

Mycological, biochemical, and histopathological studies on fungal disease on cultured *Clarias gariepinus*

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Abstract

A total fifty diseased cat fish (*Clarias gariepinus*) were collected from private fish farms in Dakahlia governorate which suffered from cumulative mortality. All collected fish were subjected to clinical, postmortem, histopathological and mycological examination. Aflatoxins were extracted and quantitated from feed and musculature of infected fish using HPLC, blood samples were collected for biochemical and haematological parameters. The clinical observation revealed that the infected fish showed opacity of the eyes, yellowish greenish gills, hemorrhagic and yellowish skin, and the fish were slow in motion. The postmortem examination showed enlargement of the gall bladder, distention of the stomach, yellowish enlarged liver and the kidneys were swollen, dark red and friable whereas, the histopathological examination revealed a severe degenerative and necrotic changes in most internal organs. On the other hand Mycological examination of infected fish yielded isolation of *Aspergillus flavus* (42%), *A. parasiticus* (34.8%), *A. niger* (12%) and *A. fumigatus* (10.9%). The mean levels of aflatoxins in feed was 108.9 ppb (46.4 ppb AFB₁, 1.5 ppb AFB₂, 58.9 ppb AFG₁, 2.1 ppb AFG₂), while the mean of AFs residues detected in musculature was 20.8 ppb (11.3, 0.15, 8.9, 0.5 ppb) respectively. Regarding to biochemical and hematological parameters, there was a significant decrease in serum Albumin and significant increase in AST, ALT and serum creatinine of infected fish which indicated liver and kidney damage. Also, there was significant decrease in Hb concentration and RBCs count which indicated the high toxic effect of aflatoxins.

Keywords: Aflatoxins, *Clarias gariepinus*, biochemical parameters, histopathological changes, mycological examination

Introduction

There is an increase attention about fish and their diseases because it is the most important source of protein, calcium and phosphorus for human consumption. Fungal contamination of fish is considered as one of the important cause of fish spoilage which led to off flavor, offensive odour and unpalatable taste as well as severe economic loss. Aflatoxins are secondary fungal metabolites produced by some strains of *Aspergillus flavus*, *Aspergillus parasiticus* (Milita et al. 2010). The increase in the incorporation

vegetal ingredients into fish feed formula enlarges the risk of contamination coming from that origin, namely fungi and their toxic metabolites (mycotoxins). Aflatoxins are known as a hepatocarcinogen in various animal species including fish, birds, rodents and non-human primates (**Abbott, 2002, Abdel – Wahhab et al., 2002, Bintvindhok, 2002 and Allameh et al., 2005**). Aflatoxin B1 is the most frequent of all aflatoxins in contaminated food (**Kennedy et al., 1998 and Hussein and Brasel, 2001**). The sensitivity to these toxins depends on age, species and development temperature where young fish are more vulnerable, warm water species are generally less sensitive to AFB₁ than cold water species. The sensitive benchmark species is the rainbow trout, which has an LD₅₀ of 0.5 – 1 ppm of AFB₁ in feed (**Lovell, 1989**). While other species including the warm water channel cat fish can tolerate increased levels up to around 10 ppm (LD₅₀ 11.5 ppm), whereas Nile tilapia is also sensitive to the toxin showing high mortality, reduced muscle mass and major organ damage when fed contaminated feed (**Abdelhamid et al., 2002c**). The clinical aflatoxin cases in fish caused severe external lesions as cloudy eye, yellow greenish infiltration near the gills, erosion of caudal fins and abdomen, slow motion and greater opercular movement due to increase oxygen demand, there is also discarded scales, hardening of the body with yellow of the body surface and haemorrhage of the skin (**Boshy et al., 2008, Durre et al., 2013**). On the other hand post mortem symptoms include enlarged gall bladder and distended stomach, yellowish liver and viscera covered by a thick layer of mucus (**Cagauan et al., 2004 and Mehrim et al., 2006**). A prolonged exposure to low concentration of AFB₁ may induce liver tumor as yellow nodules that also metastasizes to the kidney (**Russo and Yanong, 2010**). It has been shown that AFs especially AFB₁ is activated by hepatic cytochrome p₄₅₀ enzyme system to produce a highly reactive intermediate AFB_{1-8,9}epoxide which subsequently binds to nucleophilic sites in DNA (**Sharma and Farmer, 2004**). So, it induces hepatocarcinogenesis (**Preston and Williams, 2005 and Abdel-Wahaab et al., 2006**). Aflatoxin causes significant increase in liver enzymes Aspartate amino transferase (AST), Alanine amino transferase (ALT) and Alkaline Phosphatase with significant decrease in total protein, potassium, sodium and chloride (**Fernandez et al. 1995, Sahoo and Mukherjee 2001, Rauber et al., 2007, El Sayed and Khalil, 2009 and Oluwafemi and Dahunsi, 2009**). **Joner et al. (2000)** who reported that aflatoxin reacts negatively with different cell proteins which lead to inhibition of carbohydrates and lipid metabolism and protein synthesis. So, the decrease in growth rate may be due to disturbance in metabolic process. The limit of aflatoxin in the food described by the Bureau of Animal Industry is less than 20 PPb. According to Animal feed legislation in the USA, maize, corn, and Beanut products that are to be used for feeding dairy and immature animals (including fish) cannot contain more than 20 ppb of aflatoxin (**Lovell, 1992**).

