water. The amount of water provided and the amount left after drinking within 24 h was quantified daily to calculate the average amount of the extract consumed per day per bird (Table 1). The birds of group A were provided drinking water without any supplementation.

After 7 D of age, the birds of both the groups were further divided into 2 subgroups (Group A into A1 and A2, Group B into B1 and B2) of 25 and 35 birds, respectively. All the chicks of groups A2 and B2 were injected with *E. coli* O78 resuspended in the sterile normal saline solution @ 1×10^7 CFU/0.5 ml intraperitoneally, whereas chicks of group A1 and B1 were injected with 0.5 ml sterile normal saline solution intraperitoneally. The birds of the group A1 and B1 (noninfected) were kept in one room and those of groups A2 and B2 (infected) at a distance in another room with the same housing and environmental conditions. Blood was collected directly from the heart from 5 chicks from each group at 0, 7, 14, 21, and 28 D postinfection in sterilized test tubes without anticoagulant for serum separation and in sterile heparinized vials. Blood was also collected in sterile vials containing 2 mg/ml of EDTA for antioxidant enzymes studies. Thereafter, these birds from each group were sacrificed, and a thorough postmortem examination was conducted. During necropsy, tissue samples from lungs, heart, liver, spleen, proventriculus, intestines, and bursa of Fabricius were collected from all the birds in 10% buffered formalin for histopathological examination. A part of the liver from all the groups was also collected in sterilized Petri dish under aseptic conditions for viable bacterial (*E. coli*) count.

Table 1. The average extract consumed per bird per day (in ml) in different experimental groups at different intervals.

Groups	Age in D					
	1	7	14	21	28	35
B1	0.48	1.37	4.0	4.2	4.7	7.83
B2	0.47	1.41	4.0	3.56	4.8	8.67

Clinical Signs and Mortality

Each bird was closely observed daily for clinical signs and mortality, if any. The clinical signs score was calculated on a scale of 0 to 4 (No signs—0; Mild signs—1; Moderate signs—2; Moderately severe signs—3; Severe signs—4).

Body Weight

All the birds from each group were weighed at 0, 7, 14, 21, and 28 D postinfection to assess the growth response.

Biochemical Studies

The serum samples obtained at different intervals were analyzed for total protein concentration, albumin concentration, and the enzyme activities of aspartate transaminase, alanine transaminase, lactate dehydrogenase, and creatine phosphokinase using single-step reagent kits (Transasia Bio-Medicals Ltd., Mumbai, India) employing a Semiautomatic Biochemistry Analyzer.

Oxidative Stress Parameters

Preparation of Erythrocyte Lysate Blood samples collected in EDTA were centrifuged at 1,500 rpm for 10 min. The pellet was suspended in and washed thrice with normal saline solution. Finally, 10% erythrocyte lysate was prepared by adding chilled distilled water. Hemoglobin concentration of erythrocyte lysate was measured as per the method of Benjamin (1985).

Assay of Superoxide Dismutase The activity of superoxide dismutase (SOD) in erythrocyte lysate was determined by the method of Marklund and Marklund (1974). Absorbance (OD) values were read at 420 nm, and the unit of enzyme activity was defined as the amount of enzyme causing 50% inhibition of autooxidation of pyrogallol observed in control.

Assay of Catalase The activity of catalase in erythrocyte lysate was determined according to the method described by Aebi (1983). In blank test tube, only phosphate buffer (50 mM, pH 7) was taken, and the base was set zero with it. In test sample tubes, 10% erythrocyte lysate was added to phosphate buffer (50 mM, pH 7). After adding H₂O₂ (30 mM), absorbance was read at 240 nm for 1 min. The results were expressed as $\mu\text{mol H}_2\text{O}_2$ decomposed per min per mg Hb.

Assay of Glutathione Reductase The assay of glutathione reductase (GR) was performed according to the method described by Carlberg and Mannervik (1985). Absorbance (OD) was read at 340 nm for 4 min. A unit of GR activity was defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of NADPH per minute using 6.22×10^3 as the molar extinction coefficient of NADPH.

Assay of Glutathione-S-Transferase The activity of glutathione-S-transferase (GST) in erythrocyte lysate was determined by the method of Habig et al. (1974). Absorbance (OD) was read at 340 nm for 3 min. The unit

of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol conjugate of reduced glutathione and 1-Chloro-2,4-dinitrobenzene per minute per mg of Hb.

Viable Bacterial Cell Count of *E. coli* in the Liver

Viable bacterial cell count of all the groups that is infected (A2 and B2) as well as noninfected (A1 and B1) was determined in the liver at 7, 14, 21, and 28 D postinfection under aseptic conditions by the plate-counting method described by Cruickshank et al. (1975). The tissue samples were mixed with brain–heart infusion broth in the ratio of 1:2. Ten-fold dilutions were prepared, and 0.1 ml of each dilution was spread over the surface of Mackonkey's lactose agar plates in duplicate and incubated at 37°C for 24 h. The number of colonies so obtained was multiplied with respective dilution factor to get the original number of viable organisms per gram of the tissue.

Immunological Studies

Indirect ELISA

Antigen Preparation Stock culture of *E. coli* O78 was grown in brain–heart infusion broth for 24 h at 37°C and then inoculated on Mackonkey's lactose agar plates. The growth was harvested in sterile normal saline solution and after washing the pellet 3 times, and the cell suspension was sonicated (Labsonic 1510 sonifier). The sonicated bacterial cell suspension was termed as *E. coli* O78 sonicated antigen for coating the ELISA plates. The optimum concentration of sonicated bacterial cell antigen, serum dilution (1:400), and conjugate dilution (1:3,000) used in the assay was determined using checkerboard titration method.

