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Metabolomic Analyses, Toxicity Biomarkers and Histopathological Changes in the Liver of Nile Tilapia Exposed to Diazinon Toxicity

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Abstract

This study was undertaken to screen for some biomarkers of toxicity in the liver of Nile tilapia fish during subacute Diazinon toxicity (0.28 mgL-1 for 25 days) by using Targeted metabolomics analyses and quantitatively measure 17 amino acids, and also to monitor antioxidant status of liver (glutathione peroxidase, catalase, superoxide dismutase and malondialdehyde). There were significant increases in branched chain amino acids valine, leucine and isoleucine (P>0.01, P>0.05 and P>0.01) respectively. There was a significant increase in phenylalanine (an aromatic amino acid) P>0.05, a significant increase in lipid peroxidation (malondialdehyde P>0.001), and significant decreases in the activity of antioxidant enzymes (SOD, CAT, GSH-px) with p values (P>0.01, P>0.01, and P> 0.001) respectively. Histopathological examination showed diffuse hepatocellular necrosis with multifocal granuloma and massive hepatocellular vacuolation with congested sinusoids. It can be concluded that subacute toxicity of DZN in Nile tilapia is involved in proliferation and growth of tumor cells and negatively affects the antioxidant status of the liver.

KEYWORDS
Antioxidant, Biomarkers, Diazinon (DZN), HPLC, Metabolomics, Nile tilapia, subacute toxicity.

INTRODUCTION

The demand for seafood products is increasing and aquaculture production is steadily expanding year by year in order to provide enough nutrition to rapid growth population (Larsen and Roney, 2013).

Fish support consumers with protein of high quality and from their advantages that they have low cholesterol, low calories, sodium and saturated fats, on the other hand they have high quality essential minerals such as calcium (Ca), potassium (K), iodine (I), iron (Fe), zinc (Zn) and selenium (Se), and are rich in essential omega-3 fatty acids (Denton *et al*., 2010).

Tilapia species are one of the most widely cultured freshwater fish, the demand of tilapia is exponentially increasing in recent years especially in warm counties like Egypt, Middle East, Malaysia etc. (Amira, 2021). Nile tilapia fish rearing advantages in aquaculture are its price stability, marketability, and its high growth rate (Wang and Lu, 2015).

One of anthropogenic sources of pollution that is used in agriculture is pesticides, there is a strong relationship between agriculture activities and aquaculture as they are both relying in their cultivation on the drainage water (Gewaily *et al*., 2021).

A pesticide is a toxic substance or mixture of substances which is naturally or synthetically formulated to destroy or mitigate insects, weeds, rodents, fungi, or other harmful pests (Eldridge, 2008). Its usage has benefits in the agricultural sector, but their drainage into the aquatic ecosystems causes undesirable effects and threatens fish health (Dar *et al*., 2022).

Diazinon (DZN) is one of broad spectrum organophosphorus insecticides. It is flushed easily into the water and negatively affects fish health (Oruc, 2011). DZN stability can persists in water for many months, so it leads to dangerous effects on aquatic organisms' tissues due to its accumulation (Al-Ghanim, 2014).

In recent times, researchers are focusing on the dangerous effects and risk factors of xenobiotics on the ecosystems (Abdelkhalek *et al*., 2015).

Metabolomics is powerful method of analysis for dissecting mechanisms details in toxicity and is capable to discover some metabolic pathways and shedding the light on changes in different mechanism of action involved in the pesticide toxicity (Van Ravenzwaay *et al*., 2013).

A number of toxicological studies on pesticide have discovered different biomarkers by metabolomics analyses through quantitative measurement of the vigorous change in metabolites of low molecular weight such as amino acids, glucose, energy products (Yang *et al*., 2011; Du *et al*., 2013).

There are two types of metabolomics: Targeted and untargeted metabolomics. Targeted metabolomics are concentrating on a specific metabolite or metabolites (targeted) is associated with hypothesis-driven studies and involves the optimization of HPLC and MS conditions (as MS/MS transitions, retention times) using pure standards. The main advantages of targeted metabolomics are specificity, quantitative reproducibility, and a relatively high throughput. While Untargeted metabolomics are able to provide

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a profile with the largest probable number of metabolites and compounds (i.e., from hundreds to thousands) and therefore they have the capacity to reveal previously unexplored biochemical pathways (Wehrens and Salek , 2019).

