Damanhour Journal of Veterinary Sciences

Journal homepage: www.damanhourjvetsci.com

E-ISSN 2636-3003 | ISSN 2636-2996

Biochemical protective role of *Phoenix dactylifera* seeds against aflatoxicosis in broilers

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A B S T R A C T

Aflatoxin (AF) has an important concern in the poultry industry because of its serious major economic losses and health problems. Natural alternatives to chemical mycotoxin binders had been used previously as yeast cell wall. Hence, the present work aimed to study the protective role of date pits (*phoenix dactylifera*) seeds against aflatoxicosis in broilers. Two hundred and ten-one-day old Arbor Acres broiler chicks were allotted into 7 equal groups as the first control (G1) that supplemented by the basal diet, the second had the basal diet with date pits supplementation 2% (DPS2%) group (G2), the third DPS4% group (G3) fed on the basal diet with date pits 4%, in G4, Aflatoxin (AF) alone was fed a basal diet containing 100µg aflatoxin/kg feed. G5 (AF+HSCAS), fed on a basal diet containing Hydrated Sodium Calcium Aluminum Silicates (HSCAS) 0.3% plus 100µg aflatoxin/kg feed, AF+DPS2% (G6) fed a basal diet containing date pits 2% plus 100µg aflatoxin/kg feed, and finally G7 (AF+DPS4%) fed a basal diet containing date pits 4% plus 100µg aflatoxin/kg feed. The aflatoxin supplemented to the broiler ration from first day to the end of experiment at 35 days. Aflatoxins supplementation increased malondialdehyde levels in liver and muscles, apoptosis of liver cells and induced histopathological changes in the liver whereas exhibited focal hepatic necrosis with inflammatory cell infiltration, fatty degeneration of hepatocytes and congestion of portal vein with fibrocytes proliferation in portal area. However, addition of date pits (2, 4%) and HSCAS (0.3%) to broiler's diet ameliorated these negative effects of aflatoxins partially, indicating that date pits have a protective effect against aflatoxicosis and this protection is dose-related. Addition of DPS (2 and 4%) gave better results regarding mortality, lipid peroxidation, antioxidant capacity and histopathological examination of liver, gave overall compared to HSCAS concluding that date pits could be used as an effective feed additive to control aflatoxicosis in poultry avoiding harmful effect of chemical mycotoxin binders (HSCAS).

Keywords: Aflatoxins; Date pits; HSCAS; Caspase-3 gene expression levels; Malondialdehyde; Antioxidant; Histopathological changes; Broilers

1. Introduction

The poultry industry in Egypt has achieved phenomenal progress in recent decades. The quality of feed and its ingredients may affect the profit margin of the poultry economics. Mycotoxins are one of the major factors affecting poultry productivity and product quality. Among mycotoxins, aflatoxins (AF) are of more concern as they account for 25% of poultry feed samples and ingredients contamination (Mohanamba et al., 2002). Aflatoxins are toxic compounds mostly produced by some fungal species such as *Aspergillus flavus* and *parasiticus* (Rangsaz and Ahangaran, 2011). The naturally occurring aflatoxins are aflatoxin B1, B2, G1 and G2, in which AFB1 is the most abundant, toxic and carcinogenic (Qi *et al*., 2015). Aflatoxin contaminated poultry diets reduced the poultry efficiency either causing huge economic losses through poor body performance by retarding bird growth, increasing feed consumption, and reducing meat production or through sequels of aflatoxin (Nurul and Mohd, 2017; Fan *et al*., 2013). The aflatoxicosis in chickens is characterized by mortality, listlessness, anorexia, decreased growth rates, reduced efficiency of feed conversion, fatty liver, decreased egg production, poor pigmentation and increased susceptibility to other diseases (Rangsaz and Ahangaran, 2011). Producers, researchers and governments are working to develop effective prevention, management and decontamination technologies to minimize the toxic effects of AF by using physical, chemical and biological treatments (Oguz, 2011). A practical approach to detoxification is the use of sorbents in the diet that adsorb AF in the gastrointestinal tract of poultry and reduce bioavailability and toxicity (Gholami-Ahangaran and Zia-Jahromi, 2013). Various nonnutritive adsorbents have been employed for reducing or inactivating AF in poultry feeds. Hydrated Sodium Calcium Aluminum Silicates (HSCAS), which reduce AF absorption by binding with the β-carbonyl portion of AF molecules, have been shown to be effective in preventing aflatoxicosis (Ledoux *et al*., 1999). Using medicinal herbs to decrease the participation of chemicals through the worldwide tendency to return to the natural supplements has been supported by the World Health Organization (Mohamed *et al*., 2003) and many studies searched for reverse the harmful effects of aflatoxin in broiler using plant extracts (da Silva *et al*., 2016).

Egypt is considered one of the date-producing countries. The fruit of the date palm is composed of a fleshy pericarp and seed (Ahmed *et al*., 2008), and the seed represents about 15% of the total weight of the date fruits (Hussein *et al*., 1998). These date seeds (named also, pits, stones, kernels) are waste products from date industry which can be used as a functional feed ingredient because they are a good source of dietary fiber, phenolic compounds and antioxidant activity in addition to a considerable amount of feed ingredients such as protein and minerals (Barreld, 1993).

Therefore, the potential uses of date seed in different industries are promising specially with their availability at a very low cost (Golshan *et al*., 2017). Also, the high level of mannan in date pits improves the nutritive value of this product (Hoerr, 1997). Glucomannan commonly can biologically inactivates multiple mycotoxins and glucomannan type A is the main component of the cell walls of palm kernels, which in this case acts as a food reserve and disappears during germination (Navid, 2007). Date seed contains 3.1–7.1% moisture, 2.3–6.4% protein, 5.0–13.2 fat, 0.9–1.8% ash and 22.5–80.2% dietary fiber. Also, seeds contain high levels of phenolics (3102– 4430 mg gallic acid equivalents/ 100 g), antioxidants and dietary fiber (78–80 g/100 g) (Al-Farsi *et al*., 2007). Date seed powder is also used for addition to animal feed (cattle, sheep, camel, and poultry) and fish feed as it was reported to enhance growth, improve feed efficiency and meat palatability in animals (Al-Farsi and Lee, 2011).