This study was undertaken to isolate fungi and detect the levels of aflatoxin in fish feed and muscle and evaluation of the biochemical and pathological changes in infected fish.

Materials and methods

Samples:

Fish samples:

At Jun, 2015 a total fifty diseased cat fish of 750 gm – 1 kg obtained from private extensive farm in Dakahlia governorate which suffered from cumulative mortality were transported alive (with acid electric oxygen operators and the same parameters of water to minimize all stress effect) or freshly dead in plastic bags to fish laboratory of animal health research institute, El Mansoura for clinical examination, mycotic isolation and identification.

Feed samples: five samples of farm feed were collected for detection of aflatoxin

Mycological examination:

Samples were taken from kidney, liver, muscle and inoculated into duplicate plates of Sabouraud's dextrose agar incubated at 25°C, 37°C and 45°C for 3-5 days. The macroscopic examination was carried out using the morphological characters of colonies including gross appearance of culture, rate of growth, texture and color of the colonies (above and reverse side) according to **Raper and Fennel(1965)**. The microscopic examination done according to **Dovorak and Actenasek(1969)**.

Detection of mycotoxin in feeds and organs of fish:

The mycotoxins in feed and musculature of fish were extracted and measured by HPLC as (**Jemmali and Murthy, 1976**). It consists of extraction of Afs from samples with methanol, treatment of the residue with a mixture of dimethoxy methane–methanol evaporation of dimethoxymethane followed by a liquid–liquid defatting with hexane before the transfer of Afs into chloroform. The chloroform extracts are further purified by silica gel- acidic alumina-anhydrous sodium sulfate column chromatography. Quantification of AFs was done by using high performance liquid chromatography (HPLC).

Biochemical parameters:

The blood samples were collected from 20 infected fishes, the collected samples were divided into 2 portions, the first was added to anticoagulant (EDTA) for measurement

of (haemoglobin concentration ,WBCs and RBCs count) the other portion was collected without anticoagulant and centrifuged to obtain serum samples for biochemical studies included the measurement of serum albumin ,creatinine ,alanine amino transferase (ALT) ,aspartate amino transferase (AST) and total blood glucose.All tests were measured calorimetrically using standard kits supplied by Bio – Merieux (Poains, France).

Histopathological examination:

Specimens from liver, muscle, kidney, gills, spleen, intestine and skin were collected and fixed in neutral buffered formalin paraffin, sections of 5 μ thick were prepared and stained with Hematoxylin and Eosin, and examined microscopically (**Bancroft and Gamble, 2007**).

Statistical analysis:

The obtained data were analyzed using the liner model programs of SAS (1990). The differences among means were tested using Duncans Multiple range test (**Duncans, 1955**).

Results and discussion

The infected fish showed cloudy eyes, yellowish greenish infiltration near the gills, haemorrhage and yellowish color of of skin these signs were in agreement with those obtained by (**Hussein et al., 2000,Soliman et al., 2000 and Abdelhamid et al.,2002c**). whereas the post mortem lesions include enlarged stomach and gall bladder, enlarged yellowish and friable liver,kidney swollen friable and darked and viscera covered with thick layer of mucus these findings as those reported by (**Cagauan et al., 2004 and Mehrim et al.,2006**).The mortality rate in culture fish farm is aboute 45% (**Abdelhamamid et al.,2002a**)

