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Mitigative Effect of Dietary *Tinospora cordifolia* and *Andrographis paniculata* on Health and Hepato-renal Expression of Caspase-3 and TNF-α of Broiler Chickens Fed on Aflatoxin and Ochratoxin Contaminated Diet

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Abstract

Medicinal herbs are modern feed additives for poultry that have antioxidant, anti-microbial, and immune-modulatory impacts. In this study, the protective effects of medicinal natural herbs (Herb-All™ LIVER) on performance, litter, immunity, biochemical changes, and histopathological changes in broilers fed Mycotoxins-contaminated diets were assessed. One-day-old chicks were randomly assigned into 4 groups (n=120). Group A was fed a standard commercial diet; Group B was fed a standard diet + (Herb-All™ LIVER); Group C (positive control) was fed a Mycotoxins-contaminated diet, and Group D received a Mycotoxins-contaminated diet + Herb-All™ LIVER. Performance, litter hygiene, immunity, and histopathological changes were determined. Results indicated that herbal supplements (B and D) significantly improved the body weight gain and FCR of birds. Also, litter hygiene, blood indices, antibody titers, and organ functions were enhanced. In group D, liver and kidney histological architectures were mostly restored, as well as Caspase-3 and TNF expressions were moderately enhanced. It can be concluded that using Herb-All™ LIVER as a feed additive reduces the adverse effects of Mycotoxins on broilers.

KEYWORDS
Aflatoxin, Ochratoxin, *Tinospora cordifolia*, *Andrographis paniculata*, Caspase-3.

INTRODUCTION

Mycotoxins are highly toxic secondary metabolites of fungi that can contaminate poultry feed and weaken birds' immune systems. Aflatoxins and Ochratoxins are natural contaminants of feedstuff that should not exceed 20 ppb and 25 ng/g, respectively (FDA, 2001; EC, 2006). A recent survey in Egypt found that 30.6% of feed and 19.6% of ingredients exceeded Aflatoxins standards, while 91% of feed and 78.3% of ingredients exceeded Ochratoxin limits (El-Nabarawy *et al*., 2020). Co-contamination with two or more mycotoxins was reported in 38% of sampled feed and feed raw materials globally (Streit *et al*., 2013). Diets contaminated with mycotoxins can significantly affect the histological structure of birds' organs, with significant pathological changes in liver and kidney tissues represented in deterioration, inflammation, and vascular alterations (Hatab *et al*., 2022). Moreover, mycotoxin residues in animal-derived foods are considered a threat to human health.

Aflatoxicosis in broilers affects mortality, feed conversion rate, and profitability costs. Aflatoxins (AFs) are hepatotoxic compounds that impair liver function and cause lipid accumulation, liver enlargement, and interfere with protein synthesis (Quezada *et al*., 2000). Ochratoxin A (OTA) is a strong nephrotoxin and immunotoxin that can affect chickens' immune systems, cause atrophy, and decrease the weights of both primary and secondary lymphoid organs (Al-Anati and Petzinger, 2006). It can also affect the humoral response in chicks by altering the levels of specific antibodies against viruses, such as the Newcastle disease virus (Indresh and Umakantha, 2013). OTA is classified as a possible human carcinogen by the International Agency for Research on Cancer (Ostry *et al*., 2017).

Natural medicinal herbs are a recent set of poultry eco-friendly feed additives. *Tinospora cordifolia* (Guduchi) is a medicinal herb that contains multiple bioactive compounds such as phenolics, alkaloids, and polysaccharides; that have antioxidant, anti-hepatotoxic, anti-inflammatory, and immunomodulating effects (Kapil and Sharma, 1997; Raina Mehra *et al*., 2013). *T. cordifolia* also improves lipid metabolism and hepatic functions in poultry and reduces oxidative stress caused by environmental factors (Saeed *et al*., 2020). *Andrographis paniculata* is another dietary herbal supplement with numerous pharmacological effects, such as anti-inflammatory, antioxidant, and immunostimulant activities. It can also expel toxins from the body, protect the liver and kidneys from damage, and ameliorate the toxicity of aflatoxins in broilers (Sapcota *et al*., 2005; Okhuarobo *et al*., 2014; Sonwane *et al*., 2019). Hickory nuts are an oil-bearing plant product that contains 60 to 70% oil content. Hickory nut oil contains high amounts of polyunsaturated α-linolenic, α-eleostearic, and linolenic fatty acids, which have received considerable interest due to beneficial health effects associated with consumption, particularly for lowering the risk of hyperlipidemia (Zhang *et al*., 2016).

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The current study aimed to evaluate the ameliorative capacity of herbal preparation (Herb-All Liver) to improve growth performance and reduce the hepatotoxicity and nephrotoxicity of aflatoxin and ochratoxin in broiler chickens.

MATERIALS AND METHODS

Ethical approval

This study was approved by Institutional Animal Care and Use Committee (IACUC), Cairo University, Egypt with code (Vet-CU08/03/2022/427).

Birds and Location

One-day-old Avian-480 broiler chicks purchased from Sisan Misr Poultry Company were used in this study (initial weight= 42 g). The birds were housed in environmentally controlled separate pens measured 2×3 m^2 belonging to the Poultry Research Unit at the Veterinary Hygiene and Management Department, Faculty of Veterinary Medicine, Cairo University, Egypt. All birds were vaccinated against Newcastle (NDV), IB (Infectious bronchitis), Avian Influenza, and Gumboro diseases.

Experimental Design

A total of 480 one-day-old avian-48 broiler chicks were used in this experiment for 35 days in the summer season. Birds were randomly divided at one-day old into 4 equal groups each of 120 chicks and each group was subdivided into four equal replicates each of 30 birds housed in separate pens each of 2×3m^2. Group A: birds fed on commercial basal diet only and acted as the negative control group. Group B: birds received commercial basal diet + Herb-All™ LIVER (500g/ton) as natural herbs. Group C: birds fed on commercial ration contaminated with Aflatoxin (AF) and Ochratoxin (OTA) and represented the positive control. Group D: birds were fed on the mycotoxins-contaminated diet + Herb-All™ LIVER (500g/ton) (Figure 1). Birds were reared on deep litter floor bedded with shaved wood of 5 cm depth. All birds were fed a commercially formulated diet containing biological toxin-binder (Table 1). Feed and water were given ad-libitum.

