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Antioxidant activity of stem bark methanolic extract of Acacia nilotica in controlling organophosphate pesticides toxicity in mice

Mwezi, Raphael

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ANTIOXIDANT ACTIVITY OF STEM BARK METHANOLIC EXTRACT OF *Acacia nilotica* IN CONTROLLING ORGANOPHOSPHATE PESTICIDES TOXICITY IN MICE

Raphael Mwezi

A Dissertation Submitted in Partial Fulfilment of the Requirements for the Award of the Degree of Master's in Life Sciences of the Nelson Mandela African Institution of Sciences and Technology

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ABSTRACT

Organophosphate (OP) pesticides are reported to cause acute poisoning cases because of their ability to inhibit acetyl cholinesterase enzyme (AChE). Available antidotes are atropine sulfur, pralidoxime (2-pyridine aldoxime methyl chloride), and diazepam, which act to recover OP-AChE inhibition. These are controlled drugs hence not easily accessed and very expensive. In this study *Acacia nilotica* stem bark extract was assessed for its oral acute toxicity, antioxidant activity, and *in vivo* AChE depression and recovery from OP-AChE inhibition. The mice were exposed in three different OPs including chlorpyrifos 480 g/l (CPF), fenitrothion 10 g/l (FNT) and profenophos 720 g/l (PFP). The extract of had a substantial increase of absorbance readings from 2.895±0.0032 to 3.716±0.0259 compared to standard (ascorbic acid) that had advantage ranging from 0.108±0.0033 to 1.468±0.0297 at $P<0.05$. In oral acute toxicity, the results did not show significant increase of body weight at $P<0.05$ from 22.345 ± 0.068 to 24.557 ± 0.410 in control and from 20.493 ± 0.082 to 24.155 ± 0.260 in treating group. This might have been contributed to normal growth. There were no significant changes of hemoglobin concentration observed in control and treated groups. Neither mortality nor toxicity signs were observed during the first 24 h and daily. Recovery effect under crude methanolic extract from *A. nilotica*, ascorbic acid and normal feeding were compared with the untreated group. There were significant decreases of AChE level from day one to 14^{th} day in all treated groups of CPF, PFP and FNT which indicate poisoning. The significant of AChE recovery was observed only in male mice in all treatment groups. This is a first study to assess and report the antioxidant activity of stem bark methanolic extracts of *A. nilotica* in controlling organophosphate pesticide toxicity in mice, hence further studies on isolation of active compounds are recommended.

DECLARATION

I, Raphael Mwezi do hereby declare to the Senate of Nelson Mandela African Institution of Science and Technology that this dissertation is my original work and that it has neither been submitted nor being concurrently submitted for degree award in any other institution.

Name: Raphael Mwezi

Signature:…….. **Date**:……

Dr. Revocatus Machunda,

Supervisor 1

Signature:…….. **Date**:……

Dr. Hamisi Masanja Malebo

Supervisor 2

Signature: ……..…….. **Date**: ……

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CERTIFICATION

The undersigned certify that has read and hereby recommend for acceptance by Nelson Mandela African Institution of Science and Technology a dissertation entitled: **"Antioxidant activity of Stem Bark Methanolic extracts of *Acacia nilotica* in Controlling Organophosphate Pesticides Toxicity in Mice"**. The dissertation is submitted by Raphael Mwezi in partial fulfilment of the requirements for degree of Master's of Life Sciences of the Nelson Mandela African Institution of Science and Technology Arusha, Tanzania.

Dr. Revocatus Machunda

Supervisor 1

Signature:…….. **Date**:……

Dr. Hamisi Masanja Malebo

Supervisor 2

Signature: ……..…….. **Date**: ……

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DEDICATION

I dedicate this work to my mother Leonarda, to my beloved wife Leonora P. Temba and my sons Gasper and Gaston for their time, love and patience throughout my study time.

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LIST OF ABBREVIATIONS AND SYMBOLS

AChE	Acetyl Cholinesterase Enzyme
AFDB	African Development Bank
CPF	Chlorpyrifos
FAO	Food and Agricultural Organization
FNT	Fenitrothion
GDP	Gross Domestic Products
Hb	Haemoglobin
KNCHREC	Kibong'oto Infectious Diseases Hospital- Nelson Mandela African Institution of Science and Technology-Centre for Educational Development in Health, Health Research Ethical committee
NIMR	National Institute for Medical Research
OECD	Organization of Economic, Cooperation and Development
OP	Organophosphate
OS	Oxidative Stress
PFP	Profenophos
pH	Potential of Hydrogen
PPE	Personal Protective Equipment
ROS	Reactive Oxygen Species
RP	Reducing Power
S.E.M	Standard Error of the Mean
SD	Standard Deviation
UN	United Nations
UV	Ultra-Violet
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 Background of the Problem

Pesticides exposure causes adverse effects on human health (Elibariki & Maguta, 2017). Worldwide, approximately 200 000 cases are due to acute poisoning that leads to deaths each year (UN, 2009). 99% of acute poisoning occurs in developing countries (WHO, 2014). Statistics show that about 700 cases of death related to pesticide poisoning may occur annually (Gupta & Sharma, 2006). This shows the need to prevent people from the adverse effects and death associated with pesticide exposure.

In Tanzania, a recent report shows that the prevalence of occupational acute pesticide poisoning range from 50% to 96% (Lekei *et al*., 2016). The current treatment of pesticide poisoning cases available in Tanzania's hospital is an antidote, which includes use of drugs such as atropine, pralidoxime (2-pyridine aldoxime methyl chloride) and diazepam, which are generally controlled drugs. The drugs are unavailable in rural settings and are very expensive, (Eddleston *et al.,* 2008). Alternative drug products from the natural sources such as plant should be searched and developed to protect people who are exposed to pesticides and who are at risk to get pesticide poisoning. Presence of organophosphate (OP) in the human body triggers the production of reactive oxygen species (ROS), which induces Oxidative Stress (OS) such as lipid peroxidation, it also induces neurotoxic action and cause inhibition of Acetylcholinesterase Enzyme (AChE) and a decrease in the antioxidant enzyme (Verma *et al*., 2007; Oruc, 2012). These antioxidants are essential for neutralizing ROS (Sultana *et al.,* 2007), meanwhile, AChE is an essential enzyme in neuro-system which plays a great role in converting acetylcholine to Acetate and Choline, finally, Choline is taken back to the neural cell. Acetyl CoA from mitochondria combines with choline to form Acetyl-choline (Ach). Acetate at the ring is released as well as CoA in the neural (Akefe, 2017). Inhibition of AChE results into increase of acetylcholine in the body which causes a decrease of AChE level (u/mL) result into acute health effects (headache, dizziness, abdominal pain, death) or chronic health effect (cancer, loss of coordination, loss of vision) (Fayuk & Yakel, 2004). Hence there is a need to prevent the AChE level depression.

Studies have observed that antioxidants derived from vitamins have the capability to fight ROS induced by OPs (Verma *et al.,* 2007). Also, some studies reported that ROS induced by

Chlorpyrifos OP can be scavenged by vitamins A, C and E enriched antioxidants (Verma *et al*., 2007). However, other antioxidant-enriched plants should be assessed for their antioxidant properties to fight against ROS induced by other OPs. *Acacia nilotica* is a multi-medicinal plant found in the kingdom Plantae, division Magnoliophyta, Family Fabaceae and is widely found in Africa and Asia (Harmacy & Ciences, 2011). It is reported to have polyphenol antioxidant property (Johns *et al*., 1999)

The medicinal property of the plant may vary depending on part of plant taken. Barks from *A. nilotica* has been shown to contain polyphenol and flavonoids compared with leaves and roots (Sadiq *et al.,* 2015). The presence of polyphenol in *A. nilotica* gives the plant an ability to scavenge ROS induced by chemicals and protect from oxidative stress in the human body (Del *et al*., 2008; Duganath *et al*., 2010; Ravikumar & Angelo, 2015). This study was aimed to assess the antioxidant activity of *A. nilotica* stem bark methanolic extract in controlling the effects of chlorpyrifos, profenophos and fenitrothion organophosphate pesticides poisoning in mice.

1.2 Statement of the Problem

Organophosphate pesticides trigger the production of ROS, which induces oxidative stress (OS) and neurotoxic action and cause inhibition of AChE which cause accumulation of acetylcholine in the parasympathetic nerve synapses, resulting in acute pesticide poisoning symptoms such as headache, nausea, vomiting, diarrhea and eventually death (Johnson *et l*., 2014; Suarez-Lopez *et al*., 2013). The current treatment of acute pesticide poisoning is by the use of antidote drugs like atropine sulfur, pralidoxime (2-pyridine aldoxime methyl chloride), and diazepam which act to recover AChE from OP inhibition. However, these are controlled drugs which are not easily accessed thus are very expensive. The need for finding alternative treatments is inevitable.

Antioxidants found in Vitamins A, C and E have been reported to have an AChE recovery effect for rats exposed by Chlorpyrifos (Verma *et al.,* 2007). Stem barks from *A. nilotica* reported to be the best antioxidant plant having the capability of reducing (scavenging) ROS generated by body oxidative stress (Kalaivani & Mathew, 2010; Aadil *et al.,* 2014) and methanol solvent reported to extract maximum antioxidant compounds of phenols and flavonoids due to high polarity (Widyawati *et al*., 2014). But, there is limited or practically no available information on the application of *A. nilotica* extracts on how its antioxidant

activity can be used to control AChE inhibition from OP exposure. Therefore, this study intended to investigate on antioxidant activity of *A. nilotica* stem bark methanolic extracts in controlling rolling OP pesticide exposure.

