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Research Article
Protective Effect of *Urtica dioica* Seeds' Extract in Experimental Chronic Aflatoxicosis in Broiler Chickens

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Abstract

Background and Objective: Aflatoxins can lead to Aflatoxicosis in animals and humans. The aim of this study was to investigate hepatoprotective and antioxidant effect of *Urtica dioica* seeds' extract (UDSE) on chronic aflatoxicosis in broiler chickens, which were evaluated by histopathologically liver degeneration and activities/levels of the oxidative stress and antioxidant parameters. **Materials and Methods:** Thirty-two broiler chickens were randomly divided into four experimental groups: control group served as a negative control and were fed with a normal diet; AFB_1 group received aflatoxin B_1 (AFB_1) (1 mg kg^{-1} diet) for 42 days; UDSE group received UDSE (30 mL kg^{-1} diet) for 42 days and AFB_1+UDSE group received AFB_1 (1 mg kg^{-1} diet) as well as UDS extract (30 mL kg^{-1} diet) for 42 days. **Results:** In the AFB_1 group, activities of SOD, GSH-Px and CAT and the levels of TAS and BcL-2 in liver and serum were significantly lower than that of the control group ($p<0.001$), TOS and Caspase-3 levels were also significantly high ($p<0.001$). Whereas, in the AFB_1+UDSE group, the activity of GSH-Px in liver and serum and the activities of SOD and CAT and the level of TAS in serum were significantly higher than those in AFB_1 group ($p<0.001$). In the AFB_1+UDSE group, the levels of TOS and Caspase-3 were significantly lower ($p<0.001$) and the level of BcL-2 were higher in both liver and serum compared to those of the AFB_1 group ($p<0.001$). Histopathologically, diffuse hydropic degenerations, periportal fibrosis and activation of perisinusoidal myofibroblastic (*Ito*) cells were observed in the AFB_1 group. These morphological changes were reduced significantly in the AFB_1+UDSE group. **Conclusion:** The UDSE has a protective effect on chronic aflatoxicosis in broiler, which appear to be attributable to its antioxidant and anti-apoptotic activity.

Key words: Aflatoxicosis, antioxidant activity, anti-apoptotic activity, hepatoprotection, oxidative stress

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Toxicity caused by contamination of mycotoxins in food is one of the main problems in the poultry industry[1]. The most dangerous mycotoxins are aflatoxins, especially aflatoxin B_1 (AFB_1), which are produced naturally by several species of Aspergillus in agricultural products. Aflatoxins are the most researched mycotoxin group in the sensitive laboratories due to their toxic and carcinogenic effects in farm animals, acute toxicological and chronic hepatocarcinogenic effects in humans[2].

Aflatoxicosis causes damage to the liver, kidney and digestive system in the poultry, suppression of the immune system and, consequently, an increased sensitivity to many diseases. The liver, which performs the biotransformation and detoxification of various xenobiotics including steroids, eicosanoids, pharmaceuticals, pesticides, pollutants and carcinogens in the body, is the organ primarily affected by aflatoxicosis[3]. Toxication causes the enlargement, pallor and congestion of the liver and degeneration and necrosis in hepatocytes, proliferation in the bile duct[4]. Aflatoxicosis causes nephrosis in the kidney and impaired glomerular filtration and kidney function[5,6]. It impairs intestinal morphology, digestive ability, activities of digestive enzymes, intestinal microflora and intestinal natural immunity in the digestive system[7,8,9]. It causes damage to the lymphoid tissues such as thymus, bursa fabricus, spleen and bone marrow and suppression of phagocytic activity in the immune system, ultimately it reduces their disease resistance and humoral defence functions[2,10]. Additionally, it causes a decrease in feed consumption, growth rate, total body weight and egg production due to these systemic effects of AFB_1[11].

Physical, chemical and biological methods are used for detoxification of AFB_1 in feeds. Because AFB_1 causes cell damage, free radical production and lipid peroxidation, it has been reported that the use of antioxidants known as superoxide anion cleansers may aid the overall detoxification process in the liver. For this reason, it has been suggested that the use of adsorbents and antioxidants in the diet may be effective, economical and practical to prevent aflatoxicosis in poultry[12].

Because of its rich composition, nettle is one of the most researched natural antioxidants[13]. Previous studies showed that nettle has various pharmacological activities such as immunomodulator, antioxidant[14-16], antibacterial[15], anti-viral[17], analgesic, anti-inflammatory[18-20], antihypertensive, anti-hyperlipidemic and antidiabetic[21-23], hepatoprotective[24,25] and anticarcinogenic[26]. In addition, in a previous study[27] with a trial period of 21 days, *U. dioica* extract was added to food against the aflatoxicosis and very positive results have been reported. In the present study, we aimed to investigate protective and antioxidant effects of *Urtica dioica* seeds' extract on chronic aflatoxicosis in broiler chickens with a trial period of 42 days, which were evaluated by histopathologically liver degeneration, biochemically oxidative stress and antioxidant parameters, anti-apoptotic factor BcL-2 and Caspase-3 and immunohistochemically actin filaments in the liver tissue.

MATERIALS AND METHODS

Chemicals: Sodium hydrogen phosphate (Na_2HPO_4) to prepare phosphate buffer as well as aflatoxin were purchased from Sigma Aldrich Company (St. Louis, MO, United States). ELISA kits for all biochemical parameters measured in liver and serum samples were purchased from YL Biotech Co., Ltd. (Shanghai, China).

Grouping of experimental animals and nutrition program: In this study, 32 Avian breed broiler chicks, aged one day and without vaccine, were used. The ethics committee approval for the study was obtained from the Adana Veterinary Control Institute Experimental Animals Local Ethics Committee (approval protocol number: 2019-2/390). The chicks having equal live body weight were randomly divided into 4 equal groups. The groups were given water with 5% sugar for the first 4-5 h and for the next 42 days, they were given *ad libitum* access to water and food. During this period, birds were housed in compartments at a constant ambient temperature of 22°C with a 12 h light/dark cycle.

The following were the four animal groups:

- **Control group (N = 8):** This group served as a negative control and broiler chicks in this group were fed only standard diet during the whole study period
- **AFB_1 group (N = 8):** The broiler chicks in this group were fed with feed containing AFB_1 (1 mg of AF kg^{-1} of food) for 42 days
- **UDSE group (N = 8):** Broiler chicks in this group were fed with feed containing UDSE (30 mL kg^{-1} of food) for 42 days.
- **AFB_1+UDSE group (N = 8):** The broiler chicks in this group were fed with AFB_1 (1 mg of AF kg^{-1} of food) and UDSE (30 mL kg^{-1} of food) for 42 days

All the experimental chicks were weighed at the beginning of the experiment. Total feed consumptions were measured and recorded on days 7th, 14th and 21sd, 28th, 35th and 42th.