The recurrent results in Table (1) showed significant decrease in serum albumin of infected fish similar results were obtained by (**Oluwafemi and Taiwo, 2004, Rauber et al., 2007**). There is high significant increase in serum creatinine of infected fish which indicated kidney damage.The liver enzymes of infected fish showed high significant increase in AST and very high significant increase in ALT in comparison with control fish. AST enzymes associated with liver parenchymal cells and it's raised in acute liver damage, ALT is an enzyme present in hepatocytes (liver cells). When a cell is damaged, it leaks this enzyme into the blood and rises dramatically in acute liver damage (**Oluwafemi and Taiwo, 2004, Oluwafemi and Dauhsi, 2009**). The tabulated results in table (2) showed high significant decrease in Hb concentration ,very high

significant increase in WBCs count and significant decrease in RBCs count in blood of infected fish. These results come in accordance with the findings of **(Wimol et al., 1990)**.

On the other hand results in table (3) showed isolation of 164 isolates of *Aspergillus* species from infected fish which identified as *Aspergillus flavus* (42%), *A.parasiticus*(34.8%), *A.niger* (12%) and *A.fumigatus* (10.9%).whereas, the current results in table (4) showed that liver is the most affected organs(*A.flavus* 40.6%, *A.parasiticus*52.6%, *A.niger* 50%, *A.fumigatus* 44.4%) followed by muscles(33.3%,38.6%,40%,27.8%) respectively.While kidney is the lowest infected organ(26%,8.8%,10%,27.8%) respectively. These results were in agreement with those obtained by **(Ali et al., 2004)** .Results in table (5) showed that the detected level of AFs in contaminated diet were 108.9 ppb (46.4 ppb AFB₁,1.5ppb AFB₂,58.9 ppb G₁and 2.1ppb G₂) ,while the detected AFs residues in musculature were at level of 20.8 (11.3ppb,0.15ppb,8.9ppb and 0.5ppb) respectively (table 6).These results are above the permissible limit, in comparison with Egyptian standards quality control (10 ug/kg AFB₁ and 20ug/kg AFB₁,B₂,G₁,G₂).Regarding the histopathological findings in case of skin samples the epidermis cells showed proliferation of mucus and alarm cells with subepithelial leukocytic aggregation, melanomachrophage proliferation and dermal edema (Fig8). Similar results were obtained by **(Amany et al., 2009)**.while in skeletal muscle, Inter and intramuscular edema with degeneration and neumerous leukocytic aggregations were encountered (Fig9). Moreover, some muscle fibers exhibited in tense hyaline degeneration or necrosis which usually invaded by lymphocytes and melanomacrophage cells (Fig10).Whereas,all the hepatic cells of the liver suffered from degeneration or necrotic changes which characterized by vaculation of cytoplasm and pyknotic or karyolytic nuclei (Fig11). Moreover, hemorrhage hyalinized vascular wall with endotheliosis could be seen (Fig12). Similar results were reported by **(Hussein et al.,2000,Soliman et al.,2000,Abdelhamid et al., 2002c and amany et al.,2009)**. **Abdelhamid et al. (2004)** who reported that 100-200 ppm AFB₁ in the *O.niloticus* fish diet led to severe histopathological alteration in the liver.On the other hand, the renal tubular epithelia of kidney suffered from nephrotic changes mainly hydropic or hyaline degeneration beside depletion of hemopoietic centers (Fig13) and coagulative necrosis of some renal tubules with contraction of glomerular tufts were common (Fig14). while regenerative attempts in the renal parenchyma adjacent to necrotic and degenerated renal tissues were prevalent (Fig15) and scattered melanomacrophage cells of centers usually seen scattered in kidney tissues. These results nearly similar to those obtained by **(Abdelhamid et al., 2002c,Abdelhamid et al. 2004 and Merhim et al., 2006)**.Currently,both lymphoid and hemopoietic elements in spleen showed depletion and necrotic changes beside presence of melanomacrophages centers and thickening of

