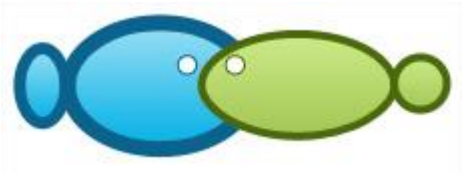

Effects of paraquat-based herbicide on acetylcholinesterase (AChE), antioxidant status and histological alterations in freshwater fish: Nile tilapia (*Oreochromis niloticus*)

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Abstract. This research aimed to study the effects of paraquat, an extensively used herbicide worldwide, on Nile tilapia (*Oremochromis niloticus*) by assessing the physiological and behavioral alterations, the acetylcholinesterase (AChE) expressions in liver and gill, and the conditions of gill and liver tissues. The toxicity as median lethal concentration (LC_{50}) was also studied. The results showed that the exposure time and concentration levels had the effects on the morphological and behavioral changes. The gill was decayed and faded out, while the external characteristics were shown as bleedings, and sores on the mouth and body. For the behavioral changes, they abnormally moved and jumped up, and their operculum were quickly opened and closed. The LC_{50} values at 24, 48, 72 and 96 h were consecutively 34.25 (32.78-35.83), 25.11 (23.94-26.29), 12.74 (11.51-13.90) and 10.23 (9.14-11.26) μL L^{-1}. The AChE expression evaluated using dot blot technique showed that the detection limit values at 96 h after exposure were 10 and 1.25 ug/μl in gill and liver, respectively. Glutathione S-transferases (GST) activity in gill and liver tissues was significantly decreased compared to control group ($p<0.05$) while superoxide dismutase (SOD) activity in gill was not different from the control group, except in the liver, which significantly decreased as compared to the control group ($p<0.05$). The alterations in the gills were blood congestion, epithelial lifting, hyperplasia and partial fusion of lamellae. The changes in liver tissue were blood congestion, vacuolation and nuclear integration. Based on our findings, gill and liver assesment of Nile tilapia could be applied to indicate the exposure of contaminated paraquat in aquatic environment.
Key Words: behavior, enzyme activity, morphology, toxicity testing.

Introduction. The application of herbicides in agricultural areas has been increasing and they cause adverse effects into ecosystems. Fishes are important and useful bioindicators of herbicides effects in aquatic environment (Glusczak et al 2011). The important purpose of aquatic toxicological studies is to determine the maximum amount of toxicants being permitted in the environment without adverse effects onto organisms. There are two roles of biomonitoring for protecting the aquatic environment: 1) continuous assessment of normal conditions and prediction of effects of new toxicants in aquatic ecosystems; 2) prediction of possible ecological effects of toxicants and

prevention of them from reaching hazardous concentrations. Fortunately, fishes can be used as bioindicators in their habitats because they can respond to even low concentrations of toxicants. Thus, they may play an important role in early warning of toxicity indicator assays (Ayanda et al 2015).

In Thailand, a large amount of pesticides has been applied to control pests or carriers of disease for increasing agricultural yields and efficiencies. In 2010, about 70,000 tons of pesticides, comprising 265 individual active ingredients, were imported (The Office of Agriculture Regulation of the Department of Agriculture 2011). The most abundant imported herbicides were glyphosate and paraquat (Sawasdee et al 2016).

Paraquat (1, 1'-dimetthyl-4, 4'-bipyridylium dichloride) is an herbicide in the group of bipyridinium, which is classified as contacts-membrane disrupters. It can knockdown a broad spectrum of weeds and is widely applied in agricultural practice and in controlling weeds in aquatic environment. Thus, paraquat has been found in aquatic environments (Sribanjam et al 2017). In addition, many studies indicated that paraquat can damage important tissues in aquatic organisms, which is washed and carried into the aquatic environment through run-off (Sawasdee et al 2016).

Bioassay techniques are applied to investigate the toxicity of chemicals or toxicants onto organisms. They are an appropriate measure to apply for investigating the toxicity of chemicals or toxicants onto aquatic organisms and humans. Especially in aquatic environments, bioassay is applied to evaluate potential toxicants into aquatic organisms and study relationship between the toxicant concentrations and its effects in aquatic organisms (Ogunwole et al 2018). Besides, bioassay techniques are applied to study the toxicity of a herbicide; however, there are many factors related to the toxicity, i.e. chemical type, chemical form, chemical concentration, organism type, exposure route, and exposure time (Walker et al 2006). Hence, there must be further studies on organisms, including aquatic organisms. Paraquat has moderate acute toxicity to organisms but high toxicity to aquatic organisms (Sribanjam et al 2017); for instance, acute toxicity of paraquat to juvenile Nile tilapia (*Oreochromis niloticus*) at 96 h was 11.84 mg L^{-1}, for *Trichogaster trichopterus* as 1.41 mg L^{-1} (Banaee et al 2013), and for *Clarias gariepinus* as 27.46 mg/L (Nwani et al 2015; Sribanjam et al 2017).

In 2005, Velkova-Jordanoska and Kostoski (2005) reported that histopathological deformities can be used as biomarkers, evaluating anthropogenic pollutants' effects on organisms for monitoring ecosystem health. The histological alteration can be used as biomarkers of stress because toxicants can disturb metabolic activation and result in inducing cellular modifications in the exposed organisms. The biochemical parameters can be applied to monitor environmental burdens in response to toxicants' exposure such as the levels of plasma proteins, glucose, and other enzymes (Ogunwole et al 2018).

Inappropriate application of substances results in residues in agricultural products and cause food safety problems. These substances, especially insecticide and herbicides, are very toxic to the nervous system and some are carcinogenic (Braz-Mota et al 2015; Ghazala et al 2016). After entering the body, these substances are transformed to cholinergic metabolites, which are toxicants in the liver and then bind to choline esterase in the body, resulting in the congestion of acetylcholine at the synapse. Therefore, the nervous system is disordered. There are 2 types of important cholinesterase enzymes in the body. The first one is found in red blood cells and nervous system tissues, called acetylcholinesterase (AChE) or true cholinesterase, which is responsible for digestion of acetylcholine (ACh), and the other one found in serum and outer nervous system tissues, called butyrylcholinesterase (BuChE) or pseudocholinesterase which has a role in toxins' elimination and hydrolysis of acetylcholine. Most of organophosphate or carbamate insecticides inhibit both BuChE and AChE. Therefore, both enzymes are used as indicators of their exposure (Colović et al 2013; Singhatong 2017).