Table 1: Effect of aflatoxin B_1 (AFB_1) and/or *Urtica dioica* seeds' extract on weekly body weight gain (BWG, g $bird^{-1}$) of the broilers

	Control	Aflatoxin (1 mg kg^{-1})	UDSE (30 mL kg^{-1})	Aflatoxin+UDSE (1 mg kg^{-1}+30 mL kg^{-1})	P
Baseline	45.63±0.74	46.19±1.21	46.23±0.66	47.10±0.76	-
7th day	175.15±4.85	154.20±6.24[a]	163.60±5.16	167.50±6.04	**
14th day	426.89±12.76	324.10±18.5[b]	374.34±26.16	390.59±19.78	0
21st day	788.23±20.23	638.65±34.22[a]	684.10±39.57	700.15±43.69	**
28th day	1235.12±19.25	113745±23.12[a]	1278.00±35.85	1187.21±35.79	**
35th day	1845.17±21.25	1680.15±20.10[a]	1925.15±20.45	1790.30±40.70	**
42th day	2445.25±15.30	2145.25±25.15[a]	2510.25±30.15	2340.20±30.45	**

[a]Significantly different (**p<0.001, *p<0.01) when compared to control values. [b]Significantly different (p<0.05) when compared to control and Aflatoxin+UDSE groups

Creating feed with aflatoxin: One mg of AFB_1 was added to one kilogram of commercial feed, which was found to have no aflatoxin by laboratory analysis (mg kg^{-1}).

Plant materials and extraction procedure: Urtica dioica seeds were used as plant material. The seeds were ground in an electric mill and their natural oil was extracted in a rotary evaporator using diethyl ether as solvent[28]. The extract (30 mL kg^{-1}) was added to the feed.

Collection of serum samples: At the end of the trial period blood samples were collected into yellow-capped biochemistry tubes and broilers were euthanized via cervical dislocation. For biochemical analysis, serum samples were obtained by centrifuging of blood at 3000 xg for 15 min. at +4°C.

Homogenization of liver tissues and supernatant production: Only 1.8 mL of phosphate buffer (50 mM) with a pH of 6.7 and was added to 200 mg of tissue and homogenized for about 15 s with a homogenizer. The mixture was then centrifuged at 3000 xg and +4°C for 30 min. The supernatant in upper phase was collected and then put in another tube and kept at -80°C for the following analysis.

Biochemical measurements in serum and liver samples: The activities of SOD, GSH-Px and CAT and the levels of TAS, TOS, BcL-2 and Caspase-3 in the serum and liver tissue homogenates were measured with commercially purchased enzyme-linked immunosorbent assay (ELISA) kits (ELISA, YL biont, Shanghai YL Biotech Co., Ltd.) in accordance with the package insert.

Histopathological examination: At the end of the experiment, necropsies of the subjects were performed and the liver tissue samples, which is considered as the main target organ for AFB_1 toxicity, were collected. The macroscopic changes were observed and recorded. The liver tissue samples were fixed in a 10% formalin solution and were then embedded in paraffin blocks. After that 4 μm sections were taken with a rotary microtome. The sections were stained with hematoxylin-eosin (HE) and, when needed, some sections were stained with Masson's trichrome and Sudan black dyes and then examined under the microscope.

Immunohistochemical examination: In immunohistochemical examination, the expression of alpha-smooth muscle actin (ASTHMA) were determined using the streptavidin/biotin immunoperoxidase kit (Histostain-Plus Bulk Kit; Zymed, South San Francisco, CA, USA) according to the streptavidin-peroxidase method (ABC). After the sections were taken on adhesive slides, they were passed through xylene and alcohol series. The sections were washed with phosphate buffer solution (PBS) and incubated in 3% H_2O_2 for 20 minutes for inactivation of endogenous peroxidase. The sections were placed in the antigen retrieval solution (citrate buffer) and covered and then heat-treated twice for 20 min. After this, they were taken out of the oven, it was waited until reached at the room temperature and then the tissues were washed again with PBS, they were blocked by protein blocking (non-immune serum) for 20 minutes. Polyclonal antibodies of ASTHMA (Novocastra-NCL-L-CD8-4B11, 1/100 dilution) were added to the tissues and incubated overnight at +4°C. After that, the sections were washed with PBS and incubated for 20 min at room temperature with the biotinylated secondary antibody. The sections washed again with PBS and then kept in streptavidin-peroxidase for 20 min. After washing with PBS, diaminobenzidine (DAB) was dropped and incubated for 1-2 min. Then all tissues were kept in Mayer's hematoxylin for 1-2 min and washed in tap water. The sections were passed again through the alcohol and xylene series and were then covered with Entellan. Negative controls were used to confirm staining. These slides were reacted with PBS instead of primary antibodies. Sections were examined and photographed under a light microscope (Nikon 80i-DS-RI2).

Statistical analysis for biochemical data: All statistical analyses were performed using the Statistical Package for

Social Science (SPSS) version 21.0 for windows (SPSS Inc., Chicago, IL, USA). Descriptive statistics of the groups were expressed as mean and standard deviation. Kruskal-Wallis non-parametric test was used to determine whether the differences between the groups were significant in the same parameter. Post-hoc test (multiple comparison testes) was performed to determine from which group the significant differences were originated. Values of p less than 0.05 were considered significant.

RESULTS

Live weight data: The effects of AFB_1-containing feed with and without UDSE on live body weight gain (BWG) of the broilers after feeding for up to 6 weeks are summarized in Table 1. The average live weight of the groups was similar at the beginning of the study (day 0). The body weight gain was significantly lower in the aflatoxin treated groups than that of the control group at all weekly measurements ($p<0.05$). However, improvement was observed in body weight gain of broiler chicks fed with AFB_1 and UDSE; there were no significant difference in terms of live body weight between AFB_1+UDSE group and the control group at any weekly measurements ($p>0.05$).

Biochemical data: In order to evaluate protective and antioxidant effects of *Urtica dioica* seeds' extract on chronic aflatoxicosis in broiler chickens, activities of SOD, GSH-Px and CAT and the levels of TAS, TOS, BcL-2 and Caspase-3 were measured in the liver homogenates as well as the serum. Effect of aflatoxin B_1 with and without *Urtica dioica* seeds' extract on the activities of SOD, GSH-Px and CAT and the levels of TAS, TOS, BcL-2 and Caspase-3 in the serum and the liver tissue is shown in Table 2 and Table 3 respectively.

