EFFECTS OF *MORINGA OLEIFERA* ON LEAD-INDUCED TOXICITY IN *PUNTIUS ALTUS*

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**A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY
(PATHOBIOLOGY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2013**

Thesis
entitled

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was submitted to the Faculty of Graduate Studies, Mahidol University
for the degree of Doctor of Philosophy (Pathobiology)
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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor, Dr. Amornrat N. Jensen for her advices, critical reading and editing of my thesis. Special thanks to Assistant Professor Dr. Wannee Jiraungkoorskul for her kindness and valuable advices throughout my study. I am grateful to my advisor committee, Dr. Tawewan Tansatit and Dr. Narin Preyavichyapugdee for their collaborations and constructive comments for my research works.

I wish to express my sincere thanks to Associate Professor Dr. Ong-ard Lawhavinit for helpful comments and suggestions for my works. I would like to express grateful appreciation to the members of the examination committee for their helpful comments and suggestions. I also would like to express my sincere appreciation to Mr. Piya Kosai, Mr. Somneuk Koota, Miss Kanjana Siridthi, Mr. Niwat Kangwanrangsan, Mrs. Tasanee Inwisai and all staffs of the Department of Pathobiology, Faculty of Science, Mahidol University for their support, assistance, and advice throughout my entire study. I also wish to thank, Mrs. Donrudee Chaisiri, staff of Faculty of Veterinary Technology, Kasetsart University for her technical advice on high performance liquid chromatography analysis and phytochemical study.

Last but not least, I would like to dedicate this thesis to my dad, my sister, and my brother. Their love and encouragement are always the great support for me throughout my study.

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ABSTRACT

Lead contamination can be found in soil, water, and food which can be accumulated into many organisms. Currently, lead exposure through dietary sources is a major public health concern. There is a growing trend worldwide on using medicinal plant as an alternative treatment for various diseases. *Moringa oleifera*, a plant in Moringaceae family, has been used in traditional medicine in many parts of the world. In this study, the protective potency of *M.oleifera*-supplemented diets protect against lead toxicity to the fish *Puntius altus* were investigated. The results showed that the gills of fish pre-administering with both dosages of *M. oleifera* diets (20 mg g^{-1} and 60 mg g^{-1}) before lead exposure showed only mild alterations to the gill filament. Interestingly, a number of mucous cells particularly the acid mucopolysaccharide cells were observed in the group of fish pre-administered with *M. oleifera*-supplemented diets. The control fish fed without *M. oleifera* supplement diet showed 94 neutral mucous cell types which indicated that the protective efficiency of this plant could be due to the role of acid mucous cells. Moreover, pre-treatment with *M. oleifera* supplement diet also reduced liver and kidney damages due to lead exposure as well as decreased in an expression of proliferating cell nuclear antigen (PCNA), a marker of cellular proliferation. Overall, these results suggest that pre-treatment with *M. oleifera*-supplemented diet is able to protect the fish against damages from lead exposure.

KEY WORDS: *MORINGA OLEIFERA*/ PHENOLICS/ FISH/ MUCOUS CELL/ CELL PROLIFERATION

105 pages

ผลของอาหารเสริมใบมะรุมต่อพิษสารตะกั่วในปลาตะเพียน

EFFECTS OF *MORINGA OLEIFERA* ON LEAD-INDUCED TOXICITY IN *PUNTIUS ALTUS*

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บทคัดย่อ

สารตะกั่วที่ปนเปื้อนทั้งในดิน น้ำ และอาหารสามารถถูกดูดซึมเข้าสู่ร่างกายของสิ่งมีชีวิตได้ ปัจจุบันพบว่าการปนเปื้อนของสารตะกั่วจากอาหารเป็นปัญหาสำคัญทางสาธารณสุข ขณะเดียวกันการรักษาโดยใช้พืชสมุนไพรได้รับความสนใจเพิ่มขึ้นอย่างต่อเนื่อง มะรุมเป็นพืชในวงศ์ Moringaceae จัดเป็นพืชสมุนไพรที่มีการใช้ประโยชน์แพร่หลายทั่วโลก การศึกษานี้เป็นการประเมินการป้องกันของอาหารเสริมใบมะรุมต่อการเกิดพิษอันเนื่องมาจากสารตะกั่วในปลาตะเพียน จากการศึกษาพบว่าปลาตะเพียนที่ได้รับอาหารเสริมใบมะรุมก่อนได้รับสารตะกั่วมีการเปลี่ยนแปลงทางพยาธิสภาพของเนื้อเยื่อเหงือกเพียงเล็กน้อยเมื่อเทียบกับปลาตะเพียนที่ไม่ได้รับอาหารเสริมใบมะรุม และจากการศึกษาเนื้อเยื่อวิทยาเคมีพบว่ามีการเพิ่มจำนวนของ acid mucous cells ในขณะที่ปลาตะเพียนในกลุ่มควบคุมพบ neutral mucous cell เป็นเซลล์ส่วนใหญ่ การศึกษานี้แสดงให้เห็นว่าประสิทธิภาพของอาหารเสริมใบมะรุมในการป้องกันพิษจากสารตะกั่วอาจมีความเกี่ยวข้องกับบทบาทของ acid mucous cells นอกจากนี้ยังพบว่าการได้รับอาหารเสริมใบมะรุมก่อนได้รับสารตะกั่ว ช่วยลดการเกิดพยาธิสภาพของเนื้อเยื่อไตและตับ อีกทั้งยังลดการเพิ่มจำนวนของเซลล์เนื้อเยื่อในเหงือกและเซลล์ตับอีกด้วย กล่าวโดยสรุปคือ อาหารเสริมใบมะรุมอาจจะสามารถป้องกันพิษจากสารตะกั่วในปลาได้

105 หน้า

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LIST OF ABBREVATIONS

%	Percentage
µg	Microgram
µL	Microliter
µm	Micrometer
µM	Micromolar
δ-ALAD	Delta-aminolevulinic acid dehydratase
Ac	Absorbance of the control
AE	Anion exchanger
ALA	Delta-aminolevulinic acid
ALAD	Aminolevulinic acid dehydratase
ALT	Alanine aminotransferase
ANOVA	Analysis of Variance
As	Absorbance of the sample
ASE	Ascorbic acid equivalent
AST	Aspartate aminotransferase
BSA	Bovine serum albumin
°C	Degree Celsius
CAT	Catalase
cm	Centimeter
dL	Deciliter
DMSA	Meso-2,3 dimercaptosuccinic acid
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EDTA	Ethylenediaminetetraacetic acid
FRAP	Ferric reducing antioxidant power
FRSA	Free radical scavenging activity

LIST OF ABBREVIATIONS (cont.)

g	Gram
GA	Gallic acid
GAE	Gallic acid equivalent
G-6-PDH	Glucose-6-phosphate dehydrogenase
GP	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Glutathione disulfide
H_2O_2	Hydrogen peroxide
HSI	Hepatosomatic index
LPO	Lipid peroxidation
M	Molar
mg	Milligram
min	Minute
mL	Milliliter
mM	Milimolar
MS-222	Tricaine methan sulphonate
NBT	Nitro blue tetrazolium
nm	Nanometer
$^1O_2$	Singlet oxygen
$O_2^{\cdot -}$	Superoxide radical
OH	Hydroxyl
Pb	Lead
PBS	Phosphate buffer saline
PCNA	Proliferating cell nuclear antigen
PMS	Post-mitochondrial supernatant
ppb	Part per billion

LIST OF ABBREVIATIONS (cont.)

ppm	Part per million
ROS	Reactive oxygen species
RP	Reducing power
rpm	Round per minute
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substance
TCA	Trichloroacetic acid

CHAPTER I
INTRODUCTION

Over a decade, heavy metals contamination in the environment continuously increases owing to anthropogenic activities and they tend to concentrate in the aquatic organisms [1, 2]. Many metal contaminations in an environment accumulate in the food chain and thus threaten animals and human beings [3]. Lead, which is commonly found in wastewater, is among the common hazardous heavy metals to human health and aquatic ecosystem [4]. Lead can be released into the environment in various ways, such as the discharge of by-products from batteries, gasoline, lead shot for fire alarm, and plastic manufacturing [5]. Lead has been known to cause adverse health effects including nephrotoxicity [6], harmful effects on the hematological [7] and cardiovascular systems [5]. Owing to its tendency to accumulate in water and sediment, lead contamination can result in a reduction of aquaculture yield [3].

Utilizing natural materials from plants for reducing toxicity of environmental metal contaminants has gained increasing attention. Among all the plant materials that have been tested, *Moringa oleifera* has been shown to be one of the most effective as a coagulant for water treatment [8]. *M. oleifera* is also known as “Maroom” in Thai [9]. It is commonly used as nutritional and medicinal plants in several tropical countries including Thailand [10]. *M. oleifera* tree has also been found to be well adapted to hot, humid, or wet condition, thus it can be cultivated under a variety of conditions [10]. It has been shown that *M. oleifera* displays various health benefits including anti-inflammation [11], anti-hepatotoxic [12], and antitumor [13]. However, the protective potency of *M. oleifera*-supplemented diet against lead toxicity has never been reported. In this study, we focused on the effects of *M. oleifera* supplement on the fish *Puntius altus* exposed to toxic level of lead contamination in the water. The use of *M. oleifera* extract as a dietary supplement may not only have an

effect on reduction of metal toxicity but may also provide growth advantages due to high proteins, vitamins, and minerals contained in the leaves [14].

The first part of this thesis demonstrated the influence of *M. oleifera* leaf extract on the histopathological changes due to lead toxicity in *P. altus*. In the second part, we investigated the antioxidant activities and phytochemical constituents of *M. oleifera* leaves which may have an influence on alterations of lead toxicity. The studies on physiological, hematological, biochemical and histopathological changes will allow us to assess the potential of using *M. oleifera* supplement in reducing lead toxicity. Herein, we demonstrated that pre-treatment with *M. oliefera* diet to the fish reduced the damages in the gill, liver, and kidney. We observed the presence of phenolics and flavonoids in *M. oleifera* extracts which may exert potent protective effects against lead-induced toxicity. Overall, *M. oleifera* diet has protective effects against lead-induced toxicity when pre-administered to fish *P. altus* before lead exposure. These protective effects of *M. oleifera* are most likely mediated through antioxidant properties of the extracts that are able to protect the cells from oxidative stress induced by lead.

CHAPTER II
OBJECTIVES

The goal of the work presented in this thesis is to assess the potential abilities of *Moringa oleifera* leaves as dietary supplementation to protect damages from lead exposure in fish.

The specific objectives are as follows:

1. To measure the protective effects of *M. oleifera* leaves as dietary supplement on lead-induced toxicity in freshwater fish, *Puntius altus* using histopathological, histochemical, hematological, and biochemical analyses.

2. To investigate whether *M. oleifera* diets can protect lead-induced cellular proliferation in gill and liver.

3. To evaluate the antioxidant potential of *M. oleifera* leaves and to screen for potential phytochemicals in the extract of *M. oleifera* leaves that can confer antioxidant activities.

CHAPTER III
LITERATURE REVIEW

3.1 Lead

3.1.1 Lead as a common pollutant

Lead (Pb) is a toxic metallic element considered as a common pollutant due to its wide distribution and persistence in all environmental media [15]. Certain human activities such as base metal mining, combustion of leaded gasoline, the use of Pb-based paints, and the uncontrolled disposal of Pb-containing products have resulted in elevated environmental level of Pb [16]. Pb exposure occurs mainly through ingestion, inhalation, and skin absorption [5]. Pb adversely affects multiple organ systems and can cause permanent damage [5]. To date, it is well accepted that an exposure to even small amounts of Pb is harmful to animals and human beings [17]. Pb shows a broad range of acute or chronic behavioral, biochemical, and physiological risks [15, 18, 19]. The Centers for Disease Control and the American Pediatric Association consider blood Pb levels $\leq$10 $\mu g\ dL^{-1}$ to be excessive for infants, children, and women of childbearing age [20, 21]. Measuring Pb level in the blood is the common biomarker for Pb exposure, however it appears that some of the adverse effects of Pb can occur at undetectable levels as well [22].

3.1.2 Toxicology of lead

Pb is well documented to cause several types of toxicities including neurotoxicity, nephrotoxicity, and deleterious effects on the hematological, cardiovascular, and reproductive systems [23, 24]. Lead can bind strongly to the enzymes and proteins containing sulfhydryl group (-SH) leading to protein inactivation and further contributing to cellular damages [25]. Particularly, the activities of delta-aminolevulinic acid dehydratase (δ-ALAD) and glutathione reductase (GR) were found reduced in both animals and humans exposed to Pb [25]. As shown in Figure 3.1, inhibition of ALAD activity by Pb leads to a decreased heme

production and an increased level of substrate delta-aminolevulinic acid (ALA) [26]. An increased amount of ALA is known to stimulate the production of reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2) and superoxide radical ($O_2^{\cdot -}$) [27]. In addition, Pb is shown to induce changes in red blood cell membrane protein and lipid leading to hemolysis of the cells [28]. GR is a crucial enzyme responsible for recycling of glutathione from the oxidized form (glutathione disulfide, GSSG) to the reduced form (reduced glutathione, GSH) [29]. In addition to GR, glutathione peroxidase (GP) and glutathione-S-transferase are also inhibited by Pb [25].

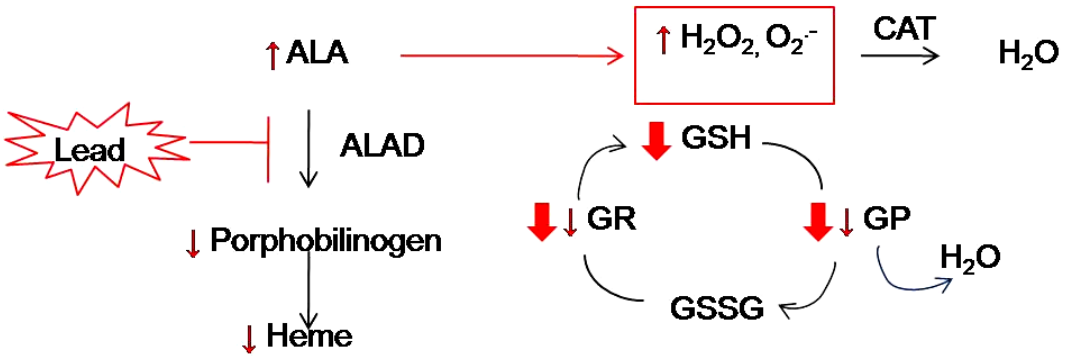


Figure 3.1 Schematic representation of lead on inhibition of ALAD and glutathione peroxidase.

Many studies have reported that lead can cause increased ROS production, DNA damage, and apoptosis in the liver by altering Bcl-2/Bax expression in liver [30, 31]. These ROS include superoxide ($O_2^{\cdot -}$), hydroxyl (OH), singlet oxygen ($^1O_2^{\cdot -}$), and hydrogen peroxide (H_2O_2) which can react with many biomolecules attributing to cellular damage and depletion of antioxidant molecules [32, 33]. However, the molecular mechanisms of lead-induced ROS are not yet completely understood. Lead can also exert its effects by reacting with one or more reactive groups (ligands) essential for normal physiological function [25]. These reactive sites are oxygen (-OH, -COO-, -OPO_3H, -CO-), sulfur (-SH, -S-S-) [34], and nitrogen (-NH_2, -NH-) [35]. Binding of lead to these reactive groups can cause disruption of the three-dimensional configuration of proteins leading to loss of catalytic functions, structural change and inhibition of transport processes of cell membrane [5, 36]. In addition, lead-induced apoptosis has also been reported (Table 3.1).

Table 3.1 Effect of different concentrations of lead on apoptosis

Lead concentration	**Effects**
Pb^{2+} (1 µM)	Selective apoptosis in rat rod cells [37] Decrease in mitochondrial membrane potential, increase in cytochrome c, and caspases-9 and -3 activated in rat rod cells [38]
$PbNO_2$ (10 µM/100g)	Apoptosis in liver of rat [39]
$PbNO_2$ (60-240 µM)	Apoptosis in rat alveolar macrophages [40]

3.1.3 Effect of lead on oxidant/ antioxidant balance

Lead can induce oxidative stress by two mechanisms. First, lead can directly catalyze the formation of ROS [41]. Second, exposure to elevated levels of lead significantly disrupts the prooxidant/antioxidant balance in tissues which leads to biochemical and physiological dysfunction [42]. Increase in generation of ROS can cause damages to cellular macromolecules, including proteins, lipids, and DNA [43]. Consequently, the fluctuations in the levels of ROS production activates signal transduction pathways [44, 45]. As indicated in Figure 3.2, there are three major roles of these reactive molecules in the liver cell response: stimulation of cell proliferation, cell adaptation, or cell death.

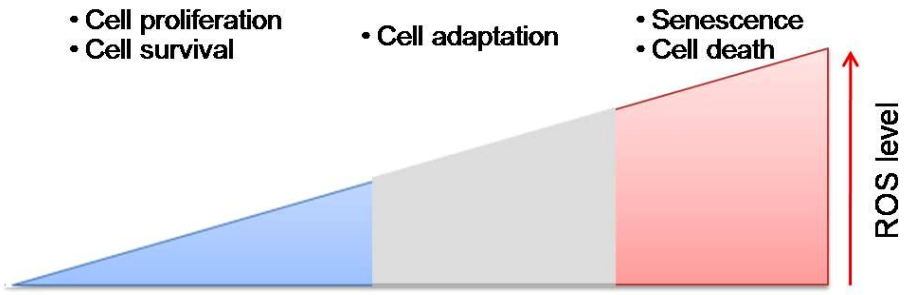


Figure 3.2 Effect of reactive oxygen species (ROS) induced by lead on liver cell fate. Elevated levels of ROS induce cell proliferation, excessive level of ROS causes oxidative stress that can ultimately lead to cell death [44, 46].

Certain enzymes as well as non-enzymatic molecules are involved in the detoxification of ROS. Non-enzymatic antioxidants are classified into two major groups; 1) endogenous antioxidants, which are derived or originated internally, 2) exogenous antioxidants originating outside of the body [47, 48]. Numerous effective exogenous antioxidants have a dietary origin. The best known are vitamins such as ascorbic acid [49], vitamin E [50], and polyphenol [51]. Examples of endogenous enzymes are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidases (GPx). SOD catalyzes the superoxide anion ($O_2^{\cdot -}$) into hydrogen peroxide (H_2O_2) and molecular oxygen [52]. The SOD activity is shown in the following reaction.

$$2O_2^{\cdot -} + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2$$

Catalase is located mostly in peroxisomes and mitochondria [53]. CAT catalyzes the decomposition of H_2O_2 to hydrogen and water as shown in the reaction below [53].

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

GPx are a group of selenium-dependent enzymes located in the cytosol and the mitochondrial matrix [54]. All GPx require reduced glutathione (GSH) as a cofactor and secondary enzymes, such as glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G-6-PDH), to function. G-6-PDH generates NADPH to recycle the GSH [54, 55]. GPx catalyzes peroxidase activity by reducing H_2O_2 to water using GSH as a hydrogen donor [55]. The GPx activity is shown in the following reaction.

$$GSSG + NADPH^+ + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

$$2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2H_2O$$

3.1.4 Lead contamination in aquatic environment

Lead contamination has been reported in aquatic organisms [2, 3]. Lead competes with calcium and disrupts calcium-regulated and calcium-mediated functions [56]. Bioaccumulation within the biota depends on its physiochemical form as well as its metabolism in the individual organism [57]. Dissolved Pb concentrations are typically <1 ppb in uncontaminated water, but may exceed 20 ppb in contaminated water [5]. Toxicity of Pb to aquatic organisms is associated with the concentration of the free dissolved ion, Pb^{2+} [58]. Waterborne Pb is more toxic and more bioavailable in soft freshwater than either hard freshwater or saltwater due to its complex formation with dissolved organic and inorganic substances [59, 60].

Among aquatic species, fish are one of the inhabitants that could not escape from Pb exposure in contaminated water sources [61]. Fish gills are the organ most affected by lead as they are the primary sites for the uptake of substances dissolved in water [62]. A number of researchers have studied the noxious effects of lead in different fish species [63-65]. Lead has been noted to affect the biosynthesis of antioxidant enzymes and disturbs the normal physiology of the fish [66]. Lead induces histopathological alterations in various organs of fish such as gills, liver, and kidney [4, 7, 60, 67].

The fish gill is a multifunctional organ (respiratory, ion regulation, acid-base regulation, and nitrogenous waste excretion) [68]. Besides its importance in gas exchange, fish gill is the major sites of lead uptake [69]. Waterborne lead can bind to the gill and disrupt its functions [70]. In teleosts, the gills are located at the rostral end of the body on either side of the pharynx and operculum [71]. They are comprised of two sets of four holobranches and each side consists of the gill arch, double rows of the gill filaments (primary lamella) forming hemibranches and gill rakers [71, 72]. The secondary lamellae, the sites of gas exchange, are regularly spaced on the upper and lower surfaces on each filament [73] (Figure 3.3). The cellular composition of gill lamellae is consisted of an envelope of simple squamous epithelium and some small goblet cells, mucous cells, supported and separated by pillar cells arranged in a row [74]. At the base of the gill lamellae, some chloride cells (acidophilic cells) which are stained bright pink with hematoxylin and eosin (H&E) are observed [75]. The lamellae epithelium is considered necessary for the normal operation of all biochemical or

physiological processes by maintaining body fluid and mineral homeostasis [71, 72]. Freshwater teleosts regulate diffusive ions loss and osmotic gain of water by absorbing Ca^{2+}, Na^{+}, and Cl^{-} and by producing large volumes of dilute urine [76]. As shown in Figure 3.4, the active ion uptake is mediated by chloride cells and pavement cells. Briefly, Na^{+} uptake is through the epithelial Na^{+} channel in combination with the electrogenic vacuolar type proton pump; Cl^{-} is absorbed by an anion exchanger in exchange for HCO_3^{-} ; and Ca^{2+} uptake is via an apical Ca^{2+} channel and basolateral Ca^{2+}-ATPase [77-79]. Importantly, numerous studies have demonstrated that these ion exchanges and acid-base regulations may have an influence on toxicants or heavy metals uptake [80-82]. Many studies revealed that the affinity of gills for lead is relatively high and there is a strong relationship between gill lead burden and level of toxicity during acute lead toxicity [70, 83, 84]. However, the impact of lead on fish is complex and also depends on physicochemical characteristics of water [71]. High concentration of calcium in the water has been shown to limit lead toxicity in the aquatic insect [85]. Cations, such as Ca^{2+} and complex ligands, such as mucus which are distributed along the gill, skin and lining the digestive tract of fish, are able to reduce lead toxicity by preventing lead from binding to the gill [86].