Many studies have been carried out on date seeds in Egypt focusing mainly on their chemical composition but, lacking the effects of date seeds on aflatoxicosis in poultry farms. Therefore, the effective use of date pits and their biochemical protective role in reducing the effects of aflatoxicosis in broiler have been investigated in the current experiment through evaluation of broiler performance, lipid peroxidation and antioxidant capacity, caspase-3 gene expression levels (Apoptotic marker), and histopathological changes in comparison to HSCAS (0.3%) supplementation in feed.

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E-ISSN 2636-3003 | ISSN 2636-2996
DOI: 10.5455/DJVS.22843

Received: November 22, 2018; Received in revised form: December 20, 2018; accepted: December 24, 2018.

2. Material and methods

2.1. Production of aflatoxin

Aflatoxin was produced from *Aspergillus parasiticus* NRRL - 2999 pure culture (National Research Centre, Cairo, Egypt) via fermentation of rice by the method of Shotwell et al., (1966). The rice powder was incorporated into the basal diet at an estimated 100 µg total AF/kg feed during the entire experimental period.

2.2. Feed additives (Mycotoxin binders)

2.2.a. Phoenix dactylifera seeds collection and preparation

Purchased from a local date pitting factory; and then to separate foreign materials, they were washed, air-dried and finely powdered with a mechanical grinder to obtain a coarse powder used in the present study. The amount of coarse powder calculated at percentage of 2% and 4% and supplemented to chickens according to each treatment mixed with basal diet.

2.2.b. Hydrated sodium and calcium aluminum Silicates (HSCAS)

The experimental adsorbent used was HSCAS, (Toxi-Mold Plus™) a commercial mycotoxin adsorbent for all types of feed obtained from Egyco Vet Company. HSCAS was weighed, calculated, added and well mixed with the basal diet according to the permissible limit required for each group by dosage 3 kg per ton.

2.3. Experimental design

The present study is affirmed by the Ethics of Animal Experiments Committee, Damanhour University, Egypt. Briefly, two hundred and ten 1-day-old unsexed Arbor Acres broiler chicks were purchased from a local commercial hatchery (NASCO Egypt, Alexandria) and randomly allocated into 7 equal groups at the first day of age. Each one group was subdivided into three replicates (10 birds per replicate) and floor reared. Feed and water were supplied *ad-libitum* for 35 days of age and optimum managemental factors were applied regarding ventilation, temperature, lighting and litter management all over the experimental period. Furthermore, the birds were vaccinated for ND & IBV at 5th day using (Polimun® ND Clon 124 + IB H120- BioTestlab Vasilkov, Ukraine). Gumbro intermediate (Bursine 2® vaccine, Zoetis, Parsippany, CA, USA) and ND (Nobilis® ND LaSota, Intervet, Netherlands) both at 12th and 20th days via eye drop. The chicks were kept on a standard commercial broiler diet based on a corn-soybean meal using the two phases feeding program. Starter feed from 1st to 21st days on the starter and grower feed from 22nd to 35th days. The diet composition represented in Table (2 and 3) and was formulated according to the recommendation of National Research Council Nutrient Requirements for Arbor Acres broiler chickens NRC (1994). The used diet was prepared without any feed additives rather than the compounds under study. A basal diet and 6 treatment diets were used as follows: the first group (G1) fed on a commercial broiler diets without supplement (control); G2 (DPS2%) fed on the basal diet with date pits 2% supplementation, G3 (DPS4%) fed on the basal diet with date pits 4%, G4 (AF) had aflatoxin as 100µg /kg feed, G5 (AF+HSCAS) fed a basal diet containing HSCAS 0.3% plus 100µg aflatoxin/kg feed, G6 (AF+DPS2%) had a basal diet containing date pits 2% plus 100µg aflatoxin/kg feed, and finally G7 (AF+DPS4%) had a basal diet containing date pits 4% plus 100µg aflatoxin/kg feed. Also, the basal diets were tested for possible residual AF before feeding and there were no detectable levels present. A recorded daily observation for health problems and mortality were carried out all over 35 days of age.

2.4. Growth Performance parameters

Chicks were weighed at the beginning and weekly during the study. Feed intake (FI) and body weight gain (BWG) were determined weekly, and feed conversion rate (FCR) was calculated. Mortality was recorded as it occurred and dead birds were necropsied to determine cause of death.

2.5. Determination of oxidative stress parameters

The frozen aliquots of liver and muscles homogenates were utilized for the colorimetric assessment of malondialdehyde (MDA) contents and total antioxidant capacity (TAC) activities.

2.5.1. Determination of lipid peroxidation

Malondialdehyde is an aldehyde by-product of lipid peroxidation that analyzed after the incubation of supernatants with thiobarbituric acid at 95°C for 30 min (pH 3.6) to form thiobarbituric acid-reactive substances, a pink colored compound. MDA levels were recognized at 532 nm and expressed as nmol MDA/mg protein (Ohkawa et al., 1979).

2.5.2. Determination of total antioxidant capacity activities

The determination of the antioxidative capacity was performed by the reaction of antioxidants in the sample with a defined amount of exogenously provided hydrogen peroxide (H2O2). The antioxidants in the sample eliminate a certain amount of the provided hydrogen peroxide, and the residual H2O2 was determined calorimetrically by an enzymatic reaction which involved the conversion of 3, 5, dichloro - 2- hydroxyl benzene sulphonate to acolored product. TAC levels were recognized at 500 nm and expressed as mmol TAC/g tissue (Koracevic, 2001).

6. Expression levels of apoptotic regulator mRNA of Caspase-3 gene by quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) assay was carried out as reported by (Chen et al., 2013). The liver homogenate collected individually from 3 birds /group at 35 days of age. Total RNA was extracted from the samples using RNeasy Mini Kit instructions. The mRNA was then reverse transcribed into cDNA using RevertAid Reverse Transcriptase for First Strand cDNA Synthesis Kit with the recombinant Ribolock RNase inhibitor. For qRT-PCR reactions, 25 µL mixtures were made by using Quantitect SYBR green qPCR Master Mix (2X) kit, containing 12.5 µL QuantiTect SYBR Green qPCR Master Mix (2X) Plus, 0.25 µL Reverse transcriptase, 0.5 µL of forward and 0.5 µL of reverse primer (Table1), 8.25 µL RNAase-free water and 3 µL cDNA. All the kits were purchased from Qiagen- GmbH Company, Germany. PCR reaction performed in a thermal cycler (qPCR) with real time PCR machine (Stratagene MX3005P). Reaction conditions were set to 1 min at 94°C, 1 min at 60°C and 1 min at 94°C for dissociation curve (cycle 1) followed by 15 sec. at 94˚C, 30 sec. at 60˚C and 30 sec. at 72˚C (40 cycles) for amplification, annealing and extension. The mRNA expression of Caspase-3 was analyzed and β- actin was used as an internal control gene. Sequence of primers was obtained from GenBank of NCBI. Primers were designed with Primer 5 and synthesized by Metabion Company (Germany). The qRT-PCR data were analyzed and fold change in expressions were calculated. Amplification curves and CT values were determined by the stratagene MX3005P software. To estimate the variation of gene expression on the RNA of the different samples, the CT of each sample was compared with that of the control group according to the "2-ΔΔCt" method stated by Yuan et al., 2006 using the following ratio: ($2^{-\Delta\Delta ct}$). Whereas ΔΔCt = ΔCt reference – Δct target, ΔCt target = Ct control – Ct treatment, and ΔCt reference = Ct control- Ct treatment.

