

## EFFECTS OF *PICRORHIZA KURROA* FEEDING ON *SALMONELLA GALLINARUM* INFECTION AND COMPARISON OF THE IMMUNE RESPONSES IN BROILER CHICKENS EXPOSED TO AFLATOXIN B1

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### ABSTRACT

The present study was undertaken to evaluate the effect of *Picrorhiza kurroa* (Kutki) feeding on immunological response and protection in chickens vaccinated and challenged with *Salmonella enterica* serovar *gallinarum* (SG) vaccine. Day-old broiler chicks (n=220) were randomly divided into six groups A, B, C, D, E and F. The chicks in groups A, B, C and D were immunized with  $2 \times 10^{10}$  cfu per  $0.4 \text{ ml}^{-1}$  SG formalin killed aluminum hydroxide adjuvant vaccine (SGFKV) subcutaneously at day zero of age. Chicks in group B were fed with *P. kurroa* @ 5 g per kg of feed, group C with aflatoxin B1 @ 2 ppm per kg feed and group D with *P. kurroa* and aflatoxin B1 with the same dose as in groups B and C from day-one of age. Group E chicks were fed *P. kurroa* @ 5 g per kg feed and group F were kept as control (no aflatoxin, no *P. kurroa*, no vaccine). At four weeks post immunization, chicks of all the six groups were challenged with  $2 \times 10^{10}$  cfu/0.5 ml/bird of SG organisms. The maximum lymphocyte transformation responses (LTR's) with mean stimulation indices of 9.60, 9.94, 9.04, 9.36 and 2.43 were observed at 4 weeks post immunization against sonicated bacterial cell protein antigen (sbcp) antigen and 20.41, 21.85, 18.51, 19.49 and 11.51 against concanavalin A (Con A) in groups A, B, C, D and E, respectively. Maximum ELISA titers of 3.42, 3.70, 2.82, 3.04 and 1.78 were observed in groups A, B, C, D and E, respectively at 3 weeks PI. It was concluded that feeding of aflatoxin B1 to broiler chicken lowered the cell mediated immunity and humoral immune response. *P. kurroa* feeding to chicks immunized with SGFKV showed an increase in HI and CMI responses. However, the immunoprotective efficacy of the SGFKV was reduced in groups C and D chickens due to immunosuppression caused by feeding of aflatoxin B1.

**Key words:** Aflatoxin B1, chickens, immune response, *P. kurroa*, *Salmonella gallinarum*

In ethno veterinary practice, various herbal and medicinal plants have been used to manage diseases in poultry (Kumari and Gupta, 2015). To combat fatal diseases in poultry, herbal formulations have been tried to promote growth, weight gain, feed efficiency and to increase livability (Nathanael and Vardhani, 2014).

*Picrorhiza kurroa* (*Scrophulariaceae*) is a small perennial herb that grows in northwest India. It has been used to treat liver and bronchial problems, dyspepsia and chronic dysentery and the aqueous rhizome extract of *P. kurroa* was also investigated for its hepatoprotective and antioxidant effects in wistar albino rats (Vinothkumar *et al.*, 2010). The most active constituents of *P. kurroa* are the cucurbitacin glycosides, apocynin, iridoid glycoside, picrosides and kutkin (Shitiz *et al.*, 2013). *P. kurroa* has hepatoprotective effect against amanita poisoning (Dwivedi *et al.*, 1992), carbon tetrachloride (Khan *et al.*, 2009) and aflatoxin B1 (Zhang *et al.*, 2012). *P. kurroa* rhizomes were also tested against various microorganisms including Gram-positive and Gram-negative bacteria and fungi (Rathee *et al.*, 2012). Bioactivity studies established its anti inflammatory (Kumar and Ramesh, 2014),

antioxidant (Rajaprabhu *et al.*, 2007) and immunomodulatory (Sane *et al.*, 2011) activities.