In sight of the previous facts, this study aimed at screening for some biomarkers of toxicity in the liver of Nile tilapia fish exposed to subacute Diazinon toxicity (0.28 mgL^{-1} for 25 days) via targeted metabolomics analyses. Moreover, the antioxidant status at the liver and the histopathological changed induced by Diazinon were also examined.

MATERIALS AND METHODS

Chemicals

Diazinon (DZN) 60% was obtained from Al-Nasr pharmaceutical company (Cairo, Egypt). Before administration, DZN was diluted with deionized water. Amino acid reference standard which used in HPLC were purchased from Sigma-Aldrich company (Darmstadt, Germany). Formalin was purchased from Al-Gomhouria company (Mansoura, Egypt), the rest of necessary kits used in completing the procedures of analysis were obtained from Biodiagnostic, Egypt.

Ethics statements

Dealing with fish during the experiment and after it was performed according to Animal Ethics Committee Guidelines, of Mansoura University.

Grouping of Fish and experimental procedure

Sixty male Nile tilapia fish were purchased from a private farm in Kafr El Sheikh Governorate, Egypt. Its body weight was about 30±8 g. The fish were transported alive; in containers contain one third water and two thirds oxygen, to the Animal Health Research Institute Mansoura province Lab. where the experiment was held. Fish were equally distributed in 6 completely set aquaria into two groups (30 Fish/group) each group of three replicates (ten fish per replicate). The acclimation period was two weeks. Water parameters were adjusted everyday according to Nile tilapia (*O. niloticus*) needs, the temperature, dissolved oxygen, and pH of water 22±2°C, 6.5±1.5 mg/L ,7.5-7.8 respectively. The aquaria were kept under constant aeration by using air pumps and light to dark hour's were12/12. The diet of the fish in all groups was a basal diet. The basal diet was prepared every week and let completely dry then preserved at 7°C for daily use. The nutritional parameters of basal diet were adjusted to contain 31.78% crude protein, 7.15% fat and 3000 KCAL. The first group (three replicates) was the control group, and the second group (three replicates) was intoxicated with DZN dose 0.28 mg/L for 20 days to induce subacute toxicity.

Sample collection

Before collection of samples, 100 mg/L of clove oil was used to anesthetize fish. Liver tissues were collected, washed with normal saline and dissected into three parts, the first part was preserved in 15 % paraformaldehyde solution then fixed in paraffin wax and sliced into 5 µm thickness until stained with hematoxylin and eosin. The stained slides were examined by light microscopy. The second part of liver tissue was homogenized by electric homogenizer in phosphate buffer saline then centrifuged in cooling centrifuge at 3000 rpm for 20 min. the upper fat layer was removed from supernatant then the supernatant was collected and preserved at freezer -80°C until determination of antioxidant enzymes activity and malondialdehyde concentration. The third part of liver tissues were stored at -80°C until be used in metabolomics analysis.

Liver antioxidant assay

The activity of superoxide dismutase was measured according to Nishikimi *et al.* (1972), catalase activity according to Aebi (1984) and glutathione peroxidase activity was according to the method of Paglia and Valentine (1967). Finally, the oxidative stress marker (MDA) was estimated according to the technique of Draper and Hadley (1990).

Procedures of amino acid extraction

For extraction of amino acids from liver tissue 50-200 mg of the liver sample was mixed with 5 mL H_2O and 5 mL of HCl, final concentration of HCl is 6 M, heated at 100°C/ 24 h then filtered. After filtration, it was dried, resuspended in 0.1 M HCl and injected after diluted it 10 times into HPLC according to Campanella *et al.* (2002) and Laurens *et al.* (2012).

HPLC conditions

The prepared liver Samples were injected into HPLC then analysis was carried out according to Jajić *et al*., (2013), by 1260 series Agilent. The separation was performed with SUPLCO Discovery® BIO Wide C18 column (4.6 mm x 250 mm i.d., 5 µm). The component of mobile phase are sodium phosphate dibasic and sodium borate buffer, with pH 8.2 and ACN:MeOH:H_2O 45:45:10 and the flow rate was 1.5 $ml.min^{-1}$. Further adjusting Fluorescence detector from 0 to 28 min at 340/450 nm (Excitation/Emission) and from 28 to 40 min at 266/305 (Excitation/Emission). The column temperature was maintained at 40°C.

