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Antiaflatoxigenic activity of chemical antioxidants (glutathione and glutathione enhancer) on *Oreochromis niloticus*

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Abstract

The aim of this study was to evaluate the efficacy of two antioxidants, namely glutathione (GSH) and glutathione enhancer (GSH-EH), for amelioration of aflatoxicosis in *Oreochromis niloticus*. Three concentrations of both of GSH and GSH-EH (0, 5 and 10 mg/kg B.W. as a single intraperitoneal administration) were tested either as a pre-or post- treatment for aflatoxin B_1 (AFB_1). Two concentration of AFB_1 (0 and 9 ppm) were tested. AFB_1 was dissolved in Dimethylsulphoxide (DMSO 25%) and injected to the fish groups. GSH and GSH-EH were dissolved in distilled water just before use directly. A total number of 224 apparently healthy *O. niloticus* were assigned to 14 treatments (T_1–T_{14}) with 2 replicates, T_1 was kept as a negative control group, T_2 (positive control AFB_1), T_3 and T_4 (fish injected with GSH at 5 &10 mg/kg B.W., respectively), T_5 and T_6 (fish injected with GSH-EH at 5 &10 mg/kg B.W., respectively), treatments T_7-T_{10} were injected at the start of the experiment by GSH and GSH-EH, then at the 2nd day were injected by AFB_1. While groups T_{11}-T_{14}, were injected by AFB_1 at the start of the experiment, then at the 2nd day were injected by GSH and GSH-EH (as a post treatment). At the end of the experiment; blood, muscles and liver samples were taken from each group to determine the glutathione activity in blood, AFB_1 residues in fish muscles and screen the AFB_1 metabolites in liver by gas chromatography (GC) and mass spectrometry (MS), and to determine the alterations in the liver of the experimental fish. The results showed that AFB_1 has significant potency for reducing the GSH values in blood *O. niloticus* either it was injected alone (T_2) or with either of GSH or GSH-EH. While, GSH and GSH-EH pre-treatments (T_7-T_{10}) reflected decrease in GSH values comparing with the post-treatments (T_{11}-T_{14}). AFB_1 residues showed that both antioxidants have the potency of vanishing the AFB_1 residues only, at the post-treatment (T_{11}-T_{14}). Microscopically, there were hepatic lesions manifested by severing congestion with large areas of hemorrhages, necrotic hepatocytes and dilatation in blood vessels in AFB_1 injected fish groups.

Keywords: Aflatoxin B1, Antiaflatoxigenic, Glutathione, Glutathione enhancer, Residues, Histopathology.

1. Introduction

Aflatoxins are secondary metabolites produced mainly by the fungus *Aspergillis flavus,* with aflatoxin B_1 (AFB_1) identified as the most toxic metabolite. AFB_1 is a potent hepatocarcinogen and hepatotoxin in some fish species, such as rainbow trout. AFB_1 has been recently classified as a group 1 carcinogen (IARC, 1993). Aflatoxin exposure may occur via contaminated food, such as moldy corn and peanuts. Jantrarotai *et al.* (1990) reported that aflatoxin is among the most common contaminants causing great economic losses in aquaculture. Research has indicated that interspecies differences in sensitivity to this hepatocarcinogen can often be explained by variations in the biotransformation of AFB_1 (Bechtel, 1989; Eaton and Gallagher, 1994 and Bailey *et al.,* 1996).

In aflatoxicosis, oxidative stress is a common mechanism contributing to initiation and progression of hepatic damage. When animals consume contaminated feeds, AFB_1 is metabolized in the liver producing the highly reactive chemical intermediaries. The binding of these intermediaries to DNA results in the disruption of transcription and abnormal cell proliferation, leading to mutagenesis and carcinogenesis (Theumer *et al.,* 2003). The activation of AFB_1 to the *exo*-AFB_1- 8,9-epoxide is thought to be responsible for its carcinogenic effects because this unstable, highly reactive intermediate can bind to cellular macromolecules, including DNA (Miller, 1978 and Essigmann *et al.,* 1982).

Glutathione *S*-transferase (GST)-catalyzed conjugation of activated AFB_1 is apparently the most important detoxification system (Hayes *et al.,* 1991). The resulting conjugate is often less toxic than the parent compound, and its increased hydrophilicity can make it more readily excretable from the body. AFB_1–glutathione (GSH) conjugation is the major detoxification pathway of aflatoxin metabolites in the liver (Allameh *et al.,* 2000). Removal of AFB_1 metabolites (predominantly AFB_1–GSH and a glucuronide conjugate) from the liver occurs via biliary excretion (Ha *et al.,* 1999). A number of hepatic metabolites of AFB_1 have been detected in urine,

including aflatoxin M_1, aflatoxin P_1, aflatoxin Q_1 (Cusumano *et al.,* 1995 and Sarr *et al.,* 1995), and AFB_1–mercapturic acids (predominantly exo-AFB_1 mercapturate, Scholl *et al.,* 1997).

Glutathione is a tripeptide synthesized from the precursor amino acids cysteine, glutamate, and glycine. It is low molecular weight sulphur containing compound (thiol), easily oxidized and can be regenerated very rapidly. These characteristics allow it to play an essential role in many biochemical and pharmacological reactions (Mates, 2000; Locigno and Castronovo, 2001 and Paolicchi *et al.,* 2002). GSH is synthesized in every cell of the body, but the liver is quantitatively the major site of synthesis (Hahn *et al.,* 1978; Lauterburg *et al.,* 1984 and DeLeve and Kaplowitz, 1990).

Therefore, the major objective of the present study was to evaluate the efficacy of both reduced glutathione (GSH) and glutathione enhancer (GSH-EH) in controlling aflatoxicosis in *O. niloticus.*

2. Materials and Methods

2.1. Preparation of aflatoxin B_1

Aflatoxin B_1 was produced on liquid medium (potato dextrose) by *Aspergillus parasiticus* (NRRL. 2999) according to Ready *et al.* (1971). Aflatoxin B_1 was dissolved in chloroform and quantitatively estimated by thin layer chromatography, TLC (AOAC, 2000). So, chloroform was evaporated to dryness on a rotary vacuum evaporator at 40ºC and redissolved in DMSO 25% (1:3 water) to the requirement of each aflatoxin concentration. AFB_1 was freshly dissolved in DMSO before injection.