The enzyme linked immunosorbent assay (ELISA) is recognized as a rapid and highly sensitive test for the detection of a number of avian pathogens including *Salmonella* (Brooks *et al.*, 2012). Aflatoxins are secondary metabolites produced by fungi *Aspergillus flavus* and *A. parasiticus*. Out of four common metabolites (B1, B2, G1 and G2), B1 is considered to be the highly toxic, biologically active, immunosuppressive and a potent carcinogen (Nathanael and Vardhani, 2014). Aflatoxins have been found to increase the susceptibility of chickens to fowl typhoid, paratyphoid, caecal coccidiosis, Marek's disease etc (Bhat *et al.*, 1997).

Efforts have been made by several workers to develop a vaccine and use of chemotherapeutics in order to control the SG infection in chicken with varying success (Agnihotri and Kumar, 2008). Hence, the present study was undertaken with the effect of *P. kurroa* feeding on immunological response and protection in chickens vaccinated and challenged with *Salmonella enterica* serovar *gallinarum* vaccine.

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## MATERIALS AND METHODS

The study was conducted in the department after prior permission and approval from Institutional Animal Ethics Committee, Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS), Hisar.

**Aflatoxins:** The aflatoxin culture material containing known levels of aflatoxins was kindly provided by Prof. George E. Rottinghaus, Veterinary Medical Diagnostic Laboratory (VMDL), University of Missouri, St. Louis, USA. The culture material on the basis of thin layer chromatography (TLC) analysis contained 2 ppm aflatoxin B1.

**Challenge Strain:** *S. gallinarum* isolated and maintained in the Department was passaged four times subcutaneously in chicks to increase its virulence and was used as a challenge strain.

**Adjuvanted Whole Cell Formalin Killed Vaccinal Preparation:** The stock culture of *S. gallinarum* was grown on brilliant green agar (BGA) in Roux flasks at 37°C for 24 h and growth was harvested by centrifugation at 2000x g for 30 min in sterile normal saline solution (NSS). The sediment was washed four times in sterile NSS by centrifugation. The sediment was plated on BGA in duplicate to determine the viable counts. The organisms so counted were suspended to an approximate concentration of  $2 \times 10^{10}$  cfu per 0.2 ml in a fixed volume of sterile NSS. Purity testing was done on BGA and the culture was inactivated using formalin (0.5%, v/v) at room temperature (20-25°C) for 24 h. The suspension was thoroughly mixed with equal volume of sterile 0.2 ml aluminum hydroxide gel adjuvant. The final vaccinal preparation contained a concentration equivalent to  $2 \times 10^{10}$  cfu per 0.4 ml. It was stored at 4°C until use.

**Sonicated Bacterial Cell Protein Antigen:** The antigen was prepared using the method of Agnihotri and Kumar (2008) with some modifications. Stock culture of SG was grown on BGA in Roux flasks for 24 h at 37°C. The growth obtained was harvested in 50 ml sterile NSS per flask by centrifugation at 2000x g for 30 min. The pellet was resuspended and washed three times in sterile NSS at 2000x g for 30 min. The cell suspension was sonicated for 3 cycles of 1 minute duration and 1 minute interval on ice in a Branson 250 sonifier. The sonicated cell suspension was centrifuged at 5000 x g for 1 hour at 4°C and supernatant was separated. The supernatant was termed as sonicated bacterial cell protein antigen (sbcp). Hyperimmune serum was raised against SG and production of precipitation arc was observed between sbcp antigen

and anti- *S. Gallinarum* HIS well, indicating antigen-antibody reaction. It was stored then in aliquots at -20°C until use.