Antioxidant status was used to evaluate effects of herbicides in freshwater fishes, especially glutathione S-transferases (GST), catalase, glutamic pyruvic transaminase (SGPT), and superoxide dismutase (SOD). GST can conjugate with various carcinogenic, mutagenic, toxic and pharmacologically active compounds with glutathione. Thus, availability of endogenous glutathione at target sites can limit GST-catalyzed conjugation

and inhibit enzyme function. Changes in this enzyme activity can disturb the activation-detoxification balance in different tissues (Timur et al 2003).

Superoxide dismutase (SOD) is an important enzyme in antioxidant defense system O_2^- dismutase in hydrogen peroxide (H_2O_2), which is used as a substrate by glutathione peroxidase (GPx) (Braz-Mota et al 2015). In fishes, this substance can be an exposure indicator.

In this study, we evaluated toxicity effects of paraquat in Nile tilapia (*Oreochromis niloticus*), using some biomarkers to investigate stressed conditions in fish. Biomarkers can be applied to predict toxicity effects of toxicants for preventing acute damages onto aquatic organisms and further regulate toxic waste discharges (OECD 2014). We also studied acute concentrations for determining sensitivity based on mortality, fish behavior and physiology from fish exposed to paraquat. Next, protein, AChE expression, GST activity, SOD activity and histological alterations were studied for effects of these chemicals at sublethal concentration.

Material and Method

Animal preparation. Research took place in March 2020 at the Department of fisheries, Faculty of Agriculture and Technology, Rajamangala University of Technology Tsan Surin Campus, Surin Province. Nile tilapia (*Oreochromis niloticus*) at 60 days age, average weight of 110±20 g, average length of 14.2±1.3 cm and width of 7.3±1.1 cm were prepared. They were acclimatized in 100 L concrete pond with aeration for 7 days. They were fed twice a day: 08.00 am and 05.00 pm.

Toxicity test. Paraquat acute toxicity test on Nile tilapia was performed by static bioassay. At first, preliminary test was done to select the appropriate concentration causing mortality in 96 h. Acute renewal bioassay was conducted in the laboratory following OECD guidelines (OECD 2019) to determine the toxicity of paraquat. Briefly, Nile tilapia was exposed to paraquat for 96 h; then, the median lethal concentration (LC_{50}) was determined. In this study, Nile tilapia was acclimated for 7 days before the test begun. Table 1 shows the conditions for animal preparation and toxicity testing applied in this study compared to OECD guidelines.

Table 1

Conditions for animal preparation and toxicity testing

Conditions	*OECD (2019)*	*Present work*
Loading	25 g of fish in 50 L water	100-120 g of fish in 500 L water
Water temperature	21-25ºC	24ºC
Oxygen concentration	Not less than 60% of air saturation value	5-7 mg L^{-1}
Feeding	None	None
Number and handling of fish	A minimum of 7 fishes	10 fishes
Salinity	< 0.2	< 0.2
pH	6.0-8.5	7.0-7.4
Photoperiod (hours light)	12-16 h	12 h

Concentration levels tested were 7 levels: 0 (control), 10, 20, 30, 40, 50 and 60 µL L^{-1}. In each level, 10 fishes were applied to exposure. The mortality rate was recorded at 0, 24, 48, 72 and 96 h and their behaviors were also noted. Dead fish were immediately removed from glass tank. After that, the acute toxicity level of the paraquat was evaluated by taking the cumulative mortality rate for further calculations to determine the LC_{50} using Minitab® 17 software (entitlement i.d.: 2ec6-9637-1508-0264-2c55-c33) and Probit analysis.

Extraction of AChE from plasma and Nile tilapia tissue and protein determination. For blood collection, Nile tilapia was cultured in water with paraquat in the 2 μL L^{-1} concentration of water for 24 - 96 h, compared to the control. Then, the blood was collected using ethylenediaminetetraacetic acid (EDTA) as anticoagulant. Next, it was centrifuged at a speed of 5,000 rpm for 5 min. The supernatant was collected for determination of AChE by using microtubes coated with 10% EDTA and 10% phenylmethyl sulfonyl fluoride (PMSF). After that, fish was fainted using ice, and surgery was then performed to collect gill and liver tissues and placed into phosphate buffer (pH 7.2) with phenylmethanesulfonyl fluoride (PMSF) 0.1 M. The ratio of the tissues used for extraction was 1 g of tissue per 2.5 ml of buffer. Then, tissue extraction was performed using homogenizer and centrifuged (Hettich Zentrifugen, Universal 320 R) at a speed of 5,000 rpm for 60 min). The supernatant was collected for determination of AChE by using tubes coated with 10% EDTA and PMSF. To determine protein concentration by using Bradford (1976) method, standard curve of bovine serum albumin (BSA) was plotted. A 10 μl of standard protein solution or protein samples (supernatant extracted from various tissues) were pipetted and mixed with 200 μl of BioRad dye (diluted with distilled water, 1:5 parts). Next, it was placed for 5 min and then was measured for optical density (OD) at wavelength of 595 nm. The measured OD was applied to calculate the protein content of the sample compared with the standard curve.

Protein form by Sodium Dodecyl Sulfate–polyacrylamide Gel Electrophoresis (SDS-PAGE). The pattern protein analysis by SDS-PAGE was slightly modified from Thanomsit et al (2018). Briefly, the ingredients of 10% separating gel and 4% stacking gel were prepared. After gel was polymerized, it was filled in electrophoresis set. Then, electrode buffer was filled. The mixture of sample and sample buffer were prepared and boiled in water for 3 min. Next, a 10 μl (80 μg of protein) was loaded in each channel. A 120 V of electricity was applied and then the gel was stained with 0.01% Coomassie Brilliant Blue R-250 for 2 h. After that, it was washed by destaining solution I and II until protein band appeared. The protein size was calculated and compared to molecular weight maker (BioRad, USA).

