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EXTRACTION AND ANALYSIS OF *A. FLAVUS* MYCOTOXINS WITH HISTOPATHOLOGICAL STUDY ON DIFFERENT ORGANS OF MALE RATS

Sadeq Obayes Kadhim[1] and Inam Badr Faleh[2]
[1]Department of Pathology and Forensic Medicine, College of Medicine, University of Misan, Iraq.
[2]Department of Pathology and Poultry Disease, College of Veterinary Medicine, University of Bagdad, Iraq.

Abstract

Mycotoxins are an important life threatening contaminants of foods and nutrients sources for animals and humans. Among these mycotoxins are *A. flavus* mycotoxins. The most serious of them are the aflatoxins, especially AFB1, which is severe toxic and carcinogenic secondary metabolite to animals and humans which get it through consumption of contaminated food, grain, peanuts or others. Therefore, in this study we extracted a crude *A. flavus* mycotoxins by culturing on yeast extract sucrose broth media (YES), then, the extracts analyzed by gas chromatography mass spectrometry (GC-MS) and histopathological study was carried out on different organs of group of 10 male albino rats which were received 2mg/kg/ body weight as single dose. After two weeks all treated animal were sacrificed and the changes were compared with animal control group (10 male rats) which only had been given distilled water. The study showed detection of essential chemical compounds that contribute in biosynthesis pathway of aflatoxins. In addition, there was severe DNA fragmentation illustrated by comet assay. Further, we present severe histopathological changes in liver, kidney, stomach and heart. The changes were included severe degenerations in liver and kidneys, gastric ulcer and hemorrhage and acute myocarditis.
Keyword **:** *A. flavus*, Aflatoxin, GC-MS and histopathology.

1. Introduction

It is believed that the mycotoxin production is a response by the fungus against the stress factors that are faced therefore, it is regarded as (stable chemical material) (Whitlow and Hagler, 2016). Therefore, great requirment to identify the different isolates of *A. flavus* which produce mycotoxins such as aflatoxins (Shephard *et al*., 2012). Gas chromatography coupled with mass spectrometry (GC-MS) is a technique that has been used in the detection and quantitation of mycotoxin in grain for over 20 years (McMaster *et al.,* 2019). Aflatoxins are derived from the difuranocoumarin compound which in turn consists from two bis-furan molecules and integrated with coumarin molecule (Fadl-Allah *et al*., 2011). Their spatial isomerism shapes are similar in all, thus, they appear as heterocyclic in their chemical structure which contain more of oxygen atoms (Zhang *et al*., 2014).

In poultry, presence of aflatoxin B1 (AFB1) in their diets reduces the hatching capacity, chick weight, rate of growth, egg and meat production and quality, efficiency of vaccination responses and increasing of diseases incidence in animals and poultry (Herzallah *et al*., 2014; Bbosa *et al*., 2013), as well as declining the feed metabolism range and make of birds more vulnerable to diseases. In broilers, (Omran, 2010) found that giving of aflatoxin contaminated diet resulted in adverse effect of immune system responses for Newcastle disease vaccine. Transfer of AFB1 with poultry products representing a health threatening to the eaters due to carcinogenic activity of AFB1 and its implication in human hepatic cancer (Fouad *et al*., 2019). This mycotoxin is potent hepatocarcinogen in animals and occasionally involved in primary cancer in liver, kidney, lung, and colon tissues in man (Amaike and Keller, 2011).

2. Materials and Methods

2.1 Culture and extraction of mycotoxins

The *A. flavus* that used in our study was isolated from clinical samples (Sputum) from sheep and humans presented with respiratory diseases. The culture and extraction were performed according to method of (Mohammed *et al*., 2016; Philippe *et al*., 2013; Khaddor *et al*., 2007) with some modification. The fungus was cultured on (100 ml) of yeast extract sucrose broth (YES), (Yeast Extract 2% and Sucrose 20%) in (250 ml) Erlenmeyer flask and incubated at 28 ºC in shaking incubator for 12 days at 120 rpm, Fig. (2-1, A). After, the culture broth was filtered by filter paper (whatmann No. 1), the metabolites were extracted by adding equal volume of chloroform to culture broth with well mixing for 10 minute and then put in shaker for 1h at 130 rpm. Then, separated by separator funnel, fig. (2-1, B) and poured in sterile petri dishes at room temperature to be dried. To suspend the residue, (1ml) of methanol was added to dry metabolites and filtered through 0.45µm syringe filter and kept at 4 ° C for 24 hours before Gas chromatography mass spectrometry analysis GC-MS.