In the liver homogenate, the activities of antioxidant enzymes (SOD, GSH-Px and CAT) and the levels of TAS and anti-apoptotic factor BcL-2 in AFB_1 group were significantly lower than that of the control group ($p<0.001$) and the levels of TOS and caspase-3 were significantly higher in AFB_1 group than that of the control group ($p<0.001$) (Table 2 and Fig. 1). In addition, the activities of SOD, GSH-Px and CAT and the level of TAS in the UDSE group were significantly higher than those of the control and AFB_1 groups ($p<0.001$). However, as in AFB_1+UDSE groups, the administration of *Urtica dioica* seeds' extract as well as AFB_1 significantly elevated activities of hepatic SOD and GSH-Px and CAT ($p<0.001$) compared to AFB_1 group. Note that, the level of TOS in AFB_1 group was higher than those in all other groups ($p<0.001$) and with the addition of UDSE, in AFB_1+UDSE group the level of TOS was lower compared to AFB_1 group ($p>0.001$).

Table 2: Effect of aflatoxin B_1 and/or *Urtica dioica* seeds' extract on the activities of SOD, GSH-Px and CAT and the levels of TAS, TOS, BcL-2 and Caspase-3 in the liver tissue

Parameters	Control	AFB_1 (1mg kg^{-1})	UDSE (30 mL kg^{-1})	AFB_1 +UDSE (1 mg kg^{-1} +30 mL kg^{-1})	p-value
SOD	4.103±0.389	3.088±0.193*	4.228±0.289$^{\varepsilon}$	3.783±0.469$^{\varepsilon}$	<0.001
GSH-Px	1.225±0.054	1.003±0.048*	1.354±0.078$^{\varepsilon}$	1.154±0.082$^{\varepsilon,\theta}$	<0.001
CAT	0.943±0.055	0.748±0.046*	1.013±0.071$^{\varepsilon}$	0.902±0.090$^{\varepsilon}$	<0.001
TAS	2.743±0.181	2.068±0.163*	2.912±0.381$^{\varepsilon}$	2.397±0.205$^{\theta}$	<0.001
TOS	0.496±0.028	0.666±0.053$^{\#}$	0.416±0.033$^{\varepsilon}$	0.501±0.068	<0.001
BcL-2	0.675±0.032	0.510±0.057*	0.675±0.053$^{\varepsilon}$	0.583±0.037$^{\theta,*}$	<0.001
Caspase-3	0.438±0.040	0.507±0.019*	0.364±0.024$^{\varepsilon,*}$	0.461±0.040$^{\theta}$	<0.001

*Significant when compared to control group ($p<0.001$), $^{\varepsilon}$Significant when compared to AFB_1 group ($p<0.001$), $^{\theta}$significant when compared to UDSE group ($p<0.001$), $^{\#}$: significant when compared to other groups ($p<0.001$). SOD: Superoxide dismutase (mU mg^{-1} protein), GSH-Px: glutathione peroxidase (ng mg^{-1} protein), CAT: Catalase (ng mg^{-1} protein), TAS: total antioxidant status (pg mg^{-1} protein), TOS: Total oxidative stress (pg mg^{-1} protein), BcL-2: Antiapoptotic gene (ng mg^{-1} protein), caspase-3 (pmol mg^{-1} protein)

Table 3: Effect of aflatoxin B_1 and/or *Urtica dioica* seeds' extract on the activities of SOD, GSH-Px and CAT and the levels of TAS, TOS, BcL-2 and Caspase-3 measured in the serum samples

Parameters	Control	AFB_1 (1 mg kg^{-1})	UDSE (30 mL kg^{-1})	AFB_1+UDSE (1 mg kg^{-1} +30 mL kg^{-1})	p-value
SOD	50.92±7.06	34.21±2.47*	84.50±9.46$^{*,\varepsilon,\Phi}$	38.96±1.12*	0.001
GSH-Px	61.05±1.28	40.20±4.95*	63.06±1.44$^{\varepsilon,\Phi}$	42.68±4.87	0.001
CAT	10.50±0.80	6.95±0.40*	13.77±2.26$^{*,\varepsilon,\Phi}$	8.40±0.54	0.001
TAS	26.47±1.92	18.01±1.04*	28.42±3.67$^{\varepsilon}$	22.10±2.06	0.001
TOS	5.10±0.40	8.40±1.33$^{\#}$	4.54±0.33	6.67±0.53*	0.001
BcL-2	5.07±0.50	3.00±0.17*	5.42±0.73$^{\varepsilon,\Phi}$	4.60±0.31$^{\varepsilon}$	0.001
Caspase-3	2.78±0.53	4.37±0.51*	2.13±0.20$^{\varepsilon,\Phi}$	4.06±0.53$^{*,\#}$	0.001

*Significant when compared to control group ($p<0.001$), $^{\varepsilon}$Significant when compared to AFB_1 group ($p<0.001$), $^{\Phi}$Significant when compared to AFB_1 +UDSE group ($p<0.001$), $^{\#}$Significant when compared to control and UDSE groups ($p<0.001$). SOD: Superoxide dismutase (mU mg^{-1} protein), GSH-Px: Glutathione peroxidase (ng mg^{-1} protein), CAT: Catalase (ng mg^{-1} protein), TAS: Total antioxidant status (pg mg^{-1} protein), TOS: total oxidative stress (pg mg^{-1} protein), BcL-2: Antiapoptotic gene (ng mg^{-1} protein), caspase-3 (pmol mg^{-1} protein)

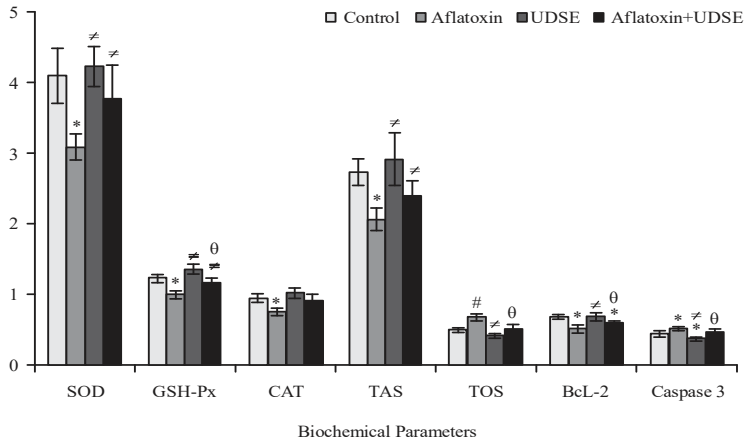

Fig. 1: Comparison of the results of biochemical parameters measured in liver tissue