Procedure Flat-bottomed ELISA plates (Nunc, Roskilde, Denmark) were coated with *E. coli* (50 μl) antigen in 0.05 M carbonate-bicarbonate buffer (pH 9.6) and incubated at 4°C overnight. The plates were washed 5 times in phosphate buffer saline containing 0.05 percent between Tween 20 (PBST, pH 7.2). Unreactive sites in well were blocked with blocking buffer (lactalbumin hydrolysate 3% v/v, new born calf serum 5%v/v, and normal rabbit serum mixed in PBST). After washing, the plates with PBST, 50 μl of each serum sample (1:400 in blocking buffer) in 2-fold serial dilution was dispensed into respective wells and incubated at 37°C for 1 h followed by washings with PBST. Then 50 μl of antichickens IgY (IgG) peroxidase conjugate diluted to 1:3,000 in blocking buffer was added to all wells of the plate. The plates were incubated at 37°C for 1 h and washed, and finally, 50 μl of substrate solution was added to the wells. The reaction was stopped by the addition of 50 μl of 1 M H₂SO₄ after the development of color in positive control well. Absorbance values were read at 492 nm using an automatic micro-ELISA reader (Tecan, Sunrise model Belgium, version 1.21). The results were expressed as endpoint titers that is the reciprocal of the serum dilution

immediately above the baseline for positivity. The baseline (cut-off) selected was 5 times the standard deviation higher than the mean optical density obtained by using serum from negative control chickens. The endpoint titers were converted to $\log_{10}x$, where x was the serum dilution.

Lymphocyte Proliferation Assay The blood samples were evaluated in the lymphocyte proliferation assay to assess cellular immune responses. Peripheral lymphocytes were harvested from blood and incubated with mitogen (phytohemagglutinin [PHA]) or *E. coli* antigen. The degree of lymphocyte proliferation was assayed by tetrazolium-based lymphocyte proliferation assay (Mosmann, 1983). The conversion of 3-(4, 5-dimethylthiazo-2-yl)-2, 5-diphenyl tetrazolium bromide to formazan is directly proportional to the number of proliferating cells and measured by a shift in the absorbance with an ELISA reader. The results were expressed as the response by stimulated cells relative to unstimulated cells.

The optimum concentration of sonicated bacterial cell antigen (1:20 dilution) and PHA (20 $\mu\text{g}/\text{ml}$) used in this assay was determined using checkerboard titration method.

Procedure Blood was collected in heparinized vials and diluted with equal volume of RPMI-1640 medium. Diluted blood was then carefully layered onto histopaque-1077 (Sigma, St Louis, MO) column and centrifuged at 3,000 rpm for 20 min at 4°C in a cooling centrifuge (Velocity R Centrifuge). The white interface layer so obtained containing lymphocytes was harvested carefully, and the cells were resuspended and washed twice with RPMI-1640 and then once with RPMI complete medium at 2,500 rpm for 10 min in the cooling centrifuge. The viability of mononuclear cells was estimated by trypan blue exclusion.

The cells were finally resuspended in RPMI (Roswell Park Memorial Institute Medium) complete medium at

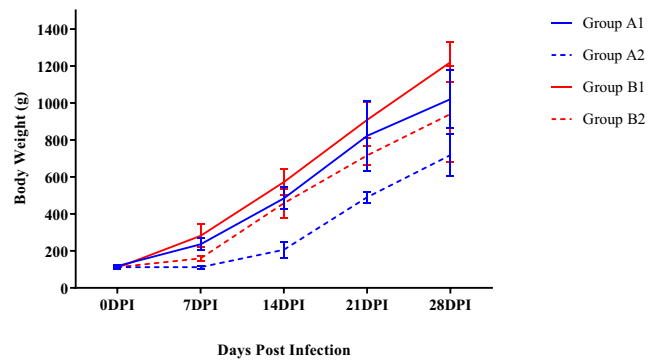


Figure 1. Mean body weight (g) of broiler chickens in different experimental groups at different intervals. Abbreviation: DPI, days post infection.

a concentration of 1×10^6 live cells/ml. To perform the lymphocyte proliferation assay, 100 μL of cell suspension (1×10^6 cells) was dispensed in each of the 3 wells of the microtiter plate. These cells were then stimulated by adding 50 μL of optimally diluted sonicated bacterial cell antigen (1:20) or mitogen (PHA [Sigma] @ 20 $\mu\text{g}/\text{ml}$). Control wells received RPMI-1640 medium only. The plates were then incubated at 37°C in CO₂ incubator for 72 h. After that, 20 μL of working solution (5 mg/ml) of 3-(4, 5-dimethylthiazo-2-yl)-2, 5-diphenyl tetrazolium bromide dye (Sigma) was added in each well, and plates were further incubated at 37°C in CO₂ incubator for 4 h. Following incubation, the supernatant of each well was discarded carefully, and 100 μL of dimethyl sulfoxide was added into each well to solubilize the formazan crystals. Finally, optical density was recorded using microplate reader (Tecan, Sunrise model, Belgium version 1.21) at 540 nm wavelength, and the stimulation index (SI) was calculated by the following formulae (Denizot and Lang, 1986):

The SI of antigen – driven proliferation

$$= \text{Absorbance of antigen – stimulated cells} / \text{Absorbance of unstimulated cells.}$$

The SI of mitogen (PHA) – driven proliferation

$$= \text{Absorbance of mitogen – stimulated cells} / \text{Absorbance of unstimulated cells.}$$

Table 2. Mean score of clinical signs of chickens of different experimental groups at different intervals (mean \pm SE).

Groups	0 DPI (n = 35)	7 DPI (n = 24)	14 DPI (n = 15)	21 DPI (n = 10)	28 DPI (n = 5)
A1	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0
A2	0.0 \pm 0.0	3.21 ^a \pm 0.19	1.8 ^a \pm 0.17	0.4 ^a \pm 0.16	0.0 \pm 0.0
B1	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0
B2	0.0 \pm 0.0	1.33 ^b \pm 0.98	0.53 ^b \pm 0.133	0.0 \pm 0.0	0.0 \pm 0.0

Abbreviation: DPI, days post infection.

^{a,b}Means with unlike superscript in the column differ significantly ($P < 0.05$).

n is no. of birds evaluated at each interval.

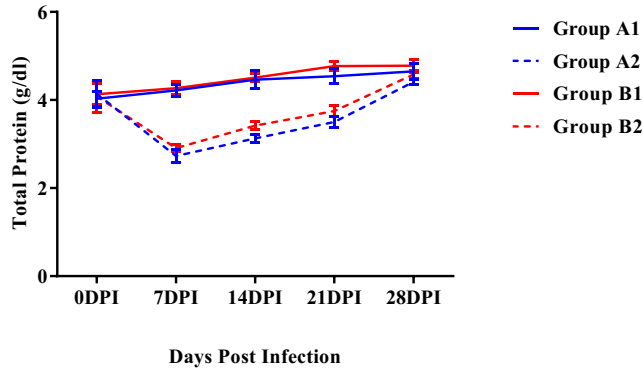


Figure 2. Mean total serum proteins concentration (g/dl) of broiler chickens in different experimental groups at different intervals. Abbreviation: DPI, days post infection.

