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Evaluation of Molasses Detoxification by *Aspergillus oryzae* NRRL 26 Using Nile Tilapia *(Oreochromis niloticus)*

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Abstract: This investigation aimed to evaluate the toxicity bioassay of fungus (*Aspergillus oryzae* NRRL 26)-treated molasses using *Oreochromis niloticus*. Mortality rate (MR %), some blood parameters (hemoglobin, total protein, albumin, globulin and enzymatic activities of liver, AST and ALT,) and the histopathological changes in Nile tilapia were investigated under laboratory conditions following treatment of molasses. 84 fish (30 g) were divided into 7 groups. G1 was the control group.G2 to G4 were exposed to three concentrations of the fungus- treated molasses (2.5, 5 and 10%), while G5, G6 and G7 were exposed to the same concentrations of untreated molasses, respectively, for a period of 4 days. The mortality rate was 100% in G7 after 48 hrs post exposure, also MR% in G5 and G6 were higher than the other groups. On the same trend, the alterations in blood parameters and histological structures of gills and livers were more severe in G5 and G6 as compared to other groups. It was concluded that *Aspergillus oryzae* has pronounced ability to detoxify molasses.

Key words: Molasses · Detoxification · Fungus · Nile tilapia

INTRODUCTION

Molasses is a sugar production byproduct with high saccharides content (48 to 50%). It has a high commercial value due to its usage as a cheap carbon source in various fermentations processes, as a biofertilizer and a feed component for domestic animals [1]. However, it was reported that the environmental impact of this molasses or its wastewater is very high, due to its dark color (melanoidin) and organic matter content. Melanoidins (dark brown pigments) are polymers which have low to high molecular weight formed as final products of Maillard reaction which is a non-enzymatic browning reaction resulting from the reaction of reducing sugars and amino compounds [2]. So, this dark color requires pretreatment before its safe disposal into the environment, since it is hazardous matter and has high pollution potential. Also, this highly colored component leads to a reduction of sunlight penetration in rivers, lakes or lagoons which in turn decrease both photosynthetic activity and dissolved oxygen concentrations causing harm to aquatic life [3].

Melanoidins are toxic for many microorganisms involved in wastewater treatment [4] due to their high bilogical oxygen demand (BOD; 40,000 mg/L), chemical oxygen demand (COD; 90,000 mg/L) and other toxic components [5,6]. Also, [7] reported that as the pigments in molasses are products of Maillard reaction between sugar and amino compounds produced by heating, it was suspected that the heating process would also produce pyrogenic compounds like polycyclic aromatic hydrocarbons (PAHs). Benzo(a)pyrene, is one of the common and most potential carcinogenic compounds. PAH that has been shown to produce severe suppression of both humoral and cell mediated immune function, which may last a significant portion of the life of the exposed animal [8]. Literature suggested that PAHs are causative agents of disease in fish [9, 10]. The adverse effects of PAH on *O. niloticus* fish immune function were studied by Holladay *et al.* [11], also, [12] investigated these effects too, but on *O. mossambicus*. While the toxicity of molasses spent wash (MSW) on *O. mossambicus* was studied by Raghukumar *et al*. [13].

Recently, de-colonization of molasses melanoidin has been attempted, but with limited success [4]. The biological treatments using certain fungi and bacteria to remove the color compound have been successfully achieved and thus can be applied

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as a bioremediation technique. This study was designed to assess the toxicity of de-colorizated sugar cane molasses by *Aspergillus oryzae* NRRL 26 using Nile tilapia fish.

MATERIALS AND METHODS

Microorganism: *Aspergillus oryzae* NRRL 26 was obtained from Microbial Properties Research Unit, National Center for Agricultural Utilization Research, Agriculture Research Service, USA. *A. oryzae* was grown at 30°C on Potato-Dextrose Agar (PDA) slants. Spore suspensions for inoculum were obtained from slants after 14 days of cultivation at 30°C, using 0.9% (W/V) NaCl, 0.1% (W/V) tween 80 solution. Spores were scraped by using 5 ml sterilized saline solution.