*p: They are significant according to the control group (p = 0.001), ᵃp: They are significant according to the aflatoxin group (p = 0.001), ᵇp: They are significant according to the UDSE group (p = 0.001), ᵗp: It is significant according to the other groups (p = 0.001). UDSE: *Urtica dioica* seed extract, SOD: Superoxide dismutase (mU mg^{-1} protein), GSH-Px: Glutathione peroxidase (ng mg^{-1} protein), CAT: catalase (ng mg^{-1} protein), TAS: Total antioxidant status (pg mg^{-1} protein), TOS: Total oxidative stress (pg mg^{-1} protein), BcL-2: Antiapoptotic gene (ng mg^{-1} protein), caspase-3 (pmol mg^{-1} protein)

AFB_1 group had a lower level of BcL-2 in liver tissue compared to the control group ($p<0.001$). Nevertheless, the group treated with UDSE significantly reversed the decrease induced by AFB_1 ($p<0.001$). Aflatoxin treatment in AFB_1 group significantly increased the level of the apoptotic factor, caspase-3 compared to the control group ($p<0.001$). This effect was reversed to some extents when UDSE was administered as in AFB_1+UDSE group; there were no significant difference in levels of caspase-3 between the control and AFB_1+UDSE groups ($p>0.05$). However, although the level of caspase-3 of the AFB_1+UDSE group were lower than that of the AFB_1 group, it was not significant ($p> 0.05$). In addition, the level of caspase-3 in UDSE group was markedly lower than those of the control and AFB_1 groups ($p<0.001$).

The measurements of the activities of SOD, GSH-Px and CAT and the levels of TAS, TOS, BcL-2 and Caspase-3 from the serum samples were comparable with those measurements taken from the liver homogenate as summarized in Table 3 and Fig. 2. Treatment with AFB_1 produced a dramatic decrease in the activities of SOD, GSH-P and CAT and the levels of TAS and BcL-2 in AFB_1 group along with a significant elevation in the levels of TOS and caspase-3 values compared to the control group ($p<0.001$). Whereas it was noteworthy that serum SOD levels in UDSE-supplemented group were significantly higher than those of the other groups, even the control group ($p<0.001$). However, the activity of SOD in AFB_1 and AFB_1+UDSE group were lower than that of the control group ($p<0.001$). However, there was no significant difference between them ($p>0.05$). In addition, this situation was similar for GSH-Px, CAT and TAS values in AFB_1 and AFB_1 +UDSE groups.

The level of the serum TOS was significantly higher in the AFB_1 group compared to the control group ($p>0.001$) and was lower in the AFB_1 +UDSE group compared to the AFB_1 group, which was not significant ($p>0.05$) though.

Serum caspase-3 value in the AFB_1 group were significantly higher than that of the control group ($p<0.001$). However, although the caspase-3 value in the AFB_1+UDSE group was lower than that of the AFB_1 group, it was not significant ($p>0.05$). AFB_1 treatment significantly decreased the level of anti-apoptotic factor BcL-2 as compared to control group ($p<0.001$). However, UDSE treatment along with AFB_1 significantly reversed the change induced by AFB_1 treatment. BcL-2 values in AFB_1+UDSE group were significantly higher than that of the AFB_1 group ($p<0.001$).

Macroscopic findings: The livers of the broilers in the aflatoxin group were larger and the edges were blunt and yellowish in colour and crisp consistency (Fig. 3c) in contrast to the normal appearances of the livers of the control (Fig. 3a) and UDSE (Fig. 3b) group of broilers. However, it was noteworthy that the livers of the animals in the AFB_1 +UDSE group were comparable to those of the control group (Fig. 3d).

Histopathological findings: The livers had normal histological appearances in the control and UDSE groups (Fig. 4a and 4b). The main morphological changes in the livers in the AFB_1 group were hydropic and fatty degenerations, which were more severe in the lobes, especially in the periacinar regions (Fig. 4c). The degenerated hepatocytes ranged from slightly

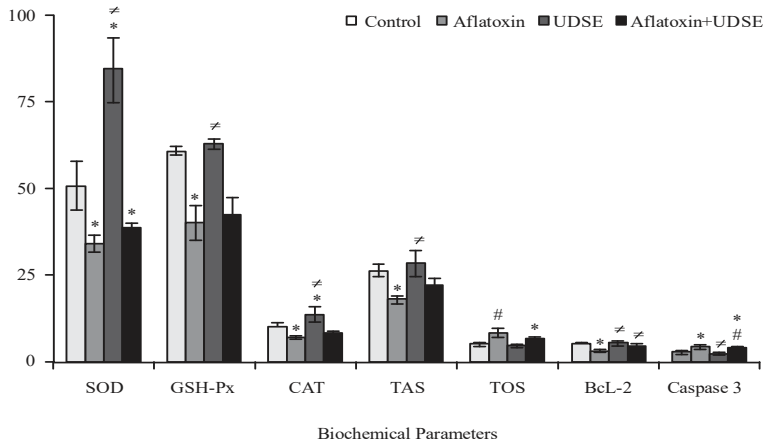

Fig. 2: Comparison of biochemical parameter values measured in serum samples

*p: They are significant according to the control group (p = 0.001), ≠p: They are significant according to the aflatoxin group (p = 0.001), #p: It is significant according to the control and UDSE groups (p = 0.001). UDSE: *Urtica dioica* seed extract, SOD: Superoxide dismutase (mU mg^{-1} protein), GSH-Px: Glutathione peroxidase (ng mg^{-1} protein), CAT: Catalase (ng mg^{-1} protein), TAS: Total antioxidant status (pg mg^{-1} protein), TOS: Total oxidative stress (pg mg^{-1} protein), BcL-2: Antiapoptotic gene (ng mg^{-1} protein), caspase-3 (pmol mg^{-1} protein)