1.3 Rationale of the Study

The findings of this present study will contribute to the information on the development of drug through the use of *Acacia nilotica* extract to treat individuals who are exposed to pesticide and assessment of the safety profile of the plant extract and to help local communities to utilize the plant for medicinal purposes where the conventional drugs are not available

1.4 Objectives

1.4.1 General Objective

Assessment of antioxidant activity of *A. nilotica* stem bark methanolic extract in controlling the effects of selected organophosphate pesticide poisoning in mice

1.4.2 Specific Objectives

(i) To evaluate *in vitro* the reducing power of the stem bark methanolic extract from *A. nilotica*

(ii) To determine the oral acute toxicity of methanolic stem bark crude extract of *A. nilotica* in mice

(iii) To assess the recovery efficacy of Acetylcholinesterase Enzyme (AChE) by methanolic stem bark extract of *A. nilotica* caused by Chlorpyrifos, Profenophos and Fenitrothion poisoning in mice

1.5 Research Questions

(i) What is the extent of reducing power of methanolic extract from *A. nilotica* extract?

(ii) Are there any oral acute toxicity effects due to the use of extracts from the *A. nilotica*?

(iii) To what extent *A. nilotica* extract has the ability to recover AChE level from the depression of OP poisoning in mice?

1.6 Significance of the Study

The knowledge from this study will contribute to providing information regarding the use of *A. nilotica* in controlling effect resulting from OP Pesticide exposure and enhance further studies towards the development of antidote drugs for the treatment OP poisoning.

1.7 Delineation of the Study

The experimental study was conducted in this present study where a plant sample of *Acacia nilotica* was collected, prepared and extracted. Mice were poisoned by chlorpyrifos 480 g/l (CPF), fenitrothion 10 g/l (FNT) and profenophos 720 g/l (PFP). The level of antioxidant and safety profile of the plant was assessed, followed by determining the level of AChE depression and recovery. The data of the reducing power were collected by using UV-Spectrophotometer while AChE level by using AChE test mate Kit where mice were employed.

CHAPTER TWO

LITERATURE REVIEW

2.1 General Pesticide Overview

Pesticide is a generic term that includes insecticides, miticides, nematicides, fungicides, herbicides, algaecides, fumigants and vertebrate poisons. The main classes of insecticides are organochlorines (e.g. DDT), organophosphates (OPs) (chlorpyrifos), carbamates (carbaryl), synthetic pyrethroids (permethrin) and neonicotinoids (imidacloprid).

Pesticides are chemicals designed for killing or controlling unwanted insects, diseases on plants, weeds, slugs and snails, birds and vertebrate mammals regarded as pests such as rodents (Watts, 2012). Pesticides are used in agricultural for controlling physiological functions in plants and public health activities in controlling pests like mosquitoes (Manyilizu & Mdegela, 2015; Kariathi *et al.*, 2016).

It was estimated that 18% of pesticides in Tanzania was used in the public health sector for malaria vector control, while 81% is used in livestock and agricultural sectors and 1% is used in other areas including protecting buildings from damage caused by insect pests (Lahr *et al*., 2016). In Tanzania, agricultural activity contributes 44.3% of the country's (Chongela, 2015) and 85% export earnings (FAO, 2016). The increase of agricultural activity tends to increase importation and use of pesticides exponentially (Elibariki & Maguta, 2017). Lekei *et al.* (2014) calculated that in the fiscal year 2013/2014, a total of 11 482 MT of pesticides was imported into Tanzania. This result in environmental contamination and accumulation of pesticides in our environment and cause diseases and death in living organisms and human, hence, initiation of risk and mitigation measure of reducing environmental and public health impact is inevitable to self-guard community health and to increase agricultural productivity, livelihood, and national economy. Organophosphates (OPs) are used widely for agriculture, vector control, and domestic purposes (Roberts & Aaron, 2007). These OPs reported to cause acute pesticide poisoning worldwide and mainly occur in rural areas agricultural communities and occupational farm workers (Abdel *et al*., 2008). Hence there is a need to control effects resulting from OPs use.

2.2 Effects of the Pesticide Exposure to Human Health

Human gets pesticide exposure through the oral, dermal, and inhalation routes. Exposure due to dermal and oral is reported to be the most common in Tanzania (Ngowi *et al.,* 2007). Globally, about 200 000 of all deaths per year are due to pesticide poisoning and about 99% occur in developing counties (UN General Assembly 2009). In Kenya, pesticide incidence cases were 1479 and 579 fatalities were reported in 2012 while in Uganda, pesticide poisoning incidents occurred in 2012 and 2013 in Wakiso and Pallisa caused 87 fatalities (WHO, 2014). In Tanzania occupational acute pesticide poisoning range from 50% to 96% (Lekei *et al*., 2016).

The use of rapid AChE test mate kit simplifies the process of getting data and early identification of people who are exposed by OP pesticides (Rajapakse *et al.,* 2008). Mostly OPs reported causing adverse health effects in Tanzania include Chlorpyrifos, Fenitrothion, and Profenophos (Ngowi *et al.,* 2001). Pesticide exposure can cause acute or chronic effects. Acute clinical signs include abdominal pain, headache, vomiting, dizziness, while chronic problems are associated with cancer, the effect on Neuro-logical, reproductive and immune-systems (John & Souza, 2017). Occupational exposure at high dose results in neuropsychiatric outcomes including increased anxiety, depression and suicide (Lekei *et al.,* 2014).

Pesticides that act through neurotoxic mechanisms that are relevant to human health include organophosphates (OPs), organochlorine (OCs), Carbamates and pyrethroid (Anger, 2015). OP causes inhibition of cholinesterase enzyme activity in the nervous tissue (Kushwaha *et al.*, 2016). Acetylcholinesterase, under normal physiological conditions, performs the breakdown of acetylcholine, which is a chemical mediator responsible for the physiological transmission of nerve impulses at different sites (El-sheikh, 2017). In the presence of OP, AChE is phosphorylated and is no longer able to break down acetylcholine into choline and acetic acid (Fig. 1). The resulting accumulation of acetylcholine in the parasympathetic nerve synapses, the motor end-plate and in the central nervous system which is responsible for all typical symptoms occurring after acute poisoning with OP (Abdel-ghany *et al.*, 2016).

In Tanzania, the treatment regime of OP poisoning includes the use of antidote drugs which are used to treat acute case only, no treatment available on a slow exposure of pesticide which mostly leads to chronic effects. Essential ways of controlling acute and chronic effects

resulting from OP exposure need to be undertaken. This present study aimed to investigate an alternative ways of controlling effects resuted from chlorpyrifos, profenophos, and fenitrothion OP exposure.

Previous studies have indicated the use of vitamins enriched antioxidants A, C and E to have the ability to combat effects resulting from Chlorpyrifos exposure (Verma *et al.*, 2007). This present study was aimed to seek information available for *A. nilotica* plants that have a similar property of having antioxidant properties (Polyphenol and Flavonoids), but it has a limitation on how it able to fight against effects generated from Chlorpyrifos, Profenophos and Fenitrothion exposure.

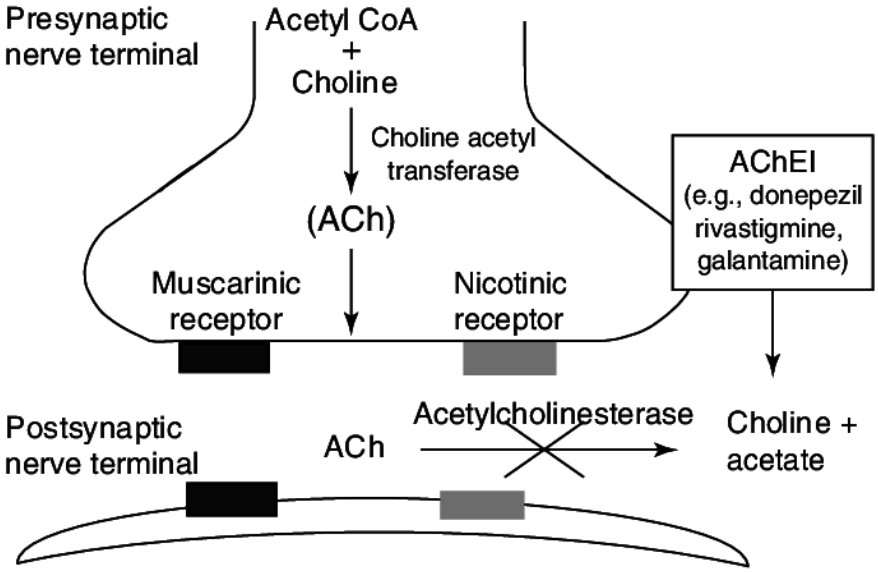

Figure 1: Mechanism of impulse flow from pre to post synaptic nerve and AChE inhibition

2.3 Applications of *Acacia nilotica*

World Health Organization (WHO) reported that more than 80% of the world's population relies on traditional medicine for their primary healthcare needs (Abarca-vargas *et al.*, 2016). *A. nilotica* (Mgunga Swahili, Language or babul acacia in English) is a multi-medicinal plant found in kingdom Plantae, division Magnoliophyta, Family Fabaceae and is widely found in Africa and Asia (Harmacy & Ciences, 2011). Genus Acacia containing 1350 species (Fig. 2) is a medium-sized tree, 15–18 m tall, with a stem diameter of 2–3 m, spreading and almost symmetrical crown. *Acacia nilotica* contains essential phytochemical compounds including, tannins, flavonoids, alkaloids, terpenes, fatty acids, gums which are potential for the

following applications, anti-inflammatory activity, anti-oxidant activity, antidiarrheal and anthelmintic activity, antihypertensive and antispasmodic activity, antibacterial and antifungal activity, antiplatelet aggregator and anti-diabetic activity, anticancer and antimutagenic properties, antiplasmodial activity, diuretic properties, milk production and prolactin release in rat, Analgesic and antipyretic activity (Rather *et al*., 2015). On other hands, this plant is commonly used by Masai and Batemi tribe in East Africa where they add it in milk and meat-based soup as a food additive (Johns *et al.*, 1999)

