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Evaluation of some tilapia species as biomarkers for pesticides accumulation levels at Lake Edku, Egypt.

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ABSTRACT

Three tilapia species; *Oreochromis niloticus, Oreochromis aureus and Tilapia zillii* were evaluated as biomarkers for five different organophosphorus pesticides (Diazenon, Lindan, Parathion, Malathion and Chlorpyrifos) at Lake Edku, Egypt. *O. niloticus* reflected highest accumulated pesticides comparing with *T. zillii*, which reflected lowest accumulation levels of Diazenon, Lindan, Parathion, Malathion and Chlorpyrifos respectively. *O. niloticus, O. aureus* and *T. zillii* showed varied antioxidant activity patterns corresponding with pesticide accumulation levels. *O. niloticus* was superior for Superoxide dismutase activity comparing with *O.aureus* and *T. zillii*, which reflected lower level respectively. Moreover, *T. zillii* reflected the lowest Catalase activity comparing with *O. aureus* that showed the highest activity. *O. niloticus* showed high glutathione Peroxidase activity comparing with *O. aureus* and *T. zillii*. Glutathione reductase reflected distinguishable variation among *O. niloticus, O. aureus,* and *T. zillii*. Glutathione - S-transferase enzyme activity was decreased for *T. zillii* comparing with O.*niloticus* and *O.aureus*. As protein fraction, Glutathione S-transferase (GST) was expressed as one single band with different protein content. Glutathione S-transferase (GST) gene sequence of *T. zillii* reflected the highest genetic similarity (92.81%) with GST reference sequence. The lowest genetic similarity for GST gene sequence remarked (75.29%) *O. niloticus. O. aureus* showed moderate genetic similarity (87.11%) comparing with the reference gene sequence. It could be concluded that, significant correlation was detected among different organophosphorus pesticides accumulation and activity levels. Protein content and sequencing of many antioxidant enzymes indicated promising potential of employing three Tilapia species *O. niloticus, O. aureus* and *T. zillii* as biomarkers.

INTRODUCTION

Tilapias consider the main source of fish consumers in Africa and the Middle East. 40–50 nominal species compose Cichlidae family have been introduced to tropical and subtropical countries around the world to increase protein content (Bo-Young *et al*. 2005). In Egypt, fish could be considered an alternative food source to capture fisheries to cover the growing demand for animal protein resources to feed

Egypt's population. Thus, Production has increased from about 92.5 thousand tonnes in 1971 to more than 1097,544 tin 2013 (Soliman and Yacout 2016).

Recently, extent of the use of pesticides and their mode of application including their abuse especially in agriculture has been of much concern to environmental scientists. Wide spectrums of pesticides have been applied in Egypt for agricultural and public health fields. Mixing River Nile or lakes with OPs pesticides cause to discharge drainage water of pesticides treated land. Among insecticides classes, vertebrates consider the most sensitive for OPs pesticides toxicity (Chambers *et al.* 2001). In Egypt, many severe symptoms especially liver and kidney diseases have a dramatic increase due to unreliable use of pesticides during the last 20 years in Egypt. Furthermore, Egypt considers the fourth largest pesticides importer among developing countries (Yamashita *et al.* 2000). Pesticides reach aquatic ecosystem via many ways like direct application, missing spray drift, also leaking from manufacturer factories. Contamination of water sources which containing fish and other aquatic organisms consider the major concern in the light of accumulation Pesticides residuals in of aquatic organism tissues and with time (Jiries *et al*., 2002). Edku Lake considers the focus of attention for environmental biologist as a result of highly concentrations of heavy metals including Iron, Zinc, Copper, Manganese, Cadmium and Lead (Fe, Zn, Cu, Mn, Cd and Pb) (Saeed *et al.,* 2008, 2011 and 2013).

