UNIVERSITI PUTRA MALAYSIA

TOXICITY EFFECTS OF COPPER ON PARTIALLY-PURIFIED CHOLINESTERASE AND VARIOUS TISSUES OF Clarias gariepinus (BURCHELL, 1822)

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By

SITI NADZIRAH BINTI PADRILAH

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DEDICATION

This thesis is dedicated to my 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

The contamination of Malaysian rivers is gradually increasing year by year. Various environmental issues associated with this problem have been reported with copper (Cu) being reported among the top pollutants in Malaysia especially in Mamut River, Sabah. Copper is a dangerous heavy metal with the ability to transform a continuous metal compound that can accumulate in water, causing imbalance to the biological system. Thus, fish was used as a biomarker in the present study to investigate the effects of Cu through its physical, behavioural, and biochemical changes. The study was carried out by exposing sub-lethal copper sulphate ($CuSO_4$) concentrations (0, 0.2, 0.5, 1.0, 2.5, 5.0, 7.5, 10.0, 15.0, 20.0 mg/L) to *Clarias gariepinus* and observing their physical and behavioural alterations based on swimming pattern, mucus secretion, skin colour and mortality. The histopathology alterations on selected organs (gill, liver, brain, muscle and blood) were analysed under light microscope, transmission and scanning electron microscopy. It was observed that the organs of untreated group showed a normal structure of cells, while histopathological abnormalities such as vacuolation, necrotic cell, pyknotic nucleus and blebbing were observed in the treated fish as the concentration of Cu increases, which also displayed an increment in damage of the cells. In fact, enzyme activity of cholinesterase (ChE) has also found to be affected by $CuSO_4$ in both *in vivo* and *in vitro* methods. During *in vivo*, ChE activity in liver and blood was mostly inhibited at 0.2 mg/L up to 41.87% and 37.1%, respectively, whereas enzyme activity was 100% inhibited at the highest concentration of 20.0 mg/L by demonstrating negative result in ChE activity. Meanwhile, ChE activity was slightly decreased in gill, brain and muscle where the enzyme was inhibited by 84.86%, 80.7% and 66.76%, respectively, at 20.0 mg/L. For *in vitro* study, the ChE enzyme was successfully partially-purified using affinity chromatography and procainamide sephacryl 6B as a resin. The optimum ChE activity of gills and muscle were determined at 40°C in 0.1 M Tris-HCl buffer with pH 8, while the optimum Che activity in liver and blood was in 0.1 M Tris-HCl buffer and pH 9, with optimum temperature of 30°C and 20°C respectively. However, optimum activity of ChE in brain was determined at 30°C in 0.1 M Phosphate-buffer, pH 7. ChE in gills, muscle, brain and blood showed a strong affinity towards substrate

acetylthiocholine iodide (ATC) while liver ChE hydrolysed butyrylthiocholine iodide (BTC) at a faster rate than other organs. Muscle has showed the lowest IC_{50} value at 1.968 mg/L compared to gill, liver, brain and blood with IC_{50} value at 1.971, 2.483, 3.358 and blood 1.975 mg/L, respectively. Therefore, the toxicology effects of Cu on the histology of selected organs have been considered as to be an alternative source for biomarker of metal toxicity, whereas the sensitivity of ChE from muscle tissues demonstrated the potential of becoming an alternative biosensor for detecting Cu.

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

KESAN KETOKSIKAN KUPRUM KE ATAS KETULINAN SEPARA KOLINESTERASE DAN KEPELBAGAIAN TISU *Clarias gariepinus* (BURCHELL, 1822)