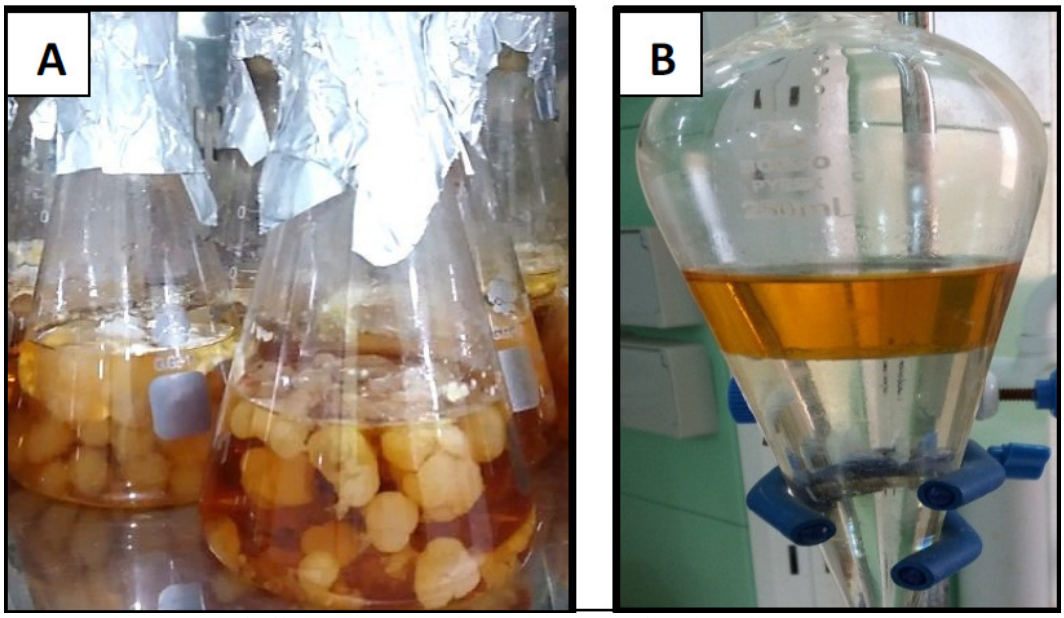


Fig. (2-1): A. Shows the characteristic fluff y ball growths of mycelia culture of *A. flavus* in (YES) broth after 12 days at 28 ºC in shaker incubator (120rpm). **B.** Shows the broth media (upper layer) after filtration by filter paper to exclude mycelia grow and mixing with chloroform containing mycotoxins (lower layer).

2.2 Analysis of extracts by Gas Chromatography-Mass Spectrometry (GC-MS)

The product was examined for analysis of chemical compounds by GC-MS (Agilent 789A) equipped with a DB-5MS column (30m×0.25mm i.d., 0.25µm film thickness, J&W scientific, Folsom, CA). The oven temperature was programmed as previous analysis. The carrier gas used was the Helium at rat 1.0 ml/min. effluent of GC column was introduced directly in the source of the MS via transfer line (250 ºC). Ionization voltage was 70 eV and ion source temperature was 230 ºC (Hameed *et al*., 2016). Scan range was 41-450 amu. The constituents were identified after compared with available data in the GC-MS library in the literature (Hamza *et al*., 2015). The categorization of metabolite components depend on differences in their mass spectra and National Institute of Standards and Technology (NIST) library of mass spectral (Altameme *et al*., 2015a).

2.3 Comet assay

Comet assay for mycotoxins were carried out in cancer center, Al-mustansiriya University according to their team procedure. The depicted images were automatically analyzed in data by (comet score professional 2.0) (rexhoover.com) and then, the data statistically analyzed by SPSS Edition (23).

2.4 Study of mycotoxins histopathologyy

The toxic dose (2mg/0.5ml/kg) was prepared in distilled water and given as single dose intraperitoneally for group of (10 male rats, 12 weeks in age) and allowed, on (Abdulmajeed, 2011) with some modification. While, the control group (10 male rats) was received only distilled water. The animals were sacrificed after two weeks and the target organs were harvested, fixed in formaldehyde (10%) for 48h and pieces from target organs had been taken and processed by auto-tissue processing machine (Dehydrated by series alcohol, Clearance by xylene and impregnation with liquid paraffin). After, the tissues were embedded in paraffin blocks, sectioned by microtome at thickness (5µm), mounted on glass slides, stained with hematoxylin and eosin, and coversliped. Finally, the changes read under light microscope comparing with normal control group (Mescher, 2016).

3. Results

3.1 Gas chromatography mass spectrometry (GC-MS) analysis

The results showed five essential chemical compounds necessary for Aflatoxins biosynthesis pathways, Charts (3-1), (3-2), (3-3), (3-4) & (3-5) at specific retain time for each compound, Table (3-1).

Table 3-1 : Shows the numbers of charts, chemical name, retain time, molecular weight and formula of each chemical compound resulted in (GC-MS) analysis.

No. of Chart	Chemical compound	Retention time (min)	Molecular weight	Formula
(3-1)	Acetic acid, dichloro-methyl ester	4.408	141.96	$C_3H_4Cl_2O_2$
(3-2)	Pentadecane-carboxylic acid	20.500	256	$C_{16}H_{32}O_2$
(3-3)	Pentadecanoic acid ethyl ester	20.625	270	$C_{17}H_{34}O_2$
(3-4)	Ethyl oleate	16.741	310.29	$C_{20}H_{38}O_2$
(3-5)	Linoleic acid ethyl ester	17.400	308.27	$C_{20}H_{36}O_2$

