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EVALUATION OF HUMIC ACID AS AN AFLATOXIN BINDER IN BROILER CHICKENS

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Abstract

The efficacy of humic acid (HA) as an aflatoxin (AF) binder in broiler chickens exposed to aflatoxin-contaminated feed from 1 to 42 days of age was assessed. A total of 200 birds were assigned to 20 pens, with 10 birds per pen. The following treatments (T) were applied: T1: basal diet (B); T2: B + AFB_1 (100 µg/kg); T3: B + AFB_1 (100 µg/kg) + HA (0.1%); T_4: B +AFB1 (100 µg/kg) + HA (0.2%); T_5: B + AFB1 (100 µg/kg) + HA (0.3%). Each treatment consisted of 4 replicates. Oxihumate was effective in diminishing the adverse effects caused by aflatoxin on body weight (BW) of broilers (P<0.05). Humic acid also showed protective effects against liver damage and some of the hematological and serum biochemical changes associated with aflatoxin toxicity (P<0.05). The supplementation of HA also enhanced the humoral immunity by counteracting the aflatoxin contamination. Results indicated that HA could alleviate some of the toxic effects of aflatoxin in growing broilers. Humic acid (0.1 to 0.3%) might, therefore, prove to be beneficial in the management of aflatoxin-contaminated feedstuffs for poultry when used in combination with other mycotoxin management practices.

Key words: humic acid, broiler, aflatoxin B_1, serum biochemistry, immunity

Aflatoxins (AF), potent mycotoxins produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus*, are a major concern in poultry production. The toxicity of AF in broilers has been widely investigated for their carcinogenic, mutagenic, teratogenic, and growth inhibitory effects (Oğuz et al., 2000; Sur and Celik, 2003). Aflatoxins contamination causes reduced feed quality and reduced animal efficiency either through poor conversion of nutrients or problems such as reproductive abnormalities (Ortatatli et al., 2002). Aflatoxicosis in poultry also causes listlessness, anorexia with lowered growth rate, poor feed utilization, decreased egg production and increased mortality (Hussain et al., 2010). Aflatoxin contamination in feed may

cause impairment of the humoral and cellular immune responses; and increased susceptibility to some environmental and infectious agents (Dhanasekaran et al., 2009). Significant changes in serum bio-hematological parameters are seen in aflatoxicosis cases in broilers and these can assist in the diagnosis of toxication (Basmacioğlu et al., 2005). Moreover, aflatoxin B_1 (AFB_1), which is known to be the most potent mycotoxin, can be found as the major residue in broiler liver and laying hen eggs after AF exposure (Denli et al., 2009; Hussain et al., 2010; Sur et al., 2011). This toxin has been classified as a carcinogenic agent to humans by the International Agency for Research on Cancer (Anonymous, 2002; Puschner, 2002). For this reason, many countries have introduced legislation restricting the levels of AF allowed in animal products such as milk (European Commission, 2010).

The presence of potential AF producer strains and AFB_1 in commercial poultry feeds has been extensively reported in previous studies (Khan et al., 2011; Anjum et al., 2012; Fareed et al., 2014). Consequently, large-scale, practical, and cost-effective methods for detoxifying aflatoxin-contaminated feedstuffs currently are in great demand. A variety of physical, chemical, and biological techniques for mycotoxin decontamination of agricultural commodities has been used, but they have had limited success (Van Rensburg et al., 2006). One of the most practical approaches is the use of non-nutritive adsorbents, which bind the mycotoxins and inhibit their absorption from the gastrointestinal tract, thus minimizing the toxic effects in poultry and the carryover of these fungal metabolites into poultry products (Ye et al., 2009). But not all adsorbents are equally effective and several adsorbents have been shown to impair nutrients utilization (Scheideler, 1993). According to Ye et al. (2009), as an ideal adsorbent, it should have a high affinity to aflatoxin, resulting in the formation of a strong complex with little risk of dissociation. It also should have a high binding capacity to prevent saturation.