Gross Pathology The chicks sacrificed or died naturally during the experiment were thoroughly examined for gross lesions, if any.

Histopathology The formalin-fixed tissues were processed by paraffin-embedding technique. After trimming and properly washing the tissues overnight in running tap water, they were dehydrated in graded ethanol, cleared in benzene, and embedded in

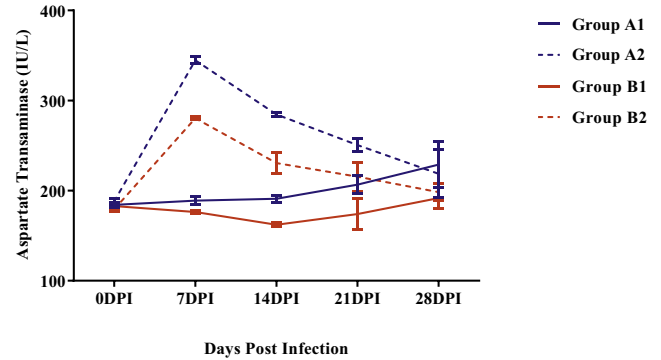


Figure 4. Mean serum aspartate transaminase activities (IU/L) of broiler chickens in different experimental groups at different intervals. Abbreviation: DPI, days post infection.

Percent mean gross and histopathological lesions were calculated as per the method described by [Witter \(1982\)](#).

Protective Effect Percent protective effect due to WSR extract supplementation in *E. coli*-infected chicks was calculated on the basis of GLS and HLS as per the method of [Witter \(1982\)](#) using the following formula:

Percent protective effect due to WSR extract supplementation in *E. coli* infected chickens

$$\frac{\text{Overall \% mean GLS or HLS in group A2} - \text{overall \% mean GLS or HLS in group B2} \times 100}{\text{Overall per cent mean GLS or HLS in group A2}}$$

paraffin wax (melting point 60°C–62°C). Sections of 3 to 4 μm thickness were cut using semiautomatic microtome and stained with hematoxylin and eosin ([Luna, 1968](#)).

Lesion Score The colibacillosis-specific gross lesion score (GLS) and histopathological lesion score (HLS) in different experimental groups were calculated for different organs/tissues at scale of 0 to 4 [No lesion—0; Mild lesions (congestion)—1; Moderate lesions (thin fibrin layer over organs)—2; Moderately severe lesions (thick fibrin layer over organs)—3; Severe lesions (very thick layer/mass of fibrin over organs, adhesions, necrosis—4)]

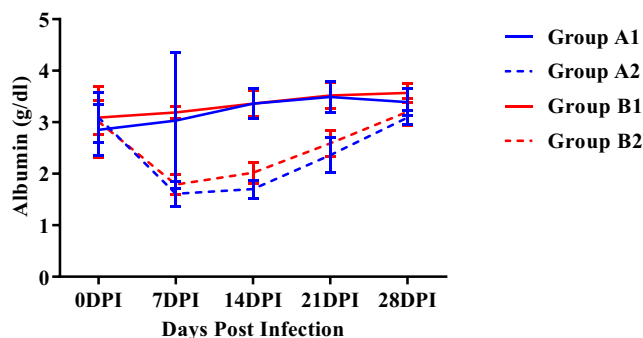


Figure 3. Mean serum albumin concentration (g/dl) of broiler chickens in different experimental groups at different intervals. Abbreviation: DPI, days post infection.

Statistical Analysis

The data of body weight and biochemical parameters including oxidative stress and lymphocyte proliferation assay were statistically analyzed by applying Analysis of Variance using Statistical Package for Social Sciences (SPSS), 17th version. Antibody titer and viable bacterial cell counts were analyzed with a t-test. Clinical signs and lesion scores were analyzed with a chi-square test. For all the parameters, the results were compared at $P < 0.05$.

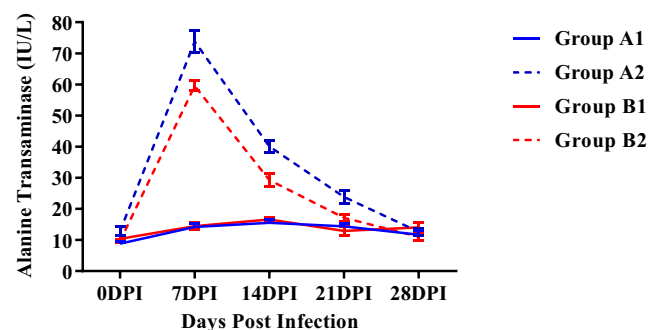


Figure 5. Mean serum alanine transaminase activities (IU/L) of broiler chickens in different experimental groups at different intervals. Abbreviation: DPI, days post infection.

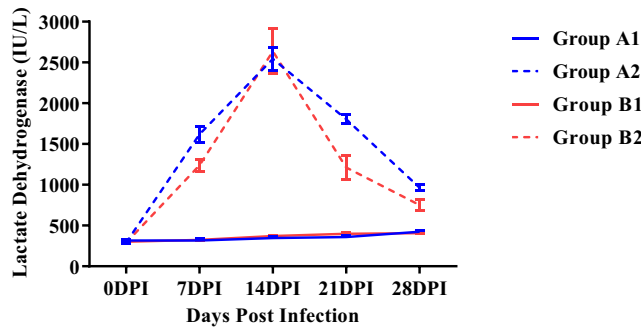


Figure 6. Mean serum lactate dehydrogenase activities (IU/L) of broiler chickens in different experimental groups at different intervals. Abbreviation: DPI, days post infection.

RESULTS

Clinical Signs and Mortality

Mean score of clinical signs of chickens in the infected groups at different intervals is given in Table 2. The mean clinical signs score was significantly lower in WSR extract supplemented and infected group B2 as compared with group A2 from 7 to 21 days post infection (DPI). Clinical signs of *E. coli* infection in group A2 (control infected, nonsupplemented) started to appear at 24 h postinfection. These were inappetence, dullness, depression, closing of eyes, anorexia, listlessness, ruffled feathers, and drooping of head and neck. They were reluctant to move and huddled together near the light source. These clinical signs were more severe at 7 DPI. After 10 DPI, the severity of clinical signs declined. The birds were almost normal at 21 DPI.

