International Journal of Advanced Research in Biological Sciences
ISSN: 2348-8069
www.ijarbs.com
DOI: 10.22192/ijarbs Coden: IJARQG(USA) Volume 6, Issue 3 - 2019

Research Article

DOI: http://dx.doi.org/10.22192/ijarbs.2019.06.03.007

# Histopathological study on the protective effect of humic acid against aflatoxins induced- oxidative stress in rats

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## Abstract

Aflatoxins (AFs) are toxic fungal metabolites associated with wide range of diseases, namely aflatoxicoses. Humic acid (HA) a natural pervasive molecule resulted from decomposition of organic matters. HA have wide range of biological activities. The current study dedicated to evaluate the role of HA in preservation of liver, kidneys, testes and brain against structural changes in male albino rats treated with low and high doses of AFs for six weeks. Tissue lipid peroxidation (LPO) (evaluated by malondialdehyde, MDA) and reduced glutathione (GSH) concentrations as well as histopathological examination were investigated at the end of experimental period. The oral administration of AFs at two doses of 1 and 2mg/kg body weight (b.w)/once/week resulted in significant dose-dependent reduction of GSH and significant dose-dependent enhancement of MDA production in examined organs. The oxidative stress was subsequently induce sever damage in liver, kidneys, testes and brain manifested by dose- dependent histopathological alterations. Fortunately, combined treatment of HA at two doses (200 and 400mg/kg b.w/daily) with AFs resulted in enhancement of antioxidant status of tested organs. Moreover HA especially high dose showed powerful protective effect against pathological changes induced by AFs in all examined organs. HA could be considered a promising natural anti-aflatoxicosis agent.

**Keywords:** Aflatoxins, histopathology, humic acid, oxidative stress, rats.

## Introduction

AFs are group of mycotoxins which have structure similarity and produced by certain pathogen fungal species of *Aspergillus* (*A. flavus*, *A. parasiticus* and *A. nominus)*as a secondary metabolites (Bintvihok, 2002 and Corcuera *et al*., 2011). Aflatoxin $B_1$ ($AFB_1$, the most ferocious), aflatoxin $B_2$ ($AFB_2$), aflatoxin $G_1$ ($AFG_1$) and aflatoxin $G_2$ ($AFG_2$) are the most common naturally produced aflatoxins (Verma, 2004). AFs represent a huge worldwide challenge due to their remarkable deleterious effects on economic and health of human and animals. AFs considered as food born toxins, they contaminate wide range of crops (peanut, corn, cereal crops, grains, beans, rice, and oil seeds crops) and improper stored foods especially under humid and worm climate which enhance mold growth and subsequent mycotoxins production (Valchev *et al*., 2013). Consumption of contaminated food and edible tissue of infected animals and poultry as well as their by-products lead to serious health problem (Groopman *et al*., 2008). Most of body systems and organs including immune system, metabolic system, liver, kidney, brain, heart and testes are vulnerable to toxic effects of AFs. Moreover AFs recorded as potent teratogenic, mutagenic, genotoxic and cancerous

agents (Groopman *et al.*, 2008, Golli-Bennour *et al.*, 2010, Yilmaz *et al*., 2018 and Hussein *et al*., 2019). Liver of human and animals is the target organ of AFs. AFs metabolized by hepatic cytochrome *P450* enzyme system to produce very toxic metabolites. Inside liver, $AFB_1$ is converted to $AFB_1$-8,9-epoxide a highly reactive intermediate which capable of binding to DNA to form $AFB_1$-DNA adducts. The major adduct is 8,9-dihydro- 8-(*N7*guanyl)-9-hydroxy-$AFB_1$ ($AFB_1$*N7*-Gua) (Smela *et al.*, 2001 and Preston and Williams, 2005). These complex processes accompanied by induction of free radicals, reactive oxygen species (ROS) like superoxide anion($O^{-2}$), hydroxyl radical ($OH^-$) and hydrogen peroxide ($H_2O_2$) and subsequent LPO which attack cellular macromolecules, in the end resulted in losing of cellular function (Towner *et al*., 2003, Berg *et al.*, 2004 and Guarisco *et al.*, 2007).

Exploring new, practical, cheep and effective strategies to control AFs toxicity are importunately needed.

Humic acid (HA) is ubiquitous around the globe as it resulted from decomposition of organic matter. Peat, dead plants, mud, sediments, soils and well waters considered as major sources of humic substances (Hartenstein, 1981). HA is one of the most important and active fraction of humic substances which recently used in animal and poultry nutrition and veterinary practice (Islam *et al*., 2005, Rath *et al*., 2006 and Hullár *et al*., 2018) for its antidiarrheal, analgesic, stimulating immunity, antioxidant and antimicrobial properties (Ipek *et al*., 2008 and Vucskits *et al.*, 2010).HA is a high-molecular weight aromatic polymer which has a complex chemical structure (aromatic rings, phenolic hydroxyl, ketone carbonyl, quinone carbonyl, carboxyl, and alkoxyl groups). HA structure varies according to geographic locations (Stevenson, 1985). The antioxidant capability, adsorption capacity and complex- forming ability of HA is owing to its reactive groups especially carboxyl and hydroxyl groups which act as electron donor and oxygen transfer molecules in biological systems (Madronova *et al.*, 2001 and Vucskits *et al.*, 2010). A recent report by Abd El-Shafea *et al.* (2014) has described that HA successfully adsorb AFs *in vitro* and reduce its bioavailability *in vivo*. Nowadays HA become a subject of interest due to lack of information about its clinical biological activities.

The current study aimed to evaluate the safety of HA and its protective effect against morphological changes associated with oxidative stress induced by AFs in liver, kidneys, testes and brain of male albino rats.

## Materials and Methods

**Chemicals:** Humic acid was provided by Loba Chemie (Wodehouse Road, Mumbai, India). The chemicals, solvent and media used for AFs production and assay were of analytical grade and purchased from Merck (Darmstadt, Germany). Kits of GSH and MDA estimation were purchased from Biodiagnostic, France.

**Microorganism:** *Aspergillus flavus* NRRL (3145) used for AFs production was provided by National Research Center (Dokki, Giza).

**Standard Aflatoxins:** $AFB_1$, $AFB_2$, $AFG_1$ and $AFG_2$ (standard aflatoxins) were obtained from Sigma Chemical Company (St. Louis, Mo USA).

**Aflatoxins production and assay:** AFs were produced from *Aspergillus flavus* according to method of Davis *et al.* (1966). Liquid medium of yeast extract sucrose (YES) was used as substrate to enhance growth of fungi. AFs were extracted from culture filtrate with chloroform (1:2,v/v) (Verma and Nair, 2001) then chloroform was evaporated by rotary evaporator till obtaining dry film. Toxins were reconstituted by dimethyl sulfoxide (DMSO) and assayed by HPLC with Flourcenses detector (Agilent 1100 Series U.S.A with column C18, Lichrospher 100 RP-18, 5µm×25cm) according to method described by Roos *et al.* (1997).

**Preparation of aflatoxins and HA doses:** The doses of AFs and HA used in the current biological experiment were based on previous study of Abd El-Shafea *et al.* (2014). Rats were treated with AFs and HA by gavage for 6 weeks.

Mixture of AFs ($AFB_1$, $AFB_2$, $AFG_1$ and $AFG_2$ in the ratio of 8: 3: 2: 1, respectively) in DMSO solution was used to prepare 2final doses of AFs, low AFs dose (1mg/6ml DMSO/kgb.w/once/week) and high AFs dose (2mg/6ml DMSO/kgb.w/once/week).

To prepare HA doses, enough amounts of HA was suspended in water to obtain 2 dose concentrations, low dose (200mg/kg b.w/day) and high dose (400mg/kg b.w/day).