2.2. Antioxidants; glutathione and glutathione enhancer

Reduced GSH was obtained from Sigma Chemical Co., and GSH-EH was produced by Sigma Pharmaceutical Industries, which contains of GSH, L-Cysteine, N-Acetyl Cysteine, L-Methionine, Vitamin C and Selenium. The indications of this drug (GSH-EH) are acute and chronic hepatitis, hepatocellular disorders and hepatic failure, fatty liver and impending cellular damage induced by stress and cancer. These antioxidants were dissolved in distilled water according to the experimental design.

2.3. Experimental design

This experiment was designed to evaluate the effects of AFB_1 on blood GSH, AFB_1 residues in muscles, GC-MS analysis of livers extract and the histopathological study of liver 4 days post-AFB_1-injection (Table 1). Two hundreds and twenty four fingerlings of *O. niloticus* with mean weight 25 g were obtained from El-Serw Station for Fishes Researches, where this study was carried out in June 2008. These fishes were randomly divided into 14 treatments (T_1-T_{14}) with 16 fish in each treatment maintained in two glass aquaria (70X40X30 cm), the fish were acclimated to aquaria conditions for a week before the experiment was initiated. The aquaria were provided with air stones, all fish were received diet twice daily at a feeding rate 3% of the actual body weight. AFB_1 was tested at levels of 0 and 9 mg/kg B.W. (being 0 and 0.25 the LC_{50}, according to EL-Barbary, 2008) at a single dose, while both of GSH and GSH-EH were used at three levels (being, 0, 5 and 10 mg/kg B.W.) at a single dose either, at the 0 day as a pre-treatment, or after 2 days from the start of the experiment. Antioxidants, DMSO and distilled water were injected alone at 0.05 ml/fish.

Table 1: Experimental design.

No. of Treatments	At 0 day of the experiment		At the 2nd day of Exp.	End of the Exp., at the	
				4th day	6th day
T_1	Control groups	DMSO 25%	D.W.	+	
T_2		AFB_1 (9 mg/kg B.W.)	D.W.	+	
T_3		GSH_1 (5 mg/kg B.W.)	DMSO 25%	+	
T_4		GSH_2 (10 mg/kg B.W.)	DMSO 25%	+	
T_5		GSH-EH_1 (5 mg/kg B.W.)	DMSO 25%	+	
T_6		GSH-EH_2 (10 mg/kg B.W.)	DMSO 25%	+	
T_7	Pre-treatment*	GSH_1	AFB_1		+
T_8		GSH_2	AFB_1		+
T_9		GSH-EH_1	AFB_1		+
T_{10}		GSH-EH_2	AFB_1		+
T_{11}	Post treatment **	AFB_1	GSH_1	+	
T_{12}		AFB_1	GSH_2	+	
T_{13}		AFB_1	GSH-EH_1	+	
T_{14}		AFB_1	GSH-EH_2	+	

* Pre-treatment means that the tested antioxidants were injected before the AFB_1- injection.
** Post-treatment means that the tested antioxidants were injected after the AFB_1- injection
D.W. = distilled water

2.4. Analytical methods

At the end of the experiment (either at the 4th or 6th day), blood samples were withdrawn from the fish heart of each treatment to determine reduced glutathione by the method of Beutler *et al.,* (1963). The obtained data were statistically analyzed by one way analysis of variance using a software (SAS, 1996).

Ten gram muscles of three fish from each treatment were homogenized and prepared to determinate the residues of AFB_1 in fish by TLC (AOAC, 2000). Also, 1g liver of each treatment was prepared to screen the AFB_1 metabolites by GC-MS, Gas chromatography and mass spectrometry, column: DB-17MS (122-4732) 30m x 0.25 mm x 0.25. This determination was carried out at Plant Pathology Lab., Agricultural Research Center, Ministry of Agric., Giza, Egypt.

The histological examination of the formalin-fixed fish liver was performed according to the technique described by Roberts (2004).

3. Results and Discussion

3.1. Determination of reduced GSH levels in blood

Data presented in Table (2) showed the GSH levels in blood of all fish treatments after 4 days from GSH and GSH-EH injection. The results showed that significant differences were observed in GSH values among some fish treatments. AFB_1 has significant potency for reducing the GSH values in *O. niloticus* blood, either it was injected alone (T_2) or with both of GSH and GSH- EH [(T_7-T_{14}) except T_{11} where no significant difference was observed between it and free AFB_1-treatments (T_3-T_6) comparing with AFB_1-control (T_2)]. In contrast, both of antioxidants led to gradual increase in blood GSH of fish treatments (T_3-T_6) comparing with control treatment (T_1). While, GSH and GSH-EH pre-treatment reflected decrease in GSH values comparing with AFB_1 pre-treatment (T_{11}-T_{14}).The high concentrations of GSH in blood can be regarded as a detoxification direct step toward active AFB_1-epoxide. There was a number of studies showing the importance of GSH coniugation of AFB_1-epoxide in the detoxification of AFB_1 (Degen and Neumann, 1978), where the increased biliary extraction of AFB_1-epoxide-GSH and the increased resistance to the acute toxicity of AFB_1 (Holeski *et al.,* 1987)

3.2. Aflatoxin B_1 residues in *O. niloticus* muscles

The residual analysis of AFB_1 in the muscles of all AFB_1-injected *O. niloticus* showed that AFB_1-control (T_2) and the antioxidants pretreatments (T_7-T_{10}) revealed presence of AFB_1 residues (17.14, 28.5, 28.0, 37.7 and 27.4, ppb respectively).These results are confirmed by previous studies of El-Barbary and Mehrim, (2009) who found that *O. niloticus* injected intraperitoneally (I.P.) with AFB_1 (9mg/kg B.W.) showed trace of AFB_1 (7.5 ppb) in their whole body. In contrast, the other AFB_1-treatments (T_{11}-T_{14}) showed absence of AFB_1 residues. These results indicate that both tested antioxidants in this study were more efficiently when used after exposure to AFB_1 (T_{11}-T_{14}) than antioxidants pre-treatment (T_7-T_{10}). So, the sensitivity of *O. niloticus* to AFB_1 can be reduced substantially by post-treatment, but not pre-treatment with antioxidants. While T_2 that injected with AFB_1 alone showed lower value of AFB_1- residues (17.14 ppb) than the treatments T_7-T_{10} which could be attributed to the stress effect of GSH and GSH-EH on O. *niloticus*; hence, their less efficiency on the conjugation of AFB_1 with these antioxidants to less toxic compounds that will be eventually excreted comparing with AFB_1 injected (T_2). However, the hepatic GSH levels are elevated within 8 h following AFB_1 treatment and remain elevated up to 5 days following daily administration of AFB_1 (Beers *et al.,* 1992).