Table 1. Primers Sequences used in this study

Gene		Primer sequence (5'-3')	Reference
Caspase3	Forward	TGGCCCTCTTGAACTGAAAG	**Huang *et al*., 2013**
	Reverse	TCCACTGTCTGCTTCAATACC	
ß. Actin	Forward	CCACCGCAAATGCTTCTAAAC	**Yuan *et al*., 2007**
	Reverse	AAGACTGCTGCTGACACCTTC	

7. Histopathological examination

At the end of experiment on day 35, three chickens from each group were euthanized, liver samples were obtained and submitted for histopathology to evaluate lesions and abnormalities. Fixation of samples was applied in 10 % buffered formalin solution for one week. Blocks and staining were carried out according to (Drury and Wallington, 1980).

8. Statistical Analysis

Data was analyzed by one-way analysis of variance (ANOVA), with Duncan's multiple range tests for significant between means ($P \leq 0.05$) by SPSS.20® (IBM Cooperation, Armonk, NY, USA).

3. Results

3.1. Growth performance parameters, FCR and mortality (%) at 35 days in all bird groups

The average final body weight and weight gain of broilers was increased significantly (P≤0.05) in G2 (DPS2%) as (1683 and 1626.7) and non-significantly in G3 (DPS4%) as (1619 and 1572) respectively, however, there was non-significant decrease or improvement (P≥0.05) in FCR in those 2 groups (G2 and G3) as 1.56 and 1.59 compared to other groups. The aflatoxicated and treated groups [G5 (AF+HSCAS), G6 (AF+DPS2%) and G7 (AF+DPS4%)] had non-significant difference (P≥0.05) between each other in final body weight (1499, 1518 and 1486 respectively) compared to other bird groups. No mortalities occurred in G1, G2 and G3, while the highest mortality rate was in birds of G4 as 6/30 (20%) which decreased to (3/30) 10% in birds of G6 and 4/30 (13.3%) in G5 and G7 (Table 4).

3.2. Determination of oxidative stress parameters (lipid peroxidation and total antioxidant capacity activities)

Liver content of MDA was significantly increased (P≤0.05) following supplementation of AF alone in G4 as 58.3 nmol/g compared to other groups. The lowest significant (P≤0.05) liver MDA level was in birds of G2 as 23.2 nmol/g. There were non-significant differences in G5, G6 and G7 respectively although the lowest level in those 3 groups was in G6 as 37.83 nmol/g. While, the breast and thigh muscle contents of MDA were significantly increased (P≤0.05) in G4 as 30 and 18.73 nmol/g respectively, compared to other groups. Also, they were reduced

Table 2. Ingredients percentages and calculated composition analysis of the experimental starter diets (as fed basis)

Ingredients	Basal diet	DPS2	DPS4	AF	AF+ HSCAS	AF+ DPS2	AF+ DPS4
Corn	57.68	55.72	53.78	57.68	57.38	55.72	53.78
SBM (CP 46%)	36.6	36.56	36.5	36.6	36.6	36.56	36.5
Sodium bicarbohydrates	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Soybean oil	1.3	1.3	1.3	1.3	1.3	1.3	1.3
Limestone	1.95	1.95	1.95	1.95	1.95	1.95	1.95
Monocalcium phosphate	1.15	1.15	1.15	1.15	1.15	1.15	1.15
L-Lysine*	0.17	0.17	0.17	0.17	0.17	0.17	0.17
Dl-methionine**	0.3	0.3	0.3	0.3	0.3	0.3	0.3
P. dactylifera seeds	0	2	4	0	0	2	4
HSCAS	0	0	0	0	0.3	0	0
Vitamins and minerals premix***	0.3	0.3	0.3	0.3	0.3	0.3	0.3
NaCl	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Total	100	100	100	100	100	100	100
Contamination levels of AF (ppb)****	0	0	0	100	100	100	100
Estimated and analyzed composition (%)							
ME	2862.86	2841.97	2821.3	2862.86	2852.81	2841.97	2821.3
CP %	22	21.96	21.91	22	21.98	21.96	21.91
Lysine %	1.3	1.3	1.29	1.3	1.3	1.3	1.29
Methionine %	0.63	0.62	0.62	0.63	0.63	0.62	0.62
Calcium %	1.05	1.05	1.06	1.05	1.05	1.05	1.06
Av. (P) %	0.39	0.39	0.39	0.39	0.39	0.39	0.39

PS: date pits. SBM: soybeanmeal. ME: metabolizable energy (Kcal/kg diet). CP: crude protein. Av. (P): available phosphorous. ⁎L-lysine, 99%feed grade. ⁎⁎Dl-methionine, 99% feed grade, China. ⁎⁎⁎Vitamin and mineral premix (Hero mix) produced by Hero pharm Co., Egypt. ⁎⁎⁎⁎HSCAS, A hydrated sodium calcium aluminosilicate (Toxi-Mold Plus^{TR}, Mycotoxin adsorbant, Egyco Vet Company for baic vet trade, Egypt). ⁎⁎⁎⁎⁎ Ground rice, total aflatoxins culture and the amount of feed contaminated with aflatoxin is expressed in ppb; premix was added to final diets to obtain 100 µg of total AF/kg of diet with or without 0.3% Toxi-Mold Plus^{TR} and with DPS 2 % or 4 %.