splenic capsule with fibrous tissue. These results were in accordance with the findings obtained by (**Kandil et al., 1991**).Regarding to stomach and intestine samples, the mucosa showed metaplasia of goblet cells or desquamation of their lining epithelium with presence of excess mucus and intense submucosal leukocytic aggregation mainly lymphocytes and the latter usually invade the muscular coat (Fig16). While some muscle fibers in the tunica muscularis may showed partial degeneration or necrosis. These results agreement with those obtained by (**Kandil et al., 1991, Amany et al., 2009**).Concedering the pathological finding in gills, the primary gill filaments showed distortion of their cartagenous structure with hyperplasia and hypertrophy of secondary lamellar epithelium forming sheets (Fig 17). Gills arch showed edema, leukocytic infiltration mainly lymphocytes and eosinophil granular cells beside metaplasia of their surface epithelium to mucous secreting cells and hyperplastic lymphoid aggregation (Fig18).while Gill racker showed hyperplasia and metaplasia to mucus secreting cells in the covering epithelium and intense subepithelial leukocytic aggregation mainly lymphocytes beside extravasated erythrocytes (Fig19). The results nearly similar to those obtained by(**El- Bouhy et al., 1993, Anjum 1994, Mohamed and Mokhbatly 1997**) who mentioned that these lesions developed as a result of immunosuppressive effect of aflatoxin.

Conclusion

From the regrding results ,we can concluded that the aflatoxins cause many dangerous effect on the health status of fish.The detected residues of aflatoxins in fish increased the awarness of use these fish for human consumption which can cause health hazard. It is indicated that the frequent examination of all environmental factors related to fish breeding are urgently must be under taken to the health of fish consumer

Table (1): mean values of some serum biochemical parameters of aflatoxicated Clarias gariepinus

Item Parameters	Control group	Contaminated group
S.Albumin (g/dl)	2.9 ± 0.19	2.2 ± 0.11*
S. Creatinine (mg/dl)	0.71 ± 0.10	1.22 ± 0.11**
S. AST (Iu/I)	161 ± 3.60	183 ± 5.38**
S. ALT (Iu/I)	25.00 ± 1.14	36.3 ± 1.32***
Glucose (mg/dl)	82.5 ± 3.11	68.4 ± 2.64**

*=significant at P < 0.05 **=high significant at P < 0.01 ***=very high significant P < 0.001

Table (2): mean values of some hematological parameters of aflatoxicated *Calarias gariepinus*

Item Parameters	Control group	Contaminated group
HB (g/dl)	8.90 ± 0.44	7.10 ± 0.38**
RBCs (x10 ⁶ /mm ³)	2.14 ± 0.16	1.44 ± 0.23*
WBCs (x10 ³ /mm ³)	30.67 ± 0.92	38.06 ± 1.07***

*=significant at P < 0.05 **=high significant at P < 0.01 ***=very high significant P < 0.001

Table (3):incidence of isolated mould genera from the examined cat fish

No.of diseased	A. flavus	A.parasiticus	A. niger	A.fumigatus	Total No.of isolets
50	69(42%)	57(34.8%)	20(12%)	18(10.9%)	164

Table (4): prevalence of isolated mould from different organ of infected cat fish

Organ Fungi	Liver		Kidney		Muscle	
	Nature	%	Nature	%	Nature	%
A. Flavus	28	40.6	18	26	23	33.3
A. parasiticus	30	52.6	5	8.8	22	38.6
A. niger	10	50	2	10	8	40
A. fumigatus	8	44.4	5	27.8	5	27.8

Table(5):The mean level of AFs (ppb) in feed of cultured *Calarias gariepinus*

No.of samples	AFs	AFB1	AFB2	AFG1	AFG2
1	107.5	44.4	1.3	59.5	2.3
2	110.6	47	1.1	60.1	2.4
3	110.9	49	1.5	58	2.4
4	108.2	46	1.5	58.6	2.1
5	107.7	46	2.1	58.7	1.1
Mean	108.9	46.4	1.5	58.9	2.1

Table(6): The mean level of AFs(PPb) residues in musculature of cultured *Clarias gariepinus*

No. of samples	AFs	AFB1	AFB2	AFG1	AFG2
1	22.05	11.9	0.15	9.6	0.4
2	20.03	10.9	0.13	8.4	0.6
3	20.7	11.4	0.14	8.7	0.5
4	21.4	11.7	0.16	9.2	0.4
5	20.07	10.5	0.17	8.8	0.6
Mean	20.8	11.3	0.15	8.9	0.5