Statistical analysis

Quantitative data were analyzed with student's t-test by using GraphPad prism (GraphPad Software, Incorporated, La Jolla, CA, USA) and were presented as mean±standard error of mean. P values of ≤0.05 were statistically significant (Scendecor and Cochran, 1969).

RESULTS

Liver antioxidant and oxidative stress markers

The oxidative stress marker (Malondialdehyde) and antioxidant enzyme activity in DZN intoxicated fish group compared with control group was illustrated in Table 1. A significant decline in hepatic SOD activity, catalase, and glutathione peroxidase activities ($P<0.01$, $P<0.01$, and $P<0.001$) respectively. On the other hand, there was a significant increase in the concentration of MDA level ($P<0.001$) in the diazinon intoxicated group.

Liver metabolomic analyses and amino acids concentration

As shown in Table 2, there was a significant elevation ($p < 0.05$) in hepatic aspartate (ASP), glutamate (GLU), serine, Histidine, Threonine, lysine and proline concentration in DZN intoxicated fish in comparison with control group and also a significant elevation of branched chain amino acids (Isoleucine, Leucine and

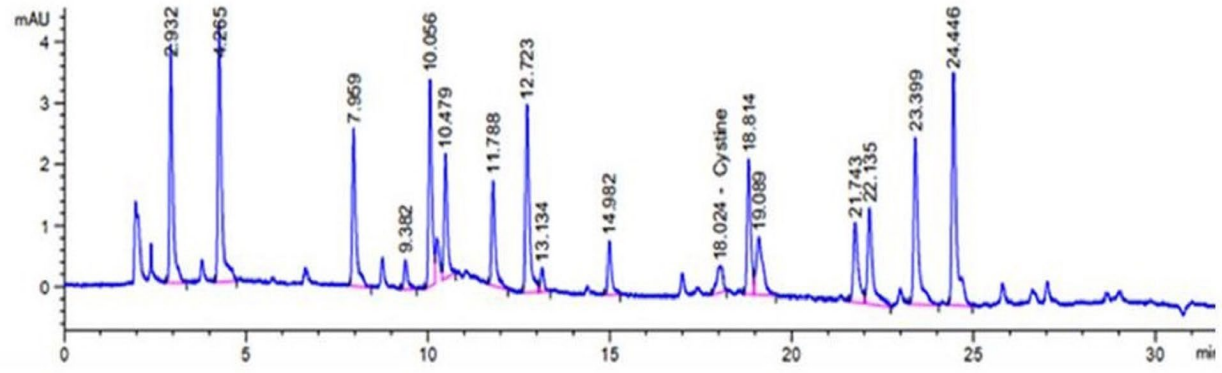

Fig. 1.A chromatogram of extracted amino acids with HPLC.

valine) with p values (p <0.01, p < 0.05 and p<0.01) respectively. There was a significant increase (p < 0.05) in Phenylalanine concentration (an aromatic amino acid). Also there was a significant elevation in cysteine (sulfur containing amino acid) (P> 0.001). A chromatogram from extracted amino acids was shown in Figure 1.

Table 1. Liver antioxidants and oxidative stress markers.

	Control	Diazinon	p value
MDA level (nmol/mg protein)	28±2.6	96.0+5.5	***
CAT activity (U/mg protein)	12.1±1.1	7.0+0.5	**
GSH-px (U/mg protein)	4.28±0.38	2.23±0.21	***
SOD (U/mg protein)	14.75±1.2	7.62±0.63	**

**p value <0.01, *** p<0.001

Table 2. Amino acids concentrations.