The positive effects of both of GSH and GSH-EH on overcoming the toxic effects of AFB_1 could be attributed to the antioxidative properties of these materials. These results showed that the ability of GSH and GSH-EH when use after aflatoxicosis to counteract the toxic effects of AFB_1 on the fish could be better than its usage before aflatoxicosis (as a pre- treatment). That may be attributed to the characteristics of GSH, which is easily oxidized and can be regenerated very rapidly (Mates, 2000; Locigno and Castronovo, 2001 and Paolicchi *et al.*, 2002); so, its efficiency to play an essential role in biochemical and pharmacological reactions to counteract the toxic effects of AFB_1 on the fish could be lower.

Table 2: Mean values of blood GSH at the 4th day of GSH- injected in *O. niloticus.*

Treatments		GSH (mol/L cells)
Control groups	T_1 Control	$1.39^c \pm 0.02$
	T_2 AFB_1	$0.99^d \pm 0.02$
	T_3 GSH_1	$2.19^b \pm 0.01$
	T_4 GSH_2	$2.98^{ab} \pm 0.01$
	T_5 GSH_1-EH	$2.19^b \pm 0.02$
	T_6 GSH_2-EH	$3.38^a \pm 0.03$
Pretreatment	T_7 $GSH_1 + AFB_1$	$0.99^d \pm 0.01$
	T_8 $GSH_2 + AFB_1$	$1.19^d \pm 0.02$
	T_9 GSH_1-EH + AFB_1	$1.79^c \pm 0.02$
	T_{10} GSH_2-EH +AFB_1	$1.39^c \pm 0.02$
Post - treatment	T_{11} AFB_1+ GSH_1	$2.58^b \pm 0.03$
	T_{12} AFB_1+ GSH_2	$2.18^b \pm 0.03$
	T_{13} AFB_1+ GSH_1-EH	$1.79^c \pm 0.02$
	T_{14} AFB_1+ GSH_2-EH	$1.98^c \pm 0.02$

a-d: Means in the same column superscripted with different letters are significantly different at ($P \leq 0.05$).

3.3. Screen of AFB_1 residues in liver by GC-MS

GC-MS analysis (Table 3 and Figures.1-3) was performed to detect AFB_1 degradation or AFB_1 metabolites. Results of GC-MS analysis of liver extract of AFB_1- injected fish either with or without the tested antioxidants comparing with liver extract of control fish treatment (T_1) at different variations of abundances and retention times were showed as peaks in Figures. 1-3. This analysis revealed absence of both AFB_1 and antioxidants residues in liver of all treatments, no large differences in peaks numbers were noted between the T_1 and T_2 (Figure1), indicating that the antioxidants (GSH and GSH-EH) activity have effects on metabolic activation of liver. While the other treatments (T_7-T_{14}) (Figures.2 and 3) showed clearly decrease in peaks numbers of each treatment at the abundance 2.000.000, especially with the high concentration (10 mg/kg B.W.) of both of GSH and GSH-EH which reflected the most decrease in peaks numbers in case pre-treatment of both of GSH and GSH-EH (Figure2; T_8 and T_{10}) comparing with the control treatments (Figure 1; T_1 and T_2).

Table 3: List of some compounds identified by GC-MS.

RT	Library / ID	Reference	CAS
4.96	Cyclohexene	31993	000138-86-3
6.67	1,3-butadiene	196646	000087-68-3
6.936	2-cyclohexan	46818	006485-40-1
7.883	Tetradecane	113289	000629-59-4
8.899	Erucic acid	288671	000112-86-7
8.727	Tributyl phosphate	207383	000126-73-8
9.450	Thymyl acetate	103015	000528-79-0
10.210	Thiosulfuric acid	56420	002937-53-3
9.281	Hydroxyl-6 cytosine	23391	000000-
10.169	Isooctyl phthalate	326920	027554-26-3
11.484	Octadecanoic acid	231322	000057-11-4
11.485	n-Hexadecanoic acid	195432	000057-10-3
13.210	n-Octadecanoic acid	231322	000057-11-4

CAS= Chemical Abstracts Service (using database, CAS and chemBlink network). RT= Retention time.