Detection of acetylcholinesterase (AChE) by dot blot technique. Dot blot technique was modified from Prasatkaew and Nanthanawat (2018). Ten concentration levels of samples: 40, 20, 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, and 0.078 μg μL^{-1}, were prepared. A 1 μL was dropped onto nitrocellulose membrane and left at room temperature until dried. Next, it was soaked in 5% skim milk in PBS for 1 h. Then, it was washed with PBS / 0.5% Tween 20 for 5 min (3 times). It was incubated in the Rabbit anti Electric Eel acetylcholinesterase (PAb-AChE, catalog number #0200-0042 from BioRad Thailand) at dilution of 1:200 for 12 h and GAR-HRP (catalog number #ab6741 from Abcam, Ltd.) at dilution of 1:2,000 for 3 h. The results were recorded at lowest concentration of antigen, to which antibodies bound.

Glutathione-S-transferases (GST) activity. GST activity was measured spectrophotometrically (Thermo Scientific, Genesys UV-VIS) at 340 nm with the rate of 1-chloro-2, 4-dinitro benzene conjugation and reduced glutathione as a function of time (Timur et al 2003). The reaction mixture was preincubated for 2 min at 25°C, which contained 1 mM glutathione (GSH), 50 mM phosphate buffer at pH 7.0 and 50 μl of an appropriate dilution of the enzyme source in the total of 3 ml. The reaction was taken for the first step by putting 0.1 ml, 30 mM 1-chloro-2, 4-dinitrobenzene (CDNB). Then, we measured the increase in A_{340} for 3 min at 25°C against a blank containing GSH and CDNB to remove non-enzymatic conjugation interferences. One unit (U) of activity was determined as the conjugated product formation of 1 μmol min^{-1}, and the extinction coefficient at 9.6 mM^{-1} cm^{-1} CDNB was used for the calculations.

Copper-zinc superoxide dismutase (SOD) (CuZn-SOD) activity. Copper-zinc SOD (CuZn-SOD) activity was determined in gill and liver samples in the accordance with the method of Braz-Mota et al (2015), using superoxide radical at 550 nm and 25°C. The

SOD activity is exhibited in U SOD per mg of protein, which one of SOD is assumed as the quantity of enzyme, promoting the inhibition of 50% of reduction rate of cytochrome C. The activities of Selenium-dependent GPx (Se-GPx) were evaluted on NADPH oxidation in the presence of GSH (0.95 mM) and H_2O_2 at 340 nm. Besides, the specific activities for the enzyme were provided as nmol NADPH transformed/min mg^{-1} protein.

Histological alterations. The histology technique was slightly adjusted from Thanomsit et al (2016). Nile tilapia samples (n=3) were randomly collected from the control and experimental groups after exposing to paraquat for 24, 48, 72 and 96 h. The collecting process of tilapia tissue samples must be performed on the ice tray all the time. All the taken tissues were measured for their length and weight; after that, they were preserved in 10% phosphate buffer formalin at least 24 h. They were then washed with tap water until the solution was being stabilized at least for 1 min and cut into pieces and fixed in 10% phosphate buffer formalin solution for 24 h. Next, they were dehydrated in a graded series of ethanol: 50%, 70%, 80%, 90%, and absolute ethanol (Merck, Germany). They were immediately cleared with xylene (Polysciences, USA), infiltrated, and embedded in paraffin (Leica Biosystems, Germany). After that, the prepared tissues were cut into a 6 µm thickness, dewaxed and stained with hematoxylin and eosin (H & E) (Sigma Aldrich, USA). The alterations in tissues were studied under the light compound microscope and photographed by OLYMPUS CX31 (Olympus Thailand, Ltd.). They were then observed for the structure at the cellular level under a microscope and photographed according to the method from Genten et al (2009).

Statistical analysis. In this study, all parameters were analyzed using descriptive statistics (mean and standard deviation). Differences of GST and SOD activities in response to exposure time of gill and liver were compared by using t-test with SAS University edition (Order number 1095069).

Results

External physiological appearance. After Nile Tilapia exposure to paraquat for 96 h, physiological changes were found as purple spots on the skin, fallen scales and lesion around the mouth and body (Figure 1).

Figure 1. External physiological alterations of Nile tilalia exposed to paraquat.

Internal morphological appearance of gill and liver. Gill of Nile tilapia exposed to paraquat at the concentration levels of 10, 20, 30, 40, 50 and 60 µL L^{-1} increasingly changed with an increasing in concentration compared to the control group. The changes found in gill were gradually discoloring, tearing, and disintegrating. After the effect of paraquat exposure in Nile tilapia was studied, it was found that the liver was changed. The color of gill became darker after 96 h of exposure at concentration levels of 10, 20, 30 and 40 µL L^{-1}. At the exposure concentration levels of 50 and 60 µL L^{-1}, the liver was paler than the control group and liver tissue was decayed (Figure 2).

Figure 2. Internal physiological alterations of liver after exposure to paraquat for 96 h.

Effect of paraquat on fish behavior. After Nile tilapia were exposed to paraquat, there were behavioral and morphological changes, that were not found in the control group. In exposing to the herbicide, there was the loss of equilibrium, startle responses, hyperactivity, abnormal swimming, haemorrhage and general restlessness. These abnormal behaviors were increased with an increasing in exposure concentration as illustrated in Table 2.

Cumulative mortality percentage (%) and median lethal concentration (LC_{50}). In this study, LC_{50} was assessed by Probit analysis using Minitab® 17 software prior to calculate cumulative mortality of Nile tilapia exposed to paraquat. The concentrations of paraquat tested were 10, 20, 30, 40, 50 and 60 µl/L compared to the control group (without exposure) and the cumulative mortality percentage was estimated at 24, 48, 72 and 96 h. The results showed that exposure concentration of 10 µL L^{-1} at 24 h resulted in 20% mortality and increasing gradually until to 96 h, with the cumulative mortality rate of 53.33±5.77%. At the exposure concentration of 20 µL L^{-1}, cumulative mortality rate increased with an increasing in exposure time. The rates at 24, 48, 72 and 96 h were 33.33±5.77%, 43.33±5.77%, 56.67±5.77% and 60.00%. At the concentration of 30 µl/L, the cumulative mortality rates at 24, 48, 72 and 96 h were 43.33±5.77%, 50.00%, 70.00% and 76.67±5.77% while they were consecutively 50.00%, 63.33±5.77%, 73.3±5.77% and 80% at the concentration of 40 µl/L. At the two highest concentration levels studied, 50 µL L^{-1} and 60 µL L^{-1}, it was found that cumulative mortality rates were highest at 96 h: 83.33±5.77% and 90.00% at the exposure concentration of 50 and 60 µL L^{-1}, respectively (Figure 3).