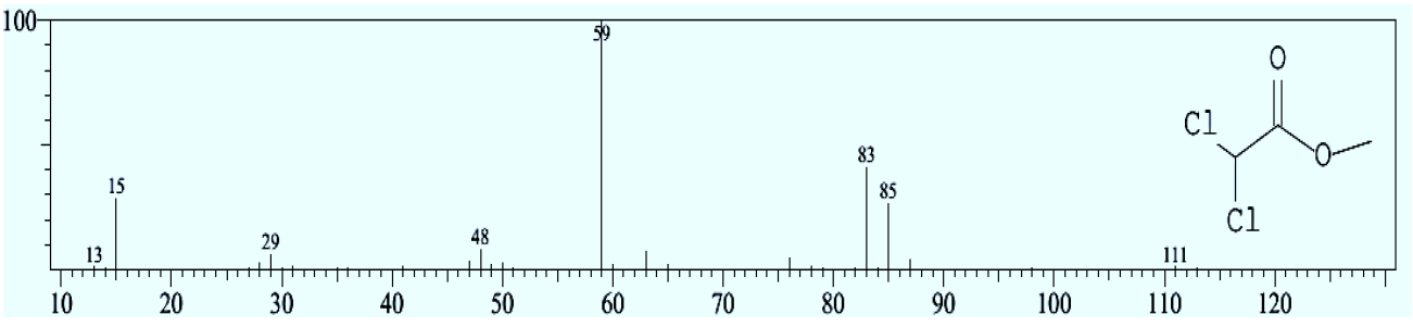


Chart (3-1) : Mass spectrum of (Acetic acid, dichloro-methyl ester) with retention time (RT)= 4.408

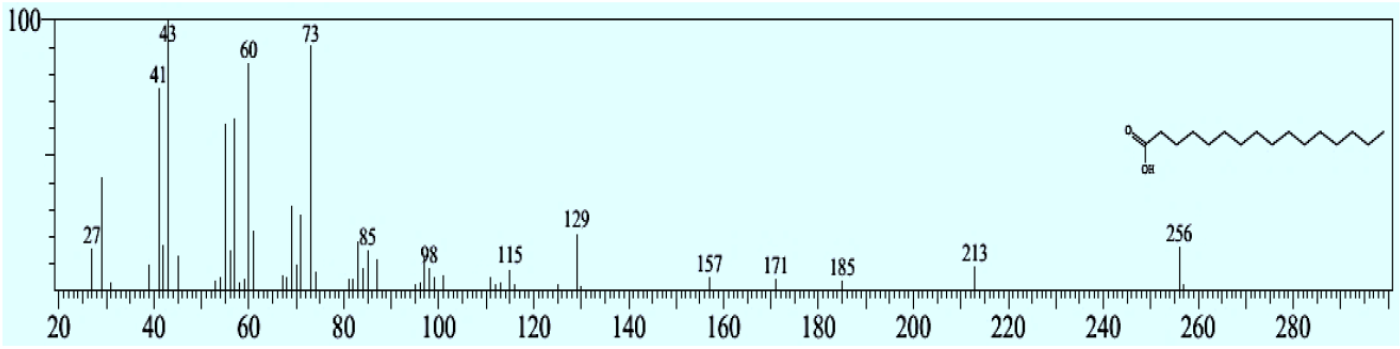
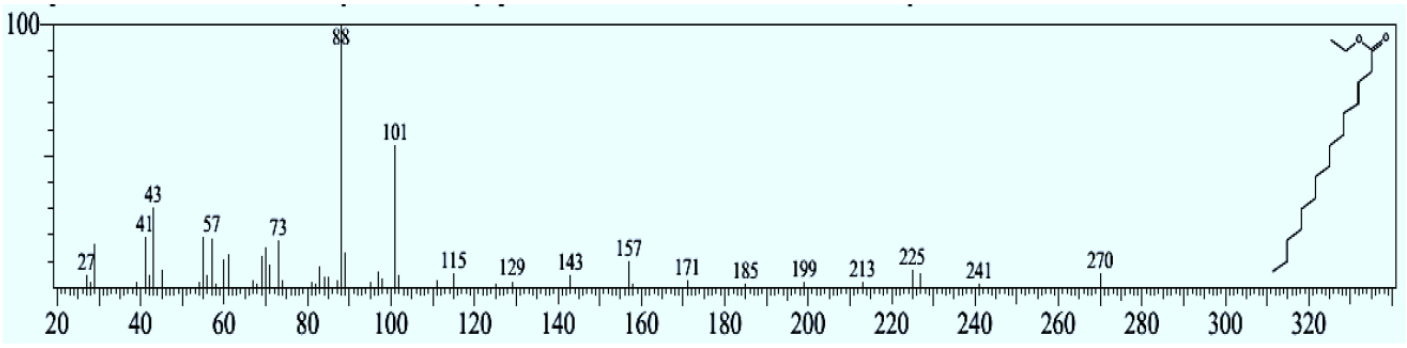


Chart (3-2) : Mass spectrum of (Pentadecane-carboxylic acid) with retention time (RT)= 20.500.



Char.(3-3) : Mass spectrum of (Pentadecanoic acid ethyl ester) with retention time (RT)= 20.625.