Oxidative process plays a key role in the metabolic responses to many xenobiotics, such as chemicals, pesticides and heavy metal which responsible for inducing Reactive Oxygen Species (ROS) and alterations in the antioxidant system (Nishida 2011). Antioxidant enzymes over expression, DNA macro and micro damages, protein oxidative and lipid peroxidation products consider basic symptoms for to vigor exposer to pesticides as oxidative stress (Li *et al.,* 2007). Super oxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione (GSH), glutathione reductase (GR) and lipid peroxidase (LPO) classified as major antioxidant enzymes (Price *et al*., 1990).

Glutathione S-transferases (EC 2.5.1.18, GSTs) belong to super family of multi-functional dimeric enzymes with roles in Phase-II detoxification and expressed in almost every species (Sheehan *et al*. 2001). Enzymic glutathione conjugation, glutathione-dependent peroxidase activity or isomerization reactions are main mechanisms for neutralize a broad range of xenobiotics and endogenous metabolic via Glutathione S-transferases (Hayes *et al.* 2005; Bamidele *et al.* 2012). Also, GSTs catalyze the conjugation of endogenous substrates, including cholesterol, prostaglandins and leukotriene as an addition detoxification mechanism (Sheehan *et al*. 2001). According to amino acid sequences similarities, Alpha, Mu, Pi, Sigma, Theta, Omega, and Zeta are main classes of mammalian cytosolic GSTs (Mannervik *et al.* 2005). Fish GST isoforms are expressed specifically in almost all the tissues (Rabahi *et al*., 1999; Thyagaraju *et al*., 2005). In some fish species, gender differences play an important role for detoxification via GST activities as directly reflect the metabolic disturbances (Carvalho-Neta and Abreu-Silva, 2013). Additionally, using Tilapia as biomarker for pesticides contamination was mentioned in many studies (Ibrahim *et al.,* 2014). They indicated that, exposure Nile Tilapia (*Oreochromis Niloticus)* to 0.3and0.8 mg/l of chlorpyrifos for 24h induced significant decreasing in metabolites parameters such as total lipid, AChE, T3, Na + and Cl-. By contrary, cholesterol, cortisol, T4 andK + revealed marked elevation during the acute period when compared to control value whereas total protein was fluctuated during acute exposure.

The purpose of this study was to evaluate *O.niloticus, O.aureus and T.zillii* capability as biomarkers for five organophosphorus pesticides (Diazenon, Lindan, Parathion, Malathion and Chlorpyrifos) at Lake Edku, Egypt. To achieve this goal, antioxidant enzyme activities and Glutathione S-transferase (GST) on gene sequence and protein expression levels were employed as effective evaluation tool.

MATERIALS AND METHODS

Fish Samples:

In this investigation, three Tilapia species *O.niloticus, O.aureus and T.zillii* were represented with one hundred individuals and collected with nets from Edku Lake (E"2'18°30 N"52'19°31) from summer 201٧ to spring 2018 (Figure 1). All samples were collected and transported immediately to the laboratory in an ice box with ice and stored at 4 °C in a refrigerator for two days before extensive analysis.

Fig. 1: Location of the Edku Lake.

Organophosphorus pesticides standards and reagents

Pesticide standards of Lindan, Diazenon, Parathion, Malathion and Chlorpyrifos were purchased from Sigma (Poole, UK). Firstly, the standard pesticide solution was prepared individually by dissolving 10 mg of each compound in 10 mL hexane and stored in amber bottles. Secondly, concentrations of 100 mg/ L of each individual standard stock solution were mixed. Through diluting 100 mg/ L of the mixed standard solution, series of calibration standards were prepared to produce a final concentration of 0.1, 0.2, 0.5, 1.0, 2.0 mg/ L in hexane and stored at 4 °C till usage. Acetone, n-hexane, methylene chloride, toluene, and acetonitrile were applied as solvents were categorized as (HPLC) grade and supplied by Sigma, USA.