Molasses: The crude sugar cane molasses was obtained from the Sugar Refinery Factory at El-Hawamdia, Giza, Egypt. Sugar cane molasses contained approximately 50% total sugars. The crude molasses was diluted to 50 g/L in water and additionally, 15 g/l glucose, 1.4 g/l urea, 1 g/l KH_2PO_4, 2 g/l NaCl and 0.5 g/l $MgSO_4$. 7 H_2O were supplemented. The addition of these nutrients came from the preliminary experiments that achieved the effective molasses de-colorization. This medium was referred to as the molasses medium.

Culture Conditions: Cultivation was made in 250 ml Erlenmeyer flasks, each containing 100 ml of sterile molasses medium at pH 5, incubated at 30°C on a rotary shaker at 150 rpm for 4 days.

Fish: Serial dilutions of molasses were prepared in dechlorinated tap water, treated and untreated molasses were used in different dilutions 0, 2.5, 5 and 10%. Eighty four fingerlings of *O. niloticus* (were obtained from El-Serw Fish Researches Station, where this study was carried out), having similar weight (average 30 g) were acclimated to aquaria conditions for one week prior to the initiation of the experiment. Fish were divided into 7 groups according to the design of experiment (Table 1). Two glass fish aquaria (70 x 40 x 30 cm) containing 20 L (of the aerated different tested dilutions) were used for each group. Behavior pattern and mortality percentage of fish groups were noted over 96 hr.

Analytical Methods: At the end of the experiment, blood samples were withdrawn from the fish heart of each group to determinate some blood parameters, mainly haemoglobin (HB), total protein (TP) and albumin (ALB) concentrations and activities of serum transferase enzymes; aspartate amino transferase (AST) and alanine amino transferase (ALT) using commercial colorimetric kits (Diamond, Diagnostic, Egypt).

Table 1: Experimental fish groups

Groups	G1	G2	G3	G4	G5	G6	G7
Treated molasses%	-	2.5	5.0	10	-	-	-
Un treated molasses%	-	-	-	-	2.5	5.0	10

The Histopathological Examination: The histopathological examination of the fish gills and livers were performed after the preparation of gills and livers which were dissected out from each group and fixed in 10% neutralized formalin solution and embedded in paraffin blocks for routine hematoxyline and eosin staining (H&E) according to the technique of Roberts [14].

Statistical Analysis: The obtained data were statistically analyzed by using a software (SAS) [15].

RESULTS

Mortality Rate: Adverse effects of molasses, either treated or untreated by *A. oryzae,* on mortality rate was reported after 48 and 96 hr (Table 2). The mortality rate gradually increased by increasing the percentage of molasses. MR% in fish groups exposed to treated molasses (G2, G3 and G4) was lower than fish groups exposed to untreated molasses (G5, G6 and G7).

Table 2: Mortality rate (%) of fish exposed to different dilutions of either *A. oryzae.* treated or untreated molasses

	Mortality rate (MR %)	
Groups	48 hr	96 hr
G1	0	0
G2	16	25
G3	16	33
G4	33	41
G5	50	58
G6	50	75
G7	100	-

Table 3: Some blood parameters of *O. niloticus* exposed to either *A. oryzae* treated or non treated molasses (Means+SE)