The medicinal property of the plant may vary depending on part of the plant. The stem barks from *A. nilotica* have shown to contain polyphenol and flavonoids compared with leaves and roots (Sadiq *et al.,* 2015). Different compounds and extracts of *A. nilotica* have been reported to possess potent antioxidant activity. *In vitro* study shows that leaf extract of *A. nilotica* has antioxidant compounds and the ability to protect DNA against oxidative stress (Mohan *et al*., 2014). Polyphenols present in green pod extracts have metal chelation as well as free radical scavenging activity (Singh *et al.*, 2009). Ethanol leaf powder extract of *A. nilotica* has potent antioxidant activity potential for hydrogen or electron-donating ability confirmed by reducing power (Kalaivani & Mathew, 2010). Also, bark fractions in different solvents were assessed by measuring reducing power, DPPH scavenging activity and lipid peroxidation assay and results were compared with standards antioxidants (Sultana *et al*., 2007). The extraction yield and the biological activities of vegetal extracts can be influenced by the solvent applied in the extraction process (Abarca-vargas *et al.*, 2016).

Figure 2: A Picture of *Acacia nilotica* plant (Picture taken by Raphael Mwezi)

2.4 Reducing Power of *Acacia nilotica*

Reducing power (RP) is the best antioxidant property of *A. nilotica* where it indicates the ability to donate hydrogen atoms to ROS and reduces from toxic to less toxic state (Sadiq *et al.,* 2015). Reducing power may vary with geographical condition and place where plant samples have been taken. In Pakistan, it is reported that *A. nilotica* has an RP of 1.99. The higher the RP value the higher the ability of the extract to scavenge ROS. Study done by Del *et al.* (2008) and Duganath *et al.* (2010) in India reported that as a concentration of crude extract from *A. nilotica* increase, absorbance reading also increased and found that at 10ug/ml (absorbance 0.50), 20 ug/ml (absorbance 0.65) and at 30 ug/ml (absorbance was 0.8). There is limited information on reducing power of *A. nilotica* growing in Tanzania. In this study, the RP of methanolic bark extract from *Acacia nilotica* was evaluated and compared with standard ascorbic acid (known antioxidant) based on different concentration.

Acacia nilotica due to its high reducing power reported being promising plant for controlling the progression and treatment of Alzheimer's Disease by targeting AChE (Crowch *et al.,* 2009). Although it has been reported that solvent used in the extraction process for example, acetyl acetate can inhibit AChE. However, no effect has been observed when ethanol and methanol were used (Eldeen *et al.,* 2005). Reactive Oxygen Species (ROS) generated by oxidative stress in human can be scavenged by antioxidant enzymes through the normal system, but high intoxication of chemicals can manifest a normal system (Osama *et al.,* 2015). This study aimed at investigating how ROS induced by chlorpyrifos, profenophos and fenitrothion OP can be scavenged by *A. nilotica* methanolic extract

2.5 Toxicity due to Use of *Acacia nilotica* Extract

The medicinal use of plant extracts has been reported to cause Acute and sub-acute effects on body physiology and structure (Alli *et al.,* 2015). In this present study, an acute toxicity test was performed based on guidelines from (Oecd/Ocde, 2008). Studies recommended that the highest dose should be 2000 mg and the lowest is 175 mg (Adewale, 2016). During acute oral toxicity study, the first 4 h after administration of a single dose of crude plant extract, the animal can present behavioral changes and clinical signs like skin changes, eyes, fur color, emotion (Kilonzo *et al.,* 2016). Variation of toxicity of the plant depends on a part of plant taken like in leaves, roots, and stem also the geographical distribution, climate condition, and nature of the soil (Sadiq *et al.,* 2015). Mukundi (2015) in Kenya reported no effects on leaf

extracts obtained from *A. nilotica*. Jigam (2011) in Nigeria reported that the use of extracts from roots of *A. nilotica* cause a decrease of packed Cell volume and Haemoglobin Concentration. But there is limited information about toxicity effects of bark extracts from *A. nilotica*.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sample Collection and Identification

Acacia nilotica stem bark was collected at Duka Bovu Village, in Monduli District at geographical coordinates at -$3^0$30’2.35”S, $36^0$44’5.04”E, Arusha Region. The stem bark pieces were collected 5 samples per population for conservation purpose (Fig. 3 and Fig. 4) and plants leaves were collected and submitted to National Herbarium of Tanzania (NHT), Tropical Pesticide Research Institute (TPRI), Arusha and given Specimen voucher RJE001 after Confirmation by a plant taxonomist. Collected stem bark were air-dried at room temperature before extraction and *in vitro* study was conducted

Figure 3: Collection of stem bark of *Acacia nilotica*

Figure 4: Preparation of cutting area

3.2 Research Design

The study involved both *in vitro* and *in vivo* experiments. *In vitro* study involved evaluation of reducing power of extracts while *in vivo* involved assessing the efficacy of crude methanolic stem bark extract of *A. nilotica* on the recovery of AChE from OP inhibition in mice. Stem bark contain antioxidants compounds of phenolic (phenolic acid, flavonoid and tannins) (Anjum *et al.*, 2013). Hence, phenolics are often extracted in a higher amount in more polar solvent like methanol (Ismail *et al.*, 2016). A pilot in vivo study was conducted to determine tolerance and toxicity doses of chlorpyrifos, profenophos and fenitrothion pesticides used in this study.

3.2.1 Preparation of Plant Extracts

Acacia nilotica stem bark methanolic extract was prepared by using Soxhlet extraction method as described by Singh, Singh and Kumar (2012) (Fig. 5). The method is preferably used when the desired compounds have limited solubility in a solvent, and the impurity is

insoluble in that solvent (Ravikumar & Angelo, 2015; Sultana *et al.*, 2007). About 20 g of powdered stem bark were uniformly packed into a thimble and extracted with 180 mL of 100% methanol. Methanol was used because of high polarity and ability to extract both polar and non-polar compounds and followed by 180 mL of 50% Methanol. Extracts were taken into beakers and kept on a hot plate and heated at 30 – 40^{o} C till all the solvent got evaporated. Dried extracts were kept in a refrigerator at 4^{o}C before they were used in the intended experiments.

Figure 5: Preparation of stem bark extract of *Acacia nilotica* under Soxhlet apparatus

3.2.2 Evaluation of Reducing Power of Methanolic Stem Bark Extract of *Acacia Nilotica*

Reducing power was performed based on the method described by Duganath *et al*. (2010). 2 mL of the crude methanolic stem bark extract of *A. Nilotica* sample was taken and diluted with normal saline as a vehicle from 2, 4, 6, 8, up to 20 mL of 10 samples in serial dilution. This dilution aimed to observe at what concentration or dilution ratio extract against the

vehicle used will show a high absorbance reading. 1 ml of distilled water was mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferric-cyanide [K_3Fe (CN) $_6$] (2.5 mL, 1%) of each sample point (Fig. 6). The mixture of each sample was incubated at 50^o C for 20 min. Then, 2.5 mL of trichloroacetic acid (10%) was added to mixtures, followed by centrifugation for 10 min at 3000 rpm. The upper layer of solution (2.5 mL) of each sample was taken and mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%). Absorbance of each prepared sample was measured in triplicate at 700 NM against a blank using UV-Vis spectrophotometer (Elico –SL 196) in comparison with ascorbic acid as a standard (Fig. 7).

Figure 6: Reagents preparation and serial dilution

Figure 7: Absorbance reading under UV- Spectrophotometer

3.3 Experimental Animals

Albino mice of both sexes, weighing between 25 and 30 g and aged 8 to 12 weeks were obtained from the Plant Protection Division (PPD) at TPRI, Arusha, Tanzania. The animals were allowed to stay in cages with sawdust litters in a controlled temperature environment of about 23 °C. Lighting was controlled to supply 12 h of light and 12 h of darkness per day. Animals were handled ethically in an experimental animal room with temperature maintained at 22° C (± 3). Animals were fed with conventional rodent laboratory diets with an unlimited supply of drinking water. Animals fasted for 2 h before dosing.

3.3.1 Acute oral toxicity of *Acacia nilotica* crude stem bark of methanolic extract in albino mice

Acute oral toxicity was conducted to investigate LD_{50} of methanolic crude extract in mice based on Protocol and OECD guideline number 425 of 2001 (OECD, 2001). The mice were acclimatized for 7 days before experimentation. Mice were divided into a control group and four experimental groups with four mice each (2 males and 2 females). Body weights of the mice were determined before dosing and later, after every 48 h and finally weekly for 14 days. The control group received rodent food (ad libitum) and water, whereas the treatment group was administered crude extract dissolved in normal saline as vehicle orally by using oral gavage at 175, 500, 1500 and 2000 mg/kg per body weight.

The animals were allowed to stay in cages with sawdust litters in a controlled temperature environment of about 23 ± 3°C. Lighting was controlled for 12 hand darkness for 12 h throughout the experiment. Each cage was identified using numbers assigned aside them. The animals were fed with standard laboratory animal food pellets with water *ad libitum* before dosing with the extract. The mice were starved for 4 h with access to adequate drinking water only. The mice were observed individually once during the first 30 minutes after dosing, periodically during the first 24 h, but much attention was first 4 h and daily for a total of 14 days. Animals were humanely killed by using the physical method of cervical dislocation for animal welfare reasons.