**Animals and treatment:** Fifty- four male adult albino rats were purchased from Laboratory Animal Center, Faculty of Veterinary Medicine, Cairo University and housed in stainless steel cages under controlled environmental conditions (12-h light/dark cycle, $22\pm2^{O}$ C). Rats (weighing about 150±10g) were feed *ad libitum* on normal basal diet during adaptation period (2 week) and experimental period (6 weeks) according to guidelines of National Research Council (1995). It worthy to note that biological experiment protocol and animal accommodations agree with recommendations of the European Union regarding animal experimentation (Directive of the European Counsel 86/609/EC). Rats were randomly divided into nine groups and treated orally by previously mentioned prepared doses of AFs and HA as follow:

**G1 (control, vehicle treated):** received 6ml DMSO/ kg b.w. once/ week.
**G2 (low HA):** received low HA dose daily + 6ml DMSO/ kg b.w. once/ week.
**G3 (high HA):** received high HA dose daily+6ml DMSO/ kg b.w. once/ week.
**G4 (low AFs):** received low AFs dose once/week.
**G5 (high AFs):** received high AFs dose once/week.
**G6 (low HA +low AFs):** received low AFs dose once/week + low HA dose daily.
**G7 (high HA+ low AFs):** received low AFs dose once/week + high HA dose daily.
**G8 (low HA+ high AFs):** received high AFs dose once/week + low HA dose daily.
**G9 (high HA+ high AFs):** received high AFs dose once/week + high HA dose daily.

**Tissue specimen and processing:** At the end of $6^{th}$ week of experiment, rats were sacrificed by cervical decapitation and liver, kidneys, testes, brain were removed. Half part of each liver & brain, one testis and one kidney were immersed in 10% buffered formalin then processed according to Banchroft *et al*. (1996) method and finally stained with haematoxylin and eosin (H&E) for histopathological examination under light microscope by suitable magnification. Whereas, the other part of liver & brain as well as kidney and testis were immediately rinsed in ice-chilled normal saline then a known weight of them were homogenized in 5.0 ml of 0.1MTris–HCl buffer (pH7.4) solution. The homogenates were centrifuged and the supernatants were used for GSH and MDA estimation.

**Estimation of GSH**: The concentration of GSH in organs homogenates was estimated according to method described by Beutler *et al*. (1963), based on fact of GSH reduce 5, 5 dithiobis (2- nitrobenzoic acid) (DTNB) to yellow compound which estimated calorimetrically at 405 nm and directly proportional to GSH concentration.

**Estimation of MDA**: Estimation of LPO in organs homogenates depends on the extent of reaction of thiobarbituric acid reactive substances (TBARS) with malondialdehyde (MDA) which measured calorimetrically at 534 nm according to method of Onkawa *et al.* (1979).

**Statistical analysis:** Results of GSH and MDA were tabulated in form of mean ± SE. Data was subjected to statistical analysis using least significant difference test (LSD) at the 5% level of probability by mean of computer Duncan institute program as described by Snedecor and Cochran (1980).

## Results

### Histopathological Results:

**Liver:** Microscopically, livers of rats of G1 (control, vehicle treated rats), G2 and G3 (HA treated rats) were revealed normal architecture structure of hepatic lobule (Figure 1). In contrary liver of rats treated with low AFs dose (1mg/kg b.w/once weekly) (G4) showed kupffer cells activation, karyomegaly of some nuclei (Figure 2), cytomegaly of hepatocytes along with hydropic degeneration (Figure 3), necrosis of sporadic hepatocytes (Figure 4) and sinusoidal leucocytosis (Figure 5). Furthermore, liver of high AFs (2mg/kg b.w/once weekly) group (G5) showed more sever lesions represented by hydropic degeneration of hepatocytes, karyomegaly of some nuclei (Figure 6), focal hepatic necrosis associated with infilammatory cells infiltration(Figure 7), cystic dilatation of bile duct and fibroblast proliferation in portal traid (Figure 8). While liver from G6 which co-treated with low HA and low AFs dose showed only hydropic degeneration of hepatocytes (Figure 9). Meanwhile liver of rats from G7 whichco-treated with high HA and low AFs dose revealed normal hepatic structure (Figure 10). Liver of rats from G8 which treated with low HA and high AFs dose showed sinusoidal leucocytosis (Figure 11), while Liver of rats from G9 which treated with high HA and high AFs dose showed normal hepatic structure except slight congestion of hepatic sinusoids (Figure 12).

