Protective Effects of *N. sativa* against the Adverse Effects of Aflatoxicosis in Ducklings

S. K. L. Karna

Trichandra Multipul Campus, Tribhuvan University, Ghantaghar, Kathmandu
e-mail: skkkth@yahoo.com

Abstract

Aflatoxin produced by *Aspergillus flavus* toxigenic strain was selected as a potent and widely distributed hepatotoxin that induces much health and economic hazards in animals and humans. *A. flavus* was cultivated on rice to produce the aflatoxin used in the present study. It was aimed to study the adverse effects of aflatoxin on some biochemical parameters in serum and liver of ducklings as well as to evaluate the possible protective effects of *N.sativa* crushed seeds against the adverse effects of this. 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 the serum of the test animal. 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 *N. sativa* to duckling's ration has a protective effect against aflatoxicosis. The result concluded that aflatoxin had hepatotoxic effects through decrease of total proteins, albumin, glutathione and glutathione S-transferase. In addition, aflatoxin induced histopathological changes of liver of the test animal and residues of aflatoxin were measured. So, we advice to use *N. sativa* as a feed additive for controlling aflatoxicosis in poultry farms.

Key words: *Aspergillus flavus*, aflatoxin, *N.sativa*, antioxidant, hepatoprotection

Introduction

Aflatoxins (AF) are produced by *Aspergillu flavus*, *A. parasiticus, A. nomius* and *A.and pseudotamarii* as as secondary metabolites (CAST 2003). Since their discovery in the 1960s, AF have 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. (Jackson 1999)**.** Corn and peanut showed that AFB_1 contamination to be higher than 50% in Egypt (Donmat 2007) *N. sativa* and its oil is 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 this plant, 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 (Salem 2000). The seeds have been used as a natural remedy for more than 2000 years to promote health and treatment of diseases of animals. Medicinal properties of black seeds have even been mentioned by the Prophet of Islam, Muhammad and its use was recommended for various ailments (Bhati 2009)*.*

Methodology

The present study was carried out on 100 Pekin ducklings each weighing 200g at (Hetauda Farm, Makawanpur) 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 commencing the experiment for acclimatization and to ensure a normal growth and behavior.

Medical plants and *Aspergillus flavus* strain

N.sativa seeds were obtained from the Department of Botany, Trichandra Campus, Tribhuvan University, Kathmandu, purified and , finally ground in mortar and

added to the diet as discribed below *A. flavus* toxigenic strain was used for the *in vitro* experiment.

Diet

Basal diet of the ducklings included (63.1% ground yellow corn, 28.2% soya bean meal, 4.3% corn gluten meal, 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).

In vitro experiment
Aflatoxin production on corn

A. flavus isolates were kept in physiological salt solution containing 0.01% Tween 80 (PST); They were inoculated onto Czapek'sDox plates and incubated at 30^0C for 7 days. After this period, the spores were harvested by washing the Czapek'sDox surface with sterile PST. The harvested spores were enumerated and used as inoculums of $(5x10^3/$ ml) spore count (Criseo 2001). *A. flavus* strain was inoculated on corn flasks (triplicate for each treatment) according to the method of (shotwell 1966) with some modifications as the following: 50 g of yellow corn and *A. flavus* inoculums (T_1), 50 g of yellow corn, *A. flavus* inoculums and *N.sativa* crushed seeds 0.5% (T_2); 50 g of yellow corn, *A. flavus* inoculums and *N. sativa* crushedseeds 1.0% (T_3) and 50 g of yellow corn, *A. flavus* inoculums and *N. 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 (AOAC 1995) with the same modifications. Then the extracted aflatoxins were measured by high performance liquid chromatography (HPLC).

Aflatoxin production on rice for *in vivo* experiment

A. flavus (spores) was cultured on rice for production of aflatoxin which was used for *in vivo* experiment by the method of (Showell 1996). Rice was collected, dried, grinded and subjected to extraction of aflatoxin which then was measured by HPLC. The extracted aflatoxin was dissolved in corn oil by using 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 Group[1] was fed with basal diet with corn oil orally and Group[2] was fed on basal diet with corn oil containing aflatoxin (30 µg/kg/ duckling daily) orally for 2 weeks. After two weeks, five ducklings from each group were scarified. Blood and liver samples of these birds 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. The experimental design will become as the following; Control (Group[3]) consists of 20 ducklings were fed a basal diet; Group[4]: consists of 25 ducklings were fed a basal diet containing crushed *N .sativa* seeds by a concentration of 2.0%; Group[5]: consists of 20 ducklings were fed a basal diet and Group[6]: consists of 20 ducklings were fed a basal diet containing crushed *N. 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 –20ºC until biochemical analysis was done. After the ducklings were scarified, the abdomens were immediately opened and the liver removed and divided into three parts: the first part was kept at –20ºC for biochemical measurements. The second part was kept at – 20ºC for extraction and quantificatation of aflatoxin residues and the last part was collected in 10% neutral buffered formalin solution (BFS) for histopathology.