**Experimental Design:** Day-old, 220, broilers chicks procured from a local hatchery were randomly divided into six groups with 36 chicks in each. The chicks were kept in cages in animal house with good management conditions. At 5th day of age i.e. day zero of the experiment and before making six groups, four chicks were randomly selected and various tests including indirect ELISA and lymphocyte stimulation test (LST) were performed and the values were used as 0 day value for all the groups. The six groups were: *Salmonella enterica* serovar Gallinarum formalin killed vaccine (SGFKV) with adjuvant immunized group (Group A), SGFKV+*P. kurroa* fed group (Group B), SGFKV+aflatoxin fed group (Group C), SGFKV+aflatoxin+*P. kurroa* fed group (Group D), *P. kurroa* fed group (Group E) and adjuvant control group (Group F). The birds in groups A, B, C and D were immunized with SGFKV @  $2 \times 10^{10}$  cfu with aluminium hydroxide adjuvant, subcutaneously at 5th day of age or day 0 of the experiment. *P. kurroa* dried powder of rhizomes and roots was fed to chicks of groups B, D and E @ 5 gram per kg of feed, respectively from day 0 of experiment to 6 weeks of age. Chicks in groups C and D were fed culture material containing aflatoxin B1 @ 2.0 ppm in feed from day one till the end of the experiment. Clean drinking water and feed was provided *ad lib* to the birds in all groups throughout the period of the experiment. Four chicks from each group were sacrificed at weekly intervals for evaluation of humoral and cellular immune responses post immunization. Cell mediated immune (CMI) response was studied by lymphocyte proliferation test using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma, USA) dye (Agnihotri and Kumar, 2008) which was assessed through SG antigen induced and mitogen induced lymphoproliferative response. Serum samples from the infected groups were analyzed for antibody titer against SG infection using indirect ELISA (El-Enbaawy *et al.*, 2012). Twelve chicks from each group were challenged with *Salmonella enterica* serovar gallinarum @  $2 \times 10^{10}$  cfu/0.5 ml at 4th week post-immunization. The challenged birds in each group were monitored for the efficacy of the vaccine in groups A, B, C and D and the immunostimulatory and protective effect of *P. kurroa* feeding in groups B, D and E. The experimental design is illustrated in Table 1.

**Table 1**  
**Experimental Design**

Group	Treatment	No. of Chicks	No. of chickens sacrificed (Weeks post immunization)							
			0	1	2	3	4	5	6	
A.	SGFKV immunized chicks	36	4	4	4	4	4	4	4	12*
B.	SGFKV + <i>P. kurroa</i> fed chicks	36	4	4	4	4	4	4	4	12*
C.	SGFKV + Aflatoxin fed chicks	36	4	4	4	4	4	4	4	12*
D.	SGFKV + Aflatoxin + <i>P. kurroa</i> chicks	36	4	4	4	4	4	4	4	12*
E.	<i>P. kurroa</i> fed chicks	36	4	4	4	4	4	4	4	12*
F.	Control	36	4	4	4	4	4	4	4	12*

\*No. of chicks challenged at 4 weeks P.I. were observed for 2 weeks post challenge.

**Lymphocyte Proliferation Assay (LPA):** The test was performed using the protocol of Agnihotri and Kumar (2008). The cells were then stimulated by adding 50 µl of sonicated bacterial cell antigen or mitogen Concanavalin A (Con A, Sigma, USA) @ 5 µg/ml. The plates were then incubated at 37°C in a CO<sub>2</sub> incubator for 72 h. After the incubation, 20 µl of working solution (5mg/ml) of MTT dye was added in each well and the plates were further incubated at 37°C in CO<sub>2</sub> incubator for 4 hours. Following incubation, the supernatant of each well was discarded carefully and 100 µl of dimethyl sulfoxide (DMSO) was added into each well to solublize the formazan crystals. Finally optical density was recorded using Multiskan Ex (Labsystems) microplate reader at 540 nm wavelength and the stimulation indices were calculated by the following formulae (Denizot and Lang, 1986):

Stimulation index (S I.) of antigen driven proliferation = Absorbance of antigen stimulated cells / Absorbance of unstimulated cells

Stimulation index (S I.) of mitogen driven proliferation = Absorbance of mitogen stimulated cells / Absorbance of unstimulated cells

**Indirect ELISA:** It was performed using the standard protocol described earlier (Barrow *et al.*, 1992). Absorbance values were read at 492 nm using an automatic micro-ELISA reader (Tecan-Sunrise Model). The results were expressed as end point titers i.e. the reciprocal of the serum dilution immediately above the base line for positivity. The base line (cut off) selected

was five times the standard deviation higher than the mean optical density obtained by using serum from negative control chickens. The end point titers were converted to log<sub>10</sub>x, where x was the serum dilution.

