

**THE INHIBITORY POTENTIALS OF SEED KERNEL METHANOL
EXTRACT OF *Mangifera indica* L. Anacardiaceae AND FRACTIONS ON
METALLOPROTEASE OF *Echisocellatus*.**

BY

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DECLARATION

I declare that the work in this dissertation entitled;“**THE INHIBITORY POTENTIALS OF METHANOL EXTRACT OF *Mangifera indica* L. Anacardiaceae AND FRACTIONS ON METALLOPROTEASE OF *Echisocellatus*,**”has been performed by me in the department of Biochemistry Ahmadu Bello University Zaria under the supervision of Prof. Sani Ibrahim and Prof. H.M. Inuwa. The information derived from the literature has been duly acknowledged in the text and the list of references provided. No part of this dissertation was previously presented for another degree or diploma at any university institution.

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CERTIFICATION

This dissertation titled “**THE INHIBITORY POTENTIALS OF METHANOL EXTRACT OF *Mangifera indica* L. Anacardiaceae AND FRACTIONS ON METALLOPROTEASE OF *Echisocellatus*”** by **PECULIAR NWANYIBUNWAOKORO** meets the regulations governing for the award of the degree in Masters of Biochemistry at Ahmadu Bello University Zaria and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to my parents Mr. and Mrs. C. Okoro and my sister Barrister Lilian Okoro for their support and encouragement throughout the study.

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ABSTRACT

Mangifera indica seed kernel methanol extract and its solvent fractions (ethyl acetate, n-butanol, and aqueous) were tested for their anti venom activities against the metalloprotease activity of *Echisocellatus* venom *in vitro* and *in vivo*. The ethyl acetate fraction was found to be the most active. To determine the mechanism of inhibition, metalloprotease was partially purified from the venom of *Echisocellatus* with a yield of 71%, a purification fold of 2.63 and a specific activity of 19.00 $\mu\text{mol}/\text{min}/\text{mg}$ protein. The enzyme appeared as a band on SDS-PAGE with a molecular weight of 23kDa. The kinetic properties of the enzyme showed a K_m of 0.31 mg mL^{-1} and a V_{max} of 9.09 $\mu\text{mol min}^{-1}$. When the enzyme was incubated with the extract, the kinetic study revealed a mixed non-competitive pattern of inhibition with a K_m values of 0.56 and 1.11 mg mL^{-1} and V_{max} values of 6.67 and 4.17 $\mu\text{mol min}^{-1}$ for 5 and 20% inhibitor concentrations respectively. An estimated K_i value of 0.168 mg mL^{-1} was obtained from a secondary plot demonstrating that the extract has a high affinity for the partially purified enzyme. Thus, could serve as an effective inhibitor. The most active ethyl acetate fraction was used to determine the *in vivo* anti venom against metalloprotease induced toxic effect on blood coagulation system (bleeding and clotting time) and some hematological parameters (Packed Cell Volume, Hemoglobin, White Blood Cell and Total Protein count) in mice. The results obtained from the *in vivo* studies showed a significant increase in the bleeding and clotting time of the mice treated with the venom alone when compared with that of the control. For in the Mice treated with venom and the extract, increase in bleeding and clotting time was observed to be significantly ($p < 0.05$) reduced. Also, the results obtained for hematological parameters showed a significant reduction, except for the WBC

count which was slightly elevated in the Mice treated with venom alone when compared to the control. Normalcy was significantly restored in the groups treated with the venom and the active fraction. This suggests that the extract has some inhibitory effect *in vivo* against the action of *Echisocellatus* venom. The relevance of these findings would be of importance in the management of snake bite envenomation and the development of anti venom.

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LIST OF ABBREVIATION, GLOSSARIES AND SYMBOLS

HGB = Haemoglobin

RBCs = Red Blood Cell

LAAOs = L-Amino Acid Oxidases

MPs = Metalloproteases

MSKE = Mango Seed Kernel Extract

SVMP = Snake Venom Metalloproteases

SDS-PAGE = Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis

S.E.M = Standard Error of Mean

TP = Total Protein

kDa = kiloDalton

K_i = Inhibition Binding Constant

V_{max} = Maximum Velocity

WBC = White Blood Cell

WHO = World Health Organisation

1.0 INTRODUCTION

Snakes are found all over the world except in the permanently frozen arctic and Antarctic regions of the world (Kasturiratne *et al.*, 2008). More than 20 families are currently recognized, comprising about 500 genera and about 3,400 species. Most species are non-venomous, they swallow prey alive or kill by constriction and those that have venom use it primarily to kill and subdue prey rather than for self-defense. Snake bite has been a common and frequently devastating environmental and occupational hazard, especially in rural areas of tropical developing countries (Alirol *et al.*, 2010). The incidence of snake bite in rural West Africa is estimated to be as high as 497 per 100,000 population, with 11–17% mortality rate (WHO, 2015).