Amino acids	Control	Diazinon	p value
ASP	6.6±0.51	9.5±0.72	*
GLU	11.08±0.5	14.6±0.9	*
Serine	3.64±0.22	5.3±0.42	*
Histidine	1.96±0.05	2.69±0.18	*
Glycine	4.07±0.2	5.24±0.42	P< 0.05
Threonine	3.76±0.23	5.56±0.35	*
Arginine	4.59±0.28	6.62±0.6	P< 0.05
Alanine	4.05 ±0.32	5.64±0.41	P< 0.05
Tyrosine	2.51± 0.21	3.78±0.35	P< 0.05
Cystine	0.94±0.05	2.7 ±0.13	***
Valine	3.74±0.23	5.78 ± 0.32	**
Methionine	1.25 ±0.07	1.36±.08	P< 0.05
Phenylalanine	3.48±0.19	5.59 ±0.42	*
Isoleucine	2.82 ±0.18	4.87 ±0.37	** p<0.01
Leucine	5.56 ±0.42	8.71 ±0.61	* p < 0.05
Lysine	6.08 ±0.52	8.68 ±0.63	* p < 0.05
Proline	3.70 ±0.24	5.38±0.45	* p < 0.05

**p value <0.01, *** p<0.001

Histopathological examination

Figure 2 shows the liver of control group with normal histological appearance of hepatocytes (h) and hepatopancrease (he), H,E, 100X and 400X, while the liver of DZN intoxicated group was shown in Figures (3and4) showing diffuse, massive hepatocellular vacuolation (v) with congested sinusoids (thin arrows), H&E, 400X in Figure 3, and diffuse hepatocellular necrosis (thick arrow) with multifocal granuloma formation (thin arrows), H&E, 100X, inset, necrotic hepatocytes with focal granuloma (thin arrow), H&E, 400X in Figure 4.

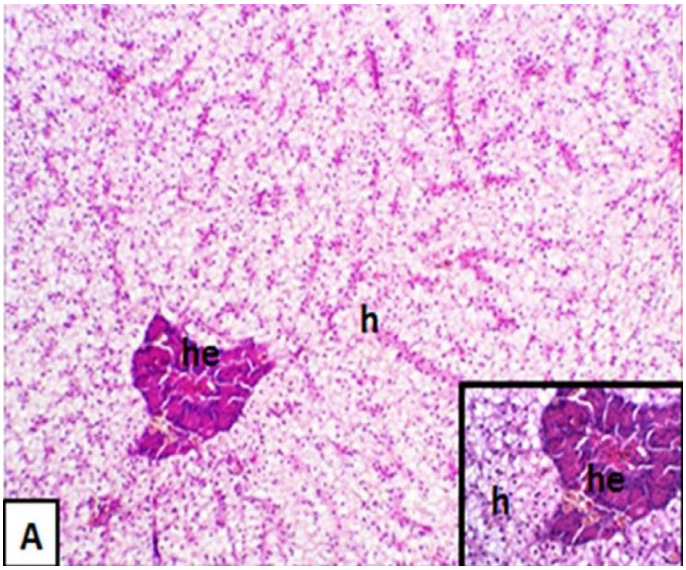

Fig. 2. control liver showing normal histological appearance of hepatocytes (h) and hepatopancrease (he), H,E, 100X and 400X.

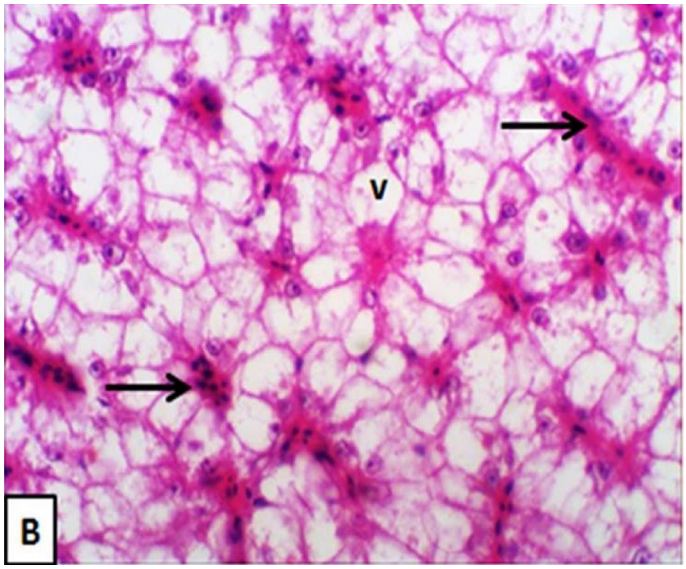

Fig. 3. Diazinon exposed liver showing diffuse, massive hepatocellular vacuolation (v) with congested sinusoids (thin arrows), H&E, 400X.