Items	G1	G2	G3	G4	G5	G6
HB, g/dl	$6.8^{A}\pm0.05$	$6.1^{B}\pm0.05$	$5.8^{BC}\pm 0.05$	$5.6^{D}\pm 0.04$	$4.3^{E}\pm0.03$	$4.1^{E}\pm 0.04$
TP, g/dl	$5.60^{A}\pm 0.11$	$5.03^{B}\pm 0.07$	$4.70^{C}\pm 0.09$	$4.70^{C}\pm0.09$	$4.56^{C}\pm 0.03$	$4.5^{C}\pm 0.06$
ALB, g/dl	$2.56^{A}\pm0.03$	$2.27^{B}\pm0.09$	$2.13^{AB}\pm0.09$	$1.93^{C}\pm0.09$	$2.03^{BC}\pm0.07$	$2.03^{BC}+\pm0.07$
GLO, g/dl	$3.03^{A}\pm 0.09$	$2.77^{B}\pm 0.03$	$2.53^{C}\pm 0.03$	$2.80^{B}\pm 0.01$	$2.53^{C}\pm 0.09$	$2.47^{C}\pm 0.03$
AST, U/I	$53.27^{D}\pm0.09$	$52.76^{D}\pm0.4$	$55.77^{C}\pm1.2$	$59.1^{B}\pm 0.08$	$63.93^{A}\pm0.09$	$66.27^{A}\pm1.0$
ALT, U/I	$22.7^{DE}\pm0.3$	$24.07^{C}\pm0.2$	$22.10^{E}\pm 0.1$	$22.9^{D}\pm0.09$	$29.7^{B}\pm 0.2$	$31.8^{A}\pm 0.2$

A-E: Means in the same row with different letters are significantly ($P \leq 0.01$) different

Blood Parameters: Table 3 illustrates the data of blood analysis of the first six fish groups, where the G7 fish were dead (MR 100% after 48 hr).

The values of hemoglobin, total protein and its main fractions, albumin (ALB) and globulin (GLO) showed significant decrease ($P<0.01$) by increasing the percentage of molasses, especially in the cases of fish groups exposed to untreated molasses (G5 and G6). Also, G5 and G6 groups reflected the lowest Hb concentrations as compared with the other groups.

Increased level of activity of hepatic transaminase enzymes (AST and ALT) in serum is a specific indicator of liver damage in fish. Exposed fish to 5 and 10% untreated molasses for 4 days showed significant increase in AST and ALT activeties comparing to all other groups. While G2 fish that exposed to 2.5% treated molasses showed non-significant difference in activity of AST and ALT compared with the control group.

Histopathological Study: Gills of the control fish (G1) have normal histological architecture (Fig. 1). Gills of fish group that was exposed to 2.5% treated molasses (G2) showed slight congestion of primary gill blood vessels with slight dilation of the vessels at the tips of secondary lamella (Fig. 2). Moderate fusion of epithelial lining and separation or lifting was noticed in G3 exposed to 5% treated molasses (Fig. 3). While Fig. 4, 5 showed curling and lifting of secondary lamellar with severe congestion of primary gill blood vessels of fish group which were exposed to 10% treated molasses. Lesions among group G5 exposed to 2.5% untreated molasses were severe in forms of proliferation of the filamenter epithelium leading to lamellar fusion that could be seen (Fig. 6) with fusion of the secondary lamellae and desquamation, particularly at the tips (Fig. 7). Gills of fish exposed to 5% untreated molasses showed severe fusion of the secondary lamellae with desquamation (Fig. 8) and congestion of primary gill blood vessels, with epithelial edema (Fig. 9).

In the control fish group, no histological changes were observed in liver. The hepatic cells appear hexagonal with centrally placed rounded nuclei and homogeneous cytoplasm without forming distinct lobules. The pancreas lies embedded in a compact manner between the hepatic cells surrounding the blood capillaries and hepatic cells surrounding the blood capillaries and thin bile canaliculi are observed between the hepatic cells.

In general, the pathological changes in the liver of the fish groups exposed to untreated molasses appeared to be more severe than those in the treated molasses, the severity of which increased with the increase of concentration. Group 2 exposed to 2.5% treated molasses showed congestion in pancreatic acini (Fig. 10) with slight hemorrhage of hepatic blood vessels (Fig. 11), hepatocyte showed normal structure and were found in regular shape. G3 (exposed to 5% treated molasses) showed thrombosis in blood vessels (Fig. 12) and severe congestion in pancreatic acini beside hemosiderin accumulation (Fig. 13). G4 (exposed to 10% treated molasses) showed moderate diffuse of hemosiderin accumulation (Fig. 14) and hemolysis in blood vessels (Fig. 15). Group 5 exposed to 2.5% untreated molasses showed severe lesions in liver in form of severe blood congestion and hemolysis with clear diffusion of Melanomacrophage (MMC) (Fig. 16) and severe thrombosis in blood vessels (Fig. 17) also some hepatocytes lost their normal polygonal structure and had prominent vacuolization with lateral situated nuclei and hydropic swelling (Fig. 18) besides severe diffusion of hemosidren accumulation. With increasing the concentration (5% untreated molasses in G6) hepatopancreas damage became more conspicuous and involved larger areas with condensed cytoplasms and loss of the contact between hepatocytes and in many cases this was associated with appearance of pyknotic and karyolysis nuclei, apoptotic, (Fig. 19) and diffused hemolysis between hepatocytes (Fig. 20) in addition to severe diffuse of hemosiderin accumulation around blood vessels.