Humic acids are ubiquitous and are found wherever matter is being decomposed or has been transposed, as in the case of sediments. Humic substances have demonstrated a strong affinity to bind various substances, such as heavy metals (Madronová et al., 2001), herbicides (Nègre et al., 2001), different mutagens (Cozzi et al., 1993), monoaromatic (Nanny and Maza, 2001) and polycyclic aromatic compounds (Kollist-Siigur et al., 2001), minerals (Elfarissi and Pefferkorn, 2000), and *Bacillus subtilis* bacteria (Moura et al., 2007). In spite of its known binding characteristics, HA have been less evaluated previously as a mycotoxin adsorbent. In recent years, it has been observed that dietary intake of humates promotes growth in poultry (Kocabağli et al., 2002; Rath et al., 2006; Mirnawati and Marlida, 2013). Although there is not enough evidence to hypothesize how humates promote the growth in poultry, it is assumed that humates might increase the uptake of nitrogen, phosphorus, and other nutrients due to their chelating properties. The objective of this study was to evaluate the effects of humic acid on growth performance, AFB_1 residues in the liver, immunity and serum bio-hematological variables in broiler chickens exposed to aflatoxins.

Material and methods

Production of aflatoxin B_1

Aflatoxins were produced via fermentation of rice by *A. parasiticus* NRRL 2999 (USDA, Agricultural Research Service, Peoria, IL). The sterile substrate, placed in Erlenmeyer flasks, was inoculated with 2 mL of an aqueous suspension of the mold containing 106 spores/mL. Cultures were allowed to grow for 7 days at 25°C in darkness. On the seventh day, the Erlenmeyer flasks were autoclaved, and the culture material was dried for 48 h at 40°C in a forced-air oven and then ground to a fine powder. The AFB_1 levels in the rice powder were measured by thin-layer chromatography and HPLC as described previously (AOAC, 2011). The milled substrate was added to the basal diet to provide a concentration of 100 μg of AFB_1/kg of feed.

Experimental design and chicks

A total of 200 Ross broiler chicks (4 replicates/treatment with 10 chicks/replicate) were individually weighed and randomly selected. Birds were fed the dietary treatments for 42 days. During the experimental period, broilers were kept under electrical heating with 23L: 1D, and they received the diet corresponding to each treatment. A grower diet was given from days 1 to 28, and a finisher diet was provided from day 29 until the end of the experiment. Care and management of the birds followed accepted guidelines (FASS, 2010).

Table 1. Composition (g/kg) and calculated nutrient content of basal diet

Ingredients	0–4 wk	5–6 wk
1	2	3
Corn	500.00	600.00
Rice, broken	50.00	-
Corn gluten meal (60%)	20.00	20.00
Canola meal	80.00	64.00
Soybean meal (47.5%)	300.00	240.00
Vegetable oil	-	30.00
Molasses	30.00	30.00
Marble chips	5.00	5.00
Dicalcium phosphate	10.00	5.00
Vitamin premix[1]	2.00	2.00
Trace mineral mix[2]	1.00	1.00
Choline Cl (60%)3	1.00	1.00
L-Lys HCl (98%)	1.00	2.00
Total	1,000.00	1,000.00
Calculated analysis		
ME (kcal/kg)	2895.65	3155.90
CP (%)	22.80	20.13
CF (%)	3.75	3.39
Ash (%)	7.17	6.39
Available phosphorus (%)	0.40	0.40

Table 1 – contd.

1	2	3
Lysine (%)	1.27	1.08
Methionine (%)	0.50	0.42
Met + Cys (%)	0.84	0.72
Sodium (%)	0.21	0.21
Chloride (%)	0.28	0.29
Lino (%)	1.16	3.04

[1]Provides per kilogram of diet: vitamin A – 7.714 IU; vitamin E – 16.53 IU; vitamin B_{12} – 0.013 mg; riboflavin – 6.6 mg; niacin – 39 mg; pantothenic acid – 10 mg; menadione – 1.5 mg; folic acid – 0.9 mg; thiamin – 1.54 mg; pyridoxine – 2.76 mg; D-biotin – 0.066 mg; ethoxyquin – 125 mg; Se – 0.1 mg.

[2]Provides per kilogram of diet: Mn (from $MnSO_4$-H_2O) – 100 mg; Zn (from $ZnSO_4$-$7H_2O$) – 100 mg; Fe (from $FeSO_4$-$7H_2O$) – 50 mg; Cu (from $CuSO_4$-$5H_2O$) – 10 mg; I from Ca $(IO_3)_2$-H_2O) – 1 mg.