Table 2

Behavioral and physiological changes on Nile tilapia exposed to paraquat

Time (h)	*Behavioral abnormality*	*Concentration (µL L^{-1})(n=3)*						
		0	*10*	*20*	*30*	*40*	*50*	*60*
24	Loss of equilibrium	-	+	+	++	++	++	+++
	Startle responses	-	+	+	+	+	++	++
	Hyperactivity	-	+	++	++	+++	+	+
	Abnormal swimming	-	+	+	++	++	+	+
	Haemorrhage	-	-	-	-	-	-	-
	Restlessness	-	-	-	-	-	-	-
48	Loss of equilibrium	-	+	+	+	+	++	+++
	Startle responses	-	+	+	+	+	++	++
	Hyperactivity	-	+	++	++	+++	+	+
	Abnormal	-	+	+	++	++	+	+

	swimming							
	Haemorrhage	-	-	-	-	-	+	++
	Restlessness	-	+	+	++	+++	-	-
72	Loss of equilibrium	-	+	+	++	++	++	+++
	Startle responses	-	+	+	+	+	++	++
	Hyperactivity	-	+	++	++	+++	+	+
	Abnormal swimming	-	+	+	++	++	+	+
	Haemorrhage	-	-	-	-	+	++	+++
	Restlessness	-	+++	++	+	-	-	-
96	Loss of equilibrium	-	-	-	-	-	-	++
	Startle responses	-	-	-	-	-	-	-
	Hyperactivity	-	+++	++	+	+	+	+
	Abnormal swimming	-	+++	++	+	+	+	+
	Haemorrhage	-	-	+	+	++	+++	+++
	Restlessness	-	++	+	-	-	-	-

Note: - Not observed, + Low expression, ++ Medium expression, +++ High expression**.**

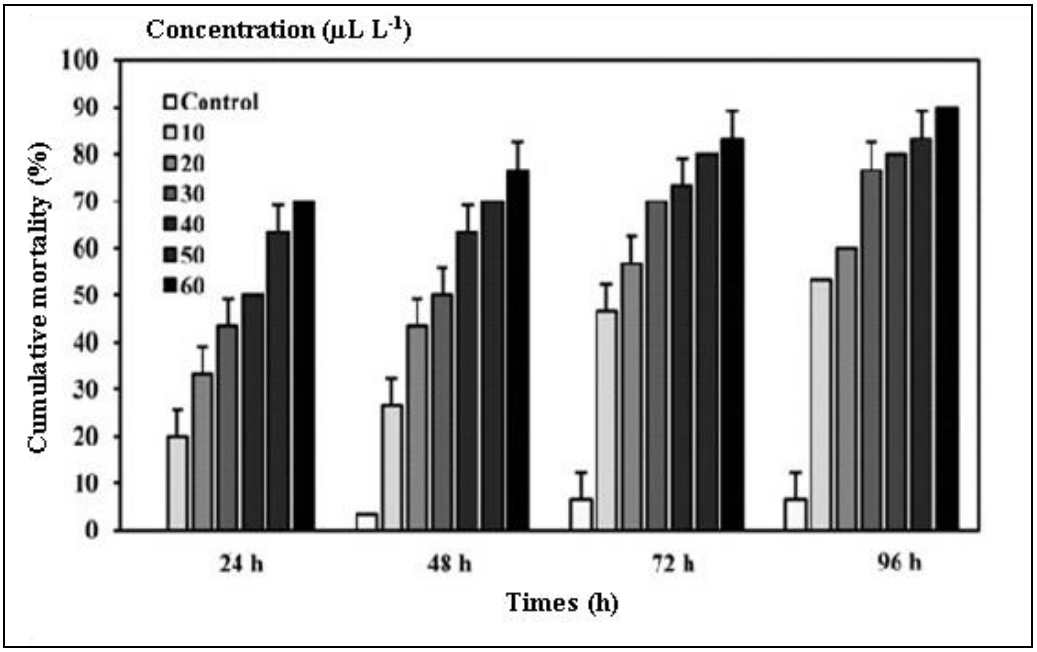

Figure 3. Cumulative mortality rate of Nile tilapia after exposure to paraquat for 24, 48, 72 and 96 h.

After being calculated by Probit analysis using Minitab software, LC_{50} values at 24, 48, 72 and 96 h were 34.25(32.78-35.83), 24.11 (23.94-26.29), 12.74 (11.51-13.90) and 10.23 (9.14–11.26) µL L^{-1}, respectively (Table 3).

Table 3

Median lethal concentration (LC_{50}) by using Probit analysis

Species	*Median lethal concentration (LC_{50})*				*Reference*
	LC_{50} (24 h)	*LC_{50} (48 h)*	*LC_{50} (72 h)*	*LC_{50} (96 h)*	
Trichogaster trichopterus	7.16±0.69 mg L^{-1}	4.46±0.43 mg L^{-1}	2.19±0.27 mg L^{-1}	1.41±0.17 mg L^{-1}	Banaee et al (2013)
Clarias gariepinus	-	-	-	27.46 mg/L	Nwani et al (2015)

Oreochromis niloticus	-	-	-	40.768 mg L^{-1}	Akinsorotan et al (2019)
Clarias gariepinus	59.95 ppm	47.59 ppm	38.12 ppm	26.18 ppm	Aghoghovwia et al (2019)
Oreochromis niloticus	34.25 uL L^{-1} (32.78-35.83) 95% confidence	24.11 uL L^{-1} (23.94-26.29) 95% confidence	12.74 uL L^{-1} (11.51-13.90) 95% confidence	10.23 uL L^{-1} (9.14–11.26) 95% confidence	Present work

Pattern of protein and AChE expression studied by dot blot technique. Total protein contents were evaluated using Bradford dry reagent from plasma of the gill and liver tissues after exposure to paraquat at the concentration of 1 µL L^{-1} for 24, 48, 72 and 96 h. It was found that exposure time did not affect the total protein contents in the tissues when compared with the control groups. It was found that exposure time did not affect the total protein contents in the tissues when compared with the control groups as shown by the result of protein contents from both groups which were not different ($p>0.05$). After studying the protein contents, protein form was then studied by SDS-PAGE. We found the expression of AChE, which had molecular weight of 71 kDa in both liver and gill, but, different in band intensity (Figure 4A and 5A).