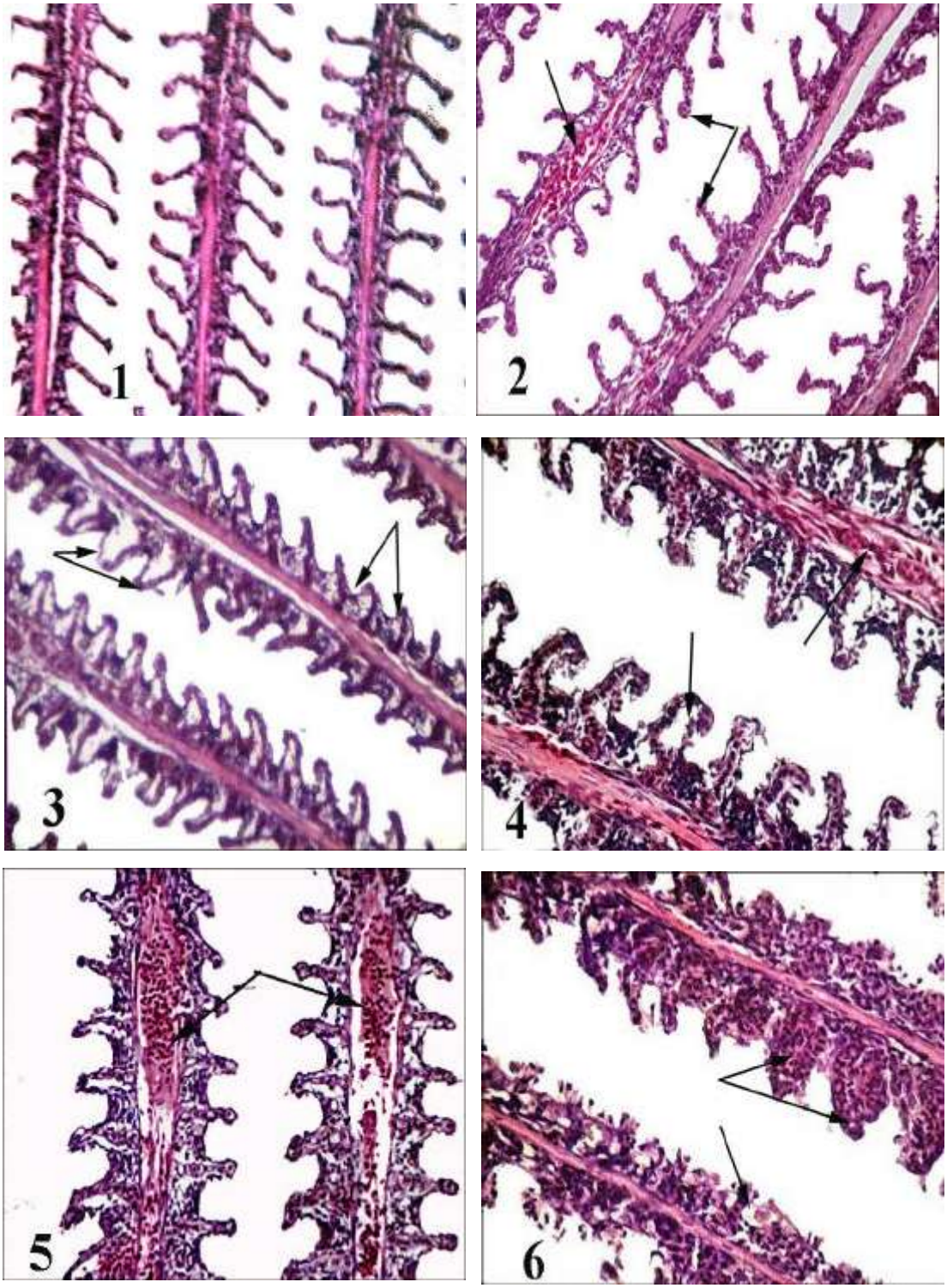

Fig. 1-6: Histological sections in gills of *O. niloticus* fish stained with H&E; (1) control fish showing normal structure (x130), (2) fish exposed to 2.5% treated molasses showing dilation in blood vessel of secondary lamellar (x150), (3) fish exposed to 5% treated molasses showing fusion and lifting of epithelial lining (x150), (4,5) fish exposed to 10% treated molasses showing curling and lifting of secondary lamellar (x400, 250 respectively), (6) fish exposed to 2.5% untreated molasses showing severe proliferation of the filamenter epithelium (x400)