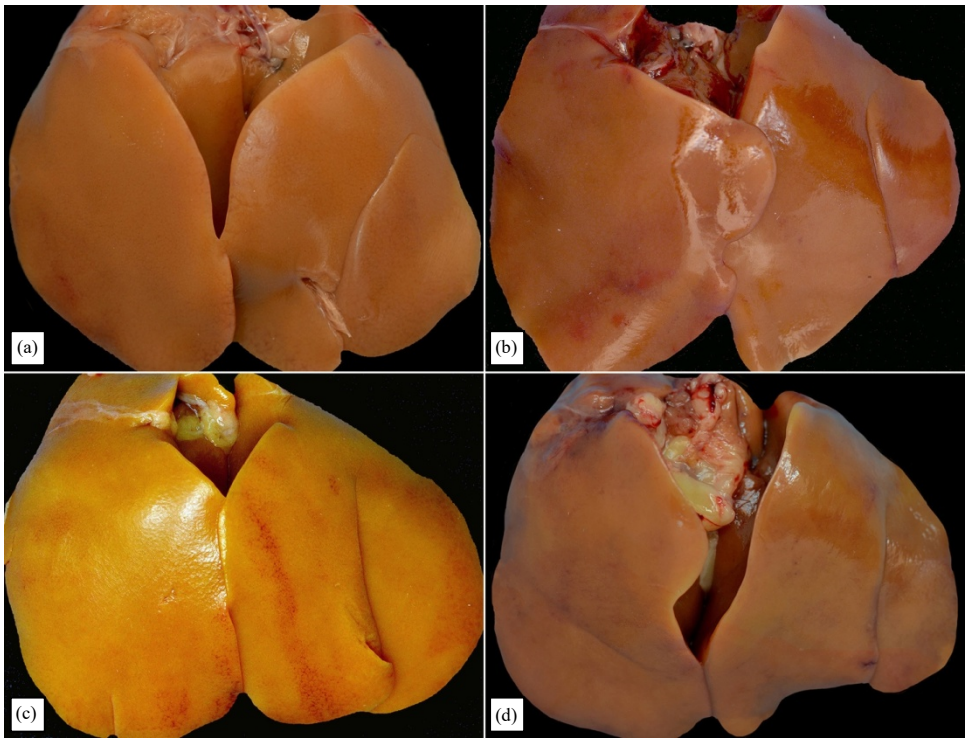

Fig. 3(a-d): Total representation of macroscopic images for all groups

The normal view of the broiler liver of the control (a) and UDSE group (b). AFB_1 group has a distinct yellowish color change (fat degeneration) in the broiler liver (c). AFB_1+UDSE group shows almost the normal appearance of the broiler liver (d)

bulging and light-coloured to severe bubble-like appearance. It was noted that the cytoplasm and nucleus of hepatocytes were pushed towards the cell wall depending on the severity of degeneration. It was also determined that necrosis occurred in the nucleus of severe balloon-like degenerated hepatocytes (Fig. 4d). The nuclei of some hepatocytes were found to be larger (karyomegaly), showing marginal hyperchromia and darker staining (dysplasia in hepatocytes). Regeneration

efforts and mitotic figures were determined in hepatocytes in some regions. As a result of these morphological changes, it was observed that the remark cord structures deteriorated and the sinusoids narrowed. It was noteworthy that activation occurred in perisinusoidal myofibroblastic cells and *Kupffer* cells in some regions and also it was noted that myofibroblastic cell proliferations (fibrotic changes) extend to portal gaps in the form of thin cords from the periacinar regions (Fig. 4e). In periportal regions, it was observed that some regions of hepatocytes formed acinar structures in the ring style. Focal mononuclear cell infiltrations were found in the parenchyma and portal intervals. A large number of proliferated small bile ducts were noticed around the vena porta (Fig. 4f).

As compared to AFB_1 group, the histological evaluation of liver sections of AFB_1+UDSE group showed only minimal morphological changes in hepatic parenchyma (Fig. 4g). However, activation in *Kupffer* cells and focal small lymphoid