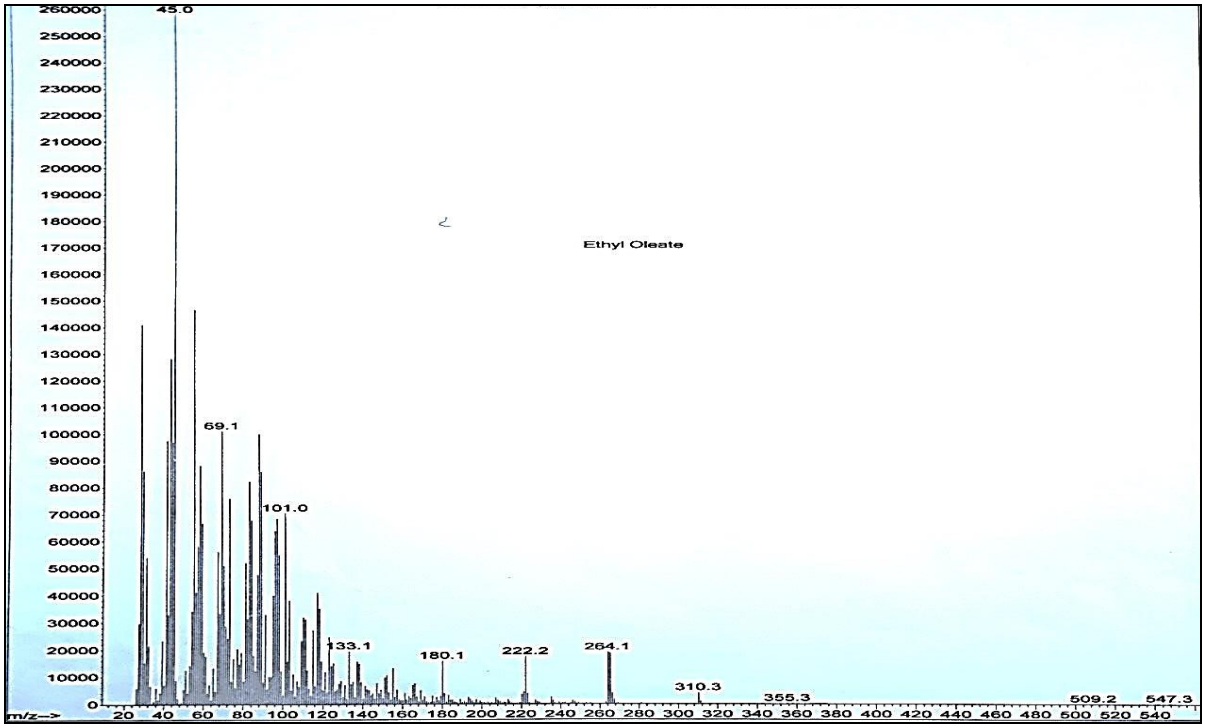


Chart. (3-4) : Mass spectrum of (Ethyl Oleate) with retention time (RT)= 16.741.

3.2. Histopathology of extracted mycotoxin

The histopathological effects of mycotoxin were involved multiple organs in the body, for instance; liver, kidneys, stomach and heart. In liver, there were severe cellular swelling, hydropic degeneration, vacuolation, nuclear pyknosis and some of necrotized cells with disappearance of sinusoids, figure(3-1,B&C) compared with normal (3-1,A). In kidneys severe tubular degeneration including cloudy swelling of proximal convoluted tubular epithelium, with some cellular necrosis, and interstitial infiltration of chronic inflammatory cells, fig (3-2,A) compared with normal field (3-2,B). In stomach, there were clear gastric ulcer surrounded with erosions of gastric mucosa with massive eosinophilic infiltration, and presence of fibrosis at the ulcer base fig.(3-3, A&B). In the heart, there is severe myocarditis characterized by massive infiltration of acute and chronic inflammatory cells including neutrophils, lymphocytes and macrophages invading the necrotized myocardial cells. In addition, there is an edematous fluids separate the myocardial muscle bundles fig.(3-4.A&B).

