THE BIOCHEMICAL PROTECTIVE ROLE OF SOME HERBS AGAINST AFLATOXICOSIS IN DUCKLINGS: II. *NIGELLA SATIVA*

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Abstract

Objective - *Our study was aimed to study the adverse effects of aflatoxin on some biochemical parameters in serum and liver of duckling as well as to evaluate the possible protective effects of Nigella sativa crushed seeds against the adverse effects of aflatoxin. Moreover, this experiment was extended to explore if these herb can decrease the production of aflatoxin in vitro by Aspergillus flavus toxigenic strain.* *Design -* *in vitro and in vivo experimentation, serum parameters, malondialdhyde (MDA), Glutathione (GSH), Glutathione S-transferase (GST) and aflatoxin residues.* *Results -* *Aflatoxin produced by Aspergillus flavus toxigenic strain was selected as a potent and widely distributed hepatotoxin that induces much health and economic hazards in animal and human. Aspergillus flavus was cultivated on rice to produce the aflatoxin used in the present study. Ducklings were allotted to control, aflatoxin treated, Nigella sativa and aflatoxin and Nigella sativa groups. Total proteins, albumin, globulins, alanine aminotransferase (ALT) (EC 2.6.1.1) and aspartate aminotransferase (AST) (EC 2.6.1.2), cholesterol and triacylglycerols were measured in serum. The levels of hepatic Malondialdhyde and Glutathione level, Glutathione S-transferase (GST) (EC: 2.5.1.18) activity, aflatoxin residues and histopathological changes were also measured. The obtained results suggest that the addition of Nigella sativa to duckling's ration has a protective effect against aflatoxicosis.* *Conclusions -* *From the obtained results, it can conclude that aflatoxin has hepatotoxic effects through decrease of total proteins, albumin, glutathione and glutathione S-transferase. Moreover, increase ALT, AST, cholesterol, triacylglycerols and lipid peroxidation levels. In addition, aflatoxin induced histopathological changes of liver and residues of aflatoxin were measured. While, addition of Nigella sativa to duckling's diet were induced a protective effect against aflatoxicosis. So, we advice to use Nigella sativa as a feed additive to control aflatoxicosis in poultry farms.*

Key words: *Aspergillus flavus*; Aflatoxin; *Nigella sativa*; antioxidant; Hepatoprotection

INTRODUCTION

Aflatoxins (AF) can be produced by the four toxic species of *Aspergillus: A. flavus, A. parasiticus, A. nomius* and *A. pseudotamarii* as secondary metabolites [1]. Since their discovery in the 1960s, AF has been demonstrated to be carcinogenic in many animal species, including rodents, non-human primates and fish. They have been also suspected to induce to human hepatocellular carcinoma, in addition, many species, including rat, turkey, duck, trout, and primates, are susceptible to the carcinogenic effects of AFB_1. [2]**.** Corn and peanut showed that AFB_1 contamination to be higher than 50% in Egypt [3].

Nigella sativa, one of the members of Ranunculaceae family, commonly grows in the Middle East, Eastern Europe and Eastern and Middle Asia. *Nigella sativa* and its oil are being used as food additives as well as natural remedies for many ailments for over

thousands of years. Many active ingredients have been isolated from *Nigella sativa*, including: thymoquinone, thymohydroquinone, dithymo- quinone, thymol, carvacrol, nigellicine and alpha-hedrin which have been identified, such as antitumour activity, antioxidant activity, anti-inflammatory activity, antibacterial activity and immunostimulant [4]. The seeds have been used as a natural remedy for more than 2000 years to promote health and treat diseases. Medicinal properties of black seeds have even been mentioned by the Prophet of Islam, Muhammad (Peace be upon him) and its use was recommended for various ailments [5].

MATERIAL AND METHOD
Animals
The present study was carried out on 100 Pekin ducklings with an average body weight of (200 g). They were given basal diet and water *ad libitum* and housed in the same place for two weeks before the beginning of experiment for acclimatization and to ensure a normal growth and behavior.