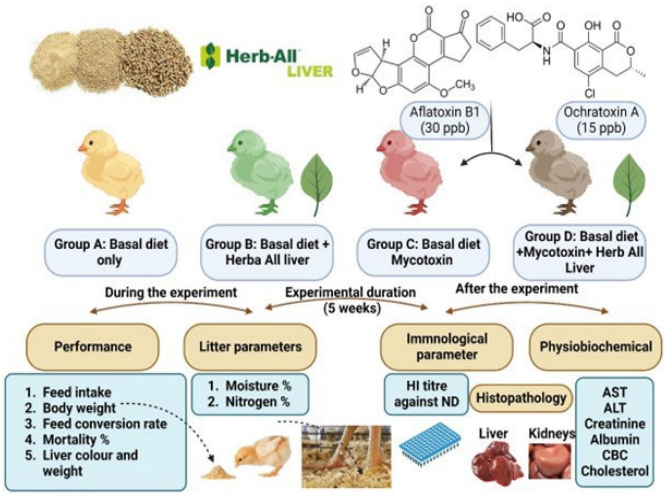

Figure 1. Schematic diagram illustrating the experimental design and parameters measured. The diagram is our own and was not taken from another source.

Natural Herbs

Herb-All™ LIVER preparation (Life Circle Nutrition AG, Hämmerli 2d, 8855, Wangen SZ, Switzerland) was used as natural herbs (500g/ton feed) for ameliorating the adverse effects of mycotoxins on bird health and performance. Herb-All™ LIVER is a mixture of medical herbal plants mainly composed of *Andrographis paniculata*, *Tinospora cordifolia*, and hickory nuts (organic carrier).

Table 1. Ingredients and chemical composition of the basal commercial diet used in the experiment.

Ingredients	Starter	Grower	Finisher
Corn- grains 7.5%	60.38	65.42	70.65
Soybean meal 46%	34.5	28.9	24.05
Wheat bran 15%	1	2.3	2.42
Fine limestone	0.85	1	1.08
Di-Calcium phosphate	1.75	0.85	0.55
Common salt	0.2	0.22	0.26
Sodium Bicarbonate	0.23	0.2	0.14
Vitamin Premix[1]	0.15	0.15	0.15
Mineral Premix[1]	0.15	0.15	0.15
D-L- Methionine 99%	0.18	0.19	0.14
L- Lysine 78.5%	0.23	0.27	0.17
L- Threonine 98.5%	0.15	0.15	0.05
Rovi-Yeast (anti-mycotoxins)	0.1	0.1	0.1
Anticoccidial	0.05	0.03	0.05
Energy enzymes	0.01	0.01	0.01
Phytase	0.01	0.01	0.01
Protease (Acid)	0.01	0.03	0.01
WinoPro (alkaline)	0.03	0.03	0.03
Betaine	0.03	0.03	0.03
Total	100	100	100
Chemical composition (calculated)			
Metabolizable Energy kcal/kg	2900	2950	3000
Crude protein %	21	19	17
Dig. Methionine%	0.47	0.46	0.39
Dig. Methionine + cysteine%	0.92	0.86	0.75
Dig. Lysine%	1.2	1.1	0.9
Dig. Threonine%	0.82	0.75	0.59
Calcium%	0.85	0.69	0.61
Avail. Phosphorus%	0.45	0.29	0.25
Sodium%	0.16	0.16	0.16
Chlorine%	0.23	0.23	0.23
Potassium%	0.87	0.8	0.72
Ether Extract%	2.67	2.84	3
Crude Fiber%	2.78	2.82	2.76

[1]Vitamin & Mineral Premix: Vitamin - mineral mixture supplied per kg of diet: Vit A, 12000IU; Vit D, 2200IU; Vit E, 10 mg; Vit K3, 2mg; Vit B1, 1mg; Vit B2, 4mg; Vit B6, 1.5mg; Vit B12, 10g; Niacin, 20mg; Pantothenic acid, 10mg; Folic acid, 1mg; Biotin, 50g; Choline chloride, 500 mg; Copper, 10mg; Iodine, 1mg; Iron, 30mg; Manganese, 55mg; and Selenium, 0.1mg.

Production and detection of mycotoxins and feed (diet) contamination

Fungal strains

Two main fungal strains were included in this study: *Aspergillus flavus* (*A. flavus*) and *Aspergillus ochraceus* (*A. ochraceus*), the main producers of Aflatoxins and Ochratoxin (Ostry *et al.*, 2013). Both are virulent and toxigenic strains that were obtained from the local Egyptian field and were isolated, identified, and provided by the Mycology Unit at the Animal Health Research Institute, Dokki, Giza, and at the Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Egypt.

Preliminary mass propagation of the obtained strains

Under complete aseptic condition, each strain was inoculated in five Erlenmeyer flasks containing 500 ml of Sabouraud's dextrose broth (SDB) and incubated for 10 days at 25°C, with regular daily observation of pure, steady, and regular growth of surface fungal mats (Gholami-Shabani *et al*., 2022). At the end of the incubation period, fungal mats were separately harvested for each strain. Harvesting was done using sterile gauze to separate the fungal growth mat phase from the submerged phase (Shih and Marth, 1975). The collected fungal mats representing each fungal strain were pooled together in a sterile mortar and then sliced into small pieces using a sterile scalpel. Using a homogenizer at 160 rpm for 10 minutes each pooled collection was homogenized with 250 ml of the previously separated fungal growth submerged phase.

Corn contamination with mycotoxins

This step aimed to; I) optimize the upcoming artificial fungal contamination condition for desired mycotoxins production, II) evaluate the actual mycotoxins production potentiality and capabilities of the obtained fungal strains, and III) prepare a nidus to be used for contaminating a larger amount of crushed corn that will be performed in the further experimentation step. Just 10 preparations each consisting of 250g of mycotoxin-free poultry prepared ration were equally spread in a sterile 30cm×15cm rectangular container with a covering lid. Then 25 ml from each strain representing previously prepared fungal homogenate was inoculated above the surface of the corn and incubated for 14 days at 25°C (Guidry and Trelles, 1962) with a daily observation of regular fungal growth and mixing using a sterile glass rod. In a way mimicking the natural occurrence of fungal growth on improperly stored poultry corn ration, this step was designed. The calculated corn amount was mixed with the previously prepared mycotoxins contaminating inoculum and spread on a plastic sheet in a dark humid chamber for 14 days, with daily mixing and observation for the proper fungal growth.

Mycotoxin detection and assessment

Aflatoxin and ochratoxin-Enzyme-linked immunosorbent assay (ELISA)-based mycotoxin assessments were performed according to the manufacturer's recommendations (MaxSignal Mycotoxin ELISA test kit, PerkinElmer, USA) in three different stages through the present work, I) at zero-day before any corn processing steps were done (pre-contamination stage), to ensure the emptiness of the used corn from aflatoxin, and ochratoxin, II) second assessment was performed at the end of the incubation period of the small scale corn contaminating inoculum (contamination stage), III) final mycotoxin assessment was performed on the whole calculated amount of corn ration that will be consumed by the tested bird groups involved in the study for the whole period of the experiment (post contamination and pre-corn ration introduction to the bird groups). The ration that was finally introduced to the birds contained 30 ppb aflatoxin B1 and 15 ppb ochratoxin as priming mycotoxin levels of cumulative mycotoxicosis cases among the bird members of the tested groups.