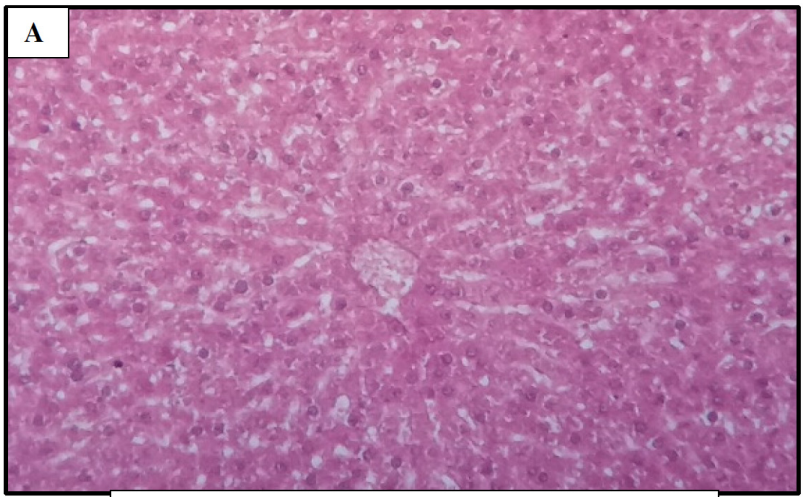


Fig. (3-1, A). 100X, Shows normal histology of liver, H&E

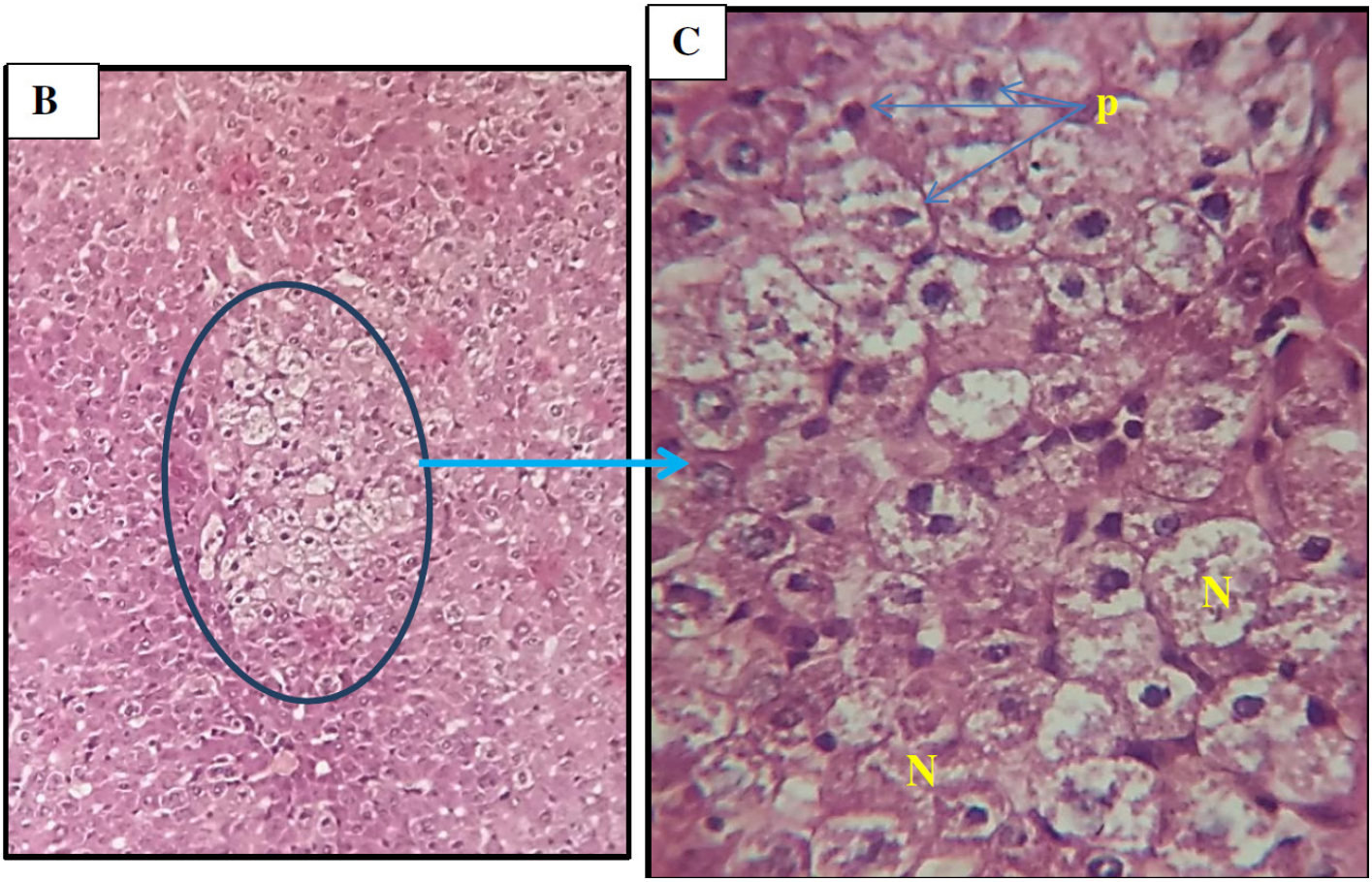


Fig. (3-1, B). 100X& C, 400X, microscopic field of liver with severe cellular swelling, hydropic degeneration, vacuolation, nuclear pyknosis (P) and some of necrotized cells (N) with disappearance of sinusoids. H&E

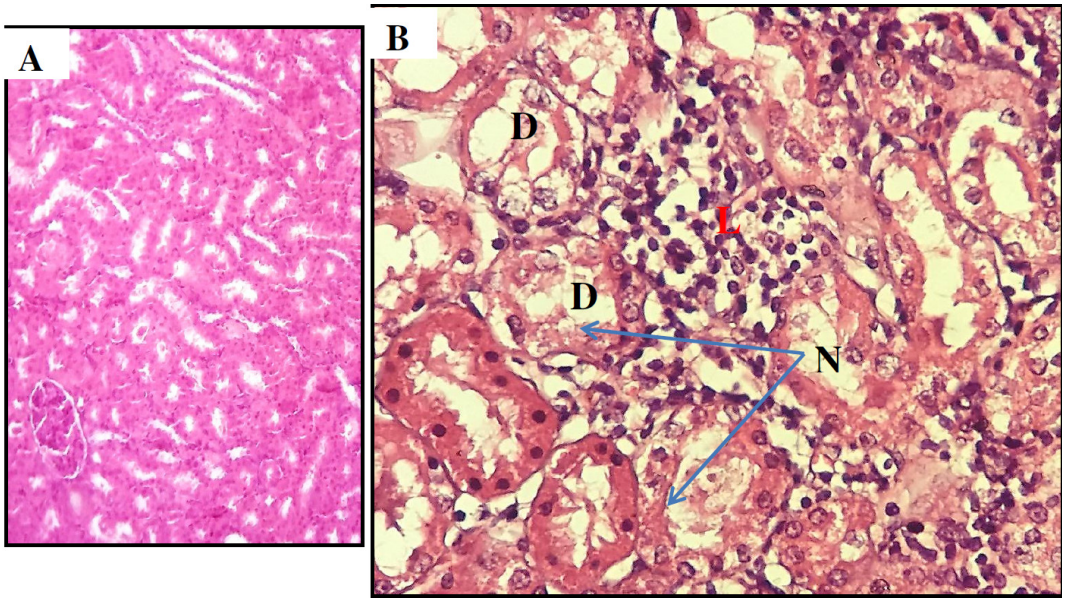


Fig.(3-2) A. Normal of renal cortex. **100X**. **B**. Interstitial nephritis shows lymphocytic infiltration (**L**), severe degenerative changes in tubular epithelium (**D**) and presence of some necrotized epithelial cells (**N**). **400X, H&E**