Oleh

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Pencemaran sungai di Malaysia semakin meningkat dari tahun ke tahun. Pelbagai isu alam sekitar yang berkaitan dengan masalah ini telah dilaporkan. Kuprum (Cu) merupakan bahan pencemar utama yang dilaporkan di Malaysia terutamanya di Sungai Mamut, Sabah. Kuprum adalah logam berat yang merbahaya dengan keupayaan untuk mengubah sebatian logam berterusan yang dapat terkumpul di dalam air, menyebabkan ketidakseimbangan sistem biologi. Oleh itu, ikan digunakan sebagai biopenanda dalam kajian ini untuk mengkaji kesan Cu melalui perubahan fizikal, tingkah laku, dan biokimia. Kajian ini dilakukan dengan mendedahkan kepekatan kuprum sulfat ($CuSO_4$) (0, 0.2, 0.5, 1.0, 2.5, 5.0, 7.5, 10.0, 15.0, 20.0 mg/L) kepada *Clarias gariepinus* dan memerhatikan perubahan fizikal dan tingkah laku mereka berdasarkan corak berenang, rembesan mukus, warna kulit dan kematian. Perubahan histopatologi pada organ terpilih (insang, hati, otak, otot dan darah) dianalisa di bawah mikroskop elektron penghantaran dan pengimbasan dan mikroskop cahaya. Diperhatikan bahawa organ-organ yang tidak dirawat menunjukkan struktur sel yang normal, sementara histopatologi yang tidak normal seperti pengosongan, sel nekrotik, nukleus piknotik dan pembengkakkan telah diperhatikan dalam ikan yang dirawat seiring dengan peningkatan kepekatan Cu, yang juga menunjukkan kenaikan kerosakan dalam sel. Malah, aktiviti enzim kolinesterase (ChE) juga didapati terjejas oleh $CuSO_4$ dalam kedua-dua kaedah *in vivo* dan *in vitro*. Semasa *in vivo*, aktiviti ChE dalam hati dan darah kebanyakannya rencat pada 0.2 mg/L sehingga 41.87% dan 37.1%, manakala aktiviti enzim 100% dihalang pada kepekatan tertinggi 20.0 mg/L dengan menunjukkan keputusan negatif dalam aktiviti ChE. Sementara itu, aktiviti ChE sedikit berkurangan dalam insang, otak dan otot di mana enzim itu rencat sebanyak 84.86%, 80.7% dan 66.76%, masing-masing, pada 20.0 mg/L. Untuk *in vitro*, enzim ChE telah berjaya ditulenkan separa menggunakan kromatografi afiniti dan procainamide sephacryl 6B sebagai resin. Aktiviti ChE optimum bagi insang dan otot ditentukan pada suhu 40°C dengan menggunakan 0.1 M penimbal Tris-HCl, pH 8 manakala untuk hati dan darah mempunyai aktiviti ChE optimum dalam 0.1 M penimbal Tris-HCl, pH 9 masing-masing pada suhu 30°C dan 20°C. Walaubagaimanapun, aktiviti optimum ChE di otak ditentukan pada 30°C dalam 0.1 M

penimbal fosfat, pH 7. ChE pada insang, otot, otak dan darah menunjukkan pertalian yang kuat terhadap substrat asetiltiokolin iodide (ATC) manakala hati ChE menghidrolisis butiriltiokolin iodide (BTC) pada kadar lebih cepat daripada organ lain. Otot telah menunjukkan nilai IC_{50} paling rendah pada 1.968 mg/L berbanding dengan insang, hati, otak dan darah dengan nilai IC_{50} masing-masing pada 1.971, 2.483, 3.358 dan darah 1.975 mg/L. Oleh itu, kesan toksikologi kuprum pada histologi organ terpilih telah dianggap sebagai sumber alternatif bagi biopenanda ketoksikan logam, manakala sensitiviti ChE daripada tisu otot menunjukkan potensi menjadi biosensor alternatif untuk mengesan Cu.

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Siti Nadzirah Padrilah, 2017

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LIST OF ABBREVIATIONS

%	Percent
ºC	Degree celcius
Al	Aluminium
Ag	Silver
Au	Gold
ATC	Acetylthiocholine iodide
BSA	Bovine serum albumin
BTC	Butyrylthiocholine iodide
ChaT	Cholineacetyltransferase
Cd	Cadmium
Cu	Copper
DTNB	5, 5-dithio-bis-2-nitrobenzoate
et al.,	And friends
G	Gram
HCl	Hydrochloric acid
Hg	Mercury
kDa	Kilo Dalton
K_m	Michaelis Menten constant
L	Litre
M	Molar
Mg	Milligram
mg/L	Miligram
mL	Mililitre
mM	Milimolar
Mg	Magnesium
MW	Molecular weight
Na	Sodium
Ni	Nickel
Pb	Lead
PMSF	Phenylmethylsulfonyl fluoride
PchE	Propionylcholinesterase
PTC	Propionylcholine iodide
SDS-PAGE	Sodium dodecyl sulfate- Polyacrylamide gel electrophoresis
SEM	Scanning electron microscope
TEM	Transmission electron microscope
TEMED	Tetramethyl-ethylene diamine
U	Unit
U/mL	Unit per mililitre
WHO	World Health Organisation
Zn	Zinc

CHAPTER 1

INTRODUCTION

The environmental monitoring of heavy metals is critically needed due to the rapid industrial development, human activities, mining and illegal plantations. Contamination occurred due to the dangerous chemicals released by heavy metals into the water bodies. A few years ago, critical river pollution has occurred in the Ranau, Sabah. This was due to the copper (Cu) mining activity at Mamut (Ali et al., 2004). Furthermore, Sabah also experienced flood in June 2015, causing the rivers to be clogged with fallen trees and boulders followed by an earthquake (The New Straits Times, 2015, June 18). This phenomenon has affected Ranau district's water treatment plant risking the people living in villages nearby the abandoned Mamut Copper Mine in getting a contaminated water supply (Joseph, 2015, June 19).