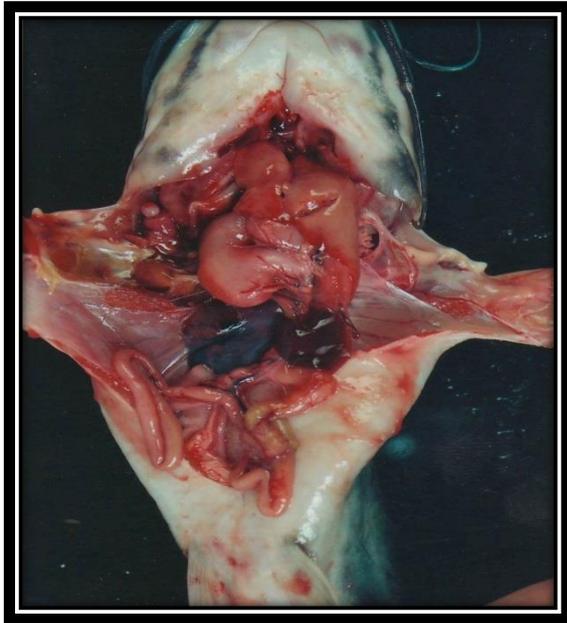


Figure (1): Skin and muscles of *Clarias gariepinus* showing that yellowish in colour, present hemorrhage on skin and fins.

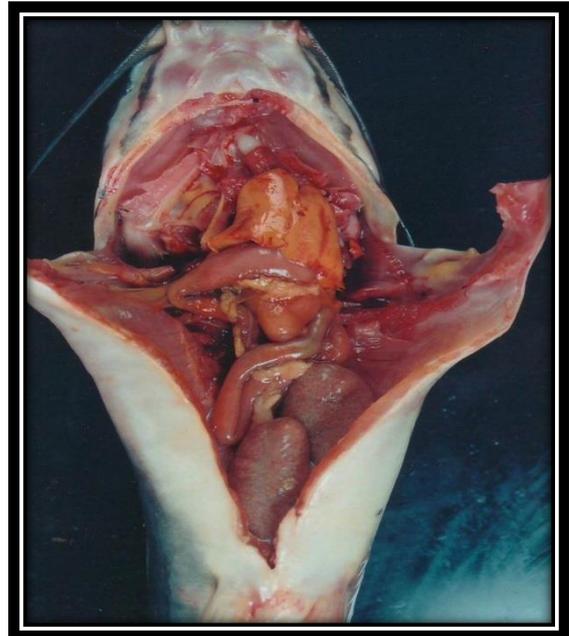


Figure (2): Gills of *Clarias gariepinus* were pale in colour and edematous.

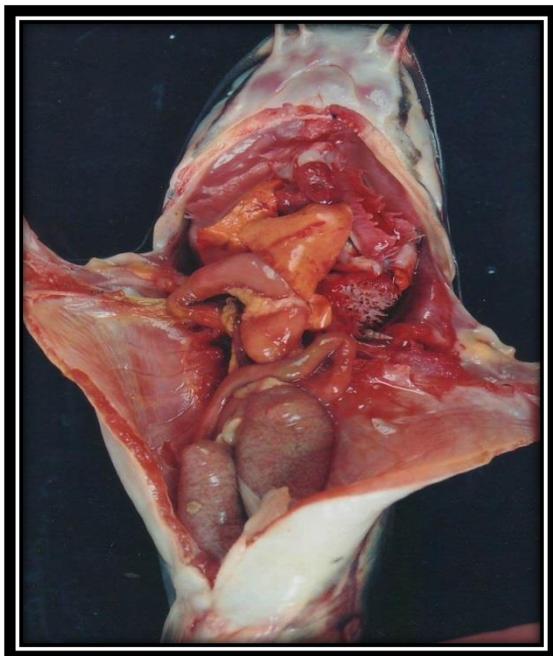


Figure (3): Female *Clarias gariepinus* showing ovary congested and yellowish in colour.



Figure (4): Kidney of *Clarias gariepinus* showing enlargement, friable and congested. Heart was congested.

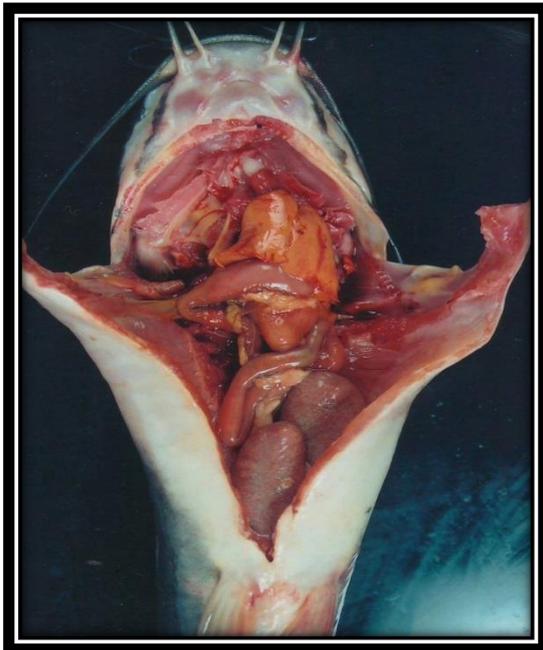


Figure (5) : Female of *Claris gariepinus* showing liver was enlarged ,friable and yellowish in color