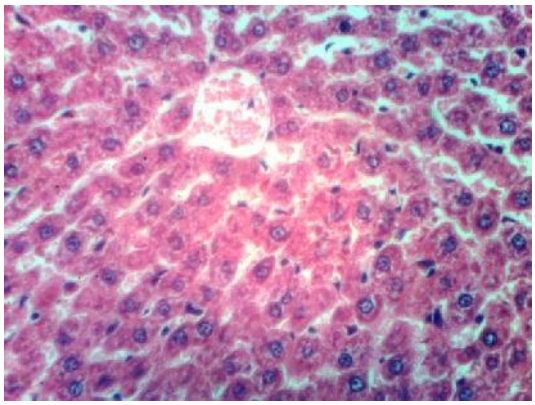

**Figure (1):** Liver of rat from G3 showing the normal histological structure of hepatic lobule (H & E X 400).

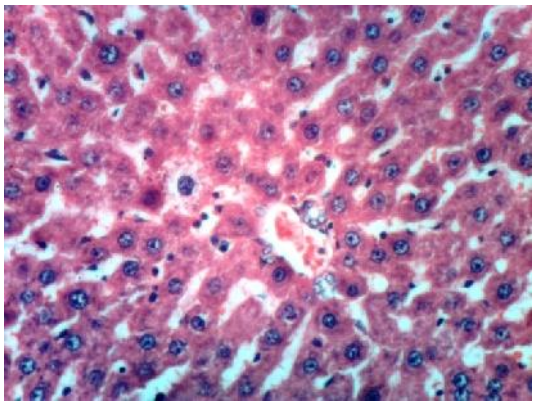

**Figure (2):** Liver of rat from G4 showing kupffer cells activation and karyomegaly of some nuclei (H & E X 400).

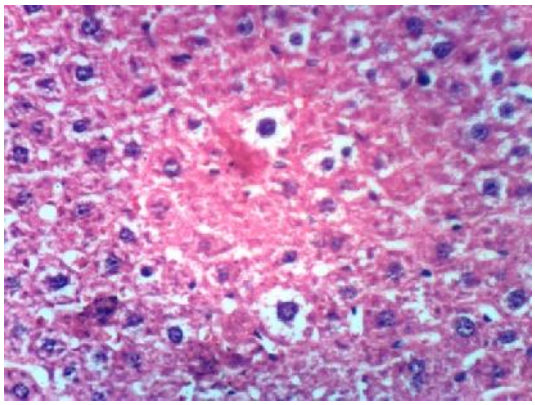

**Figure (3):** Liver of rat from G4 showing cytomegaly of hepatocytes with hydropic degeneration (H & E X 400).

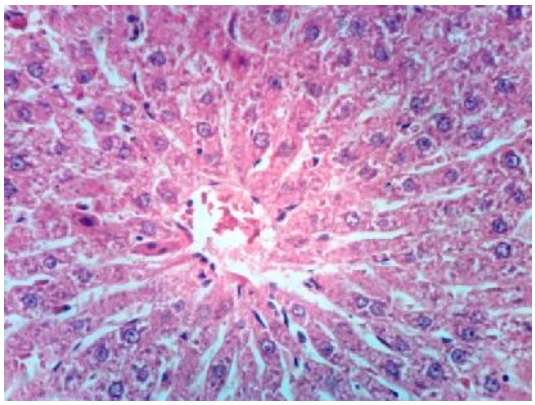

**Figure (4):** Liver of rat from G4 showing necrosis of sporadic hepatocytes (H & E X 400)**.**

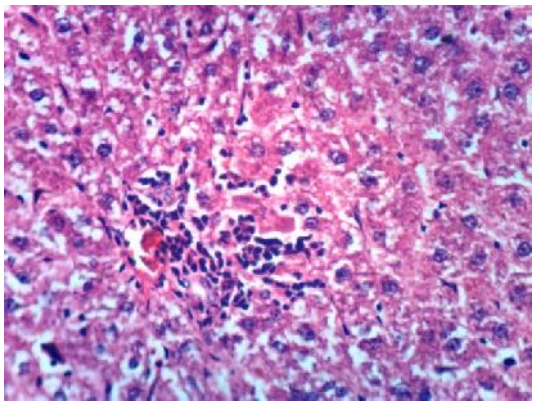

**Figure (5):** Liver of rat from G4 showing sinusoidal leucocytosis (H & E X 400).

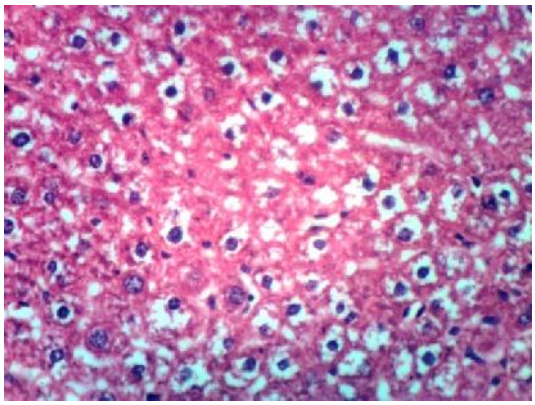

**Figure (6):** Liver of rat from G5 hydropic degeneration of hepatocytes and karyomegaly of some nuclei(H & E X 400).

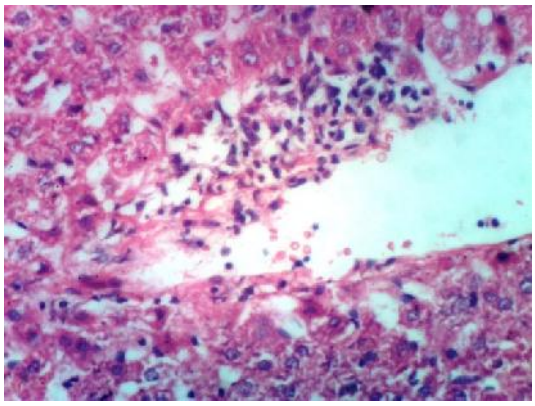

**Figure (7):** Liver of rat from G5 showing focal hepatic necrosis associated with infilammatory cells infiltration (H & E X 400).

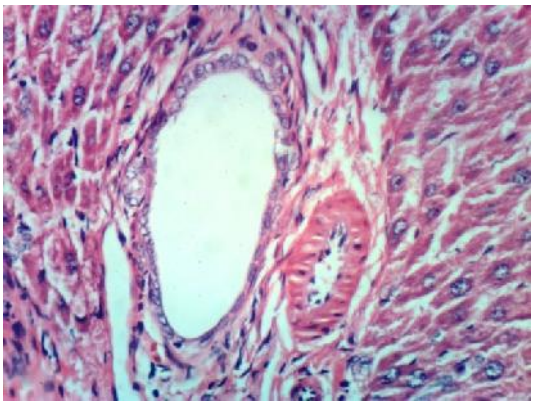

**Figure (8):** Liver of rat from G5 showing cystic dilatation of bile duct and fibroblast proliferation in portal traid(H & E X 400).

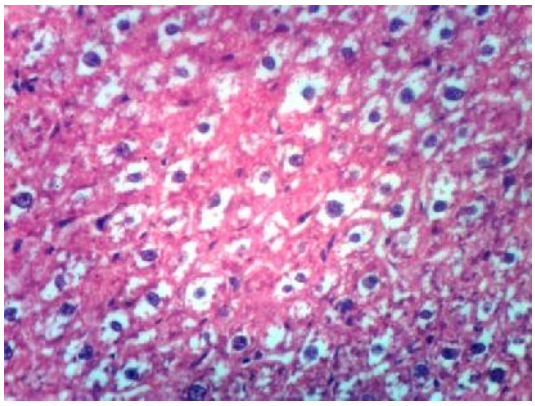

**Figure (9):** Liver of rat from G6 showing hydropic degeneration (H & E X 400).

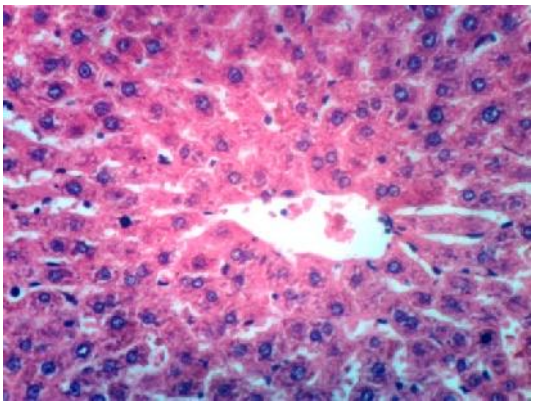

**Figure (10):** Liver of rat from G7 showing apparent normal hepatocytes (H & E X 400).

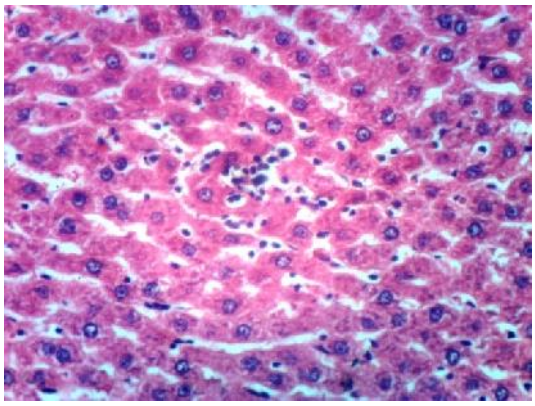

**Figure (11):** Liver of rat from G8 showing sinusoidal leucocytosis (H & E X 400).

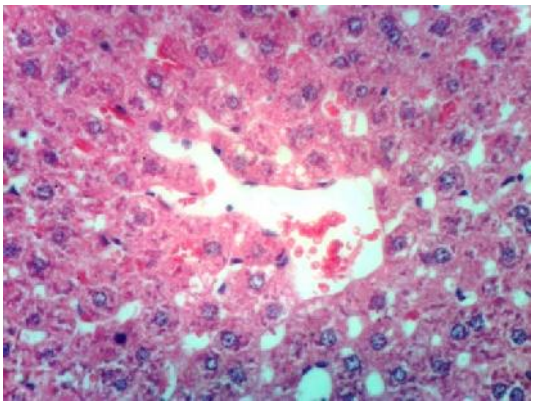

**Figure (12):** Liver of rat from G9 showing slight congestion of hepatic sinusoids (H & E X 400).

**Kidneys:** Microscopically, kidneys of rats from G1 (control), from G2 and G3 which treated with HA (200 and 400mg/kg b.w/daily, respectively) revealed the normal histological structure of renal parenchyma (Figure 13). In contrary, kidneys of rats from G4 (low AFs) showed vacuolation & congestion of glomerular tuft and vacuolation of epithelial lining renal tubules (Figure 14) and peritubular inflammatory cells infiltration (Figure 15). While kidney of rats from G5 (high AFs) revealed atrophy of glomerular tuft, distension of Bowman′s space (Figure 16), congestion of glomerular tuft, peritubular inflammatory cells infiltration (Figure 17), vacuolation of epithelial lining of glomerular tuft, presence of protein cast in the lumen of renal tubules (Figure 18), interstitial nephritis and cystic dilatation of some renal tubules (Figure 19). Fortunately, kidneys of low AFs intoxicated-rats' treatment with low and high HA doses (G6 and G7, respectively) revealed normal structure of renal parenchyma (Figure 20). Meanwhile kidney of rats (G8) which treated with low HA and high AFs dose revealed apparent normal histopathological structure except of slight congestion of glomerular tuft (Figure 21). Kidney of rats of G9 which treated with high HA and high AFs dose revealed apparent normal histological structure (Figure 22).