3.3.2 Determination of Haemoglobin (Hb) Concentration

Hemoglobin concentration was assessed to evaluate whether the use of methanolic stem bark extract from methanolic stem barks *A. nilotica* affects the blood or not. Animals from both sexes were measured their level of blood Hb concentration before dosing followed by the weekly test by using an AChE test mate kit where blood samples were collected from the tail of mice of all sex.

3.3.3 Assessment of the Recovery Efficacy of AChE by Methanolic Stem Bark Extract of *Acacia nilotica* caused by Chlorpyrifos, Profenophos and Fenitrothion Poisoning in Mice

Three different active ingredients of OPs were assessed in mice, including chlorpyrifos 480 g/l (CPF), fenitrothion 10 g/L (FNT) (Fig. 13) and profenophos 720 g/L (PFP) (Fig. 12). OPs were prepared by dissolving into normal saline whereby 3 mL of CPF in 200 mL, 3 mL of PFP in 200 mL and 3g of FNT in 200 mL, differently. These 3 mL or 3g in 200 ml doses were established as a tolerance dose for mice to produce results without getting harm that was developed during the pilot study. Three categories of pesticides of CPF, FNT and PFP were grouped based on recovery, treatment including normal feeding, ascorbic acid, and methanolic crude extract. Blood samples were collected from the mice tail before and after treatments (Fig. 9). Blood samples were analyzed for the level of AChE (u/mL) by using AChE test mate kit (Fig. 10 and Fig. 11) as described in Test-mate ChE Cholinesterase Test System (Model 400) - Instruction Manual.," (2003) where 10 uL of blood filled in capillary and placed into assay tube followed by vigorously shaking before inserting into the analyzer. Mice were poisoned with CPF, FNT and PFP from day one, 7^{th} and 14^{th} OPs (Fig. 12 and Fig. 13) bottles were removed manually and induce AChE recovery treatments including methanolic crude extracts from *A. nilotica,* ascorbic acid and normal feeding for 21^{st} and 28^{th} days. The control group did not receive anything except food and water. The amount of dose administered was 1500 mg/kg per body weight (LD_{50} dose obtained during oral toxicity study) of both Crude extracts of *A. nilotica* and ascorbic acid.

Figure 8: Procedure for blood sample collection from the mice

Figure 9: Poisoning by CPF, FNT

Figure 10: AChE test mate kit

Figure 11: AChE reagents

Figure 12: Profenophos (PFP)

Figure 13: Fenitrothion (FNT)

3.4 Data Handling and Analysis

The results were entered into an Excel spreadsheet and expressed as the mean ± STDEV. The comparison t-test was used to compare mean absorbance readings of crude methanolic stem bark extract of *A. nilotica* against ascorbic acid, mean haemoglobin concentration of treatment and control, mean body weight, the level of Acetylcholinesterase level (AChE) of treatment and control by using MedCalc ® Version 12.7.1.0

3.5 Ethical Consideration

Ethical approval was obtained from Kibong'oto Infectious Diseases Hospital, Nelson Mandela African Institution of Science and Technology, Centre for Educational Development

in Health and Research Ethical committee (KNCHREC) and given Approval No. KNCHREC009

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

4.1.1 Evaluation of Reducing Power of *Acacia nilotica* Stem Bark Extract

There is a significant difference in the increase of the mean absorbance readings of the crude methanolic extracting of *A. nilotica* and ascorbic acid at P<0.05. Absorbance readings of the crude methanolic extract of *A. nilotica* were observed to be increasing as diluted with normal saline decreasing from 1:10, 2.895 ± 0.006 up to the ratio of 1:1, 3.716 ± 0.045 (Table 1) meanwhile absorbance readings of ascorbic acid at dilution ratio 1:10 was 0.108 ± 0.006 and 1:1 was 1.468 ± 0.052 (Table 1).

Table 1: Mean Absorbance of Methanolic Stem Bark Extract of *Acacia nilotica* In comparison to Ascorbic Acid

S.ID	G1	G2	G3	Mean diff	Sign Level	95% CI	
						Lower Bound	Upper Bound
S1	1:1	3.716±0.045	1.468±0.052	2.248	0.0001	2.1378	2.3582
S2	1:2	3.394±0.057	0.648±0.003	2.746	0.0001	2.6545	2.8375
S3	1:3	3.236± 0.013	0.535± 0.006	2.701	0.0001	2.678	2.724
S4	1:4	3.366±0.011	0.425±0.003	2.941	0.0001	2.9227	2.9593
S5	1:5	3.254±0.011	0.347±0.006	2.907	0.0001	2.8869	2.9271
S6	1:6	3.217±0.003	0.323±0.003	2.894	0.0001	2.8872	2.9008
S7	1:7	3.117±0.001	0.286±0.005	2.831	0.0001	2.8228	2.8392
S8	1:8	3.066±0.002	0.215±0.001	2.851	0.0001	2.8474	2.8546
S9	1:9	2.979±0.006	0.186±0.008	2.793	0.0001	2.777	2.809
S10	1:10	2.895±0.006	0.108±0.006	2.787	0.0001	2.7734	2.8006

Value expression: Mean ± Standard deviation, S.ID: Sample Identification number,

G1: Volume ratio between crude extract / ascorbic and normal saline, **G2**: Absorbance readings of crude extract of *A. nilotica*, **G3**: Absorbance readings of ascorbic acid.

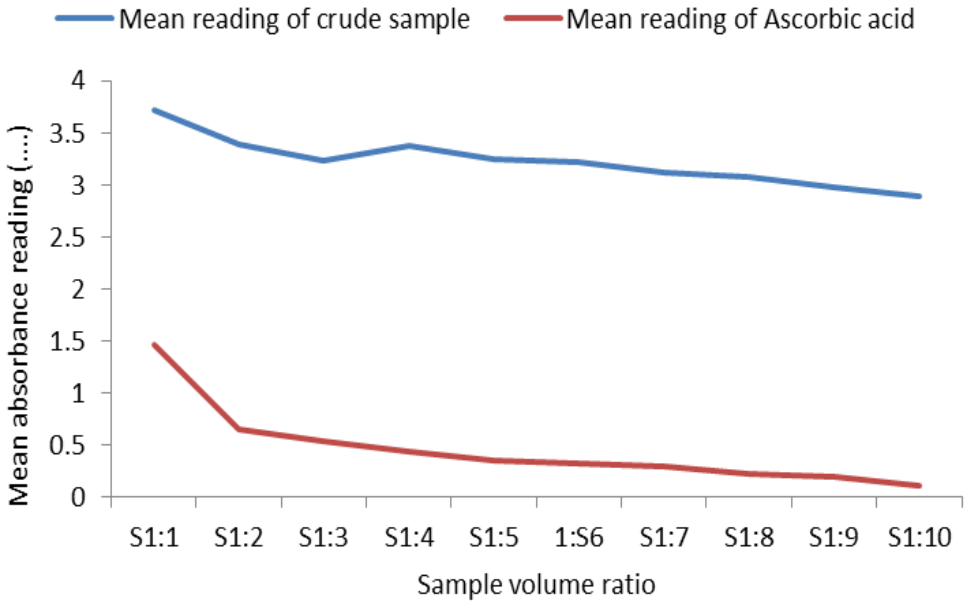

Figure 14: Comparison of absorbance readings between crude methanolic extract of *A. nilotica* stem bark and ascorbic acid

4.1.2 Acute Oral Toxicity of *Acacia nilotica* Extract in Albino Mice

This study aimed to determine the safety profile of the aqueous stem bark extracts of *A. nilotica* in albino mice. The findings of this study indicated that there were no clinical signs of toxicity in mice of both control and treated groups up to a dose level of 2000 mg/kg body weight. All animals remained normal throughout the study period and they survived until the end of the 14th day of experimentation. Neither signs of toxicity nor death were recorded at first 4 h after dosing within 24 h and other days for both control and treated groups up to a dose level of 2000 mg/kg per body weight. The results of body weight in Table 2 indicate that both control and treatments increased significantly from the first week up to the second week at $P<0.05$. In control group mean body weight increased from 22.345 ± 0.068 to 24.557 ± 0.410 for male mice and 20.316 ± 0.045 to 23.316 ± 0.319 for female mice on the other hand, in the treatment group at 175 mg/kg body weight was increased from 21.221 ± 0.057 to 24.317 ± 0.072 for male mice and 20.348 ± 0.054 to 22.910 ± 0.147 for female mice. This gradual increase of weight was similar in all treatment groups (Fig. 15)

Table 2: Mean Body weight (g) values of control mice and those treated with *Acacia nilotica* methanol stem bark extract

Dose(mg/kg BW)	Sex	First Week	Second week	P-value
Control	M	22.345 ± 0.068	24.557 ± 0.410	0.000548
	F	20.316 ± 0.045	23.316 ± 0.319	0.000557
175	M	21.221 ± 0.057	24.317 ± 0.072	0.000321
	F	20.348 ± 0.054	22.910 ± 0.147	0.00421
500	M	20.120 ± 0.070	24.309 ± 0.099	0.000222
	F	19.411 ± 0.045	23.389 ± 0.089	0.000119
1500	M	213292 ± 0.105	24.738 ± 0.257	0.000882
	F	20.149 ± 0.050	23.360 ± 0.119	0.000046
2000	M	20.493 ± 0.082	24.155 ± 0.260	0.000253
	F	20.537 ± 0.120	24.602 ± 0.349	0.002109