Table 3. Ingredients percentages and calculated composition analysis of the experimental grower diets (as fed basis)

Ingredients	Basal diet	DPS2	DPS4	AF	AF+ HSCAS	AF+ DPS2	AF+ DPS4
Corn	59	55.67	52.3	59	58.7	55.67	52.3
SBM (CP 46%)	34.8	35.13	35.5	34.8	34.8	35.13	35.5
Sodium bicarbohydrates	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Soybean oil	2	2.82	3.82	2	2	2.82	3.82
Limestone	1.95	1.95	1.95	1.95	1.95	1.95	1.95
Monocalcium phosphate	0.96	1.15	1.15	0.96	0.96	1.15	1.15
L-Lysine*	0.14	0.13	0.12	0.14	0.14	0.13	0.12
Dl-methionine**	0.3	0.3	0.31	0.3	0.3	0.3	0.31
P. dactylifera seeds	0	2	4	0	0	2	4
HSCAS	0	0	0	0	0.3	0	0
Vitamins and minerals premix***	0.3	0.3	0.3	0.3	0.3	0.3	0.3
NaCl	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Total	100	100	100	100	100	100	100
Contamination levels of AF (ppb)****	0	0	0	100	100	100	100
Estimated and analyzed composition (%)							
ME	2928.54	2942.16	2971.18	2928.54	2990.65	2942.16	2971.18
CP %	21.27	21.27	21.30	21.27	21.24	2127	21.30
Lysine %	1.23	1.22	1.21	1.23	1.22	1.22	1.23
Methionine %	0.62	0.62	0.62	0.62	0.62	0.62	0.62
Calcium %	1.02	1.05	1.05	1.02	1.02	1.05	1.05
Av. (P) %	.034	0.38	0.38	0.34	0.34	0.38	0.38

DPS: date pits. SBM: soybeanmeal. ME: metabolizable energy (Kcal/kg diet). CP: crude protein. Av. (P): available phosphorous. ⁎L-lysine, 99%feed grade. ⁎⁎Dl-methionine, 99% feed grade, China. ⁎⁎⁎Vitamin and mineral premix (Hero mix) produced by Hero pharm Co., Egypt. ⁎⁎⁎⁎HSCAS, A hydrated sodium calcium aluminosilicate (Toxi-Mold Plus^{TR}, Mycotoxin adsorbant, Egyco Vet Company for baic vet trade, Egypt). ⁎⁎⁎⁎⁎ Ground rice, total aflatoxins culture and the amount of feed contaminated with aflatoxin is expressed in ppb; premix was added to final diets to obtain 100 µg of total AF/kg of diet with or without 0.3% Toxi-Mold Plus^{TR} and with DPS 2 % or 4 % .

Table 4. Effect of DPS dietary supplementation on growth performance parameters (g), FCR and mortality (%) at day 35 in all bird groups

Group	Initial Weight	Final Weight	Weight Gain	Feed Intake	FCR	Mort. %
G1 (Control)	43.66 ± 0.3^{a}	1577 ± 20^{bc}	1533.34 ± 19^{bc}	2477 ± 28^{ab}	1.61 ± 0.02^{a}	0
G2 (DPS 2%)	46.3 ± 0.6^{a}	1683 ± 30^{a}	1636.7 ± 35^{a}	2553.93 ± 32^{a}	1.56 ± 0.04^{a}	0
G3 (DPS 4%)	46.6 ± 1.2^{a}	1619 ± 20^{ab}	1573 ± 21^{ab}	2503.01 ± 28^{ab}	1.59 ± 0.03^{a}	0
G4 (AF)	45.33 ± 0.3^{a}	1388 ± 39^{d}	1342.67 ± 40^{e}	2231.98 ± 25^{c}	1.66 ± 0.06^{a}	6/30 (20%)
G5 (AF+HSCAS)	45.66 ± 0.6^{a}	1499 ± 8^{c}	1453.34 ± 8^{cd}	2385.11 ± 36^{abc}	1.64 ± 0.03^{a}	4/30 (13.3%)
G6 (AF+DPS2%)	45.3 ± 0.8^{a}	1518 ± 21^{c}	1472.7 ± 20^{cd}	2409.2 ± 43^{ab}	1.63 ± 0.03^{a}	3/30 (10%)
G7 (AF+DPS4%)	44.6 ± 0.6^{a}	1486 ± 21^{c}	1441.4 ± 21^{d}	2359 ± 26^{c}	$1.63 \pm .01^{a}$	4/30 (13.3%)

G: group. DPS: date pits (2 or 4%). AF: aflatoxin (100 µg/kg feed). HSCAS: hydrated sodium calcium aluminosilicate (0.3%). FCR: feed conversion rate. Mort. %: mortality %. Means within the same column under the same category carry different superscripts are significantly different ($P<0.05$). Values are expressed as means ± SE.

significantly ($P\leq0.05$) to the lowest level in bird of G2 and G3 (G2 had superior reduction as 1.2 and 3.8 nmol/g, respectively). In the aflatoxicated and treated groups, there were a significant decrease ($P\leq0.05$) in G6, G5 and G7 as 8.6, 9.2 and 15 nmol/g, respectively for MDA in breast muscle and as 8.16, 9.6 and 12.5 nmol/g, respectively in thigh muscle (Table 5).

Regarding the levels of total antioxidant capacity in liver, there was significantly increased ($P\leq0.05$) levels of TAC following DPS supplementation as 10.1 and 8.6 Mn/g in G2 and G3 respectively as compared to all other groups. The lowest level of TAC was in birds of G4 as 2.6 Mn/g, however, it was non-significantly different ($P\geq0.05$) from other groups (either control G1 or the aflatoxicated and treated groups G5, G6 and G7). In breast muscles, TAC was significantly ($P\leq0.05$) in the maximum level as 1.22 Mn/g in birds of G2 (DPS2%) compared to the minimum level in the AF treated G4 as 0.54 Mn/g and all other groups were non-significantly ($P\geq0.05$) lower than G2 or higher than G4. TAC levels didn't differ significantly ($P\geq0.05$) in thigh muscles of all bird groups.