Biochemical analysis of serum and liver

Serum samples were subjected to biochemical analysis for serum total proteins and albumin (Henry 1964), serum globulins (Coles 1974), ALT (EC 2.6.1.1) and AST (EC 2.6.1.2) (Reitman 1957), cholesterol (Zak 1954) and triacylglycerols (Fossati 1982). Liver samples were used for measurement of malondialdhyde (MDA) (Placer 1966), Glutathione (Sedlak 1968) and Glutathione S-transferase (GST) (EC: 2.5.1.18) activity(Hebig 1974). Residues of aflatoxin in liver samples of all groups before and after withdrawal of aflatoxin were determined by the method of (AOAC 1995).

Histopathological studies

Liver specimen were 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 60°C. 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 (Culling 1983).

Results and Discussion

In vitro experiment

Addition of *N.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). 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 *N. 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 (AL- Quarashi 2007) against *A.flavus*. *N.sativa* oilcompletely inhibited aflatoxin production by *A.flavus* at 3% (v/v) concentration, while crude extract of *N.sativa* inhibited three types of aflatoxins at 5% (w/v) concentration. The inhibitory effect of *N.sativa* on aflatoxin production ability of *A.flavus* might be related to several components known to have biological activities, such as á-pinene and thymol and high phenolic content (Maraqa 2007)**.**

Table 1. Effect of *N. Sativa* on aflatoxin production from *Aspergillus flavus*

	AFB1 production level (ppb)	AFB1 production inhibition %	AFB2 production level (ppb)	AFB2 production inhibition%
T1	1152±3.90	14.75	54±1.25	7.14
T2	936±0.56*		50±0.24	
T3	830±5.43*	27.95	47±1.51	12.96
T4	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 (*N.sativa* 0.5% and *A. flavus*); T_3 (*N.sativa* 0.5% and *A. flavus*); T_4 (*N.sativa* 0.5% and *A. 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 (Toulah, 2007) Japanese quail (Madheswaran 2004) and ducks (Han 2008). The decrease in total serum proteins might be contributed to the binding of aflatoxin to DNA. Therefore, aflatoxin hinder transcription and translation in return decreased 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 (Cavin 2008)**.**

Table 2. Effect of aflatoxin on total proteins, albumin, ALT, AST, cholesterol and triacylglycerols level

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

The data illustrated in Table 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. 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 *N.sativa*. These observations are agreed with that of [AL-Gaby 1998] who used *Nigella* cake protein as feed supplement. *N.sativa* crushed seeds[Le 2004) and thymoquinone (Badary 2000) were significantly

decreased serum triacylglycerol level. This result might be due to the antioxidant effect of *N.sativa* against aflatoxin. On 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 group 5. But, hepatic glutathione levels were significantly increased in group4 and significantly decreased in group5 than control. *N.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.

Table 3. Effects of *N.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

	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 (*N.sativa*); Group5 (Aflatoxicated birds); Group6 (AF+ *N.sativa*)

Table 4. Effects of *N.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 (N.sativa); Group5 (Aflatoxicated birds); Group6 (AF+ *N.sativa*)

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)

Table 6. Effects of *N. 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

	6 week			8 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< 0.05Group3 (Control untreated); Group4 (N.sativa); Group5 (Aflatoxicated birds); Group6 (AF+ *N.sativa*)

Aflatoxin treatment caused a significant increase in lipid peroxidation in liver of aflatoxin-treated mice (Choudhary 2005), rabbit's liver (Guerre 1999) and plasma MDA of quail (Citil 2005)**.** After 30 days of aflatoxin treatment in chicks, hepatic GSH levels and GST activities concentration were significantly decreased (Karaman 2010) this decrease is obtained by the oxidant effect of aflatoxin as a xenobiotic substance. *N.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 (EL-Far 2003). Pretreatment with thymo-quinone and *N.sativa* oil were induced a significant decrease in MDA level as compared with ischemic group (Hosseinzadeh 2007)**.**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 become 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 were 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 (Bintvihok 2002). The highest levels of AFB_1 residues were found in the liver, followed by the crop and muscle tissue (Madden 1995). AFB_1 residues were observed only in muscle and liver of birds given dietary AFB_1 (Bintvihok 2006). While, in group6 supplementation of ducklings by *N.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 *N.sativa* might be return to the antioxidant effect of *N.sativa*.