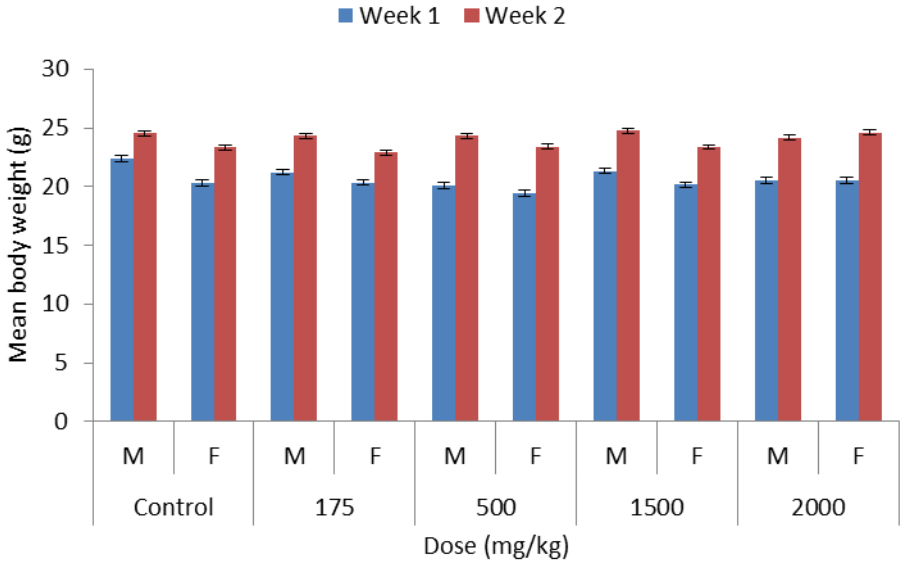

Figure 15: Body weight of mice in all treatment groups and control

Table 3: Mean Haemoglobin Concentration (g/dl) values of control and mice treated with *A. nilotica* methanol bark extract measured during the acute toxicity study

Dose	Sex	Week 1	SD	Week 2	SD	P-value
Control	M	15.7±1.80	2.545584	14.8±0.55	0.777817	0.663862
	F	15.3±0.20	0.282843	15.3±0.45	0.636396	0.928389
175	M	15.9±1.60	2.262742	15.7±0.50	0.707107	0.915934
	F	14.6±1.25	1.767767	14.4±0.30	0.424264	0.91777
500	M	15.7±0.85	1.202082	15.7±0.85	1.202082	0.641021
	F	16.1±2.80	3.959798	16.1±2.80	3.959798	0.90289
1500	M	15.2±1.65	2.333452	15.0±0.55	0.777817	0.918956
	F	14.6±0.30	0.424264	14.3±0.20	0.282843	0.492907
2000	M	16.4±0.45	0.636396	15.5±0.05	0.070711	0.185178
	F	14.4±0.05	0.070711	15.4±0.45	0.636396	0.157848

Where SD: standard deviations, Values are expressed as mean ± S. E. M

The results from Table 3 above were obtained during the acute oral toxicity test were collected blood samples of mice before and after administering crude methanolic extract of the stem bark of *A. nilotica*. The results show that there is no statistical significant difference between the mean haemoglobin of the first week and the second week in all control and treatment groups (175, 500, 1500 and 2000 mg/kg body weight) at $P>0.5$

4.1.3 Assessment of AChE Depression and Recovery

(i) Depression of AChE Level by Fenitrothion (FNT) and Recovery Efficacy

A 3g of FNT diluted in 200 ml of normal saline was used to poison mice both sexes. The poisoning assessment was compared with the untreated group (A1) (Table 4). The results from table 4 show that at day one (before poisoning with FNT), mean AChE level in both sexes, male and female mice were observed to be significantly decreased compared to the untreated group at $P<0.05$ in A2 (group poisoned with FNT from day one to day14 and recovery by methanolic stem bark extract of *A. nilotica* at the rate of 1500 mg/kg per body weight from 21^{st} to 28^{th}) group, while no significant differences of the means observed in groups A3 (a group poisoned with FNT from day one to $day14^{th}$ and recovery by ascorbic acid at the rate of 1500 mg/kg per body weight from day 21^{st} to 28^{th}) and A4 (a group poisoned with FNT from day one to day14 and recovery through normal feeding (food and water) from day 21^{st} to 28^{th}) at $P>0.05$. For the 7^{th} day, in treatment groups, A2, A3 and A4 the mean AChE level was significantly decreased compared to the untreated group at $P<0.05$. But, in the 14^{th} day, all treated mice were very weak. These findings indicate poisoning effect

and depression of AChE in the mice. The recovery effect was observed when poisoning (FNT) was removed after the 14th day. Whereby, on the 28th day it was observed that the mean AChE level of the A2 treatment was the same as the untreated group (A1) in male mice which is an indicative of recovery similar to A3 and A4 groups. Hence, the recovery effect observed only in male mice group was significant in all treatments including methanolic bark extract of *A. nilotica*, ascorbic acid, and normal feeding.

Results as presented in Table 5 indicate that there is no significant difference in the mean ± SD AChE level between crude methanolic extract from stem barks of *A. nilotica* and ascorbic acid at P>0.05 from day one to 28th. This indicates that poisoning with FNT from day one to 14th was affected the mean AChE level equally in both groups (A2 and A3) and recovery efficacy of mean AChE level was the same from day 21st to 28th at P>0.05. These results as presented in Table 5 were similar to the those in Table 6 where there is no significant difference between the means of crude methanolic extract of stem barks of *A. nilotica* and normal feeding acid at P>0.05 from day one to 28th.

Table 4: Mean AChE level (u/ml) of mice poisoned with fenitrothion (FNT) and treated by methanolic stem bark extract of *A. nilotica*, Ascorbic acid and normal feeding

Interval	Treatments		A1	A2	A3	A4
	Days	Sex				
Poisoning	0	F	1.735 ± 0.21920	0.915 ± 0.06364*	1.340 ± 0.31113	1.550 ± 0.16971
		M	1.705 ± 0.04949	1.235 ± 0.12021*	1.070 ± 0.25456	1.595 ± 0.37477
	7	F	1.710 ± 0.07071	0.285 ± 0.04949*	0.670 ± 0.28284*	0.545 ± 0.13435*
		M	1.390 ± 0.07071	0.375 ± 0.09192*	0.730 ± 0.26870	0.640 ± 0.11314*
	14	F	1.630 ± 0.05657	-0.165 ± 0.4030	0.330 ± 0.16971*	-0.15 ± 0.537401*
		M	1.450 ± 0.14142	-0.22 ± 0.01414*	0.005 ± 0.33234*	-0.065 ± 0.54447
Recovery	21	F	1.530 ± 0.09899	0.115 ± 0.00707*	0.330 ± 0.12728*	0.125 ± 0.007071*
		M	1.105 ± 0.17678	0.045 ± 0.38891	-0.160 ± 0.70711	-0.125 ± 0.75660
	28	F	1.520 ± 0.18385	0.665 ± 0.16264*	0.390 ± 0.08485*	0.325 ± 0.14849*
		M	1.090 ± 0.32527	0.225 ± 0.16264	0.330 ± 0.14142	0.250 ± 0.19799

Values are expressed as mean ± STDEV, STDEV: standard deviation, day one: the day treatment started. A1- untreated group, A2 - The group poisoned with FNT from day one to 14th and recovery by methanolic stem bark extract of *A. nilotica* at the rate of 1500 mg/kg per body weight from day 21st to 28th. A3: The group poisoned with FNT from day one to day14th and recovery by ascorbic acid at the rate of 1500 mg/kg per body weight from day 21st to 28th, A4: The group poisoned with FNT from day one to 14th and recovery through normal feeding (food and water) from day 21st to 28th: Superscript "*" indicate significance at $P < 0.05$ compared to the untreated group for each treatment.

Table 5: Comparison of mean AChE (u/ml) between mice group poisoned with FNT and treated by methanolic stem bark extract of *Acacia nilotica* (A2) and ascorbic acid (A3)

Interval	Treatments		A2	A3	Mean diff	Sign Level	95 % Confidence	
	Days	Sex					Lower Bound	Upper Bound
Poisoning	0	F	0.915 ± 0.06364	1.340 ± 0.31113	0.425	0.1989	-0.54119	1.39119
		M	1.235 ± 0.12021	1.070 ± 0.25456	-0.165	0.4943	-1.02149	0.69149
	7	F	0.285 ± 0.04949	0.670 ± 0.28284	0.385	0.1984	-0.48859	1.25859
		M	0.375 ± 0.09192	0.730 ± 0.26870	0.355	0.2191	-0.50901	1.21901
	14	F	-0.165 ± 0.4030	0.330 ± 0.16971	0.005	0.9903	-1.46894	1.47836
		M	-0.22 ± 0.01414	0.005 ± 0.33234	-0.215	0.4572	-1.22704	0.79704
Recovery	21	F	0.115 ± 0.00707	0.330 ± 0.12728	0.215	0.1398	-0.17284	0.60284
		M	0.045 ± 0.38891	-0.160 ± 0.70711	0.115	0.8589	-2.34026	2.57026
	28	F	0.665 ± 0.16264	0.390 ± 0.08485	-0.275	0.1681	-0.83311	0.28311
		M	0.225 ± 0.16264	0.330 ± 0.14142	0.105	0.5620	-0.55072	0.76072

Values are expressed as mean ± STDEV, STDEV: standard deviation, day one: the day treatment started. A2 - The group poisoned with FNT from day one to 14^{th} and recovery by methanolic stem bark extract of *A. nilotica* at the rate of 1500 mg/kg per body weight from day 21^{st} to 28^{th}. A3: The group poisoned with FNT from day one to 14^{th} and recovery by ascorbic acid at the rate of 1500 mg/kg per body weight from day 21^{st} to 28^{th}.