Medical Plants and *Aspergillus flavus* strain
Nigella sativa seeds were identified (Department of Plant, Faculty of Agriculture, Damanhour university), purified, finally ground in mortar and added to the diet along the period of experiment as which will be shown below. Moreover, *Aspergillus flavus* toxigenic strain was used for *in vitro* experiment.

Diet
Basal diet contains (63.1% ground yellow corn, 28.2% soya been meal (44% CP), 4.3% corn gluten meal (60% CP), 0.6% vegetable oils, 1.8% dicalcium phosphate, 1.1% ground limestone, 0.4% common salts, 0.3% mineral and vitamin premix, 0.1% lysine and 0.1% methionine) was used for feeding the duckling.

In vitro experiment
Aflatoxin Production on Corn
Aspergillus flavus isolate was kept in physiological salt solution containing 0.01% Tween 80 (PST); they were inoculated onto Czapek's Dox plates and incubated at 30C for 7 days. After this period, the spores were harvested by washing the Czapek's Dox surface with sterile PST. The harvested spores were enumerated and used as inoculums of $(5 \times 10^3/\text{ml})$ spore count [6].

Aspergillus flavus strain was inoculated on corn flasks (triplicate for each treatment) according to the method of [7] with some modifications as the following; 50 g of yellow corn and *Aspergillus flavus* inoculums (T_1); 50 g of yellow corn, *Aspergillus flavus* inoculums and *Nigella sativa* crushed seeds 0.5% (T_2); 50 g of yellow corn, *Aspergillus flavus* inoculums and *Nigella sativa* crushed seeds 1.0% (T_3) and 50 g of yellow corn, *Aspergillus flavus* inoculums and *Nigella sativa* crushed seeds 2.0% (T_4).

Aflatoxin Extraction and Quantification
Residues of AFB_1, AFB_2 in corn were determined using the method of Association of Official Analytical Chemists [8] with same modifications. Then the extracted aflatoxins were measured by high performance liquid chromatography (HPLC).

Aflatoxin Production on Rice for *in vivo* Experiment
Aspergillus flavus (spores) was cultured on rice for production of aflatoxin which will be used for *in vivo* experiment by the method of [7]. Rice has been collected, dried, grinded and subjected to extraction of aflatoxin which then measured by HPLC. The extracted aflatoxin has been dissolved in corn oil by using chloroform by dissolving the aflatoxin extract in chloroform then adding to the corn oil with continuous mixing by magnetic stirrer at 60°C to evaporate chloroform leaving aflatoxin mixed with corn oil.

In vivo Experiment
Hundred ducklings were allotted into two equal groups 50 ducklings classified as following: Group1: (It fed basal diet with corn oil orally) and Group2: (It fed basal diet with corn oil contains aflatoxin (30 µg/kg ducklings daily) orally for 2 weeks (five ducklings were die during this period). After two weeks five ducklings from each group were scarified. Blood and liver samples were collected for biochemical analysis and histopathological examination of liver. After withdrawal of aflatoxin the remaining birds of each group were allotted into two subgroups. So, the experimental design will become as the following; Control (Group3) consists of 20 ducklings fed a basal diet; Group4: consists of 25 ducklings fed a basal diet containing crushed *Nigella sativa* seeds by a concentration of 2.0%; Group5: consists

of 20 ducklings fed a basal diet and Group6: consists of 20 ducklings fed a basal diet containing crushed *Nigella sativa* seeds by a concentration of 2.0%. Blood and liver samples were collected at 6th and 8th weeks after aflatoxin withdrawal.

Blood and Liver Samples

At 6th (2 weeks after AF withdrawal) and 8th week (4 weeks after AF withdrawal) blood samples from each group were collected by animal scarification and centrifuged at 3000 rpm for 10 min. Separated sera were collected in eppindorff tubes and kept at –20°C until biochemical analysis was done.