Sample extraction

The extraction and clean-up technique employed in this work was according to Chen et al. (2009). Fifteen grams of each fish muscle samples vortexes for 1 min with 3.0 mL of double distilled water. Then, 20 mL of acetonitrile was added as an extraction solvent. Additionally, 5 g sodium chloride was added to the mixture and vortexes for another 2 min and centrifuged for 5 min at 4000 rpm. To precipitated total lipids as pale yellow, 10 mL of the extraction solution was stored in 100 mL freezed flask at -24 °C for 20 min. then, precipitated lipid was dissolved in 10 mL of acetonitrile and finally concentrated to 1 mL by rotary evaporation to follow

SPE procedure. NH2 cartridge supplemented with anhydrous sodium sulfate was applied for sample Clean-up according to Yahia and ELsharkawy (2013).

GC\ MS analysis for Malathion

Different organophosphorus pesticides were detected for *Oreochromis niloticus* muscles samples through Agilent 7890 instrument equipped with 5975 insertion source mass detection system (Agilent Technologies, USA). Ten grams of each fish muscles were extracted and cleaned up according to Chen *et al*. (2009). After that and before sample application, anhydrous sodium sulfate was placed on top of NH2 cartridge according to Yahia and ELSharawy (2013).

Antioxidant enzyme assays

Six Antioxidant enzymes were evaluated as Malathion indicators. One gram of *Oreochromis niloticus* muscles which collected from different five locations from Edku Lake was homogenized through Kinematica Polytron™ PT2100 Benchtop homogenizer for 5 min in ice cold 0.1M Tris-HCl buffer solution. Then, centrifuges at 6000 rpm for 20 min. finally, supernatant was removed and stored for -20°C for antioxidant enzyme assays. Superoxide dismutase, Catalase, glutathione peroxidase, Glutathione reductase, Glutathione a were used as different pesticides biomarker according to Kakkar et al. (1984), Maehly and Chance (1954), Lawrence and Burk (1976), David and Richard (1983), Patterson and Lazarow (1995) respectively.

Purification Glutathione S-transferases (GSTs) using affinity chromatography

GST enzyme was coupled to Epoxy-activated Sepharose 6B according to Simons and Jagt (1977). 5 grams of each *O. niloticus*, *O. aureus*, and *T. zillii* muscles were homogenized in 50% (w/v) 25 mMTris-HCl buffer, pH 8.0 containing 1 mM EDTA and 1 mM DTT. Then, homogenates were centrifuges at 10,000 xg for 15 min. crude extracts was obtained via filtration and kept at -20°C to purify GST using affinity chromatography. 15 mL of GSH-Sepharose matrix was mixed for 30 min at 4°C with shaking separately with Crude extracts. Then, packed to a column (15 × 1 cm i.d.) and eluted with 50 mMTris-HCl buffer, pH 8.0 containing 10 mM GSH at a flow rate of 1 mL/min.

GST electrophoretic patterns

Purified Glutathione S-transferase (GST) from *O. niloticus*, *O. aureus* and *T. zillii* were examined by 7% polyacrylamide gel electrophoresis (PAGE) followed by staining for protein using Coomassie brilliant blue according to Laemmli (1970). A single band corresponding to a molecular mass of 27.5 kDa was detected and remarked GST.

Glutathione S-transferase gene amplification and sequencing

Genetic variation for GST DNA sequences was applied as bioindicator for pesticides accuilation. Thus, total RNA were extracted for *O. niloticus*, *O. aureus* and *T. zillii* muscles which exposer to different organophosphorus pesticides through GeneJET RNA Purification Kit, (#K0731, Thermo Scientific). Nanodrop ND-1000 spectrophotometer (Nano Drop Products, Thermo Fisher Scientific, Schwerte, Germany) was applied to check the quantity and purity of extracted RNA. High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA) was applied to convert purified RNA to complementary DNA from purified. Complementary DNA (cDNA) products were stored at -20°C for PCR. GST specific primer For: ATG TCA AGA CTG AAG CTA TAC TTT G and Rev: TTA AAT CTT GGA TGC CAG GAA GTG Rhee *et al.*, (2007) were applied to amplify Glutathione S-transferase gene. The PCR products with 480 bp were determined with1.5% agarose gel electrophoresis. Gel docum-