Table 6: Comparison of mean AChE (u/ml) between mice group poisoned with FNT and treated with methanolic stem bark extract of *Acacia nilotica* (A2) and normal feeding (A4)

Interval	Treatments		A2	A4	Mean diff	Sign Level	95 % Confidence	
	Days	Sex					Lower Bound	Upper Bound
Poisoning	0	F	0.915 ± 0.06364	1.550 ± 0.16971	0.635	0.0384	0.08356	1.18644
		M	1.235 ± 0.12021	1.595 ± 0.37477	0.360	0.3251	-0.8374	1.55743
	7	F	0.285 ± 0.04949	0.545 ± 0.13435	0.260	0.1240	-0.1756	0.69560
		M	0.375 ± 0.09192	0.640 ± 0.11314	0.265	0.1238	-0.1786	0.70851
	14	F	-0.165 ± 0.4030	-0.15 ± 0.537401	-0.015	0.9777	-2.0587	2.02867
		M	-0.22 ± 0.01414	-0.065 ± 0.54447	-0.155	0.7263	-1.8121	1.50207
Recovery	21	F	0.115 ± 0.00707	0.125 ± 0.007071	0.010	0.2929	-0.0204	0.04042
		M	0.045 ± 0.38891	-0.125 ± 0.75660	0.080	0.9064	-2.5081	2.66821
	28	F	0.665 ± 0.16264	0.325 ± 0.14849	-0.34	0.1607	-1.0100	0.33003
		M	0.225 ± 0.16264	0.250 ± 0.19799	0.025	0.9029	-0.7546	0.80455

Values are expressed as mean ± STDEV, STDEV: standard deviation, day one: the day treatment started. A2 - The group poisoned with FNT from day one to 14th and recovery by methanolic stem bark extract of *A. nilotica* at the rate of 1500 mg/kg per body weight from day 21st to 28th, **A4**: The group poisoned with FNT from day one to 14th and recovery through normal feeding (food and water) from day 21st to 28th

(ii) Depression of AChE Level by Profenophos (PFP) and Recovery Efficacy

Acetylcholinesterase Enzyme depression was achieved through poisoning of mice of both sexes into a solution made by 3 ml of PFP to 200 ml of normal saline from day one to 14th while AChE recovery from 21st to 28th day through administering 1500 mg/kg body weight of crude methanolic extract of the stem bark of *A. nilotica* (B2), ascorbic acid (B3) and normal feeding (B4) (Table 7). All treatment groups were compared with the untreated group (B1).

Results from Table 7 show that there is a significant decrease of the mean AChE level of female mice during poisoning with PFP in the crude methanolic extract of the stem bark of *A. nilotica* group (B2) compared to the untreated group at days one, 7th and 14th, while there was no significant difference was observed in male mice, however, on 14th day all treated mice were very weak due to pesticide exposure. Results presented in Table 4 show that there is sex variation. The recovery effect was observed when poisoning (PFP) was removed after the 14th day. Whereby, on the 28th day it was observed that the mean AChE level of the B2 treatment was the same as the untreated group (B1) in male mice which is an indicator of recovery, similar to B3 and B4 groups. Hence, the recovery effect observed only in male mice was significant in all treatments (B2, B3, and B4) including methanolic bark extract of *A. nilotica*, ascorbic acid, and normal feeding. Comparison of the means of the AChE level between B2 and B3 show that there was no significant difference from day one to 28th (Table 8), similar to Table 9 where B2 and B4 were compared.

Table 7: Mean AChE level (u/ml) of mice poisoned by profenophos (PFP) and treated by methanolic stem bark extract of *A. nilotica*, ascorbic acid and normal feeding

Interval	Treatments		B1	B2	B3	B4
	Days	Sex				
Poisoning	0	F	1.735 ± 0.21920	1.025 ± 0.02121*	1.265 ± 0.47376	1.150 ± 0.35355
		M	1.705 ± 0.04949	1.050 ± 0.63639	1.010 ± 0.26870	1.150 ± 0.07071*
	7	F	1.710 ± 0.07071	-0.15 ± 0.07071*	0.450 ± 0.21213*	0.100 ± 0.28284*
		M	1.390 ± 0.07071	-0.05 ± 0.63639	0.100 ± 0.70711	0.100 ± 0.56569
	14	F	1.630 ± 0.05657	-0.15 ± 0.35355*	0.100 ± 0.28284*	-0.10 ± 0.28284*
		M	1.450 ± 0.14142	-0.25 ± 0.63639	0.050 ± 0.35355*	-0.20 ± 0.14142*
Recovery	21	F	1.530 ± 0.09899	0.200 ± 0.14142*	0.100 ± 0.42426*	-0.05 ± 0.35355*
		M	1.105 ± 0.17678	0.250 ± 0.21213*	0.350 ± 0.07071*	0.150 ± 0.07071*
	28	F	1.520 ± 0.18385	0.250 ± 0.07071*	0.250 ± 0.21213*	0.200 ± 0.14142*
		M	1.090 ± 0.32527	0.450 ± 0.35355	0.350 ± 0.21213	0.150 ± 0.07071

Values are expressed as mean ± STDEV, STDEV: standard deviation, day one: the day treatment started. B1- untreated group, B2 - The group poisoned with PFP from day one to 14th and recovery by methanolic stem bark extract of *A. nilotica* at the rate of 1500 mg/kg per body weight from day 21st to 28th. A3: The group poisoned with PFP from day one to 14th and recovery by ascorbic acid at the rate of 1500 mg/kg per body weight from day 21st to 28th, B**4**: The group poisoned with PFP from day one to 14th and recovery through normal feeding (food and water) from day 21st to 28th: Superscript "*" indicate significance at $P < 0.05$ compared to an untreated group for each treatment group.

Table 8: Comparison of mean AChE (u/ml) between mice group poisoned with PFP and treated with methanolic stem bark extract of *A. nilotica* (B2) and ascorbic acid (B3)

Interval	Treatments		B2	B3	Mean diff	Sign Level	95 % Confidence	
	Days	Sex					Lower Bound	Upper Bound
Poisoning	0	F	1.025 ± 0.02121	1.265 ± 0.47376	0.24	0.5485	-1.20283	1.68283
		M	1.050 ± 0.63639	1.010 ± 0.26870	-0.04	0.9422	-2.14169	2.06169
	7	F	-0.15 ± 0.07071	0.450 ± 0.21213	0.30	0.1982	-0.38030	0.98030
		M	-0.05 ± 0.63639	0.100 ± 0.70711	0.05	0.9475	-2.84431	2.94431
	14	F	-0.15 ± 0.35355	0.100 ± 0.28284	-0.05	0.8902	-1.42751	1.32751
		M	-0.25 ± 0.63639	0.050 ± 0.35355	-0.20	0.7351	-2.41491	2.01491
Recovery	21	F	0.200 ± 0.14142	0.100 ± 0.42426	-0.10	0.7818	-1.46061	1.26061
		M	0.250 ± 0.21213	0.350 ± 0.07071	0.10	0.5917	-0.58030	0.78030
	28	F	0.250 ± 0.07071	0.250 ± 0.21213	0.00	1.0000	-0.68030	0.68030
		M	0.450 ± 0.35355	0.350 ± 0.21213	-0.10	0.7643	-1.35442	1.15442

Values are expressed as mean ± STDEV, STDEV: standard deviation, day one: the day treatment started. B2 - The group poisoned with PFP from day one to 14th and recovery by methanolic stem bark extract of *A. nilotica* at the rate of 1500 mg/kg per body weight from day 21st to 28th. B3: The group poisoned with PFP from day one to 14th and recovery by ascorbic acid at the rate of 1500 mg/kg per body weight from day 21st to 28th

Table 9: Comparison of mean AChE (u/ml) between mice group poisoned with PFP and treated with methanolic stem bark extract of *A. nilotica* (B2) and normal feeding (B4)

Interval	Treatments		B2	B4	Mean diff	Sign Level	95 % Confidence	
							Lower Bound	Upper Bound
	Days	Sex						
Poisoning	0	F	1.025 ± 0.02121	1.150 ± 0.35355	0.125	0.6672	-0.95259	1.202587
		M	1.050 ± 0.63639	1.150 ± 0.07071	0.100	0.8457	-1.84809	2.04809
	7	F	-0.15 ± 0.07071	0.100 ± 0.28284	-0.05	0.8310	-0.93701	0.837006
		M	-0.05 ± 0.63639	0.100 ± 0.56569	0.050	0.9414	-2.54054	2.640535
	14	F	-0.15 ± 0.35355	-0.10 ± 0.28284	-0.05	0.8902	-1.42751	1.327508
		M	-0.25 ± 0.63639	-0.20 ± 0.14142	-0.05	0.9235	-2.03341	1.933406
Recovery	21	F	0.200 ± 0.14142	-0.05 ± 0.35355	-0.15	0.6335	-1.30851	1.008514
		M	0.250 ± 0.21213	0.150 ± 0.07071	-0.10	0.5917	-0.78030	0.580303
	28	F	0.250 ± 0.07071	0.200 ± 0.14142	-0.05	0.6985	-0.53105	0.431047
		M	0.450 ± 0.35355	0.150 ± 0.07071	-0.30	0.3604	-1.39696	0.796955

Values are expressed as mean ± STDEV, STDEV: standard deviation, day one: the day treatment started. B2 - The group poisoned with PFP from day one to 14th and recovery by methanolic stem bark extract of *A. nilotica* at the rate of 1500 mg/kg per body weight from day 21st to 28th, B4: The group poisoned with PFP from day one to 14th and recovery through normal feeding (food and water) from day 21st to 28th

(iii) Depression of AChE Level by Chlorpyrifos (CPF) and Recovery Efficacy

Acetylcholinesterase Enzyme depression was observed when mice were poisoned with CPF solution made by dissolving 3 ml of CPF active ingredient to 200ml of normal saline administered to the mice orally from day one to 14th and recovery was attempted through administering the methanolic stem bark extract of *A. nilotica* (C2), Ascorbic acid (C3) and normal feeding (C4) from day 21st to 28th (Table 10). Results show that the mean AChE level on day one, male mice of the treatment groups C3 and C4 were decreased significantly compared to an untreated group at $P<0.05$ while no significant difference observed in female mice in both groups at $P>0.05$, but on 14th day all treated mice were very weak. AChE depression continued up to 21st day for all treatment groups, despite CPF was removed on the 14th day and the presence of the recovery indication on day 28th day for male mice was observed to be significant equal to an untreated group at $P<0.05$ in treatment groups C2, C3 and C4. Results indicate that there is sex variation in mean AChE recovery effects due to CPF poisoning. From Table 11 results show that there is no significant difference between

the mean AChE level for both treatment groups C2 and C3 at P>0.05, similar to treatment groups in Table 12 where C2 and C4 were compared at P>0.05.