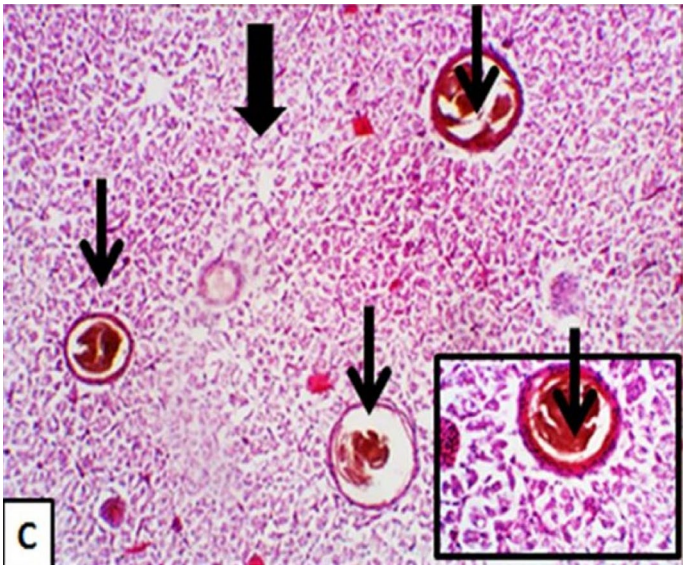

Fig. 4. Diazinon exposed liver showing diffuse hepatocellular necrosis (thick arrow) with multifocal granuloma formation (thin arrows), H&E, 100X, inset, necrotic hepatocytes with focal granuloma (thin arrow), H&E, 400X.

DISCUSSION

Oxidative stress is resulting from imbalances between antioxidants and prooxidants equilibrium status which lead to free radicals amplification that are complemented with the number of progressive pathological conditions including cancer (Lupoli *et al*., 2018). Diazinon has the ability to perform oxidative damages that lead to alteration in various metabolic pathways as redox enzymes and also affect the electron permeability of the mitochondria (Abdel-Daim, 2016).

The mitochondrial leakage leads to elevation in the production of free radicals which affect the hepatocyte permeability (Srivastava *et al*., 2004) and this may explains the diffuse and massive hepatocellular vacuolation in this study.

The excessive oxidative stress in the tissue causes depression of liver antioxidant assay. In this study liver antioxidant enzymes (SOD, CAT and GSH-px) were significantly decreased in DZN intoxicated group, inhibition of catalase enzyme also was studied by Gultekin *et al*. (2000) and indicated that catalase activity was decreased by organophosphorus pesticide, which also inhibits the creation of superoxide radical leading to an inhibition in the activity of glutathione peroxidase enzyme. In this study, there was a significant increase in malondialdehyde levels in diazinon intoxicated groups, and this is a distinctive response during toxicity (Akturk *et al*., 2006).

Branched chain amino acids and aromatic amino acids are crucial precursors of synthesis of protein and production of energy (Sears *et al*., 2009; Somashekar *et al*., 2011) this may explain the significant increase in branched chain amino acids BCAAs (leucine, valine and isoleucine) and phenylalanine (an aromatic amino acid) in this study. The elevated levels of BCAAs and aromatic amino acids in hepatic tissues of diazinon treated fish might leads to a risk of many lesions that include the onset of cancer because BCAAs contribute to growth and proliferation of tumor cells during anaplerosis (Dwivedi *et al*., 2012) which may explain diffuse hepatocellular necrosis with multifocal granuloma in this study.

In this study there was a marked elevation of glutamate concentration which observed in Diazinon treated fish. This elevation of glutamate was a proof of anoxia and hypoxia (Bak *et al*., 2006). Hypoxia and subsequent deficiency in energy may cause failure of membrane ion pump leading to the intracellular accumulation of sodium, calcium and water causing cell edema (Perlman, 2006) also may explain the edema and vacuolation in hepatocyte in our study.

CONCLUSION

The obtained findings illustrate the negative toxic effect of diazinon on fish health by screening the metabolism of liver of Nile tilapia fish with metabolomics analyses which is a highly effective approach to explain the toxicological effects and the understanding of mechanisms of pesticides with discovering toxicity biomarkers. And can conclude that DZN has a role in hepatic tumor and also able to induce Oxidative damage the liver of Nile tilapia as well as lipid peroxidation.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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