entation system (Geldoc-it, UVP, England), was applied for data analysis using Totallab analysis software, ww.totallab.com, (Ver.1.0.1). Specific GST gene fragments were eluted from agarose gel through E.Z.N.A.® Gel Extraction Kit (omega BioTEK, USA). Sequence analysis was employed using the ABI PRISM® 3100 Genetic Analyzer (Micron-Corp. Korea). Aligned sequences were analyzed on NCBI website (http://www.ncbi.nlm.nih.gov/website) using BLAST to confirm their identity. Phylogenetic tree constructed based on Glutathione S-transferase gene sequence homology, and birds were computed by Pairwise Distance method using ClusteralW software analysis (www.ClusteralW.com).

Statistical Analysis

Our obtaining findings of antioxidant enzyme quantifications from five locations were statistically analyzed to evaluate significant differences by using independent t-test sample (SPSS version 7.0.1 copyright SPSS INC1997).

RESULTS

This investigation was carried out to evaluate employment *O.niloticus, O.aureus and T.zillii* as a biomarker for organophosphorus pesticides at Edku Lake, Egypt. Different accumulation levels for organophosphorus pesticides were detected for *O.niloticus, O.aureus and T.zillii.* As shown by Table (1) and Figure (2), for five organophosphorus pesticides, *O.niloticus* accumulated highest pesticides levels (2.81±0.25, 1.25±0.21, 7.15±0.85, 0.51±0.11 and 0.37±0.25) comparing with *T.zillii* which reflected lowest accumulation levels (1.98±0.45, 0.26±0.62, 4.55±0.55, 0.21±0.36 and 0.14±0.19) for Diazenon, Lindan, Parathion, Malathion and Chlorpyrifos respectively. Additionally, O.aureus showed moderate accumulation level with 2.14±0.74, 0.65±0.68, 5.25±0.280.35±0.17 and 0.28±0.65 for Diazenon, Lindan , Parathion , Malathion and Chlorpyrifos respectively.

Table 1: Average concentration (µg/Kg) of the different types of pesticide residues in the examined tilapia samples (n = 10 pooled samples with five fish each). In area

Tilapia species	Average concentration of Pesticides				
	Diazenon	Lindan	Parathion	Malathion	Chlorpyrifos
O. aureus	2.14±0.74	0.65±0.68	5.25±0.28	0.35±0.17	0.28±0.65
O. niloticus	2.81±0.25	1.25±0.21	7.15±0.85	0.51±0.11	0.37±0.25
T. zillii	1.98±0.45	0.26±0.62	4.55±0.55	0.21±0.36	0.14±0.19

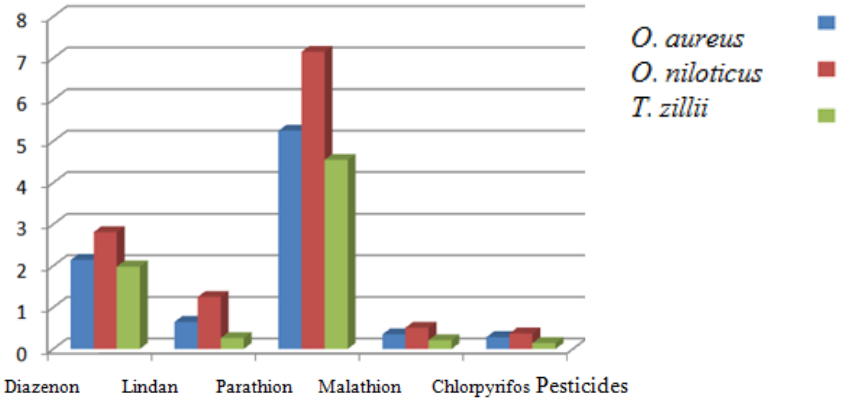


Fig. 2: Average concentration (µg/Kg) of five pesticide residues for, and Where:

Evaluation of antioxidant enzyme activities

As shown by Table (2), as a direct response for different accumulation levels of organophosphorus pesticides, antioxidant enzyme activities reflected significant variation among three Tilapia species. Generally, *O. niloticus* expressed highly significant antioxidant enzyme activities which consequential descendingly for *O. aureus* and *T. zillii* respectively. For Superoxide dismutase, *O. niloticus* was superior for activity with 151.6 ± 10.5 comparing with *O. aureus* and *T. zillii* which reflected 137.5 ± 6.8 and 86.6 ± 9.4 respectively. Also, T.zillii reflected lowest Catalase activity with 92.5± 58.6 comparing with *O. aureus* which showed the highest activity (191.5± 96.2). *O. niloticus* showed high glutathione Peroxidase activity (1.84±0.52) comparing with *O. aureus* and *T. zillii* which reflected 1.77±0.61 and 0.87±0.17 respectively. Glutathione reductase reflected distinguish variation among *O. niloticus*, *O. aureus* and T.zillii which showed 1.78± 0.12, 1.65± 0.08 and 1.12± 0.14. finally, significant dramatic decrease was detected for Glutathione -S-transferase enzyme activity for T.zillii (0.34±0.34) comparing with *O. niloticus* and *O. aureus* (0.81±0.08 and 0.75±0.14 respectively).

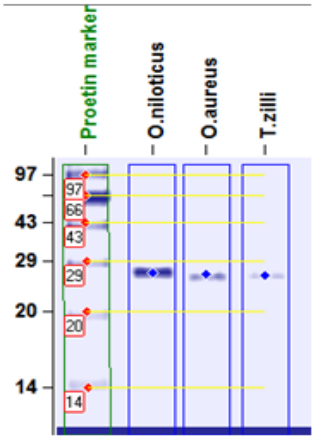
Table 2: Antioxidant enzyme activities (µM/min/g/ wet weight tissue) for tilapia species

Tilapia species	Superoxide dismutase	Catalase	glutathione Peroxidase	Glutathione reductase	Glutathione -S-transferase
O. aureus	137.5 ± 71	184.2± 22.5	1.77± 0.84	1.65± 0.07	0.75± 0.11
O. niloticus	151.6 ± 12.5	191.5± 39.2	1.84± 0.22	1.78± 0.23	0.81± 0.10
T. zillii	86.6 ± 3.5	92.5± 14.3	0.87± 0.17	1.12± 0.19	0.34± 0.24

Purification GST Polyacrylamide gel electrophoresis

Glutathione S-transferase (GST) of *O. niloticus, O. aureus and T. zillii* were purified by affinity chromatography. Then, fractionated via 7% polyacrlyamide gel electro-phoresis (PAGE) to detect and quantify protein content. Glutathione S-transferase (GST) was expressed as one single band with different protein content for *O. niloticus*, *O. aureus* and *T. zillii* as tested by 7% PAGE (Fig.3). *O. niloticus* expressed Glutathione S-transferase (GST) as 1.5 and 4.7 folds than *O. aureus and T. zillii* respectively.

(A)



(B)

O.niloticus (2)		O.aureus (3)		T.zilli (4)	
Lane %	MW	Lane %	MW	Lane %	MW
3.00	26.325	1.90	26.103	0.63	25.884

Fig. 3: Polyacrylamide gel electrophoresis for affinity purified GST of the three tilapia species (C)

GST Gene sequencing analysis

Comparing with reference GST gene sequence, *O. niloticus, O. aureus and T. zillii* GST gene amplification and sequencing were employed as molecular biomarker to evaluate direct different OP influence through comparing with reference GST gene (Oreochromis niloticus glutathione S-transferase (LOC100534425), mRNA NCBI Reference Sequence: NM_001279634.1). As shown by Figure (4), GST amplicons were obtained for *O. aureus, O. niloticus and T.zillii* with remarkable fragment length (~480bp). Then, GST gene were purified and sequenced for each species and different GST gene sequences identity % were recorded. *O. niloticus*, *T. zillii* and *O. aureus* GST gene sequences identified as Oreochromis niloticus glutathione S-transferase (LOC100534425), mRNA NCBI Reference Sequence: NM_001279634.1 with 89.89%, 100 % and 94.02 of identity % respectively (Figure 5A, B and C).