**Statistical Analysis:** Data generated during the experiment were analyzed statistically by using two way analysis of variance techniques (completely randomized design) and student's t-test (Snedecor and Cochran, 1968). Data were analyzed using OPSTAT and SPSS statistical software version 17.

## RESULTS AND DISCUSSION

**LPA:** The LTR's to sbcp antigen in various groups of chickens following immunization and challenge with *S. Gallinarum* are presented in Figs. 1 and 2. The results of LTR's to sbcp antigen revealed a progressive increase in the SI in various groups of chickens at various intervals post immunization (PI) and post challenge (PC).

The birds of groups C and D exhibited higher LTR's to sbcp antigen as compared to groups E and F. The birds of groups A and B exhibited higher LTR's to sbcp antigen as compared to the aflatoxin fed groups C and D at all intervals and the difference was statistical significant from 3 weeks PI. The SI values in group D in comparison to group A and B were lower at all day PI, without statistical difference. The peak SI was recorded at 4 weeks PI in all the groups.

The SI was significantly higher in group B as compared to group C two weeks post challenge and significantly higher to group A at one week PC. The birds of control group (group F) failed to express significant SI during 6 weeks experimental period. The SI in groups E and F was significantly lower than groups A, B, C and D at all the intervals.

Observations of LTR's to Con A in various groups of birds following immunization are presented in Fig. 3. A progressive rise in the SI against Con A was observed in birds of groups A, B, C and D following immunization but it was higher than sbcp. The LTR's in groups C and D were low as compared to groups A and B birds at different intervals post immunization and the difference was statistically significant at 5th and 6th week PI. The LTR's observed in group E birds were also lower as compared to groups A and B birds at various intervals PI

Following challenge, the maximum LTR's in groups A and B birds were observed at 2 weeks PC A lower LTR's was seen in groups C and D chicks at 2 weeks

PC, respectively as compared to groups A and B birds. The SI in groups B and D were higher than their respective controls i.e. groups A and C at two weeks PC with significant difference in groups C and D.

**ELISA:** The ELISA titers observed following immunization and challenge are presented in Figs. 5 and 6. The cut-off OD value was calculated as the mean obtained from titration of a reference negative serum plus three times the standard deviation and was 0.250. The results are expressed as mean ELISA titers ( $\log_{10}$ ) against sbcp antigen.

A gradual rise in the mean antibody titers was observed in groups A and B chicks following immunization upto 3 weeks PI. The birds in aflatoxin fed groups C and D exhibited steady but lower rise in the antibody titre following immunization as compared to groups A and B birds. The ELISA titres in *P. kurroa* fed groups B and D were higher at all intervals PI as compared to their respective non-*P. kurroa* fed groups (A and C) but without statistical difference. Challenged birds of groups A and B exhibited a secondary antibody response with mean ELISA titer reaching a maximum at 2 weeks PC. The ELISA titres of groups A and B were significantly higher than groups C and D at all intervals.

The result of experimental study revealed that birds of group B exhibited comparatively higher LTR's to sbcp antigens than SGFKV immunized group (A), SGFKV immunized and aflatoxin fed group (C), SGFKV immunized and aflatoxin and *P. kurroa* fed group (D) and *P. kurroa* group (E) chickens at various intervals PI and PC. Agnihotri and Kumar (2008) observed the highest LTR's against sbcp antigen and against Con A at 4 week in chickens immunized with adjuvanted formalin killed vaccinal preparation of SG. The LTR's post challenge as observed by these authors also showed patterns similar to the present study. In a study, Roy (2010) observed the highest LTR's against sbcp antigen and against Con A at 4 weeks in chickens immunized with adjuvanted formalin killed vaccinal preparation of *S. Enteritidis*. These findings are suggestive of the cell mediated immune responses induced by SG vaccinal preparation.