On the other hand, the clinical signs of *E. coli* infection in group B2 (infected, WSR extract supplemented) appeared at 36 h postinfection. Clinical signs were almost identical to those observed in group A2, but they were less severe as compared with group A2. The birds of group B2 chicks appeared almost normal after 14 DPI.

There was no mortality in noninfected groups A1 and B1. The overall mortality in group A2 and B2 was 25.71 and 17.14%, respectively.

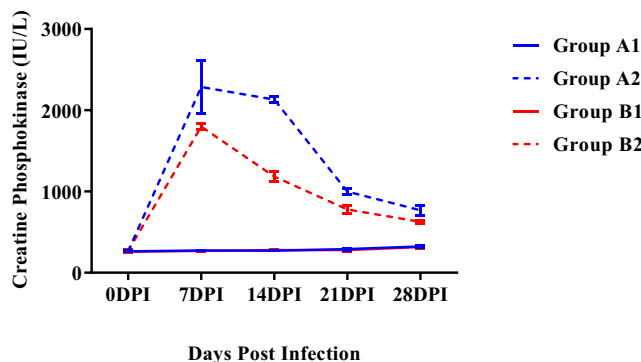


Figure 7. Mean serum creatine phosphokinase activities (IU/L) of broiler chickens in different experimental groups at different intervals. Abbreviation: DPI, days post infection.

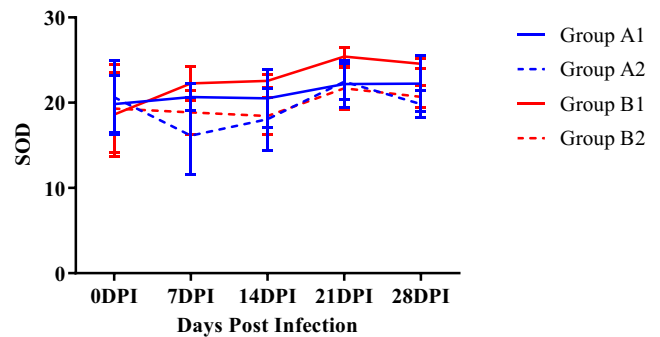


Figure 8. Mean superoxide dismutase levels (U/mg Hb) of broiler chickens in different experimental groups at different intervals. Abbreviations: DPI, days post infection; SOD, superoxide dismutase.

Body Weight

Mean body weights of different groups are illustrated in Figure 1. Mean body weight of WSR supplemented groups (B1 and B2) was significantly higher ($P < 0.05$) as compared with corresponding control groups (nonsupplemented) that is A1 and A2 from 7 DPI onward, indicating the ameliorating effect of WSR extract on body growth.

Biochemical Studies

Total Serum Protein Concentration Figure 2 showed that a significant decrease ($P < 0.05$) in mean total serum protein concentrations was observed in both the infected groups (A2 and B2) as compared with noninfected groups from 7 to 21 DPI. This decrease was less in group B2 as compared with group A2 though the difference was nonsignificant.

Albumin Concentration A significant decrease ($P < 0.05$) in mean serum albumin concentrations was observed in both the infected groups as compared with corresponding control groups from 7 DPI onward (Figure 3). However, the mean serum albumin concentration in group B2 was significantly higher ($P < 0.05$) as compared with group A2 on 14 DPI.

Aspartate Transaminase A significant increase ($P < 0.05$) in mean serum aspartate transaminase (AST) activity was observed in both the infected groups

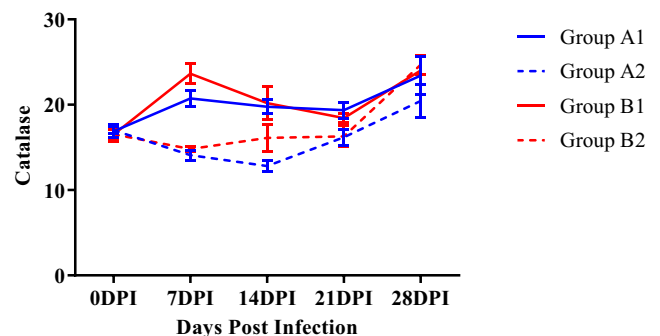


Figure 9. Mean catalase levels (U/mg Hb) of broiler chickens in different experimental groups at different intervals. Abbreviation: DPI, days post infection.

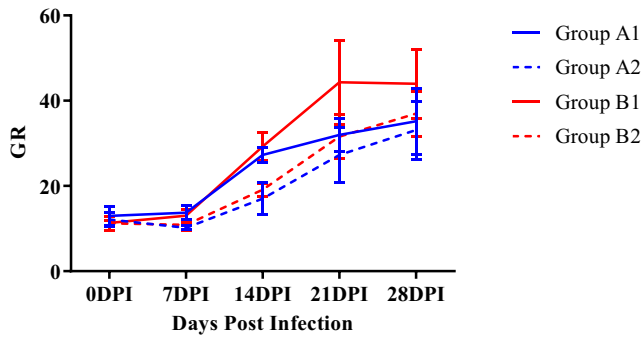


Figure 10. Mean glutathione reductase levels (U/mg Hb) of broiler chickens in different experimental groups at different intervals. Abbreviations: DPI, days post infection; GR, glutathione reductase.

A2 and B2 as compared with noninfected control groups A1 and B1 respectively, from 7 to 21 DPI (Figure 4). Moreover, the serum AST activity of group B2 was significantly low ($P < 0.05$) as compared with group A2 at 7 and 14 DPI.

Alanine Transaminase Mean serum alanine transaminase (ALT) activities were significantly higher ($P < 0.05$) in both the infected groups as compared with respective control groups from 7 DPI onward (Figure 5). This increase in serum ALT activity persisted up to 21 DPI in group A2 while only up to 14 DPI in group B2. Moreover, the mean serum ALT activity was significantly low ($P < 0.05$) in group B2 as compared with group A2 from 7 to 21 DPI.

Lactate Dehydrogenase Figure 6 revealed that mean serum lactate dehydrogenase (LDH) activities were significantly higher ($P < 0.05$) in both the infected groups as compared with respective control groups from 7 DPI onward. Mean serum LDH activity of group B2 was significantly lower ($P < 0.05$) as compared with group A2 at 7, 21 and 28 DPI.