Table 10: Mean AChE level (u/ml) of mice poisoned with chlorpyrifos (CPF) and treated by methanolic stem bark extract of *A. nilotica*, ascorbic acid and normal feeding

Interval	Treatments		C1	C2	C3	C4
	Days	Sex				
Poisoning	0	F	1.735 ± 0.21923	1.285 ± 0.06362	1.600 ± 0.39598	1.600 ± 0.08485
		M	1.705 ± 0.04952	1.450 ± 0.49492	1.410 ± 0.05657*	1.375 ± 0.06364*
	7	F	1.710 ± 0.07071	0.210 ± 0.04243*	0.805 ± 0.19092*	0.275 ± 0.07778*
		M	1.390 ± 0.07071	0.305 ± 0.28993*	0.400 ± 0.05657*	-0.21 ± 0.45962
	14	F	1.630 ± 0.05657	-0.03 ± 0.06364*	0.170 ± 0.08485*	-0.11 ± 0.32527*
		M	1.450 ± 0.14142	-0.12 ± 0.15556*	0.010 ± 0.18385*	-0.54 ± 0.16264*
Recovery	21	F	1.530 ± 0.09899	0.340 ± 0.11314*	0.235 ± 0.14849*	-0.2 ± 0.50912
		M	1.105 ± 0.17680	0.275 ± 0.09192*	0.315 ± 0.28991	-0.38 ± 0.06364*
	28	F	1.520 ± 0.18385	0.440 ± 0.14142*	0.380 ± 0.24042*	0.120 ± 0.09899*
		M	1.090 ± 0.32527	0.495 ± 0.09192	0.375 ± 0.33234	0.17 ± 0.24749

Values are expressed as mean ± STDEV, STDEV: standard deviation, day one: the day treatment started. C1- untreated group, C2 - The group poisoned with CPF from day one to 14th and recovery by methanolic stem bark extract of *A. nilotica* at the rate of 1500 mg/kg per body weight from day 21st to 28th. C3: The group poisoned with CPF from day one to 14th and recovery by ascorbic acid at the rate of 1500 mg/kg per body weight from day 21 to 28, C**4**: The group poisoned with CPF from day one to 14th and recovery through normal feeding (food and water) from day 21st to 28th: Superscript "*" indicates significance at P < 0.05 compared to the untreated group for each treatment group

Table 11: Comparison of mean AChE (u/ml) between mice group poisoned by CPF treated with methanol stem bark extract of *Acacia nilotica* (C2) and ascorbic acid (C3)

Interval	Treatments		B2	B4	Mean diff	Sign Level	95 % Confidence	
	Days	Sex					Lower Bound	Upper Bound
Poisoning	0	F	1.285 ± 0.06362	1.600 ± 0.39598	0.315	0.3823	-0.90519	1.53519
		M	1.450 ± 0.49492	1.410 ± 0.05657	-0.04	0.9200	-1.55557	1.47557
	7	F	0.210 ± 0.04243	0.805 ± 0.19092	0.595	0.0500	-0.00003	1.19003
		M	0.305 ± 0.28993	0.400 ± 0.05657	0.095	0.0543	-0.80373	0.99373
	14	F	-0.03 ± 0.06364	0.170 ± 0.08485	0.140	0.2029	-0.18269	0.46269
		M	-0.12 ± 0.15556	0.010 ± 0.18385	-0.11	0.5845	-0.84271	0.62271
Recovery	21	F	0.340 ± 0.11314	0.235 ± 0.14849	-0.105	0.5098	-0.67297	0.46297
		M	0.275 ± 0.09192	0.315 ± 0.28991	0.040	0.8696	-0.88531	0.96531
	28	F	0.440 ± 0.14142	0.380 ± 0.24042	-0.06	0.7897	-0.90862	0.78862
		M	0.495 ± 0.09192	0.375 ± 0.33234	-0.12	0.6713	-1.16909	0.92909

Values are expressed as mean ± STDEV, STDEV: standard deviation, day one: the day treatment started. C2 - The group poisoned with CPF from day one to 14th and recovery by methanolic stem bark extract of *A. nilotica* at the rate of 1500 mg/kg per body weight from day 21st to 28th. C3: The group poisoned with CPF from day one to 14th and recovery by ascorbic acid at the rate of 1500 mg/kg per body weight from day 21st to 28th.

Table 12: Comparison of mean AChE (u/ml) between mice group poisoned with CPF and treated with methanolic stem bark extract of *Acacia nilotica* (C2) and normal feeding (C4)

Interval	Treatments		C2	C4	Mean differ	Sign. Level	95 % Confidence	
	Days	Sex					Lower Bound	Upper bound
Poisoning	0	F	1.285 ± 0.06362	1.600 ± 0.08485	0.315	0.0523	-0.00766	0.63766
		M	1.450 ± 0.49492	1.375 ± 0.06364	-0.075	0.8514	-1.59316	1.44316
	7	F	0.210 ± 0.04243	0.275 ± 0.07778	0.065	0.4085	-0.20456	0.33456
		M	0.305 ± 0.28993	-0.21 ± 0.45962	-0.095	0.8278	-1.74833	1.55833
	14	F	-0.03 ± 0.06364	-0.11 ± 0.32527	0.080	1.0884	-0.92838	1.08838
		M	-0.12 ± 0.15556	-0.54 ± 0.16264	0.42	0.1186	-0.26472	1.10472
Recovery	21	F	0.340 ± 0.11314	-0.2 ± 0.50912	-0.14	0.7407	-1.72675	1.44675
		M	0.275 ± 0.09192	-0.38 ± 0.06364	0.105	0.3154	-0.23515	0.44515
	28	F	0.440 ± 0.14142	0.120 ± 0.09899	-0.32	0.1199	-0.84519	0.20519
		M	0.495 ± 0.09192	-0.17 ± 0.24749	-0.325	0.2238	-1.12823	0.47823

Values are expressed as mean ± STDEV, STDEV: standard deviation, day one: the day treatment started. C2 - The group poisoned with CPF from day one to 14th and recovery by methanolic stem bark extract of *A. nilotica* at the rate of 1500 mg/kg per body weight from day 21st to 28th, C4: The group poisoned with CPF from day one to 14th and recovery through normal feeding (food and water) from day 21st to 28th

4.2 Discussion

4.2.1 Reducing Power

This study evaluated *in vitro* reducing power of crude methanolic extract from *A. nilotica*. Increase in reducing power of crude methanolic extract from *A. nilotica* stem bark compared to ascorbic acid was observed as indicated by Table 1. Increase of absorbance readings (Table 1). The current results of this study indicate that the crude methanolic extract from *A. nilotica* has high reducing power compared to ascorbic acid (Fig. 14), which is similar to previously reported result for *A. nilotica* lignins (Aadil *et al*., 2014). The solvent used in extraction may influence reducing power of extracts (Kalaivani & Mathew, 2010). In this study maximum reducing power was 3.716 ± 0.045 where methanol was used while a study in Pakistan by Sadiq *et al*. (2015) reported 2.53 ± 0.06 when ethanol was used. These variations are based on changes in polarity of the solvent, consequently the polarity of the extractives.

The better results have shown at a dilution of 1:1 (S1) Table 1 to be 3.716 ± 0.045 for crude methanolic extract of *A. nilotica* and 1.468 ± 0.052 for ascorbic acid and poor results have

shown at dilution of 1:10 where for the crude extract was 2.895 ± 0.006 while 0.108 ± 0.006 for ascorbic acid. This is similar to the study done by Del *et al.* (2008) and Duganath *et al.* (2010) in India where they reported that as a concentration of crude extract *A. nilotica* increase also absorbance reading increase and found that at 10ug/ml (absorbance 0.50), 20ug/ml (absorbance 0.65) and at 30ug/ml (absorbance was 0.8). Also for an *in vivo* study to attain the maximum effect of reducing the power the dilution ratio between crude extract and vehicle should be considered.