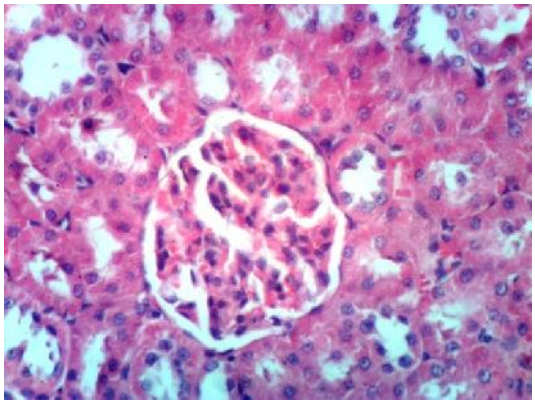

**Figure (13):** kidney of rat from G3 showing normal histological structure of renal parenchyma (H & E X 400).

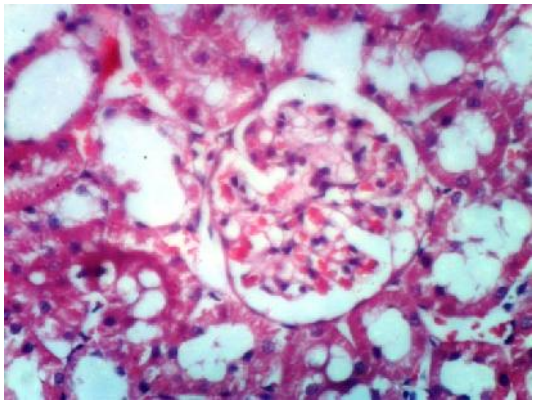

**Figure (14):** kidney of rat from G4 showing vacuolation& congestion of glomerular tuft and vacuolation of epithelial lining renal tubules(H & E X 400).

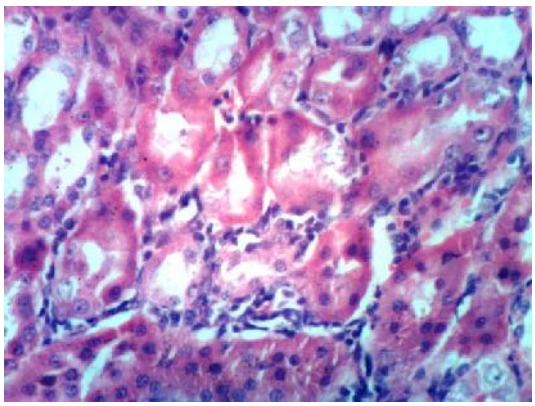

**Figure (15):** Kidney of rat from G4 showing peritubular inflammatory cells infiltration(H & E X 400).

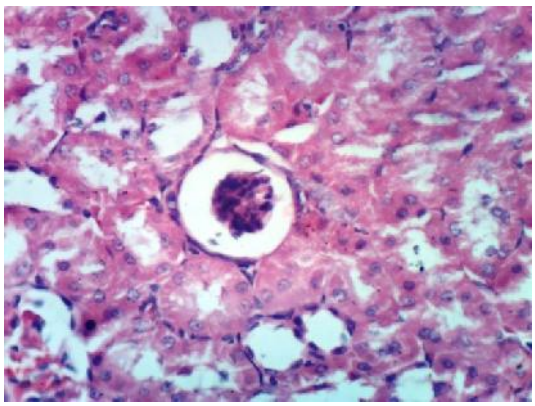

**Figure (16):** Kidney of rat from G5 showing atrophy of glomerular tuft and distension of Bowman′s space (H & E X 400)

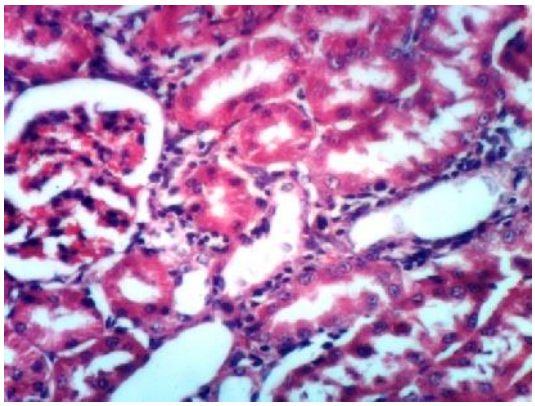

**Figure (17):** Kidney of rat from G5 showing congestion of glomerular tuft and peritubular inflammatory cells infiltration (H & E X 400).

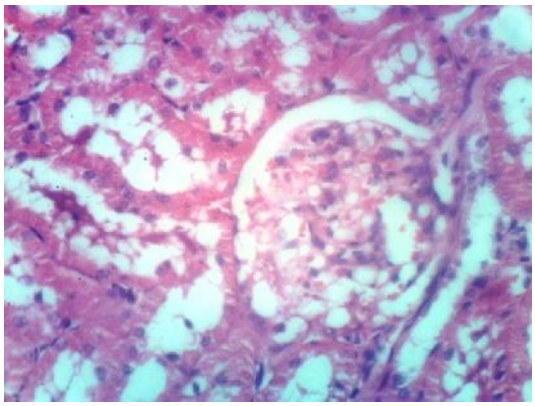

**Figure (18):** Kidney of rat from G5 vacuolation of epithelial lining of glomerular tuft and presence of protein cast in the lumen of renal tubules (H & E X 400).

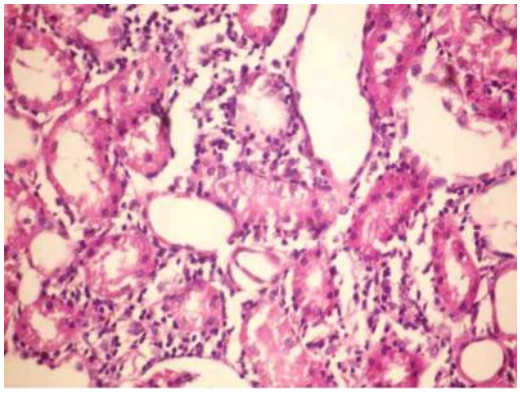

**Figure (19):** Kidney of rat from G5 showing interstitial nephritis and cystic dilatation of some renal tubules (H and E X 200).