Creatine Phosphokinase Mean serum creatine phosphokinase (CPK) activities were significantly higher ($P < 0.05$) in both the infected groups as compared with respective control groups from 7 DPI onward (Figure 7). Mean serum CPK activity of group B2 was significantly low ($P < 0.05$) as compared with group A2 from 7 DPI onward up to 28 DPI.

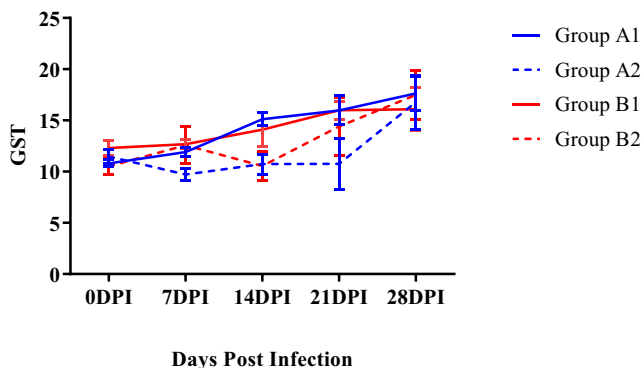


Figure 11. Mean glutathione-S-transferase levels (U/mg Hb) of broiler chickens in different experimental groups at different intervals. GST, glutathione-S-transferase. Abbreviation: DPI, days post infection.

Oxidative Stress Parameters

Assay of Blood Superoxide Dismutase Figure 8 shows that the mean SOD activities of group A2 at 7 DPI were significantly lower ($P < 0.05$) as compared with respective control group A1 indicating oxidative stress because of *E. coli* infection. However, the SOD activity of group B2 was significantly low than that of group B1 at 21 and 28 DPI. There was significantly higher activity of SOD in group B1 as compared with group A1 at 21 DPI indicating increased antioxidant scavenging activity in WSR extract-supplemented chicks.

Assay of Blood Catalase The mean catalase activities of infected groups were significantly lower as compared with respective noninfected control groups (Figure 9). Furthermore, the mean catalase activities of group B2 were considerably higher as compared with group A2 from 7 to 28 DPI, though the differences were nonsignificant.

Assay of Blood Glutathione Reductase The mean GR activities of control WSR supplemented group was significantly higher ($P < 0.05$) as compared with control nonsupplemented group (Figure 10). Moreover, the GR activities of infected groups A2 and B2 were significantly lower as compared with respective control groups from 7 to 21 DPI.

Assay of Blood Glutathione-S-Transferase The mean GST activity of the infected group A2 was lower compared with the corresponding control group (A1) at 14 DPI (Figure 11). However, the mean GST activity of infected WSR supplemented group B2 did not decrease significantly as compared with corresponding noninfected group B1.

Viable Bacterial Cell Count

Table 3 shows that mean viable bacterial (*E. coli*) cell counts in the liver of group B2 was lower as compared with group A2 from 7 DPI onward though the difference was nonsignificant. The noninfected groups (A1 and B1) did not reveal any viable bacterial cell.

Immunological Studies

Antibody Titer Mean reciprocal log₁₀ antibody titer against *E. coli* in group B2 was higher as compared with group A2 from 7 DPI onward though significant difference ($P < 0.05$) was noticed only at 21 DPI (Figure 12).

Lymphocyte Proliferation Assay The SI means for antigen (*E. coli*)-driven lymphocyte proliferation responses in group B2 were found to be higher as compared with group A2 from 14 DPI onward though a significant difference was present only at 21 DPI (Figure 13).

The SI means for the PHA-driven lymphocyte proliferation responses were significantly higher ($P < 0.05$) in groups A1 and B1 as compared with groups A2 and B2, respectively, from 7 DPI onward (Figure 14). Moreover, group B1 exhibited significantly higher ($P < 0.05$) values of SI as compared with group A1 at 28 DPI.

Table 3. Mean viable bacterial cell count ($\times 10^6$ CFU/g of liver, mean \pm SE) in different experimental groups at different intervals.

Groups	Days postinfection			
	7	14	21	28
A1	0.00 \pm 0.00 ^a	0.00 \pm 0.00 ^a	0.00 \pm 0.00 ^a	0.00 \pm 0.00 ^a
A2	1.22 \pm 0.32 ^a	3.06 \pm 1.00 ^a	0.014 \pm 0.008 ^a	0.002 \pm 0.001 ^a
B1	0.00 \pm 0.00 ^a	0.00 \pm 0.00 ^a	0.00 \pm 0.00 ^a	0.00 \pm 0.00 ^a
B2	1.08 \pm 0.40 ^a	1.29 \pm 0.32 ^a	0.005 \pm 0.002 ^a	0.00 \pm 0.00 ^a

^aMeans with superscript in the column did not differ significantly ($P < 0.05$).

Gross and Histopathological Lesions

No pathological changes could be observed in chicks from noninfected groups (A1 and B1) at different intervals throughout the experiment.

Group A2 (*E. coli* Infected) On 7 DPI, there was fibrinous perihepatitis associated with coagulative necrosis of hepatocytes at focal areas, fibrinous pericarditis, and myocarditis. Congestion was noticed in various visceral organs such as liver, heart, lungs, spleen, proventriculus, and kidneys. Lungs revealed bronchopneumonia along with pleuritis. In spleen, depletion of lymphocytes was noticed in the white pulp along with congestion and focal necrotic areas. Bursa of Fabricius revealed atrophy of bursal follicles, depletion of lymphocytes, and increase in inter follicular space, infiltration of heterophils, and edema. On 14 DPI, the heart, liver, and spleen lesions were more severe along with adhesions with other abdominal organs because of thick fibrin mass on the surface of the liver. In spleen, depletion of lymphocytes was noticed in the white pulp along with congestion and focal necrotic areas. On 21 DPI onward, the lesions in different organs were of mild magnitude as there was only a thin fibrin layer covering on the surface of heart and liver.

Group B2 (*E. coli* infected + WSR extract supplemented) On 7 DPI, heart and liver surface revealed mild inflammatory lesions and lungs and spleen revealed only congestion. On 14 DPI onward, the lesions in different organs were of very mild degree. On 21 DPI, the bursa of Fabricius was almost normal with the prominent demarcation between the medulla and cortex maintaining the normal architecture of follicles along with presence of lymphocytes in the follicles.