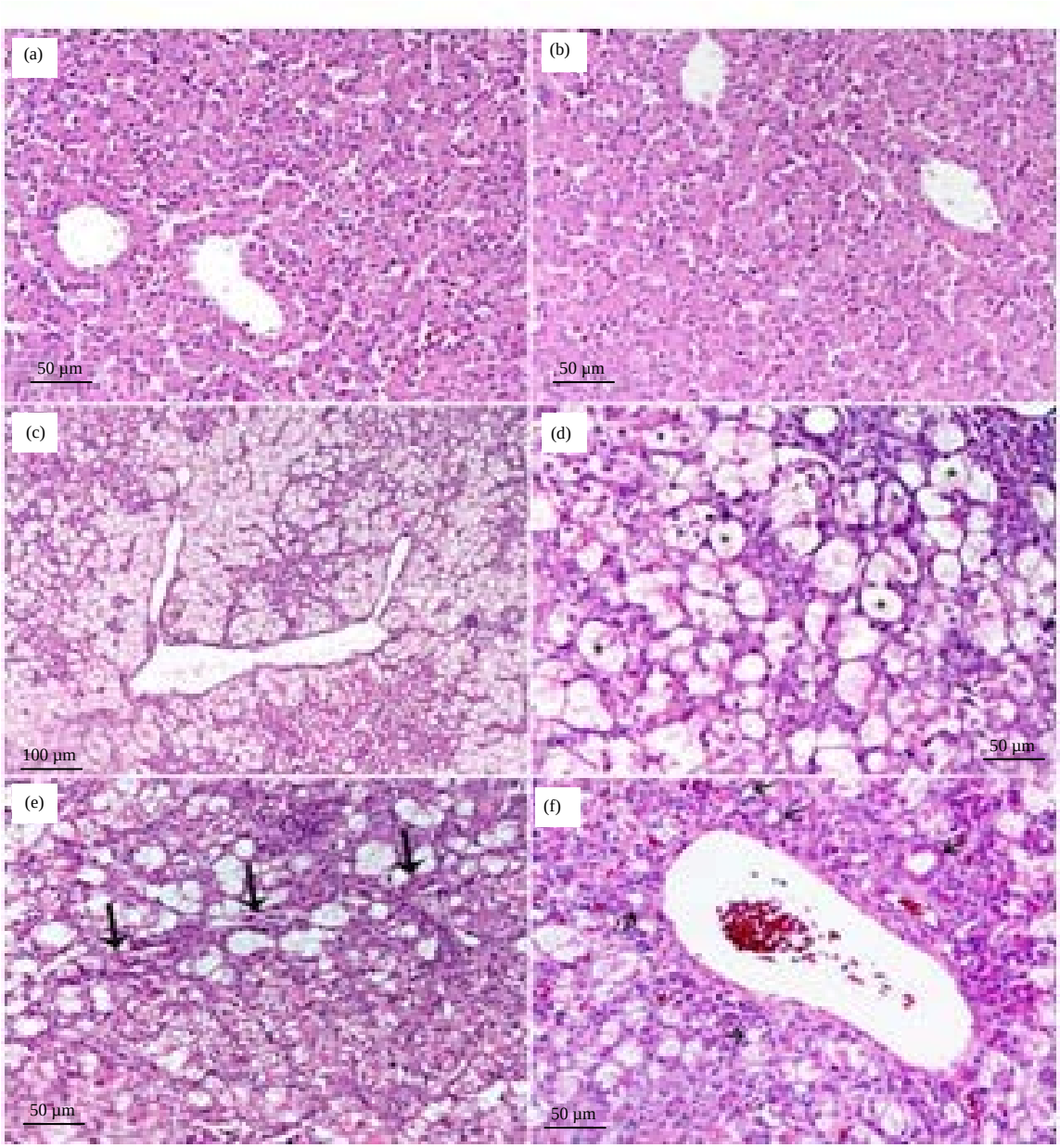

Fig. 4(a-j): Continue

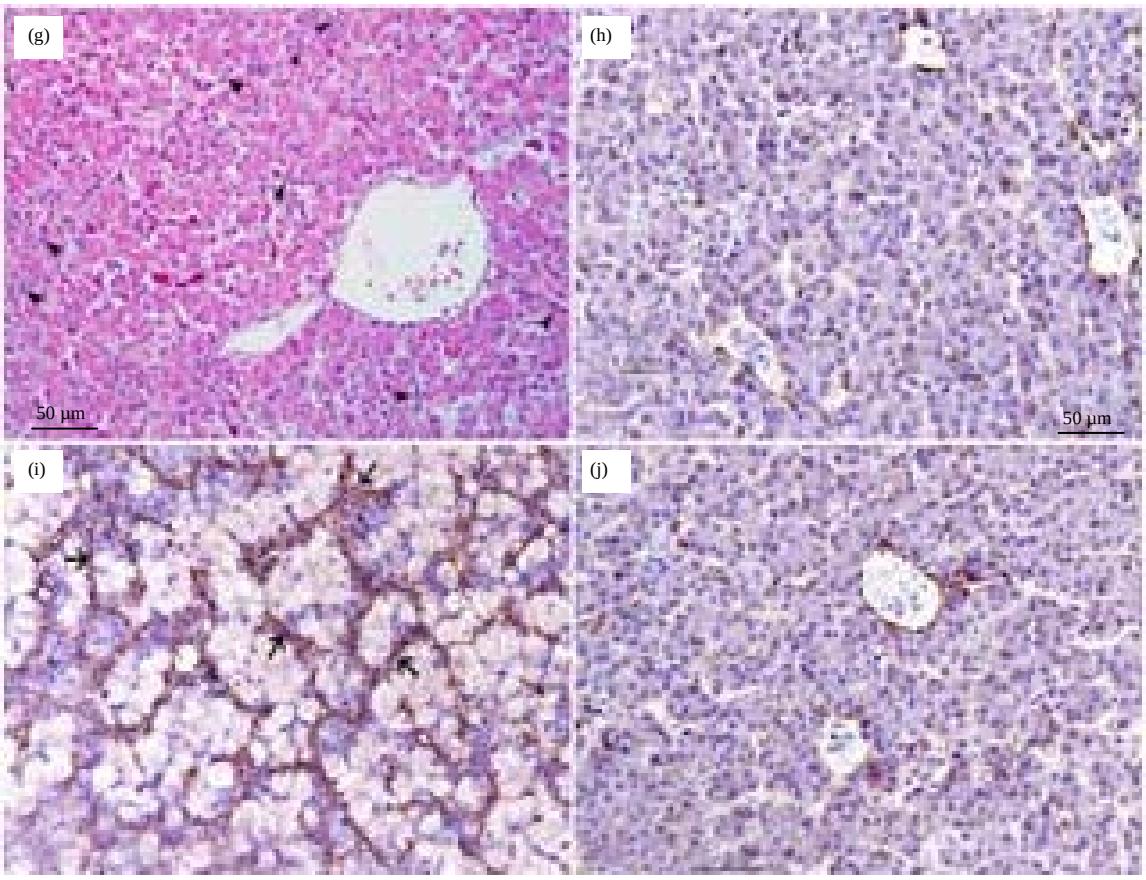

Fig. 4(a-j): Collective representation of microscopic images for all groups

Normal histological view of the liver belonging to the control (a) and UDSE group (b), (H&E. Bar; 50 µm). Widespread severe hydropic and fatty degeneration in hepatocytes belonging to aflatoxin group (H&E. Bar; 100 µm) (c). In AFB_1 group, note that the cytoplasm and nucleus of severe balloon-like degenerate hepatocytes were pushed towards the cell wall and the sinusoids narrowed (stars) (H&E. Bar; 50 µm), (d). Fibrotic changes (arrows) spreading in thin cords in the liver parenchyma, showing the activation of perisinusoidal cells in AFB_1 group (H&E. Bar; 50µm) (e). Numerous proliferating small bile ducts around the portal vein in AFB_1 group (H&E. Bar; 50 µm) (F). Almost normal histological appearance in the liver parenchyma of AFB_1 +UDSE group, but significant activation (arrowheads) in *Kupffer* cells is noteworthy (H&E. Bar; 50 µm) (g). Sparse immunohistochemical positive reaction for alpha-smooth muscle actin in perisinusoidal cells of the control group (Immunoperoxidase staining, Bar; 50 µm) (h). A more pronounced immunoreactivity in narrowed sinusoids around severely degenerated hepatocytes in the AFB_1 group, showing the activation of perisinusoidal myofibroblastic cells (arrows) (Immunoperoxidase staining, Bar; 50 µm), (I). The immunohistochemical reactions in the AFB_1+UDSE group, being an almost similar appearance to those of the control group (Immunoperoxidase staining, Bar; 50 µm) (j).

cell infiltrations were observed in the parenchyma. The main morphological changes detected in the liver are shown in Table 4.

Immunohistochemical findings

Alpha-smooth muscle actin (ASTHMA): Sparse staining was detected in perisinusoidal cells of the control and UDSE groups (Fig. 4h). A more pronounced immunoreactivity in sinusoids around severely degenerated hepatocytes was determined in the AFB_1 group, showing the activation of perisinusoidal myofibroblastic cells (Fig. 4I). The immunohistochemical reactions in the AFB_1+UDSE group, control and UDSE groups were almost similar (Fig. 4j).