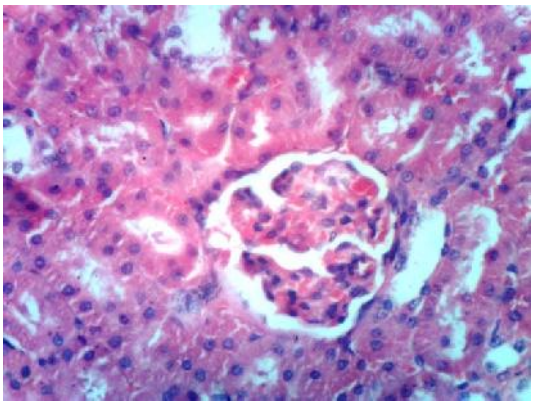

**Figure (20):** Kidney of rat from G7 showing apparent normal histopathological structure (H & E X 400).

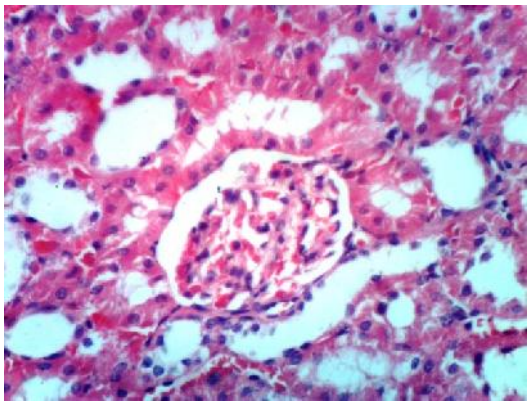

**Figure (21):** Kidney of rat from G8 showing slight congestion of glomerular tuft (H & E X 400).

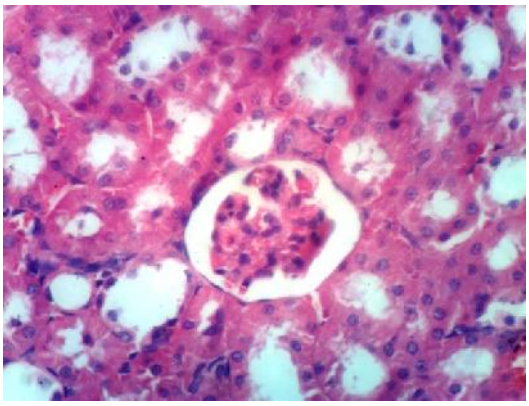

**Figure (22):** Kidney of rat from G9 showing apparent normal histopathological structure (H & E X 400).

**Testes:** Histopatholgical examination of testes of G1, G2 and G3 rats (control, low HA and high HA, respectively) revealed the normal histological structure of seminiferous tubules with complete spermatogenesis (Figure 23). Meanwhile, testes of low AFs treated rats (G4) showed degeneration and necrosis of spermatogoneal cells lining seminiferous tubules (Figure 24). While testes of high AFs treated rats (G5) showed interstitial edema (Figure 25), degeneration of spermatogoneal cells lining seminiferous tubules (Figures 25 and 26) and hyperplasia of Lyedig cells (Figure 27). Normal structure of seminiferous tubules was revealed in testes of low and high AFs-rats which co-treatment with both HA dose (G6, G7, G8 and G9) (Figures 28 and 29).

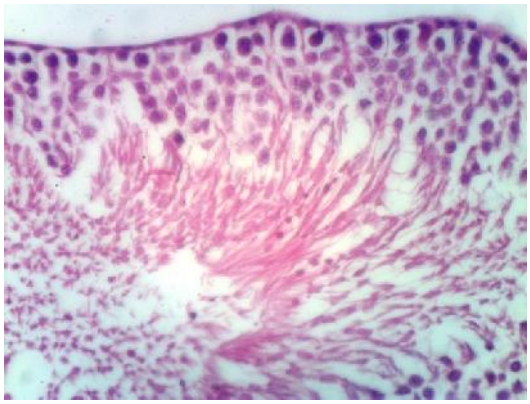

**Figure (23):** Testis of rat from G2 showing normal histological structure of seminiferous tubule with complete spermatogenesis (H & E X 400).

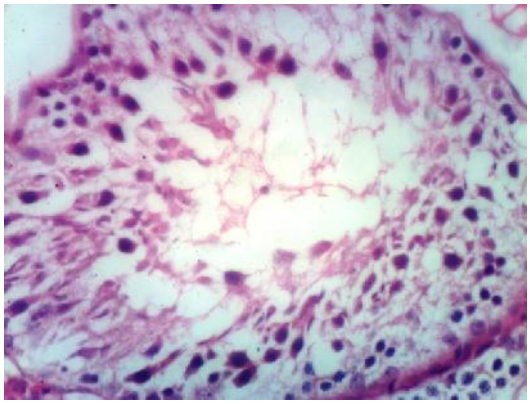

**Figure (24):** Testis of rat from G4 showing degeneration and necrosis of spermatogoneal cells lining seminiferous tubules (H & E X 400).

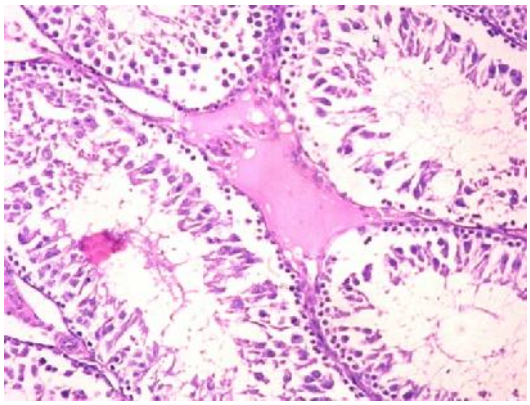

**Figure (25):** Testis of rat from G5 showing interstitial edema and degeneration of spermatogoneal cells lining seminiferous tubules (H & E X 200).

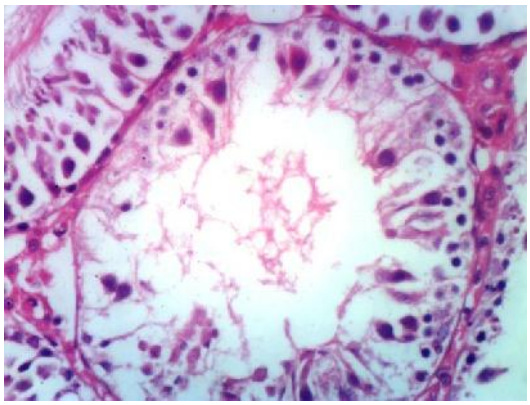

**Figure (26):** Testis of rat from G5 showing degeneration of spermatogoneal cells lining seminiferous tubules (H & E X 400)

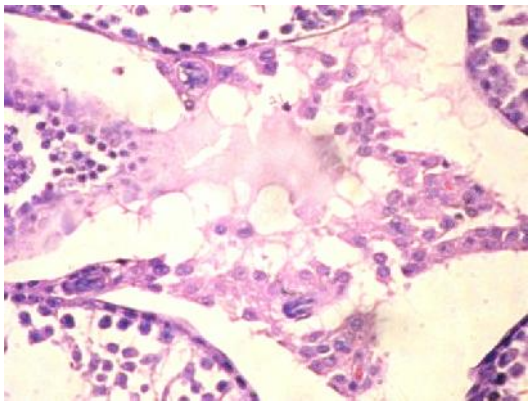

**Figure (27):** Testis of rat from G5 showing hyperplasia of Lyedig cells (H & E X 400).

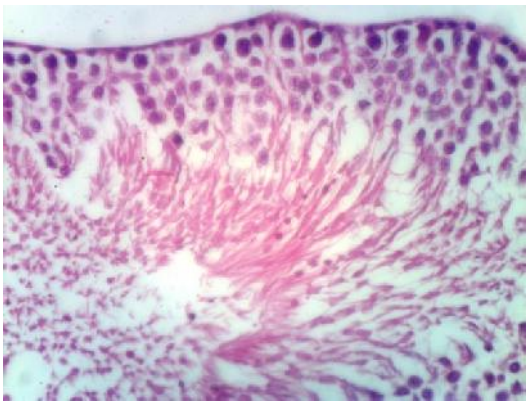

**Figure (28):** Testis of rat from G7 showing no histopathological changes (H & E X 400).

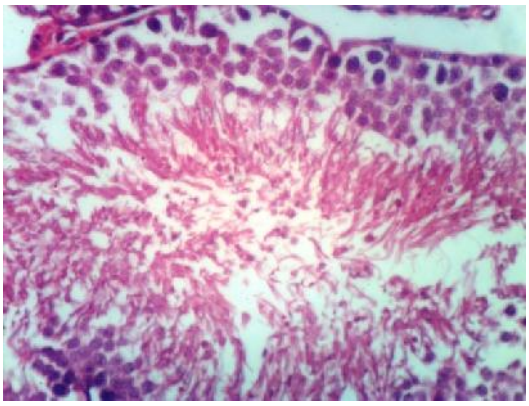

**Figure (29):** Testis of rat from G9 showing no histopathological changes (H & E X 400).

**Brain:** histolopathological examination of brain of rats from G1 (control), G2 and G3 (HA treated rats) revealed no histopathological changes (Figure 30). While examined sections from low AFs treated rats (G4) revealed neuronophagia of pyknotic neurons (Figure 31), congestion of cerebral blood vessels (Figure 32) and focal cerebral hemorrhage (Figure 33). Examined sections of brain of rats treated with high AFs dose (G5) revealed necrosis of neurons, neuronophagia (Figure 34), congestion of meningeal blood vessel (Figure 35) and congestion & hemorraghe in Virchow space (Figure 36). Brain of rats from G6 and G7 which simultaneously treated with HA (low and high) and low AFs dose revealed normal histological structure (Figure 37). While, examined sections from G8 which simultaneously treated with low HA and high AFs dose pyknosis of some neurons (Figure 38). Brain of rats of G9 which simultaneously treated with high HA and high AFs dose revealed apparent normal structure (Figure 39).