Measurement of growth performance

Weekly measurements of feed intake, live body weight, weight gain, Feed Conversion Ratio (FCR), and mortality rate were recorded and calculated during the 5-week experimental period. While the total measurements were calculated at the end of the experiment. On day 35, a total of 40 birds per treatment (10 birds/replicate) were sacrificed quickly by slaughtering through neck cutting, where jugular veins, carotid arteries, trachea, and the oesophagus were severed (Zaman *et al*., 2017; Banaszak *et al*., 2021). Neck cutting is one of the fastest humane approaches to induce brain death (Zaman *et al*., 2017).

Litter hygiene assessment

Litter samples were collected from each replicate for physical and chemical examinations. Moisture was determined by hot air drying 10g of litter samples at 100±5°C for 24-48h in the hot air oven (Dumas *et al*., 2011). Litter moisture % was calculated by subtracting dry weights from the initial weights. Additionally, the total nitrogen content of litter samples was determined as total Kjeldahl nitrogen (Jackson, 1973).

Physiological and biochemical assay

Blood samples were collected on the 35th day and sera were separated and kept in the refrigerator at 4°C for the quantitative determination of blood biochemical tests using BIOMED Diagnostics® kits. Total cholesterol and HDL-cholesterol (HDL-C) were measured at 500/520 and 546 nm, respectively. Creatinine, albumin, aspartate aminotransferase (AST/GOT), and alanine aminotransferase (ALT/GPT) were determined.

Measuring Newcastle disease virus vaccinal Immunity

Antibody titers of Newcastle disease virus (NDV) were assessed by Haemagglutination Inhibition (HI) test on the 28th and 35th days (OIE, 2021). In 99-microwell plates, 25µl of each serum sample was subjected to two-fold serial dilutions. Then, four haemagglutination units (4HAU) of ND-Lasota antigen were prepared and 25µl were added to each well. After 20 minutes, 25µl of 1% chicken-RBC suspension was added to each well. NDV Antibody titers were described as mean $\log_2$ HI titers.

Liver weight and colour

At the end of the experiment, on the 35th day, 40 birds per treatment (10 birds/replicate) were slaughtered and livers were separated and weighed with the aid of a sensitive digital weighing scale. The weights of the organs were expressed on final body weight as a percentage. Macroscopical examination of liver colour and texture was performed according to Anjos *et al*. (2016).

Histopathological changes

General histological examination

A total of 32 samples were collected (16 liver and 16 kidney specimens) and sliced to 3-4 mm thick, fixed in 10% neutral buffered formalin (10% NBF). The technique for histological preparations is demonstrated by Bancroft and Stevens (2016). Sections examined and photographed under different powers to investigate any pathological alteration in the examined sections.

Immunohistochemistry Staining Protocol

Immunohistochemistry was applied on paraffin tissue sections and fixed on positively charged slides by using the avidin-biotin-peroxidase complex (ABC) method (Hsu *et al*., 1981).

Rabbit Anti Caspase 3 Antibody, Polyclonal Antibody (LSBio (LifeSpan) Cat# LS-B3404-50, RRID: AB_10627102), Dil.: 10 µg/ml) and Rabbit Anti-Tumor Necrosis Factor (TNF α) antibody (Gold), polyclonal antibody (Biorbyt Cat# orb13033, RRID: AB_10750851) have been tested. Sections from each studied group were incubated with the formerly stated antibodies, subsequently, the chemicals involved in the ABC technique (Vectastain ABC-HRP kit, Vector laboratories) were inserted. Marker expression was identified with peroxidase and stained with diaminobenzidine (DAB, produced by Sigma) to distinguish antigen-antibody complex. Negative controls were integrated using non-immune serum instead of the primary or else secondary antibodies. Immuno-stained sections were checked and photographed using a Leica microscope under different magnification powers (CH9435 Hee56rbrugg) (Leica Microsystems, Switzerland) at the Faculty of Veterinary Medicine, Cairo University, Giza, Egypt.

Quantitative scoring of immunohistochemical results "Area Percentage"

Six high-power fields (x400) exhibiting positive brown immunostaining were selected for evaluation in each serial section of the studied groups for liver and kidney organs. Area % was determined for Caspase 3 and TNF-alpha sections via Leica QWin 500 image analyzer computer system (England). This image analyzer entails a Leica microscope, a coloured video camera, a coloured monitor and a hard disc of a Leica IBM personal computer linked to the microscope and managed by Leica QWin 500 software. Records of each antibody were statistically described in terms of mean and standard deviation (mean ± SD) for area %.

Statistical analysis

Data were examined for normality via the Kolmogorov-Smirnov test. The outcomes of this test pointed out that most data were normally distributed (parametric data). Accordingly, descriptive analysis, One Way-ANOVA, and Tukey's post-hock tests were operated for intergroup relationships. P-values less than 0.05 were represented as statistically significant. Statistical analysis was conducted with SPSS 21.0 (Statistical Package for Scientific Studies, SPSS, Inc., Chicago, IL, USA) for Windows.

RESULTS

Performance parameters

Table 2 presents the cumulative performances of broiler chicks' groups during the 35 days. The C group exhibited the worst performance parameters, with low body weight (BW), reduced body weight gain (BWG), and elevated feed conversion ratio (FCR) with -10.3%, -10.5%, and +13.8% differences from control, respectively (P <0.05). The D group showed significantly better BW, BWG, and lower FCR with +7%, +7%, and -9% differences from C, respectively (P <0.05). Feed intake was more efficient in B and D groups than in the C group (-41 and -59 g feed/bird, respectively). No significant differences in feed intake and mortality rates were recorded between treatments (P > 0.05).

Litter parameters

Table 3 summarizes the results of litter quality. On day 35, group C had the highest litter moisture level (P < 0.05), which was +25% higher than group D. In contrast, litter nitrogen was significantly reduced in groups B and D compared to group C (P < 0.05).

Biochemical blood parameters

Table 4 shows the liver and kidney function evaluation in the studied groups. Groups supplemented with Herb-All (B and D) had better liver enzymes and kidney functions than groups supplemented with basal di*et al*one. However, the group C fed basal diet contaminated with mycotoxin showed a statistically significant elevation of liver and kidney functions (P < 0.05). Meanwhile, group D receiving Herb All with a diet contaminated with mycotoxin revealed improved results compared to group C.

Table 2. Cumulative performance of broiler chicks fed on mycotoxin contaminated ration and supplemented with Herb-All Liver as natural Herbs (All weeks).