[3]Provides 1.040 mg choline per kilogram of diet.

All diets were formulated to meet or exceed NRC (1994) recommendations for essential amino acids in starter (0 to 4 weeks) and finisher (5 to 6 weeks) feeding periods (Table 1). The basal diet contained 2 µg of AFB_1/kg of diet, as determined by the techniques described above. Humic acid was purchased from Enrich feed (imported and marketed by Almuttahar, Shan Arcade Barkat Market, New Garden town, Lahore, Pakistan). It contained 70% HA, 14.50% moisture, 3.6% crude protein, 0.05% crude fat, 17.35% total ash and 9.0% sodium. The experimental diets for each treatment (T) were as follows: T1: basal diet (B); T2: B + AFB_1 (100 µg/kg); T3: B + AFB_1 (100 µg/kg) + HA (0.1%); T4: B + AFB_1 (100 µg/kg) + HA (0.2%); T5: B + AFB_1 (100 µg/kg) + HA (0.3%). Broilers were monitored daily for signs of morbidity and mortality. At the end of the assay, the efficacy of HA in preventing aflatoxicosis was evaluated by measuring productivity parameters (i.e., body weight (BW), feed consumption, feed conversion ratio, mortality rate and dressing percentage).

Analysis of aflatoxin B_1 residue in livers

For this assay, 5 livers from each treatment (at least 1 for each replicate) were selected. Aflatoxin B_1 in the liver tissue was extracted and partially purified according to the method described previously by Tavčar-Kalcher et al. (2007). Briefly, the ground liver sample (50 g) was mixed thoroughly with 5 mL of a 20% aqueous citric acid solution and diatomaceous earth (10 g). The mixture was extracted with 100 mL of dichloromethane by shaking for 30 min. The filtered extract was dried by addition of Na_2SO_4 and filtered again, and an aliquot (20 mL) was evaporated to dryness. The concentrate was mixed with acetonitrile: H_2O (75:25, vol/vol) and hexane and centrifuged, and 10 mL of the aqueous phase was taken and evaporated to dryness. The concentrate was mixed with methanol:water (80:20, vol/vol). The solution was applied to an Oasis cartridge (60 mg, Waters Corporation, Milford, MA) that was previously conditioned with methanol and water. Aflatoxin B_1 in the extract was detected and quantified by liquid chromatography-mass spectrometry (HPLC-MS/MS; Sørensen and Elbaek, 2005). Dried extracts were dissolved in mobile phase [acetonitrile: H_2O (20:80, vol/vol)] and injected into the HPLC-MS/MS instrument.

The calibration range was from 0.05 to 0.5 ng of injected AFB_1. The quantification and detection limits of the analytical technique used to determine AF residues in livers were 0.025 and 0.0025 ng/g, respectively; the percentage of AFB_1 recovered was 99±13%.

Serum biology

On day 42, three chicks from each replicate were bled by puncture of the brachial vein. Blood sample was collected from these birds in tubes without anticoagulant. Serum was obtained from these samples and analyzed for total serum protein, serum albumin, calcium, phosphorus, aspartate aminotransferases and γ-glutamyltransferase. Serum protein was determined by biuret method (Coles, 1974) and serum albumin was determined by using Bromocresol green method at pH 4.2 (Webster, 1974). Inorganic phosphorus was determined by the method described as Fiske and Subba Row (1925), and calcium was analyzed by atomic absorption spectrophotometry using a Perkin-Elmer Model 5100 PC.5. Serum enzyme activities of aspartate aminotransferases and γ-glutamyltransferase were measured using UV visible spectrophotometer (Shimadzu Corp., Tokyo, Japan) and estimated by using the NADH oxidation reaction method described by Neeley et al. (1985). Another blood sample was collected in EDTA blood tubes for determination of red blood cell, white blood cell, heterophil, lymphocytes, monocyte and hematocrit according to the procedure of MAFF (1984).