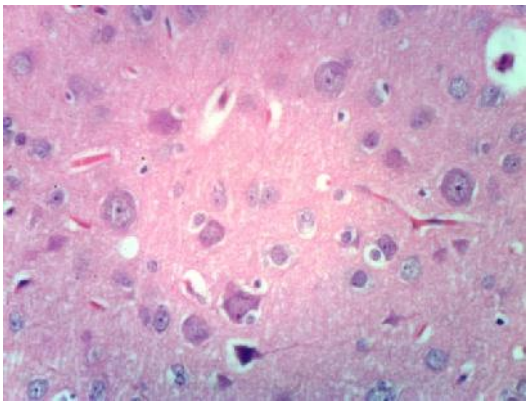

**Figure (30):** Brain of rat from G2 showing normal histological structure (H & E X 400).

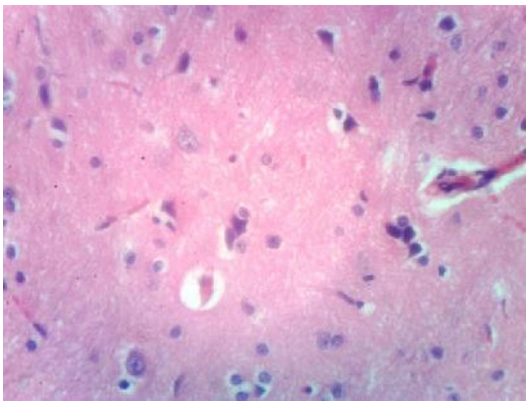

**Figure (31):** Brain of rat from G4 showing neuronophagia of pyknotic neurons (H & E X 400).

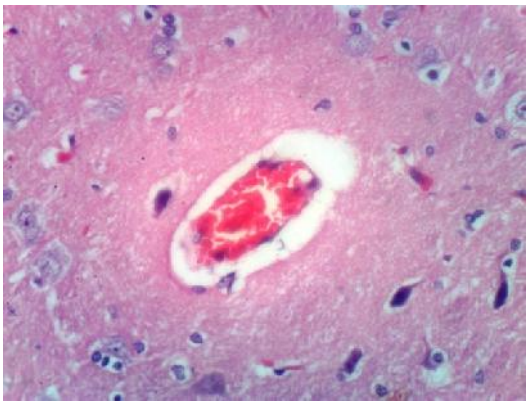

**Figure (32):** Brain of rat from G4 showing congestion of cerebral blood vessels(H & E X 400).

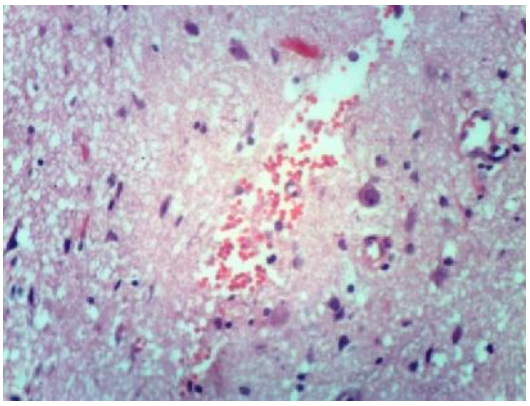

**Figure (33):** Brain of rat from G4 showing focal cerebral hemorrhage (H & E X 400)

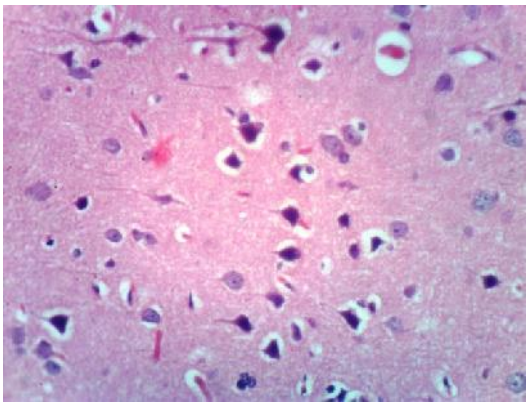

**Figure (34)**: Brain of rat from G5 showing necrosis of neurons andneuronophagia (H & E X 400).

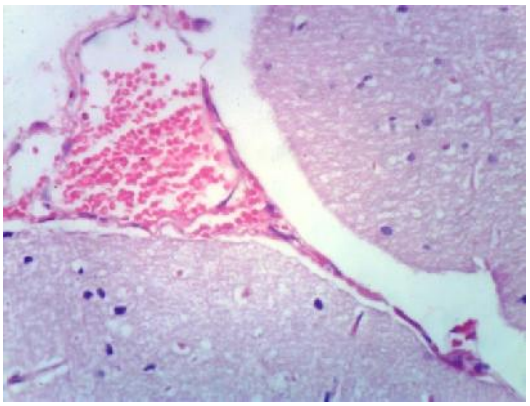

**Figure (35):** Brain of rat from G5 focal showing congestion of meningeal blood vessel (H & E X 400).

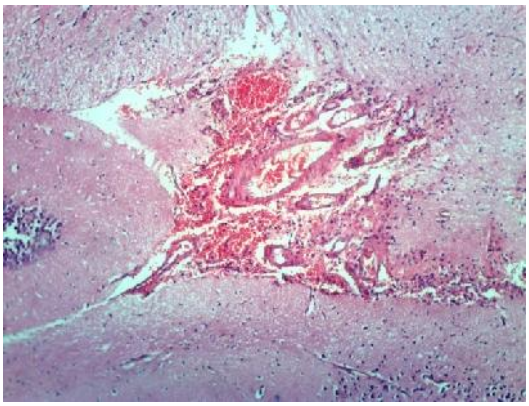

**Figure (36):** Brain of rat from G5 showing congestion and hemorraghe in Virchow space (H & E X 400).

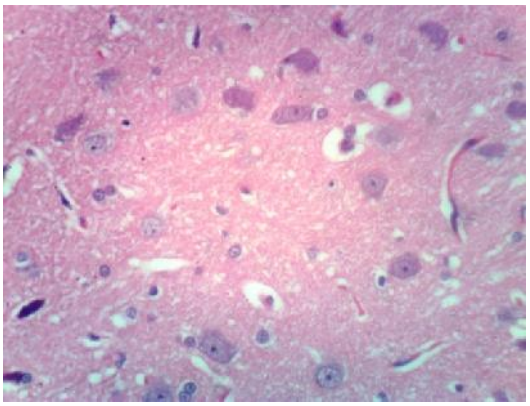

**Figure (37):** Brain of rat from G7 showing no histopathological changes (H & E X 400).

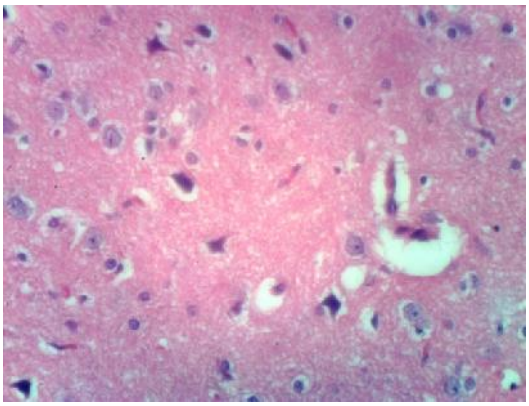

**Figure (38):** Brain of rat from G8 showing pyknosis of some neurons (H & E X 400).

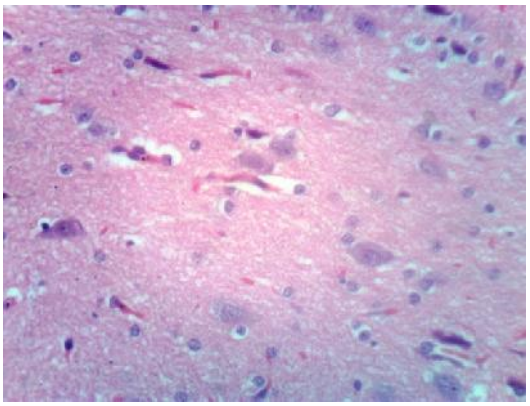

**Figure (39):** Brain of rat from G9 showing no histopathological changes (H & E X 400).

**GSH concentration in liver, kidney, testis and brain:** Table (1) demonstrates that HA treatment at both doses didn't have any negative impact on GSH concentration in all examined organs comparing with control group (G1) ($P<0.05$). Meanwhile AFs treatment significantly reduce ($P<0.05$) GSH concentration in all examined organs in dose-dependent manner (comparing with control). Fortunately, the depleted level of GSH was elevated significantly ($P<0.05$) as a result of coincide treatment of AFs- intoxicated groups with HA (G6, G7, G8 and G9) when compared with corresponding AFs groups (G4 and G5). Furthermore, intake of low dose of HA restores GSH concentration in testis and brain of low AFs dose- treated rats (G6). While, intake of high dose of HA restores GSH concentration in liver, kidney, testis and brain of low AFs dose- intoxicated group (G7) and in testis and brain of high AFs dose-intoxicated group (G9) in comparison with control group (G1) ($P<0.05$).

**MDA concentration in liver, kidney, testis and brain:** LPO results were tabulated in table (2) from measured MDA formation. Obtained date revealed that both HA dose didn't affect MDA concentration in all examined organs in comparison with control group (G1) ($P<0.05$). Meanwhile, LPO production was significantly enhanced ($P<0.05$) after AFs administration for 6 weeks in dose- dependent manner comparing with control. However, the co- treatment of AFs- intoxicated rats with HA resulted in significant reduction ($P<0.05$) of MDA production (comparing with AFs groups). Such effect was more pronounced with high HA dose as intake of high dose of HA depress LPO concentration in liver, testis and brain of low AFs dose- intoxicated group (G7) and in testis of high AFs dose-intoxicated group (G9)in comparison with control group (G1) ($P<0.05$).

**Table (1): Reduced Glutathione (GSH) in organs of control and treated rats (means ± SE).**

| Organs / Groups | GSH (mg/g tissue) | | | |