Groups	Total Feed intake (g)	One-day body weight (g)	Final body weight (g)	Total body weight gain (g)	FCR (g/g)	Mortality No. (%)
A	3075.0±100.0	42	1963.0±21.0[a]	1920.0±34.0[a]	1.60.0±0.05[c]	1(0.83)
B	3091.0±51.0	42	1962.0±21.0[a]	1920.0±21.0[a]	1.60.0±0.02[c]	1(0.83)
C	3132.0±17.0	42	1760.0±23.0[c]	1718.0±23.0[c]	1.82.0±0.02[a]	1(0.83)
D	3073.0±30.0	42	1883.0±18.0[b]	1841.0±18.0[b]	1.66.0±0.00[b]	1(0.83)

[a,b,c] Data are presented as Mean ± SE, small alphabetical letters denote statistical significance difference between groups within the same column, P < 0.05.
Groups A: control basal diet; B: basal diet + herb (Herb-All Liver); C: basal diet + mycotoxins only (Aflatoxin and Ochratoxin); D: basal diet + Mycotoxins (Aflatoxin and Ochratoxin) + herb (Herb-All Liver).

Table 3. Litter moisture content and Nitrogen excretion of broiler chicks fed on mycotoxin contaminated ration and supplemented with Herb-All Liver as natural Herbs.

Group	Moisture % at day 21	Moisture % at day 35	Nitrogen % at day 35
A	21.63 ± 0.36	17.49 ± 3.27[b]	0.97 ± 0.06[d]
B	21.06 ± 1.38	15.53 ± 4.03[b]	1.36 ± 0.01[c]
C	22.39 ± 0.80	47.54 ± 0.82[a]	3.20 ± 0.56[a]
D	21.85 ± 1.05	22.79 ± 5.18[c]	1.99 ± 0.35[b]
P-value	0.813	0.000	0.015

[a,b,c] Data are presented as Mean ± SE, small alphabetical letters denote statistical significance difference between groups within the same column, P < 0.05.
Groups A: control basal diet; B: basal diet + herb (Herb-All Liver); C: basal diet + mycotoxins only (Aflatoxin and Ochratoxin); D: basal diet + Mycotoxins (Aflatoxin and Ochratoxin) + herb (Herb-All Liver).

Table 4. Biochemical parameters (liver and kidney functions) of broiler chicks fed on mycotoxin contaminated ration and supplemented with Herb-All Liver as natural Herbs.

Groups	ALT (U/L)	AST (U/L)	Creatinine (mg/dl)	Albumin (g/dl)	Cholesterol (mg/dl)	HDL (mg/dl)
A	160.25^{c}	186.81^{c}	0.255±0.03^{c}	1.79±0.27^{c}	128.302±9.66^{b}	32.24±0.85^{c}
B	156.25^{d}	168.01^{d}	0.215±0.01^{d}	1.71±0.11^{d}	110.453±7.27^{d}	30.73±0.89^{d}
C	269.25^{a}	312.76^{a}	0.363±0.05^{a}	2.20±0.13^{a}	152.305±6.23^{a}	58.83±1.28^{a}
D	204.25^{b}	224.09^{b}	0.285±0.01^{b}	1.83±0.05^{b}	121.896±5.09^{c}	41.31±1.38^{b}
P-value	<0.0001	<0.0001	0.019	0.011	0.128	<0.0001

^{a,b,c} Data are presented as Mean ± SE, small alphabetical letters denote statistical significance difference between groups within the same column, P < 0.05.
Groups A: control basal diet; B: basal diet + herb (Herb-All Liver); C: basal diet + mycotoxins only (Aflatoxin and Ochratoxin); D: basal diet + Mycotoxins (Aflatoxin and Ochratoxin) + herb (Herb-All Liver).

Newcastle disease virus vaccinal antibody titers

Table 5 displays the antibody titers of Newcastle disease virus (NDV). On days 28 and 35, antibody titers were statistically different between groups. Group B showed the highest antibody titers, followed by A and D groups, then C. Supplementing the mycotoxin-contaminated diet with Herbals (D) raised the antibody titters to the same level as that of the control group (A) and to 27% and 42% enhancement than mycotoxicated group (C) on days 28 and 35, respectively (P<0.05).

Table 5. Vaccinal HI titers for NDV of broiler chicks fed on mycotoxin contaminated ration and supplemented with Herb-All Liver as natural Herbs.

Group	HI antibody titer (log_2)	
	28 days	35 days
A	3.75 ± 0.47^{b}	4.75 ± 0.48^{b}
B	5.00 ± 0.41^{a}	5.50 ± 0.41^{a}
C	2.75 ± 0.48^{c}	3.00 ± 0.41^{c}
D	3.50 ± 0.28^{b}	4.25 ± 0.48^{b}
P-value	0.018	0.009

^{a,b,c} Data are presented as Mean ± SE, small alphabetical letters denote statistical significance difference between groups within the same column, P < 0.05.
Groups A: control basal diet; B: basal diet + herb (Herb-All Liver); C: basal diet + mycotoxins only (Aflatoxin and Ochratoxin); D: basal diet + Mycotoxins (Aflatoxin and Ochratoxin) + herb (Herb-All Liver).

Liver weight and colour

Table 6 lists the liver weights and macroscopical findings. Group B exhibited the largest livers, exceeding control (A) by 6.4% (P<0.05). The lowest liver weight was reported in the mycotoxicated birds (C) with a -16.4% decrease compared to control (A) (P<0.05). Group D birds showed enhancement in liver weights with 13.2% higher weight than group C (P<0.05). Regarding the liver macroscopical findings, 85% of B's and 77.5% of A's and D's livers were normal, with colour ranging from tan to deep mahogany. While in group C, only 55% of livers were normal, and 25% were pale yellowish in colour and fragile in texture.

Histological findings

The results of hematoxylin and eosin-stained sections of the liver and kidneys were displayed in Figures (2) and (3). Groups A and B showed the normal architecture of the liver (Fig. 2A and 2F). Group C displayed degenerative changes in the liver with massive inflammatory cell infiltration (Fig. 2B-D). Group D revealed improvement in liver tissue with nearly normal architecture. However, congestions in some hepatic sinusoids were noticed (Fig. 2E). In kidneys, groups A and B exhibited the normal histological structure of the renal cortex (Fig. 3A and 3F). Group C highlighted severe degeneration along the renal cortex area with loss of their normal structure, as well as necrotic changes (Fig. 3B-D). Group D displayed evident tissue recovery with normal renal corpuscle except glomerulus detected with few vacuolations (Fig. 3E).

Immunohistochemistry

Figures 4 and 5 display immunohistochemical findings of liver and kidney sections tested by Caspase-3. The immunoreactivity of Caspase-3 in liver tissue sections of A and B groups (Fig. 4) showed non-significant (P=0.985) expressions with mean area%= 3.12±0.624 and 2.75±0.52, respectively. However, group C showed significant (P<0.0001) intense positive nuclear expression of hepatocytes compared to groups A and B, with a mean area%= 42.48±1.11. Additionally, group D revealed a marked significant (P<0.0001) reduction in expression with moderate nu-

Table 6. Liver weight and macroscopical examination of liver of broiler chicks fed on mycotoxin contaminated ration and supplemented with Herb-All Liver as natural Herbs.