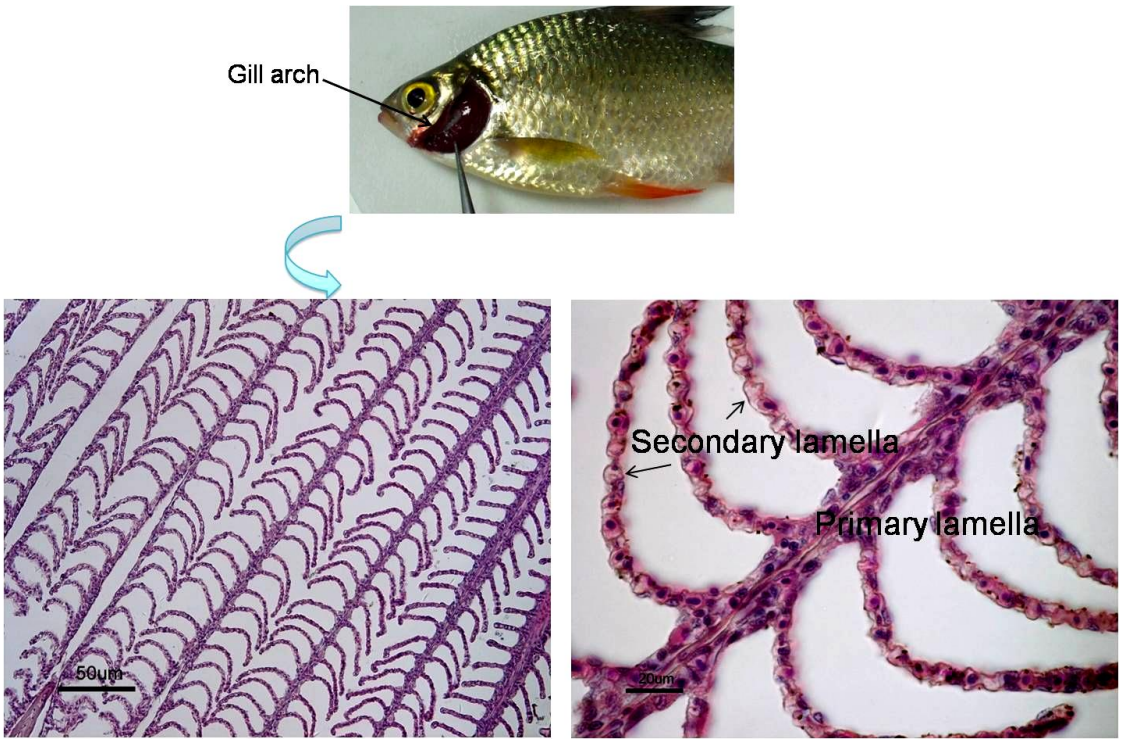


Figure 3.3 Diagrammatic structure of the fish gills showing gill arch and gill filament. The fish gills are protecting beneath the operculum from which pairs of gill filaments arise.

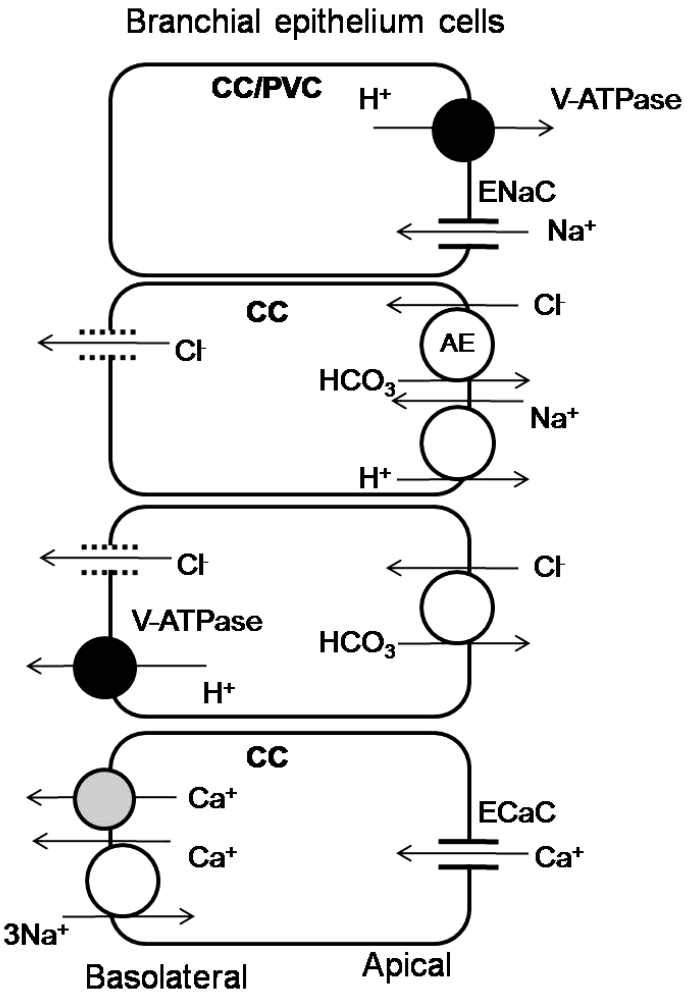


Figure 3.4 Possible mechanisms of ion uptake by gill epithelium of freshwater fish engage in active ion uptake to maintain gill homeostasis.

The liver is also considered as a target organ of Pb toxicity as it has a large volume of blood supply leading to high level of toxicant exposure and accumulation [7]. Fish liver is required for internal homeostasis and survival due to its function in metabolisms. Fish liver is found in the anterior part of the body cavity as brownish red mass. Like other animals, it produces many types of enzyme which are stored in the gall bladder [87, 88]. However, certain features of fish liver are quite different from those of mammalians [88, 89]. There are fewer tendencies for disposition of the hepatocytes in cord or lobules of the fish when compared with mammalians [89]. A

few sinusoids are lined by endothelial cells with prominent nuclei [90]. Fish liver is also a glucose-utilizing, glucose-producing and glucose storage organ [91, 92]. As such, it acts as a glucostat by maintaining blood glucose levels in vertebrate organisms [91]. Glycogen levels in fish liver vary over a wide range of concentrations, and can make up to more than four percent of body weight [93]. Previous studies reported that hepatic glycogen is rapidly mobilized during stress conditions [94]. Shaffi et al. demonstrated that lead nitrate exposure can cause several biochemical parameter changes including glycogenolysis in various species of freshwater fish [18]. It appears that there is a direct relationship between lead levels in water and levels of serum glucose and lactose [18, 95]. Under lead exposure most of antioxidant enzymatic defense system in the liver is induced [96]. The mechanism of lead toxicity in the liver is not clearly known but maybe a consequence of direct binding of lead to thiol groups resulting in enzyme-inactivating activity [96]. Several studies have shown a variety of changes in the liver of fish, resulting from exposure to different lead concentrations and the length of exposure [97-99]. In addition, lead decreases cytochrom P450 activity [100] and depletion of cellular glutathione levels in carp [101].

3.1.5 Effect of lead on *Puntius altus*

P. altus has been placed in a superclass Neopterygii and classified as follows:

Phylum	Chordata
Subphylum	Vertebrata
Superclass	Neopterygii
Class	Teleostei
Subclass	Euteleostei
Order	Cypriniformes
Suborder	Cyprinoidei
Family	Cyprinidae
Genus	Puntius
Species	Altus

Figure 3.5 Taxonomy of *Puntius altus*

Figure 3.6 External anatomy of *P. altus*

P. altus is economically important ornamental fish and widely cultured in Singapore and Malaysia for an export [102] (Figure 3.5 and Figure 3.6). It is also an important fish product in Thailand [103]. *Puntius spp.* (syn. Barbodes) has been widely cultivated due to its rapid growth and the ability to be commercially cultivated in cages, raceways as well as in open ponds [104]. These species are also distributed widely in freshwater regions of Thailand. With the remarkable increase in fish production demand and their relatively low prices, *P. altus* are expected to become an important source of animal protein [102].

Lead contamination is a serious concern because of its toxicity, persistence and bioaccumulation in the food chain [16, 23]. The presence of lead in river, lakes, and seas has considered undesirable effects to fish [2, 63]. For all the above reasons, it is important to determine the toxicity of lead and the influence of *M. oleifera*-supplemented diets on lead exposure.

3.1.6 Role of antioxidant in alleviation of Pb-induced pathology

The current therapeutic approach of Pb toxicity is to increase the excretion of Pb by using chelation therapy [105, 106]. Ethylenediaminetetraacetic acid (EDTA) and meso-2,3 dimercaptosuccinic acid (DMSA) are chemical chelating agents used for Pb poisoning treatment [107, 108]. However, EDTA-chelated Pb burden have been shown to cause renal dysfunction, peripheral nerve damage, and neurobehavioral symptoms [110, 111]. Chelation of Pb by DMSA has been shown to be more effective than by EDTA, but the long-term use of DMSA has also been shown to decrease renal function resulting in nephrotoxicity [112-114].

One of the current approaches to minimize Pb toxicity is the use of antioxidants which act as free radical scavengers [47]. Antioxidants, specifically, vitamin C, vitamin E, zinc, and selenium have been shown to minimize the damaging effects of Pb-generated ROS [114-116]. In Pb-exposed rats, administration of vitamin C in drinking water has been shown to inhibit lipid peroxidation and reduce ROS levels by 40 percent [116, 117]. Other studies indicate that vitamin C is efficient to chelate Pb with have the ability to reverse heme production and normalized blood ALAD levels of Pb-exposed rats [118]. In addition to acting as an antioxidant, vitamin C also has inhibiting effect on Pb uptake as seen in mammalian cell co-culture with Pb

and vitamin C [119]. Similar to vitamin C, vitamin E and its-related compounds have been shown to prevent lipid peroxide-related Pb toxicity in Rbc, liver, and kidney of Pb-exposed rats [6, 120].

Zinc is known to compete with Pb in binding to metallothionein-like transport protein, cysteine rich proteins that bind Pb ions in the gastrointestinal tract [121, 122]. As a result, when zinc was given the presence of Pb, elevated urine ALA and decreased in ALAD levels from Pb toxicity were improved [123]. Similarly, supplementation with selenium in Pb-exposed rats improved SOD, GR, and GSH activities in liver and kidney [122]. Selenium is required mineral for metalloenzyme GP, which play a key role in glutathione metabolism [124]. The proposed mechanism of selenium in reducing Pb toxicity is due to its affinity to form selenium-Pb complexes [125, 126]. Nevertheless, further study is need in utilizing these antioxidants alone or in combination with chelating agents in reducing Pb toxicity.

3.1.7 Natural antioxidants in herb as alternative antioxidant sources

To attenuate the effect of oxidative stress, the use of natural antioxidant such as phenolic compounds, vitamin C, vitamin E, and carotenoids has increased interest [127, 128]. Phenolic compounds are mainly composed of phenolic acids and flavonoids [128, 129]. These compounds have been shown to possess extensive biological properties such as antioxidant, anti-aging, anti-inflammation, and inhibition of angiogenesis and cell proliferation [130, 131]. Phenolics are substituted with one to three hydroxyl groups on the aromatic ring in different positions (Figure 3.7). The antioxidant capacity depends mainly on the number and the position of hydroxyl groups and identity of the main substituent [132]. A variety of herbs have known to be sources of phenolic compounds [133]. These phytochemicals possesses significant antioxidant capacities that are associated with lower incidence of toxicity of chemicals [134-136].

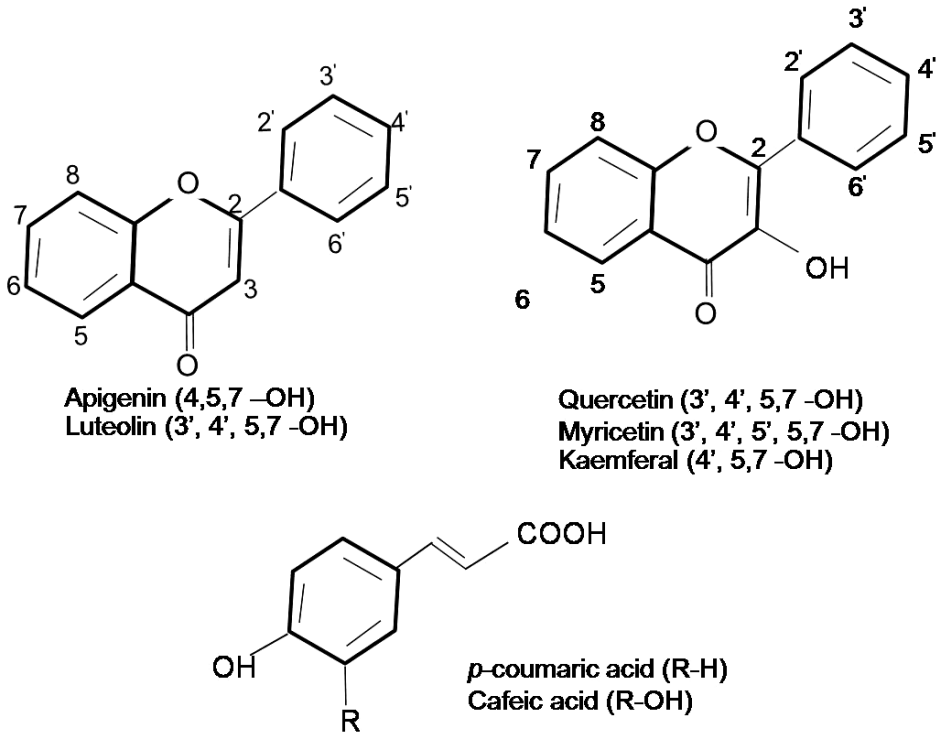


Figure 3.7 Structures of major phenolics in herbs [128].

3.2 *Moringa oleifera*

3.2.1 Taxonomy, distribution, nutrient composition and uses

Moringa oleifera Lam. (syn. *Moringa pterygosperma*) is a plant of the family Moringaceae, which is a fast-growing plant widely distributed in the tropical and subtropical countries [10, 137].

Kingdom	Plantae
Phylum	Tracheobionta
Class	Magnoliophyta
Subclass	Magnoliopsida
Order	Dilieniidae
Family	Moringaceae
Genus	Moringa
Species	Oleifera

Figure 3.8 Taxonomy of *Moringa oleifera*

The anatomical structure of *M. oleifera* is shown in Figure 3.9. Generally, Moringa species is comprised of 13 species [137]. Among those species, *M. oleifera* is the most economically and medicinally important species due to its compositions and is widely cultivated throughout Southeast Asia, including Thailand [9]. Most part of the tree including leaves, long bean-like pods, flowers and seeds are edible and exceptionally nutritious [10].

Figure 3.9 Morphology of *M. oleifera*

Moringa species was used as animal forage (from leaves and seed-cake), biogas (leaves), fertilizer (seed-cake), foliar nutrient (juice expressed from the leaves), green manure (leaves), gum (tree trunks), honey and sugar cane juice-clarifier (powdered seeds), honey (flower nectar), medicine (all plant parts), pulp (wood), rope (bark), tannin for tanning hides (bark and gum) and also provide materials for industrial applications, such as water filtration and purification (powdered seeds), and the production of insecticide [119].

In addition, *M. oleifera* is also considered a medicinal plant [12, 138, 139]. Parts of the tree are applied in folk medicine for the treatment of human diseases due to its potential health benefits such as hypocholesterolemic activity [140], cardio protective effect [141], and anti-microbial property [142]. Recently, it is used for treatment of enlarged liver and shown to be effective in cancer prevention in rat [143]. Various parts of *M. oleifera* are also known for their multiple biological activities including hepatoprotective [12], hypolipidemic [140], and antioxidant [143]. *M. oleifera* leaves have been used as nutritional supplement and growth promoters due to the significant presence of protein, selenium, phosphorus, calcium, β-carotene and α-tocopherol [14].

Moringa species are a rich source of various phytochemicals such as a simple sugar, rhamnose, and a unique group of glucosinolate and isothiocyanate compounds [144, 145]. Glucosinolates and their relative compounds have been demonstrated to have a wide range of biochemical and pharmacological effects [9, 137]. There are also reports of various nitriles, thiocarbamates, and carbamates which

have been shown to have strong hypotensive and spasmolytic effects present in the leaves of plants in Moringa species [146]. Moringa plants have been shown to display anti-carcinogenic activities [147]. The proposed anti-carcinogenic mechanism is via modulations of the activities of phase I (cytochrome P450s) and phase II (glutathione-S-transferase, UDP-glucuronosyl-transferase, and quinone reductase) enzymes [148, 149]. In addition, sinigrin, one of the abundant glucosinolates showed similar effects on enzyme activities [150]. Moreover, it has been demonstrated that sinigrin can stimulate glutathione-*S*-transferases which play a key role in phase II metabolism.

CHAPTER IV
MATERIALS AND METHODS

4.1 Fish and *in vivo* treatments

4.1.1 Animal maintenance

Freshwater fish, *Puntius altus* (35-40 g in body weight and 12-15 cm in total length) was chosen for this study because it is commonly available in local fish farm in Thailand. Juvenile *P. altus* were acclimated under laboratory conditions for 30 days (29±1ºC, total hardness 68 to 88 mg L^{-1} and alkalinity 75 to 80 mg L^{-1}) and were supplied with dechlorinated tap water prior to experimentation. All the necessary precautions for maintaining the fish followed the American Water Works Association recommendations [151]. Water quality conditions (pH, temperature and salinity) were measured daily. The amount of food fed was approximately equal to 2% of their body mass and was given twice daily with commercial fish food (Charoen Pokphand Group, Bangkok, Thailand). Abnormality and mortality at each test group were recorded once every 24 hours. The fish were watched carefully for signs of disease, stress, physical damage and mortality. A 16-hour light and 8-hour dark photoperiod was maintained. This study was approved beforehand by the Mahidol University-Institutional Animal care and Use Committee (MU-IACUC, protocol no. 210).

4.1.2 Plant material and supplemented food preparation

The commercial fish food was supplemented with 0, 20 and 60 mg of *Moringa oleifera* leaves powder per g of fish food. All ingredients were mixed with grounded commercial fish food and distilled water. Then, the prepared diets were extruded through minced-meat machine and allowed to dry at 70°C for 48 hr and stored at room temperature until use. These doses of *M. oleifera* are selected based on the previous report that they do not show toxicity to the fish [152].

4.1.3 Experimental design

The healthy fishes were assigned into six groups each group consists of 14 each in duplicate groups. The fish were fed with the formulated diet containing various levels of *M. oliefera* leaves for 28 days due to their rapid growth. All fish were weighted at the start and at the end of the experiment. Fish were fed at 2% of their body mass and the diets were offered to each tank twice daily. Every five days, fish in each tank were weighed and the amount of feed was re-adjusted accordingly. At the last administration of feeding program, fish were exposed to 93.8 ppm of $Pb(NO_3)_2$ for another 24 hours (Table 4.1). Contaminant was directly exposed in fish using $Pb(NO_3)_2$ diluted as freshly prepared mixture in new tank. The dose of lead exposure was selected based on the previous published studies [153]. Subsequently, the fish were anesthetized with 0.2 g L^{-1} of MS-222 for blood collection. After euthanasia with overdose of MS-222, gill, liver, and kidney were removed and preserved in 10% neutrally buffered formalin for future analyses.

Table 4.1 Experimental design

	1	**2**	**3**	**4**	**5**	**6**
			(20 mg g^{-1} of fish food)		(60 mg g^{-1} of fish food)	
Commercial fish food	+	+	+	+	+	+
Supplemented with *M. oleifera*	-	-	+	+	+	+
Exposed to 93.8 ppm of $Pb(NO_3)_2$	-	+	-	+	-	+

4.2 Blood collection and Hematological parameters

All fish were anesthetized before blood sampling. Blood sample was collected from the caudal vein. The sample was taken at the midline of the anal fin.

The needle was inserted via the musculature into the ventral surface of the fish spine (Figure 4.1). Samples for hematological analysis were collected in heparin tubes and were immediately centrifuged. The remaining blood was allowed to clot at room temperature and subsequently centrifuged for 10 minutes. Plasma was withdrawn and stored at -20°C until assayed.

Figure 4.1 Blood collection from *Puntius altus* at caudal vein.

4.2.1 Hemoglobin concentration was determined according to cyanmethemoglobin method [154]. Hemoglobin in red blood cells reacts with Drabkin's reagent to form cyanmethemoglobin, which its absorbance can be measured by spectrophotometry. Twenty microliter of blood sample was added to 5 ml of Drabkin's solution. Then, the absorbance was measured at 540 nm (Hitachi Model 150-20, Japan).

4.2.2 Pack red cell volume (PCV) or hematocrit was determined employing a micromethod by holding blood in the capillary tube (3/4 of height) centrifuged at 10,000 to 12,000 rpm for 5 min (BOECO, Germany). Then, place the tube in the microhematocrit reader and read the hematocrit by following the instruction on the micro capillary reading device (IEC Micro Capillary Reader No 2210, USA).