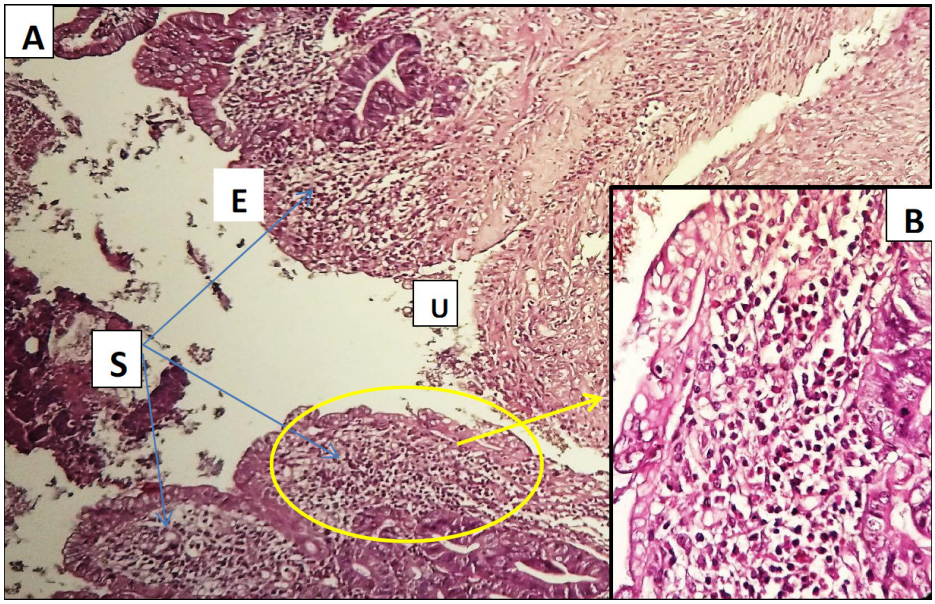


Figure (3-3) A. Revealed mucosal gastritis with severe erosion (**E**) and ulcer (**U**) associated with massive infiltration of eosinophils (**S**) in lamina propria of gastric mucosa 100X. **B.** focus on high magnification **400X, H&E**

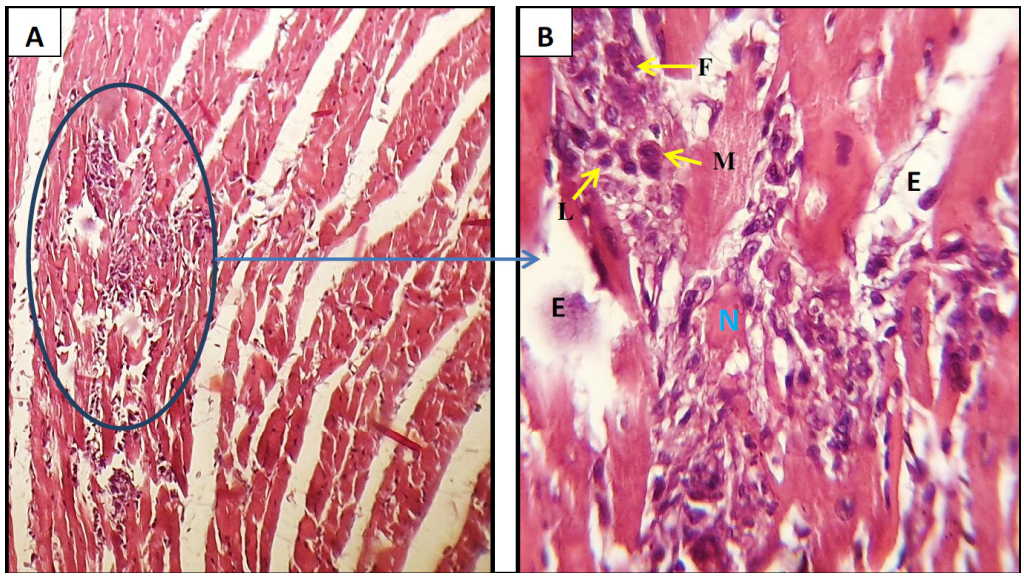


Fig.(3-4) A. Shows of severe myocarditis (**circles**) due to mycotoxicity, 100X. **B**, The same figure with high magnification, characterized by edema (**E**), inflammatory exudate with neutrophilic infiltration (**F**) and necrotized myocardial cells (**N**). Also presence of lymphocytes (**L**) and macrophages (**M**). **400X. H&E**

3.2. Comet assay

The comet assay revealed significant differences between mycotoxin group and control group in six important parameters. table (**3-2**) and fig.(**3-5A&B**):

Table 3-2. Shows comet assay for mycotoxin effects on six DNA parameters

Parameters / **Groups**	**Tail DNA %**	**Tail length**	**Tail moment**	**Head DNA%**	**Head mean**	**Comet mean**
Myc.	39.703±2.347 **A**	79.112±10.545 **A**	33.377±7.243 **A**	60.296±2.347 **A**	63.205±2.298 **A**	69.150±2.323 **A**
Con.	2.073±0.236 **B**	1.223±0.306 **B**	3.746±2.133 **B**	93.938±1.411 **B**	103.033±3.065 **B**	105.773±2.884 **B**