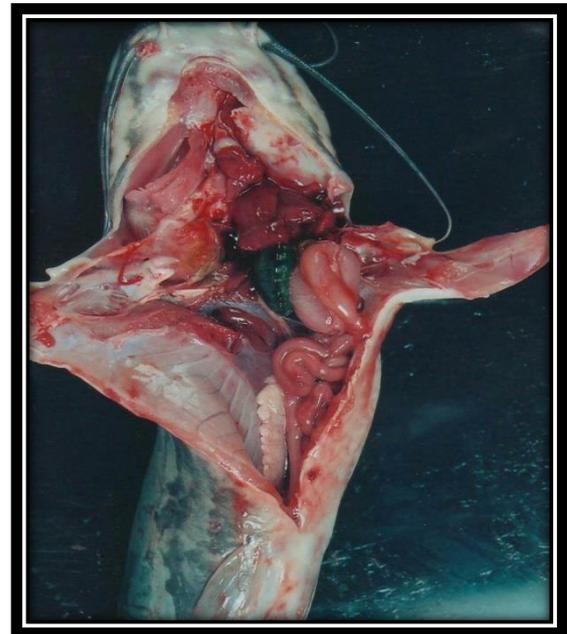


Figure (6): Male of *Clarias gariepinus* showing spleen was enlarged and congested. Testes were congested.



Figure (7): Gall bladder of *Clarias gariepinus* was enlarged and congested.

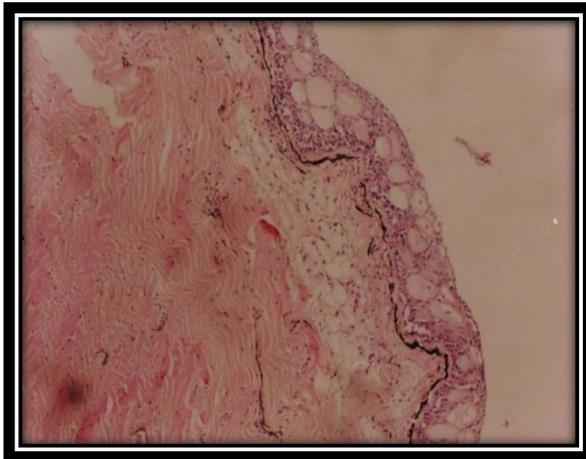


Figure (8): Skin of *Clarias gariepinus* fed on contaminated ration with AFB₁ showing proliferation of alarm and mucus cells in epidermis and dermal edema. H&E X120

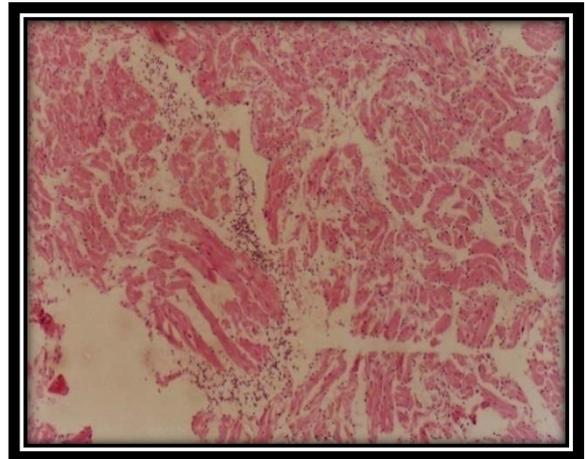


Figure (9): skeletal muscle of *Clarias gariepinus* fed on contaminated ration with AFB₁ showing muscular edema and leucocytic aggregation. H&E X120