Table 5. Effect of DPS dietary supplementation on MDA (nmol/g tissue) and TAC (Mm/g tissue) levels in broiler's liver, breast and thigh muscles which exposed to 100 μg of aflatoxin / kg diet

Group	Malondialdehyde (MDA)			Total Antioxidant Capacity (TAC)		
	Liver	Breast muscle	Thigh muscle	Liver	Breast muscle	Thigh muscle
G1 (Control)	34.96 ± 5.6^{bc}	4.03 ± 1.5^{cd}	5.83 ± 1.4^{b}	5.1 ± 0.46^{b}	0.84 ± 0.32^{ab}	2.54 ± 0.02^{a}
G2 (DPS 2%)	23.2 ± 6.54^{c}	1.2 ± 0.42^{d}	3.8 ± 0.33^{b}	10.1 ± 1.1^{a}	1.22 ± 0.34^{a}	2.54 ± 0.18^{a}
G3 (DPS 4%)	33.56 ± 5.2^{bc}	2.6 ± 0.79^{d}	5.3 ± 0.34^{b}	8.6 ± 1.91^{a}	1.10 ± 0.2^{ab}	2.55 ± 0.15^{a}
G4 (AF)	58.3 ± 2.89^{a}	30 ± 4.21^{a}	18.73 ± 9^{a}	2.6 ± 1.17^{b}	0.54 ± 0.02^{b}	2.48 ± 0.03^{a}
G5 (AF+HSCAS)	40.0 ± 4.87^{bc}	9.2 ± 0.52^{bc}	9.6 ± 0.8^{ab}	3.3 ± 0.28^{b}	0.68 ± 0.04^{ab}	2.5 ± 0.143^{a}
G6 (AF+DPS2%)	37.83 ± 5.63^{bc}	8.6 ± 1.9^{c}	8.16 ± 2^{ab}	4.2 ± 0.37^{b}	0.74 ± 0.05^{ab}	2.51 ± 0.03^{a}
G7 (AF+DPS4%)	48.1 ± 4.9^{ab}	15 ± 0.49^{b}	12.5 ± 0.6^{ab}	4.9 ± 1.42^{b}	0.75 ± 0.03^{ab}	2.52 ± 0.11^{a}

G: group. DPS: date pits (2 or 4%). AF: aflatoxin (100 µg/kg feed). HSCAS: hydrated sodium calcium aluminosilicate (0.3%). Means within the same column under the same category carry different superscripts are significantly different (P<0.05). Values are expressed as means ± SE.

Table 6. Gene expression of both β. Actin and Caspase -3 in the examined liver homogenate of 3 birds/group

Group	Sample No.	β. actin				Caspase-3			
		Individual CT	Mean CT	Fold change (Gene expression)		Individual CT	Mean CT	Fold change (Gene expression)	
				Individual	Mean			Individual	Mean
G1 (Control)	L1.1	20.98	20.73	1	1^{a}	23.09	22.763	0.94	1.0 ± 0.00^{d}
	L1.2	20.69		1		22.82		0.93	
	L1.3	20.52		1		22.37		1.13	
G2 (DPS2%)	L2.1	19.73	20.46	1	1^{a}	22.03	22.86	0.83	0.77 ± 0.36^{d}
	L2.2	20.48		1		22.85		0.79	
	L2.3	21.17		1		`23.70		0.70	
G3 (DPS4%)	L3.1	20.92	20.73	1	1^{a}	23.61	23.56	0.63	0.58 ± 0.40^{d}
	L3.2	19.82		1		22.85		0.50	
	L3.3	21.46		1		24.21		0.60	
G4 (AF)	L4.1	20.78	20.47	1	1^{a}	19.61	19.05	9.19	11.08 ± 1.05^{a}
	L4.2	20.09		1		18.63		11.23	
	L4.3	20.55		1		18.90		12.81	
G5 (AF+ HSCAS)	L5.1	21.61	21.47	1	1^{a}	20.76	20.97	7.36	5.86 ± 0.74^{b}
	L5.2	21.87		1		21.54		5.13	
	L5.3	20.92		1		20.60		5.09	
G6 (AF+DPS2%)	L6.1	19.58	20.64	1	1^{a}	19.94	21.05	3.18	3.06 ± 0.73^{c}
	L6.2	20.92		1		21.40		2.93	
	L6.3	21.41		1		21.82		3.07	
G7 (AF+DPS4%)	L7.1	22.01	21.91	1	1^{a}	23.42	23.12	1.53	1.77 ± 0.15^{cd}
	L7.2	22.12		1		23.11		2.05	
	L7.3	21.60		1		22.84		1.72	

G: group. DPS: date pits (2 or 4%). AF: aflatoxin (100 µg/kg feed). HSCAS: hydrated sodium calcium aluminosilicate (0.3%). L: liver. Means within the same column under the same category carry different superscripts are significantly different (P<0.05). Values are expressed as means ± SE.

3.3. Expression levels of apoptotic regulator mRNA of Caspase-3 gene by qRT-PCR

The gene expression levels of Caspase-3 in liver of broilers were increased significantly (P≤0.05) in birds of G4, G5 and G6 and non-significantly (P≥ 0.05) in birds of G7 compared to G1, G2 and G3. The maximum value observed in G4 (AF) as 11.08 and the minimum value observed in G3 as 0.58. Also, the data showed that gene expression levels of Caspase-3 in G7, G6 and G5 as 1.77, 3.06 and 5.86 respectively were decreased significantly (P≤0.05) in relation to G4 treated with AF alone (Table 6).

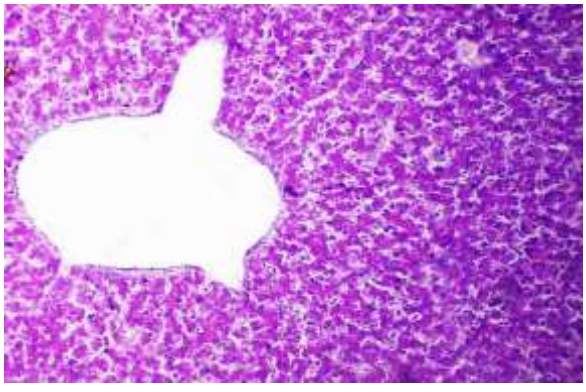

Figure (1): Liver of a chicken of control group (G1) showing normal histological structure. H&E. (x160).

3.4. Histopathological examination

The liver of birds in G1, G2 and G3 showed normal histological appearance and structure (Fig. 1, 2 & 3, resp.). In the examined birds of G4, liver exhibited focal hepatic necrosis with inflammatory cell infiltration and fatty degeneration of hepatocytes (Fig. 4.A). Additionally, the portal area showed congestion of portal vein with inflammatory cell infiltration and fibrocytes proliferation (Fig. 4.B). The liver of the examined birds in G5 showed moderate congestion of blood vessels and portal vein (Fig. 5) while, the liver of birds in G6 showed mild activation of lymphoid aggregation (Fig. 6) and only focal hepatic necrosis with moderate infiltration of inflammatory cells was seen in the examined liver of birds in G7 (Fig. 7).