4.2.3 Total red cell count was counted manually by using hemacytometer. Blood sample was diluted 1:200 with Natt and Herrick solution. Erythrocytes were counted in the loaded hemacytometer chamber and total numbers were reported as 10^6 cells cu.mm^{-1}. The derived erythrocytes of mean corpuscular volume (MCV: µm^3), mean corpuscular hemoglobin (MCH: pg), and mean corpuscular hemoglobin concentration (MCHC: g dl^{-1}) were calculated using following formulars:

$$MCV = \frac{\text{Hct (\%) x 10}}{\text{Rbc in million ml}^{-1}\text{ blood}}$$

$$MCH = \frac{\text{Hb (g dl}^{-1}\text{) x 10}}{\text{Rbc in million ml}^{-1}\text{ blood}}$$

$$MCHC = \frac{\text{Hb (g dl}^{-1}\text{) x 100}}{\text{Hct (\%)}}$$

4.2.4 Total white cell count was also determined by using hemacytometer. Two milliliter of Natt and Herrick diluting solution and 10 µl of blood were mixed and dropped onto the hemocytometer at the edge of the cover slip. The suspension was allowed to flow under the cover slip by capillary action onto the counting chamber. Then, white bold cells (WBC) were counted under light microscope.

4.2.5 Blood smear analyses were allowed to quantitative difference types of WBC and detection of morphologic abnormalities of blood cells constituents [155]. Briefly, a drop of blood was dropped on the end of the slide. A clean slide, held at 45ºC angle, was touched to the slide with the drop of blood and gently spreads the blood across the slide. The smear was allowed to air dry, fixed with absolute methanol, and then stained with Wright Instant Staining Set (Polysciences, Inc, Germany) according to the protocol. The stained smear was determined under light microscope (Nikon DMX 1200 digital camera, Tokyo, Japan).

4.3 Blood chemical analyses

4.3.1 Aspartate aminotransferase (AST)

The enzymatic activity of AST was measured using Reitman-Frankel method [156].

$$\text{Alpha-ketoglutarate + L-aspartate} \xrightarrow{\text{AST}} \text{glutamate + oxaloacetate}$$

Oxaloacetate was measured colorimetrically after the reaction with 2, 4-dinitrophenylhydrazine at 505 nm. Briefly, 0.25 ml of aspartate transaminase substrate was warmed for 5 min in a water bath at 37^{o}C. Then, sample serum was added mixed, covered with parafilm, and incubated for 60 minutes. Later, color reagent (0.25 ml) was added, shaken gently, allowed at room temperature for 20 minutes, NaOH (0.25 ml) was then added and mixed the absorbance was measured by spectrophotometer at 505 nm against distilled water as a blank.

4.3.2 Alanine aminotransferase (ALT)

The enzymatic activity of ALT was measured by monitoring pyruvate produced in the reaction by Reitman-Frankel method [156].

$$\text{Alpha-ketoglutarate + L-alanine} \xrightarrow{\text{ALT}} \text{glutamate + pyruvate}$$

Pyruvate was measured colorimetrically after the reaction with 2,4-dinitrophenylhydrazine at 505 nm. The procedures for the analysis of glutamic pyruvic transaminase activity were performed similar to those of oxaloacetic transaminase, substituting with the alanine transaminase substrate, and incubated at 37^{o}C for 30 min.

4.4 Antioxidant parameter analyses

Immediately after removal, liver of fish was weighed and chopped with scissors, homogenized on ice using Potter Elvehjem homogenizer with 3 mL of 1.17% potassium chloride (KCl). The homogenate was centrifuged at 1,000 rpm for 10 minutes , followed by centrifugation of the resulting supernatant at 20,000 rpm at 4^{o}C for 30 minutes to obtain the post-mitochondrial supernatant (PMS). The antioxidant enzymes activities were determined in the supernatant fraction.

4.4.1 The activity of CAT was determined following the method of Aebi [157]. Briefly, 20 µl of PMS was added to 80 µl of 10 mM Tris-HCl pH 8.0 and 900 µl of 9 mM H_2O_2, to a final volume of 1 ml. CAT activity was determined spectrophotometrically at 240 nm. The results were expressed as U mg^{-1} protein. Total proteins are measured in both fractions, using the Bradford method [158].

4.4.2 Reduced glutathione (GSH) determination was carried out according to Akerboom and Sies [159]. Briefly, an aliquot of 1.0 ml of 10% PMS was precipitated with 1.0 ml of 4% sulphosalicylic acid and precipitated proteins were separated by centrifugation for 15 min at 1,200 g, 4°C. The mixture assay contained 0.2 ml of 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB, 40 mg in 10 ml of 0.1 M PBS, pH 7.4) in 0.1 M PBS, pH 7.4 (2.7 ml), to a final volume of 3 ml. The DTNB reduction rate was measured spectrophotometrically at 412 nm. GSH was expressed as nmol of H_2O_2 consumed min^{-1} mg^{-1} protein.

4.4.3 Glutathione reductase (GRx) determination was determined following previous published procedure [160]. Briefly, 1.65 ml of 0.1M PBS (pH 7.6) was mixed with 0.1 ml of 0.5mM EDTA, 0.05 ml of 1mM oxidized glutathione, 0.1 ml of 0.1mM NADPH, and 0.1ml of PMS, to a final volume of 2 ml. GRx activity was determined spectrophotometrically at 340 nm and was calculated as nmol NADPH oxidized min^{-1} mg^{-1} protein using a molar extinction coefficient of 6.22 x 10^3 M^{-1} cm^{-1}.

4.4.4 Glutathione peroxidase (GPx) determination was determined following previous published procedure [160]. Briefly, the assayed consisted of 1.44 ml of 0.05 M PBS (pH 7.0), 0.1 ml of 1mM EDTA, 0.1 mM sodium azide (NaN_3), 0.05 ml of GR (1 U ml^{-1}), 0.1 ml of 1mM GSH, 0.1 ml of 2 mM NADPH, 0.01 ml of 0.25 mM H_2O_2 and 0.1 ml of 10% PMS in a total volume of 2 ml. GPx activity was determined spectrophotometrically at 340 nm and was calculated as nmol NADP reduced min^{-1} mg^{-1} protein using a molar extinction coefficient of 6.22 x 10^3 M^{-1} cm^{-1}.

4.5 Lipid peroxidation analyses

The assay for products of lipid peroxidases was performed spectrophotometrically at 535 nm by the thiobarbituric acid reaction at pH 3.5. PMS sample was mixed with 10 mM n-butanol, and 1% orthophosphoric acid, and then the mixture was incubated at 90°C for 45 min before absorbance measurement. Lipid peroxidation was expressed as thiobarbituric acid reactive substances (TBARS) mg^{-1} protein [161].

4.6 Histological analyses

Gill, liver, and kidney were carefully removed, weighed, and recorded. The liver mass was then calculated as hepatosomatic index (HSI) and kidney index (KI) for each specimen.

$$\text{Hepato-somatic index (HSI)} = \frac{\text{Weight of liver (g)}}{\text{Body weight of fish (g)}} \times 100$$

$$\text{Kidney index (KI)} = \frac{\text{Weight of kidney (g)}}{\text{Body weight of fish (g)}} \times 100$$

Then, gill, liver, and kidney were sampled for comparative histological analyses. The samples were immediately fixed after the dissection in 10% neutrally buffered formalin for 24 hours. The samples were then dehydrated through graded series of alcohols, cleared in xylene and embedded in paraffin wax. The embedded samples were sectioned at 5 µm thickness using microtome (Histo STAT, Reichert, USA). Sections were prepared for light microscopy analyses using hematoxylin and

eosin (H & E) staining. One of the sections was submitted to the alcian blue (AB) and periodic acid Schiff's (PAS) stain for identification of certain mucous cell types in the gill tissue. The PAS alone was used to examine the amount of glycogen composition in fish liver.

The sections were immersed in 1% periodic acid, washed in distilled water, stained in Schiff reagent, counter stained in hematoxylin, washed in distilled water, and dehydrated through ascending alcohol series. They were mounted with permount and examined under light microscope. Mucous cell was quantified along gill filament and results are present as a proportion of the total number of mucous cell counted. All stained sections were examined and photographed using the Nikon DMX 1200 digital camera (Tokyo, Japan).

4.7 Immunohistochemical analyses of PCNA

Tissue samples were quickly dissected, fixed in Davinson's fixative for 24 hours, dehydrated in a graded ethanol, cleared in xylene, and embedded in paraffin. The 5 µm section was subjected to the indirect immunohistochemistry technique to examine the PCNA protein distributions.

Sample section was deparaffinized, rehydrated and washed in phosphate buffered saline (PBS, pH 7.1). Endogenous peroxidase was blocked by immersing section in 0.3% hydrogen peroxide for 30 min and then washing in PBS. Then, primary antiserum: monoclonal anti-mouse PCNA, PC 10 (Santa Cruz, USA) was then applied at concentration 1:200 and incubated for 16–18 hr at 4°C. Afterwards, rinsed section twice with PBS and incubated with HRP conjugated rabbit anti-mouse IgG (Sigma-Aldrich Canada Ltd) for 1 hr (1:500) at room temperature. PCNA-positive cells were visualized using 3,3-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich Canada Ltd) and peroxidase substrate as chromogen. In addition, hematoxylin stain was used for visualization of the general tissue structure. Sections serving as negative controls were treated similarly except that normal rabbit serum are used instead of anti-PCNA.

4.8 Antioxidant capacities, total phenolic, total flavonoid contents of *Moringa oleifera* and its phytochemicals

Leaves of *M. oleifera* were collected from Nakhon Pathom, Thailand. The whole leaves were washed several times with tap water, shade dried (Thelco®, GCA/Precision scientific, USA) at 45^{o}C for 72 hours and were grounded into powder using a mixer blender (Otto, Thailand).

Sample preparations were optimized by varying the types and ratio of extraction solvents and mixing time. *M. oleifera* leaves powder (2 g) was extracted in 12.5 ml of following solvents: water, 70% ethanol, 50% acetone, and absolute methanol. Each mixture was stirred at 250 rpm for 1, 3, 6, and 24 h at room temperature with shaker (Germmy Orbit Shaker model VRN-480, Taiwan). The extracts were filtered then through Whatman No. 1 filter paper and were centrifuged at 10,000 g for 10 min, and the supernatants were kept at -20^{o}C for further analysis.

4.8.1 Determination of antioxidant constituents in *M.oleifera* extracts

a) Total phenolic content was determined by Folin–Ciocalteu assay [162] using gallic acid (GA) as a standard and expressed as mg g^{-1} gallic acid equivalent (GAE). Briefly, 0.5 ml of crude extract (2.5 mg ml^{-1}) was mixed with 2.5 ml of 10% Folin–Ciocalteu's reagent and 2 ml of 0.7 M sodium carbonate (Na_2CO_3). Then, the mixture was allowed to stand for 2 h at room temperature and the absorbance was measured by spectrophotometer at 765 nm against distilled water as a blank. The results were expressed as gallic acid equivalents (GAE) mg g^{-1} all samples were analyzed in triplicate.

b) Flavonoid contents were determined based on colorimetric aluminium chloride ($AlCl_3$) method [162]. Briefly, 0.5 ml of leaves extract was mixed with 0.1 ml of 10% $AlCl_3$, 0.1 ml of 1M potassium acetate, and 2.8 ml of distilled water, and then left at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm. Total flavonoid contents were calculated as quercetin from a calibration curve. Quercetin in methanol was used for calibration curve preparation.

c) DPPH radical scavenging activity was measured by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution according to previous published procedures with slight modification [163]. Briefly, 2 ml of leaves extract was mixed in 0.5 ml of 1 mM methanolic DPPH solution and was incubated at room temperature in the dark for 30 min. Thereafter, the absorbance of the sample (As) and control (Ac) were read at 517 nm. The percentage inhibition of the DPPH radical by the samples was calculated using the following equation:

$$\text{Scavenging activity (\%)} = [(Ac-As)/ Ac] \times 100$$

Ac = absorbance of DPPH radical + ethanol

As = absorbance of DPPH radical + extracted sample or ascorbic acid

d) Reducing capacity (RP) was determined by ferric reducing antioxidant power assay [164], using quercetin as reference standard and expressed as ascorbic acid equivalent (1mM = 1 ASE). The ASE ml^{-1} value is inversely proportion to RP. Two milliliter of extract was added to potassium fericyanide (2.5 ml) and was incubated at 50°C for 20 min. Trichloroacetic acid (2.5 ml) was added to the mixture and then centrifuged at 1000 rpm for 10 min. The supernatant (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml). The absorbance was measured at 700 nm. Higher absorbance indicated greater reducing capacity [164].

4.8.2 Reverse phase HPLC analysis of glucosinolates and flavonoids in leaves extract of *M. oleifera*

Five milliliters of milli-Q water was added into the stored sample extract. The sample was centrifuged at 10,000 rpm for 10 min. All sample solutions were filtered through 0.45 µm membrane filter (Millipore, Germany) before the analysis. The quantitative analysis of phenolic compounds was carried out on Water e2695 HPLC system equipped with a 2998 photodiode array detector (Agilent Technologies, Inc). The column used was Phenomenex Luna C_{18} reversed-phase column (250 x 4.6 mm, 5µm, Torrance, CA, USA) and the mobile phase a mixture of two solvents of A (20mM ammonium acetate containing 0.05% trifluoroacetic acid) and B (MeOH). Elution was achieved at 30 °C with a gradient of 0% B in 8 min (1ml min^{-1}) and 0-

60% B in 15 min (1ml min^{-1}1.0 ml min^{-1}). The compounds were monitored at 335 nm and the injection volume was 10µL.

In order to determine glucosinolates concentrations, sinigrin was used as internal standard, which can be detected at a UV wavelength of 227 nm. The samples were injected into HPLC system. The mobile phases consisted of gradient HPLC with A (20mM ammonium acetate containing 0.05% trifluoroacetic acid) and B (MeOH) at flow rate of 1ml min^{-1}. The thirty min run consisted of 0% B (8 min), 0-60% B (12 min), and a 10 min hold at 60-80% B. Sinigrin was identified by comparison of the retention time and UV spectra to the purified standard and quantitative by relative to the sinigrin standard. All samples were analyzed in duplicate.

4.9 Statistical analyses

All data were calculated as mean ± SD. Analysis of variance (ANOVA) with least significant difference (LSD) post-hoc test was performed for each groups. Significance of differences was considered when $p \leq 0.05$.

CHAPTER V
RESULTS

A growing concern for the effects of environmental contaminants on the fish health has started a search for an approach to alleviate the toxic effects and thus improve both the quantity and the quality of the fish. In this study, we hypothesize that *Moringa oleifera* can improve the health of fish *Puntius altus* against acute and chronic stress from lead toxicity. Many research studies have demonstrated multiple biological activities of *Moringa oleifera* including hepatoprotective [165], hypolipidemic [166], and antioxidant [14, 143, 167]. To test the protective role of *M. oleifera* on fish health, the fish were divided into three groups. The first group was fed with only basic fish diet (control fed) and the other two groups were fed with diets supplemented with two different concentrations (20 and 60 mg g^{-1}) of *M. oleifera* leaves powder for 28 days. Then, all fish were exposed to 93.8 ppm of $Pb(NO_3)_2$ for 24 hours. Twenty four hours of lead exposure is too short to be strongly related with effects from lead exposure in the environment. For instance, we observed sublethal responses in fish to lead which provides direct relative toxicities. Analysis of growth, blood and biochemical parameters, enzymes, and histopathological, and histochemical characteristics by various methods were performed.

5.1 *In vivo* study of the potential effect of *M. oleifera* dietary supplementation against lead-induced toxicity in *P. altus*

5.1.1 Growth performance

We have monitored the possibilities of *M. oleifera* as a supplement for fish. There was no increase in mortality in *P. altus* fed with the *M. oleifera* supplement observed throughout the experiment. All fish were active and appeared healthy. Regarding the effects of *M. oleifera*-supplemented diets on fish growth, it was found

that the highest fish growth was markedly detected in the group of fish fed with *M. oleifera* diets (Figure 5.1 B). This finding is consistent with several studies showing that the use of plant materials as dietary supplement can improve the growth of fish [168, 169]. Furthermore, hepatosomatic index (HSI) is increased with suggesting that *M. oleifera* leaves may have an effect on the fish liver. As previously of Atlantic salmon *Salmo salar* fed soybean oil found that soybean supplemented diets increased considerably the fat composition and affected morphology of liver [170]. Overall, *M. oleifera* extract appears to increase the growth of fish receiving the extract as a diet supplement.

5.1.2 Effects of *M. oleifera* on hematological parameters and liver functions in lead-treated fish

Hematological indices are useful tools in assessing various aspects on the health of fish exposed to contaminants. Results of hematological parameters are shown in Figure 5.2. Exposure of fish to waterborne lead significantly ($p<0.05$) decreases the white blood cells (WBC) count but has no effect on erythrocytes count, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration among treatments. It is noteworthy that an administration of either low dose of *M. oleifera* (20 mg g^{-1} of fish food) or high dose of *M. oleifera* (60 mg g^{-1} of fish food) alone had no affect on any blood parameters. Fish consumed diet containing *M. oleifera* before lead exposure shows increases in WBC levels compared the group without the herbal supplement.

To determine whether *M. oleifera*-supplemented diets can attenuate liver damages in the lead-treated fish, the alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total protein were measured (Figure 5.3). In lead exposed fish, the activity of ALT and AST were significantly increased where as total protein was decreased when compared with non-lead exposed group. Pre-treatment with *M. oleifera* can alleviate liver toxicity as shown by reductions in ALT and AST activities, and an increased total protein (Figure 5.3B). These results suggested that pre-administration of fish with *M. oleifera*-supplemented diet is able to decrease lead-induced liver toxicity.

5.1.3 Effects of *M. oleifera* on hepatic oxidative stress, antioxidant parameters, and lipid peroxidation

Exposure to lead has been described to enhance oxidative stress and causes hepatotoxicity [171, 172]. Thereby, the level of lipid peroxidation (LPO) and the activities of catalase (CAT), reduced glutathione (GSH), glutathione reductase (GR), and glutathione peroxidase (GPx) showing the oxidative stress and antioxidant status respectively were measured from hepatic tissue to evaluate the protective property of *M. oleifera*-supplemented diets.

The level of LPO was significantly increased in the lead-exposed fish (Figure 5.4A). Pre-treatment with high dosage (60 mg g^{-1}) of *M. oleifera* diets appeared to slightly decrease cellular lipid peroxidation (Figure 5.4A) Administration of both doses of *M.oleifera* diet alone showed significant increases in CAT, GSH, GR, and GPx activities when compared to the control group (Figure 5.4B). Exposure of fish to lead significantly decreases CAT, GSH, GR, and GPx activities. Noticeably, pre-treatment with *M. oleifera*-supplemented diets before lead exposure can prevent the fish from losing these hepatic antioxidant enzyme activities due to lead toxicity (Figure 5.4B). These findings indicate that supplementation of *M. oleifera* to the fish diet exhibits protective effects on lead-induced hepatic oxidative stress.

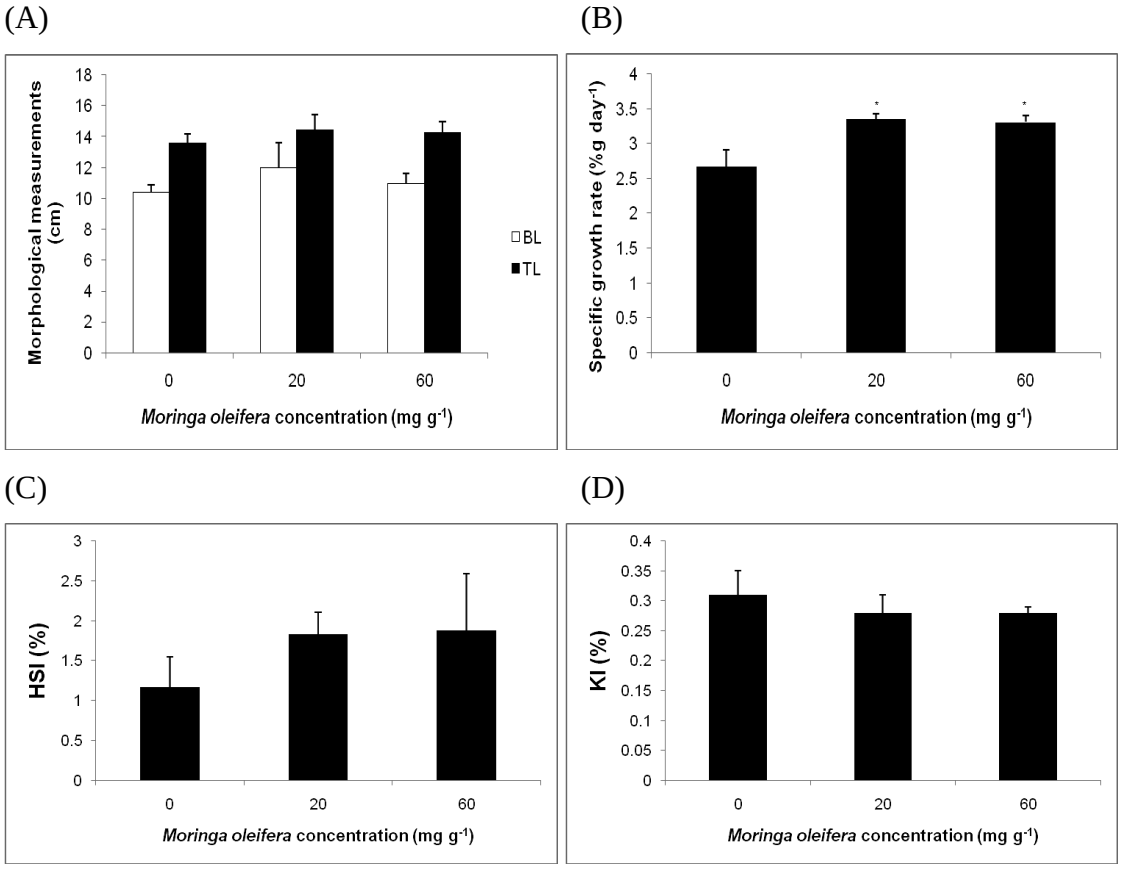


Figure 5.1 Effects of *M. oleifera*-supplemented diets on growth performance in *P. altus.*

Note:

BL = body length (cm);

TL = total length (cm);

SGR = specific growth rate (%g day^{-1}) = [ln final weight (g) – ln initial weight (g)]/ time (days) x100;

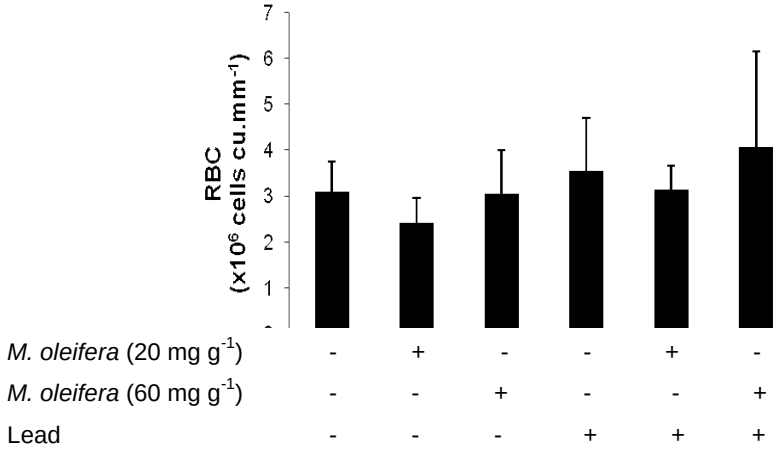
HSI = hepatosomatic index (%) = [liver weight/body weight] x100;

KI = kidney index (%) = [kidney weight/body weight]x100;

All values represent as mean ± SD N=84 (7 fish x duplicate)

* Indicates $p<0.05$ compared with control group (non-lead exposed group).

