UNIVERSITI PUTRA MALAYSIA

In Vivo EFFECTS OF COPPER ON FISH TOXICITY AND CHOLINESTERASE OF Oreochromis mossambicus (W. K. H. PETERS,1852) (BLACK TILAPIA)

AIN AQILAH BINTI BASIRUN

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By

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Thesis Submitted to the School of Graduate Studies,Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

December 2017

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DEDICATION

This thesis is dedicated to my beloved family.

Abstract of thesis presented to Senate of Universiti Putra Malaysia in fulfillment of the requirements for the degree of Master of Science

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Chairman: Siti Aqlima Binti Ahmad, PhD
Faculty : Biotechnology and Biomolecular Sciences

Heavy metals including copper (Cu) has recently become an overwhelming pollutant towards the environment especially aquatic system. Many current researchers are focusing on cholinesterase (ChE) for biomarker and biosensor development as preliminary screening to prove the existence of xenobiotic in the aquatic system. In this study, an inhibitive assay for Cu was developed using the partially purified fraction of ChE from *Oreochromis mossambicus*. In addition, the biochemical, morphology and histopathology changes of *O. mossambicus* were observed as the biomarker for Cu exposure *in vivo* method. Five selected organs namely brain, blood, gills, liver and muscle depicted the alterations upon 96 h sub-acute exposure of $CuSO_4$. Common anomalies observed include the karyohexis and keryolysis in brain cell strutures, gills hyperplasia, melano macrophage centre (MMC) and hemosiderin formation in liver, blood cell alterations, massive formation of macrophagic cell in blood system and degeneration of muscle bundle in muscle. $CuSO_4$ has also inhibited ChE in *in vivo* analysis. ChE from five selected organs was inhibited starting from the concentration of 5 mg/L $CuSO_4$. Liver ChE showed the fluctuation of ChE inhibition. *In vitro* analysis took place where ChE of untreated *O. mossambicus* was partially purified through affinity chromatography using Procainamide-Sephacryl 6B as ligand. The folds of purification of ChE from brain, blood, gill, liver and muscle ChE were 5.8, 4.9, 3.6, 7.2, and 3.5, respectively. The optimisation of all ChEs were studied. Substrate specificity has specified ChE extracted from all five organs, which are brain (ATC 3.0 mM), blood (PTC 3.0 mM), gill (ATC 2.0 mM), liver (BTC 2.0 mM) and muscle (PTC 2.5 mM). The optimum pH and temperature studies of those organs recorded brain (pH 9, 20°C), blood (pH 9, 40°C), gill (pH 8, 30°C), liver (pH 9, 30°C), and muscle (pH 9, 30°C). In this case, the optimisation of ChEs from five *O. mossambicus* organs was not much different with each organ. Half maximal inhibitory concentration (IC_{50}) was studied to determine the Cu potency toward inhibiting *O. mossambicus* ChEs. IC_{50} of Cu on brain, blood, gill, liver and muscle were 2.65, 0.297, 0.935, 7.66 and 10.58 mg/L. IC_{50} categorised the blood PrChE of *O. mossambicus* as very sensitive and suitable to be used as biosensor for Cu pollution monitoring. Those alterations as well as cholinesterase inhibition of fish upon heavy metal exposure can contribute to

potential biomarker for aquatic pollution monitoring. Development of biomarker will work as preliminary screening of toxicants prior to second validation through high technology instruments followed by water rehabilitation.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Sarjana Sains

KESAN *In Vivo* LOGAM KUPRUM KE ATAS KETOKSIKAN IKAN DAN KOLINESTERASE DARI *Oreochromis mossambicus* (W. K. H. PETERS, 1852) (TILAPIA HITAM)