Overall severity of lesions in different organs in WSR extract-fed infected group B2 was significantly less than control infected group A2. On 28 DPI, there were no lesions in any of the organs.

Lesion Score The colibacillosis-specific mean GLS and HLS in various organs (heart, liver, spleen, bursa of Fabricius, lungs, and intestines) in different experimental groups are shown in Table 4 and Table 5. The mean GLS was considerably lower in WSR extract-supplemented infected group B2 as compared with group A2. Similarly, HLS was also lower in group B2 in comparison to group A2. The overall percent mean GLS and HLS in different organs irrespective of postinfection period and organs in different experimental groups (Table 6) were also lower in supplemented infected groups as compared with the nonsupplemented infected group.

Percent protection due to WSR extract against *E. coli* infection on the basis of gross and histopathological lesions (Table 7) was 31.48 and 34.38%, respectively.

DISCUSSION

The present experiment was undertaken to evaluate the in vivo effect of aqueous WSR extract on *E. coli* infection in broiler chicken with respect to biochemical, immunological, and pathological parameters. The clinical signs of colibacillosis in WSR extract-supplemented group appeared later, comparatively of less intensity and persisted for a shorter period as compared with control infected group. Mortality was also reduced. These findings suggest that the ameliorating effect of the WSR extract on severity of

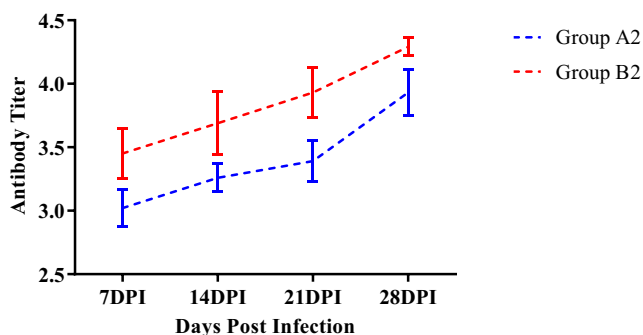


Figure 12. Mean reciprocal log₁₀ antibody titer against *E. coli* infection in all the infected groups. Abbreviation: DPI, days post infection.

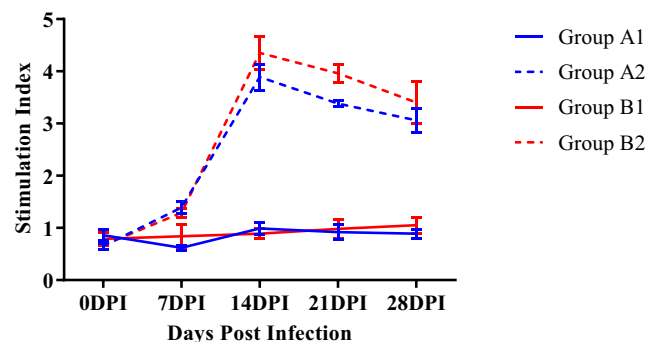


Figure 13. Mean stimulation index of antigen-driven lymphocyte proliferation responses of broiler chickens in different experimental groups. Abbreviation: DPI, days post infection.

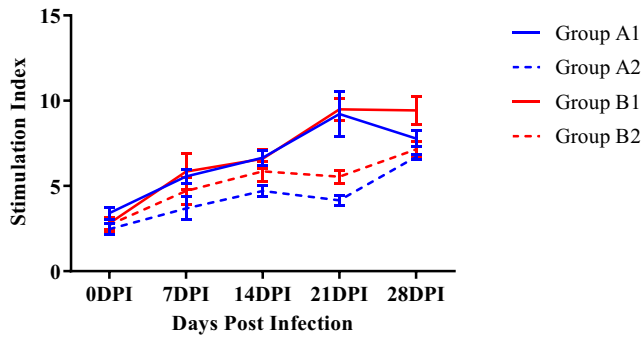


Figure 14. Mean stimulation index of mitogen (phytohemagglutinin)-driven lymphocyte proliferation responses of broiler chickens in different experimental groups. Abbreviation: DPI, days post infection.

colibacillosis may reduce the economic losses associated with the disease. These findings are in accordance with those of Kumari et al. (2015b) and Owais et al. (2005) who observed an increase in the survival rate in Balb/C mice and Salmonella-infected broiler chicks, respectively, when they were treated orally with WSR. The protective effect of WSR on the disease manifestations might be because of immunomodulatory and antimicrobial effects as observed in the present study and literature (Akotkar et al., 2007; Gupta and Rana, 2007). Body weight gain in WSR extract supplemented groups (infected as well as noninfected) was significantly higher as compared with their respective control groups indicating an accelerated growth response in the WSR extract supplemented birds. The enhanced growth rate because of WSR supplementation might be attributed to improved feed conversion ratio and free radical scavenging effect of WSR supplementation (Akotkar et al., 2007; Vasanthakumar et al., 2015). The significant reduction in serum total protein concentration in all the infected groups was due to hypoalbuminemia. More or less similar results have been reported by other workers because of *E. coli* infection (Jindal et al., 2003; Zaki et al., 2012; Kumari et al., 2014). This might be because of severe hepatic lesions observed in the infected groups in the present study. According to Kaneko (1997), during inflammation, the

synthesis of albumin (negative acute phase protein) in the liver is inhibited. Serum albumin concentration in WSR extract-supplemented infected group which might be attributed to variance in the severity of hepatic lesions of both the infected groups. Dhenge et al., (2009) also reported a significant increase in serum total protein in broilers supplemented with *W. somnifera*. According to Udayakumar et al., (2009), the phytochemicals present in WSR extract might have contributed to the reversal of changes in serum total protein and albumin concentration.

The significantly higher activities of serum aspartate transaminase and alanine transaminase observed in *E. coli*-infected groups as compared with control have also been reported by other workers (Eleiwa et al., 2011; Zaki et al., 2012; Verma and Swamy 2013; Kumari et al., 2014). The restoration of the normal activity of these enzymes was evident in WSR extract supplemented infected group as compared with control infected group indicating the hepatoprotective effect of WSR supplementation. Nevertheless, pathological changes in liver due to *E. coli* infection in WSR supplemented group was of lower magnitude as compared with nonsupplemented infected group of the present study. More or less, similar ameliorating effects of WSR supplementation have been reported in rats (Harikrishnan et al., 2008; Udayakumar et al., 2009), which could be due to presence of alkaloids, flavonoids, and free radical scavenging activity of WSR.