(A)



(B)

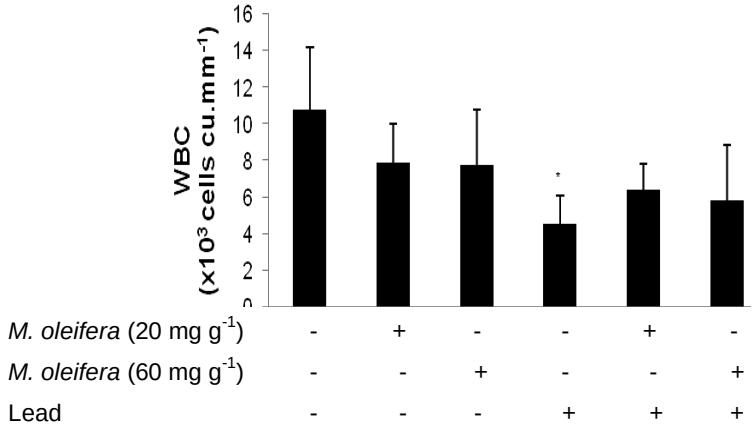
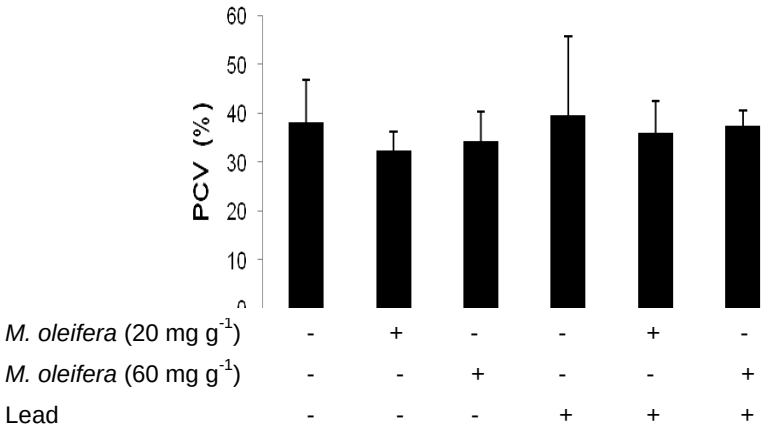


Figure 5.2 Effects of *M. oleifera*-supplemented diets on hematological variable in *P. altus* (n=84).

* Indicates $p<0.05$ compared with control group (non-lead exposed group).

(C)



(D)

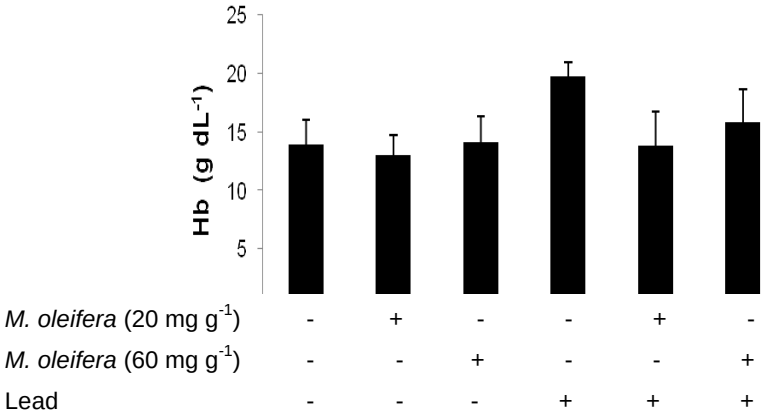


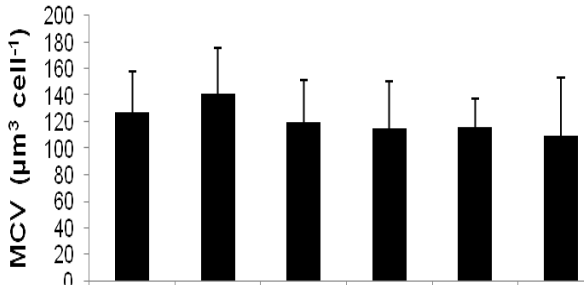
Figure 5.2 (cont.) Effects of *M. oleifera*-supplemented diets on hematological variable in *P. altus* (n=84).

* Indicates $p<0.05$ compared with control group (non-lead exposed group).

Figure 5.2 (cont.) Effects of *M. oleifera*-supplemented diets on hematological variable in *P. altus* (n=84).

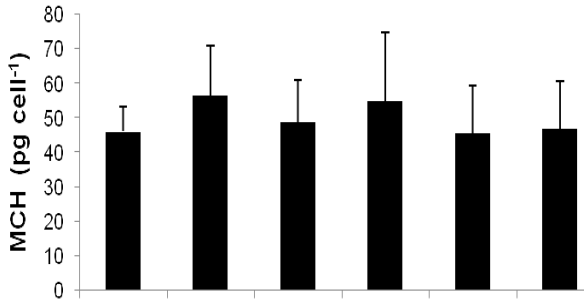
* Indicates $p<0.05$ compared with control group (non-lead exposed group).

(E)



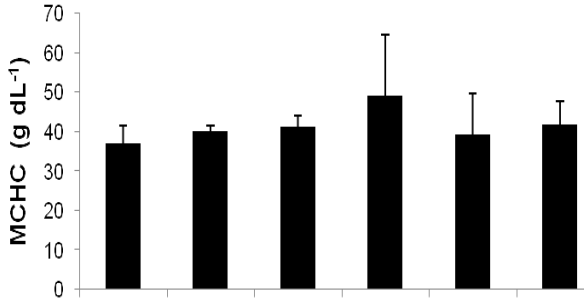
M. oleifera (20 mg g^{-1})	-	+	-	-	+	-
M. oleifera (60 mg g^{-1})	-	-	+	-	-	+
Lead	-	-	-	+	+	+

(F)



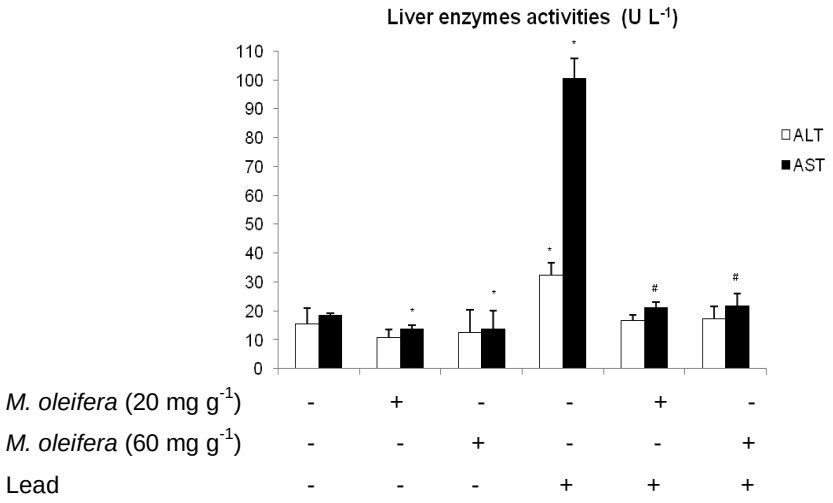
M. oleifera (20 mg g^{-1})	-	+	-	-	+	-
M. oleifera (60 mg g^{-1})	-	-	+	-	-	+
Lead	-	-	-	+	+	+

(G)



M. oleifera (20 mg g^{-1})	-	+	-	-	+	-
M. oleifera (60 mg g^{-1})	-	-	+	-	-	+
Lead	-	-	-	+	+	+

(A)



(B)

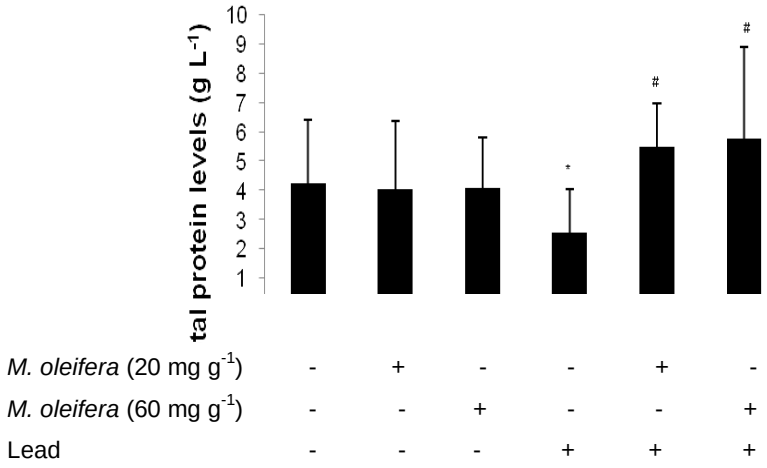
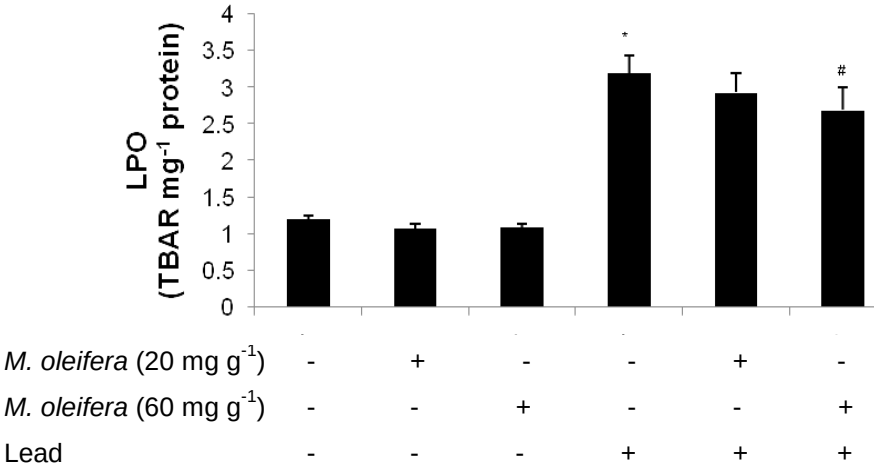


Figure 5.3 Effects of *M. oleifera*-supplemented diets on liver function related enzymes activities in *P. altus* (n=84).

* Indicates $p<0.05$ compared with control group (non-lead exposed group).

# Indicates $p<0.05$ compared with lead exposed group

(A)



(B)

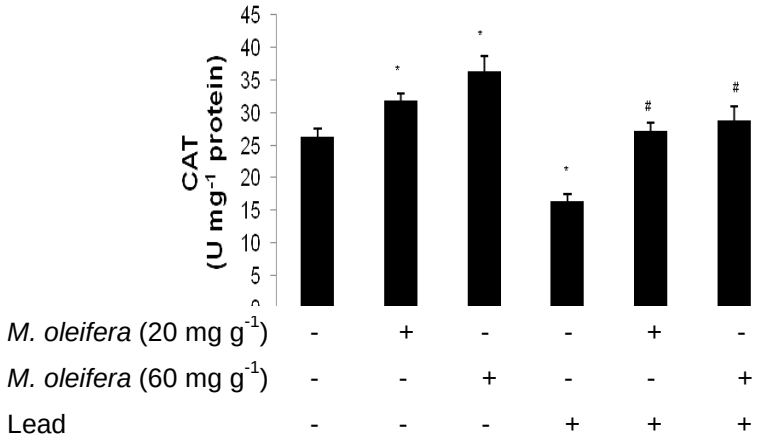


Figure 5.4 Effects of *M. oleifera*-supplemented diets on liver LPO and antioxidant enzymes activities in *P. altus* (n=84).

* Indicates $p<0.05$ compared with control group (non-lead exposed group).

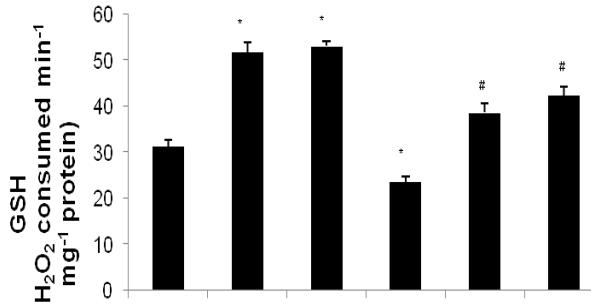
# Indicates $p<0.05$ compared with lead exposed group (C)

Figure 5.4 (cont.) Effects of *M. oleifera*-supplemented diets on liver LPO and antioxidant enzymes activities in *P. altus* (n=84).

* Indicates $p<0.05$ compared with control group (non-lead exposed group).

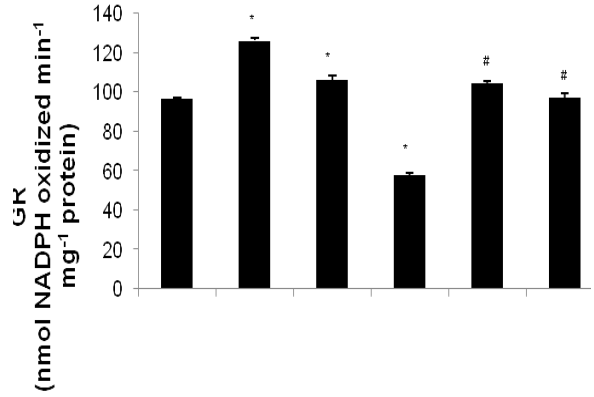
# Indicates $p<0.05$ compared with lead exposed group.

(C)



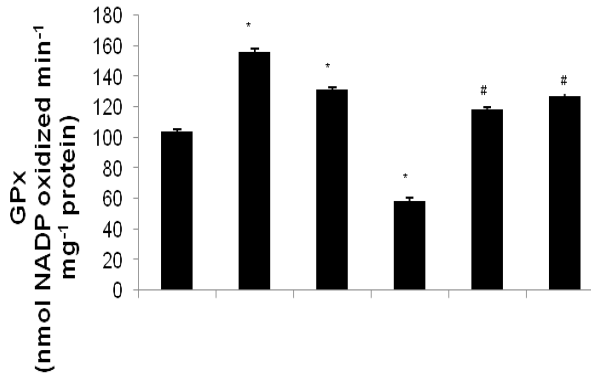
M. oleifera (20 mg g^{-1})	-	+	-	-	+	-
M. oleifera (60 mg g^{-1})	-	-	+	-	-	+
Lead	-	-	-	+	+	+

(D)



M. oleifera (20 mg g^{-1})	-	+	-	-	+	-
M. oleifera (60 mg g^{-1})	-	-	+	-	-	+
Lead	-	-	-	+	+	+

(E)



M. oleifera (20 mg g^{-1})	-	+	-	-	+	-
M. oleifera (60 mg g^{-1})	-	-	+	-	-	+
Lead	-	-	-	+	+	+

5.2 Histopathological examination of gill, liver, and kidney

5.2.1 Gills

Gills are the first target of waterborne lead toxicity due to their constant contact with external environment. As illustrated in Figure 5.5, the groups of fish with no lead exposure show normal gill structure. The gill appearance is consisted of primary gill filament and secondary gill filament, which are comprised of various cell types such as mucous cells that located along the gill filament [75]. Fish exposed to waterborne lead alone show the most alterations including increased filament thickness (Figure 5.6B), blood congestion at secondary filament (Figure 5.6C) and lifting of the outer layer of the lamellae epithelium with space under the epithelium (Figure 5.6C). In contrast, the gills of fish pre-administering with both dosages of *M. oleifera* diets (20 mg g^{-1} and 60 mg g^{-1}) show only mild changes which are thickening of primary gill filament and bedding of secondary gill filament (Figure 5.7A and Figure 5.7B). These results indicate that pre-administration of *M. oleifera* diets can protect the gill damages upon lead exposure.

We monitored whether *M. oleifera*-supplemented diet can influence the types of mucous cell in fish gill filament. To determine certain mucous cells, special staining method using combined alcian blue and periodic acid schiff (AB/PAS) was performed. AB was used to stain acid mucopolysaccharide mucous cell, which is detected in blue. PAS was used to stain neutral mucous cell, which is detected in magenta. Mucous cells play an important role in protecting fish against waterborne heavy metals such as lead [173]. The presence of difference types of mucous cell, especially acid mucous cells (contained sulfated and carboxylated mucoproteins), seems very important for fish, living in a stressor environment [174, 175]. An increased in mucous cell containing acidic mucin is bound or trapped with cation metal resulting in reduction of it absorption [176, 177]. Here we found that neutral mucous cells were mainly observed in the gill filament with non-lead exposure (Figure 5.8). As prior to non-lead exposure, neutral mucous cell continue to be found in the group of fish with lead exposure (Figure 5.9A). Interestingly, several acid mucous cells were detected in fish pre-administered with *M. oleifera*-supplemented diets (Figure 5.9B and Figure 5.9C). This indicated that the protective effects of *M. oleifera* diets to the gill could be due to

the defense mechanism of acid mucous cell that is located along the gill filament against water born lead exposure.

5.2.2 Liver

The liver plays a primary role in the metabolism and excretion of lead [7, 178]. Lead exposure induces hepatic enzyme activities and can lead to histopatological changes in the liver [179] As seen in Figure 5.12A and Figure 5.12B, administration of either 20 mg g^{-1} or 60 mg g^{-1} of *M. oleifera* alone had no affect on liver structure as the fish display normal hepatocytes and nuclei. Hepatocytes are located among sinusoids forming cord-like structures, known as hepatic cell cords. Many of blood sinusoids were observed and separated the hepatic cords one from another. Exposure of fish to waterborne lead exhibited various changes such as abundant cytoplasmatic vacuolation and hepatic cord disorganization with scatter of hypertrophy hepatocytes nuclei (Figure 5.13). In contrast, with pre-treatment of 20 mg g^{-1} or 60 mg g^{-1} of *M. oleifera* before lead exposure, the liver of fish showed significant reduction in hepatic pathological alterations induced by lead intoxication (Figure 5.14A-D). Similar to the results observed in gill tissue, pre-treatment of *M. oleifera*-supplemented diets before lead exposure can protect hepatic changes due to lead-induced hepatotoxicity.

The liver also serves as storages of energy, glycogen and lipids [87, 88]. Glycogen is depleted in response to various physiological processes such as sexual maturation, temperature, or in response to environmental toxicants such as heavy metals exposure [180, 181]. To detect and quantify the amount of glycogen in the cytoplasm of hepatocytes, the periodic acid-Schiff (PAS) staining was performed. Non-lead exposed fish revealed glycogen staining remained constant in all groups (group1, 3, and 5) (Figure 5.15A, Figure 5.16A and Figure 5.16C). Exposure of fish to waterborne lead showed a dramatic reduction in glycogen content (Figure 5.15B). Remarkably, fish pre-administered with both 20 mg g^{-1} and 60 mg g^{-1} of *M. oleifera* before lead treatment showed only a slight reduction in glycogen levels (Figure 5.16B and Figure 5.16D). Overall, *M. oleifera* as dietary supplementation appears to protect the loss of glycogen content in the liver from lead-induced hepatotoxicity.

5.2.3 Kidney

The kidney of fish is comprised of three distinctive systems; endocrine, hematopoietic, and excretory systems [182]. As in all vertebrates, kidney is one of the major targets of waterborne lead because it is a major route for the excretion of lead [182]. The administration of either 20 mg g^{-1} or 60 mg g^{-1} of *M. oleifera* alone had no affect on kidney structure as the kidney showed normal structure of glomerulus and renal tubules (Figure 5.18A and Figure 5.18C). Exposure of fish to waterborne lead alone showed the most alterations including glomerulus atrophy and an increase in the number of lymphocytes in the parenchyma. Cloudy swelling, tubular narrowing and hyaline droplet were also found in the renal tubule of lead-exposed fish (Figure 5.17A). In contrast, fish pre-administered with both 20 mg g^{-1} and 60 mg g^{-1} of *M. oleifera* showed decreases in renal pathological changes induced by lead exposure (Figure 5.18B and Figure 5.18D).