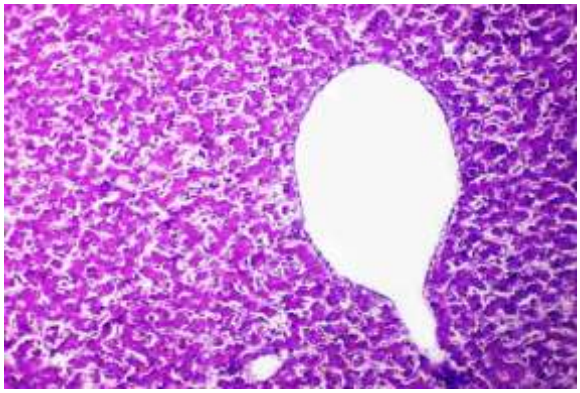

Figure (2): Liver of a chicken of G2 (DPS2%) showing normal histological structure. H&E. (x160).

4. Discussion

Seeking effective ways to alleviate the negative effects of AF has attracted more and more attention and nowadays, researchers found that many feed additives had the ability to relieve aflatoxicosis, but few researches are carried out on incorporation of date seed powder into the diet for poultry production. Therefore, this investigation provides a strategy to resolve the problems of aflatoxicosis in poultry industry by introducing date pits in to the broiler's diets.

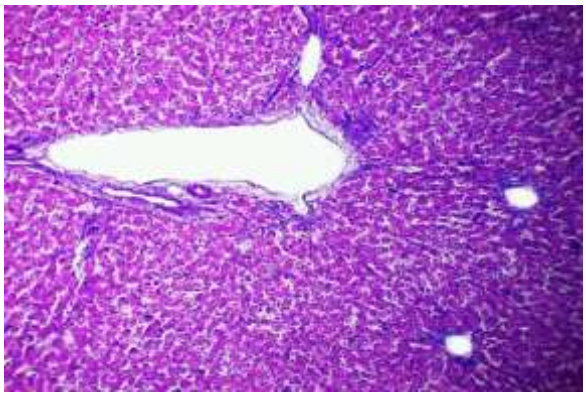

Figure (3): Liver of a chicken of G3 (DPS4%) showing normal histological structure. H&E. (x160).

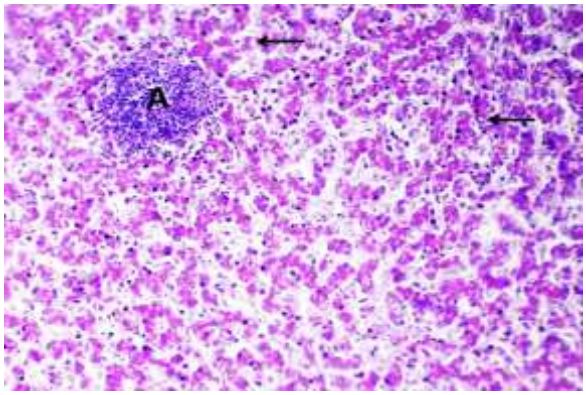

Figure (4.A): Liver of a chicken of AF (G4) showing focal hepatic necrosis with inflammatory cell infiltration (A) and fatty degeneration of hepatocytes (arrows). H&E. (x160).

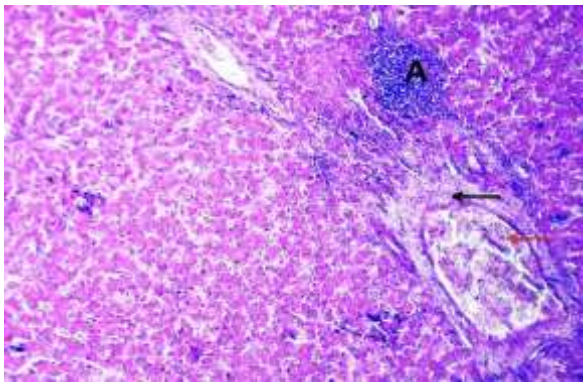

Figure (4.B): Liver of a chicken of AF (G4) showing congestion of portal vein (red arrow) with inflammatory cell infiltration (A) and fibrocytes proliferation (black arrow) in portal area. H&E. (x160**).**

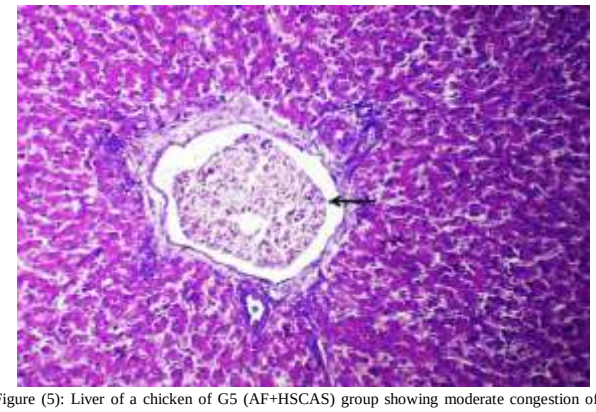

Figure (5): Liver of a chicken of G5 (AF+HSCAS) group showing moderate congestion of portal vein (arrow). H&E. (x160).

From the obtained results, we noticed that supplementation of broilers with ration containing DPS at levels 2% and 4% is of great beneficial improvements in broiler health producing healthy birds with significantly higher (P≤0.05) body weights, whereas there were significant higher BW and BWG in G2 than negative control G1. While, FI increased non-significantly (P≥ 0.05) in G2 and FCR improved non-significantly (P≥ 0.05) in broilers of G2 and G3 than other groups which may be attributed to mannan-oligosaccharides content found in date pits as mentioned by Daneshyar et al. (2014) who used high levels of AF and recorded that mannanoligosaccharides in date pits can prevent the negative effects of aflatoxin B1 on broilers' performance and their carcass characteristics The present results are in accordance with those of earlier studies, which investigated DPS containing diets at levels of 2 and 4 % showed higher significant increases (P≤0.05) in BW and BWG in broilers (El-Far *et al*., 2016). Also, quail diet supplemented with 2.5 % and 5% CDP improved BW, BWG, RGR% and FCR of quail (Kamel et al., 2016). This may be owed to supplementation of date pits with the exogenous enzymes which improve the utilization and the nutritional value of the date pits, by the degradation of beta-galactomannan polysaccharide (Hassan and Al Aqil, 2015). Other results were obtained by (Kamel et al (1981) who reported that the replacement of maize by whole dates resulted in decreased growth performance.