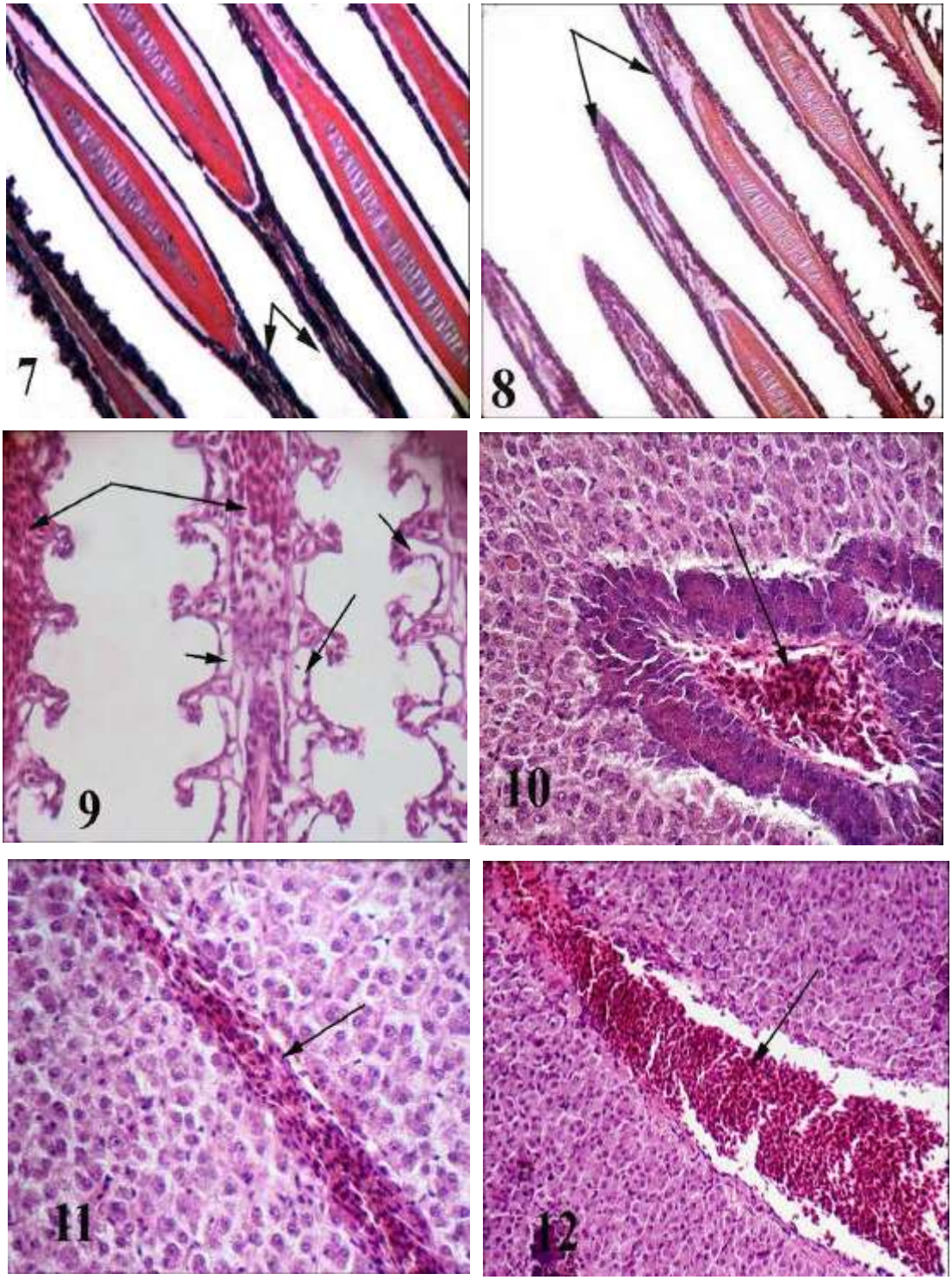

Fig. 7-9: Histological sections in gills of *O. niloticus* fish stained with H &E; (7) fish exposed to 2.5% untreated molasses showing fusion and desquamation of secondary lamellae (x150), (8-9) fish exposed to 5% untreated molasses showing fusion and desquamation of secondary lamellae(8,x130) and epithelial edema (9,x400). Figs. (10-12) histological sections in liver of *O. niloticus* fish stained with H&E. (10)fish exposed to 2.5% treated molasses showing slight congestion in pancreatic acini (x600) with slight hemorrhage of hepatic blood vessels(11,x800), (12) fish exposed to 5%treated molasses showing thrombosis in blood vessels(x400)