- Values are expressed as mean ± SE.
- n= 10 rat/group,
- Different letters refers to presence of significant differences (P<0.05) within a column.
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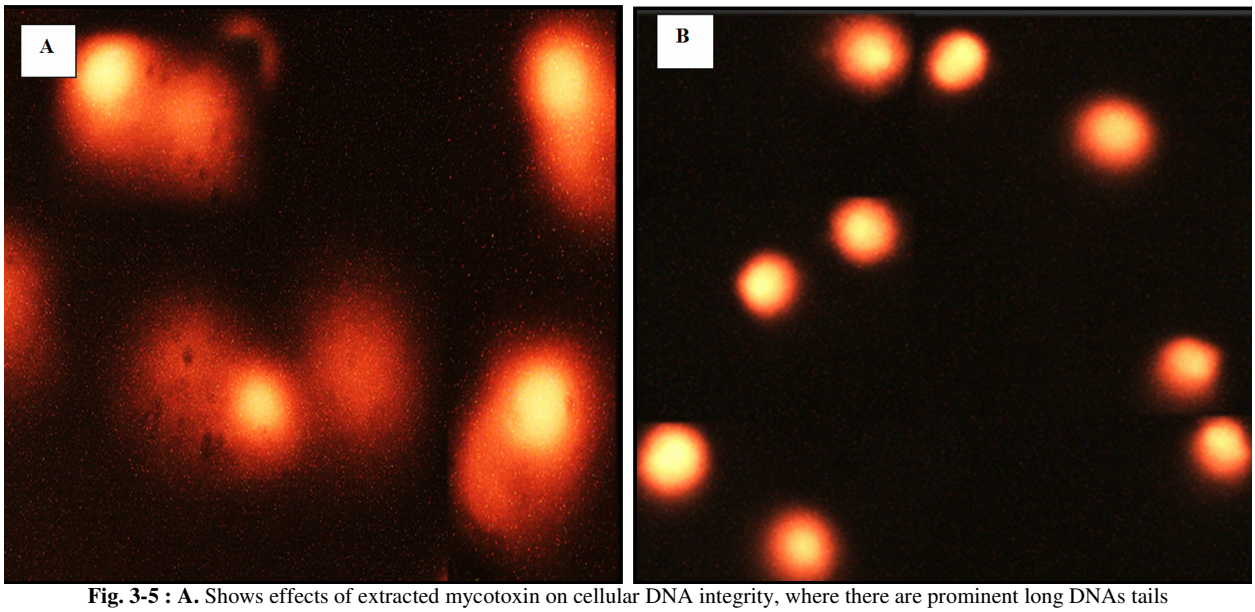

Fig. 3-5 : A. Shows effects of extracted mycotoxin on cellular DNA integrity, where there are prominent long DNAs tails compared with normal control one (**B**).

4. Discussion

4-1. Mycotoxins extraction

In this study, the fungal culture were performed on YES broth, because this media is considered as optimal media for aflatoxin production (Medina *et al*., 2015; Abdel-Hadi *et al*., 2012), where they found that this media promote high concentration of mycotoxins production. The characteristic fluffy ball growths of mycelia Fig.(2-1,A) was similar to that presented by (Hussain *et al*., 2015).

4-2. Gas Chromatography-Mass Spectrometry (GC-MS) analysis of mycotoxins

In our study, GC-Ms analysis results were included acetates including (Acetic acid, dichloro-methyl ester), Char. (3-1), the most important chemical compounds that considered as the building blocks or starter substances of initial step in aflatoxins biosynthesis and this was agreed with Gu *et al.* (2007). The acetates are convert into polyketide, which inturn convert into anthraquinone, then in to axanthones and finally in to Aflatoxins (Degola *et al*., 2007; Yu *et al*., 2004). The conversion of acetate in to polyketide mediated by important enzymes (polyketide synthase and fatty acids synthases encoded by three genes (*PksA*) and (*fas-1*& *fas-2*) respectively, then the polyketide convert into anthaquinones which in turn form rings of bisfuran (Yu, 2012).

Other compounds of GC-MS analysis are unsaturated fatty acids (Ethyl oleate and Linoleic acid ethyl ester), char.(3-4&5), and this result was comported with other studies which considered the unsaturated fatty acids as sources of acetates, where the fungal peroxisomes will oxidize these fatty acids forming acetates units (Chanda *et al*., 2010; Chanda *et al*., 2009). According to this information, the oxidation of unsaturated fatty acids also may be other biosynthesis pathway for aflatoxins production (Maggio-Hall *et al*., 2005). Pentadecanoic acid ethyl ester, Char.(3-3) was other important compound in GC-MS analysis in present study. This was in consistence with result of (Asuncion and Angeles, 2006) where this fatty acid is play main role in synthesis of lactone group, which is formed by fatty acids hydroxylation. (Kourist and Hilterhaus (2014) were confirmed the formation of lactone group in high concentration from hydroxylation of decanoic acid ethyl ester derivatives (hexadecanoic acid ethyl ester). Moreover, Watanabe *et al*. (1996) were biochemically evidenced the role of fatty acids initiators in the aflatoxin production. Formerly Lee *etal*. (1981) found that cleaving of cyclic lactone structure in aflatoxin B1 resulted in reduce about (450 fold) toxicity than of intact AFB1, in adition to reduced mutagenicity and other biological activity and also supported by Mao *et al*. (2016), therefore, corporation of lactone associated coumarin is required to insert high toxicity for AFB1, and degradation or removing of lactone group will

resulting in reduce or complete loss of its toxicity (Bandyopadhyay *et al*., 2019).