The semi-quantitative scoring of the gill, liver, and kidney lesion are summarized in Table 5.1. Overall, our findings here indicate that pre-administration of fish with *M. oleifera*-supplemented diets can protect the fish from the gill, liver, and kidney changes due to lead-induced toxicity.

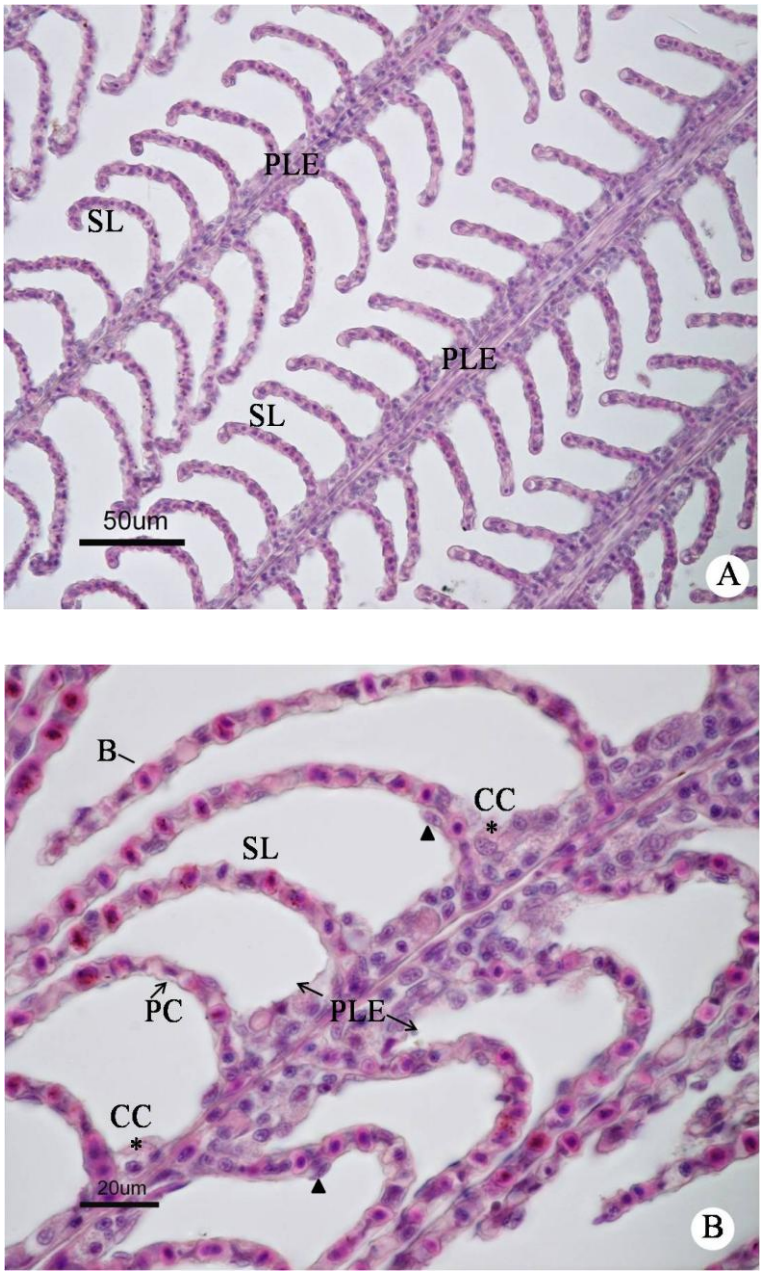


Figure 5.5 Representative of light micrographs of gill filament in *P. altus* illustrating basic gill structure *(H&E stain)*

A Overall structure of the gill containing primary gill filament (PLE) and secondary gill filament (SL). *Scale bar = 50 µm.*

B Cross section of the gill showing typical morphology of pillar cells (PC), chloride cells (CC), blood channel (B), and pavement cells (squamous epithelial cells: ▲) *Scale bar = 20 µm.*

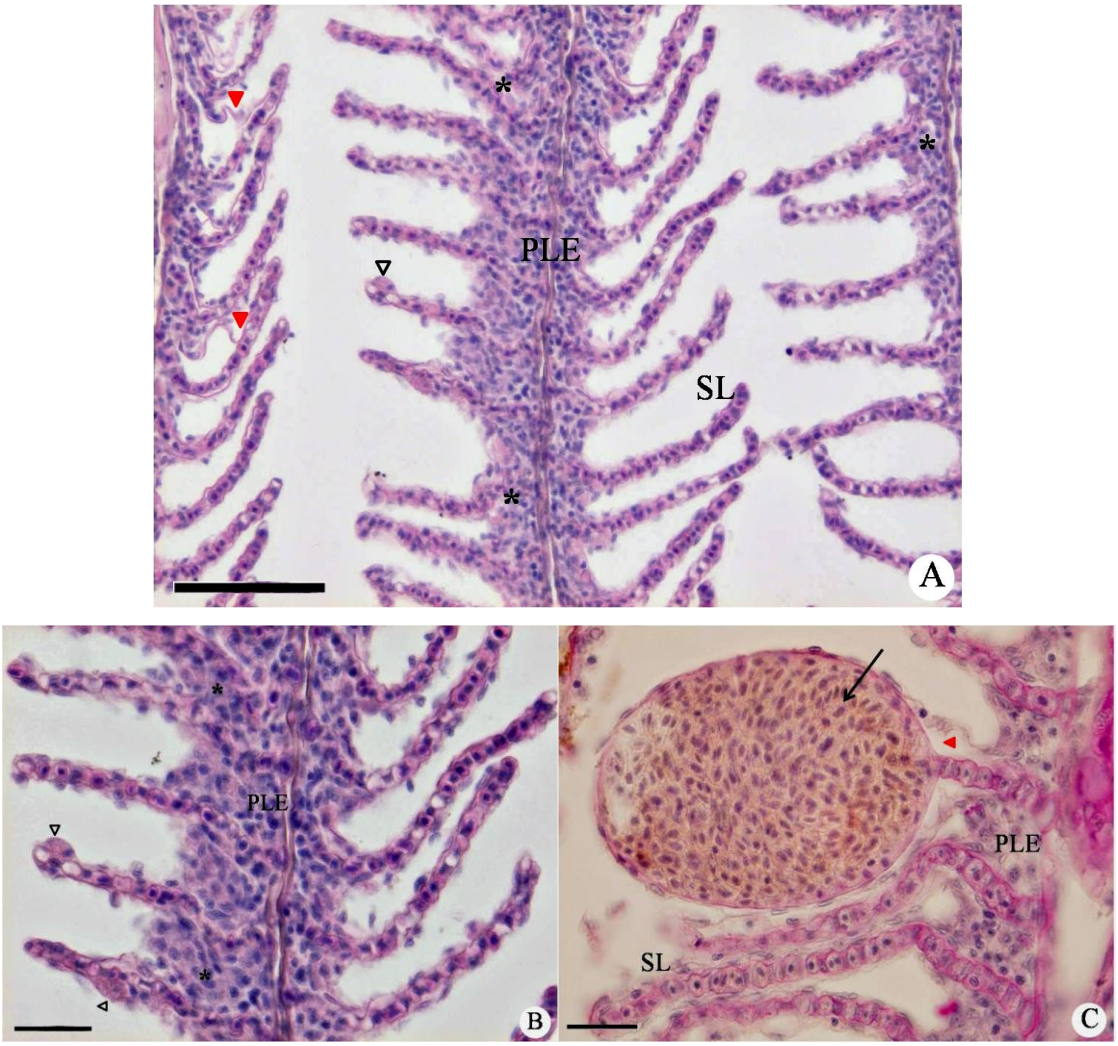


Figure 5.6 Histological changes of the gill of *P. altus* showed gill alterations *(H and E stain)*

(A) Low magnification of lead-exposed fish showed an increase of the primary filament thickness (*), hypertrophy of the lamellae epithelium (Δ), and and epithelial lifting (▲) *Scale bar = 50 µm.*;

(B) High magnification of lead-exposed fish showed hypertrophy of the lamellae epithelium (Δ) *Scale bar = 20 µm*;

(C) High magnification of lead-exposed fish showed vascular congestion or aneurisms (↓) at secondary gill filament and epithelial lifting (▲) *Scale bar = 20 µm.*

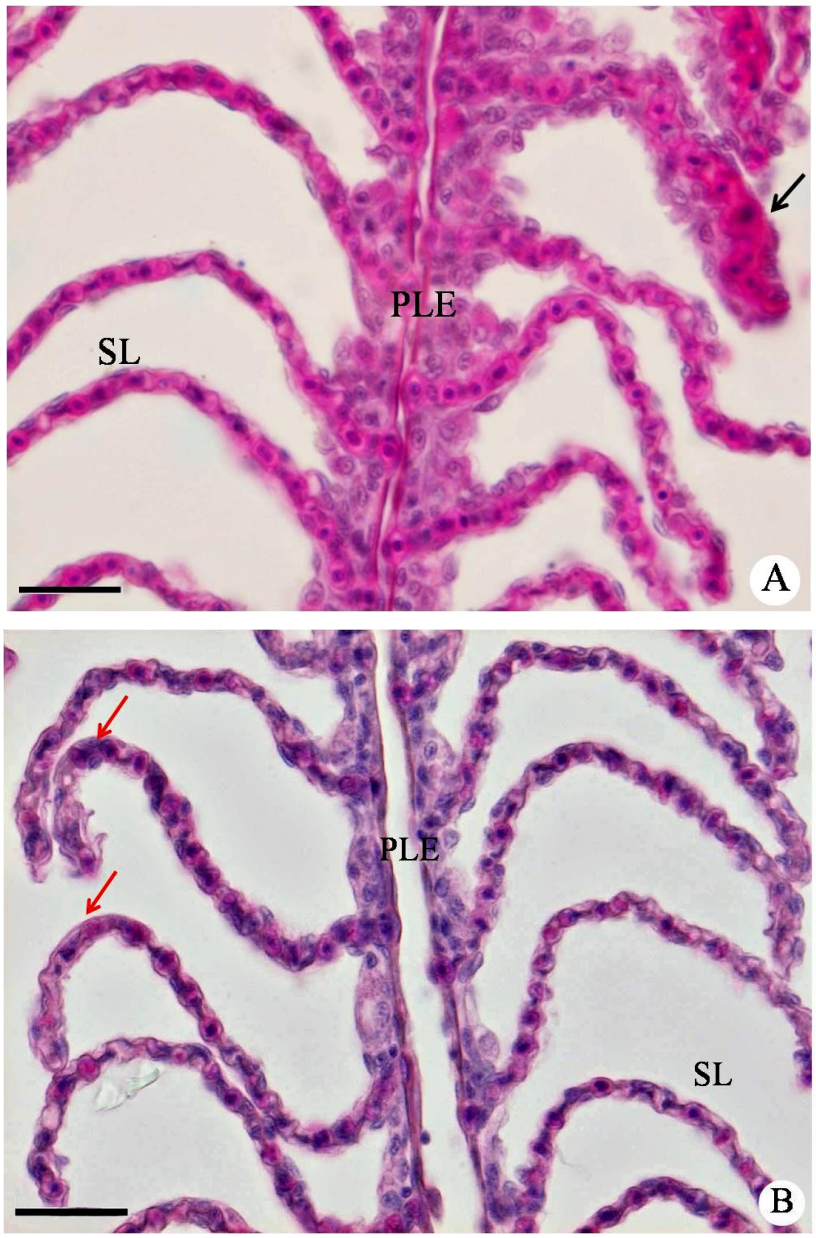


Figure 5.7 Histological changes of the gill of *P. altus* showed gill alterations *(H and E stain)*

(A) Fish fed with 20 mg g^{-1} of *M. oleifera* before lead exposure showed partial vascular congestion or aneurisms (↓ *Scale bar = 20 µm*;

(B) Fish fed with 60 mg g^{-1} of *M. oleifera* before lead exposure showed bend of secondary filament (↓) *Scale bar = 20 µm.*

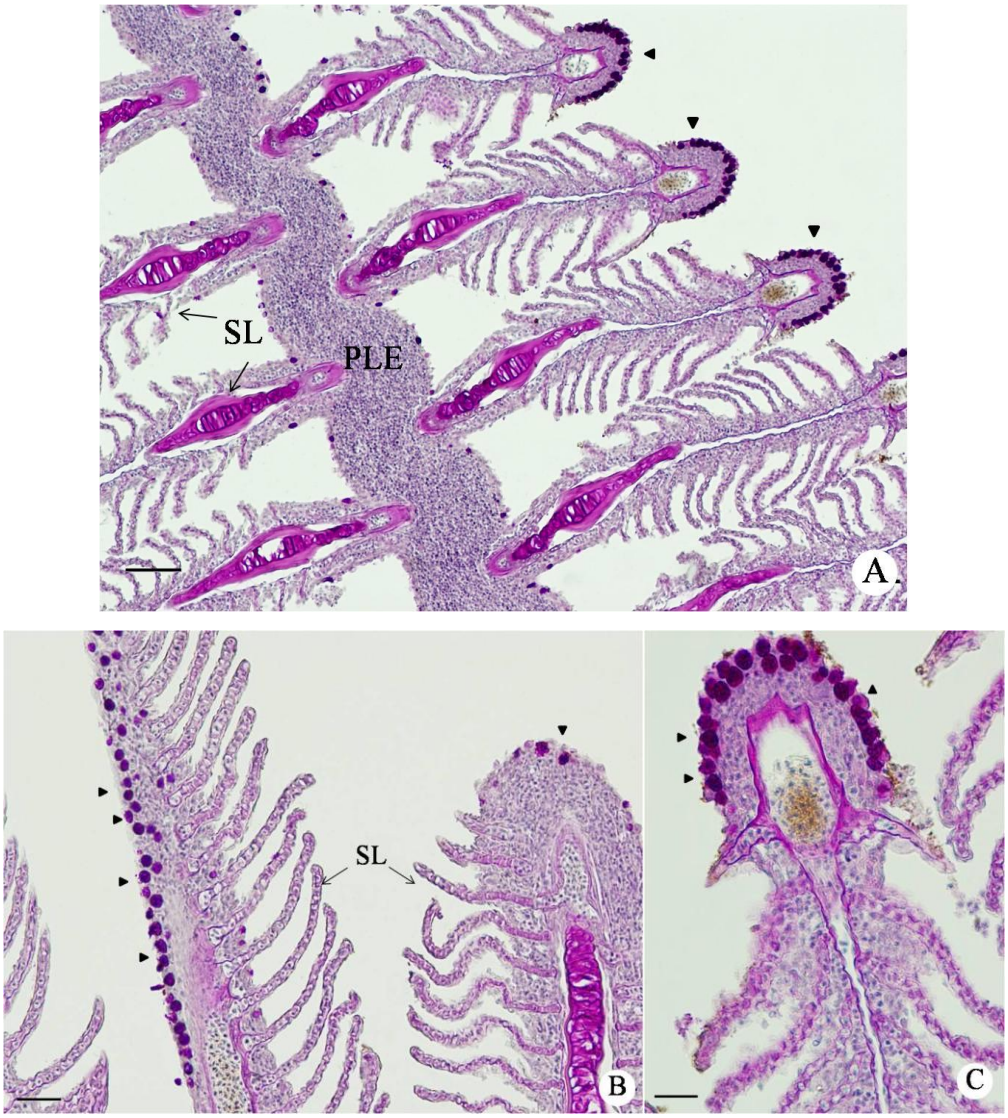


Figure 5.8 Representative of light micrographs of gill filament in *P. altus* in controlled fish (Non-lead exposed) illustrating a number of neutral mucus cells (▲) *(AB/PAS stain)*

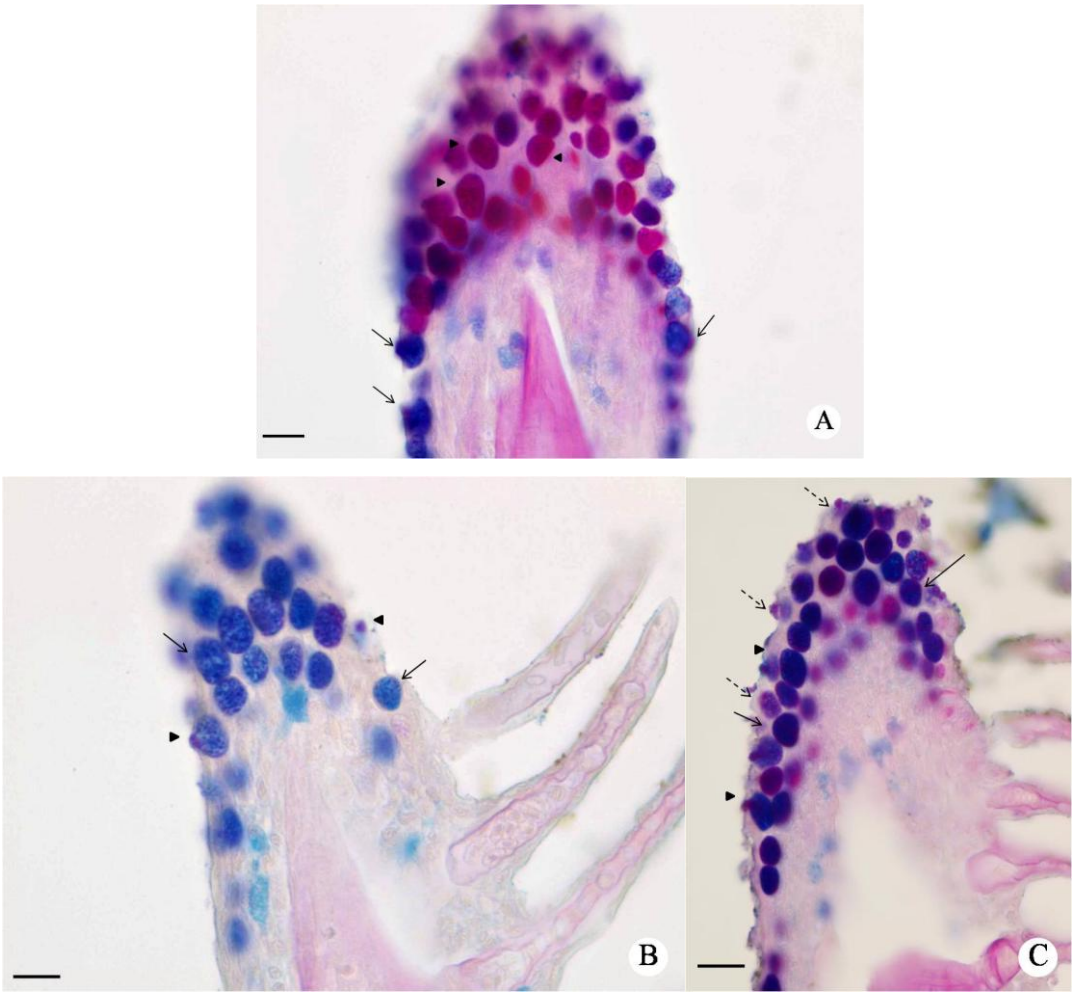


Figure 5.9 Higher magnification view of the gill filament of *P. altus* showed certain mucous cell types *(AB/PAS stain)*

(A) Lead exposed fish showed neutral mucous cells (▲) and scattering of acid mucous cells along gill filament (→);

(B) Lead exposed fish supplemented with 20 mg g^{-1} of *M. oleifera* mainly showed acid mucous cells (▲);

(C) Lead exposed fish supplemented with 60 mg g^{-1} of *M. oleifera* showed acid mucous cells (▲) and neutral-acid mucous cells ( ). *Scale bar = 20 µm.*

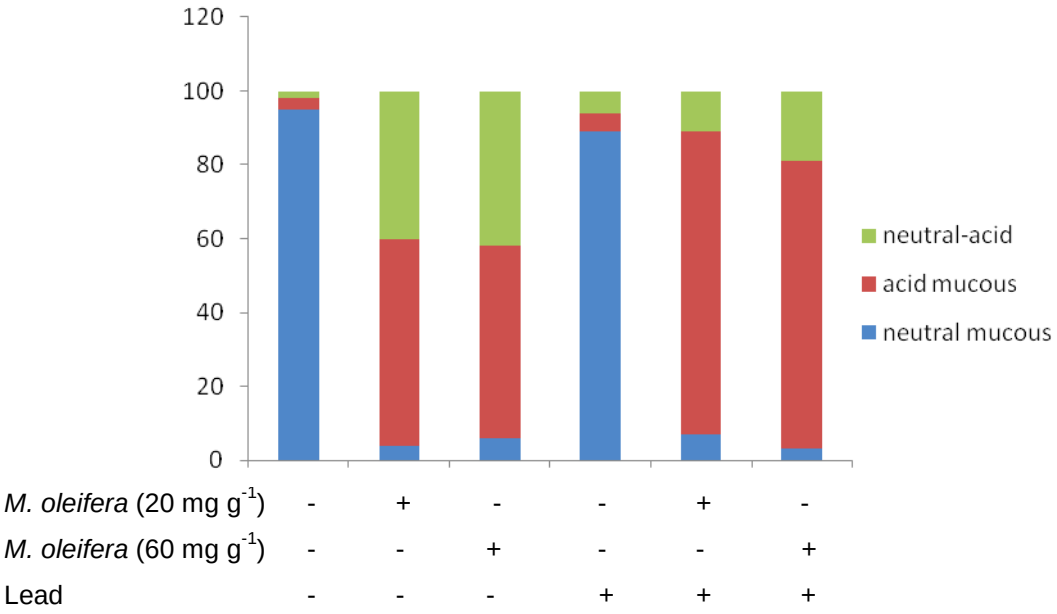


Figure 5.10 Percentage neutral, acidic, and neutral-acidic mucous cells of the gill filament of *P. altus*. Data are expressed as percentage of the total number of mucous cell quantified.