Groups	Liver weight (g) Mean + SE	Number of examined sample	Macroscopically examination Liver color and texture					
			Normal		Moderate		Pale yellowish and fragile	
			No	%	No	%	No	%
A*	52.61 ± 1.34^{b}	40	31	77.5	7	17.5	2	5
B**	55.98 ± 0.89^{a}	40	34	85	4	10	1	2.5
C***	43.98 ± 0.62^{d}	40	22	55	8	20	10	25
D	49.78 ± 0.56^{c}	40	31	77.5	6	15	3	7.5
P-value	<0.0001							

^{a,b,c} Data are presented as Mean ± SE, small alphabetical letters denote statistical significance difference between groups within the same column, P < 0.05.
Groups A: control basal diet; B: basal diet + herb (Herb-All Liver); C: basal diet + mycotoxins only (Aflatoxin and Ochratoxin); D: basal diet + Mycotoxins (Aflatoxin and Ochratoxin) + herb (Herb-All Liver).
Liver colour is categorized according to Anjos *et al*. (2016) into 3 types: *Normal liver: colour ranged from tan to deep mahogany. **Moderate liver: Two thirds of the liver is pale yellow in colour. ***Pale and yellow liver: All liver is pale yellow in colour and fragile in texture.

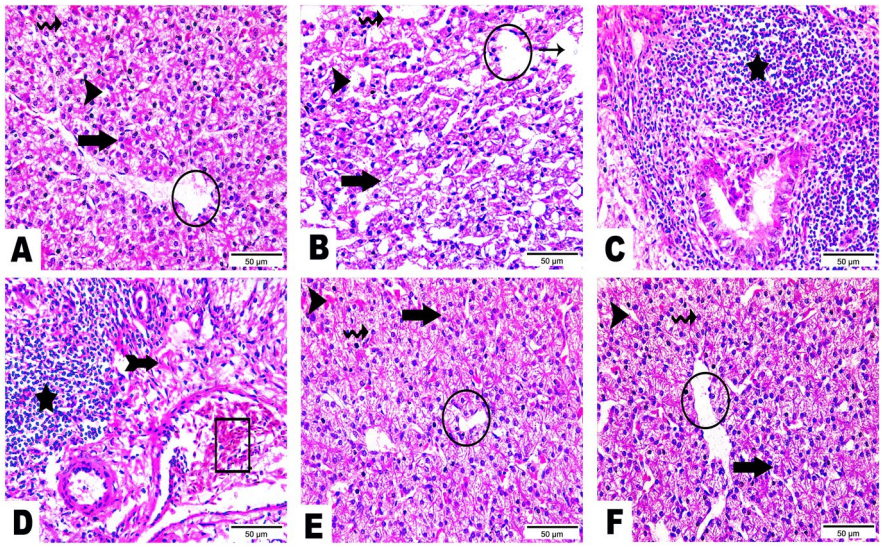

Figure 2. Photomicrographs displayed the histopathological variations in liver tissue sections between broilers groups as follows: (A) Negative control group and (F) Herb-All liver group demonstrating the normal architecture of central vein (circle), large sheets (wave arrow) of polygonal hepatocytes with central, spherical, and vesicular nuclei (thick arrow). Hepatocytes separated from each other by sinusoids with their von Kupffer cells (arrowhead). (B, C and D) Positive control group highlighting severe degenerative changes including loss of regular hepatic organization (circle) with hydropic disintegration (thick arrow), vacuolations and apoptotic hepatocytes (wave arrow), dilated sinusoids (arrowhead), edema (thin arrow), congested portal vein (cube), obvious increase in fiber amount besides massive infiltration of inflammatory cells encircling portal area (star). (E) Treated Group revealing a marked improvement with nearly normal central vein (circle), restore the organizations of hepatic sheets (thick arrow) and normal existence of light vesicular hepatocytes (wave arrow). Conversely, congested hepatic sinusoids were noticed (arrowhead). (Hematoxylin and Eosin Stain, Magnification Power= x400 and Scale Bar= 50µm).

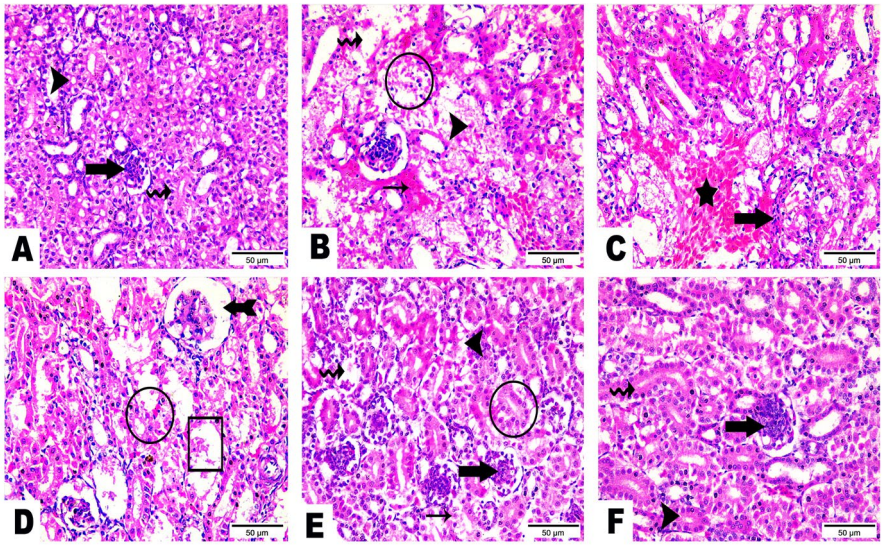

Figure 3. Photomicrographs presented the histopathological alterations in renal cortex area of kidney tissue sections between broilers groups as follows: (A) Negative control group and (F) Herb-All liver group exhibiting the normal histological structure of renal cortex comprising renal corpuscle (thick arrow), proximal convoluted tubules (arrowhead), and distal convoluted tubules (wave arrow). (B, C and D), Positive control group highlighting severe degeneration with loss of their normal structure (circle) as well as necrotic changes (thin arrow). Most renal tubules damaged, and others existed with epithelial desquamation (cube) and apoptotic nuclei (arrowhead). Interstitial hemorrhage (star), inflammatory cells infiltration (thick arrow), and edema (wave arrow) were observed. Additionally, some renal corpuscle marked with dilated interglomerular space and vacuolated glomerulus (arrow with tail). (E) Treated group displayed obvious tissue recovery with normal renal corpuscle except glomerulus detected with few vacuolations (thick arrow). Renal tubules marked either as in normal structure (circle) or with detached basal membrane and vacuolations (arrowhead), besides few looked degenerated (thin arrow). Little interstitial edema is yet noted (wave arrow). (Hematoxylin and Eosin Stain, Magnification Power= x400 and Scale Bar= 50µm)

clear reactivity of hepatocytes compared to groups A and B, with mean area% = 26.81±0.57 as presented in Fig. 8.