Antibody response against Newcastle virus

Antibody response against Newcastle disease virus was determined by Hemagglutination Inhibition (HI) test (Thayer and Beard, 1998) at University Diagnostic Laboratory, University of Veterinary and Animal Sciences, Lahore, Pakistan. Blood samples from each of ten birds of each group were collected on days 7, 14, 21, 28 and 42 of post-vaccination. Serum was separated and processed for HI test.

Statistical analysis

In this experiment, all statistical analyses were conducted in accordance with a completely randomized design using the SPSS version 16 (SPSS, Cary, NC, USA) statistical analysis program. P value of <0.05 was considered for significant differences among groups and the comparison of means was made by using Duncan's Multiple Range Test (Steel and Torrie, 1984).

Results

Broiler performance

The effect of different levels of HA on BW, feed conversion, mortality and dressing percentage of broilers during the experimental period is shown in Table 2. For the toxicity study, significant ($P<0.05$) differences were noted between birds receiving HA and those who did not receive HA, for all parameters measured in both phases

of growth. A concentration of 100 µg of AFB_1/kg of feed depressed all the performance parameters of the broilers in both phases of growth. Supplementation of HA improved ($P<0.05$) all the performance parameters after neutralization of aflatoxin toxicity. The mortality rate did not differ significantly ($P>0.05$) among different levels of HA treatments. The basal diet group had the lowest mortality rate, with a mean of 0.25 bird per pen (2.50±0.05%). The highest mortality rate observed was for the group fed AFB_1, with a mean of 1.75 birds per pen (17.50±0.29%). Inclusion of HA in basal diet reduced ($P<0.05$) mortality rate in broilers as compared to diet containing AFB_1.

Table 2. The effect of humic acid on body weight, feed conversion ratio, mortality and dressing percentage

Items	Age (d)	Treatments*				
		T1	T2	T3	T4	T5
Body weight (BW; g)	1–28	967.60±41.50 a	860.00±23.10 b	967.00±39.00 a	963.00 ±40.00 a	955.06±41.00 a
	29–42	920.09±24.30 a	795.09±15.90 b	916.60±29.50 a	915.08±35.00 a	898.10±32.00 a
	1–42	1884.00±65.00 a	1655.06±49.80 b	1883.00±62.10 a	1882.00±62.10 a	1845±52.30 a
Feed:gain ratio	1–28	1.67 b	1.86 a	1.72 b	1.69 b	1.69 b
	29–42	2.29 b	2.43 a	2.26 b	2.21 b	2.28 b
	1–42	1.98 b	2.15 a	1.99 b	1.95b	1.99 b
Mortality (%)	1–42	2.50±0.05 c	17.50±0.29 a	9.13±0.09 b	8.88±0.06 b	8.75±0.08 b
Dressing percentage	42	72.85±0.52 a	56.85±0.16 b	72.30±0.42 a	71.96±0.24 a	68.07±0.10 a

a, b, c – values with different superscripts in rows were significantly different ($P>0.05$).

*T1: Basal diet (B); T2: B + AFB_1 (100 µg/kg); T3: B + AFB_1 (100 µg/kg) + HA (0.1%); T4: B +AFB_1 (100 µg/kg) + HA (0.2%); T5: B + AFB_1 (100 µg/kg) + HA (0.3%).

Table 3. The effect of humic acid on liver weight and aflatoxin B_1 (AFB_1) residues in the liver of broiler chicks

Items	Treatments*				
	T1	T2	T3	T4	T5
Relative liver weight (% BW)	2.43 ± 0.20 b	4.24 ± 0.20 a	2.40± 0.20 b	2.30 ± 0.20 b	2.26 ± 0.20 b
AFB1[1] (ng/g)	ND	1.00 ± 0.48 a	0.42 ± 0.15 b	0.22 ± 0.11 bc	0.17 ± 0.10 c

a, b, c – values with different superscripts in rows were significantly different ($P>0.05$).

*T1: Basal diet (B); T2: B + AFB_1 (100 µg/kg); T3: B + AFB_1 (100 µg/kg) + HA (0.1%); T4: B +AFB_1 (100 µg/kg) + HA (0.2%); T5: B + AFB_1 (100 µg/kg) + HA (0.3%).

[1]Mean levels of AFB_1 in the liver (ng/g) were obtained from 5 liver samples. ND = non-detectable levels (detection limit: 0.0025 ng/g).