**Table 7. AFB1 and AFB2 residues of duckling's liver before and after aflatoxin withdrawal**

	4th week			6th week		8th week	
	AFB1	AFB2		AFB1	AFB2	AFB1	AFB2
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 (*N.sativa*); Group5 (Aflatoxicated birds); Group6 (AF+ *N.sativa*); ND (Non detectable level)

Histopathological studies

Fig.1 showed normal healthy hepatocytes of control (group1). While, Fig. 2 showed diffuse hydropic degeneration due to oral administration of aflatoxin for two weeks.Histopathologically, in group4 at 6thweek Fig.3 showed normal healthy hepatocytes. While at 8th week after aflatoxin withdrawal, *N.sativa* succeeded to induce an activation of lymphoid aggregation in liver (Fig.4). Fig.5 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. 6) .Fig. 7 cleared that; addition of *N.sativa* in group6 at 6th week induced a focal infiltration of inflammatory cells besides mild hydropic degeneration. While addition of N.sativa (8th week) induced a complete regeneration of the hepatic parenchyma (Fig. 8). 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 (Bintvihok 1991). In addition, aflatoxin induced a liver injury of ducklings such as bile duct proliferation and fatty degeneration (Abdolamin 2005). Poultry diets containing 2.0% *N.sativa* crushed seed is too useful to birds. Histologically, *N.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 (EL-Gazzar 2006).
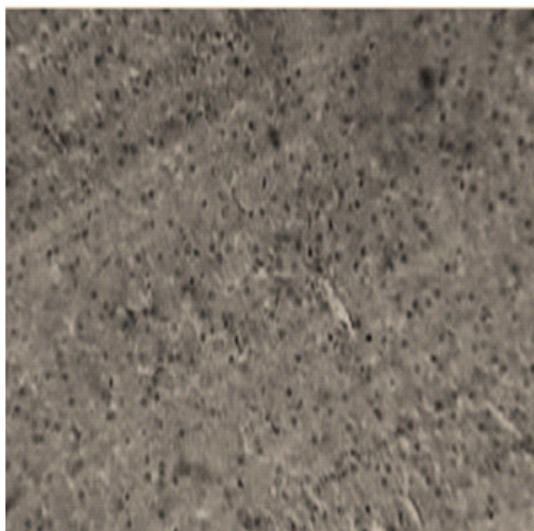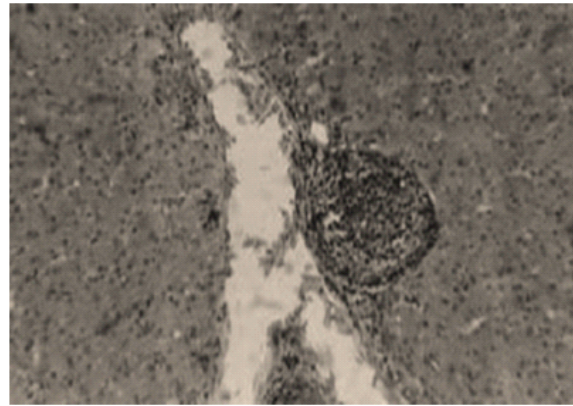

Fig. 1. liver of duck in group1 (control) showing normal and healthy hepatocytes. H&E (X250)
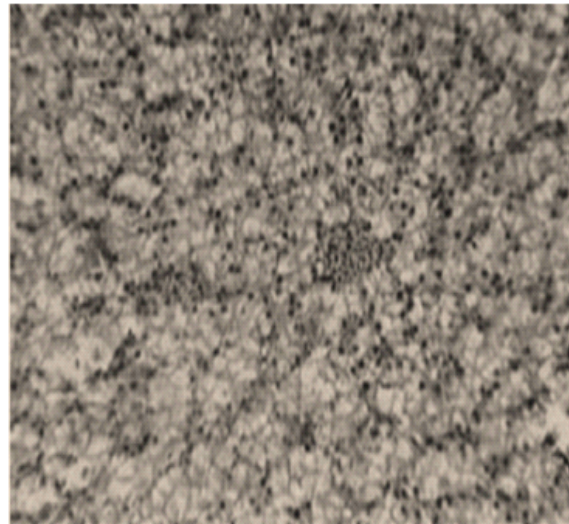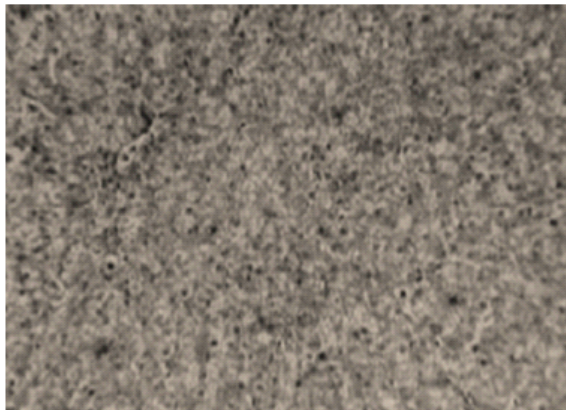