Dot blot technique was used to study the sensitivity and specificity of antibodies with AChE to evaluate AChE expression in gill and liver tissues of Nile tilapia after exposed to paraquat at different times. AChE expression was decreased with the exposure time in liver. The limitation of detection in gill was 10 µg uL^{-1} , while it was 1.25 ug uL^{-1} in liver (Figure 4B and 5B).

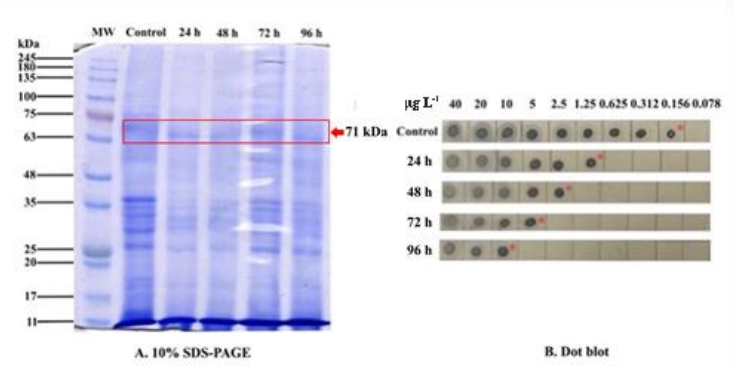

Figure 4. 10% SDS-PAGE showing protein form (A) and AChE expression studied by Dot blot technique (PAb-AChE 1:200) (B) from gill tissue of Nile tilapia after exposed to paraquat at sub lethal concentration (1 µL L^{-1}) for 24, 48, 72 and 96 h compared to control group.

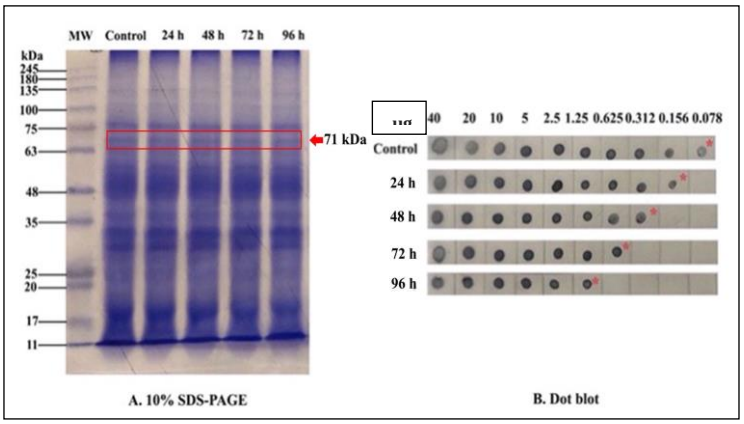

Figure 5. 10% SDS-PAGE showing protein form (A) and AChE expression studied by dot blot technique (PAb-AChE 1:200) (B) from liver tissue of Nile tilapia after exposed to paraquat at sub lethal concentration (1 µL L^{-1}) for 24, 48, 72 and 96 h compared to control group.

Glutathione S-transferases (GST) activity. Figure 8 showed GST activity values in gill and liver tissues of Nile tilapia after exposure to paraquat at the concentration of 1 µL L^{-1} (sublethal concentration) for 24, 48, 72 and 96 h compared to the control group. We found that GST activity value in both gill and liver tissues were lower than that of the control in all the studied exposure times. In gill tissue of fish exposed to paraquat for 48, 72 and 96 h, the measured GST activity values in the experimental group were significantly different from the control ($p<0.05$). The GST activity values in gill at 48, 72 and 96 h were respectively 1.05±0.05, 1.04±0.04 and 0.82±0.06 U min^{-1} mg $protein^{-1}$.

In liver tissue, the GST activity values in Nile tilapia exposed to paraquat for 24, 48, 72 and 96 h were decreased with an increasing in exposure time as found also in gill. However, there were only 3 exposure times: 24, 48 and 96 h, which were significantly different to the control ($p<0.05$). The measured GST activity values were respectively 4.07±0.05, 3.99±0.02, 3.88±0.29 and 3.50±0.02 U min^{-1} mg $protein^{-1}$ (Figure 6).

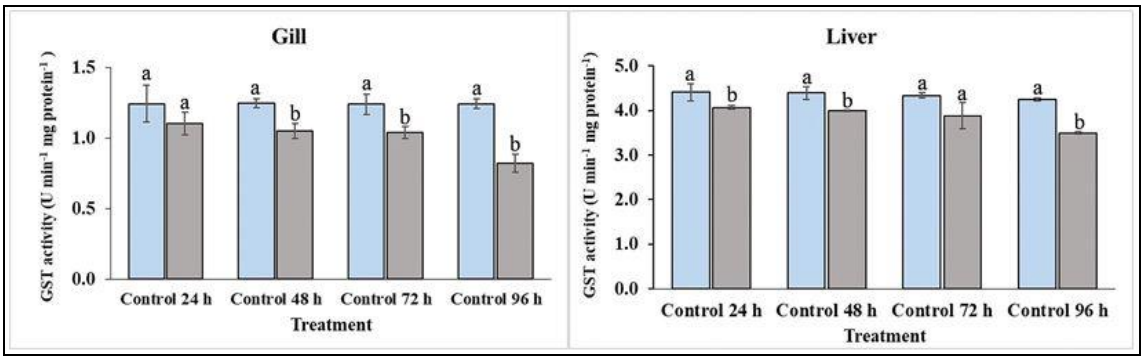

Figure 6. GST activity values reported in mean±std in gill and liver tissues of Nile tilapia exposed to paraquat at sublethal concentration (1 µL L^{-1}) for 24, 48, 72 and 96 h compared to control groups; different characters expressing statistically significant differences ($p<0.05$).