After ducklings were scarified the abdomen was immediately opened and the liver removed and divided into three parts; the first part was kept at –20°C for biochemical measurements. The second part was kept at –20°C for extraction and quantificatation of aflatoxin residues and the last one was collected in 10% neutral buffered formalin solution (BFS) for histopathology.

Biochemical analysis of serum and liver

Serum samples were subjected to biochemical analysis of serum total proteins and albumin [9], serum globulins [10], ALT (EC 2.6.1.1) and AST (EC 2.6.1.2) [11], cholesterol [12] and triacylglycerols [13]. Liver samples were used for measurement of malondialdhyde (MDA) [14], Glutathione [15] and Glutathione S-transferase (GST) (EC: 2.5.1.18) activity [16]. Residues of aflatoxin in liver samples of all groups before and after withdrawal of aflatoxin were determined by the method of [8].

Histopathological studies

Liver specimen was collected and rapidly fixed in 10% BFS for at least 24 hours. The fixed specimens were processed through the conventional paraffin embedding technique (dehydration through ascending grades of ethanol, clearing in chloroform and embedding in paraffin wax at 60C. Paraffin blocks were prepared from which 5 microns thick sections were obtained and stained by Hematoxyline and Eosin (H&E) according to the method described by [17].

Statistics

All calculations and analysis was done by [18].

RESULTS AND DISCUSSIONS
In vitro experiment

Addition of *Nigella sativa* to yellow corn by concentrations of 0.5%, 1.0%, and 2.0% were decreased the AFB$_1$ production levels significantly with inhibition percentages of 14.75%, 27.95%, 57.55%, respectively when compared with control positive group (Table 1 and Fig. 1). At the same concentrations AFB$_2$ production were inhibited by percentages of 7.14%, 12.96%, and 72.22%, respectively. Aflatoxin production by *in vitro* study indicated that *Nigella sativa* crushed seeds have an inhibitory effect on growth and aflatoxin production by *Aspergillus flavus*. Results from this experiment were come in accordance with studies of the antifungal effects of thymoquinone [19] against *Aspergillus flavus*. *Nigella sativa* oil completely inhibited aflatoxin production by *Aspergillus flavus* at 3% (v/v) concentration, while crude extract of *Nigella sativa* inhibited three types of aflatoxins at 5% (w/v) concentration. The inhibitory effect of *Nigella sativa* on aflatoxin production ability of *Aspergillus flavus* might be related to several components known to have biological activities, such as α-pinene and thymol and high phenolic content [20].

Table 1: Effect of *Nigella sativa* on aflatoxin production from *Aspergillus flavus*.

	AFB$_1$ production level (ppb)	AFB$_1$ production inhibition %	AFB$_2$ production level (ppb)	AFB$_2$ production inhibition%
T$_1$	1152±3.90		54±1.25	
T$_2$	936±0.56*	14.75	50±0.24	7.14
T$_3$	830±5.43*	27.95	47±1.51	12.96
T$_4$	489±4.89*	57.55	15±0.28*	72.22

* Values are significant compared to control (P< 0.05).
T$_1$ (control flasks); T$_2$ (*Nigella sativa* 0.5% and *Aspergillus flavus*);
T$_3$ (*Nigella sativa* 0.5% and *Aspergillus flavus*); T$_4$ (*Nigella sativa* 0.5% and *Aspergillus flavus*).
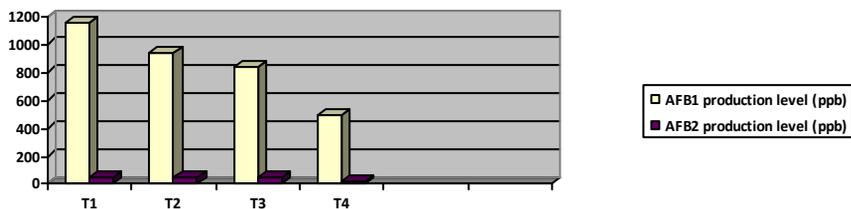
Fig. 1: Effect of *Nigella sativa* on aflatoxin production from *Aspergillus flavus*.