|---|---|---|---|---|
| | Liver | kidney | Testis | Brain |
| G1 Control | 5.60±0.14$^{a}$ | 4.63±0.15$^{ab}$ | 3.78±0.14$^{ab}$ | 3.00±0.09$^{a}$ |
| G2 Low HA | 5.76±0.13$^{a}$ | 4.90±0.16$^{a}$ | 3.88±0.13$^{a}$ | 2.97±0.10$^{a}$ |
| G3 High HA | 5.57±0.15$^{a}$ | 4.95±0.18$^{a}$ | 3.93±0.17$^{a}$ | 2.94±0.09$^{a}$ |
| G4 Low AFs | 3.52±0.17$^{d}$ | 2.14±0.14$^{f}$ | 2.47±0.12$^{c}$ | 1.81±0.11$^{b}$ |
| G5 High AFs | 2.30±0.14$^{e}$ | 1.31±0.20$^{g}$ | 0.94±0.13$^{d}$ | 0.64±0.11$^{c}$ |
| G6 Low HA+ Low AFs | 4.45±0.18$^{c}$ | 3.88±0.12$^{c}$ | 3.63±0.13$^{ab}$ | 2.84±0.09$^{a}$ |
| G7 High HA+ Low AFs | 5.47±0.18$^{a}$ | 4.38±0.17$^{b}$ | 3.83±0.12$^{a}$ | 2.92±0.11$^{a}$ |
| G8 Low HA+ High AFs | 4.07±0.11$^{c}$ | 2.62±0.16$^{e}$ | 2.35±0.15$^{c}$ | 1.94±0.10$^{b}$ |
| G9 High HA +High AFs | 4.98±0.14$^{b}$ | 3.23±0.14$^{d}$ | 3.39±0.14$^{b}$ | 2.67±0.14$^{a}$ |
| $LSD_{0.05}$ | 0.43 | 0.46 | 0.39 | 0.30 |

Within the same column, various superscript letters indicate significant differences (Duncan, *P <0.05*).

**Table (2): Lipid peroxidation (Malondialdehyde levels, MDA) in organs of control and treated rats (means ± SE).**

| Organs / Groups | MDA (nmol/g tissue) | | | |
|---|---|---|---|---|
| | Liver | kidney | Testis | Brain |
| G1 Control | 12.47±0.39$^{fg}$ | 8.24±0.33$^{g}$ | 6.05±0.24$^{e}$ | 4.21±0.24$^{f}$ |
| G2 Low HA | 12.15±0.32$^{fg}$ | 7.96±0.22$^{g}$ | 5.86±0.26$^{e}$ | 4.00±0.27$^{f}$ |
| G3 High HA | 11.73±0.35$^{g}$ | 7.78±0.28$^{g}$ | 6.00±0.25$^{e}$ | 3.90±0.26$^{f}$ |
| G4 Low AFs | 19.76±0.32$^{c}$ | 16.89±0.25$^{b}$ | 10.86±0.26$^{b}$ | 8.75±0.25$^{b}$ |
| G5 High AFs | 26.21±0.34$^{a}$ | 21.01±0.27$^{a}$ | 18.48±0.27$^{a}$ | 13.92±0.31$^{a}$ |
| G6 Low HA+ Low AFs | 15.50±0.33$^{e}$ | 12.62±0.29$^{d}$ | 8.05±0.25$^{d}$ | 6.20±0.22$^{d}$ |
| G7 High HA+ Low AFs | 12.82±0.41$^{f}$ | 9.69±0.25$^{f}$ | 6.17±0.29$^{e}$ | 4.50±0.20$^{ef}$ |
| G8 Low HA+ High AFs | 21.38±0.30$^{b}$ | 14.49±0.33$^{c}$ | 9.77±0.28$^{c}$ | 7.76±0.21$^{c}$ |
| G9 High HA +High AFs | 18.11±0.32$^{d}$ | 11.42±0.26$^{e}$ | 6.31±0.25$^{e}$ | 5.14±0.24$^{e}$ |
| $LSD_{0.05}$ | 0.98 | 0.79 | 0.74 | 0.70 |

Within the same column, various superscript letters indicate significant differences (Duncan, *P <0.05*).

## Discussion

The current study was performed to evaluate oxidative stress- induced pathological alterations in liver, kidney, testis and brain of rats by exposure to different doses of AFs (1and 2 mg/kg b.w/once weekly) for 6 weeks as well as beneficial ameliorating effect of HA (200 and 400mg/kg b.w/daily) against aflatoxicosis.