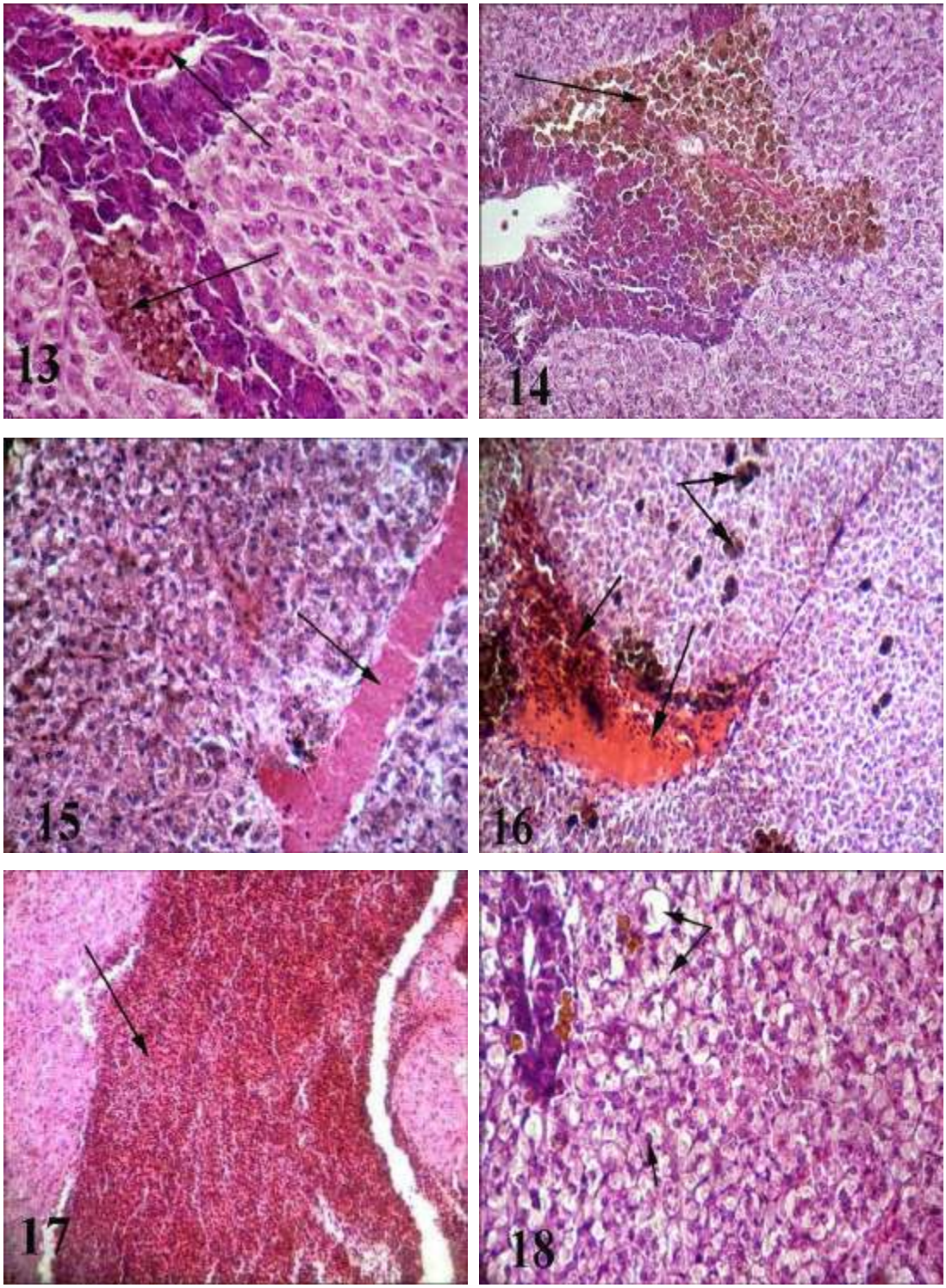

Fig. 13-18: Histological sections in liver of *O. niloticus* fish stained with H&E. (13)fish exposed to 5% treated molasses showing hemosiderin accumulation (x 800), (14, 15) fish exposed to 10% treated molasses showing hemosiderin accumulation (14, x600) and hemolysis in blood vessels (15, x800), (16, 17, 18) fish exposed to 2.5% untreated