Oleh

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Logam berat seperti kuprum (Cu) pada masa kini telah menjadi bahan toksik yang mencemarkan alam sekitar terutama sistem akuatik. Kajian terkini lebih tertumpu kepada kolinesterase (ChE) untuk perkembangan biopenanda dan biosensor sebagai penyaringan awal untuk mengesan kewujudan bendasing dalam sistem akuatik. Dalam kajian ini, ujian perencatan untuk Cu telah dijalankan dengan menggunakan sample hasil daripada separa penulenan ChE dari *Oreochromis mossambicus*. Di samping itu, perubahan biokimia, morfologi, dan histopatologi pada *Oreochromis mossambicus* dikaji untuk dijadikan sebagai biopenanda untuk pencemaran Cu dalam kaedah *in vivo*. Lima organ yang dipilih ialah otak, darah, insang, hati, dan otot ikan menunjukkan perubahan pada pendedahan separa-akut $CuSO_4$ selama 96 jam. Beberapa perubahan yang terdapat di dalamnya termasuk *karyorrhexis* dan *karyolisis* dalam struktur sel otak, *hiperplasia*, *melano macrophage centre* (MMC) dan pembentukan hemosiderin dalam sel hati, perubahan sel darah, pembentukan sel makrofaj secara besar-besaran dalam sistem darah dan kerosakan dalam tisu otot. $CuSO_4$ juga merencat ChE dalam analisis *in vivo*. ChE daripada lima organ yang dipilih terencat bermula dari kepekatan 5mg/L $CuSO_4$. ChE daripada hati menunjukkan turun naik perencatan ChE. Analisis *in vitro* berlaku di mana ChE daripada *O. mossambicus* yang tidak terdedah kepada Cu diasing dan disepara tulenkan melalui kromatografi afinasi menggunakan Procainamide-Sephacryl 6B sebagai ligan. Faktor penulenan ChE dari otak, darah, insang, hati, dan otot ChE masing-masing adalah 5.8, 4.9, 3.6, 7.2 dan 3.5. Pengoptimuman semua ChE telah dikaji dan substrat yang spesifik telah menunjukkan ChE yang diekstrak dari semua organ termasuk otak (ATC 3.0 mM), darah (PTC 3.0 mM), insang (ATC 2.0 mM), hati (BTC 2.0 mM), dan otot (PTC 2.5 mM). Kajian pH dan suhu optimum organ-organ tersebut mencatatkan nilai; otak (pH 9, 20°C), darah (pH 9, 40°C), insang (pH 8, 30°C), hati (pH 9, 30°C) dan (pH 9, 30°C). Dalam kes ini, pengoptimuman ChEs daripada organ *O. mossambicus* tidak banyak berbeza di antara setiap organ. Kepekatan separa perencatan (IC_{50}) dikaji untuk menentukan potensi Cu ke arah merencat *O. mossambicus* ChEs. IC_{50} pada otak, darah, insang, hati, dan otot masing-masing adalah 2.65, 0.297, 0.935, 7.66, 10.58 mg/L, IC_{50} mengkategorikan darah PrChE of *O. mossambicus* sangat sensitif sebagai biosensor untuk pemantauan pencemaran Cu. Perubahan serta perencatan terhadap kolinesteres ikan apabila

didedahkan logam berat menyumbang kepada biopenanda yang berpotensi untuk pemantauan pencemaran akuatik. Perkembangan biopenanda akan berfungsi sebagai pemeriksaan awal toksik sebelum pengesahan kedua melalui instrumen teknologi tinggi dan diikuti dengan pemulihan air.

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Ain Aqilah binti Basirun, 2017.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirements for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

%	Percent
<	Less than
>	Greater than
µL	Microlitre
µm	Micrometre
µM	Micromolar
Abs	Absorbance
Ach	Acetycholine
AChE	Acetylcholinesterase
Ag	Silver
Al	Aluminium
ALAT	Alanine transferase
ANOVA	Analysis of variance
APS	Aluminum persulphate
ARD	Acid rock drainage
As	Arsenic
ASAT	Aspirate amino transferase
ASP	Aspartate
ATC	Acetythiocholine iodide
ATPase	Adenosine Tryphosphatase
Au	Gold
Ba	Barium
BCh	Butyrylcholine
BChE	Butyrylcholinesterase
BSA	Bovine serum albumin
BTC	Butyrylthiocholine iodide
Ca	Calcium
CAT	Catalase
Cd	Cadmium
ChE	Cholinesterase
Cm	Centimetre
CNS	Central nervouse system
Co	Cobalt
Cr	Chromium
Cu	Copper
Cu^{2+}	Copper ion
CuO	Copper oxide
CuO_2	Copper dioxide
$CuSO_4$	Copper sulphate
Da	Dalton
dH_2O	Distilled water
DO	Dissolved oxygen
DTNB	5,5-dithio-bis-2-nitrobenzoate
EDTA	Ethylene diamine tetra acetic acid
EDX	Energy dispersive X-ray
et al	and friends
FAO	Food and Agricultural Organisation

