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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(BLACK TILAPIA)

By

AIN AQILAH BINTI BASIRUN

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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By

AIN AQLILAH BINTI BASIRUN

December 2017

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 CuSO₄. 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. CuSO₄ has also inhibited ChE in in vivo analysis. ChE from five selected organs was inhibited starting from the concentration of 5 mg/L CuSO₄. 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 Procaïnamide-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 (IC₅₀) was studied to determine the Cu potency toward inhibiting O. mossambicus ChEs. IC₅₀ of Cu on brain, blood, gill, liver and muscle were 2.65, 0.297, 0.935, 7.66 and 10.58 mg/L. IC₅₀ 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

AIN AQILAH BINTI BASIRUN

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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 CuSO₄ 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. CuSO₄ juga merencat ChE dalam analisis in vivo. ChE daripada lima organ yang dipilih merencat bermula dari kepekatan 5mg/L CuSO₄, ChE daripada hati menunjukkan turun naik perencatan ChE. Analisis in vitro berlaku di mana ChE daripada Oreochromis 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 Oreochromis mossambicus tidak banyak berbeza di antara setiap organ. Kepekaan separa perencatan (IC₅₀) dikaji untuk menentukan potensi Cu ke arah merencat Oreochromis mossambicus ChEs. IC₅₀ pada otak, darah, insang, hati, dan otot masing-masing adalah 2.65, 0.297, 0.935, 7.66, 10.58 mg/L, IC₅₀ mengkategoriikan darah PrChE of Oreochromis 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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I certify that a Thesis Examination Committee has met on 5 December 2017 to conduct the final examination of Ain Aqilah binti Basirun on her thesis entitled "In Vivo Effects of Copper on Fish Toxicity and Cholinesterase of Oreochromis mossambicus (W. K. H. Peters, 1852) (Black Tilapia)" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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<table>
<thead>
<tr>
<th>Symbol</th>
<th>Abbreviation</th>
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<tr>
<td>%</td>
<td>Percent</td>
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<tr>
<td>&lt;</td>
<td>Less than</td>
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<td>Alanine transferase</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>APS</td>
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<td>ARD</td>
<td>Acid rock drainage</td>
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<td>Aspirate amino transferase</td>
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<td>ATPase</td>
<td>Adenosine Tryphosphatase</td>
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<tr>
<td>Au</td>
<td>Gold</td>
</tr>
<tr>
<td>Ba</td>
<td>Barium</td>
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<td>Butyrylcholine</td>
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<tr>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>Calcium</td>
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<td>Catalase</td>
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<td>Central nervous system</td>
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<td>Cr</td>
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<tr>
<td>Cu</td>
<td>Copper</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>Copper ion</td>
</tr>
<tr>
<td>CuO</td>
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<tr>
<td>CuO₂</td>
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<td>Copper sulphate</td>
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<td>Da</td>
<td>Dalton</td>
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<tr>
<td>dH₂O</td>
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<td>DO</td>
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<tr>
<td>DTNB</td>
<td>5,5-dithio-bis-2-nitrobenzoate</td>
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<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>EDX</td>
<td>Energy dispersive X-ray</td>
</tr>
<tr>
<td>et al</td>
<td>and friends</td>
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<td>FAO</td>
<td>Food and Agricultural Organisation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
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<td>Fe</td>
<td>Iron</td>
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<td>Granular layer</td>
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<tr>
<td>Glu</td>
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</tr>
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<td>Gluthathione S-transferase</td>
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<tr>
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<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
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<td>Hg</td>
<td>Mercury</td>
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<td>HgCl₂</td>
<td>Mercury chloride</td>
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<td>IC₅₀</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>K</td>
<td>Potassium</td>
</tr>
<tr>
<td>kb</td>
<td>Kilo base</td>
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<td>Kilo Dalton</td>
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<tr>
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<tr>
<td>LC₅₀</td>
<td>Half maximal lethal concentration</td>
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<td>Li</td>
<td>Lithium</td>
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<tr>
<td>LM</td>
<td>Light inverted microscope</td>
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<tr>
<td>LOEC</td>
<td>Low observed effects of concentration</td>
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<tr>
<td>M</td>
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<tr>
<td>MB</td>
<td>Muscle bundle</td>
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<td>Mg</td>
<td>Magnesium</td>
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<td>mg</td>
<td>Miligram</td>
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<td>min</td>
<td>Minute</td>
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<td>mL</td>
<td>Mililitre</td>
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<tr>
<td>MMC</td>
<td>Melano-macrophage centre</td>
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<td>Manganese</td>
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<tr>
<td>MT</td>
<td>Metallothionine</td>
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<td>MW</td>
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<td>NaOH</td>
<td>Sodium hydroxide</td>
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<td>OP</td>
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<td>Lead</td>
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<td>Phenylalanine</td>
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<td>PNS</td>
<td>Peripheral nervous system</td>
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<td>PrCh</td>
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<td>Pt</td>
<td>Platinum</td>
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<td>PTC</td>
<td>Propionylthiocholine iodide</td>
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<td>RBC</td>
<td>Red blood cell</td>
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<td>Rₚ</td>
<td>Retention factor</td>
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<td>RNA</td>
<td>Ribosomal nucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
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<td>Rotation per minute</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
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<td>--------</td>
<td>--------------------------------------</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
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<td>SEM</td>
<td>Scanning electron microscope</td>
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<tr>
<td>Ser</td>
<td>Serine</td>
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<tr>
<td>SH-</td>
<td>Sulfhydryl</td>
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<tr>
<td>SL</td>
<td>Secondary lamella</td>
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<tr>
<td>Sn</td>
<td>Selenium</td>
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<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
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<tr>
<td>Sr</td>
<td>Srontium</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscope</td>
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<tr>
<td>Trp</td>
<td>Tryptophan</td>
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<tr>
<td>Tyr</td>
<td>Tyrosine</td>
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<tr>
<td>U</td>
<td>Enzyme unit</td>
</tr>
<tr>
<td>V</td>
<td>Voltage</td>
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<tr>
<td>Vmax</td>
<td>Maximum velocity</td>
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<tr>
<td>Zn</td>
<td>Zinc</td>
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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 enhance 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 (Cu²⁺) and copper sulphate pentahydrate (CuSO₄·5H₂O) 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 (CuSO₄) 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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