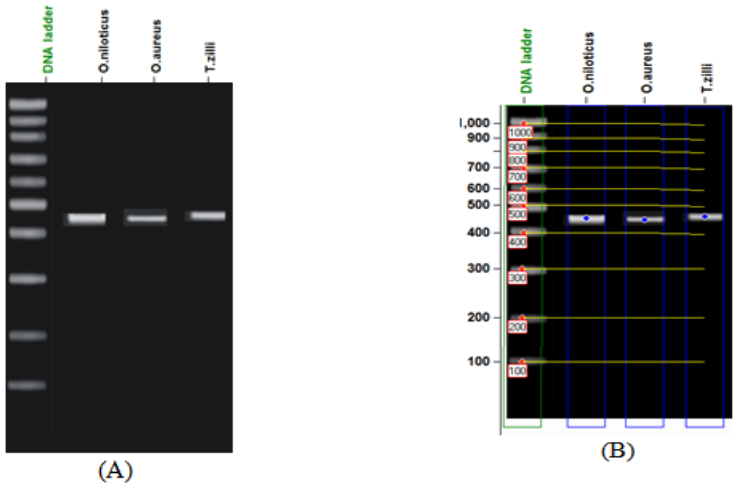


Fig. 4: Glutathione S-transferases (GST) amplicons (A) and computerized fragments length calculation (B) for *O.aureus, O.niloticus and T.zillii.*

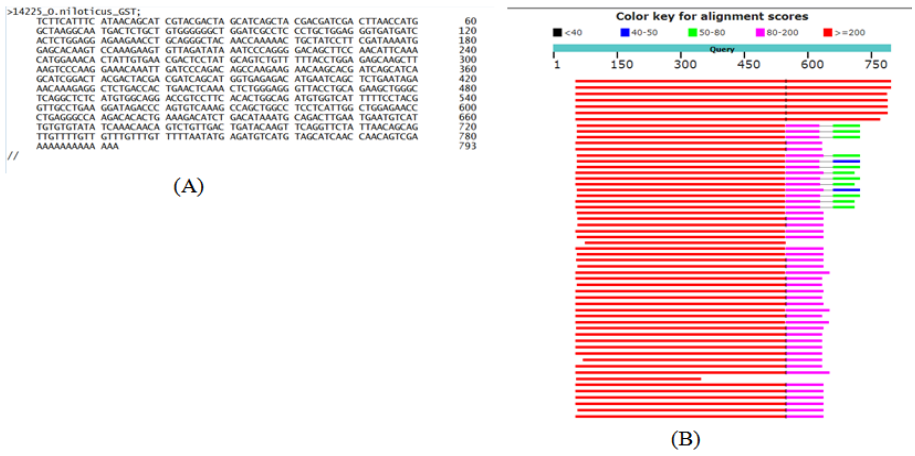


Fig. 5A: *O. niloticus* Glutathione S-transferases (GST) sequence (A) and alignment data (B).

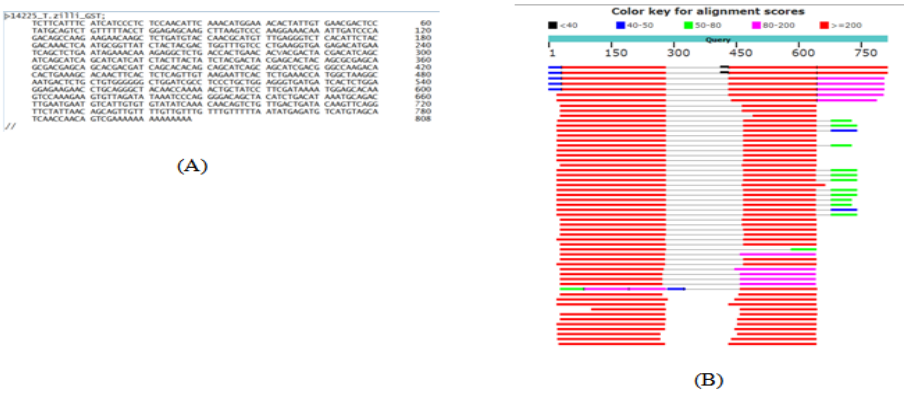


Fig. 5B: *T.zillii* Glutathione S-transferases (GST) sequence (A) and alignment data (B).