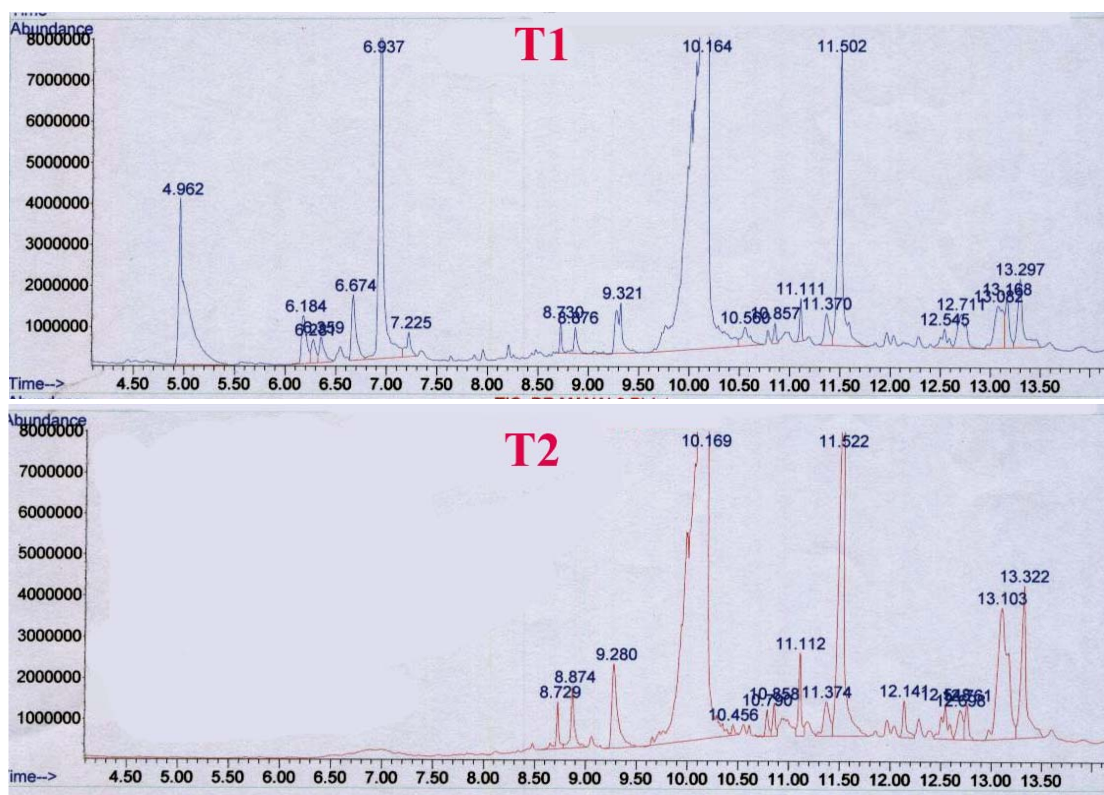

Figure 1: Chromatogram of fractions of the extracted compounds of *O. niloticus* liver of both control (T_1) and AFB_1 treated fish (T_2).

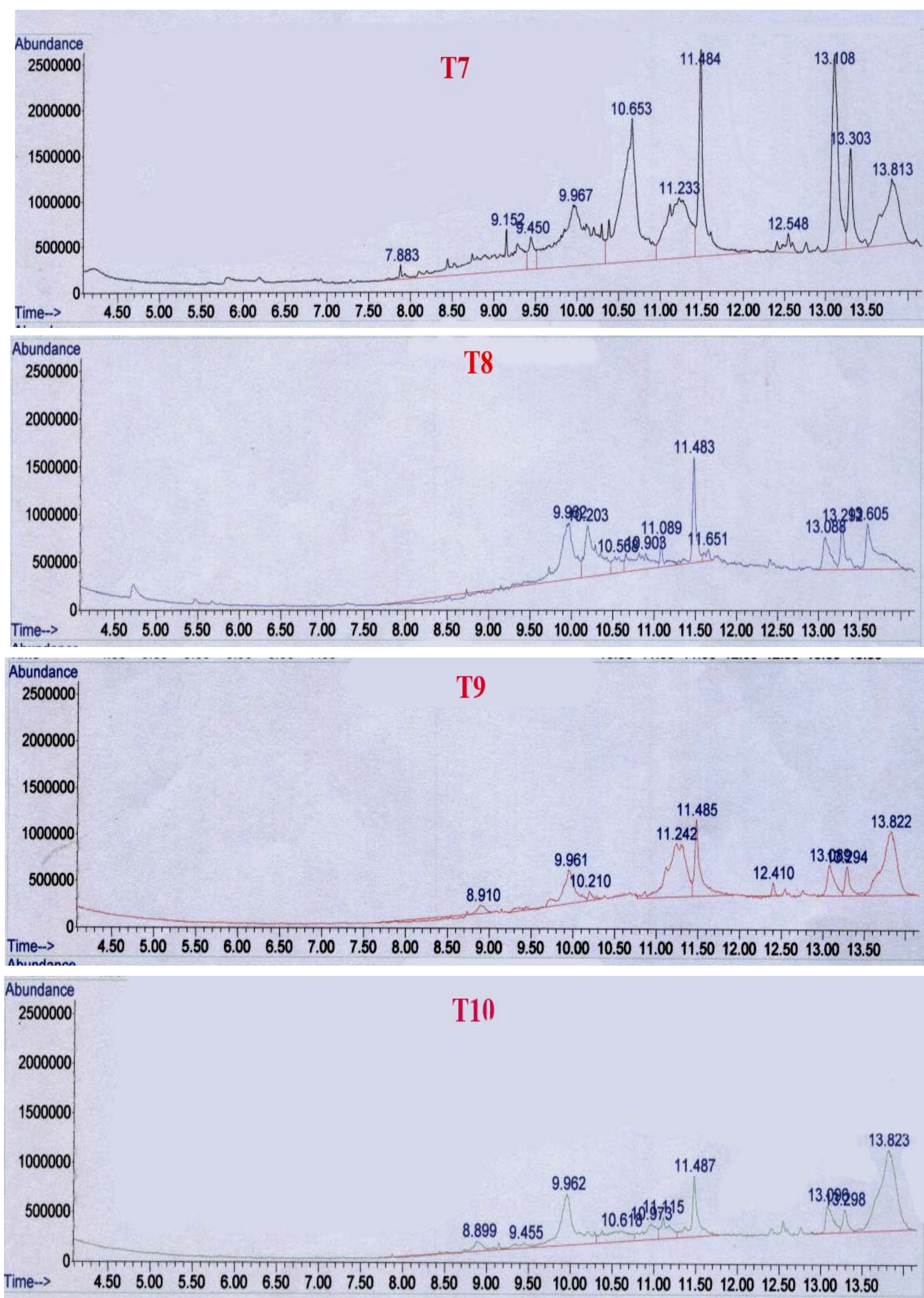

Figure 2: Chromatogram of fractions of the extracted compounds of *O. niloticus* liver of both GSH (T_7 and T_8) and GSH-EH (T_9 and T_{10}) pretreatment at the two levels of them (5 & 10 mg/kg B.W. respectively).