In vivo **experiment**

Table, (2) revealed that in group2 the levels of serum total proteins and serum albumin were significantly decreased. On the other hand, the levels of ALT, AST, cholesterol and triacylglycerols were significantly increased in comparable to control group. The same results were obtained by the studies on broiler chicks [21], Japanese quail [22] and ducks [23]. The decrease in total serum proteins might be contributed to the binding of aflatoxin to DNA. Therefore, aflatoxin hinder transcription and translation in return decrease the protein synthesis, as the exo-epoxide product of aflatoxin metabolism reacted with N_7-guanine in DNA and contributed to protein adduct formation of aflatoxin [24].

Table 2: Effect of aflatoxin on total proteins, albumin, ALT, AST, cholesterol and triacylglycerols levels

	Total proteins	Albumin	ALT	AST	Cholesterol	Triacylglycerol
Group1	7.54 ±0.22	5.09±0.43	17±0.07	18±2.41	120.6±6.25	138.24±7.12
Group2	6.21±0.08*	3.79±0.07*	42±2.37*	48±1.46*	198±4.27*	172.04±8.40*

* Values are significant compared to control (P< 0.05).
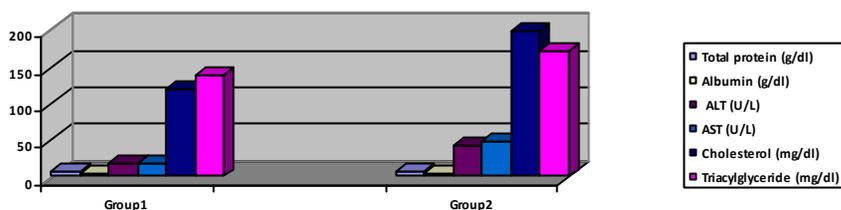Group1 (Control); Group2 (Aflatoxin treated)

Fig. 2: Effect of aflatoxin on total proteins, albumin, ALT, AST, cholesterol and triacylglycerols levels

The data illustrated in Table (3) and Fig. (3) stated that at 6th week of age the serum levels of total protein and albumin were significantly increased while cholesterol and triacylglycerol levels were significantly decreased in group4 in comparison to control one. Group5, serum total protein levels were significantly decreased. In contrary, levels of ALT, AST, cholesterol and triacylglycerol were significantly increased. In group6, all parameter levels were reverted near control levels except ALT enzyme activity. The data illustrated in Table (4) and Fig. (4) revealed that at 8th week total proteins and albumin levels were significantly increased, while cholesterol and triacylglycerol levels were significantly decreased in group4 in comparison with control one. In group5 cholesterol and triacylglycerol levels were significantly increased. In regard to group6 all tested parameters were nearly reverted around normal values this finding might be due to the

antioxidant effect of *Nigella sativa*. These observations are agreed with that of [25] who used *Nigella* cake protein as feed supplement. *Nigella sativa* crushed seeds [26] and thymoquinone [27] were significantly decreased serum triacylglycerol level. This result might be due to the antioxidant effect of *Nigella sativa* against aflatoxin.

Table 3: Effects of *Nigella sativa* on serum total proteins (g/dl), albumin (g/dl), ALT (U/L), AST (U/L), cholesterol (g/dl) and triacylglycerol (g/dl) two weeks after aflatoxin withdrawal.

	6th week of age (2 weeks post- aflatoxin withdrawal)					
	Total proteins	Albumin	ALT	AST	Cholesterol	Triacylglycerol
Group3	7.77±0.11	4.28±0.01	17±1.10	22±1.40	150.75±1.25	130.64±2.01
Group4	8.85±0.03*	5.86±0.11*	16±0.97	21±1.52	129.80±2.14*	112.12±0.10*
Group5	5.74±0.47*	3.08±0.14	42±3.12*	46±3.41*	193.05±4.11*	166.01±0.14*
Group6	7.19±0.78	3.78±0.25	30±2.41*	28±2.56	166.41±3.48	137.37±1.74