Liver weight and aflatoxin B_1 residues in the liver of broiler chicks

The effect of different levels of HA on liver weight and AFB_1 residues in the liver of broilers is shown in Table 3. The liver weight and AFB_1 residues in the liver increased in group of basal diet containing AFB_1 as compared to other treatments. The present data indicate that the relative weights for the livers were significantly increased by almost 47% for the chicks consuming diets contaminated with AFB_1. The supplementation of humic substances (HS) reduced ($P>0.05$) relative liver weight and AFB_1 residues in the liver of broilers.

Serum biochemical parameters

Serum chemistry after 6 weeks of treatment is shown in Table 4. The reduction ($P>0.05$) in serum concentration of total protein, albumin, calcium and phosphorus was observed in all groups fed aflatoxin. The serum aspartate aminotransferase activity was significantly ($P<0.05$) increased in birds fed aflatoxin-contaminated diets. However, high level of HA reduced ($P<0.05$) the concentration of serum aspartate aminotransferase activity. Furthermore, aflatoxin-contaminated diets in this study significantly ($P<0.05$) increased serum γ-glutamyltransferase enzyme activity. The HA supplementation could not reduce concentration of serum γ-glutamyltransferase enzyme.

Table 4. Effect of humic acid (HA) on serum biochemical values of 6-week-old chickens (n=5 per treatment)

Items	Treatments*				
	T1	T2	T3	T4	T5
Protein (g/L)	30.20±1.01 a	26.03±1.00 b	25.12±1.02 b	26.29±1.01 b	27.03±1.01 b
Albumin (g/L)	14.18±0.45 a	10.59±0.40 b	10.38±0.43 b	10.75±0.39 b	10.85±0.4 b
Ca (mg/dL)	11.10±0.30 a	10.10±0.20 b	9.93±0.19 b	9.90±0.21 b	9.87±0.20 b
P (mg/dL)	6.50±0.33 a	5.50±0.20 b	4.98±0.25 b	5.00±0.19 b	5.10±0.21 b
Aspartate aminotransferase (IU/L)	98.50±3.0 b	107.60±2.90 a	105.07±2.99 a	104.80±2.99 a	99.8±2.95 b
γ-Glutamyl transferase (IU/L)	6.00±0.80 b	9.72±0.82 a	8.78±0.81 a	8.70±0.84 a	8.60±0.83 a

a, b, c – values with different superscripts in rows were significantly different ($P>0.05$).

*T1: Basal diet (B); T2: B + AFB_1 (100 µg/kg); T3: B + AFB_1 (100 µg/kg) + HA (0.1%); T4: B +AFB_1 (100 µg/kg) + HA (0.2%); T5: B + AFB_1 (100 µg/kg) + HA (0.3%).

Hematological parameters

Blood differential counts after 6 weeks of treatment are shown in Table 5. In this trial, supplementation of HA had significant ($P<0.05$) effect on white blood cell, red blood cell, monocyte, and lymphocyte counts, or hematocrit values. Supplementation of HA improved ($P<0.05$) blood parameters as compared to basal diet containing AFB_1.

Table 5. Effect of humic acid (HA) on blood differential counts of 6-week-old chickens (n=5 per treatment)

Items	Treatments*				
	T1	T2	T3	T4	T5
White blood cell (10^3/μL)	36.50±4.50 a	23.10±2.76 b	38.74±3.98 a	40.34±2.09 a	46.36±5.60 a
Red blood cell (10^3/μL)	2.55±0.09 a	1.90±0.02 b	2.49±0.03 a	2.50±0.07 a	2.53±0.08 a
Heterophil (%)	25.15±5.00 a	7.99±1.89 b	17.55±2.61 a	18.00±2.50 a	18.90±2.63 a
Lymphocyte (%)	67.89±4.50 a	57.50±2.38 b	70.53±2.99 a	74.38±3.81 a	74.76±3.70 a
Monocyte (%)	8.17±1.78 a	4.95±0.54 b	6.98±0.65 a	7.13±0.66 a	7.65±0.79 a
Hematocrit (%)	34.05±0.58 a	31.08±0.50 b	33.10±0.65 a	33.80±0.65 a	33.85±0.50 a

a, b, c – values with different superscripts in rows were significantly different (P>0.05).