Fig. 2. liver of duck exposed to aflatoxin (group2) for two weeks showing diffused hydropic degeneration. H&E (X250)
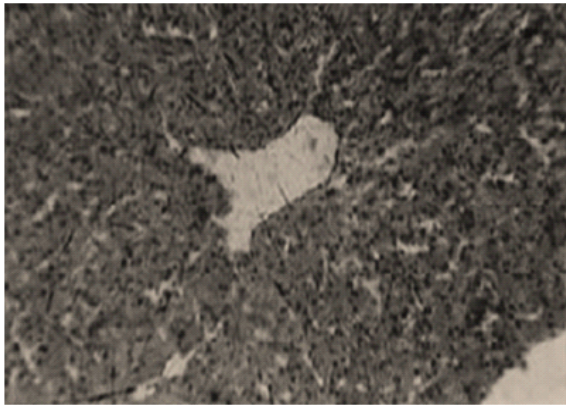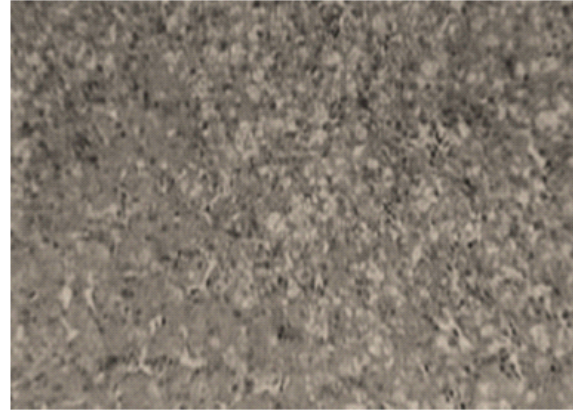
Fig. 3. liver of ducklings in *N. sativa* at 6 th week after withdrawal of aflatoxin showing normal hepatocytes. (H&E (X 250)
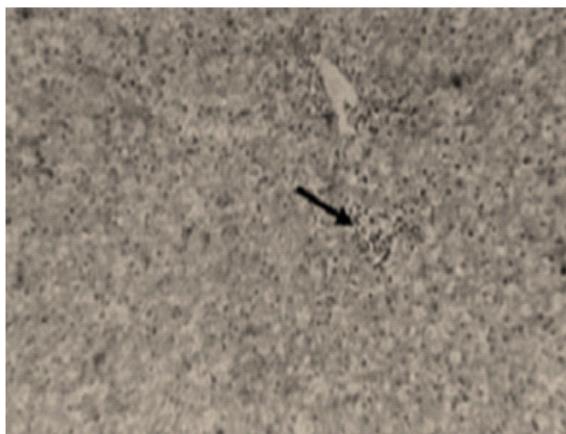

Fig. 4. liver of ducklings *N. sativa* 8th week after withdrawal of aflatoxin showing an activation of lymphoid aggregation (H&E (X 250)
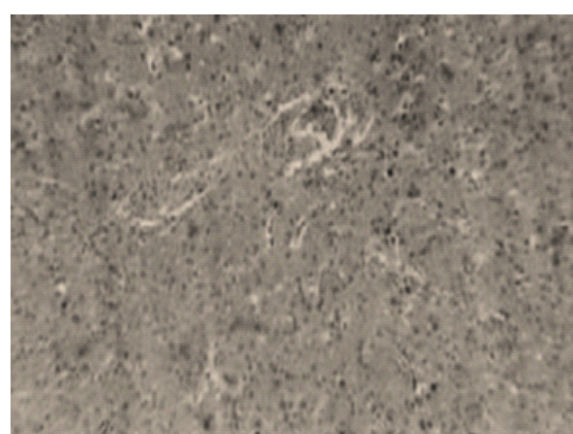

Fig. 5. liver of ducklings in the group5 at 6 th week after withdrawal of aflatoxin showing diffuse hydropic degeneration H&E (X 250)

Fig. 6. liver of ducklings in the group5 at 8 th week after withdrawal of aflatoxin showing degenerative changes in the hepatocytes with granular cytoplasm H&E (X 250)

Fig. 7. liver of ducklings in the group6 at 6 Th week after withdrawal of aflatoxin showing focal infiltration of inflammatory cells (arrow) beside mild hydropic degeneration H&E (X 250)

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

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

Acknowledgements

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