Heavy metals especially Cu have become major environmental hazards despite their great biological significance as micronutrients (Sabullah et al., 2015a). Copper is a trace element considered to be essential for animals and plants, which is important in metalloenzymes and respiratory pigments (Thangam, 2016). Copper also helps in maintaining homeostasis as it is important for cellular growth. In fish metabolism, Cu is needed as micronutrient to activate several functions in the fish or act as a cofactor of enzyme involved in the biochemical reaction of fish. However, the abundance in amount of Cu is toxic to aquatic environment where it could be accumulated in the body of an organism, disrupting the food chain and ultimately threatened the human life. The toxicity studies of Cu have been reported by many researchers (Sabullah et al., 2014a; Thangam, 2016; Hedayati and Ghaffari, 2013; Singh et al., 2008) where the accumulation of Cu in fish body damages and weakens the mechanisms concerned leading to physiological, pathological and biochemical disorders (Joshi, 2011).

Copper is considered unsafe for all living organisms including aquatic organisms and human. Specifically, aquatic systems have a high sensitivity level towards heavy metal pollutants and the gradual increase in the level of such metal in aquatic environment due to anthropogenic sources (Ashraf et al., 2012). Fish is one of the aquatic organisms with high economic value and it has become the major economic source for Malaysian industry. However, the current pollution issues have be a concern for Malaysian government. Thus, precautionary step is needed to prevent this situation from being worse and fish itself can be manipulated in the formation of biomarker since it is very suitable for finding pollution indicator. Fish also one of the major protein sources and important in human nutrition and making it a suitable biomarker of heavy metals contamination. In addition, fish is a good bioaccumulator since it has the potential to accumulate metals, having the optimum size for analysis, long lifespan, easy to be obtained in large quantity and easy to be sampled (Batvari et al., 2008; Ashraf et al., 2012). The metal ion presents in water will enter the fish body and accumulated in various organs like liver, kidney, blood and gills (Balambigai and Aruna, 2011; Sabullah et al., 2014a; Singh et al., 2008).

Previous study stated that heavy metals can be accumulated in various organs of a fish and concentrated at various levels within the fish body (Balambigai and Aruna, 2011; Papagiannis et al., 2004). Ronagh et al. (2009) showed that the highest concentration of Cu was accumulated in liver of fish. Liver is the main part of Cu deposition before being distributed to other organs. The fish blood is susceptible to contamination-induced stress and its interchanging caused by the haematological parameters can be utilised as toxicity indices of xenobiotics (Hedayati and Ghaffari, 2013). Besides, enzyme cholinesterase (ChE) found in the fish organs also act as biomarker to monitor the accurate conclusion on the effects of pollutant exposure toward fish. Previous study has stated that ChE play the important roles in two function determinants for the survival and performance or organism, neurotransmission and detoxification (Cunha et al., 2007).

A number of pollutants including heavy metals such as Cu were found to exhibit anticholinesterase activity in some animals whether *in vivo* or *in vitro*. Copper is one of the dangerous heavy metals that could turn into a continuous metal compound causing abnormalities in fish such as the reduction in swimming performance and less feed intake (Ali et al., 2003; Kumar et al., 2015). A few studies have showed inhibition of ChE in fishes exposed to heavy metals including that by Sabullah et al. (2014a) mentioning that the inhibition of ChE by heavy metals could cause the accumulation of acetylcholine at the synaptic cleft that may interrupt the nervous transmission and leads to death.

Therefore, in this study, *Clarias gariepinus* species was suggested to be an indicator species for biomonitoring aquatic pollution due to their wide spread in Malaysian rivers.

This study was conducted through the following objectives:

1. To determine the physical and behavioural changes of *C. gariepinus* inhibited by Cu.
2. To identify the histopathological changes of *C. gariepinus* exposed by different concentrations of Cu.
3. To examine the minimal concentration and half maximal inhibitory concentration (IC_{50}) of Cu that would give significant changes to *C. gariepinus*.

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