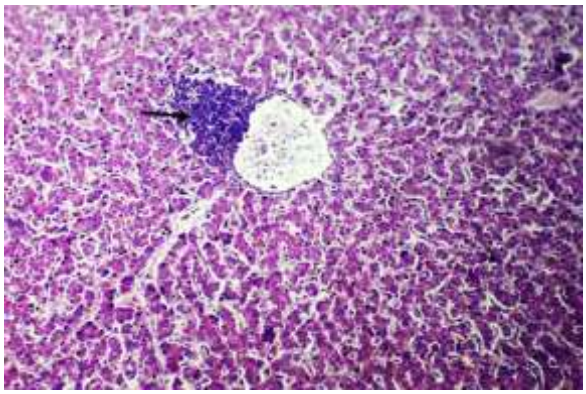

Figure (6): Liver of a chicken of G6 (AF+DPS2%) showing moderate activation of lymphoid aggregation (arrow). H&E. (x160).

The poor performance in broilers with dietary aflatoxin supplementation at level of 100 µg/kg feed (G4) as severely reduced BW, BWG and FI of and had explained in many studies concluding that AF impaired the activation and utilization of nutrients due to maldigestion or malabsorption and metabolic disorders of proteins, starches and fats (Chen et al., 2014; da Silva et al., 2016; Fan et al., 2013; Pasha et al., 2007 and Zhao et al., 2010). On the other hand, significantly higher (P≤0.05) BW and BWG in G6 (AF+DPS2%) and G7 (AF+DPS4%) when compared to G4 treated with AF alone, indicating the ability of date pits used in the current experiment to diminish significantly the growth inhibitory effects caused by AF as it could have adsorbed in the gastrointestinal tract biologically and enzymatically through mannanoligosaccharides content of date pits which act also as a growth promoter increasing body weight and performance of broilers. The addition of HSCAS to the aflatoxicated broilers (G5) improved the BW and BWG and decreased FI compared to AF alone in G4 which was supported by Harvey et al. (1993) who reported that addition of 0.5% of the HSCAS compounds significantly decreased the growth inhibitory effects caused by AF by 39–90% in 3 different experiments.

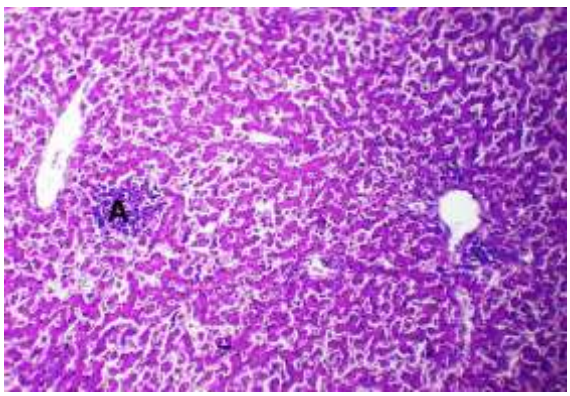

Figure (7): Liver of a chicken of G7 (AF+DPS4%) showing focal hepatic necrosis with mild infiltration of inflammatory cells (A). H&E. (x160).

Oxidative stress occurs when the level of reactive oxygen species (ROS) exceeds the tolerance capacity of the cellular antioxidant defense system. As a strong indicator of oxidative stress and toxicity, lipid peroxidation (LPO) is an auto-catalytic free-radical mediated destructive process, whereby polyunsaturated fatty acids in cell membranes undergo degradation to form lipid hydroperoxides (Palanivel et al., 2008) and MDA is formed at the end of lipid peroxidation, reflects the degree of the whole lipid oxidation in the body. However, these levels of free radical molecules and lipid peroxidation are controlled by an antioxidant defense system of enzymatic components such as SOD, CAT, and GR, and non-enzymatic components such as GSH and vitamin E (Delles et al., 2014).

In the present study, the results of increased MDA and reduced TAC level in the liver, breast and thigh muscles in the birds of G4 indicated that the level of AF (100 µg/kg) could increase the oxidative status in the liver, breast and thigh muscles of broilers, as resulted from an increase of ROS due to the oxidative stress condition in the broilers with AF intoxication. These observations were consistent with the previous reports, as MDA content were significantly increased and antioxidant enzyme activities and GSH level were significantly decreased in the liver and spleen of broilers fed a diet contaminated with AFB1 than those in the control group (Li et al., 2014 and Liu et al., 2016).

The lowest significant ($P \leq 0.05$) liver, breast and thigh muscles MDA levels were in birds of G2 which had also the highest significant ($P \leq 0.05$) TAC in the same organs indicating the obvious antioxidant activity of DPS2% in birds. Regarding G5, G6 and G7 aflatoxicated and treated groups, there were non-significant differences ($P \geq 0.05$) in MDA and TAC levels, however, the lowest level in those 3 groups was in G6 as 37.83, 8.6 and 8.16 nmol/g for MDA and in G5 as 3.3, 0.68 and 2.5 for TAC in liver, breast and thigh muscles, respectively. So, the addition of date pits is contributed to make a difference in the total antioxidant capacity of the body as the antioxidant activity and phenolic content of DPS had been well investigated by researchers (Sharifi et al., 2017). In addition, Habib and Ibrahim (2011) studied the effect of DPS on antioxidant status in rats and reported that including 7% or 14% of DPS to their diet had a significant effect on MDA in both liver and plasma, while not affecting SOD, GSH-Px and catalase in the liver. Also, significant decrease in MDA level in the testicular tissue of DPS treated male rats in comparison with the control one (Orabi and Shawky, 2014). Similar results that HSCAS stimulate antioxidant functions by relieving oxidative stress during aflatoxicosis (Chen et al., 2014).