*Values are significant compared to control (P< 0.05).
Group3 (Control untreated); Group4 (*Nigella sativa*); Group5 (Aflatoxicated birds);
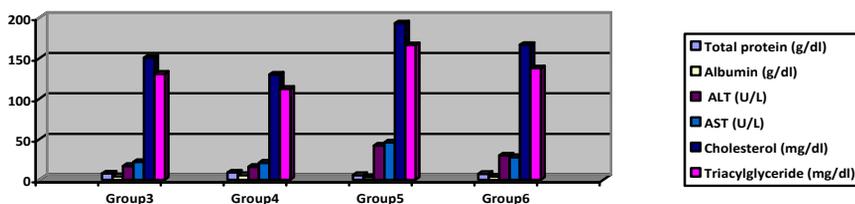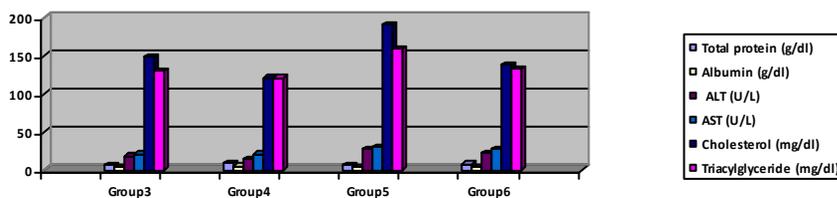Group6 (AF+ *Nigella sativa*)

Fig 3: Effects of *Nigella sativa* on serum total proteins, albumin, ALT, AST, cholesterol and triacylglycerol two weeks after aflatoxin withdrawal.

Table 4: Effects of *Nigella sativa* on serum total proteins (g/dl), albumin (g/dl), ALT (U/L), AST (U/L), cholesterol (g/dl) and triacylglycerol (g/dl) four weeks after aflatoxin withdrawal.

	8th week of age (4 weeks post- aflatoxin withdrawal)					
	Total proteins	Albumin	ALT	AST	Cholesterol	Triacylglycerol
Group3	6.48±0.24	4.25±0.74	18±1.42	21±0.88	149.17±4.23	131.13±3.12
Group4	8.61±0.22*	5.27±0.54*	15±2.40	21±0.16	121.45±1.47*	120.76±0.50*
Group5	6.59±0.14	4.07±0.47	28 ±1.16	31 ±1.33	190.6±4.20*	158.78±4.79*
Group6	7.38±0.81	4.20±0.30	22±0.78	27±1.27	138.6±5.10	133.5±3.12

*Values are significant compared to control (P< 0.05).
Group3 (Control untreated); Group4 (*Nigella sativa*); Group5 (Aflatoxicated birds);
Group6 (AF+ *Nigella sativa*)

Fig 4: Effects of *Nigella sativa* on serum total proteins, albumin, ALT, AST, cholesterol and triacylglycerol four weeks after aflatoxin withdrawal

Observed data in Table, (5) revealed that, in group2 (2 weeks post-treatment) MDA level was increased significantly. While, glutathione levels and GST activities were significantly decreased in comparable to control one. After 2 and 4 weeks of withdrawal of aflatoxin (6th and 8th week) Table (6) revealed a significantly decrease in MDA levels in group4 and a significant increase in group5. But, hepatic glutathione levels were significantly increased in group4 and significantly decreased in group5 than control. *Nigella sativa* significantly increased GST activity at 6th week and 8th week (group4). In contrary its activities were significantly decreased in group5 when compared with control. At 6th week group6, GST activity has no differences but at 8th week, its level was significantly increased.