Regarding kidney tissues, groups A and B recorded few expressions (Fig. 5) besides non-significant (P=0.883) differences between them with mean area% = 3.25±0.26 and 3.69±0.26, respectively. Although significant (P<0.0001) strong positive nuclear and cytoplasmic expression of Caspase-3 was observed along renal corpuscle and tubules of group C than that of groups A and B with mean area% = 34.29±0.58. Additionally, group D verified an obvious significant (P<0.0001) decrease in Caspase-3 expression and appeared with moderate nuclear reactivity lining renal corpuscle and tubules than A and B groups with mean area% = 13.44±0.54 as shown in Fig. 8.

Figures 6 and 7 display immunohistochemical findings of liver and kidney sections tested by TNFα markers. The expression of TNF-α in liver tissue (Fig. 6) was not significantly different between groups A and B (P>0.05) with mean area% = 3.43±0.41 and 3.9±0.44, respectively. However, group C showed a significant (P<0.0001) increase in TNF-α expression with intense positive cytoplasmic expression to hepatocytes in all tissue sections compared to groups A and B with mean area% =67.84±1.12. Group D showed a significant (P<0.0001) decrease in TNFα expression with moderate cytoplasmic reactivity of hepatocytes compared to groups A and B with mean area% = 33.04±0.62 (Fig. 8).

In kidney tissue (Fig. 7), TNFα expression was not significantly different between groups A and B (P=0.996) with mean area% =1.75±0.21 and 2.01±0.22, respectively. However, group C showed a significant (P<0.0001) increase in TNF-α expression with strong positive cytoplasmic expression along renal corpuscle and tubules compared to groups A and B with mean area%

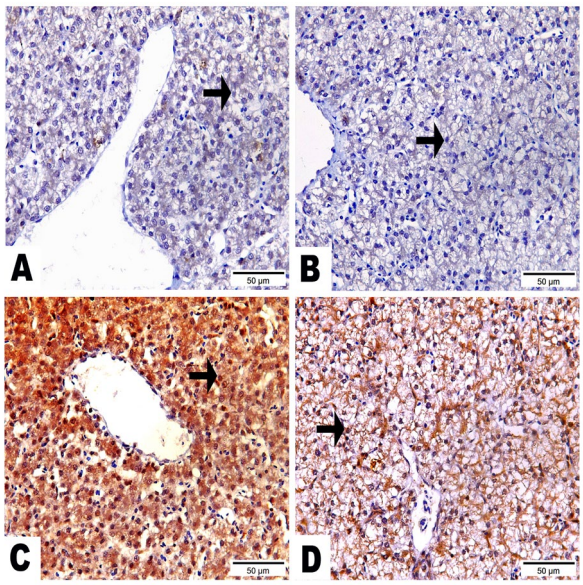

Figure 4. Photomicrographs displayed the immunoreactivity of Caspase 3 in liver tissue sections between broilers groups as follows: (A) Negative control group and (B) Herb-All liver group showing the normal negative expression of caspase 3 along hepatic tissue (arrows). (C) Positive control group highlighting intense positive nuclear expression of hepatocytes in all hepatic tissue (arrow). (E) Treated group revealing a marked reduction in expression with moderate nuclear reactivity of hepatocytes to Caspase 3 antibodies (arrow). (Caspase 3 Antibody, Magnification Power= x400 and Scale Bar= 50µm).

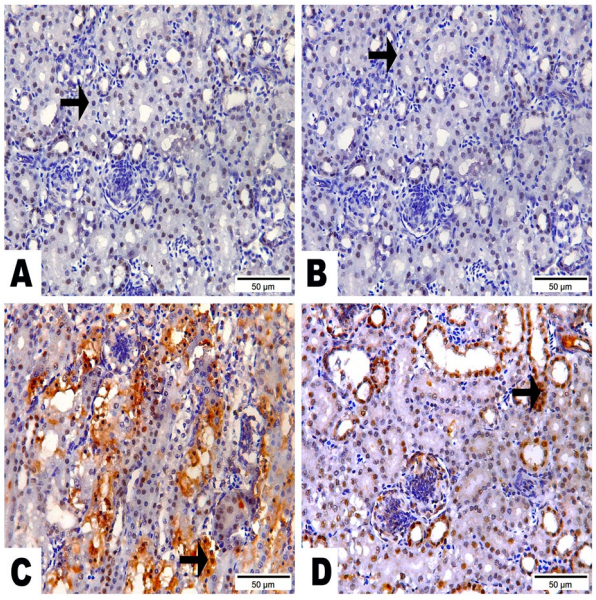

Figure 5. Photomicrographs presented the immunoreactivity of Caspase 3 in kidney tissue sections between broilers groups as follows: (A) Negative control group and (B) Herb-All liver group existing with normal negative expression of caspase 3 along renal cortex area (arrows). (C) Positive control group emphasizing strong positive nuclear and cytoplasmic expression of along renal corpuscle and tubules (arrow). (E) Treated group demonstrating obvious reduction in caspase 3 expression and appeared with moderate nuclear reactivity lining renal corpuscle and tubules (arrow). (Caspase 3 Antibody, Magnification Power= x400 and Scale Bar= 50µm).

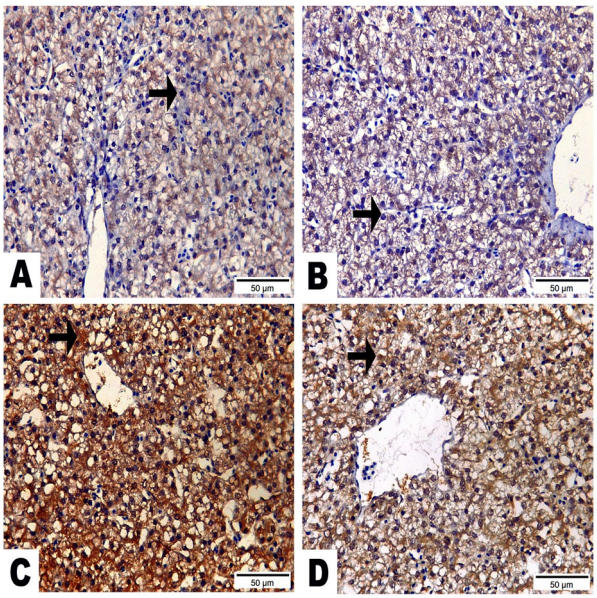

Figure 6. Photomicrographs demonstrated the immunoreactivity of Tumor Necrosis Factor Alpha (TNFα) in liver tissue sections between broiler groups as follows: (A) Negative control group and (B) Herb-All liver group showing the normal few expressions of TNFα along hepatic tissue (arrows). (C) Positive Control group highlighting intense positive cytoplasmic expression of TNFα to hepatocytes in all tissue section (arrow). (E) Treated group revealing a strong decrease in expression with moderate cytoplasmic reactivity of hepatocytes to TNFα antibody (arrow). (TNFα Antibody, Magnification Power= x400 and Scale Bar= 50µm).