Comparatively higher LTR's observed in birds of group B than group A was suggestive of augmentation of cellular immune response induced in group B probably due to *P. kurroa* feeding following vaccination. The immune enhancing effects of four probiotic fermented herbs (PFH) combination were studied against SG

infection in experimentally infected broiler chicks (Jung *et al.*, 2010). Continuous ingestion of PFH increased peripheral blood mononuclear cell proliferation and antibody production level. The immunomodulatory potential of the rhizomes extract of *P. kurroa* was also studied (Hussain *et al.*, 2013) and showed stimulatory effects on cell mediated immunity.

Evaluation of the immune responses of chickens against SG infection (Alvarez *et al.*, 2003) and showed that immunized birds showed the highest antibody titres ( $P < 0.05$ ) as well as the highest peripheral blood lymphocytes stimulation indices with Con A and SG antigens.

The present study also revealed immunosuppressive action of aflatoxin B1 in birds. Our observations are in accordance with the findings of Otim *et al.* (2005) who also observed maximum immunosuppressive effects of aflatoxins on CMI and various growth parameters after 7 days post immunization in chickens. Hassan *et al.* (2012) observed that the frequencies of IgA, IgG and IgM bearing cells were significantly lower in bursa of progeny chicks obtained from hens fed the ochratoxin A (5 mg/kg)+aflatoxin B1 (5 mg/kg) mixed diet.

The titers in birds of group B were higher than group A at all intervals PI which suggested the augmenting property of *P. kurroa* on HI response. Dwivedi *et al.* (2008) also evaluated immunomodulatory activity of *P. kurroa* in BALB/c mice. T cell proliferation as well as antibody production in their study revealed that picroliv helped in evoking strong immuno-potentiating response in the immunized mice. Hussain *et al.* (2013) also studied the immunomodulatory potential of the rhizomes extract of *P. kurroa* and reported that plant extract ameliorated both CMI and humoral antibody responses.

The strong and rapid serological response PC obtained in birds of group B could be due to invasive nature of *S. gallinarum* in poultry. The findings of Rana and Kulshreshtha (2006) are also in close agreement with the observation of present study. They recorded high SI for cellular and humoral immune responses PI and PC with virulent plasmid cured mutant strain of *S. enterica* serotype gallinarum 9. Their findings also indicated that the live attenuated mutant vaccine induced a strong cellular and humoral immunity which played a key role in the protection of fowl typhoid in broiler chickens. Aflatoxin plays an important role in the inhibition of protein synthesis and it could be the reason for lowered ELISA titres in groups C and D chickens in the present study. Ebrahimi

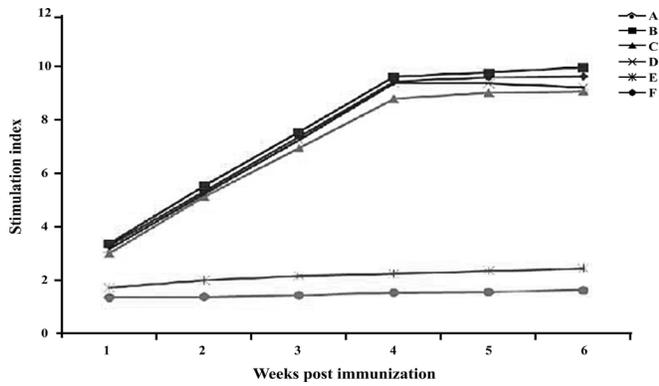


Fig. 1: Effect of *Picrorhiza kurroa* feeding on lymphocyte transformation responses against sonicated bacterial cell protein antigen in chicks immunized with *Salmonella gallinarum* formalin killed vaccinal preparation.