Aflatoxin treatment caused a significant increase in lipid peroxidation in liver of aflatoxin-treated mice [28], rabbit's liver [29] and plasma MDA of quail [30]**.**After 30 days of aflatoxin treatment in chicks, hepatic GSH levels and GST activities concentration were significantly decreased [31] this decrease is obtained by the oxidant effect of aflatoxin as a xenobiotic substance. *Nigella sativa* crushed seeds significantly increased the activity of hepatic GST and GSH concentration in rabbit's liver. In contrary, lipid peroxidation product (MDA) levels were significantly decreased [32]. Pretreatment with thymo-quinone and *Nigella sativa* oil were induced a significant decrease in MDA level as compared with ischemic group [33]**.**
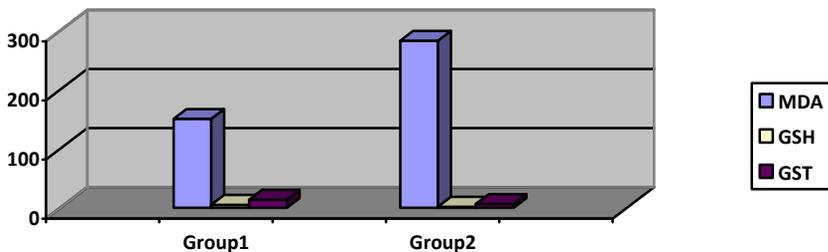
Table 5: Effect of aflatoxin on hepatic MDA (nM MDA / g wet liver) and glutathione (µM / g wet liver) levels and GST activity (mmol/min/g wet liver) at 4th weeks of age (2 weeks post-treatment).

	MDA	GSH	GST
Group1	150.54±15.31	5.26±0.13	13.9±2.41
Group2	283.09±23.46*	2.01±0.24*	6.45±1.50*

* Values are significant compared to control (P< 0.05).
Group1 (Control); Group2 (Aflatoxin treated)

Fig. 5: Effect of aflatoxin on hepatic MDA (nM MDA / g wet liver) and glutathione (µM / g wet liver) levels and GST activity (mmol/min/g wet liver) at 4th weeks of age

Table 6: Effects of *Nigella sativa* on MDA level (nM MDA / g wet liver), glutathione level (µM / g wet liver) and glutathione S- transferase activity (mmol/min/g wet liver) after withdrawal of aflatoxin

	6th week			8th week		
	MDA	GSH	GST	MDA	GSH	GST
Group3	146.43 ± 1.12	4.67±0.12	9.73 ± 1.03	152.7±7.15	4.82±0.14	21.74±2.45
Group4	113.41±1.02*	9.79±0.02*	17.77±2.78*	95.04±5.40*	11.14±0.45*	39.33±4.01*
Group5	226.15±0.11*	1.14±1.47*	6.14 ±2.14	203.37±1.45*	2.69 ±0.25	11.87±2.69*
Group6	154.80±1.23	6.17±0.25*	7.90 ±1.01	138.80 ± 0.45	5.43±1.21*	24.33±1.51

* Values are significant compared to control (P< 0.05).
Group3 (Control untreated); Group4 (*Nigella sativa*); Group5 (Aflatoxicated birds);
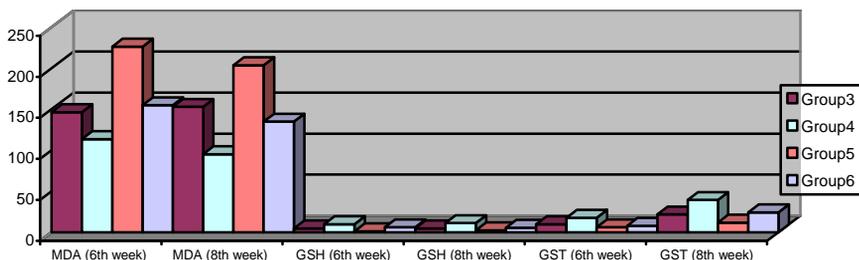Group6 (AF+ *Nigella sativa*)

Fig. 6: Effects of *Nigella sativa* on hepatic MDA, glutathione and glutathione S- transferase activity after withdrawal of aflatoxin