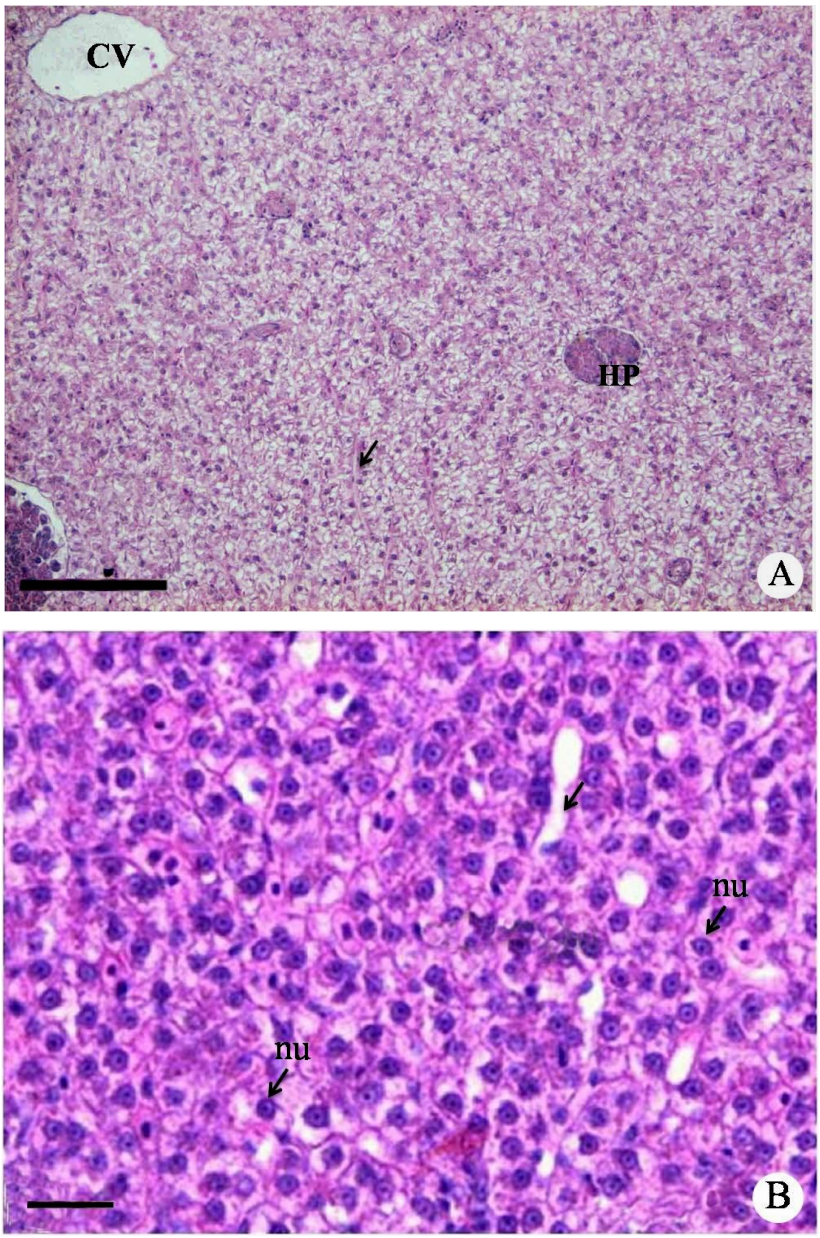


Figure 5.11 Representative of light micrographs of the liver of *P. altus* (*H&E stain*)
(A) Low magnification of non-lead-exposed fish showed hepatocytes with sinusoidal lumen (→), central vein (CV), and hepatopancrease (HP) *Scale bar = 50 µm.*;
(B) High magnification of non-lead-exposed fish showed, showed hepatocyte nucleus (nu) with sinusoidal lumen (→) *Scale bar = 20 µm.*

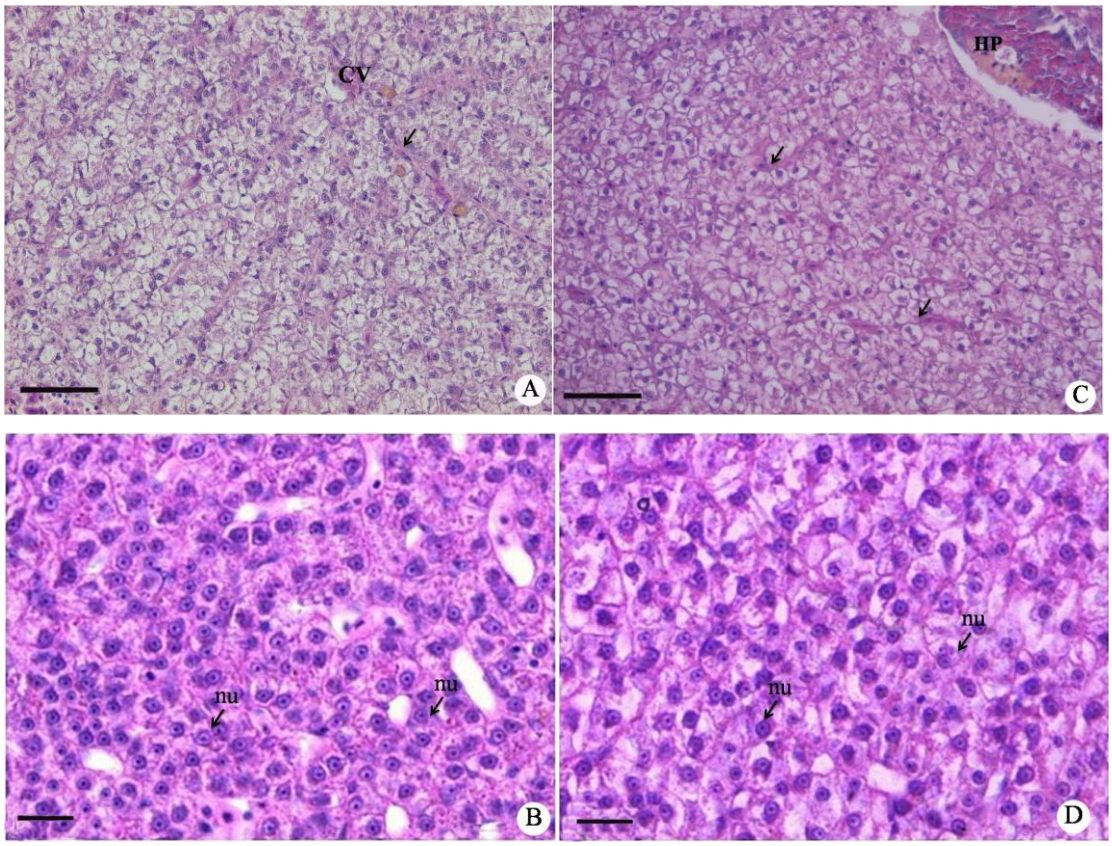


Figure 5.12 Representative of light micrographs of the liver of *P. altus* (*H&E stain*) (A and C) Fish fed with 20 mg g^{-1} and 60 mg g^{-1} of *M. oleifera* showed normal hepatocytes with sinusoidal lumen (→) and central vein (CV) *Scale bar = 50 µm*; (B and D) Hepatocytes are located among sinusoidal lumen (hepatocyte nucleus: nu) *Scale bar = 20 µm.*

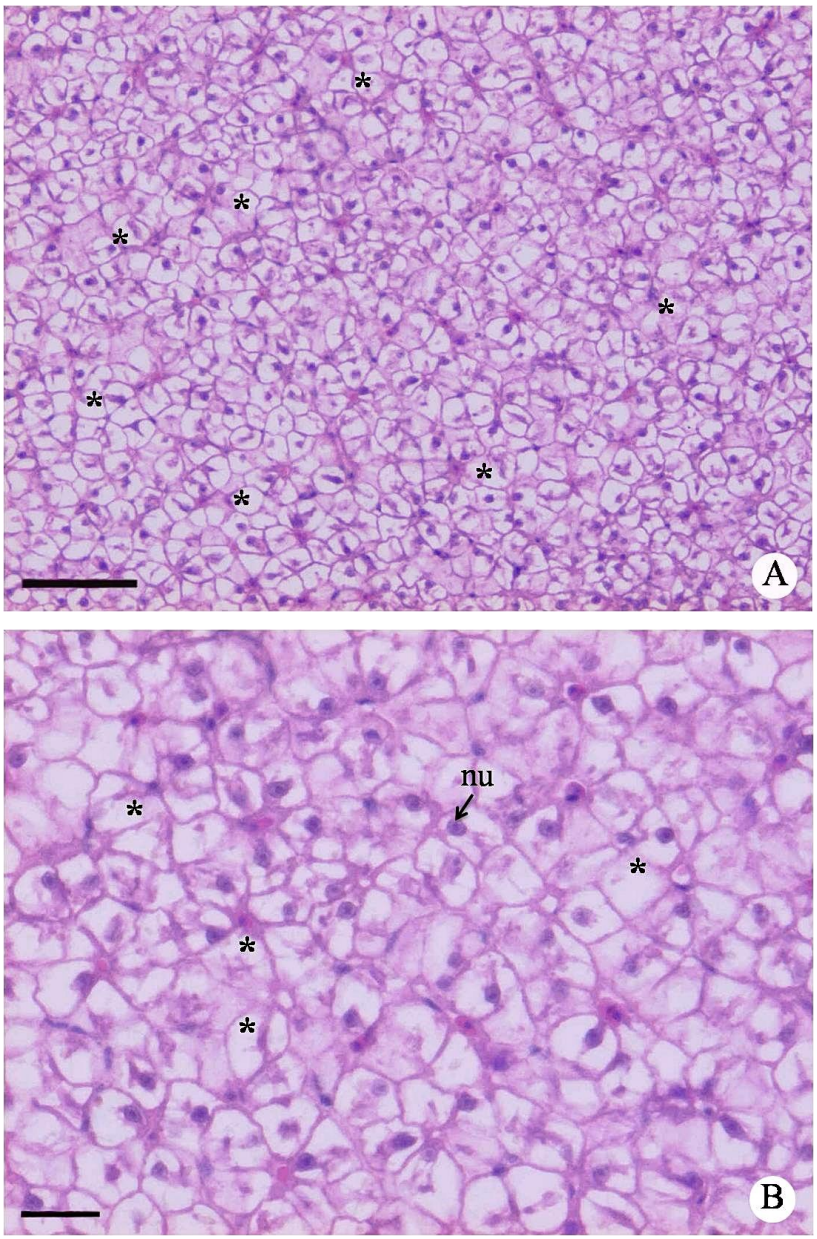


Figure 5.13 Representative of light micrographs of the liver of *P. altus* (*H&E stain*)
(A) Lead-exposed fish showed an abundant of cytoplasmatic vacuolation (*) and hepatic cord disorganization *Scale bar = 50 µm*;
(B) Lead-exposed fish showed hepatocyte nucleus (nu) with sinusoidal lumen (→) *Scale bar = 20 µm.*

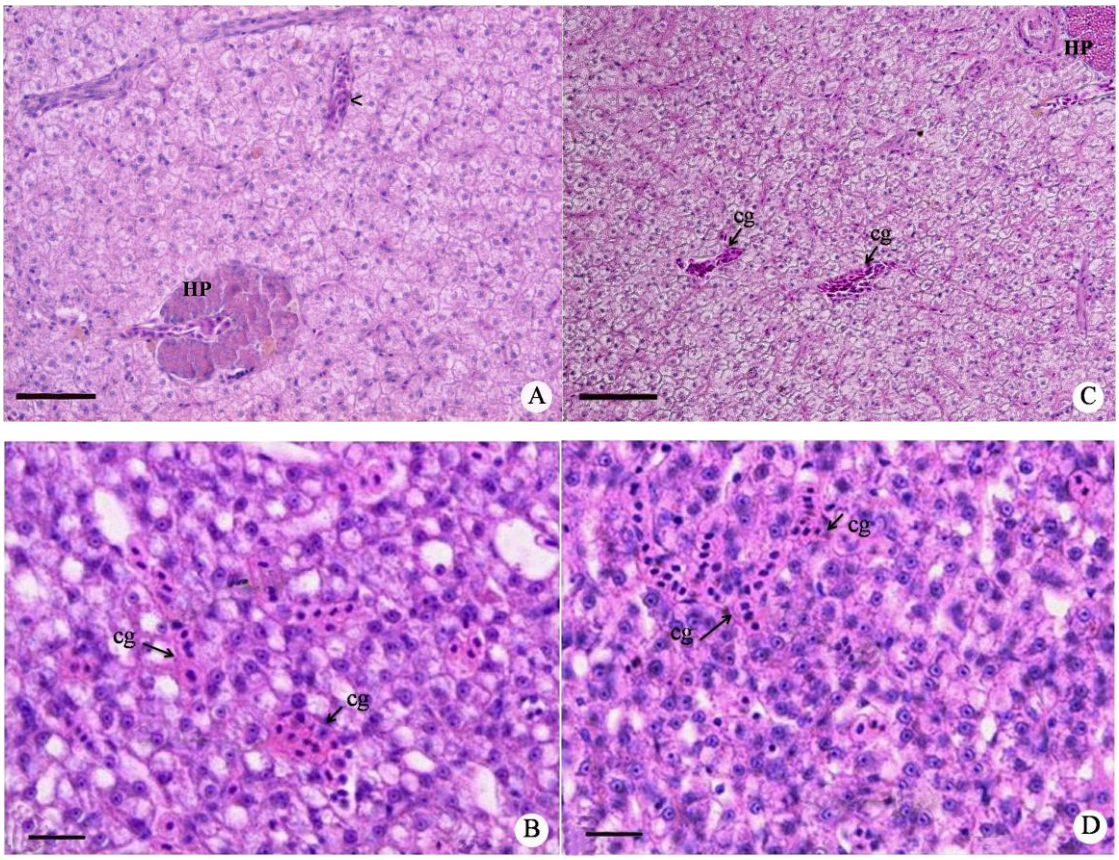


Figure 5.14 Representative of light micrographs of the liver of *P. altus* with lead exposure (*H&E stain*)

(A) Fish fed with 20 mg g^{-1} of *M. oleifera* showed normal hepatocytes with sinusoidal lumen (→) and central vein (CV);

(B) High magnification;

(C) Fish fed with 60 mg g^{-1} of *M. oleifera* showed normal hepatocytes with sinusoidal lumen (→) and central vein (CV);*Scale bar = 100 µm.*

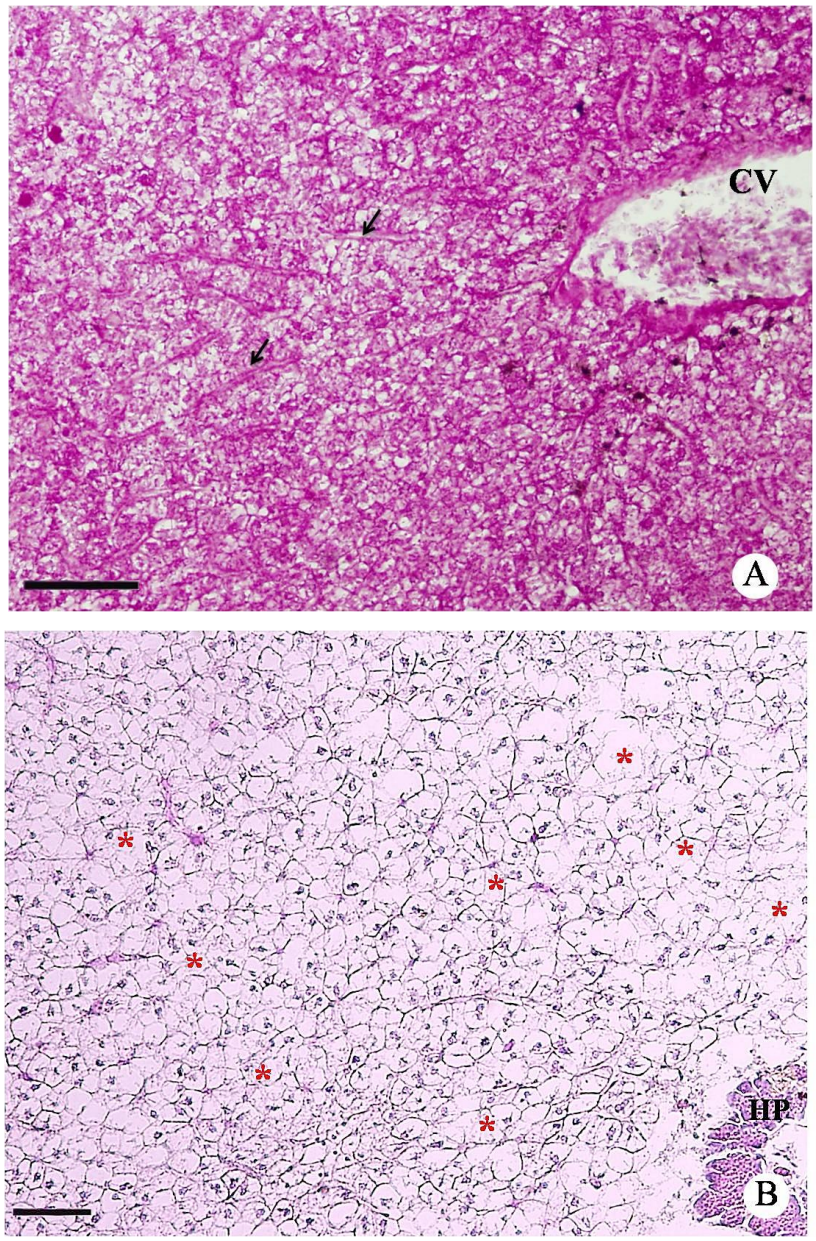


Figure 5.15 Representative of light micrographs of the liver of *P. altus* (*PAS stain*)
(A) Non-lead exposed fish showed glycogen content represented as purple fine granule in the cytoplasm of hepatocyte;
(B) Lead-exposed fish showed hepatocytes with reductions of glycogen.
Note cytoplasmatic vacuolation (∗); central vein (CV); hepatopancrease (HP). *Scale bar = 100 µm.*

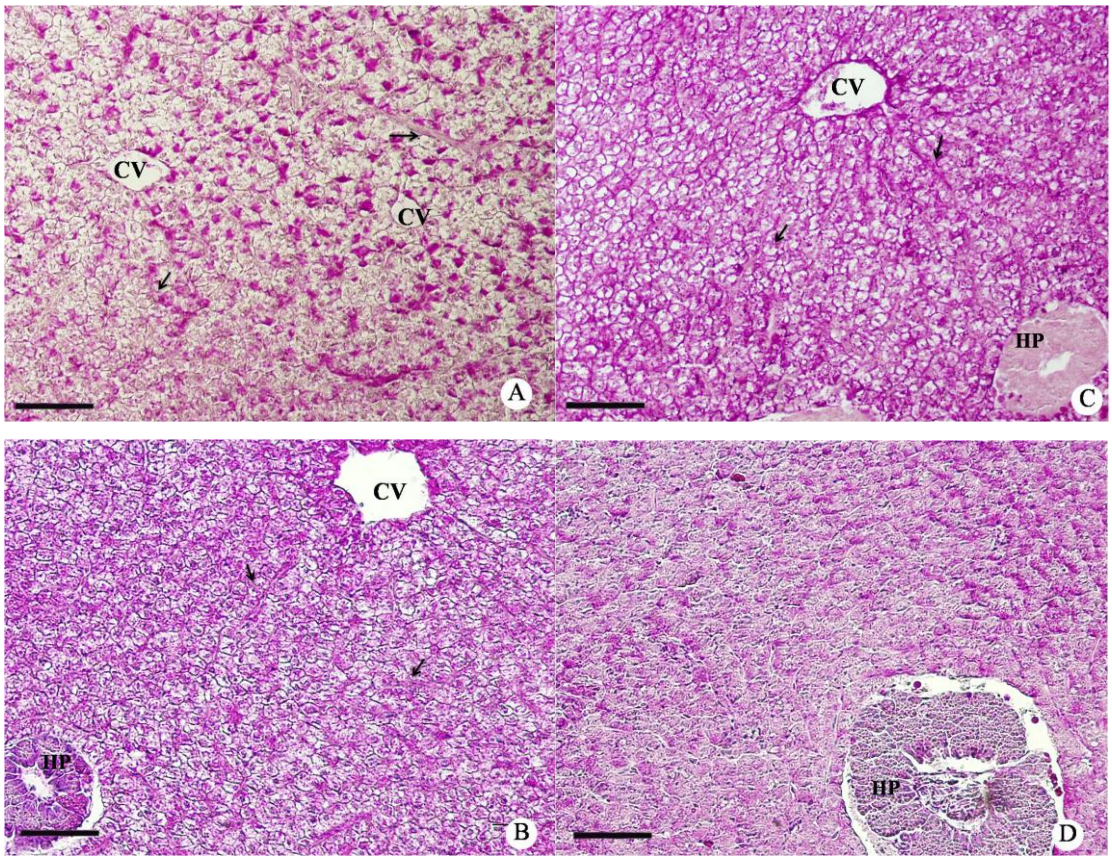


Figure 5.16 Representative of light micrographs of the liver of *P. altus* (*PAS stain*)
(A and C) Non-lead exposed fish fed 20 and 60 mg g^{-1} of *M. oleifera*, respectively, showed glycogen content represented as purple fine granule in the cytoplasm of hepatocyte.
(B and D) Lead-exposed fish showed hepatocytes with reductions of glycogen.
Note cytoplasmatic vacuolation (∗); blood cell congestion (→); hepatic vessel (v); hepatopancrease (HP). *Scale bar = 100 µm.*