Also, apoptosis or programmed cell death phenomenon is an important indicator of toxicity which characterized by a series of typical morphological features, such as shrinkage of the cell, fragmentation into membrane-bound apoptotic bodies and rapid phagocytosis by neighboring cells (Saraste and Pulkki, 2000). Caspase-3 (cysteinyl aspartate proteinase) is one of cysteine proteases which play a major role in the execution of apoptosis (Nicholson, 1999). A number of genetic and biochemical studies suggest that caspase activation is essential for the occurrence of the apoptotic phenotype of cell death (Janicke *et al*., 1998). Results of Caspase-3 expression levels as apoptotic regulator mRNA gene as detected by qRT-PCR indicated that its expression levels in liver of broilers were increased significantly ($P \leq 0.05$) with maximum value in birds of G4 as 11.08 indicating sever apoptosis in the hepatic cells as a result of aflatoxicosis. The increased caspase-3 activity was associated with biochemical disturbances in oxidant/antioxidant balance system as it was positively correlated with LPO while negatively correlated with GSH in rat liver tissues treated with AFB1 (Meki et al., 2004). This may be ultimately interlinked in the pathogenic network of the AFB1 toxicity whereas an imbalance between ROS and the antioxidant enzymes may lead to the alteration of structure and function of proteins, lipids, and DNA, and then induce the damage of lipid membranes, cellular catalytic reactions, and finally cell apoptosis (Jorgenson et al., 2013).

Dietary AFB1 exposure is able to induce excessive apoptosis in chicken by triggering mitochondrial and death receptor mediated apoptosis pathways leading to oxidative DNA damage as there are two major pathways that lead to apoptosis: (1) death receptor (DR), and (2) mitochondrial, both pathways lead to the activation of caspase-3, the effector apoptotic protein (Canbay et al., 2002; Elmore, 2007 and Peng et al., 2016). Also, AFB1 is bioactivated by P450 enzymes to generate AFB1exo-8, 9-epoxide, which can react with DNA, forming trans-8, 9-dihydro-8-(N7-guanyl)-9-hydroxyaflatoxin B1 (AFB1-N7-Gua) leading to oxidative effect (Wang and Groopman, 1999).

Moreover, in the current study, gene expression levels of Caspase-3 in G7, G6 and G5 as 1.77, 3.06 and 5.86 respectively were decreased significantly ($P \leq 0.05$) in relation to G4 treated with AF alone (11.08). The minimum value was obtained in G3 as 0.58., indicating that Caspase-3 gene expression levels significantly decreased following date pits supplementation and the higher supplementation of date pits reduced the expression of apoptotic dysregulation Caspase-3 in the liver indicating better hepatic cell regeneration and inhibited liver apoptosis through correcting the disturbance of oxidant/antioxidant balance system and hence ensures early and complete recovery from Aflatoxicosis. Similary, dates (flesh and pits) have hepatoprotective effects and increasing hepatocytes resistance to apoptosis, through their ability to attenuate the modulating levels of Fas, caspase-3, Bax and Bcl2 and to induce HO-1 expression in liver cells; however, their anti-apoptotic effects have not been investigated (Al-Rasheed et al., 2017). This protection of dates could be attributed to the high content of antioxidants including phenolics and different classes of flavonoids (Hamad et al., 2015), this antioxidant effects of date decreased the toxicity of aflatoxin and improved the liver histopathologically.

This beneficial effect of date pits supplementation appeared obviously through diminishing the mortality rate from 20% (6/30) in birds of G4 (AF) to 10% (3/30) in birds of G6 (AF+DPS2%) and 13.3% (4/30) in G7 (AF+DPS4%) which was the same as G5 (AF+HSCAS). This effect could be also explained histopathologically through the graded protection of bird liver in G6 and G7 fed DPS 2% or 4% plus AF from mild to moderate lesions compared to those of G4 supplemented by 100 ppb aflatoxin which had severe liver lesions as indicative for aflatoxicosis exhibited through liver congestion, fatty degeneration, and necrosis which may be due to increase the expression of caspase-3 in liver and induction of apoptosis leading to disturbance of oxidant/antioxidant balance. Previous researches recorded the toxic effect of aflatoxins in liver as Bovo et al. (2015) and Gholami-Ahangaran et al. (2016) who recorded congestion, degeneration, and necrosis in chicken's liver up on receiving 3 ppm aflatoxin and owed this to the cellular macromolecules damages (lipid, DNA, and protein) that leads to lipid peroxidation and oxidative damage of DNA (Allameh et al., 2005). Also, it seems that this effect of mannanoligosacharides in DPS on AF not only related to the binding capacity but also these MOS have the ability to prevent colonization of opportunistic bacterial pathogens in the gastrointestinal tract (Olsen, 1995).

However, the addition of HSCAS to diet contain 100ppb AF in G5 couldn't fully improve the hepatic function as there were moderate congestion of liver blood vessels and portal vein which indicated that HSCAS didn't completely protect broilers against aflatoxicosis but partially ameliorated its effect which may be attributed to the dose of HSCAS as mentioned previously by Neeff et al. (2013) who reported that HSCAS didn't completely protect broilers against aflatoxicosis, but was effective in reducing aflatoxin residues in liver and kidney of chicks fed 2.5 mg of AFB1/kg of diet from 0-21 days. Also, Phillips (1999), said that the protective effect of HSCAS resulted from the rapid binding capacity of HSCAS to aflatoxins in the gastrointestinal tract of chickens, thus preventing its absorption and normal distribution to the liver. This effect could be increased through higher HSCAS dose supplemented in feed. This agreed with Pasha et al (2007) who reported that the mortality increased significantly in broilers to 40% with the addition of 100 mcg/kg AF and was restored to 16.6%, with the dietary inclusion of 0.5% simple sodium bentonite.

Finally, no significant toxic microscopic lesions were evident in liver sections of birds in G1 (control) or G2, G3 fed DPS 2% and 4% respectively and all these 3 groups had no mortalities.

5. Conclusion

Addition of date pits (2, 4%) to broiler's diet ameliorated the negative effects of aflatoxins and induced a partial protective effect against aflatoxicosis and this protection is dose-related as 2% supplementation gave better protection than the higher dose 4%. Also, incorporation of DPS in broiler chicken's diet produces a muscle of lower levels of MDA that of several beneficially effects on human health. Date pits can be an attractive alternative for aflatoxin binders, since they are efficient, applicable and cheap. So, we advise to use date pits as a feed additive to control aflatoxicosis in poultry farms, avoiding the harmful chemical mycotoxin binders causing appreciable losses in nutritive value and palatability. Concomitantly, further studies on the combinations of date pits and other medicinal plants having protective effect against aflatoxicosis in poultry should be applied. Also, other researches should be done targeting the spectrum of activity of date pits regarding another types of mycotoxins.

Competing Interests

The authors have no conflict of interest.

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