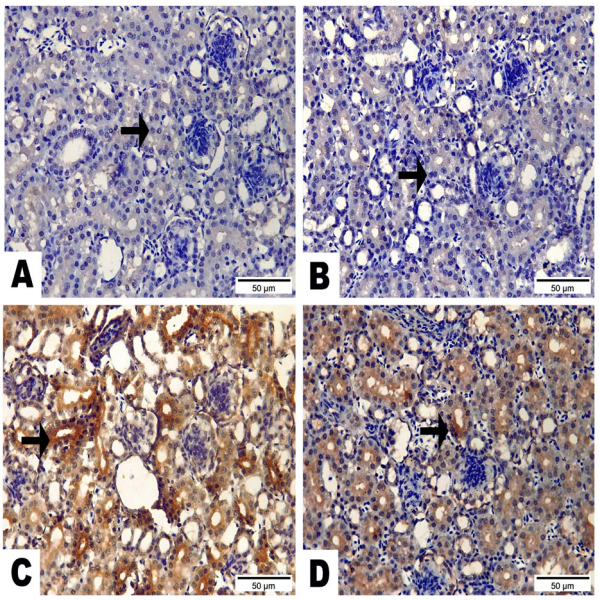

Figure 7. Photomicrographs highlighted the immunoreactivity of Tumor Necrosis Factor Alpha (TNFα) in kidney tissue sections between broilers groups as follows: (A) Negative control group and (B) Herb-All liver Group existing with normal few expressions of TNFα along renal cortex area (arrows). (C) Positive control group emphasizing strong positive cytoplasmic expression of along renal corpuscle and tubules (arrow). (E) Treated group demonstrating obvious reduction in TNFα expression and emerged with moderate cytoplasmic reactivity lining renal corpuscle and tubules (arrow). (TNFα Antibody, Magnification Power= x400 and Scale Bar= 50µm).

=55.96±1.26. Group D demonstrated a significant (P<0.0001) reduction in TNFα expression with moderate cytoplasmic reactivity lining renal corpuscle and tubules compared to groups A and B with mean area% =22.82±1.01 (Fig. 8).

DISCUSSION

In this experiment, chickens were fed either a commercial diet or a diet contaminated with both aflatoxin (30 ppb) and ochratoxin (15 ppb) to induce mycotoxicosis. Herb-All Liver was added to the diets of both groups as a growth promoter and an anti-mycotoxin supplement for six weeks. The FDA restricts aflatoxin levels in food and animal feeds to 20 ppb, while the EU limits aflatoxin levels to 15 ppb (Yang *et al.*, 2020). In Egypt, in 2014 and 2018, El-Nabarawy *et al.* (2020) reported that all the 37 (100%) analyzed compound broiler feed samples were positive for aflatoxin and ochratoxin and their levels ranged from 1 to 55 ppb and 1.8 to 71 ppb, respectively. The combined toxic effects of aflatoxin and ochratoxin in feed and food might pose veterinary and public health risks.

Results of group C fed on the mycotoxicated diet showed the lowest growth performance, which agreed with previous reports (Andretta *et al.*, 2011; Elnabarawy *et al.*, 2020b) which revealed that birds fed mycotoxin-contaminated diets (4-ppb aflatoxin

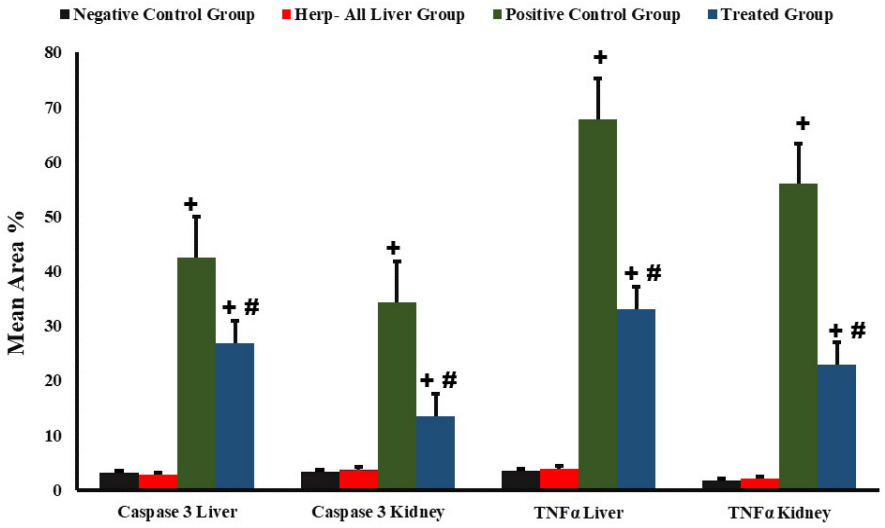

Figure 8. Graph highlighted the mean area% of Caspase-3 and TNF-α immune scoring in liver and kidney tissue sections between broilers groups. Values were expressed as Mean ± SE. (+): reveals significant difference from negative control group, (#): reveals to significant difference from positive control group at ($P < 0.0001$); using One-way ANOVA followed by Tukey's post-hoc test.

and 3-ppb ochratoxin) during the starter and growing periods showed a significant 14% reduction in broiler growth when compared with the control groups. On the other hand, Herb-All Liver significantly enhanced the growth parameters in groups B and D birds. These findings are in line with earlier studies (Bhardwaj *et al*., 2011; Singh *et al*., 2018, Priya *et al*., 2020), which stated that the higher growth efficiency in the broiler chicks supplemented with *T. cordifolia* at the dose rate of 0.5g/Kg body weight is due to tinosporine, the active principles of *T. cordifolia*, which limits the metabolic signs of stress and alleviate its physiological consequences. Moreover, *A. paniculata* diet supplementation either in a single form (30%) or in an herbal combination (with a lower dosage of 15 mg per kg of feed) improved broiler performance (Hidanah *et al*., 2020; Jahja *et al*., 2023). The dietary polyherbal combination could shift the intestinal microbiota profile towards beneficial bacteria such as *Lactobacillus* spp. and reduce the pathogenic bacteria.