A=*Salmonella enterica* serovar *gallinarum* formalin killed vaccine (SGFKV), B=SGFKV+*P. kurroa* fed group, C=SGFKV+Aflatoxin fed group, D=SGFKV+Aflatoxin+*P. kurroa*, E=*P. kurroa* fed group, F=Control

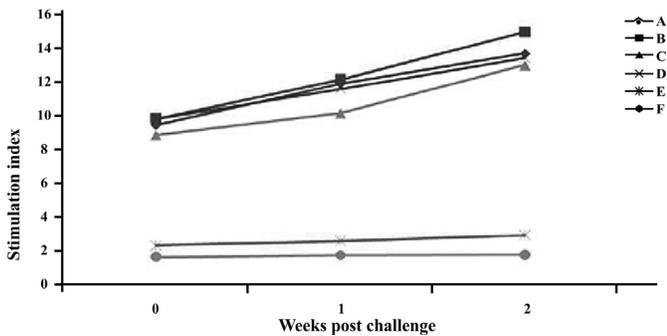


Fig. 2: Effect of *Picrorhiza kurroa* feeding on lymphocyte transformation responses against sonicated bacterial cell protein antigen in chicks immunized with formalin killed *Salmonella gallinarum* vaccine following challenge with *Salmonella gallinarum*.

A=*Salmonella enterica* serovar *gallinarum* formalin killed vaccine (SGFKV), B=SGFKV+*P. kurroa* fed group, C=SGFKV+Aflatoxin fed group, D=SGFKV+Aflatoxin+*P. kurroa*, E=*P. kurroa* fed group, F=Control

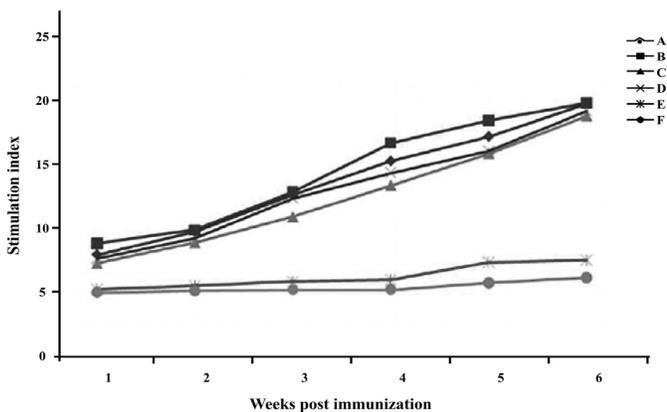


Fig. 3: Effect of *Picrorhiza kurroa* feeding on lymphocyte transformation response against Con A in chicks immunized with killed *Salmonella gallinarum* vaccinal preparation.

A=*Salmonella enterica* serovar *gallinarum* formalin killed vaccine (SGFKV), B=SGFKV+*P. kurroa* fed group, C=SGFKV+Aflatoxin fed group, D=SGFKV+Aflatoxin+*P. kurroa*, E=*P. kurroa* fed group, F=Control

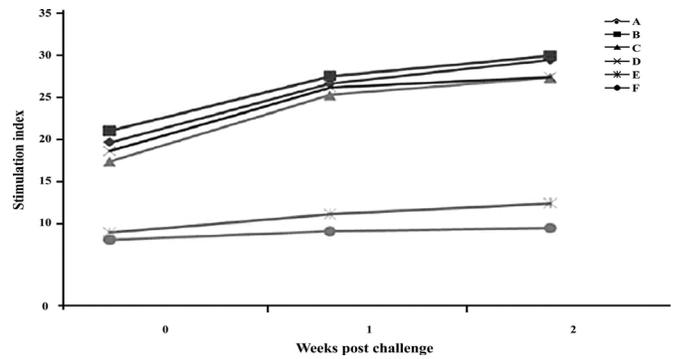


Fig. 4: Effect of *Picrorhiza kurroa* feeding on lymphocyte transformation response against Con A in chicks immunized with formalin killed *Salmonella gallinarum* vaccine following challenge with *Salmonella gallinarum*.