Fe	Iron
g	Gram
GL	Granular layer
Glu	Glutamine
GST	Gluthathione S-tranferase
h	Hour
H&E	Hematoxylin and Eosin
Hg	Mercury
$HgCl_2$	Mercury chloride
His	Histidine
IC_{50}	Half maximal inhibitory concentration
K	Potassium
kb	Kilo base
kDa	Kilo Dalton
Kg	Kilogram
L	Litre
LC_{50}	Half maximal lethal concentration
Li	Lithium
LM	Light inverted microscope
LOEC	Low observed effects of concentration
M	Molar
MB	Muscle bundle
Mg	Magnesium
mg	Miligram
min	Minute
mL	Mililitre
MMC	Melano-macrophage centre
Mn	Manganese
MT	Metallothionine
MW	Molecular weight
Na	Sodium
NaOH	Sodium hydoxide
Ni	Nickel
^oC	Degree Celsius
OD	Optical density
OP	Organophosphate
Pb	Lead
Phe	Phenylalanine
PL	Primary lamella
PNS	Peripheral nervous system
PrCh	Propionylcholine
PrChE	Propionylcholinesterase
Pt	Platinum
PTC	Propionylthiocholine iodide
RBC	Red blood cell
R_f	Retention factor
RNA	Ribosomal nucleic acid
ROS	Reactive oxygen species
RPM	Rotation per minute
SDS	Sodium dodecyl sulphate

SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Scanning electron microscope
Ser	Serine
SH-	Sulfuhydryl
SL	Secondary lamella
Sn	Selenium
SOD	Superoxide dismutase
Sr	Srontium
TEM	Transmission electron microscope
Trp	Tryptophan
Tyr	Tyrosine
U	Enzyme unit
V	Voltage
Vmax	Maximum velocity
Zn	Zinc

CHAPTER 1

INTRODUCTION

The vital environmental pollutants are those that can accumulate and are persistent due to the chemical stability and poor biodegradability (Yousafzai et al., 2017). Heavy metals effluences have become a great concern due to their multifunction, toxicity and resistance to degradation as well as their potential of bioaccumulation (Naji et al., 2014). The transformation of heavy metals into persistent metallic composites can cause bioaccumulation in an organism's body, disrupt the biological food chain and eventually give several adverse effects to the ultimate consumer, which is humankind that utilises aquatic sources to live (El-Moselhy et al., 2014; Zhou et al., 2008). Previous studies reporting several freshwater systems in Malaysia including Klang River, Langat basin, and also Mamut River in East Malaysia, Sabah, have raised a concern on their level of contamination by heavy metals such as copper, cadmium, zinc and lead (Abalaka, 2015; Ali et al., 2014; Naji et al., 2014).

The occurrence of heavy metals spillage is due to misappropriate and inconsiderate disposal of metal-rich wastes from human activities such as rapid industrial development and urbanisation, abandoned mining site and mining activities as well as agricultural and deforestation (Ibemenuga, 2016). Heavy metals accumulation in aquatic systems can affect the sustainability and productivity of aquatic organisms (Javed and Usmani, 2016; Sabullah et al., 2015a). In general, the accumulation of heavy metals in organism's tissues can enhace the production of reactive oxygen species (ROS) and eventually cause the biochemical, molecular and morphological alterations (ElGazzar et al., 2014). Heavy metals such as copper (Cu), zinc (Zn) and cadmium (Cd) are the vast metal ions those were excessively accumulate in water.

Copper is among the heavy metals that can be naturally found in several forms like copper ion (Cu^{2+}) and copper sulphate pentahydrate ($CuSO_4.5H_20$) or known as "bluestone" (Yanong, 2009). Copper plays crucial role in several integral parts of enzymes regarding respiration, collagen synthesis and to reduce free radicals (Acosta et al., 2016). Organisms require small amount of Cu to regulate body metabolism. According to Mashifane and Moyo (2014), several Cu compounds were effectively utilised in water treatment as they preserve the discolouration of water and monitor or eliminate algae development and fish parasites in freshwater and marine systems.