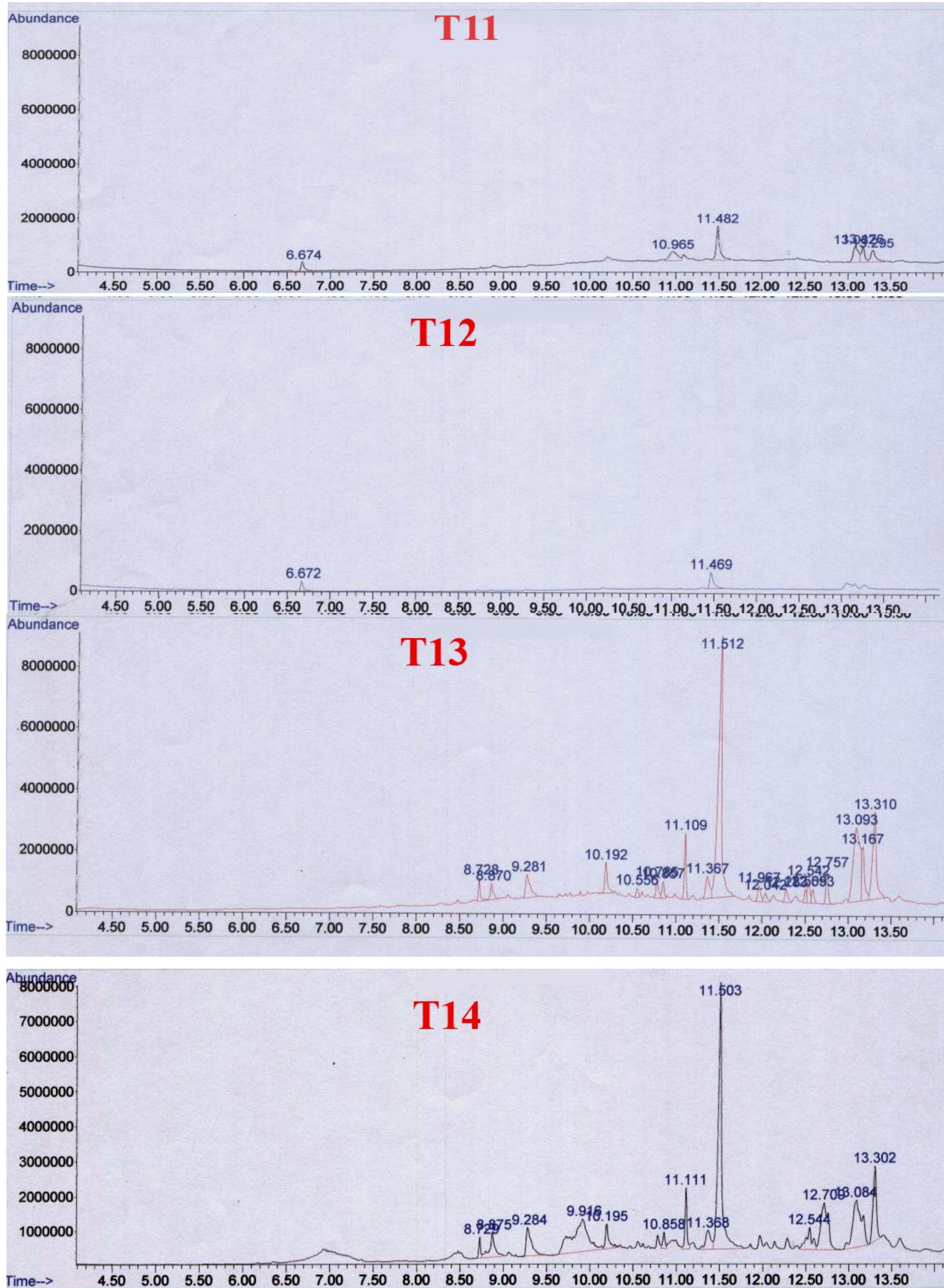

Figure 3: Chromatogram of fractions of the extracted compounds of *O. niloticus* liver of both GSH (T_{11} and T_{12}) and GSH-EH (T_{13} and T_{14}) treated fish (after aflatoxicosis) at the two levels of them (5 & 10 mg/kg B.W. respectively).

3.4. Histopathological alterations of *O. niloticus* liver

The histopathological lesions in liver of *O. niloticus* fish injected with AFB_1 with and without either antioxidant were observed in the present study, comparing with the control (T_1). In the control fish group, no histological changes were observed in liver (Figure 4a). T_2 (fish injected with 9mg AFB_1 / kg B.W.) showed severe lesions in the liver in form of thrombosis in blood vessels and focal areas of necrosis between the hepatocytes (Figure 4b), some of the hepatocytes showed pycnosis (Figure 4c), and the hepatocytes lost their normal polygonal structure and had prominent vacuolization with lateral situated nuclei and hydropic swelling (Figure 4d). T_3 and T_4 (fish injected with both of 5 and 10mg /kg B.W. GSH alone, respectively) showed normal structure of hepatocyte (Figure 4e) besides severe diffusion of hemosidren accumulation around blood vessels (Figure 4f), respectively. While T_5 and T_6 (fish injected with both of 5 and 10mg GSH-EH /kg B.W., respectively), showed diffusion of slight hemorrhage between hepatocytes associated with slight degeneration vacuoles in hepatocytes (Figure 5a), in addition to, congestion in blood sinusoids associated with appearance of pycnotic nuclei in the hepatocytes (Figure 5b), respectively. T_7 and T_8 (fish was pre-injected with the two levels of GSH before injection with AFB_1, respectively) showed vacuolar degeneration in the hepatocytes (Figure 5c) with focal areas of necrosis between the hepatocytes (Figure 5d), respectively. So, these results showed that the pathological changes in the liver of the fish treatments injected with the high level of both glutathione and glutathione enhancer appeared to be more than those in the fish treatments injected with the low level of them, these pathological changes increased with GSH-enhancer comparing to GSH.

The lesions of T_9 and T_{10} (fish were pre injected with the two levels of GSH-EH before injection with AFB_1, respectively) were intravascular haemolysis and necrosis between hepatocytes (Figure 5e), dilatation and congestion in blood sinusoids and vacuolar degeneration in the hepatocytes (Figure 5f), respectively.