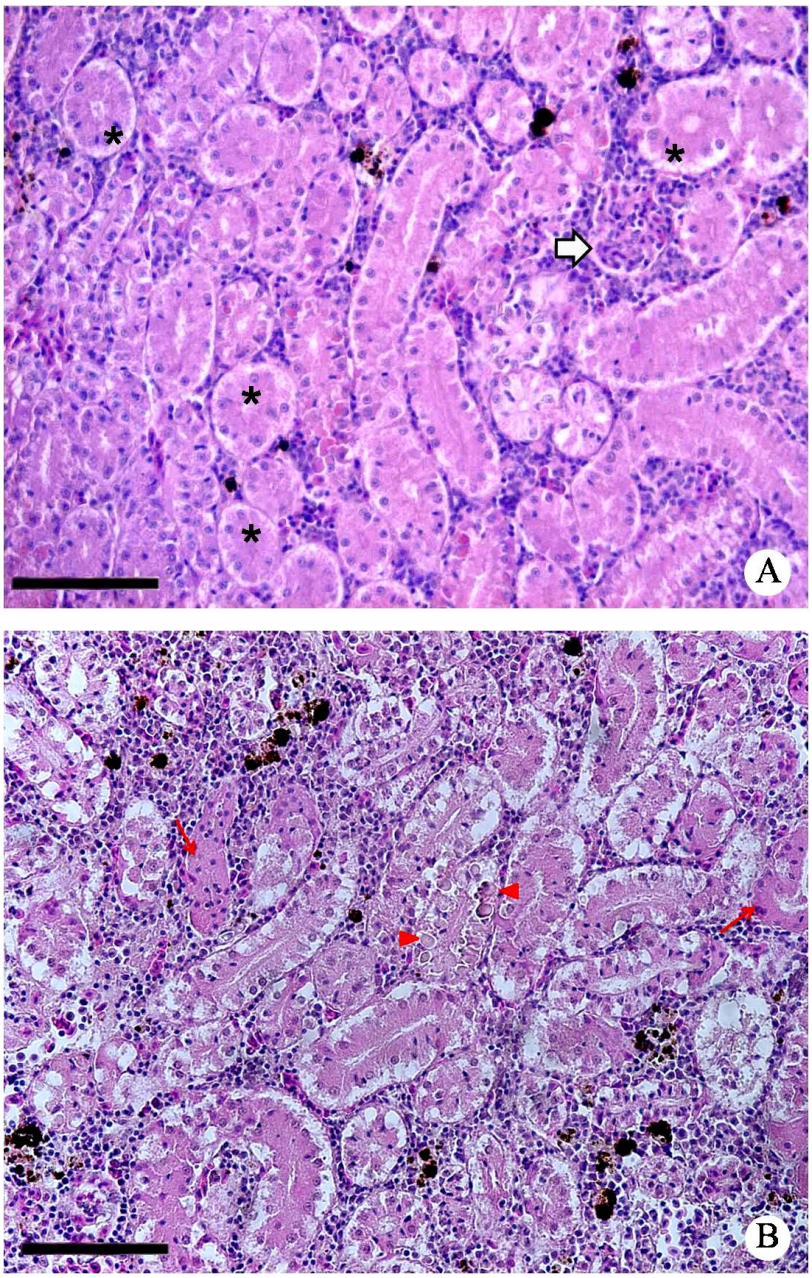


Figure 5.17 Representative of light micrographs of kidney of *P. altus* (*H&E stain*)
(A) Non-lead exposed fish showed normal renal corpuscle, glomerulus, Bowman's space (arrow) and renal tubule (*)
(d) Lead-exposed fish showed renal tubule cells with hypertrophy nucleus, cloudy swelling degeneration (→) and tubule with hyaline droplet (Δ);

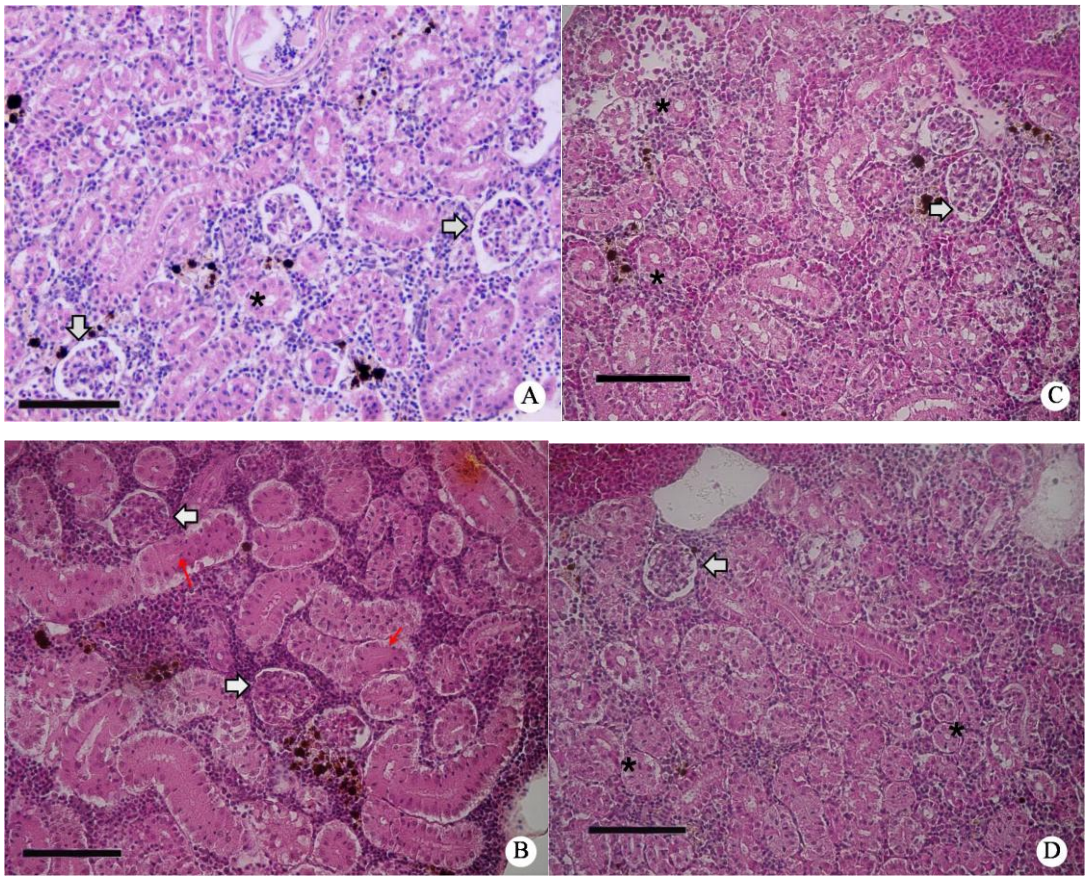


Figure 5.18 Representative of light micrographs of kidney of *P. altus* (*H&E stain*)
(A and C) Non-lead exposed fish showed normal renal corpuscle, glomerulus, Bowman's space (arrow) and renal tubule (*) in fish fed 20 mg g^{-1} and 60 mg g^{-1} of *M. oleifera* groups, respectively;
(B and D) Lead exposed fish that were pre-treated with 20 mg g^{-1} and 60 mg g^{-1} of *M. oleifera* pre-treated) showed mild cloudy swelling with cellular occlusion of the tubular lumen and slight melano-macrophages aggregation (white arrow). *Scale bar = 100 µm.*

Note: The lesion was evaluated semi-quantitatively by ranking tissue lesion severity. Ranking from – to +++ depending on the degree and extent of the alteration as follows: (-) no histopathology, (+) histopathology in <20% of fields; (++) histopathology in 20 to 60% of fields; (+++) histopathology in >60% of fields. Five slides were observed from each organ and treatment [183].

Group 1: control diet

Group 2: control diet + Pb exposure

Group 3: 20 mg g^{-1} of *M. oleifera*

Group 4: 20 mg g^{-1} of *M. oleifera* + Pb exposure

Group 5: 60 mg g^{-1} of *M. oleifera*

Group 6: 60 mg g^{-1} of *M. oleifera* + Pb exposure

Table 5.1 Histopathologic analysis of gill, liver, and kidney of *P. altus*.

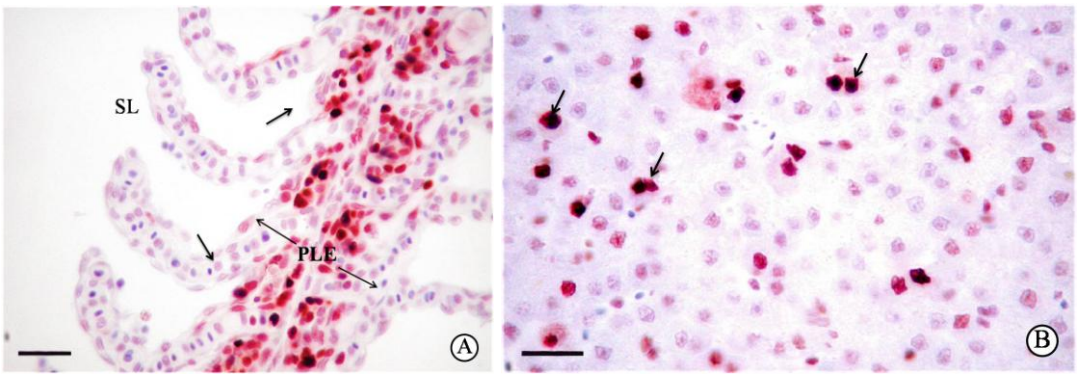
Tissue and histopathology	**Groups**					
	1	2	3	4	5	6
Gill						
Primary filament hyperplasia	+	+++	-	+	-	-
Lamella hypertrophy	-	++	-	+	-	-
Epithelial lifting of lamellae	-	+++	-	+	-	-
Kidney						
Cloudy swelling	-	+++	-	+	-	-
Hyaline droplet degeneration	-	++	-	+	-	-
Liver						
Pyknotic nucleus	-	+	-	-	-	-
Cytoplasmatic vacuolation	+	+++	+	+	+	+
Focal necrosis	-	+	-	-	-	-

5.3 Immunohistochemical analyses

Lead has been reported to induce proliferations of liver cells and epithelium cells of the kidney [184, 185]. To address whether protective role of *M.oleifera* diet is associated with the change in cell proliferation induced by lead, proliferating cell nuclear antigen (PCNA) was monitored.

PCNA-positive cells with brown stained nuclei were detected in primary gill filament (Figure 5.19A). Non-lead exposed fish showed less than 10% PCNA-positive cells. Fish exposed with waterborne lead alone displayed nearly 40% PCNA-positive cells in the filament epithelium. Whereas, pre-administration of either 20 or 60 mg of *M. oleifera* remarkably decreased the percentage of PCNA-positive cells in fish gill (Figure 5.19C).

PCNA-positive nuclei were also examined in the liver cells (Figure 5.19B). As like in the gill, the number of liver PCNA-positive cells was found increased in fish treated with lead only and was reduced with *M. oleifera* pre-administration. These results indicate that pre-administration of 20 and 60 mg of *M. oleifera* can effectively reduce cell proliferation induced by lead in both gills and liver.



C

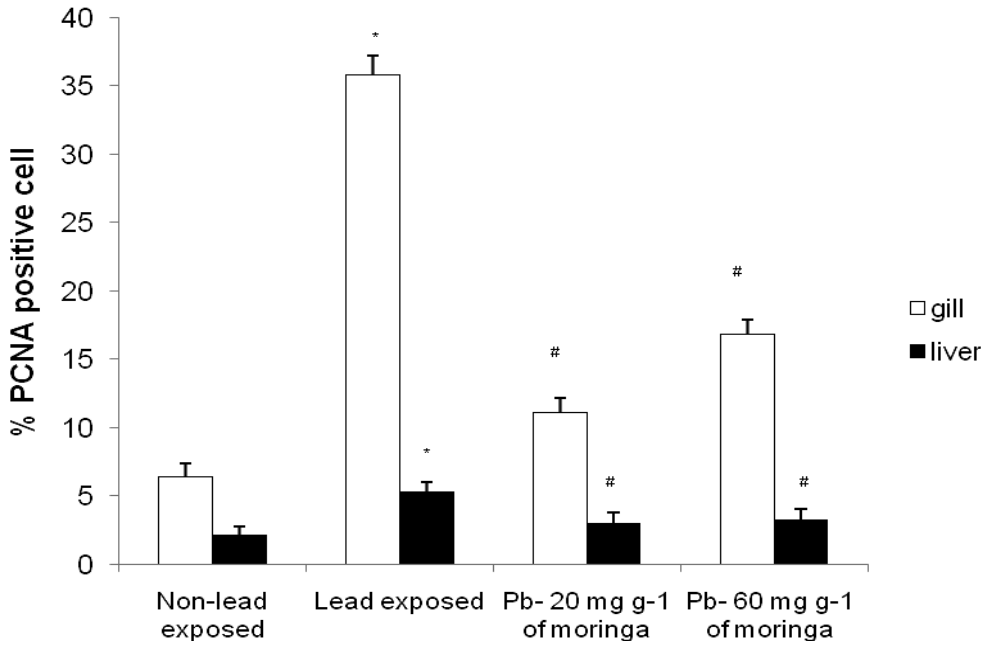


Figure 5.19 Immunohistochemical staining patterns for PCNA in gill and liver. Section was immunostained with anti-PCNA monoclonal antibody. Signal detection was accomplished with DAB (brown, positive signal) and 100 cells were evaluated for PCNA labeling.

(A) High number of PCNA-positive nuclei in the primary gill filaments, *Scale bar = 20 µm*;

(B) Low number of PCNA-positive nuclei in hepatocytes, which scattered around liver lobules, *Scale bar = 20 µm*;

(C) Percentage of PCNA-positive cells in the gill and the liver. Sections were examined and the results are shown as mean±sd.

* Indicates $p<0.05$ compared with control group (non-lead exposed group);

# Indicates $p<0.05$ compared with lead exposed group.

5.4 *In vitro M. oleifera* leaves antioxidant studies

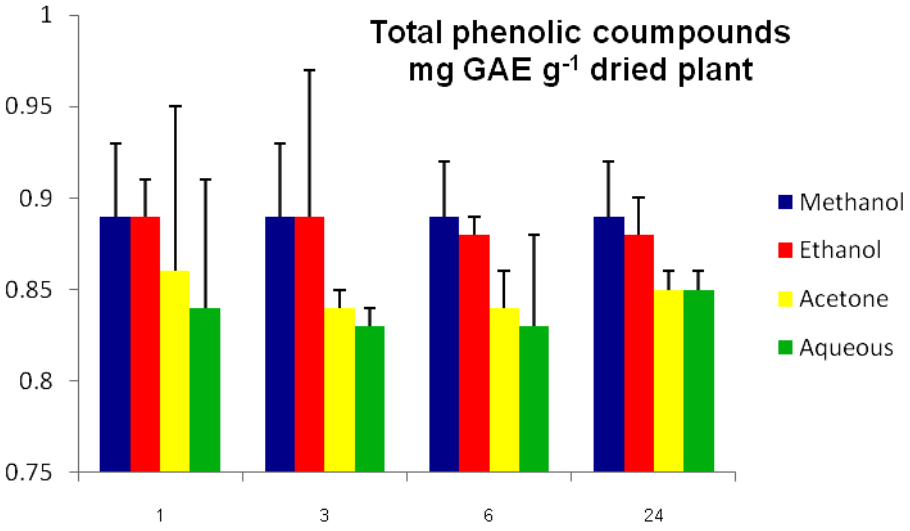
M. oleifera has been recognized to have broad-spectrum of therapeutic functions such as hepatoprotective [165], hypolipidemic, and anti-atherosclerotic properties [14, 143, 167]. In this study, we investigated the antioxidant activities of the extracts from *M. oleifera* leaves through various measurement methods as described in chapter IV.

The antioxidant activity of water, acetone, ethanol, and methanol *M.oleifera* leaves extracts revealed the presence of phenolic and flavonoid contents which have been shown to have antioxidant activities. Figure 5.20 shows the total phenolic and flavonoid contents of each extracted sample. Most of the extracts display antioxidant activities with best activities found in the methanolic extract of *M. oleifera* leaves. The highest concentration of total phenolics was observed in methanolic and ethanol extracts (Figure 5.20A).

Methanolic leaves extract of *M. oleifera* also exhibited the highest flavonoids concentration followed by those in ethanol extract, acetone extract and aqueous extract (Figure 5.20B).

Two methods were used to determine the antioxidant capacity of *M. oleifera* leaves; reducing power (RP) and DPPH radical scavenging activity (DPPH) radical scavenging assay. RP was used for a determination of ferric reducing activity based on reduction of a ferric tripyridyltriazine complex to its ferrous colored form [164]. Increasing in the absorbance at 700 nm indicates an increment of antioxidant capacity. As seen in Figure 5.21A, ethanolic extract possessed the highest RP followed by acetone extract, aqueous extract, and methanol extract, respectively. The DPPH method was used for a rapid screening of radical scavenging in complex mixtures [163]. The greatest free radical scavenging activity was also found in 24 hour-ethanol extract (40.23%), followed by methanol extract (32.92%), aqueous extract (31.07%) and acetone extract (23.25%) (Figure 5.21B). These findings indicate that the antioxidant activity of *M. oleifera* extracts is different depending on the solvent used.

(A)



(B)

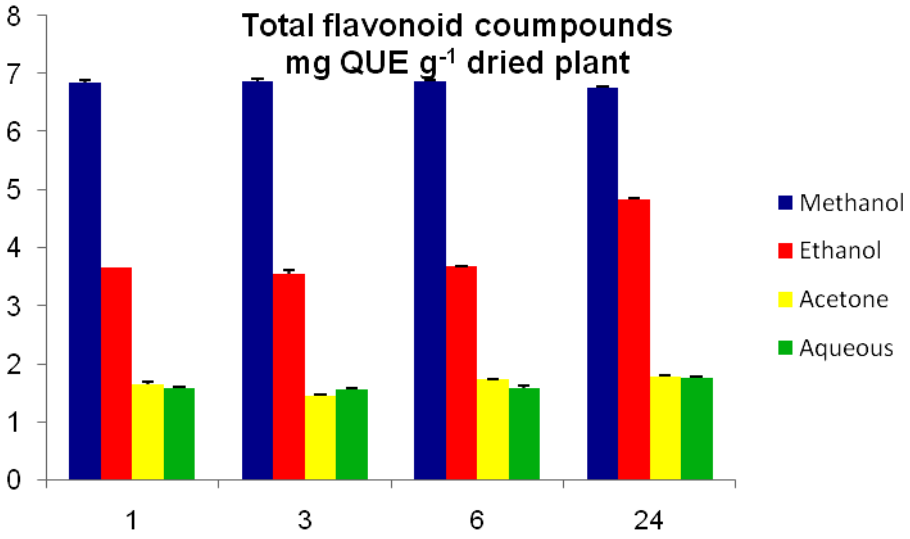
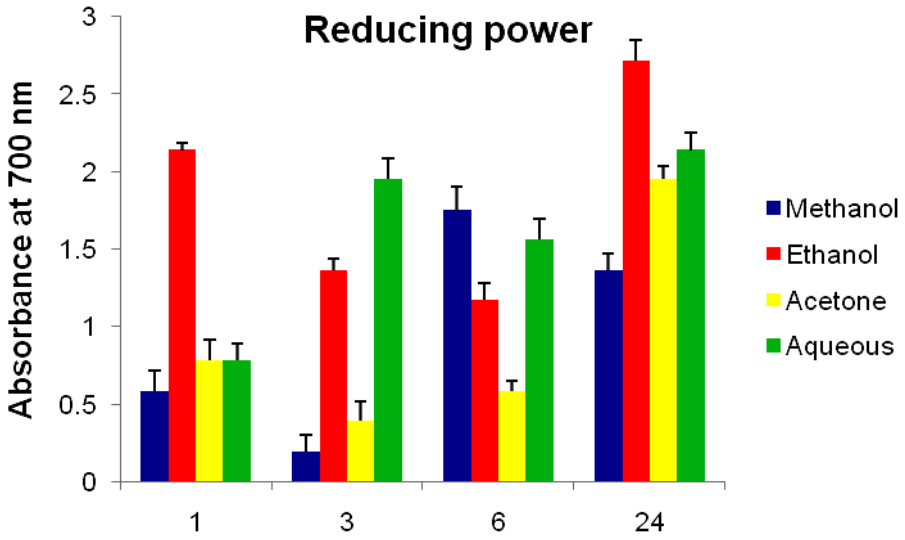


Figure 5.20 Relative levels of total phenolic compounds (A), flavonoid compounds (B) concentrations in *M. oleifera* leaves determined by spectrophotometry. Methanolic leaves extract of *M. oleifera* exhibited the highest both phenolic and flavonoids concentration. The results are from three independent analysis.

Note: blue bar represented methanol extract; red bar represented ethanol extract; yellow bar represented acetone extract; and green bar represented aqueous extract at 1, 3, 6, and 24 hrs, respectively.