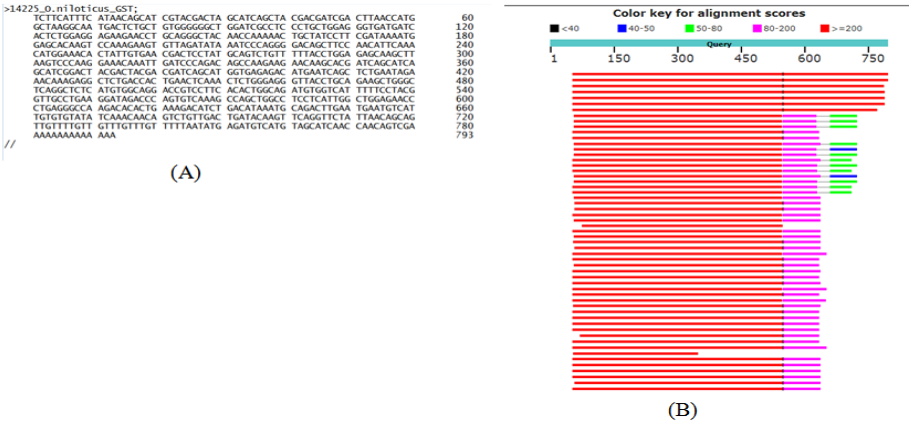


Fig. 5C: O.aureus Glutathione S-transferases (GST) sequence (A) and alignment data (B).

To estimate genetic similarity among GST gene sequences of *O. niloticus*, *O. aureus* and *T. zillii* and reference GST gene (Oreochromis niloticus glutathione S-transferase (LOC100534425), phyllogenetic tree was constructed (Figure 6). Corresponding with GST gene sequence data, as shown by Table (3) *T.zillii* GST gene sequence reflected the higest genetic similarity (92.81%) with GST reference sequence. Also, lowest genetic similarity for GST gene sequecen remarked (75.29%) *O. niloticus*. *O. aureus* showed moderate genetic similarity (87.11%) comparing with reference gene sequence.

Fig. 6: phyllogenetic tree for *O. niloticus*, *O. aureus and T. zillii* based on GST gene sequences.

Table 3: Percent Identity Matrix for *O.niloticus, O.aureus and T.zillii* based on GST gene sequences.

Tilapia species	*O. niloticus*	*O. aureus*	*T. zillii*	Gst reference gene
O. niloticus	100	87.58	71.84	75.29
O. aureus	87.58	100	68.05	87.11
T. zillii	71.84	68.05	100	92.81
Gst reference gene	75.29	87.11	92.81	100

DISCUSSION

Our findings provided significant addition for Egyptian ecology though employment different biochemical and molecular markers as biomarker for accumulated pesticides of Some Egyptian Tilapia Species. More light was added to our findings for applied Tilapia sp. as a biosensor for pesticides stresses by Grillitsch *et al.* (1999). They indicated that, *Tilapia guineensis* reflected behavioral responses to acute and sublethal toxicity of pesticides. Furthermore, in accordance with our results for direct response of organophosphorus pesticides different accumulation levels, Chindah *et al*. (2001) noted that aquatic organisms (shell and fin fishes) will be vulnerable to respiratory tract damage and other organs of the body.