molasses showing diffusion of MMC (16, x400) with severe thrombosis in blood vessels(17, x 400) and necrosis and vaculation (18, x 800)

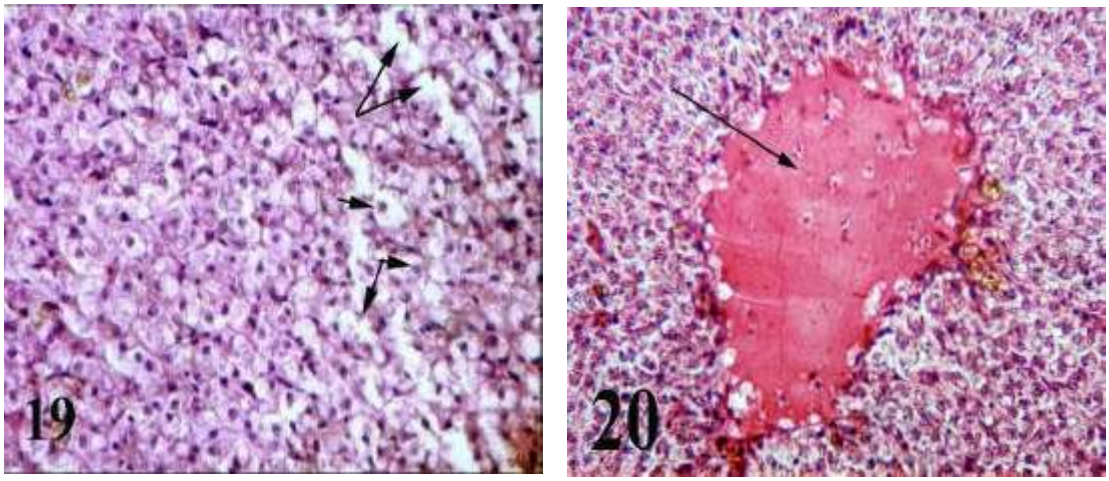

Fig. 19,20: Histological sections in liver of *O. niloticus* fish stained with H&E exposed to 5% untreated molasses showing necrosis (19, x800) and diffuse of hemolysis between hepatocytes (20, x 400)