*T1: Basal diet (B); T2: B + AFB_1 (100 μg/kg); T3: B + AFB_1 (100 μg/kg) + HA (0.1%); T4: B +AFB_1 (100 μg/kg) + HA (0.2%); T5: B + AFB_1 (100 μg/kg) + HA (0.3%).

Immunity

The maternal antibody titer was 6.50±0.23. The effects of dietary treatments on antibody production against ND in broilers from day 7 to 42 are presented in Table 6. On the 7th day of the study, there was no difference among antibody titer of experimental groups. The feeding of AFB_1 reduced the antibody production against ND in broilers from 28 to 42 days of age (P<0.05). The supplementation of HA improved the antibody production against ND in broilers from 28 to 42 days of age (P<0.05).

Table 6. The effect of humic acid on the production of antibody titers against Newcastle virus in broiler chicks

Antibody titers	Treatments*				
	T1	T2	T3	T4	T5
7th d	5.766±0.22	5.634±0.25	5.909±0.32	6.050±0.24	6.194±0.32
14th d	4.563±0.26	4.401±0.41	4.630±0.37	4.646±0.35	4.654±0.25
21st d	4.621±0.39	4.529±0.35	4.809±0.45	5.049±0.36	5.055±0.46
28th d	6.811±0.43 a	4.976±0.57 b	6.879±0.39 a	6.976±0.46 a	7.401±0.44 a
42nd d	6.311±0.20 a	4.390 ± 0.34 b	5.679±0.45 a	5.699±0.39 a	6.432±0.33 a

a, b, c – values with different superscripts in rows were significantly different (P>0.05).

*T1: Basal diet (B); T2: B + AFB_1 (100 μg/kg); T3: B + AFB_1 (100 μg/kg) + HA (0.1%); T4: B +AFB_1 (100 μg/kg) + HA (0.2%); T5: B + AFB_1 (100 μg/kg) + HA (0.3%).

Discussion

Broiler performance

In this study, no significant differences were observed between broilers fed the basal diet and those fed the diet containing HA, indicating that the adsorbent was inert and nontoxic in agreement with previous findings of Van Rensburg et al. (2006). These results support various researchers, who found that aflatoxin ingestion inhibits growth in chickens (Miazzo et al., 2000; Van Rensburg et al., 2006). The decrease in BW caused by the addition of AFB_1 in feed was diminished at both phases of growth by the addition of 0.1 to 0.3% of HA to the diet. Taklimi et al. (2012) reported that broilers fed diets with HA (0.3%) had higher mean live weight gain and better feed conversion ratio. Kocabağli et al. (2002) reported an improvement in feed conversion in birds that were given 0.25% HA either from 0 to 42 days or during growing periods only, between days 21 and 42. In the recent studies, the addition of HA (0.1 to 0.19%) improved the broilers performance without affecting their carcass characteristics (Nagaraju et al., 2014; Edmonds et al., 2014*)*. A similar conclusion was drawn by Arafat et al. (2015), who showed a better feed conversion in hens supplemented with 0.2–0.6% HA, and it did not affect body weight. On the contrary, no significant performance and carcass traits were observed with broilers fed the HA at 0.10, 0.19 and 0.28% during a 49-day test period (Karaoglu et al., 2004). Moreover, Rath et al. (2006) found that HA-treated chickens showed a reduction in body weight, and the feed conversion ratio was numerically higher. However, Ozturk et al. (2010) reported that the higher level of HA supplementation (1.0 and 1.5%) appeared to have assessable impact on live performance improving feed efficiency and broiler carcass characteristics. The inconsistent results and different responses to supplementary HS from other studies might be due to the species, age, sex, plane of nutrition, nutrient composition of the diet, levels of HS in the diet, the duration of supplementation or environmental conditions. Inclusion of HA in basal diet reduced ($P<0.05$) mortality rate in broilers as compared to diet containing AFB_1. The results of the present study are in agreement with those of some researchers who found that mortality rate in quails and broilers was reduced by the supplementation of HA in basal diets (Abdel-Mageed, 2012; Edmonds et al., 2014). However, Van Rensburg et al. (2006) reported that HA did not affect mortality rate of broilers, when diet contained 1000 to 2000 µg of AFB_1/kg. These toxin values in diets were very high compared to the present study.