Our investigation for assessment pesticides toxicity referring to aquatic biota, especially fish was employed in many previous studies (Kumar et al., 2015). Employment glutathione S-transferase (GST) as biomarker for environmental marine contamination was supported by Pathiratne *et al.,* (2009). They indicated that GST activities in fish from highly contaminated Bolgoda Lake were induced 4.2-16.6 folds compared with the control fish. In accordance with our findings for using many biochemical parameters as biomarker for pesticides accumulation, additional enzymes such as, glucose-6-phosphate dehydrogenase (G6PDH) and lactate dehydrogenase (LDH) were applied as biomarker for pollutants prediction in Nile tilapia tissues (Osman 2012).

In accordance with our represented data for using antioxidant activity as biomarker for pesticides accumulation, Jiminez and Stegeman, (1990) showed that, activity of antioxidant may be increased or inhibited under chemical stress depending on the intensity and duration of stress applied as well as susceptibility of exposure species. Diffraction for SOD activity among *O. niloticus, O. aureus and T.zillii* under organophosphorus pesticides In consistence with Vander Oost *et al*., (2003) findings which clear that, SOD is responsible for the removal of hydrogen peroxide which is metabolized to oxygen and water. Our presented findings to evaluate Superoxide dismutase, Catalase, glutathione peroxidase, Glutathione reductase, Glutathione as pesticides biomarkers was in accordance of many studies which recorded the same antioxidant enzymes within the same range for other fresh water fishes (Oruce and Usta, 2007; Talas *et al*., 2008; Metwaly 2009; Wenju *et al*., 2009 and Gad and Yacoub 2009). Similar results for employment antioxidant enzymes as contaminant indicators have been monitored in gilthead sea bream (*Sparus aurata*) and *Carassius auratus* exposed to polyaromatic hydrocarbons as phenanthrene (Sun *et al*., 2006 and Correia *et al*., 2007). Varied values of GST in the . O.niloticus, *T.zillii* and *O.aureus* tissues were found to be within the same range of other freshwater fishes (Oruce and Usta 2007; Talas *et al*., 2008;Wenju *et al*., 2009 and Gad 2009).

To get better understanding between Glutathione S-transferase (GST) and pesticide accumulations for three Tilapia species, *O. niloticus, O. aureus and T.zillii,* Glutathione S-transferase (GST) was purified and fractionated and recorded as one single band of specific molecular weight (27 KDa) with different protein expression

content. Superior Glutathione S-transferase (GST) protein expression of *O. niloticus* comparing with *O. aureus and T. zillii* could be explained in the light of highly accumulation levels for different organophosphorus pesticides under study corresponding with dramatic increase of antioxidant activity. Our presented findings are in agreement with specified Glutathione S-transferase (GST) molecular weights which mentioned by the major isoforms of GST from different fish species expressed as ranging molecular mass from 22.4 to 26.9 KDa (Nova-Valinas et al. 2002; Hamed *et al*. 2004). Furthermore, more support was added to our findings Huang et al. (2008). They detect similar GST protein pattern. Also, condensed subunits of molecular mass equal 24.8 kDa was fractionated for predominant GST for salmon fish livers.

In the present study, Glutathione S-transferase (GST) gene were amplified, eluted, sequenced and alignments for *Tilapia* species, *O. niloticus, O. aureus and T. zillii*. Then, genetic similarity and phyllogenetic tree was constructed comparing with GST reference gene sequence. Glutathione S-transferase (GST) gene sequence finding added more light to clear resistance and adaptation mechanism of organophosphorus pesticides for Tilapia species.

CONCLUSION

This investigation carried out to evaluate employment *O. niloticus, O. aureus and T. zillii* as biomarker for different organophosphorus pesticides at Edku lake, Egypt. *O. niloticus, O. aureus and T.zillii* reflected varied accumulation levels for Diazenon, Lindan , Parathion , Malathion and Chlorpyrifos. Antioxidant activity and Glutathione S-transferase gene sequences and protein expression levels clear better understanding of pesticides accumulation molecular base, resistance and adaptation for *O. niloticus, O. aureus* and *T.zillii*. our previous data could be concluded as potential probability for applying *O. niloticus, O. aureus and T. zillii* as biomarker for organophosphorus pesticides accumulation.

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