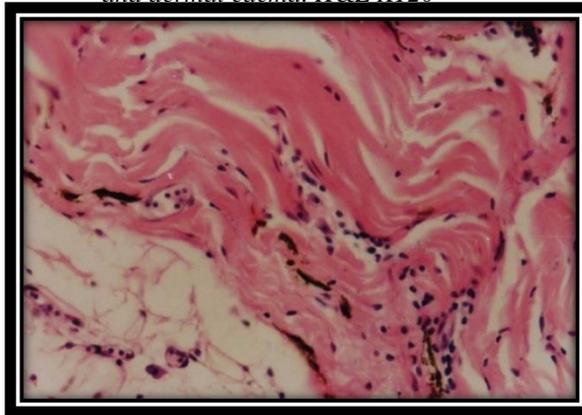


Figure (10): Skeletal muscle of *Clarias gariepinus* fed on contaminated ration with AFB₁ showing hyaline degeneration or necrosis of some muscle fibers. H&E X300

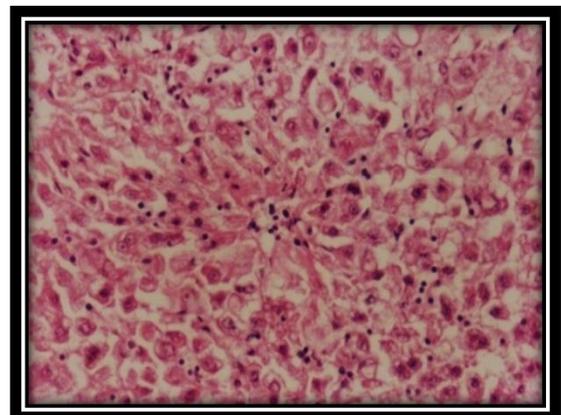


Figure (11): Liver of *Clarias gariepinus* fed on contaminated ration with AFB₁ showing degenerated or necrotic changes of the hepatic cell. H&E X300

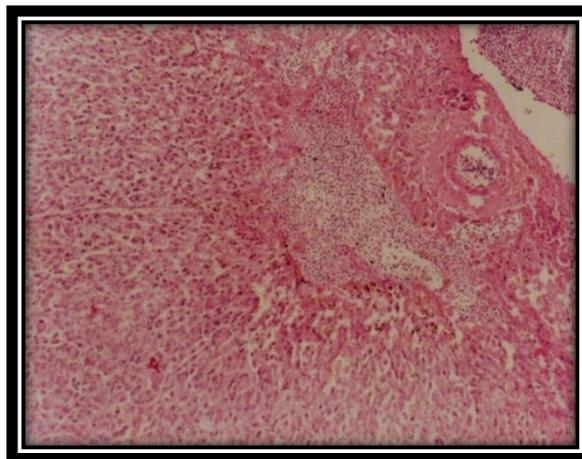


Figure (12): Liver of *Clarias gariepinus* fed on contaminated ration with AFB₁ showing haemorrhages, hyalinized vascular wall and endotheliosis. H&E X120

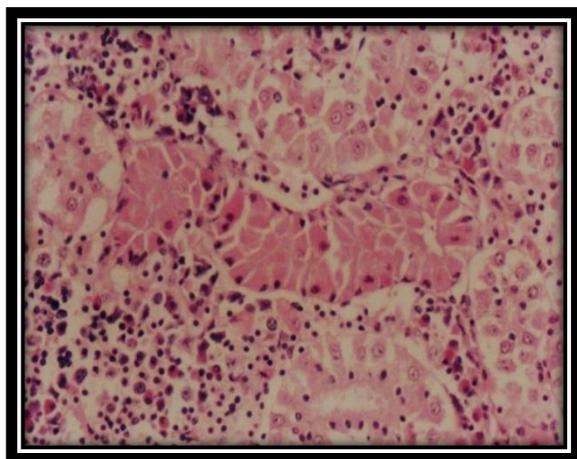


Figure (13): Kidney of *Clarias gariepinus* fed on contaminated ration with AFB₁ showing necrosis of tubular epithelium and depleted hemopoietic tissues. H&E X300

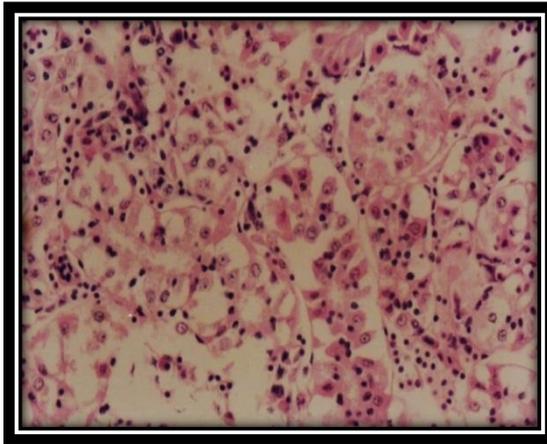


Figure (14): Kidney of *Clarias gariepinus* fed on contaminated ration with AFB₁ showing intense coagulative necrosis of renal tubular epithelium. H&E X300