Superoxide dismutase (SOD) activity. SOD activity is another indicator of the study. We found that the SOD activities in gill and liver in the experimental groups were lower than that of control group. However, in the gill tissue it was found that the SOD activity did not differ from the control in all exposure time ($p>0.05$). The SOD activity values in gill tissue at 24, 48, 72 and 96 h were 163±3.20, 161.36±2.01, 159.34±1.00 and 157.45±3.96 U min^{-1} mg $protein^{-1}$, respectively. In the liver tissue, the SOD activity values were 260.11±1.00, 258.10±2.78, 252.67±4.97 and 251.34±6.99 U min^{-1} mg $protein^{-1}$ after exposure to paraquat for 24, 48, 72 and 96 h. However, there were only 3 exposure times: 24, 72 and 96, which were significantly different to the control ($p<0.05$) (Figure 7).

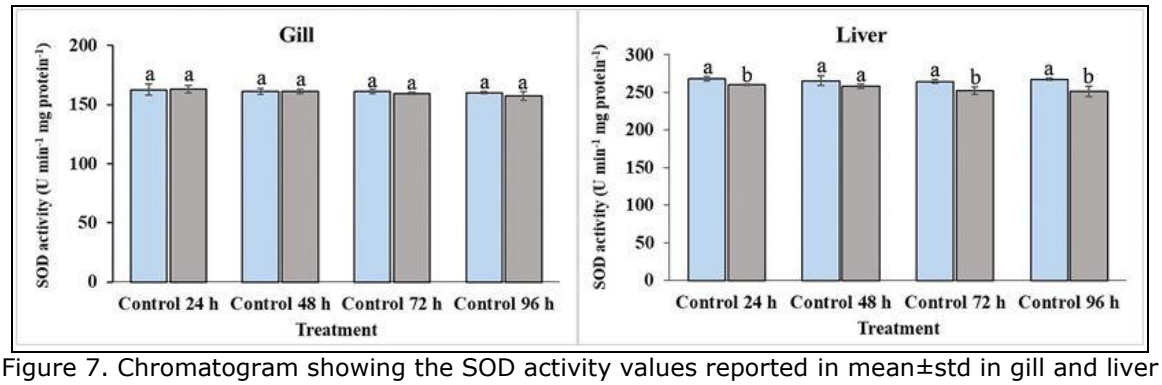

Figure 7. Chromatogram showing the SOD activity values reported in mean±std in gill and liver tissues of Nile tilapia exposed to paraquat at sublethal concentration (1 µL L-1) for 24, 48, 72 and 96 h compared to control groups; different characters expressing statistically significant differences ($p<0.05$).

Histological alterations. Gill is the first organ to be studied in histological alteration because it firstly exposes to paraquat herbicides. According to the sub-lethal study, the

concentration of 1 µL L^{-1}, we found that the alteration was depended on exposure time. The observed symptoms were blood congestion, epithelial lifting and partial fusion of gill lamella (Figure 8). When studying the histological alterations of liver in Nile tilapia exposed to sublethal concentration of paraquat, we found that the alteration was also depended on the exposure time. The observed changes were blood congestion, cell and nuclei integration, and vacuolation (Figure 9).

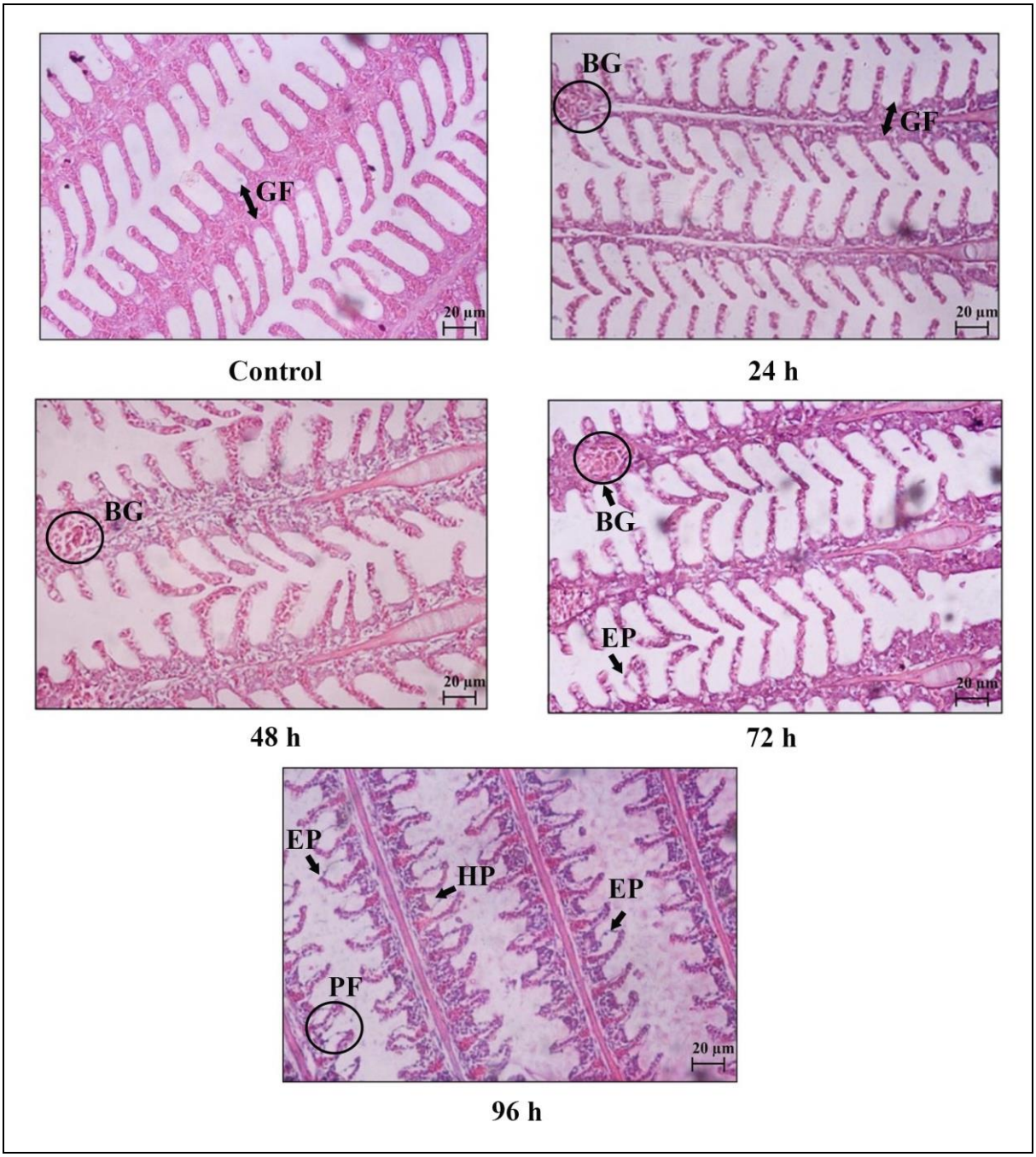

Figure 8. Alterations of gill tissue of Nile tilapia exposed to paraquat at sub lethal concentration (1 µL L^{-1}) for 24, 48, 72 and 96 h compared to control group (40x) where as GF: gill filament, BG: blood congestion, EP: epithelial lifting, HP: hyperplasia, PF: partial fusion of lamella.