The mechanism by which HS affect poultry performance is largely unknown. There are limited numbers of articles which show that HS promote growth by altering partitioning of nutrient metabolism (Abdel-Mageed, 2012; Taklimi et al., 2012). It is assumed that, due to the chemical compositions, proteins, water soluble vitamins, digestive enzyme and many other immune stimulating agents and antibacterial substances in HA, they will have significant role in productivity of birds. This could be mainly attributed to their ability to change the gut microflora (by increasing the concentrations of beneficial bacteria) in the intestine (Schepetkin et al., 2003). As reported earlier by Taklimi et al. (2012), HA had significant effect on crypt depth of villi in jejunum of treated birds compared to non-supplemented groups. It is obvi-

ously known that growth of villi is generally dependent on pH, microflora and toxic substances in the intestine, although HA have the ability to reduce pH and concentration of harmful bacteria in intestine. There is evidence that HA could have positive effect on poultry performances via digestive tract ecosystems (Taklimi et al., 2012).

Liver weight and aflatoxin B_1 residues in the liver of broiler chicks

In poultry, the relative weight of the liver is increased by aflatoxin ingestion more than that of any other organ (Van Rensburg et al., 2006). The livers of these chicks also appeared to be friable and pale yellow as a result of fat accumulation in the cytoplasm of the hepatocytes. However, Magnoli et al. (2011) reported that the relative weight of livers remained unchanged when toxin levels were relatively low (50 μg/kg of dietary AFB_1), at least for a broiler production period of 46 days. In the current study, supplementation of HA showed significant protective effects with respect to liver damage, as indicated by an inhibition of liver enlargement. Van Rensburg et al. (2006) showed a concentration-dependent increase in the rate of hemolysis, indicating AFB_1-induced cytotoxicity, which could be due to lipid peroxidation of plasma membranes, permeability alterations, and cell lyses. These workers reported that additions of oxihumate (0.35%) inhibited this effect at the contamination level of 1000 μg of AFB_1/kg of feed. This contamination level is very high compared to the present study. The affected birds of basal diet containing AFB_1 retained significantly higher residues of the AF in their livers as compared to other treatments. Other authors observed that at different levels of AF in diets (50 to 100 and 3,000 μg/kg), the residual AFB_1 from livers were similar in all broilers (0.13 and 0.15 ng/g, respectively; Bintvihok et al., 2002; Bintvihok and Kositcharoenkul, 2006). In the present study the mean levels observed in T2 were higher than those reported previously. The present results showed that an important percentage of the toxin diet remained in the liver. In an experiment carried out to assess the transfer of ochratoxin A in the pork product chain, Bertuzzi et al. (2013) reported that feeding pigs with diets containing slightly less than 50 μg/kg of ochratoxin A led to the consequent presence of the toxin in muscle at concentrations close to 1 μg/kg, which represents the guideline value for meat products recommended by the Italian Ministry of Health. Humic acid has binding capacity for many molecules. In the current study, all levels of HA as binding agent decreased ($P<0.05$) adverse effect of AFB_1 on liver. These findings are in line with a study that evaluated oxihumate as AFB_1 binder, *in vitro* and *in vivo* (Van Rensburg et al., 2006). Oxihumate showed a high *in vitro* affinity for AFB_1. *In vivo* trial showed that oxihumate decreased adverse effects caused by AFB_1 on broiler body weight and also protective effect against liver damage.

Serum biochemical parameters

The observed decline in serum concentration of total protein and albumin in all groups fed aflatoxin indicates impaired protein synthesis in the liver caused by the blockage of RNA synthesis (Tung et al., 1975), resulting from the hepatotoxicity seen in aflatoxicosis (Bailey et al., 1998). The reduction in the serum concentrations of calcium and phosphorus in HA groups may be due to a metal chelating effects of HA, which is affected by large number of carboxylic acid side chains (Klocking,