However, massive usage of Cu can lead to Cu pollution that would give several negative influences to the aquatic living systems. One of the causes of Cu exposure into stream systems is the abandoned copper mining site. For instance, extreme accumulation of Cu in Malaysia has become alarming especially in Mamut River near Ranau, Sabah. Copper pollution has occurred in the headwater of Mamut River due to an open pit Cu mine operated since 1975 and ceased its operation in 1999. Beyond this time range, this mine became a source of heavy metals accompanied by the increase in number of environmental problems beyond Ranau areas. The main source of heavy

metals was originated from the runoff of mine site, in addition to the floatation process used in preparing Cu concentrates (Ali et al., 2014).

Copper is tremendously toxic to aquatic life (Ezeonyejiaku et al., 2011). Cu exposure can generally disrupt the neural processes, protein function and chemosensory abilities (Dew et al., 2012). Consequences of Cu poisoning include several organ defects to organisms. As such, Cu can encourage larval mortality that will endanger the productivity of aquatic living systems, movement limitation of organisms in their habitat and cells degeneration (Gandhewar et al., 2012; Sabullah et al., 2015a). Meanwhile, exposure and accumulation of Cu toward aquatic organisms especially fish would give substantial effects. Physiological and histopathological alterations of fish upon exposure would aid in clarifying the health status of the fish as the food sources to humans. Aquatic environment makes up the major part of the human resources and is said to be interrelated to human (Ballesteros et al., 2017). Hence, fish has become a sentinel species to be included as a tool of biomonitoring for preliminary assessment of the toxicant in the aquatic systems. It is a potential biomarker for monitoring the heavy metals including Cu in aquatic living system. Fish can be a good biomarker by referring several parts such as liver, gill, muscle and brain that change due to exposure. Gills are the primary organ exposed to toxicant exposure (Sabullah et al., 2014a). Fish biomarkers are necessary to track environmentally induced alterations in accessing the effects of xenobiotics especially Cu on fish (Authman, 2015).

Biomarkers are deliberated to be one of the most capable tools for ecotoxicological applications as they could offer an early recognition of noxious waste exposure and a primary indication of possible effects at advanced levels of biological organisation such as population and ecosystem effects (Quintaneiro et al., 2015). Biomarkers have been considered as the reliable tool to screen alterations in biological responses toward environmental threat ranging from molecular through cellular and physiological response to behavioural changes, which are correlated to the exposure of xenobiotic and highly toxic compounds (Kaviraj et al., 2014; Sabullah et al., 2015a). Apart from the morphology of organism, biochemical alterations can also present as pollution biomarker. Cholinesterase (ChE), a ubiquitous enzyme was selected as the best biomarker for heavy metal detection as the response of inhibition towards vast range of inhibitors was closely accompanied by a rise in mortality and survival of aquatic organism, which was impaired due to inhibition (Nunes, 2011).

In this study, *O. mossambicus* was chosen as a test subject as the previous study mentioned about the capability of this species as an alternative biomarker for detecting of selective heavy metals known as Cu using the behavioural and histological alterations as well as inhibitive assay of ChE activity (Sabullah et al., 2015a). Therefore, this study can be perhaps referred to as another basis for future application of this species, whether in aquaculture management and production or in environmental monitoring.

In response to that, the sublethal concentration of copper sulphate ($CuSO_4$) acute exposure in various concentrations and the behavioural response and physiological changes of the exposed *Oreochromis mossambicus* was done in this study as the most

sensitive indication of potential toxic effects. The use of local *Oreochromis* sp. is expected to increase the potential of this fish to become a sentinel species that permits the recognition of lower contamination level of heavy metals especially Cu. Therefore, the objectives of this study are:

1. To determine the physical, behavioural, and morphological changes of *O. mossambicus* inhibited by Cu using *in vivo* method.
2. To purify and characterise ChE activity from different organs of untreated *O. mossambicus.*
3. To examine half maximal inhibitory concentration (IC_{50}) of Cu that would give inhibit ChE extracted from different organs of untreated *O. mossambicus* through *in vitro* analysis.

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