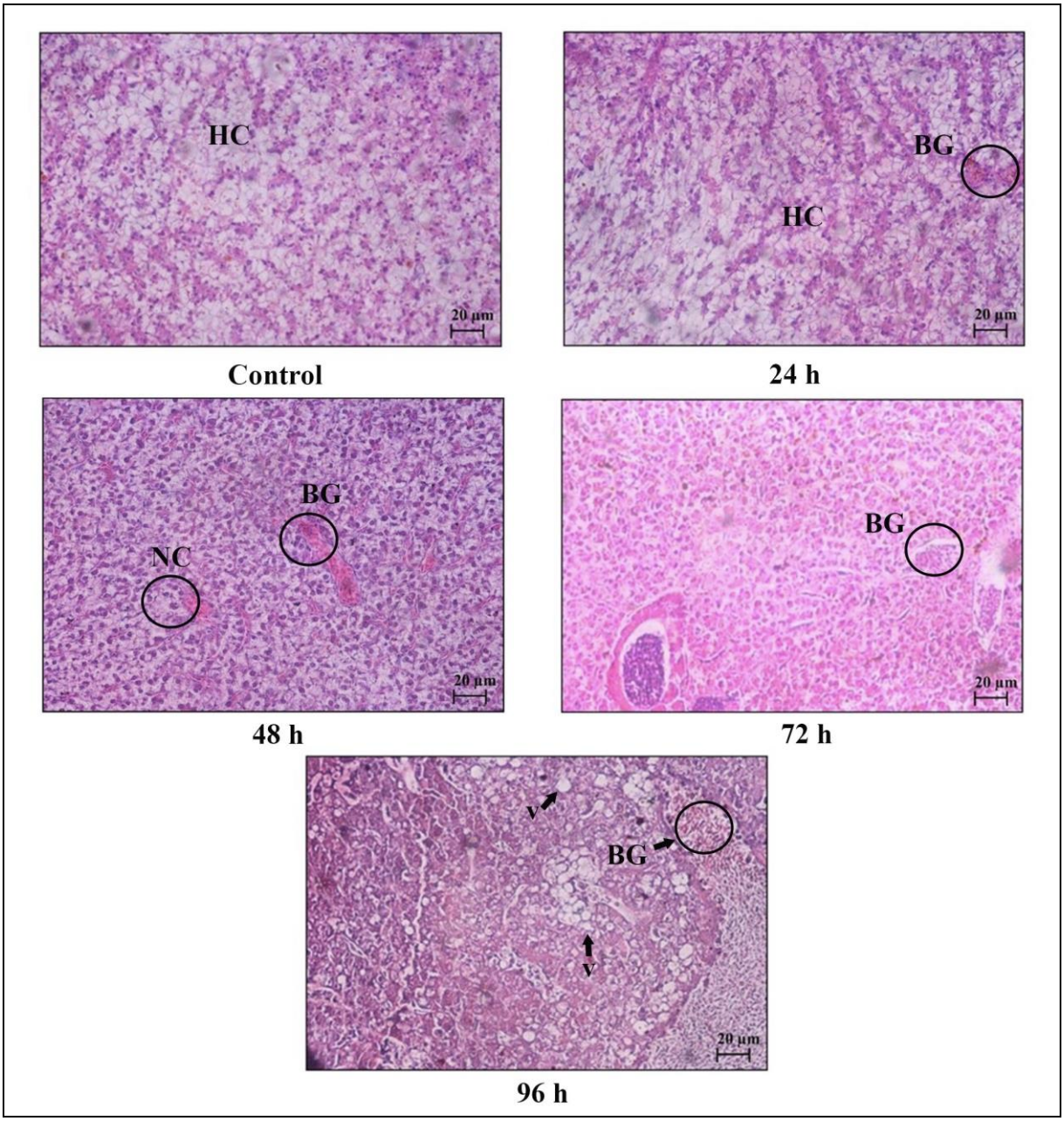

Figure 9. Alterations of liver tissue of Nile Tilapia exposed to paraquat at sub lethal concentration (1 µL L^{-1}) for 24, 48, 72 and 96 h compared to control group (40x), where as, HC: hepatocyte, BG: blood congestion, NC: nuclei integration, V: vacuolation.

Discussion. Paraquat is an herbicide, widely applied in Thailand; however, the study of its contamination and effect in aquatic environment is still very limited. This study aimed to investigate its effects on Nile tilapia (*Oreochromis niloticus*), an economic freshwater fish and favorite aquatic food, consumed as a model in the toxicity study.

For measuring the stressed conditions in fish: physiological, morphological and behavioral alterations, and histopathology were studied. Behavioral response is an important indicator for natural and/or external influencer. It is the first notable sign of stress in an organism. It is a very useful tool in ecotoxicology study. Behavioral and morphological changes in fish have been used to screen and differentiate substances based on their mode of action (Isaac et al 2017).

Paraquat affected the physiological characteristics and behavior of Nile tilapia when exposed to in the concentration inducing acute toxicity. The observed changes were loss of equilibrium, startle response, hyperactivity, abnormal swimming, haemorrhage and restlessness. These changes were clearly associated with concentration and exposure

time. These changes were also in agreement with the study of Ogunwole et al (2018), who examined the effects of paraquat in *Clarias gariepinus* and also found the similar behavioral changes (loss of equilibrium and rapid gill movement) caused by fenitrothion in Nile tilapia (Abu Zeid & Khalil 2014). These changes were relevant to nervous system, especially pesticides have high potential to directly alter behavior, since they are neurotoxins. Thus, they can cause neurotoxic effects on aquatic organisms including inhibition of AChE activity.