DISCUSSION

The present study showed antioxidant and hepatoprotective effects of *Urtica dioica* seeds' extract on chronic aflatoxicosis in broiler chickens. The broiler chickens fed with aflatoxin B_1 (AFB_1) developed significant hepatic damage as manifested by diffuse hydropic degenerations, periportal fibrosis and activation of perisinusoidal

Table 4: Comparison of histopathological lesions observed in the liver

Liver lesions		Control	Aflatoxin	UDSE	Aflatoxin+UDSE	p-values
Degeneration		-/8[b]	8/8[a]	-/8[b]	-/8[a]	*
	Mild	*	0	*	*	
	Moderate	*	3	*	*	
	Severe	*	5	*	*	
Myofibroblastic proliferation		-/8[b]	8/8[a]	-/8[b]	5/8[ab]	**
	Mild	*	0	*	5	
	Moderate	*	4	*	*	
	Severe	*	4	*	*	
Bile duct proliferation		-/8[b]	8/8[a]	-/8[b]	-/8[b]	*
	Fibrosis	-/8[b]	8/8[a]	-/8[b]	-/8[b]	*
	Mild	*	0	*	*	
	Moderate	*	4	*	*	
	Severe	*	4	*	*	
Dysplasia in hepatocytes		-/8[b]	4/8[a]	-/8[b]	-/8[b]	*
	Mild	*	4	*	*	
	Moderate	*	*	*	*	
	Severe	*	*	*	*	

[ab]The difference between the values carrying different letters in the same line are significant, ($p<0.05$) . *$p<0.05$

myofibroblastic (*Ito*) cells. Aflatoxin B_1 treatment also decreased the activities of SOD, GSH-Px and CAT and the levels of TAS and BcL-2 and increased of the levels of TAS and caspase-3 in liver tissue and serum. Administration of *Urtica dioica* seeds' extract restored the changes induced by aflatoxin B_1 to a large extent.

The most harmful, reactive and electrophilic metabolites of aflatoxins is exo AFB_1-8, 9-epoxide derivative (exo-AFB_1-8,9-epoxide = AFBO)[29]. It has been reported that the main enzyme responsible for the conversion of aflatoxin to aflatoxin-O in poultry is the hepatic microsomal cytochrome CYP-450 enzyme, which is found mainly in the liver, especially in hepatocytes around the vena centralis[29,30]. AFBO can be detoxified by conjugating with endogenous glutathione (GSH) by a reaction catalysed by glutathione S-transferases (GSTs). GSH thus reduces the toxic effects of AFBO and helps it to be expelled from the body. GSH is one of the most important enzymes that protect from potentially toxic chemicals such as drugs and carcinogens[31-33]. The sensitivity of the poultry species to aflatoxin is determined by the inadequate level of conjugation of AFBO by GSH[34]. Among poultry species, it has been noted that turkeys are the most susceptible to aflatoxins, but chickens are relatively more resistant[35].

When AFBO is detoxified, they react with DNA, RNA, functional proteins, enzyme cofactors and chemical structures in the membrane and inhibit biosynthesis pathways and energy production. As a result, by inhibition of protein synthesis in the cell and disruption of cellular integrity the cellular injury occurs in tissues[29-31,36]. Since various toxic substances are metabolized and detoxified by the liver, it is the most affected organ in aflatoxicosis[3,37]). Aflatoxicosis cause a range of pathological changes including necrosis and fatty degeneration especially in hepatocytes in the centrilobular regions and bile duct proliferation and inflammatory cell infiltration in the liver[4] and also fibrosis and hepatocellular carcinoma in chronic cases[38,39].

Aflatoxins have been reported to cause wilt, enlargement and congestion in the liver macroscopically[4,40]. In the study presented here, it was observed that there were significant macro-morphological (enlargement and yellowish colour) changes in the livers of broilers in the aflatoxin group, but the livers of the broiler in the AFB_1 +UDSE group were quite similar to the control group. Morphological changes observed in the present study were consistent with previous studies[27,41]. The finding that the histopathological changes were reversed by treatment with *Urtica dioica* seeds' extract may imply that the *Urtica dioica* seeds' extract added to the feed protects the liver due to its strong antioxidant effect or decreased absorption of aflatoxin by binding of aflatoxin in the gastrointestinal tract. There are evidences presented in the current study for antioxidant effect, but detailed studies are needed regarding the binding of aflatoxin, especially in the gastrointestinal tract.

In experimental aflatoxicosis studies in broiler chicks, it has been reported that hepatocellular degenerations in the liver show especially periacinar localization[16,27,42]). It was noteworthy that this study was also compatible with the above studies[16,27,42] in terms of localization of the lesions. In aflatoxicosis, morphological changes such as increasing enlargement in the nucleus and cytoplasm of hepatocytes (megalocytosis), condensation of nuclear chromatin in the nuclear membrane (hyperchromasia), increasing number and prominence in nucleus were also observed[16,27] and the results of the present study are compatible with these previous studies.

Proliferation of bile ducts and hyperplasia in ductus epithelium in portal areas were formed in cases of aflatoxicosis[16,27,43]. It has been suggested that the severity of these morphological changes in bile ducts is closely related to the dose of AFB_1 and the duration of experimental period and that it is a reaction against parenchymal damage in the liver, followed by degenerative-necrotic changes in hepatocytes[44]. In current study, proliferation in bile ducts was also observed in aflatoxin group.

During the development of chronic parenchymal damage, the proliferation and collagen production by myofibroblastic change of *Ito* cells is crucial in the pathogenesis of liver fibrosis. It has been reported that fibrosis previously appeared only in portal areas, but over time, fibrous septa that extend from portal areas to vena centralis have appeared[45]. The activation of *Ito* cells was also observed in the liver as a result of aflatoxicosis[44]. In the present study, the activation, which was histologically evident in perisinusoidal cells, was observed especially in the aflatoxin group and it was observed that these proliferations extended as thin cords (fibrous bands) from the periacinar regions to the portal spaces. In immunohistochemical staining, strong smooth muscle actin reaction was noted in these areas. These reactive cells are thought to be perisinusoidal myofibroblastic *Ito* cells. However, remarkably, it was determined that these recurrent cells showed a very limited increase in the AFB_1 +UDSE group. As a result, it is thought that the development of degenerative-necrotic changes in the liver tissue is prevented by the effect of many bioactive components in *U. dioica* seeds' extract and thus the fibrosis formation is suppressed. These findings show that *U. dioica* seeds' extract has a strong hepatoprotective effect. Indeed, it has been also demonstrated in many studies that *U. dioica* seeds' extract has a strong hepatoprotective effect[15,16,24,27]. In one of these studies, it was noted that the UDSE prevented lipid peroxidation by 98%, therefore it is a powerful natural antioxidant[15]. In another study, it has been reported that in experimentally CCl_4-induced liver fibrosis, UDSE has a strong antifibrotic effect and this may be related to the antioxidant and immunomadulatory properties of UDSE[24]. In other studies where experimental aflatoxicosis was induced in rats[16] and broiler chicks[27], it has been emphasized that UDSE has a strong hepatoprotective effect against aflatoxins. In addition, it has been suggested that this protective effect may be achieved by preventing lipid peroxidation or by activating antioxidant defence systems through various bioactive components of UDSE.