A=*Salmonella enterica* serovar *gallinarum* formalin killed vaccine (SGFKV), B=SGFKV+*P. kurroa* fed group, C=SGFKV+Aflatoxin fed group, D=SGFKV+Aflatoxin+*P. kurroa*, E=*P. kurroa* fed group, F=Control

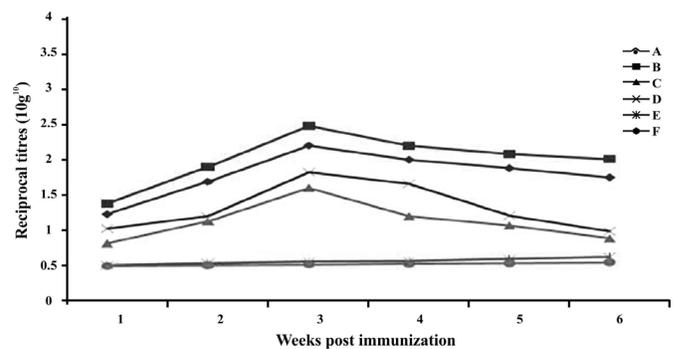


Fig. 5: Effect of *Picrorhiza kurroa* feeding on ELISA antibody titers against sonicated bacterial cell protein antigen in chicks immunized with killed *Salmonella gallinarum* vaccinal preparation.

A=*Salmonella enterica* serovar *gallinarum* formalin killed vaccine (SGFKV), B=SGFKV+*P. kurroa* fed group, C=SGFKV+Aflatoxin fed group, D=SGFKV+Aflatoxin+*P. kurroa*, E=*P. kurroa* fed group, F=Control

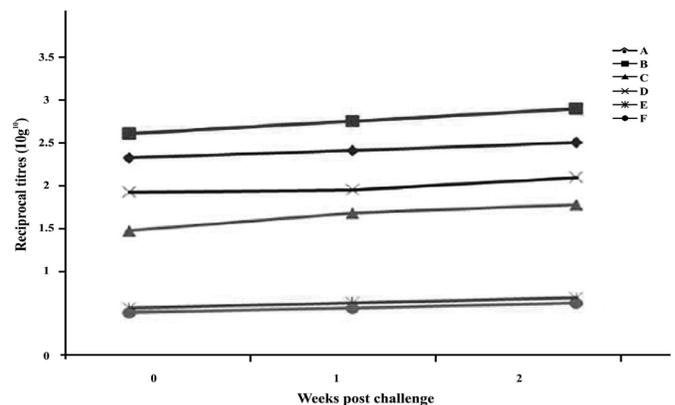


Fig. 6: Effect of *Picrorhiza kurroa* feeding on ELISA antibody titers against sonicated bacterial cell protein antigen in chicks immunized with formalin killed *Salmonella gallinarum* vaccine following challenge with *Salmonella gallinarum*.

A=*Salmonella enterica* serovar *gallinarum* formalin killed vaccine (SGFKV), B=SGFKV+*P. kurroa* fed group, C=SGFKV+Aflatoxin fed group, D=SGFKV+Aflatoxin+*P. kurroa*, E=*P. kurroa* fed group, F=Control

and Shahsavandi (2008) recorded significant reduction in antibody titers of chickens fed 200 ppb aflatoxin from 10 days of age to 42 days. Sirajudeen *et al.* (2011) also reported decrease in humoral and cell mediated immune responses in growing chicks fed aflatoxin contaminated diet (1 mg/kg feed) for 40 days.

The present study thus revealed that *P. kurroa* feeding significantly improved the SI in immunized birds (group B) followed by groups A and group D. The findings also suggested that the CMI response was hampered by aflatoxin B1 feeding and was augmented by *P. kurroa* feeding. The observations suggested the possible role of humoral immunity in protection against fowl typhoid.

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