The results tabulated in Table (7) showed that the level of AFB_1 and AFB_2 accumulation in duckling's liver of group2, in which aflatoxin B_1 and B_2 residues are 2.21 ppb and 0.27 ppb respectively. In the group5 aflatoxin B_1 residues were decreased to 0.69 ppb and 0.24 ppb than that of group2 at 6th and 8th weeks respectively. Also, the level of aflatoxin B_1 residues were decreased in group6 to became 0.21 ppb at 6th week and in non detected amount at 8th week. In respect to AFB_2 residues were became in non detectable level in the group5 and group6 all over the experimental period. Administration of aflatoxin to ducklings led to accumulation of aflatoxin in liver (Table, 7) which extracted and measured by HPLC. A variety of domestic poultry species fed with diets containing 3000 ng/kg AFB_1 for a 7day period and showed that levels of AFB_1 and its metabolites were greater in liver than in muscle for all bird species tested [34]. The highest levels of AFB_1 residues were found in the liver, followed by the crop and muscle tissue [35]. AFB_1 residues were observed only in muscle and liver of birds given dietary AFB_1 [36]. While, in group6 supplementation of ducklings by *Nigella sativa* crushed seeds decreased the residues of aflatoxin until 6th week and this decreased continued until the level of aflatoxin in liver became in non-detectable level at 8th week. This protective effect of *Nigella sativa* might be return to the antioxidant effect of *Nigella sativa.*

Table 7: AFB_1 and AFB_2 residues of duckling's liver before and after aflatoxin withdrawal

	4th week			6th week		8th week	
	AFB_1	AFB_2		AFB_1	AFB_2	AFB_1	AFB_2
Group1	ND	ND	Group3	ND	ND	ND	ND
			Group4	ND	ND	ND	ND
Group2	2.21 ppb	0.27 ppb	Group5	0.69 ppb	ND	0.24 ppb	ND
			Group6	0.21 ppb	ND	ND	ND

* Values are significant compared to control (P< 0.05).
Group1 (Control); Group2 (Aflatoxin treated); Group3 (Control untreated); Group4 (*Nigella sativa*);
Group5 (Aflatoxicated birds); Group6 (AF+ *Nigella sativa*); ND (Non detectable level)

Histopathological studies

Fig. 7 showed normal healthy hepatocytes of control (group1). While, Fig. 8 showing diffuse hydropic degeneration due to oral administration of aflatoxin for two weeks. Histopathologically, in group4 at 6th week Fig. 9 showed normal healthy hepatocytes. While at 8th week after aflatoxin withdrawal, *Nigella sativa* succeeded to induce an activation of lymphoid aggregation in liver (Fig., 10). Fig. 11 showed the liver of ducklings in the group5 at 6th week after withdrawal of aflatoxin showing diffuse hydropic degeneration. While a degenerative changes in the hepatocytes with granular cytoplasm were noticed at 8th week (Fig., 12). Fig., (13) cleared that; addition of *Nigella sativa* in group6 at 6th week induced a focal infiltration of inflammatory cells beside mild hydropic degeneration. While addition of *Nigella sativa* (8th week) induced a complete regeneration of the hepatic parenchyma (Fig., 14). Oral administration of aflatoxin for two weeks induced liver injuries including diffuse

hydropic degeneration. The microscopic changes including diffuse degeneration in parenchymal cells, enlarged nuclei and extensive bile duct proliferation was the most common acute toxicity of aflatoxin in chicken and ducklings livers respectively [37]. In addition, aflatoxin induced a liver injury of ducklings such as bile duct proliferation and fatty degeneration [38]. Poultry diets containing 2.0% *Nigella sativa*

crushed seed is too useful to birds. Histologically, *Nigella sativa* induced an activation of lymphoid aggregation in liver at 6th week ended by complete regeneration of liver at 8th week. A marked increase in the number of mucus-secreting goblet cells in the airway mucosa, blocking of lung tissue eosinophilia and goblet cell hyperplasia were induced by thymoquinone [39]**.**
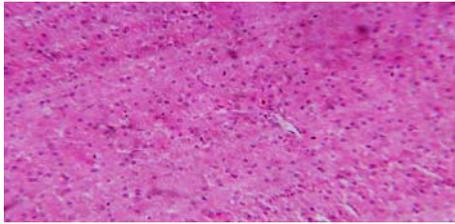
Fig. 7: liver of duck in the group1 (control) showing normal healthy hepatocytes. H&E (X250).
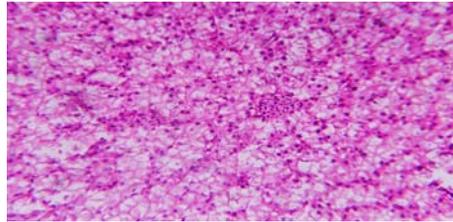