(A)



(B)

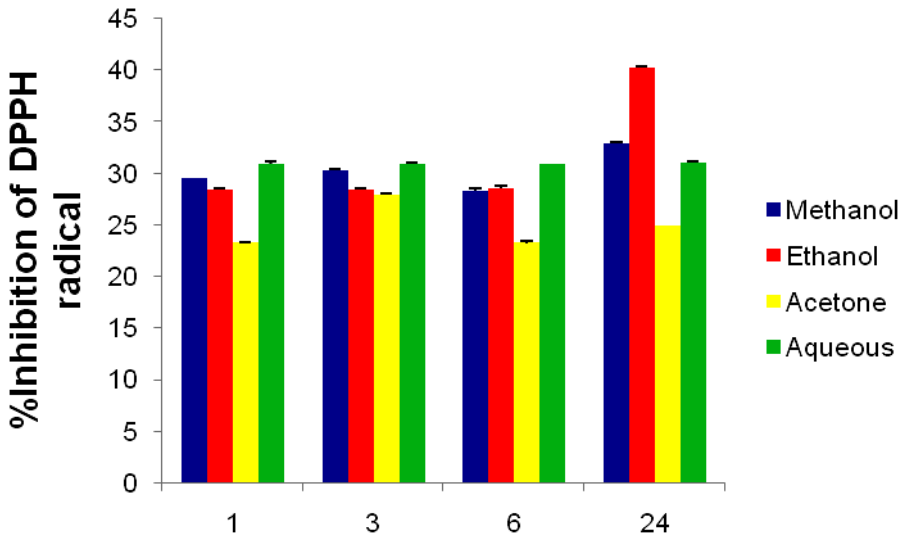
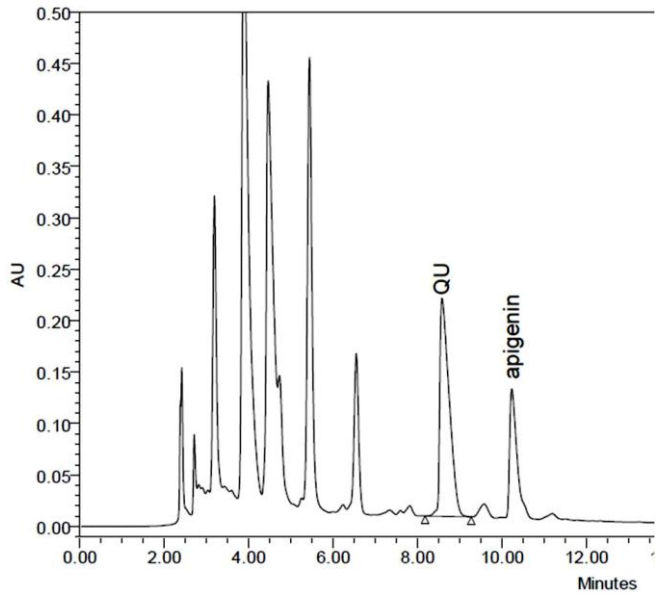


Figure 5.21 Relative reducing power (A) and DPPH radical scavenging activity (B) of different solvent extracts of *M. oleifera* leaves extract. Ethanolic extract possessed high both RP follow by acetone extract, ethanol extract, and aqueous extract, respectively. Whereas, slightly differs DPPH activity were found depending on the solvent used, however ethanol extract showed strongest DPPH capacity. Both assays were used ascorbic acid as a reference standard, the activities based on ascorbic calibration curve.

A modified reverse phase high performance liquid chromatography (RP-HPLC) was used to provide the chromatographic profiles of the phenolics and glucosinolates in the leaves of *M. oleifera* extracts. According to the retention time of calibration standards, the main compounds in the *M. oleifera* leaves extracts are composed of two phenolic compounds including quercetin and apigenin, as well as a glucosinolate compound, sinigrin (Figure 5.22). It is noteworthy that the chromatogram of the *M. olifera* extracts also showed some other chromatographic peaks apart from those three mentioned; however we could not identify those compounds due to the limitation of the internal standard available. The selection of the three standards used in the assay is based on the medicinal properties reported. Quercetin and apigenin are strong antioxidants which have been proposed to have anti-inflammatory, hepato-protective, or anti-carcinogenic actions [186-188]. Besides their antioxidant capacities, quercetin and apigenin have also been shown to possess metal chelating capacities [189]. Sinigrin has contributed to modulation of the activities of phase I (cytochrome P450s) and phase II (glutathione-S-transferase, UDP-glucuronosyl-transferase, and quinone reductase) detoxification enzymes [150, 190, 191].

Above results indicated that *M. oleifera* leaves extract significantly promote beneficial activities as natural antioxidant. Taken together, overall results suggested that protective efficacy of fish pre-administered with *M. oleifera*-supplemented diets before lead exposure is likely involved in the constituent of phenolics and glucosinolate present in the leaves. It is possible that these phyto-molecules may serve as natural antioxidant and prevent the fish from the harmful effects of lead.

(A)



(B)

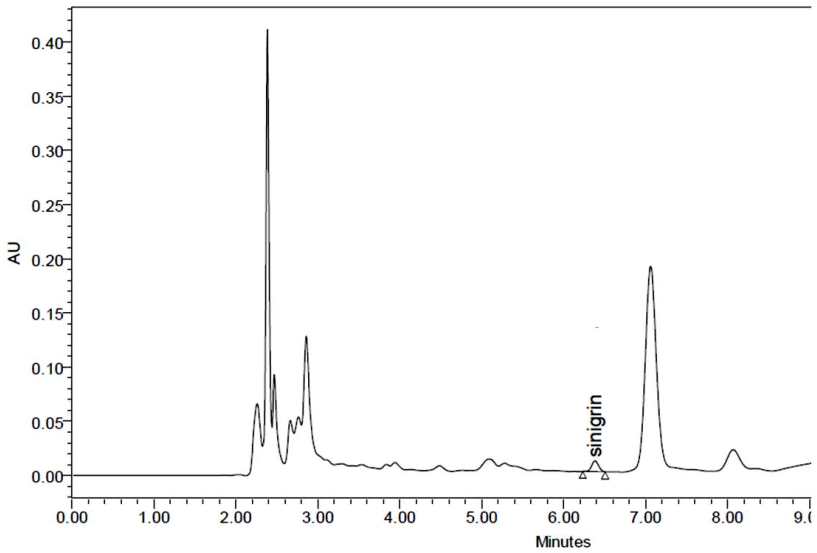


Figure 5.22 Representative HPLC chromatograms of methanolic extract constituent of *M. oleifera* leaves under different detection wavelength 335 nm for (A) and 227 nm for (B).

CHAPTER VI
DISCUSSION

A variety of medicinal plants are being studied for their potential protective effects against environmental toxicants including *Moringa oleifera*. *M. oleifera* has been considered as a valuable herb due to its broad spectrum of biological activities [138, 143]. *M. oleifera* has been shown to reduce the hepatic injury from carbon tetrachloride (CCl_4) toxicity in albino rats [192]. Recent research studies have focused on the protective effects of *M. oleifera* on cardiovascular system and liver in rabbits and rats [12, 143]. Due to the high contents of antioxidants found in *M. oleifera*, it is possible that a consumption of *M. oleifera* is able to alleviate the toxicity of lead that has been shown to cause cellular oxidative stress. Lead is an important environmental pollutant affecting aquaculture productions [1, 193]. Therefore, in this study, we investigate the protective effect of *M. oleifera* against deleterious effects from waterborne lead exposure in fish *Puntius altus*.

Although the toxic effects of lead have been known for centuries, harmful lead exposure to the aquatic animals is still widespread [3]. Herein, we demonstrated that exposure to lead causes gills, liver, and kidney damages in fish within a short period of time. The effects of lead on the morphology of gills were similar to those reported previously [194-196]. Large area of epithelial cell lifting, lamella fusion and thickening of gill filament were observed in fish exposed to lead. These changes serve as a protection mechanism by increasing the distance from which waterborne lead can diffuse to the bloodstream [196]. However, such alterations may impede gas exchanges and reduce the oxygen uptake leading to damage to the gill. We have found that pre-treatment with *M. oleifera*-supplemented diets can reduce these lesions in the gill. The reduction in gill damage may be due to an increase in the number of acid mucous cells along the gill filament, which was observed in our study. It has been shown in rainbow trout exposed to zinc that an acid mucous can bind and precipitate zinc, thus reducing the zinc absorption into the body [197]. We hypothesized that an

increase in the number of acid mucous cells in fish receiving *M. oleifera*-supplemented diets potentially helps in reducing lead absorption and thus decreases the toxicity from subsequent lead exposure.

Alterations of the liver from lead exposure including cytoplasmatic vacuolation, hepatic cord disorganization with scattered hypertrophy hepatocytes nuclei, and increased leakage of hepatic enzymes were observed. These alterations are more likely due to an increase in free radicals and impaired endogenous antioxidant activities. In this study, we observed a significant increase in lipid peroxidation in lead-exposed fish similar to the previous reports [96, 198]. This change was also accompanied by decreasing of catalase (CAT), reduced glutathione (GSH), glutathione reductase (GR), and glutathione peroxidase (GPx) activities in lead-exposed fish. It has been shown that a decrease in catalase activity causes increased lipid peroxidation and oxidative stress from lead exposure [199]. GSH and its related enzymes are also shown to be important molecules protecting the cells from lead induced toxicity [200]. The reduction in the activities of these antioxidant molecules may be due to oxidative damage to the enzymes or proteins.

In this study, we found that pre-treatment of *P.altus* with *M. oliefera* before lead exposure was able to reduce the severity of hepatic injuries observed with both histological and biochemical analyses including a reduction of liver enzymes leakage and increases in the antioxidant enzymes activities (CAT, GSH, GR, and GPx) when compared to fish exposed to lead alone. The increases in antioxidant activities such as CAT, GSH, GR, and GPx maybe consider as a protective mechanism against lead-induced oxidative damage. This finding suggests that the activities of these endogenous antioxidant enzymes are enhanced due to the role of exogenous antioxidant constituents that contain in *M. oleifera* supplementation. These results are in agreement with the previous study demonstrating that the methanolic extract of *M. oleifera* leaves can protect the rats from liver injury caused by acetaminophen toxicity [166]. The protective effects of *M. oleifera* are due to an antioxidant action from the phenolics, flavonoids, and related antioxidant constituents. We found that the extract of *M. oleifera* leave contains high levels of phenolics and flavonoids. The levels of phenolic content in *M. oleifera* extracts found in this study is approximately four times higher than the phenolic level present in the reference standard, gallic acid and is

comparable to those of other medicinal plants including *Alcea kurdica* (Malvaceae), *Stachys lavandulifolium* (Lamiaceae), *Valeriana officinalis* (Valerianaceae), *Lavandula officinalis* (Lamiaceae) and *Melissa officinalis* (Lamiaceae). Phenolic compounds are known to be potent free radical scavengers and are considered as agents that can reduce toxicity from chemicals [128]. We therefore propose that the phenolics and flavonoids found in this herb may exert potent protective effects against lead-induced oxidative stress in the liver. Phenolic compounds identified in the *M. oleifera* leaves extracts in this study are quercetin and apigenin. Nonetheless, unknown phenolic compounds were also detected in HPLC chromatograms leading to further identification of these compounds. Quercetin and apigenin are strong antioxidant that has been proposed to have various biological properties such as anti-inflammation [187], hepato-protectant [188], and anti-carcinogen [186]. Interestingly, in addition to the antioxidant activities, quercetin and apigenin also display metal chelating capacities [189]. Overall, these results suggest that *M. oleifera* displays antioxidant properties that potentially counteract the oxidative stress induced by lead and also contains phenolic compounds that may chelate the toxic metal and therefore, reducing the cellular damages from lead exposure. However, further studies are needed in order to elucidate the exact mechanisms of how *M. oleifera* play a role in protecting cellular damages from lead and to identify the major constituents that play a major role in this protection.

M. oleifera can also protect nephrotoxic effect induced by lead. This finding is consistent with the previous study in rats showing that the extract of *M. oleifera* leaves can attenuate renal injury induced by gentamicin [201]. The nephroprotective effect of *M. oleifera* observed may also occur via its antioxidant action similar to the observations found in the liver. Lead is known to affect cell proliferations of liver cells and epithelium cells of the kidney [184, 185] and interfere with localization and expression of proliferating cell nuclear antigen (PCNA, marker for cell proliferation). So, we next addressed whether protective effect of *M. oleifera* diet is associated with the change in cell proliferation induced by lead. The present investigation reveals that cell proliferation in gill and liver were increased after lead treatment. However, pre-treatment with *M. oleifera* diets effectively decrease numbers of cell proliferation of gills and liver cells in fish exposed to lead possibly by

protecting the cells from oxidative stress that can lead to increase cell proliferation. Recent studies have shown that phenolic compounds, particularly quercetin and apigenin, are important compounds involved in attenuation of cell proliferation [202-204]. We therefore propose that the action of *M. oleifera* supplement in reducing cell proliferation upon lead exposure is by the antioxidation property of phenolic compounds found in *M. oleifera* extract.

Additionally, we monitored the protective effects of *M. oleifera*-supplemented diets against waterborne lead exposure on hematological parameters of fish *P. altus*. Due to intimate contact between the fish circulatory system and lead-contaminated water, both hematology and blood chemistry assessment are important parameters used for an evaluation of physiological status of the fish [205]. A significant decrease in white blood cell count was detected in fish exposed to lead alone consistent with the known immunotoxicity of lead [206]. The decrease in white blood cell has direct consequences on the fish immune response as well as altering the host resistant against infectious pathogens [207]. However, fish pre-administered with *M. oleifera* diet showed an increase in the number of white blood cells when compared to the group without herbal supplement. Although the exact mechanism of how *M. oleifera* diet increases the level of white blood cells is not yet known, we propose that this may also come from the antioxidant activity of phenolic compounds present in *M. oleifera*. The observed values of erythrocytes count, pack red cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) in all fish fall within the range previously reported for teleost [208]. Slightly increase in erythrocytes count with increase in PCV and MCV indicated that small red blood cells circulate in fish as results from lead exposure [209].

In summary, the present study demonstrated that *M. oleifera* has protective effects against lead-induced toxicity when pre-administered to *P.altus* before lead exposure. The results presented in this thesis shed a light on a possibility of using *M. oleifera* leaves as natural supplement in the fish food diet in aquaculture farm to help alleviate the toxicity of lead that may contaminate the water sources. The dosage of 20 mg g^{-1} *M. oleifera* supplementation seems to be able to protect the fish from lead induced toxicity. These protective effects of M. oleifera are most likely mediated

through antioxidant properties of the phenolic constituents that are able to protect the cells from oxidative stress induced by lead.

CHAPTER VII
CONCLUSION

Many research studies have focused on using herbs as dietary supplements for aquaculture in order to promote growth as well as prevent of chemicals toxicity [210, 211]. *Moringa oleifera* has been known to have various biological properties and is considered to be a highly nutritive herb [10, 143]. In this study, we investigated the protective potency of *M. oleifera*-supplemented diets against lead toxicity to the fish *Puntius altus*.

This study demonstrated that waterborne lead exposure causes marked oxidative damage in the liver, alters the activities of antioxidant enzymes, alters histological structures of gill, liver, as well as kidney, and induces the proliferations of gill and liver cells. Pre-treatment with *M. oleifera*-supplemented diets to the fish before exposing to a toxic level of lead can reduce these alterations. Moreover, we have shown the extract of *M. oleifera* leaves contains both phenolic and flavonoid compounds and possesses high antioxidant activities when compared with other studies medicinal antioxidant plants [130, 131]. We have identified quercetin and apigenin as potential major sources of antioxidant found in the leave extract. These findings indicate that *M. oleifera* contains compounds that are capable of preventing cellular oxidative damage induced by lead.

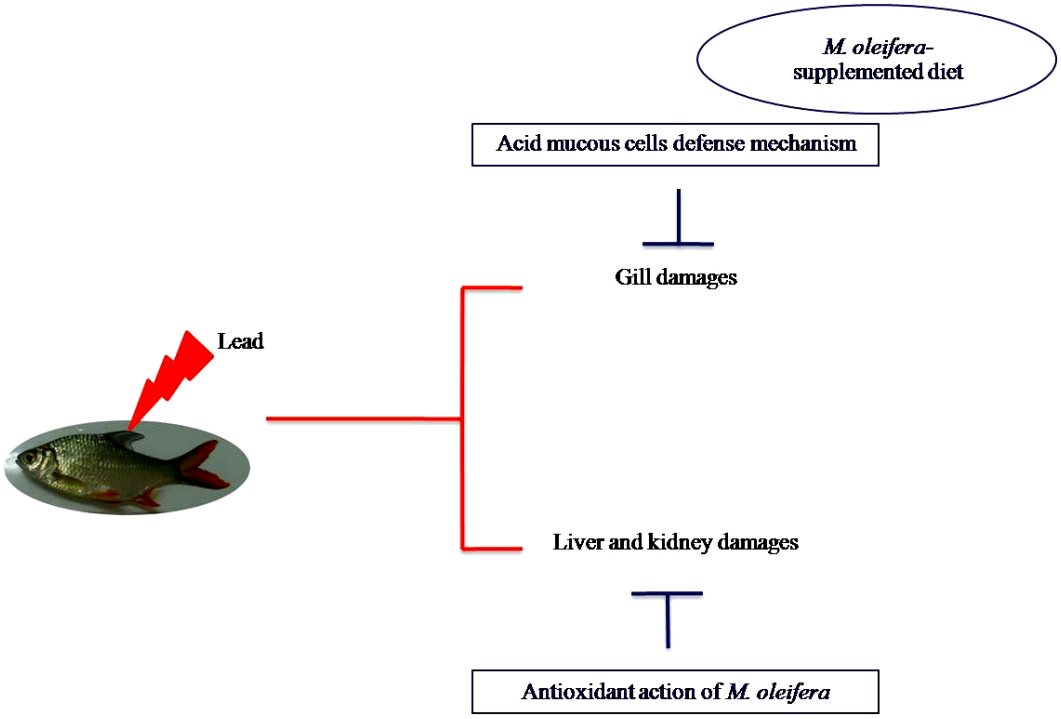


Figure 7.1 schematic diagram of the possible protective mechanisms of *M. oleifera* diet against lead-induced toxicity in fish.

Once lead is absorbed, it can generate reactive oxygen species resulting in various physiological changes and cellular damage in fish [184, 212, 213]. However, pre-treatment with *M. oleifera*-supplemented diets can reduce these adverse affects to the fish by restoring biochemical parameters, improving hepatic structure, enhancing hepatic antioxidant enzyme activities, and reduction of gill and liver cell proliferations. Specifically, *M. oleifera* diet attenuated lead-induced gill damages perhaps by defensive action of acid mucous cells as well as inhibiting liver and kidney damages possibly through its antioxidant properties (Figure 7.1). *M. oleifera* extract showed high amounts of quercetin, apigen, and sinigrin which have been reported to not only exert strong antioxidants, but also display chelating capacities [189, 214, 215]. We proposed that the protective effects of *M. oleifera* dietary supplement could be mainly due to the presence of these constituents. Overall, the research herein demonstrated *M. oleifera* as a potential supplement utilized in aquaculture to protect the aquatic organisms against the toxicity of lead that may contaminate the water system. The diets supplemented with *M. oleifera* could potentially help improving the quality of aquatic animals as well as increasing the aquaculture yield.

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APPENDIX

Light microscope study

1. Reagent preparation

1.1Preservative solution (10% buffered Formalin)

1. Formalin (40% Formaldehyde)	1000 ml
2. Distilled water	9000 ml
3. Di-sodium-hydrogen-phosphate, anhydrous	65 g
4. Sodium-di-hydrogen-phosphate, monohydrate	40 g

1.2 Haris's haematoxylin Stain

1. Aluminium alum	40 g
2. Distilled water	400 ml
3. Haematoxylin	2 g
4. Absolute alcohol	20 ml
5. Mercuric Oxide	1 g
6. Glacial Acetic Acid	5 ml

1.3Eosin Stain

1. 1% Eosin in water	100 ml
2. 1% Phloxin-B in water	20 ml
3. 95% alcohol	780 ml
4. Glacial Acetic Acid	4 ml

2. Schedule for histological process

No	Method	Duration	
1	Dissection		Removal and fixing tissues in 10% buffered formaldehyde 24 hours.
2	10% buffered formaldehyde	24 hours	
3	70% alcohol	24 hours	Washing out the fixative
4	80% alcohol	40 minutes	Dehydration
5	95% alcohol	40 minutes	
6	95% alcohol	40 minutes	
7	Absolute alcohol	40 minutes	
8	Absolute alcohol	40 minutes	
9	Xylene	1 hour	
10	Xylene	1 hour	
11	Paraffin	40 minutes	
12	Paraffin	40 minutes	
13	Embedding		
14	Sectioning	24 hours	Sectioning, placing on glass slides, drying overnight
15	Staining		Staining, mounting, and examination

3. Schedule for staining sections (*H & E*)

No	Method	Duration
1	Xylene (I)	5 minutes
2	Xylene (II)	5 minutes
3	Xylene (III)	5 minutes
4	Absolute alcohol (I)	3 minutes
5	Absolute alcohol (II)	3 minutes
6	Absolute alcohol (III)	3 minutes
7	95% alcohol	3 minutes

8	80% alcohol	3 minutes
9	70% alcohol	3 minutes
10	Running water	5 minutes
11	Hematoxylin	6 minutes
12	Running water	6 minutes
13	Eosin	1 minutes
14	95% alcohol (I)	3 minutes
15	95% alcohol (II)	3 minutes
16	95% alcohol (III)	3 minutes
17	Absolute alcohol (I)	3 minutes
18	Absolute alcohol (II)	3 minutes
19	Absolute alcohol (III)	3 minutes
20	Xylene (I)	5 minutes
21	Xylene (II)	5 minutes
22	Xylene (III)	5 minutes
23	Mounting	
24	Examination	

4. Schedule for staining sections (*PAS/AB*)

No	Method	Duration
1	Xylene (I)	5 minutes
2	Xylene (II)	5 minutes
3	Xylene (III)	5 minutes
4	Absolute alcohol (I)	3 minutes
5	Absolute alcohol (II)	3 minutes
6	Absolute alcohol (III)	3 minutes
7	95% alcohol	3 minutes
8	80% alcohol	3 minutes
9	70% alcohol	3 minutes
10	Distilled water	5 minutes

11	Alcian blue	5 minutes
12	Running water	3 minutes
13	Schiff's reagent	15 minutes
14	Running water	5 minutes
15	Hematoxylin	1 minutes
16	Acid rinse	1 minutes
17	Running water	5 minutes
18	95% alcohol (I)	3 minutes
19	95% alcohol (II)	3 minutes
20	95% alcohol (III)	3 minutes
21	Absolute alcohol (I)	3 minutes
22	Absolute alcohol (II)	3 minutes
23	Absolute alcohol (III)	3 minutes
24	Xylene (I)	5 minutes
25	Xylene (II)	5 minutes
26	Xylene (III)	5 minutes
27	Mounting	
28	Examination	

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