The significant increase in serum lactate dehydrogenase activity observed in both the infected groups as compared with control in the present study might be because of injury to cardiac lesions. The restoration of LDH activity in WSR supplemented group may indicate the cardiac and hepatoprotective activity of WSR. These findings are in agreement to the observations of Mohanty et al. (2004) in rats.

The results of CPK activity revealed that the WSR supplementation had contributed to significant restoration of cardiac lesions caused by *E. coli* infection. Mohanty et al. (2004) reported that hydroalcoholic extract of *W. somnifera* restored myocardial damage in rats.

Table 4. Mean gross lesion score in various organs of chickens of different experimental groups.

Organs	Groups	Days postinfection				
		0	7	14	21	28
Heart	A2	0.00 ± 0.00	3.20 ± 0.20 ^a	3.20 ± 0.37 ^a	1.20 ± 0.37 ^a	0.00 ± 0.00
	B2	0.00 ± 0.00	3.00 ± 0.32 ^a	2.80 ± 0.37 ^a	0.40 ± 0.24 ^a	0.00 ± 0.00
Liver	A2	0.00 ± 0.00	3.40 ± 0.24 ^a	3.20 ± 0.37 ^a	0.60 ± 0.24 ^a	0.00 ± 0.00
	B2	0.00 ± 0.00	2.60 ± 0.51 ^a	2.40 ± 0.24 ^a	0.40 ± 0.24 ^a	0.00 ± 0.00
Lungs	A2	0.00 ± 0.00	2.40 ± 0.24 ^a	2.40 ± 0.51 ^a	0.60 ± 0.24 ^a	0.00 ± 0.00
	B2	0.00 ± 0.00	2.20 ± 0.37 ^a	1.80 ± 0.37 ^a	0.40 ± 0.24 ^a	0.00 ± 0.00
Spleen	A2	0.00 ± 0.00	2.40 ± 0.24 ^a	2.00 ± 0.32 ^a	0.80 ± 0.37 ^a	0.00 ± 0.00
	B2	0.00 ± 0.00	1.60 ± 0.40 ^a	1.40 ± 0.24 ^a	0.20 ± 0.20 ^a	0.00 ± 0.00
Bursa	A2	0.00 ± 0.00	2.20 ± 0.20 ^b	2.20 ± 0.37 ^b	0.40 ± 0.24 ^a	0.00 ± 0.00
	B2	0.00 ± 0.00	1.00 ± 0.32 ^a	1.20 ± 0.20 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00
Intestine	A2	0.00 ± 0.00	1.00 ± 0.32 ^a	1.00 ± 0.32 ^a	0.20 ± 0.20 ^a	0.00 ± 0.00
	B2	0.00 ± 0.00	0.20 ± 0.20 ^a	0.40 ± 0.24 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00

^{a,b}Means with unlike superscript in the column differ significantly ($P < 0.05$).

Table 5. Mean histopathological lesion score in various organs of chickens of different experimental groups.

Organs	Groups	Days postinfection				
		0	7	14	21	28
Heart	A2	0.00 ± 0.00	3.00 ± 0.32 ^a	2.20 ± 0.49 ^a	1.00 ± 0.45 ^a	0.20 ± 0.20 ^a
	B2	0.00 ± 0.00	3.20 ± 0.37 ^a	1.80 ± 0.37 ^a	0.20 ± 0.20 ^{ab}	0.00 ± 0.00 ^a
Liver	A2	0.00 ± 0.00	3.00 ± 0.45 ^a	1.80 ± 0.37 ^a	1.00 ± 0.45 ^a	0.20 ± 0.20 ^a
	B2	0.00 ± 0.00	2.00 ± 0.32 ^a	1.80 ± 0.20 ^a	0.60 ± 0.24 ^{ab}	0.20 ± 0.20 ^a
Lungs	A2	0.00 ± 0.00	2.60 ± 0.24 ^a	2.40 ± 0.51 ^a	1.00 ± 0.32 ^{ab}	0.40 ± 0.24 ^a
	B2	0.00 ± 0.00	1.60 ± 0.40 ^a	1.60 ± 0.24 ^a	0.20 ± 0.20 ^{ab}	0.00 ± 0.00 ^a
Spleen	A2	0.00 ± 0.00	2.20 ± 0.20 ^a	2.40 ± 0.24 ^b	0.60 ± 0.24 ^a	0.00 ± 0.00
	B2	0.00 ± 0.00	1.80 ± 0.58 ^a	1.20 ± 0.37 ^a	0.20 ± 0.20 ^{ab}	0.00 ± 0.00
Bursa	A2	0.00 ± 0.00	3.00 ± 0.32 ^b	2.80 ± 0.58 ^b	1.20 ± 0.20 ^b	0.00 ± 0.00
	B2	0.00 ± 0.00	1.80 ± 0.20 ^a	1.00 ± 0.32 ^a	0.20 ± 0.20 ^a	0.00 ± 0.00
Intestine	A2	0.00 ± 0.00	0.40 ± 0.24 ^a	0.60 ± 0.40 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00
	B2	0.00 ± 0.00	0.80 ± 0.37 ^a	0.60 ± 0.40 ^a	0.20 ± 0.20 ^a	0.00 ± 0.00

^{a,b}Means with unlike superscript in the column differ significantly ($P < 0.05$).

A number of serum antioxidant enzymes were analyzed to assess the role of WSR extract in the prevention of oxidative stress due to *E. coli* infection. A significant reduction in mean SOD level in control infected group A2 as compared with noninfected control group A1 indicates overwhelmed free radical production and less free radical scavenging activity of SOD, resulting in oxidative stress due to *E. coli* infection in the birds. However, B1 revealed significantly higher SOD levels as compared with group A1 suggesting an increase in the free radical scavenging activity of SOD in WSR extract-fed chicken. Other workers have also reported the antioxidant effect of *W. somnifera* in mice (Panda and Kar, 1997), rats (Mohanty et al., 2004; Hosny and Farouk, 2012), and broiler chicken (Gupta et al., 2008; Bharavi et al., 2010; Vasanthakumar et al., 2015).