Fig. 8: liver of duck exposed to aflatoxin (group2) for two weeks showing diffuse hydropic degeneration. H&E (X250).
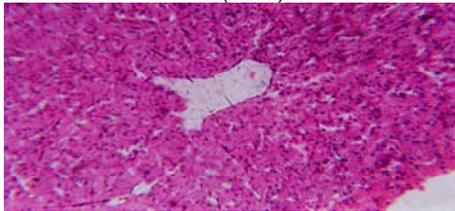

Fig. 9: liver of ducklings in *Nigella sativa* at 6th week after withdrawal of aflatoxin showing normal hepatocytes. H&E (X 250).
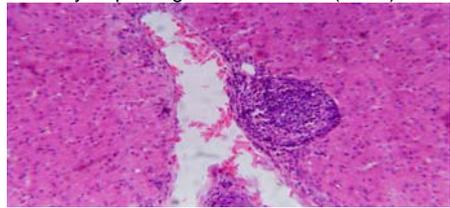

Fig. 10: liver of ducklings *Nigella sativa* 8th week after withdrawal of aflatoxin showing an activation of lymphoid aggregation H&E (X 250).
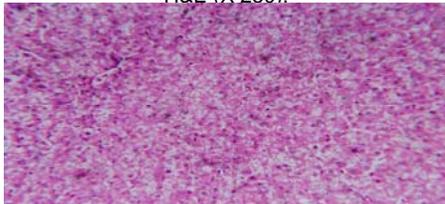

Fig. 11: liver of ducklings in the group5 at 6th week after withdrawal of aflatoxin showing diffuse hydropic degeneration. H&E (X 250).
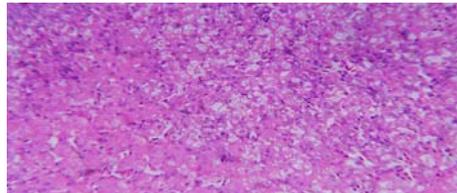

Fig. 12: liver of ducklings in the group5 at 8th week after withdrawal of aflatoxin showing degenerative changes in the hepatocytes with granular cytoplasm. H&E (X 250).

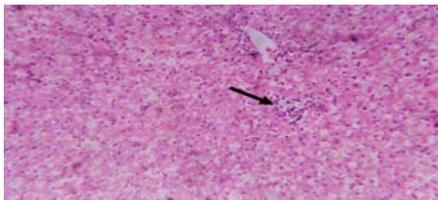
Fig. 13: liver of ducklings in the group6 at 6th week after withdrawal of aflatoxin showing focal infiltration of inflammatory cells (arrow) beside mild hydropic degeneration. H&E (X 250).
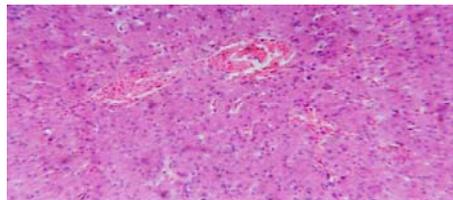

Fig. 14: liver of ducklings in the group6 at 8th week after withdrawal of aflatoxin showing a complete regeneration of the hepatic parenchyma. H&E (X 250).

CONCLUSIONS

From the obtained results, it can conclude that addition of *Nigella sativa* as a feed additive in poultry diet products the hepatotoxicity induced by aflatoxin as *Nigella sativa* inhibit the growth and aflatoxin production by *Aspergillus flavus*.

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