In intensive production systems, litter condition plays a crucial role in bird welfare. The current study found that mycotoxicated birds (group C) had extremely moist litter (45%) with high nitrogen content compared to other groups. Once litter moisture exceeds 250 g/kg (25%), its cushioning, insulating and water-holding capacity is compromised (Collett, 2012). The increased water excretion could result from nutritionally induced pathological changes, which compromise water recovery and aggravate polyuria or cause enteritis and diarrhoea (Collett, 2012). Ochratoxin A has been commonly associated with ochratoxin-induced wet litter in birds due to renal pathology resulting in polyuria. Ochratoxin A has also been associated with catarrhal enteritis, malabsorption, and diarrhoea (Hoer, 2003). Additionally, the herbal supplement in the broiler diet (groups B and D) reduced the excreted total nitrogen (N) content of the litter. Dietary supplements to broiler diet could help decrease litter nitrogen content (Ismael *et al*., 2022) and consequently reduces ammonia emissions to the environment, besides reducing ammonia's harmful effects on birds. Our results agreed with Park and Kim (2020), who stated that birds' excreta ammonia emissions decreased as dietary herbal supplementation increased (linear, $P<0.05$).

Additionally, results of liver and kidney functions revealed better liver enzymes and kidney functions in groups supplemented with Herb-All compared to groups supplemented with basal diet alone, which highlighted its positive effect. *A. paniculata* demonstrated protection of the liver from damage brought by substances with various hepatotoxic pathways, indicating that *A. paniculata* and its contents may not be agent-specific and may have general hepatoprotective properties (Ding *et al*., 2014). Blood indices of group D were improved and approached the negative control (group A), assuring the useful influence of the Herb All supplement. These observations were supported by Sharma and Pandey (2010) report, in which the administration of *Tinospora cordifolia* extract with aflatoxin B1 simultaneously had a substantial impact on nearly all blood parameters. *Tinospora cordifolia* has been described as a blood purifier in Ayurvedic literature (Kirtikar and Basu, 1993). It may work by activating the liver and spleen, which eliminates damaged and faulty RBCs from peripheral blood circulation.

The recorded HI results revealed that the positive control group (mycotoxicated birds) experienced low antibody response to NDV vaccination. Elnabarawy *et al*. (2016) confirmed that the vaccinal immunity in properly NDV-vaccinated flocks was broken down due to the potent immune suppressive effect of mycotoxins contamination in feed. Mycotoxins cause aplasia of the bursa of Fabricius, thymus, and spleen in chicken, which markedly decreases the cellular and antibody responsiveness of the immune system (Karaman *et al*., 2005). However, in group D, when the mycotoxin-contaminated diet was supplemented with the herbal mix, the antibody levels were improved and resembled that of the negative control. Findings agreed with Kapil and Sharma (1997), who indicated that the immunopotentiating effect of *T. cordifolia* was due to the augmentation of IgG antibodies and that their impact was dose dependent. Additionally, the polyherbal supplement, which contained *A. paniculata* increased the levels of serum IgG in broiler birds, suggesting that it can enhance immunity by protecting the immune system from damage and suppressing the production of inflammatory cytokines (Gao *et al*., 2022).

The histological changes in the mycotoxicated group were similar to previous research (Aneesh *et al*., 2021; Zabiulla *et al*., 2021), with liver tissues displaying degenerative changes and hepatocytes showing significant positive nuclear and cytoplasmic expressions of caspase-3 and TNF-α. The proliferation of hepatocytes is inhibited by the cytotoxic effect of mycotoxins (Yarru *et al*., 2009). Hence, hepatocytes grow and swell through the accumulation of metabolic products (Abdel-Wahhab *et al*., 2002). As well, cytoplasmic vacuolations could be attributable to impaired lipid transport, macromolecular damage of cells, the oxidative loss of deoxyribonucleic acid (Gholami-Ahangaran *et al*., 2016; Hassanen *et al*., 2023), aggregation of calcium in hepatocytes and thus mitochondrial dysfunction coupled with diminished production of adenosine triphosphate (Lakkawar, 2015). Moreover, the hyperplasia of bile duct epithelium that has been reported, may be caused by the direct impact of mycotoxins on biliary epithelial cells or by an excessive amount of prostaglandin synthesis (Hashem and Mohamed, 2009).

Findings in the mycotoxicated group supplemented with Herb-all liver (D) revealed an improvement and restoration of liver tissue architecture. Additionally, the herbal mix significant-

ly reduced hepatocyte nuclear and cytoplasmic expressions of caspase-3 and TNFα. These results confirm the hepatoprotective effect of *Andrographis paniculata* and its ability to inhibit serious liver injury and decline the severity of mycotoxins (Sonwane *et al*., 2019; Rajendrakumar *et al*., 2020). The hepatoprotective proficiency of *Andrographis paniculata* might be due to the antioxidant ability of its flavonoid's constituent, which enriches hepatic glutathione peroxidase, superoxide dismutase and glutathione-S transferase activities (Aneesh *et al*., 2018). Herb-all liver supplement group (B) showed identical liver immunoreactivity of Caspase-3 and TNF-α scores to the negative control, confirming previous data reported by Zabiulla *et al*. (2021).

The Herb-all liver supplemented group exhibited a normal appearance of the renal cortex, while the mycotoxicated group (C) showed severe degeneration, loss of normal structure, necrotic changes, and strong positive nuclear and cytoplasmic expressions of Caspase-3 and TNFα as previously reported (Zabiulla *et al*., 2021; Hassanen *et al*., 2023). Mycotoxins are eradicated across the kidneys, and the gathering of a high concentration of toxins restricts the transportation of tubular cells, leading to impaired excretory function and nephrotoxicity (Sharma *et al*., 2011). The kidneys of the mycotoxicated group supplemented with Herb-all liver (D) displayed obvious tissue recovery with normal renal corpuscle except glomerulus detected with few vacuolations. Also, a noticeable reduction in Caspase-3 and TNF-α expressions in cells lining renal corpuscle and tubules was indicated, which was attributed to Andrographolide's antioxidant properties (Sivakumar and Rajeshkumar, 2015).

CONCLUSION

Both healthy birds and those fed a mycotoxin-contaminated diet showed notable improvements in body weights, FCR, blood indices, antibody titers, and organ functions when supplementing their diets with natural herbs. Dietary natural herbs could enhance litter quality by enhancing intestinal nutrient utilization, gut health, and kidney function. Liver and kidney histological architectures could be restored, with enhanced expression levels of cellular Caspase-3 and TNF-α by the hepatoprotective and nephroprotective effects of *Andrographis paniculata* and *Tinospora cordifolia* herbs. Conclusively, using natural herb mixtures (Herb-All™ Liver) as feed additives can ameliorate the harmful effects of aflatoxin and ochratoxin in broilers.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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