In fish injected with 9mg AFB_1/kg B.W. before injection with both of 5 and 10 mg GSH /kg B.W. (T_{11} and T_{12}), the hepatocytes showed some loss in their normal polygonal structure and disappearance of hepatocyte wall and karyolitic necrosis (Figure 6a), some of the hepatocytes showed pycnosis besides congestion in portal blood vessels (Figure 6b). While, T_{13} and T_{14} (fish injected with 9mg AFB_1/kg B.W. before injection with both of 5 and 10mg GSH-EH /kg B.W., respectively) showed severe hemorrhage in blood vessels and vascular degeneration in hepatocytes (Figure 6c), hepatocytes arranged around the central vein associated with pycnosis in most of the hepatocytes (Figure 6d). The histopathological alterations in liver agree with those lesions described by Hussein *et al.* (2000), Abdelhamid *et al*. (2002), El-Barbary and El-Shaieb (2006) and El-Barbary and Mehrim (2009), who reported similar histopathological lesions in the liver of *O. niloticus* injected with 9 and 18 mg AFB_1/ kg B.W.

In this study, the histopathological findings revealed that the chemical antioxidants at the tested levels could not have potency to overcoming the side effects of AFB_1 on the liver histology. However they reflected a positive effect in reducing AFB_1 residues in fish muscles.

4. Conclusion

It could be concluded that no residues of aflatoxin B_1 was found in the aflatoxicosed *O. niloticus* liver after the 4th day of its injection, but it was detected in the fish muscles, both of glutathione and glutathione enhancer have the ability to conjugate with AFB_1 and to be excreted from the body only in case of its use after aflatoxicosis. The optimal levels of these antioxidants for detoxification of aflatoxin effects need more studies.

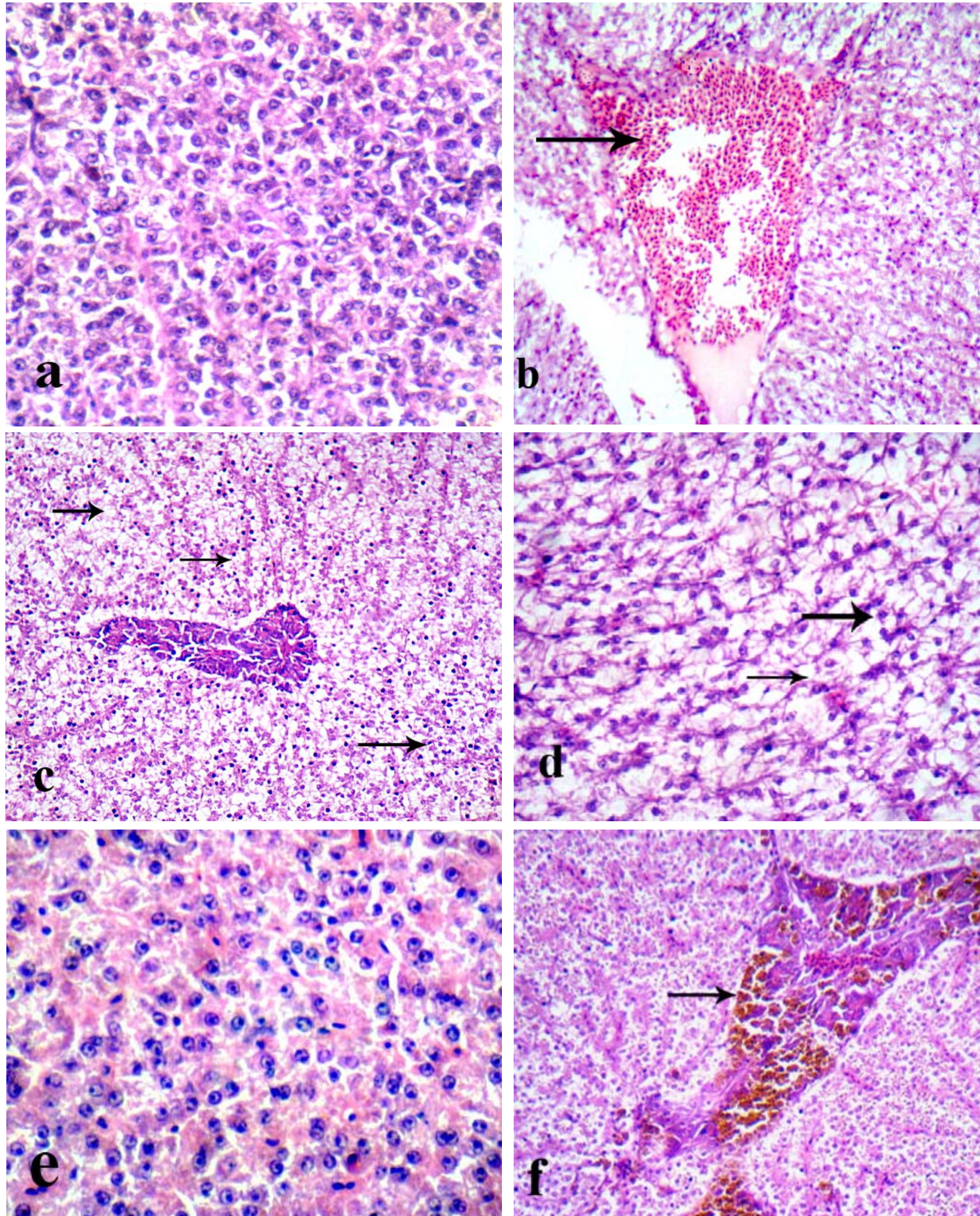

Figure 4: Histopathological changes in liver of *O niloticus* injected with AFB_1 as compared to control (stained with H&E). (a): The control fish group showing normal structure (T_1, x350). (b-d); fish injected with AFB_1 (9mg/kg B.W., T_2) showing thrombosis in blood vessels (b, x200) besides necrosis in hepatocytes (c, x200), vaculation and necrosis in hepatocytes (d, x400) ; (e) fish injected with GSH (5mg /kg B.W.,T_3) showing normal structure (x400). (f): fish injected with GSH (10mg /kg B.W., T_4) showing hemosidren accumulation around blood vessels (x200).