According to data presented by Nasidi (2007), globally over 3 million humans are bitten annually by venomous snakes, resulting in more than 150,000 deaths. Nigeria is reported to have one fifth of all West African region cases occurring in the country. This number does not account for unreported incidences, and those who survive envenomation will often deal with secondary effects such as necrosis or limb amputation.

The venomous snakes in Africa are known to belong to four main families – the Colubridae, Elapidae, Viperidae and Hydrophidae. In Nigeria, the most common poisonous snakes are the *Elapidae* and *Viperidae* (Mebs, 2002). These include the *Naja nigricolis* (spitting cobra), the *Echis ocellatus* (carpet viper) and *Bitis arietans* (puff adder). However, *Echis ocellatus*, the West African carpet viper, is considered the single most medically important snake species in West Africa and constitutes a severe economic and public health problem, particularly to farming communities. It accounts for 90% of bites and 60% of the fatalities in Nigeria, which add up to 20% of all African cases (WHO, 2011).

In humans, envenomation by *Echis ocellatus* causes severe blistering, oedema and necrosis at the bite site, and life threatening systemic effects including haemorrhage, coagulopathy and occasionally hypovolaemic shock (WHO, 2010).

The composition of most viper venoms contains at least 30% of metalloproteases, suggesting their potentially significant roles in envenomation-related pathogenesis, such as bleeding, intravascular clotting, edema, inflammation and necrosis (Fox et al., 2009). Metalloproteases are zinc-dependent proteins, known as snake venom metalloprotease (SVMP) (Soichi et al., 2012). One of the most devastating effects of SVMPs is their ability to disrupt microvessels, provoking local and systemic hemorrhage by interfering with the blood coagulation and hemostatic plug formation or by degrading the basement membrane or extracellular matrix components of the victims (Gutiérrez *et al.*, 2010). Additionally, leakage of blood from affected vessels also helps spread other venom toxins to their target tissues. Scarcity and delay of administration of anti-venom, poor health services and difficulties with transportation from rural areas to health centers are major factors that contribute to high fatality ratio of snakebite envenomation (Pinho and Pereira, 200). Snake antivenom is a biological product that typically consists of venom neutralising antibodies derived from a host animal. Antivenoms are produced by immunizing animals with whole venoms or isolated venom components (Gutiérrez *et al.*, 2011). They are the most effective pharmaceutical preparations in treatment of bites from venomous snakes (Theakston *et al.*, 2003; Fry *et al.*, 2003; Laing *et al.*, 2004). However, administration of animal-derived antivenins aimed at the neutralization of venoms and toxins in humans is prone to potential risks due to activation of the immune system by the heterologous protein (Devi *et al.*, 2002; Panfoli, 2010). This has led to the consideration of alternatives, especially, from plant sources for antivenom production.

The use of medicinal plants play a significant role to cover the basic health needs not only in the developing countries, but also in developed countries (Selim et al., 2013). Medicinal plants contain many compounds, such as polyphenols (phenolics), flavonoids, flavonols, alkaloids, saponnins and steroids which are among the other phytochemical compounds that have been shown to exhibit therapeutic activities such as antivenom and anti-microbial, antidiabetic as well as antiinflammatory properties (Dey and De, 2012; Selim et al., 2013; Barkatullah et al., 2013). For example, *Ceiba pentandra* Leaves was effective in the neutralization of *Echis ocellatus* Venom (Ibrahim et al., 2012), leaf extract of *Guiera senegalensis* has a detoxifying action against the venoms of *Echis carinatus* and *Naja nigricollis* (Abubakar et al., 2000; Sallau et al., 2005), and the antiinflammatory, analgesic and hypoglycemic effects of *Mangifera indica* Linn. (Anacardiaceae) stem-bark aqueous extract has also been reported (Ojewole, 2005).

The ethanol and methanol extracts of *Mangifera indica* L. (Anacardiaceae) have a relatively high phenolic content and have been shown to exhibit potent antioxidant (Rocha et al., 2007), anti-inflammatory (Garrido et al., 2004), hepatoprotective (Prasad et al., 2007) as well as anti-enzymatic activities (Leanpolchareanchai et al., 2009; Humphery et al., 2014).