Also, presence of Pentadecane-carboxylic acid in GC-MS analysis, char.(3-2), was other evidence to presence of other essential chemical compounds in the aflatoxin biosynthesis. This result was in agreement with (Tikhomirov *et al*., 2013), where the carboxylic acid compounds contribute with anthraquinone, diketones and furans in the formation of (lactone) group, which play important role in the aflatoxin toxicity (Phillips, 1999).

4.3. Histopathological effects of mycotoxins

The results of mycotoxin histopathology were involved multiple organs, for instance in liver there were severe cellular swelling, vacualation, hydropic degeneration leading to loss of sinusoids, fig.(3-1,B&C). These findings were in agreement with results of (Zarev *et al*., 2019; Omran *et al*., 2019) in rats and. Also, presence of some necrotized cells Fig. (3-2,C) were resemble to results of (Ruggeberg *et al*., 2020; Olonisakin *et al*., 2019) in rats. Corcuera *et al*. (2015) also were reported necrosis and severe pyknosis associated with infiltration of inflammatory cells in liver of AFB1 treated rats. The mentioned hepatic lesions mostly due to exhaustion of glutathione storages which play an important role in mycotoxins detoxification and because aflatoxins induced reactive species (ROCs) and decreased antioxidant and anti-inflammatory chemicals this result in increased proinflammatory chemokines providing peroxidation of fatty stored hepatocytes (Wang *et al*., 2019; Muhammad *et al*., 2018). Biotransformation of aflatoxins in liver will formed DNA and protein high affinity adducts 8-9-epoxide (Pasha *et al*., 2007). Liu *et al*. (2020) were found in their study that aflatoxin upregulate gene expression of proinflammatory cytokines especially interleukin-6 (IL-6) stimulating hepatic inflammatory response (Hasso and Al-Janabi, 2019).

Kidney changes induced by mycotoxin in this study Fig. (3-2,B) including degenerative changes of tubular epithelium and necrotic of tubular epithelial cells were in concordance with results of (Olonisakin *et al*. (2019); El-Mahalaway, (2015) in rats, they presented clear degenerative changes including cloudy swelling of proximal tubular epithelium. They ascribe these changes to toxic injurious effect of aflatoxins to cellular structures, tissue and immune defenses. Presence of focal chronic interstitial nephritis with predominantly the lymphocytes in the renal cortex was described by Anand *et al*., 2013 in *A. flavus* infected mice they concluded increasing level of proinflammatory cytokine in renal milieu mostly IFN-Y and IL-6, which may due to mycotoxin production.

Severe damage of gastric mucosa and underlying layers Fig. (3-3,A,B) induced by *A. flavus* mycotoxins especially AFB1 were in consistent with that confirmed by other researchers (Koohi *et al*., 2017; Akinrinmade *et al*., 2016; Singh *et al*., 2014) in rats, but in our study the damage was more severe inducing prominent gastric ulcer with eosinophilic infiltration which may due to differences in periods and mycotoxin doses. They ascribe these damage to increase level of proinflammatory cytokins (TNF-α and IL1β) and nitrogen free radical (NO) follow aflatoxins administration. These mediators were responsible for initiated severe inflammatory process leading to pathological damage of gastric mucosa (Yu *et al*., 2018; Koohi *et al*., 2017; Akinrinmade *et al*., 2016) in rats.

Aflatoxin can interfere with many metabolic activities including biosynthesis of proteins affecting multiple body organs especially the heart (Mohammed and Metwally, 2009) in rats. In the current study, mycotoxin toxic effects in the heart were in consistent with that presented by (Yilmaza *et al*., 2018) in rat, they were evidenced hemorrhage and infiltration of inflammatory cells, but in our study there were severe destructive myocarditis associated with infiltration of different types of inflammatory cells including neutrophils, macrophages and lymphocytes, fig.(3-4,A&B). In rats, Ge *et al*. (2017) was observed that aflatoxins had been induced damage of mitochondrial membranes and cristae. Kudayer *et al*. (2019) also was recorded cardiac edema in their study on rats.

4.4. Comet assay for mycotoxin genotoxicity

There severe DNA damage, detected by comet assay in AFB1 treated rats, Fig. (3-5,A) evidenced by depicted microscopic fields and statistical analysis compared with control rats, Fig. (3-5,B). These results was in concordance with earlier results obtained by (Corcuera *et al*., 2015) on AFB1 treated rats and Zavala-Franco *et al*. (2020) in human blood sample in vitro.

The significant DNA damages usually attributed to AFB1 induced oxidative stress which resulted in stopping of cell cycle and cell death. Furthermore, some studies observed that AFB1 induced 8,9-epoxide can form DNA-adduct leading to DNA fragmentation. Versicolorin A and AFB1 poses two bonds in furan groups which is the site of biologically AFB1 activation by cytochrome P450 (CYP450) (Gauthiera *et al*., 2020 on human intestinal cells; Kim *et al*., 2016 on intestinal cells).

We conclude that the YES broth media is may be the optimum broth media for *A. flavus* mycotoxin (particularly Aflatoxin) production. In addition, the GC-MS analysis is efficient to analyze and diagnose the components of *A. flavus* mycotoxins. Moreover, we concluded that these mycotoxins are severe toxic and may constitute main serious and life threatening problem on animal and human health and other environmental compartments, due to their severe toxic effects on blood and body tissues including liver, kidney, stomach, lungs, spleen and heart in acute exposure. Also they can induce severe DNA defects or even mutations if there is chronic exposure. Therefore, this problem must be awarded more attention to reduce the dangerous consequences of these important fungal mycotoxins.

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