Results clearly indicated that AFs administration resulted in dose- dependent pathological changes in liver, kidney, testis and brain associated with dramatic dose-dependent depleting of GSH content and dose-dependent increase in MDA production.

The main lesions in liver in our experimental model were kupffer cells activation, karyomegaly of some nuclei, cytomegaly of hepatocytes along with hydropic degeneration, necrosis of sporadic hepatocytes, sinusoidal leucocytosis, focal hepatic necrosis associated with infilammatory cells infiltration, cystic dilatation of bile duct and fibroblast proliferation in portal traid. Such hepatotoxic effect was previously mentioned by Darwish *et al.* (2011) and Gupta *et al.* (2011) in mice as a result of AFs and $AFB_1$ administration, respectively. Yilmaz *et al*. (2017) recorded hydropic degeneration of hepatocytes and necrosis in liver of rats injected by single intraperitoneal (i.p.) dose of $AFB_1$. Hammoud *et al*. (2018) reported sever liver damage after oral administration of rats with different doses of AFs mixture. The injurious effect of AFs was concomitant with failure of antioxidant defense mechanism and increased LPO production in liver (Sivanesan and Begum, 2014 and Hussein *et al*., 2019) which attributed to reactive AFs epoxide formation in liver and consequent initiation of peroxidative damage (Preston and Williams, 2005 and Guarisco *et al.*, 2007).

Kidneys of AFs treated rats in the current biological experiment showed distortion of renal architecture manifested by vacuolation & congestion of glomerular tuft and vacuolation of epithelial lining renal tubules, peritubular inflammatory cells infiltration, atrophy of glomerular tuft, distension of Bowman′s space, presence of protein cast in the lumen of renal tubules, interstitial nephritis and cystic dilatation of renal tubules. Devendran and Balasubramanian (2011) and Hammoud *et al*. (2018) reported variable degree of renal degeneration as a result of treatment of rats with different doses of AFs for 8 days (i.p.) and for 6 weeks (orally), respectively hence the severity of lesions was dose-dependent. Previous researches by Darwish *et al.* (2011) and Gupta *et al.* (2011) in mice and Yilmaz *et al*. (2017) in rats reported that AFs induce renal damage through inhibition of antioxidant system and enhancement of LPO in kidney.

Examination of H&E stained sections of testes of AFs-intoxicated rats revealed degeneration and necrosis of spermatogoneal cells lining seminiferous tubules, interstitial edema and hyperplasia of Lyedig cells. AFs disrupt spermatogensis and induce testicular changes (Murad *et al.*, 2015, Althnaian *et al*., 2016 and Hammoud *et al*., 2018) accompanied with decrease of total antioxidant capacity, GSH, antioxidant enzymes, increase LPO (Ahmed *et al*., 2015 and Althnaian *et al*., 2016) and biochemical changes in testis (Verma and Nair, 2001) as well as decrease plasma testosterone level (Ortatatli *et al*., 2002).

In view of current results AFs induce brain damage characterized by neuronophagia, pyknotic neurons, congestion of cerebral blood vessels, focal cerebral hemorrhage, necrosis of neurons, congestion of meningeal blood vessel and congestion & hemorraghe in Virchow space. Our results were in agreement with Lakkawar *et al.* (2004) who reported brain damage in young rabbits fed on $AFB_1$ contaminated diet, Wangikar *et al.* (2004) who reported brain damage of fetus as a result of treatment of pregnant rats with $AFB_1$and Hammoud *et al*. (2018) who reported that the severity of degenerative brain changes were dose-dependent. AFs induce brain damage mediated by ROS production and further oxidative stress (Mohamed *et al.*, 2014).

LPO could be considered as pathway by which AFs induce histological damage. AFs activate in the liver by cytochrome *P450* enzyme which in turn resulted in cascaded events started with formation of reactive highly toxic intermediate namely, epoxide (Niki *et al.*, 2005),associated with elaborating of ROS and consequent oxidative damage (Guarisco *et al.*, 2007). Free radicals and ROS attack polyunsaturated fatty acids (PUFA) in the cell membrane lipid bilayer causing oxidative damage of cellular membrane and initiating further chain reaction of LPO resulted in damage of macromolecules & DNA and eventually cell alterations and death (Choudhary and Verma, 2005). Moreover, the $AFB_1$–8,9-epoxideis detoxified by GSH to form $AFB_1$-epoxide-GSH conjugate which explain decrease of the intracellular GSH content (Raney *et al.*, 1992).

Despite uses of HA in folk medicine from long time (Lotosh, 1991) as humic substances used during Roman Empire (Priegnitz, 1986) and HA used in China to treat wide range of disease, the full knowledge about its medicinal properties still limited (Vetvicka *et al.*,2014). Current research studied the effect of HA on reduction of AFs toxicity.

Results of our study revealed that, the examined doses of HA are safe and didn't induce any negative impact on histology of tested organs or antioxidant status.

Luckily, in the current study both HA doses were able to alleviate oxidative stress and pathological alterations induced by AFs evidenced by elevation of GSH levels in tissue and depression of LPO production. It worthy to note that, the low dose of HA was able to preserve architecture structure of kidney and brain of low AFs- treated rats, while high dose of HA was able to protect liver, kidney and brain of low-AFs treated rats and kidney and brain of high AFs treated- rats. HA at both doses was able to preserve normal structure of testes of rats treated with both AFs doses. The beneficial properties of HA against some of toxic and injurious agents were evaluated in many previous studies. HA reduces bioavailability and toxicity of heavy metal such lead toxicity in chicken (Zralý *et al*., 2008) and cadmium in *Oreochromis niloticus* and brown trout (Osman *et al.*, 2009 and Alak *et al.*, 2013, respectively) and improves hematological, physiological and immunological state. Vetvicka *et al.* (2014) reported that HA exhibits hepatoprotective effect against lipopolysaccharide and ethanol treatment in mice through enhancement of GSH level in liver. Similar to our results, Ghahri *et al.* (2010) reported that HA acts as adsorbent for AFs in AFs- contaminated diet and suggested that HA reduces toxic effect of AFs on liver, bursa of Fabricius, serum enzyme activities and feed efficiency through decrease AFs absorption by body. Santosa *et al* (2011) reported that HA inactivate AFs in monogastric animals by absorption of AFs in digestive system (oral cavity, stomach and intestines) at different pH. The *in vitro* trials of Abd El-Shafea *et al.* (2014) revealed that HA bind AFs and form a stable adsorption complex, moreover it reduces the bioavailability of AFs *in vivo.* The exceptionally chemical structure of HA is responsible for its great chelating& adsorbing capacity and colloidal characteristics(Livens, 1991 and Van Rensburg *et al.* 2006). Moreover, HA possess antioxidants activity and anti-inflammatory properties (Van Rensburg and Naude, 2009 and Abd El-Shafea *et al.*, 2014). Vašková *et al.* (2011) suggested that HA enhance antioxidant defensive mechanism via capturing free radicals and redox regulation.

## Conclusion

The results of the current experimental trial indicated that aflatoxins induce pathological changes in liver, kidney, testes and brain via disruption of antioxidant defense mechanism and excessive generation of lipid peroxidation. On the other hand, humic acid ameliorates toxic effect of aflatoxins and showed robust powerful anti-aflatoxicosis properties.

## Conflicts of Interest

The authors declare no conflict of interest.

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| DOI:10.22192/ijarbs.2019.06.03.007 | |

How to cite this article:
Gihan M. Hammoud, Nivin S. Nail, Yasser M. Abd El-Shafea and Asmaa A. Salem. (2019). Histopathological study on the protective effect of humic acid against aflatoxins induced- oxidative stress in rats. Int. J. Adv. Res. Biol. Sci. 6(3): 111-127.
DOI: http://dx.doi.org/10.22192/ijarbs.2019.06.03.007