4.2.2 Acute Oral Toxicity of Acacia nilotica Extract

In this present study no clinical signs of toxicity were observed which is contrary to Mohammed (2011) reported on slight decrease of alertness and locomotion, slight animals' spontaneous activity where ethanol extract of *A. nilotica* was used at lower doses administered while Jimoh *et al.* (2015) reported major observable clinical signs in rats which are treated with ethyl acetate and n-Butanol extracts of leaves of *A. nilotica* for 14 days with early deaths recorded after 12 h and late deaths 48 h after fractions administration similarly to Umaru *et al*. (2016) reported clinical signs of toxicity Depression, anorexia and dyspnea in rats treated with the aqueous pod extract of *A. nilotica*. Medani *et al*. (2016) reported clinical signs of toxicity such as salivation, staggered gait, intermittent loss of voice, low appetite and death (between day 4 and day 8 at dose of 5 g/kg/d) in Nubian goats fed with whole pods of *A. nilotica* at doses of 1 to 5 g/kg/day for 35 days. In this present study no mortality of mice where recorded up to oral administration of 2000 mg/kg body weight similarly to results reported by Khan *et al*. (2015) and Mohan *et al*. (2014) but contrary to the studies reported by Mohammed (2011) where 20-100% mortality was reported in rats treated acutely with 50-500 mg/kg of extract on intra-peritoneal administration.

Studies reported that a decrease of body weight more than 10% of the initial weight indicates the toxicity of extract administered to an animal (Kandhare *et al.*, 2015). Change in body weight is an indicator of the adverse effect of drugs. In this present study the use of methanolic stem bark extract of *A. nilotica* shown to be significance increase of body weight in both control for the average of 22.345 ± 0.068 to 22.345 ± 0.068 and the treated group with average from 20.493 ± 0.082 to 24.155 ± 0.260 Similar results reported by Alli *et al.* (2015) where body weight of rats increased from 140.80 ± 1.22 to 177.10±1.20 in control group and 140.10 ± 1.20 to 175.90 ± 1.12 and 164.70 ± 1.15 in the treated group and Lompo-Ouedraogo *et al*. (2004) reported to be increased from 7·83 ± 0·39 to 17·65 ± 1·54 for the controls, from

8·17 ± 0·66 to 22·94 ± 0·57 for treated group. This provides evidence that the administration of the crude extract has a negligible level of toxicity on the growth of the animals. These results are contrary to the findings from Mohan *et al. (*2014), who reported a decrease in body weight in rats fed with 2% and 8% leaves aqueous extract of *A. nilotica* in the diet for 2 and 4weeks. Jigam *et al*. (2011) reported that the use of methanol root extract of *A. nilotica* was significantly contributing to a decrease in the bodyweight of mice treated over 5 weeks.

The findings of present results have shown no significant changes in hemoglobin level at P>0.05 in mice for both control and treated group, similarly to the findings reported by Umaru *et al*. (2016) where no significant alteration of the levels of hemoglobin concentration recorded, contrary to the findings of studies from Saudi Arabia, reported that over long-term use of *A. nilotica* as diet may induce hematological changes, where haemoglobin levels were reduced in rats fed 8% acacia diet for 4 weeks compared with those in control (Al-Mustafa & Dafallah, 2000) and findings from Nigeria where the use of roots from causes increase of red blood cell, hemoglobin and packed cell volume (PCV). The use of *A. nilotica* in goat reported lowering PCV and haemoglobin level in the blood (Ab *et al*., 2016). The differences can be associated with the part of the plant taken, differences of geographical allocation, a solvent used during extraction processes and species variation of animals used however further studies are recommended.

4.2.3 AChE Depression and Recovery

Organophosphate pesticides are reported to inhibit AChE in the blood (Chidiebere, 2012). Inhibition of AChE activity is an important indicator of OP poisoning (Malaysiana *et al*., 2017). Different OPs exert different adverse effects by irreversible inhibition of AChE at the cholinergic synapses in the central and peripheral nervous systems (Maitra, 2018). In this current study, the poisoning and recovery effect FNT, PFP and CPF were assessed through testing the AChE level of mice's blood. This evidence has been proven from result table 4 – 12 where there is a significance of AChE depression, which is an indication of AChE inhibition due to exposure from FNT, CPF and PFP from day one to 14^{th} day to all mice sexes for all treatment groups. The inhibition of AChE causes excessive accumulation of Acetyl Choline (Ach) at the synapses and neuromuscular junction, leading to overstimulation of ACh receptors (Jindal & Kaur, 2014). Recovering of AChE from OP-inhibition after being removed from OP exposure is very crucial to resolving the over-accumulation of ACh in both

control and treated group. Results of this present study show that there is a sex variation which indicates that the female sex group is at higher risk to be affected by OPs compared to the male mice because their body physiological, Life style and behavior, a similar statement as reported by Comfort and Re (2017) and Ngowi *et al*. (2017).

Fenitrothion has also been reported to exert their primary toxic effects through the inhibition of AChE (Malaysiana *et al*., 2017). The findings of this study have demonstrated that the 3ml of FNT can inhibition and cause depression of AChE in mice and AChE recovery can occur naturally with or without using any induced treatment. This observation matches with that of a previous study were 2.6 mg/kg bodyweight of Fenitrothion contaminated in beans used and cause slightly AChE depression and a complete AChE recovery observed at the end of the feeding period (Taylor *et al.*, 2007) and study by Farghaly (2008) reported the same effect upon 1.9 ppm of Fenitrothion, on other hand 10 mg/mL which corresponds to 10 mg/kg body weight of Fenitrothion (Sumithion 50, 500 mg/mL) cause depression of AChE within 7 days similarly to the study conducted on fish which was exposed to different levels of FNT where at 0.04 ppm FNT produced a 64% depression of AChE activity at 96 h exposure, whereas 0.02 ppm of FNT produced only a 44% reduction in AChE at 96 h and recovery occurred when submerged into fresh water (Sancho, Ferrando and Andreu, 1997) but contrary to the Malaysiana *et al*. (2017) reported that the use of Palm oil which is a major sources of vitamin E that consist both tocopherol and tocotrienol tocotrienol-rich fraction capable in protecting the oxidative toxic effects and AChE recovery effect from FNT poisoning.

The inhibition of AChE by PFF produce similar effects as described in FNT. Previous studies have stipulated that antioxidants have a significant protective role against OP pesticides against damage from lipid peroxidation (Morsy, 2003). Ascorbic acid (vitamin C) is major circulating water-soluble antioxidant. The findings of this current study demonstrated that 3ml of PFP can induce inhibition of AChE and recovery of AChE can occur through the usual body process without inducing neither methanolic stem bark extract of *A. nilotica* nor ascorbic acid. Similarly AChE recovery effect reported in fish exposed to OP induced the same effect despite species variation (Oruc, 2012; Venkateswara Rao, 2006).

Chlorpyrifos is known to be the AChE inhibitor (Altuntas *et al.*, 2003). It may induce oxidative stress and inhibit antioxidative and physiological activities (Mevlüt, 2013) through its non-systemic pathway to AChE inhibition at the end prevent breakdown of the

neurotransmitter-acetylcholine (ACh) which lead to accumulation of ACh in the synaptic cleft and causes overstimulation of the neuronal cells, which leads to neurotoxicity and death (Akefe, 2017). In the current study, AChE depression induced by CPF poisoning from day one to 14th (Table 10) was significant, this is due to the ability to inhibit AChE activities that lasts several weeks (Ambali *et al.*, 2012; Mevlüt, 2013). AChE recovery from CPF inhibition from this study demonstrated to be recovered and matches with an untreated group on 28th day only on males in all recovery treatments of methanolic stem bark extract of *A. nilotica*, Ascorbic acid and normal feeding (Table 10) which gave a clue that at a small dose of exposure recovery of AChE to normal level can be induced by the body itself through the normal body physiology of enzyme regeneration and other proteins through the signaling system and the feedback loop. These results are contrary to the study reported by Ambali *et al.* (2012) where the use of vitamin E was proven to have improved restoration of AChE level due to its antioxidant efficiency. On the other hand, Verma *et al*. (2007) reported that the use of a combination of vitamins A, E and C gives better results of AChE recovery from CPF inhibition. It has been reported that AChE recovery by induced antioxidant extract is based on its antioxidant property of containing polyphenol compounds like flavonoids, alpha-tocopherol and carotenoids which suggest having redox properties, which play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides generated by OPs (Kalaivani & Mathew, 2010), however, in this study further studies of searching more plant enriched antioxidants are recommended.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Ethno-pharmacology information about *A. nilotica* has been reported to be used on scavenging ability against ROS based on its antioxidant activity. The findings of this present study suggested that the use of methanolic stem barks from *A. nilotica* proven evidence of increasing reducing power at high concentration and nontoxic effects in mice due to lack of mortality and sign of toxicity up to 2000 mg/kg body weight. This implies that the plant is safe for utilization for rural communities for the medicinal purpose where conventional drugs are unaffordable due to high costs.

This is a first study to assess and report the antioxidant activity of stem bark methanolic extracts of *A. nilotica* in controlling OPs pesticide toxicity in mice, hence assessment is also warranted, despite OPs reported to inhibit AChE and cause AChE depression, recovery of AChE through the use of antioxidants from *A. nilotica* extract is not promising, however, at the end of the experiment male mice were safer than female mice. This sex variation indicates that the female sex group is uniquely susceptible to pesticide exposure compared to the male because of their body physiological characteristics, lifestyle and behavior.

5.2 Recommendations

Based on the results output from this present study, the following recommendations are encouraged to be employed:

(i) Since only methanol is used in this study, other solvents are encouraged to be employed.

(ii) In this present study no specific antioxidant chemical compound has been revealed due to the use of crude sample, hence further studies are needed to isolate and identify specific antioxidant active compound from crude extract of *Acacia nilotica*.

(iii) Since the only single plant is used in this present study, more antioxidants enriched plants must be searched and assessed.

(iv) Further toxicological assessment of the plant (sub-acute toxicity) must be conducted

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