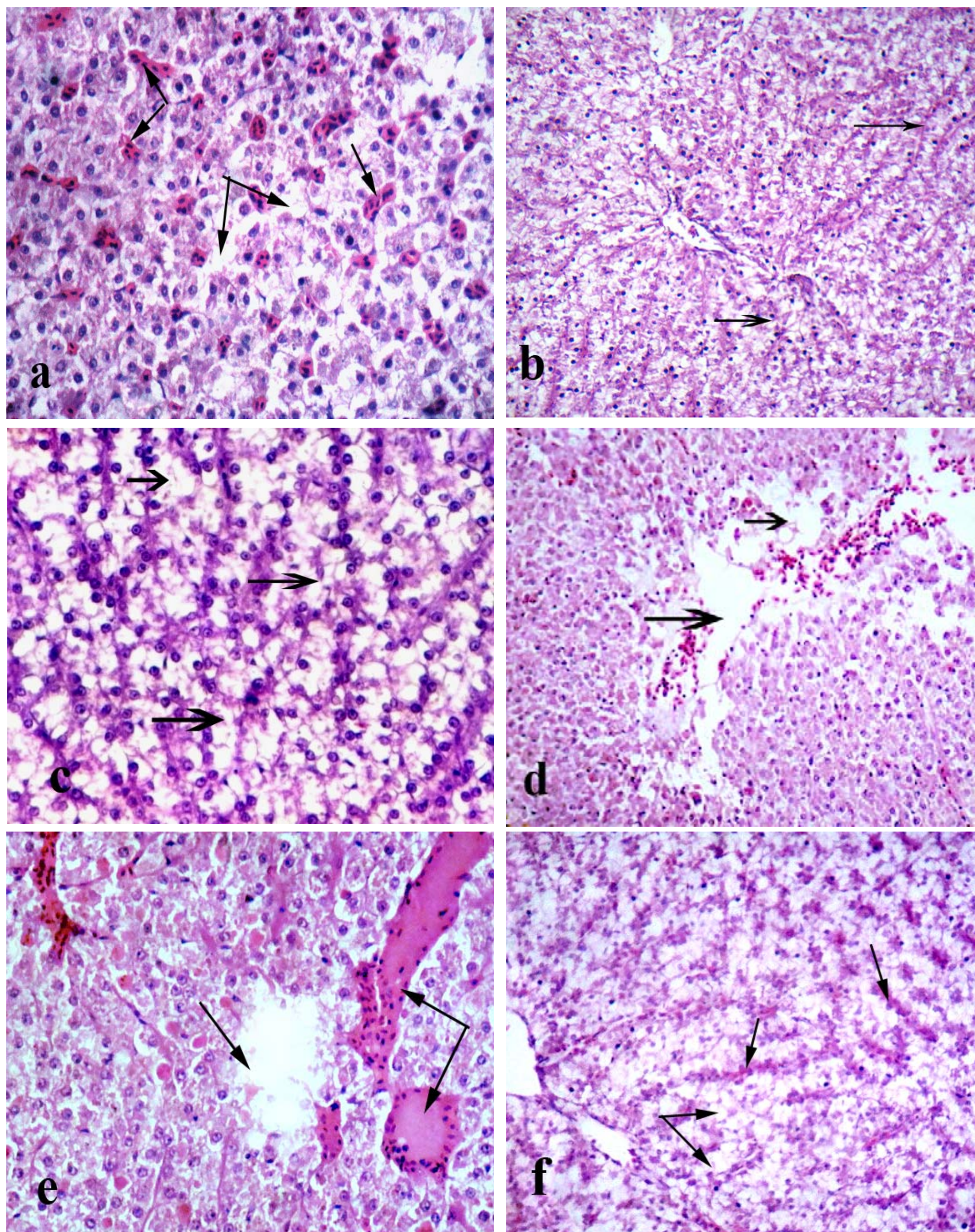

Figure 5: Histopathological changes in liver of *O niloticus* injected with antioxidants with or without AFB_1 (stained with H&E). (a); fish injected with GSH-EH (5mg/kg B.W., T_5) showing slight hemorrhage between hepatocytes associated with slight degeneration vacuoles in hepatocytes (x300). (b); fish injected with GSH-EH (10mg/kg B.W., T_6) showing, congestion in blood sinusoids with pycnotic nuclei in the hepatocytes (x200). (c); T_7 showing vacuolar degeneration in the hepatocytes (x400). (d); T_8 showing focal areas of necrosis between the hepatocytes(x200). (e); T_9 showing haemolysis and necrosis between hepatocytes(x300). (f); T_{10} showing congestion in blood sinusoids and vacuolar degeneration in the hepatocytes (x250).