1994). Aflatoxin-contaminated diets in this study significantly ($P<0.05$) increased serum aspartate aminotransferase activity which is in contrast to the findings of Huff et al. (1992), in which aflatoxin caused a decrease in the activity of aspartate aminotransferase. Van Rensburg et al. (2006) did not find aflatoxin to have any effect on serum activity of either γ-glutamyltransferase or aspartate aminotransferase. These variable results might be due to different levels of aflatoxin used in poultry diets. In the current study high level of HA neutralized the effect of toxin showing reduction in aspartate aminotransferase activity in chickens. Serum γ-glutamyltransferase enzyme activity is a sensitive indicator of liver disease, whether the disorder involves liver inflammation, lesions, or obstruction to the biliary tract (Kubena et al., 1990 a, b). In the present study, serum γ-glutamyltransferase activity was significantly increased in birds consuming AF diets. This observation is in agreement to studies in which an increase in γ-glutamyltransferase activity in the serum was reported (Kubena et al., 1990 a, b). Numerically, supplementation of HA declined enzyme activity in chickens and neutralized the effect of toxin.

Hematological parameters

The supplementation of HA improved blood parameters, probably as a result of effective adsorption in the gut to reduce the amount of aflatoxin absorption by the body. Ipek et al. (2008) reported that HA (0.04, 0.05 or 0.06%) increased red blood cell but did not have any effect on white blood cell in nine-week-old female Japanese quails. On the contrary, Rath et al. (2006) reported that HA (1.0 or 2.5%) did not have any effect on white blood cell, red blood cell, monocyte, and lymphocyte counts, or hematocrit values. This variation in results may be caused by effects of several factors such as HA preparations due to different sources, poultry species, rearing of birds in various regions of the world differing in the climate.

Immunity

The present results showed that the feeding of AFB_1 reduced the antibody production against ND in broilers. This was similar to the results in other studies (Ghosh and Chauhan, 1991; Hegazy et al., 1991; Gabal and Azzam, 1998) showing the immunotoxic effects of AF with 100 to 2500 µg AF/kg in the diet. The immunosuppressive effect of AF has been related to its direct inhibition of protein synthesis (Oğuz et al., 2000) including those with specific function such as immunoglobulin G (IgG) and A (IgA), inhibition of migration of microphages (Ibrahim et al., 2000), interference with the hemolytic activity of complement, reduction of number of lymphocytes (Ghosh and Chauhan, 1991) through its toxic effect on the bursa of Fabricius (Ortatatli et al., 2002) and impairment of cytokines formation by lymphocytes (Gabal and Azzam, 1998). This study agrees with previous findings by Oğuz et al. (2003), who reported that ND titers were significantly lower ($P<0.05$) in 100 µg AF/g fed chicks, while no significant differences were seen in 50 µg AF/kg group compared to the control group ($P<0.05$).

In the current study, addition of HA to the AFB_1-containing diet ameliorated the adverse effect of AF on antibody production. Similarly, Hasan et al. (2010) reported that addition of 0.4, 0.6, 0.8 and 1.0% of HA to the AF-containing diet, significantly

ameliorated the adverse effect of AF on antibody production against ND in broilers from 28 to 35 days of age ($P<0.01$). This effect could be attributed to the role of HA as binding agent to the AF molecules in gastrointestinal tract and precluding their absorption that can alleviate the toxicity of AF in poultry (Van Rensburg et al., 2006). They further reported that HA was able to absorb (*in vitro*) about 10.3, 7.4 and 11.9 mg of AFB_1/g of oxihumate at pH 3, 5 and 7, respectively. These results clearly demonstrate that 100 µg of AFB_1/kg diet-treatment significantly affects the HI against ND and simultaneous addition of HA (0.1 to 0.3%) to the AFB_1 containing diet provides significant reduction to the immunotoxic effects of AF.

It is concluded that HA was effective in diminishing the growth inhibitory effects of aflatoxin and there was apparent protection noted for liver organ and minimized the aflatoxin residues in liver. The biochemical and hematological changes associated with aflatoxin toxicity improved with HA supplementation. Aflatoxin treatment significantly affects the antibody production against Newcastle disease and simultaneous addition of HA to the aflatoxin containing diet provides significant reduction to the immunotoxic effects of aflatoxin. These results suggest that HA (0.1 to 0.3%) might be sufficient to counteract the adverse effects of AFB_1.

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