The mean catalase activity of WSR extract supplemented infected group B2 was higher than control infected group A2, though the difference was nonsignificant. Panda and Kar (1997) reported an increase in catalase activity in mice because of supplementation of WSR powder. Other workers have also reported increase in catalase activity in rats (Bhattacharya et al., 2001; Mohanty et al., 2004; Hosny and Farouk 2012) and broilers (Gupta et al., 2008; Bharavi et al., 2010; Vasanthakumar et al., 2015) because of supplementation of *W. somnifera*.

The higher mean GR activity of the WSR extract supplemented and infected group as compared with control infected group is in accordance with the findings of Malik et al. (2013), who reported enhanced GR activities in

mice with the administration of aqueous root extract of *W. somnifera*.

The mean GST activities of control infected group decreased significantly as compared with control placebo, but WSR extract supplemented infected group did not reveal significant decrease in GST activity as compared with control indicating antioxidant activity of WSR extract. Enhanced activity of GST because of the administration of *W. somnifera* has also been reported in rats (Sabina et al., 2013).

Antioxidant activity of WSR may be contributed to its active principles such as glycowithanolides, sitoindoside VII-K, and withaferin A (Bhattacharya et al., 2000; Mishra et al., 2000).

The lower viable *E. coli* count observed in the liver of the supplemented infected group as compared with the nonsupplemented group may reflect the antibacterial activity of WSR (Sonwane et al., 2017). Nevertheless our in vitro experimental results have also demonstrated a remarkable antibacterial activity of aqueous extract of WSR against *E. coli* (Kumari and Gupta, 2014). Iuvone et al., (2003) have reported that that WSR extract enhanced nitric oxide synthetase activity of the macrophages, which in turn increased the microbial killing power of these immune cells. The significantly higher mean *E. coli*-specific antibody titer in WSR extract supplemented infected group suggests enhancement of humoral immune response due to supplementation of aqueous WSR extract. Bhardwaj et al. (2012) reported significantly higher immunoglobulins in Japanese quails supplemented with 1.5% *W. somnifera* as

Table 6. Overall percent mean lesion scores irrespective of post-infection period and organs in different experimental groups.

Lesion score	Groups	Overall percent mean lesion scores irrespective of postinfection period and organs
GLS	Group A2	27.00 ± 3.95
	Group B2	18.50 ± 4.33
HLS	Group A2	26.67 ± 4.50
	Group B2	17.50 ± 2.6

Abbreviations: GLS, gross lesion score; HLS, histopathological lesion score.

Table 7. Percent protection due to *W. somnifera* extract against *E. coli* infection in broiler chicken.

Group	Percent protection	
	GLS	HLS
B2 (WSR extract supplemented and infected)	31.48	34.38

Abbreviations: GLS, gross lesion score; HLS, histopathological lesion score.

compared with the control group. Arivuchelvan et al. (2013) reported reversal of enrofloxacin-induced immunosuppression in broilers because of supplementation of crude extract of *W. somnifera*. Similarly, enhanced humoral immune response in *Salmonella gallinarum*-infected broiler chicks because of the administration of WSR has also been reported (Kumari et al., 2015a). Ghosal et al. (1989) suggested that the increase in humoral immune response might be because of the immunomodulatory activity of glycowithanolides found in *W. somnifera*.

Regarding the cell-mediated immune response, the significantly higher SI of PHA-driven lymphocyte proliferation responses in control WSR extract-supplemented group B1 as compared with control placebo A1 revealed enhancement of cell-mediated immune response. Furthermore, the significantly higher mean SI of *E. coli* antigen-driven lymphocyte proliferation responses in group B2 as compared with group A2 suggests that the immune system was less compromised because of *E. coli* infection in group B2. Enhanced cell-mediated immune response due to supplementation of WSR powder has also been reported by other workers in the broiler chicken (Akotkar et al., 2007; Kumari et al., 2015a) and rats (Gupta et al., 2006). Raghavan et al. (2011) also reported that supplementation of *W. somnifera* to broilers improved the immune status and reduced stress by improving the total leukocyte count and reducing the heterophil:lymphocyte ratio. The role of interferon gamma, interleukin-2, and granulocyte macrophage colony-stimulating factor in immunopotentiating effect of WSR has been reported in mice (Iuvone et al., 2003). The gross and histopathological lesions in WSR extract supplemented infected group at different intervals were of lower magnitude as compared with control infected group (nonsupplemented), which indicates less injury to the visceral organs of chicks supplemented with WSR extract.

The results of lesion scores revealed that the protective effect of WSR extract on *E. coli* infection was 31.48% in gross lesions and 34.38% in histopathological lesions. The differences in the severity of hepatic and cardiac lesions between the control infected and WSR supplemented infected group might be because of antioxidant principles of *W. somnifera* such as withanolides, which reduces lipid peroxidation, improves antioxidant status (Bharavi et al., 2010), antilipoperoxidative activity (Bhattacharya et al., 2001; Hosny and Farouk, 2012), and antiinflammatory activity (Mahmoud et al., 2012), which work together to reduce the cellular injury in these organs. The hepatoprotective and cardioprotective potential of WSR has also been demonstrated by different workers in rats/mice (Mohanty et al., 2004; Harikrishnan et al., 2008; Kaithwas et al., 2011) and chickens (Waihenya et al., 2002; Kumari et al., 2015a). The recovery of the lesions in the spleen and bursa of Fabricius in WSR-supplemented infected group indicates an immunomodulatory effect of WSR. *W. somnifera* has also been reported to provide immunoprotection against *E. coli* infection in Guinea

pigs (Teixeira et al., 2006) and *S. gallinarum* infection in broilers (Kumari et al., 2015a).

The overall pathological findings in different organs suggested that WSR extract resulted in considerable reduction in inflammation and injury to various organs due to colibacillosis. Rasool and Varalakshmi (2006) reported that *W. somnifera* activated and mobilized macrophages for rendering increased phagocytic activity.

It is concluded that administration of 20% aqueous WSR extract @ 20 ml/liter of water to broiler chicken caused a significant reduction in the severity and course of *E. coli* infection and enhanced humoral and cellular immune responses, suggesting a protective effect on the pathology and pathogenesis of *E. coli* infection.

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