Aflatoxins inhibit the digestion of fats by inhibiting enzymatic reactions and bile secretion in living beings, thereby disrupting the body's energy metabolism because of the decreased rate in utilization of fat[46]. Aflatoxins also react with fat-soluble vitamins and cause a deficiency of these vitamins in the body and therefore lesions occur especially due to vitamin A, D and K deficiencies [47,48]. In a previous study, it has been reported that there is a decrease in the body weight of broiler chicks with aflatoxicosis by 12-57%[47]. These decreases in body weight gain have been associated with inhibition of protein and lipid synthesis due to decreased appetite and feed intake and hepatotoxicity[9]. Consistently, in our study, as can be seen in Table 1, the weekly body weights of the broilers in the aflatoxin group were significantly ($p<0.05$) lower than those of the control group. At the end of the experiment, the body weight increase in the aflatoxin group was found to be less by approximately 20% when compared to the control group. It was observed that this rate of growth retardation in broilers in our study was similar to earlier studies conducted in broilers[47,49]. In the current study, it was noteworthy that the mean body weight at the end of the experimental period (day 42) in the control, UDSE and AFB_1 +UDSE groups was comparable. These results showed that addition of 30 mL of UDSE to kg of feed restores the negative effects of aflatoxin on live weight.

Aflatoxin has been found to stimulate the release of free radicals called as reactive oxygen species that cause damage to protein, lipid and DNA[36,50,51]. Increased lipid peroxidation in hepatotoxicity associated with AFB_1 leads to increased sensitivity, leading to damage to cell membranes[52,53]. Due to these damages, levels of ALT, AST and lipid peroxidation increase and the activity of antioxidant enzymes such as SOD, CAT and GSH-Px decreases[16.27,54]. It has been noted that SOD, catalase and deforaximin inhibit the destructive effect of AFB_1 on rat liver cells[53]. This information proves that reactive oxygen species play an important role in cell toxicity induced by AFB_1. *U. dioica* has been reported to have strong antioxidant properties compared to well-known antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), vitamin E and quercetin[15]. Yener *et al.*[16] showed that the use of *U. dioica* in rats treated with aflatoxin significantly improved GR, SOD, CAT and GSH-Px activities and GSH levels compared to aflatoxin group in erythrocyte, brain and liver tissues. In a previous study Erboğa *et al.*[55] investigated the protective effects of *U. dioica* on inhibition of SOD, CAT and GSH-Px activities with doxorubicin administration and reported that *U. dioica* restored SOD, CAT and GSH-Px

activities. Vajic *et al.*[56] showed that extract from *U. dioica* leaves increased SOD, CAT and GSH-Px activities in erythrocytes of rats with high blood pressure. Joshi *et al.*[57] showed that in rats with CCl_4-induced liver damage, UDSE increased CAT activity as well as reduced glutathione (GSH) levels. In our study, there was a significant increase in the activities of SOD, GSH-Px and CAT in the serum and the liver of UDSE group compared to the control group. However, this increase was found to be significant only for serum SOD values. In addition, in the aflatoxin group, SOD, GSH-Px and catalase levels decreased significantly compared to the control group. However, SOD, GSH-Px and catalase levels in the group using UDSE with aflatoxin were significantly higher than the aflatoxin group. These results are compatible with the previous studies mentioned above[55-57], indicating that UDSE along with aflatoxin restores the activities of SOD, GSH-Px and catalase significantly.

TAS and TOS levels has been widely studied in recent years to determine oxidative stress and antioxidant capacity[58,59]. Bitiren *et al.*[58] found that in rats with CCl_4-induced liver injury, administration of UDSE along with CCl_4 significantly increased serum TAS levels compared to CCl_4 group and decreased TOS levels. Terzi *et al.*[59] showed that *U. dioica* increased TAS levels and decreased oxidative stress index (TOS / TAS) in rats with liver ischemia reperfusion injury. In our study, aflatoxins significantly decreased the liver and serum TAS levels and increased TOS levels compared to the control group. However, administration of UDSE along with aflatoxin increased TAS levels compared to the aflatoxin group and decreased TOS levels. These results show that administration of UDSE along with aflatoxin may increase TAS levels compared to aflatoxin group and decrease TOS levels. These findings strongly indicated that the supplementation of UDSE in combination with aflatoxin can maintain antioxidant capacity in serum and liver tissue and prevent liver dysfunction.

Induction, cell death in apoptotic pathways is associated with activation of caspases[60]. Caspase-3 is one of the key players of apoptosis. Activation of caspase-3 is an important sign of apoptosis following ischemic brain injuries[61]. In the current study, the level of caspase-3 in aflatoxin treated subjects was higher than that of the control group in both serum and liver tissues. However, supplementation of UDSE along with aflatoxin slightly lower caspase-3 levels compared to the aflatoxin group, indicating that the use of UDSE restore the increased level of caspase-3 induced by aflatoxin.

One of the key regulators in the molecular mechanisms of apoptosis is the BcL-2 family[62]. The BcL-2 multigen super family includes anti-apoptotic genes such as BcL-2, BcL-xL and proapoptotic genes such as Bax and Bad[61]. Apoptosis is suppressed by BcL-2, which can inhibit Bax gene expression and free radical production[62]. Bax's proapoptotic mechanism is triggered by the release of cytochrome c, which can activate caspase-3 and form a diplo polymer with BcL-2. The release of cytochrome-c after mitochondria damage leads to DNA damage and apoptosis by activation of the caspase-3-mediated pathway[62]. In a study, BcL-2 and BcL-xL levels were relatively unchanged in neurons that remained alive after ischemic deformation, while the immune reactivity of BcL-2 and BcL-xL was low and Bax immune reactivity was high in damaged-neurons after focal and global ischemia (Bax)[61]. Consistently, in the current study, we observed that aflatoxin significantly decreased the level of BcL-2, but the use of UDSE restored BcL-2 levels.

As a whole, UDSE prevented tissue damage due to an effective antiapoptotic and strong antioxidant effects in broiler aflatoxicosis, which is supported by histopathological and immunohistochemical results.

CONCLUSION

Biochemical analysis showed that *Urtica dioica* seeds' extract efficiently restores the activities of SOD, GSH-Px and CAT and the levels of TAS, TOS, BcL-2 and Caspase-3, which is further supported by histopathological and immunohistochemical observations. Therefore, it could be recommended that UDSE could possibly be used in broiler breeding for the purpose of protection from aflatoxicosis.

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