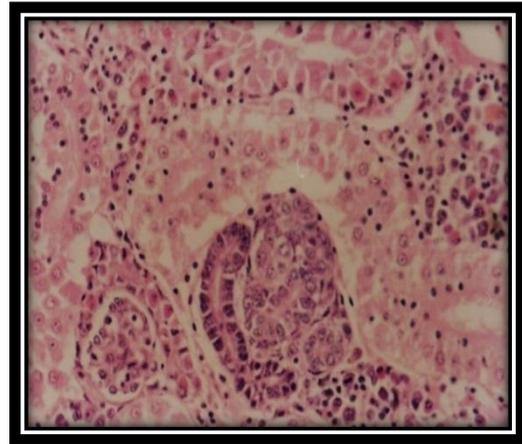


Figure (15): Kidney of *Clarias gariepinus* fed on contaminated ration showing regenerative attempts in some tubular epithelium. H&E X300

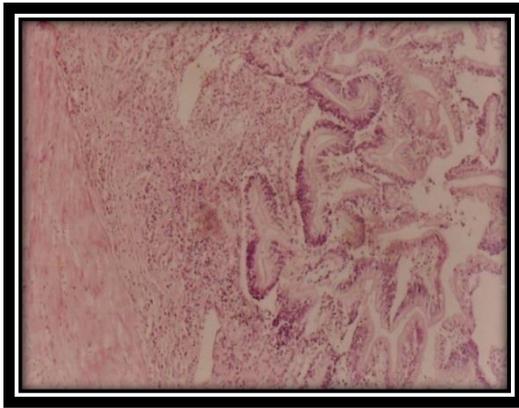


Figure (16): Stomach of *Clarias gariepinus* fed on contaminated ration with AFB₁ showing submucosal leukocytic aggregations, excess mucus and goblet cells. H&E X120



Figure (17): Gills of *Clarias gariepinus* fed on contaminated ration with AFB₁ showing hyperplasia and hypertrophy of secondary lamellar epithelium. H&E X120

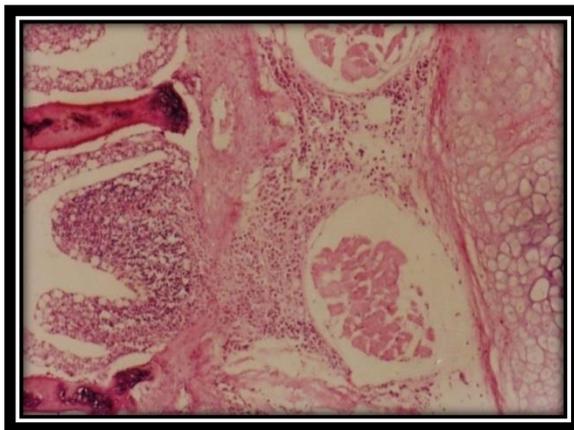


Figure (18): Gills of *Clarias gariepinus* fed on contaminated ration with AFB₁ showing edema and leukocytic infiltration in gill racker. H&E X120

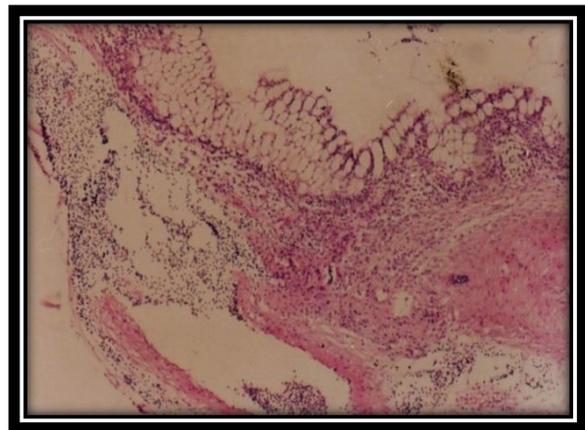


Figure (19): Gills of *Clarias gariepinus* fed on contaminated ration with AFB₁ showing hyperplasia and metaplasia of covering epithelium to mucus cells and intense leucocytic aggregations beside hemorrhages. H&E X120

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