DISCUSSION

The results of the toxicity assessment suggested that untreated molasses caused nonspecific toxic action on Nile tilapia which could be due to the high BOD and COD values and the presence of such pollutants as PAHs as byproducts during melanoidin formation [16] and metals as iron where molasses is very rich in iron [17]. PAHs are known to have many adverse effects on biological systems. In aquatic organisms, the gills are consider a vital organ, since they play an important role in the transport of respiratory gases and regulate the osmotic and ionic balance. Toxic substances may cause damage to gills tissues, thereby reducing the oxygen consumption and disrupting the osmoregulatory function of aquatic organisms [18]. In fish, some PAHs have been demonstrated to have mutagenic/carcinogenic [19], genotoxic [20] and cytotoxic [21] effects as well as to alter reproduction [22], energy metabolism [23], growth [24] and immune function [25]. Moreover total body iron is classically viewed as being distributed among three compartments; strong iron, that are bound to ferritin and hemosiderin,transport iron and functional iron, consists of iron that is bound to hemoglobin and others [26, 27]. While [28] reported that one of the most important potential side effects of iron supplements is iron overload. The mechanism of iron toxicity involves the production of free radical species that can oxidize a wide array of lipids and proteins.This eventually leads to tissue damage and fibrosis [29]. Also, [30] reported that the increased iron deposition may cause hemosidrosis. Hemosiderin that often forms after bleeding when blood leaves a ruptured blood the cell dies and hemoglobin of the red blood cells is released into the extracellular space. Thus macrophages engulf the hemoglobin to degrade it, producing hemosiderin. MMC have been described by Ferguson [31] as repositories for the end products of cellular breakdown and in addition to MMC tended to aggregate in the proximity of hepatic portal blood vessels. The accumulation of iron can cause liver failure and hepatocellular carcinoma [32]. In this study, the reduction of the values of hemoglobin, total protein and its main fractions, especially in the cases of fish groups exposed to untreated molasses (G5 and G6) may be due to the damage of the hemopiotic organs and the gills as a result of exposure to untreated molasses which was confirmed histopathologically (Fig. 6 to 9) leading to inhibition of blood synthesis, where the gills play an important role in this process, as the untreated molasses is characterized by extremely high COD and BOD [5] that may cause stress on fish respiration. Moreover, the disturbances in values of TP, AST and ALT may be attributed to the hepatotoxic effect of molasses, especially untreated and consequently hepatic cell damage and liver dysfunction; since protein synthesis usually occurs in liver. Moreover, iron inclusion led to increased activity of both transaminases, AST and ALT [33, 34]. The physicochemical analyses of treated (de-colorized) molasses revealed that melanoidin de-colorization was accompanied by simultaneous reduction in different physicochemical parameters *A. oryzae* reduced the BOD (85.3%), COD (89.2%), color reduction 74.4%, pH was 4.5, PAHs before and after treatment was 16.9% and 13% respectively while benzo (a) pyrene was 14.8 and 3.9% (from PAHs) before and after treatment, respectively. Also the values of melanoidin iron were reduced after

treated by bacterial de-colorization [18]. These positive alterations in the chemical composition of de-colorized molasses decreased its toxicity as confirmed by previous studies, [13] using a marine fungus, *Flavodon flavus* for combined de-olorization and detoxification of 10% molasses spent wash by using estuarine fish *O. mossambicus,* where the results showed no liver damage in fish exposed to treated effluent in contrast to untreated which showed moderate liver damage, Also benzo(a)pyrene was detected in the MSW and appeared as a one of the causes of toxicity of the MSW. In another study, [17] reported that the de-colorization of synthetic melanoidin by three *Bacillus spp.* significantly reduced the toxicity to the tubificid worm (*Tubifex tubifex*, Muller). In conclusion *Aspergillus oryzae* has pronounced ability to detoxificate the molasses.

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