In the aquatic toxicological study, the LC_{50} at 96 h is one of the most essential parameters to assess toxicity of a toxicant (Ogunwole et al 2015). Thus, we also tested toxicity of paraquat to Nile tilapia and found that LC_{50} at 24, 48, 72 and 96 h were consecutively 33.33±5.77%, 43.33±5.77%, 56.67±5.77% and 60.00%. Our findings were different to previous studies performed on *Trichogaster trichopterus* (Banaee et al 2013), *Clarias gariepinus* (Nwani et al 2015) and Nile tilapia (Akinsorotan et al 2019). These may be caused by the difference of paraquat form being applied, including species and size of fish. Cheng et al (2019) indicated that AChE can hydrolyze neurotransmitter acetylcholine in cholinergic synapse of aquatic organisms. This mechanism can be irreversibly inhibited by organophosphate and carbarmate pesticides. Thus, AChE is then presented as specific biomarker for this toxicant group (Walker et al 2006; Li et al 2007). In this study, Nile tilapia exposed to paraquat in sublethal at 1 μL L^{-1} and then was taken to study AChE expression by using SDS-PAGE technique. We found AChE with molecular weight of 71 kDa in both gill and liver tissues. But, the detectection in each organ was differently limited. Our results showed that when the exposure time incresed the AChE expression decreased, which were in agreement with the study of Li et al (2007) who investigated the effect of methomyl in topmouth gudgeon (*Pseudorasbora parva*) and found a decreasing of brain AChE activity with an increasing of exposure time (until the end of experimental duration at 96 h) in all studied concentrations. Furthermore, the study of Braz-Mota et al (2015) reported that Roundup® inhibited AChE in Amazon fish after exposure for 96 h.

For antioxidant level, the GST and SOD activities were applied as biomarkers of paraquat exposure in Nile Tilapia at sub-lethal concentration. GST is mostly found in animals and most of them are in multiple isoenzymic forms. They play an important role in a significant intracellular mechanism of detoxification. In this study, we found significant decreasing of GST activity in fish gill compared to control group ($p<0.05$) after 48 h of exposure. This may be due to the toxicity of paraquat, which destroy the cells, resulting in GST activity. In the liver, GST activity was significantly lower than the control group in all exposure time ($p<0.05$). This finding showed that paraquat exposure affects GST activity and the liver has an important role in toxicant detoxification (Li et al 2007; Timur et al 2003). This study is consistent with the study of GST activity in the Amazon fish exposed to Roundup® herbicide for 96 h, which found that GST activity in both gill and liver were lower than that in the control group. And, this result was in agreement with the study of Ogunwole et al (2018), which found that GST activity in liver tissue of *C. gariepinus* was reduced after exposed to paraqout compared to the control.

Superoxide dismutase (SOD) activity is a part of anti-oxidative stress system which inhibit oxy-radical formation and detoxify free radicals by transforming to non-reactive molecules (Cheng et al 2019). There were many studies applying SOD activity as biochemical biomarker for pesticide exposure (Braz-Mota et al 2015; Li et al 2007). In this study, we found only SOD activity in liver tissue different from the control group, and the exposure time affected to SOD activity. This result might be explained that liver is an important organ in eliminating toxicants thus we found obvious changes compared to gill. For decreasing of SOD activity in the period of 24-96 h found in this study is in agreement with the study performed in Amazon fish (Braz-Mota et al 2015) and topmouth gudgeon (Li et al 2007). However, this result was different from the study performed Chinese mitten crab (*Eriocheir sinensis*) which might be caused by the difference of phylum and their immunological and detoxification systems (Cheng et al 2019).

The study of tissue alteration is the last technique applied in this study, Nile tilapia exposed to sublethal levels, the severity of alteration increased with an increasing in

exposure time in both gill and liver tissues. Banaee et al (2013) indicated that gill was an important organ, contacting to pollutants and any kinds of damage to the gill tissue. After exposure, the toxicant can disorder gas exchange process and decrease an ion regulation efficiency via this organ. Thus, histopathological alteration in gill is a useful bio-indicator to pollution monitoring. In this study, the changes of gill were blood congestion, epithelial lifting and partial fusion of gill lamella. The reason for choosing to study the change in gill tissue of Nile tilapia that was exposed to paraquat is that the organism contacts with surrounding water through their gills, which cover significat body surface area. Therefore, gill is directly related to exposure (Begum et al 2007). The changes in the tissues detected were similar to the study of Banaee et al (2013) and Begum et al (2007).

Liver is important in metabolism and detoxification of harmful substances, such as herbicides, atrazine, as shown in the study of Mela et al 2013 which tested neotropical catfish (*Rhamdia quelen*); therefore, we chose to study the changes in this tissues. We found blood congestion, nuclear integration and vacuolation, which are similar to the study in Nile tilapia exposing to fenitrothion (Abu Zeid & Khalil 2014) and abamectin (Thanomsit et al 2016). An increasing in cytoplasmic vacuolation in liver is caused by disturbances in lipid metabolism or in cytoskeleton structure. Vacuolation of hepatocyte found in Nile tilapia could be a protection mechanism because these lipid droplets can sequester the fat soluble pesticide and minimize toxicity; for examples, protein synthesis inhibition, energy depletion, microtubules disaggregation, substrate utilization shifts in hepatic tissue as also found in the study Mela et al (2013).

All studies have found that paraquat is an herbicide that has a great impact on Nile tilapia. Thus, it should be more studied in field environment. Our results can be applied as a base line in planning aquatic environment and fish resources management. Moreover, in the case of consuming Nile tilapia contaminated with paraquat, it will affect human health (Walker et al 2006; Thanomsit et al 2020).

Conclusions. Paraquat affects Nile tilapia at both the sublethal and lethal level concentrations. The observed effects found in this study were morphological and behavioral alterations, AChE expression, changes of antioxidant status (GST activity and SOD activity) and tissues. These results can be applied to assess paraquat exposure in Nile tilapia (*Oreochromis niloticus*). This study presents very useful information in field studies and planning of paraquat application to reduce contamination in natural water sources and accumulation in fish.

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Conflict of interest. The authors declare that there is no conflict of interest.

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