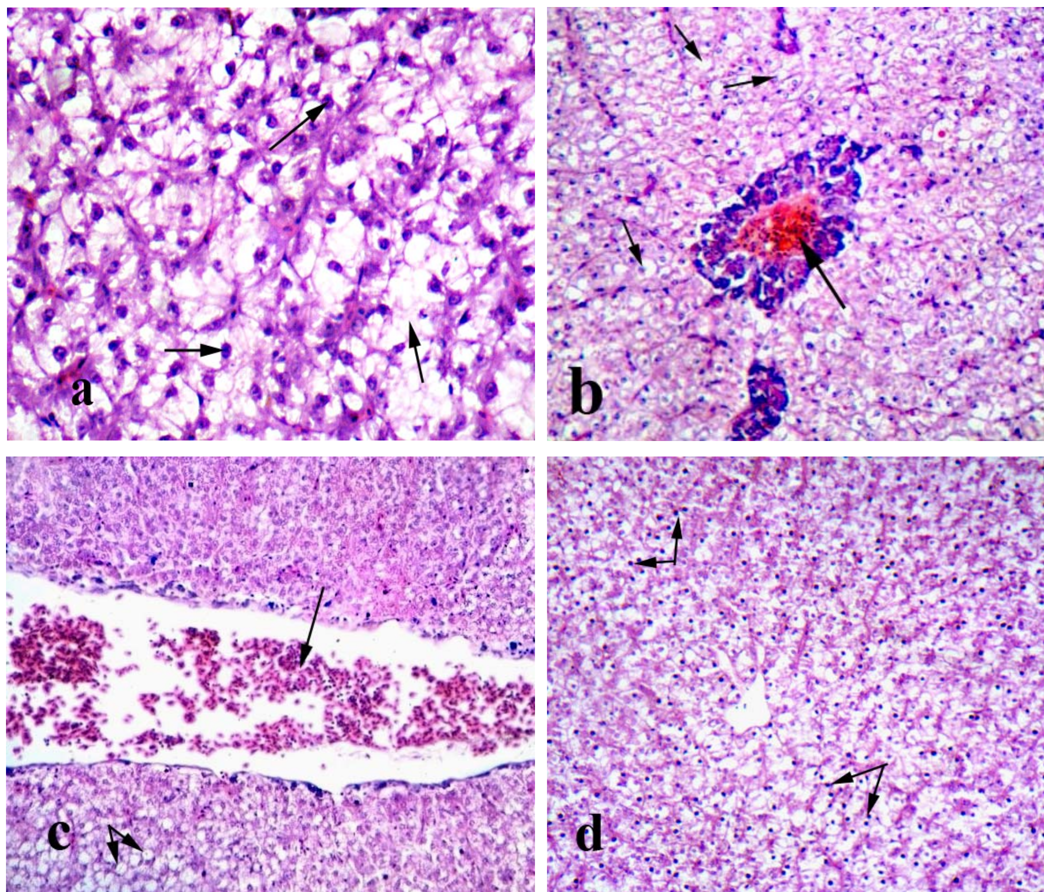

Figure 6: Histopathological changes in liver of *O niloticus* injected with either antioxidant (after aflatoxisosis) (stained with H&E). (a); T_{11} showing disappearance of hepatocyte wall and karyolitic necrosis (x250). (b); T_{12} showing pyicnosis besides congestion in portal blood vessels(x250). (c); T_{13} showing severe hemorrhage in blood vessels and degeneration vacuoles in hepatocytes (x200). (d); showing pycnosis in most of the hepatocytes (x250).

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فاعلية مضادات الأكسدة الكيميائية (الجلوتاثيون – محفز الجلوتاثيون) كمضادات للتسمم الأفلاتوكسيني في أسماك البلطي النيلي

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أجريت هذه الدراسة بهدف تقدير فاعلية كلا من الجلوتاثيون(GSH) ومحفز الجلوتاثيون (GSH-EH) علي مواجهه التسمم الأفلاتوكسيني في أسماك البلطي النيلي وذلك بإستخدام 3 تركيزات من كل منهما (0-5- 10مجم /كجم وزن جسم) كجرعة وأحدة عن طريق الحقن في الغشاء البريتوني للأسماك، سواء في صورة معاملة أولية (قبل الحقن بالأفلاتوكسين ب1) أو معاملة ثانوية (بعد الحقن بالأفلاتوكسين ب1). الأفلاتوكسين استخدم بتركيز 9 مجم /كجم وزن جسم عن طريق الحقن في البريتوني أيضا، وذلك بعد إذابته في داي ميثيل سلفوكسيد 25%، بينما مضادات الأكسدة تم إذابتها كل علي حده في ماء مقطر وذلك قبل الحقن مباشرة.

استخدم في هذه التجربة 224 من إصبعيات البلطي النيلي، قُسّمت إلي14 معاملة (T_1-T_{14}) , المعاملة رقم 1 كانت تمثل مجموعة المقارنة (الكنترول السالب) , المعاملات من T_2-T_6 مجاميع الكنترول لكل من الأفلاتوكسين, الجلوتاثيون 5 مجم , الجلوتاثيون 10مجم , محفز الجلوتاثيون 5 مجم, محفز الجلوتاثيون 10مجم /كجم وزن جسم علي التوالي. المعاملات من T_7-T_{10} كانت محقونة عند بداية التجربة بكل من الجلوتاثيون ومحفز الجلوتاثيون (بتركيزات 5 و 10 مجم /كجم لكل منهما) كمعاملة أولية قبل الحقن بالأفلاتوكسين الذي يُحقن به نفس المعاملات (7-10) بعد اليوم الثاني من بداية التجربة. بينما المعاملات الأخيرة (11-14) كانت تُحقن أولاً بالأفلاتوكسين عند بداية التجربة، وفي اليوم الثاني كانت تُحقن بكل من مضادات الأكسدة بنفس التركيزات السابقة كمعاملة ثانوية. في نهاية التجربة تم أخذ عينات دم وعضلات وكبد من كل معاملة وذلك لتقدير نشاط الجلوتاثيون في الدم، وتقدير المتبقي من الأفلاتوكسين ب1 في عضلات الأسماك، وتقدير نواتج الميتابولزم للتوكسين في كبد الأسماك عن طريق جهازGC-MS (الكروماتوجراف الغازى / طيف الكتلة)، وكذلك دراسة التغيرات النسيجية في الكبد، وبالتالي تقدير دور مضادات الأكسدة في مواجهة التأثيرات المرضية للتسمم الأفلاتوكسيني لأسماك البلطي النيلي. ولقد أظهرت نتائج قياس الجلوتاثيون في الدم بصفة عامة أن الأفلاتوكسين يؤدي إلي خفض قيم الجلوتاثيون سواء بدون (T_2) أو مع مضادات الأكسدة، بينما المعاملات (T_7-T_{10}) عكست انخفاض في قيم جلوتاثيون دم الأسماك مقارنة بقيم المعاملات الثانوية الحقن بالجلوتاثيون (T_{11}-T_{14}). كما أظهرت النتائج أن كل من مضادات الأكسدة المستخدمة أدت إلي عدم ظهور أي متبقيات للأفلاتوكسين ب1 في عضلات الأسماك في حالة استخدامها كمعاملة ثانوية (T_{11}-T_{14}) بعد الحقن بالأفلاتوكسين فقط. كما أظهر التحليل الكروموتوجرافي

الغازى وطيف الكتلة لمستخلصات الكبد للأسماك المعاملة بالأفلاتوكسين سواء مع أو بدون مضادات الأكسدة المختبرة إلي عدم ظهور أي نواتج ميتابولزمية للأفلاتوكسن ب1 في الكبد. أما بالنسبة للتغيرات النسيجية في كبد أسماك المعاملات المختبرة فقد أدي الأفلاتوكسين بمفرده إلي إحداث تغيرات أكثر شدة من التي يسببها الأفلاتوكسين ب1 مع كل من الجلوتاثيون أو محفز الجلوتاثيون، وقد شملت التغيرات النسيجية في الكبد احتقان ونزف شديد بالإضافة لموت الخلايا الكبدية، وكذلك اتساع و تمدد في الأوعية الدموية في الأسماك المصابة بالتسمم الأفلاتوكسيني .