1.1 Statement of Research Problem

Snakebite envenomation is a major public health problem among rural communities of the Nigerian savanna region. The carpet viper (*Echis ocellatus*) and, to a lesser extent, the African cobras (*Naja* spp.) and puff adders (*Bitis arietans*) have proved to be the main cause of mortality and morbidity in the country. Most incidences of snake bites occur mostly in developing countries like Asia and Africa (Nigeria) and are responsible for over 100,000 deaths worldwide annually (WHO, 2015). In Nigerian alone, 0.174% of every hospital admissions are attributed to snake bite envenomation and those who survive envenomation often times deal with secondary effects such as necrosis, skin ulceration or limb amputation (Goje *et al.*, 2013; Gutierrez *et al.*, 2006). Antivenom immunotherapy which has been the only specific treatment against snake bite envenomation is burdened with various side effects such as anaphylactic shock, pyrogen reaction and serum sickness, and unfortunately, sensitivity tests for the antivenom dosage are unreliable and have no predictive value for the occurrence of early reactions or even severe systemic anaphylaxis (Bawaskar, 2004; Abdulrazaq, 2011). In addition, antiserum development in animal is time consuming, expensive and requires ideal storage condition. In Africa, especially in Nigeria, there seems to be restricted availability of these antivenoms and usage, thus, limiting the use of serum therapy (Joseph and Afolabi, 2012). *Mangifera indica* seed extracts has a relatively high phenolic content and have been reported to exhibit potent antioxidant, anti-inflammatory and hepatoprotective activities (Rocha *et al.*, 2007; Garrido *et al.*, 2004; Prasad *et al.*, 2007; Leanpolchareanchai *et al.*, 2009; Humphery *et al.*, 2014). Therefore, there is the need to evaluate its potentials in the treatment and management of snake bite envenomation as an alternative to serum therapy.

1.2 Justification

The use of natural venom inhibitors from *Mangifera indica* seed extracts, which are widely known by many could complement or substitute for the action of sheep serum anti-venoms. It would be an alternative way to minimize the socio-medical problem of snake bite in tropical countries, especially in Nigeria. *Mangifera indica* seed kernel extract may provide this cheap and readily accessible alternative.

1.3 Aim

The aim of this study is to determine the inhibitory potentials of *Mangifera indica* Seed kernel extract and its effects on metalloprotease in *Echis ocellatus* venom.

1.4 Specific Objectives

- To extract and carry out assay guided solvent fractionation of *Mangifera indica* Seed kernel.
- To partially purify metalloprotease from *Echis ocellatus* venom.
- To determine whether or not the extract will inhibit the metalloprotease activity from *Echis ocellatus* venom *in vitro* and study the possible mechanism of inhibition.
- To determine the inhibitory effect of the active fraction on the coagulopathy and haematotoxicity induced by metalloprotease from *Echis ocellatus* venom in Albino mice (*in vivo*).

2.0 LITERATURE REVIEW

Snake bite is a common and frequently devastating environmental and occupational disease, especially in rural areas of tropical developing countries with significant morbidity and mortality which has been found to occur more among farmers, plantation workers and other people who dwell outdoors especially in rural and poor communities (Alirol *et al.*, 2010; Warrel, 2008). In Nigeria, it commonly affects the rural population of the savannah region of the country where farming and animal husbandry are the major occupation (Omogbai *et al.*, 2006).

Though it is difficult to be precise about the number of cases due to unreported incidences, it is estimated that worldwide incidence of snake bite is in excess of 5 million per year with more than 120,000 deaths. In Nigeria, an estimated one million snakebites occur annually with an estimated 10,000 per year in the savanna region of Nigeria (Mustapha, 2003; WHO, 2015)

For instance, between 2005 and 2010, a total of 12,398 snakebite victims were attended to at the Snake Bite Treatment and Research Centre, Kaltungo in Gombe State Nigeria. From this figure, 55 deaths were recorded. Many of the snakebite victims were from Gombe, Bauchi, Taraba and Adamawa states of Nigeria (Ademola-Majekodumi *et al.*, 2010).

Common culprit of these bites in Nigeria is the carpet viper (*Echis ocellatus*), which is seen everywhere but highest in the Middle Belt particularly Plateau, Gombe, Bauchi, Northern Enugu, Kwara, Kogi, Kaduna and Taraba States. Gombe has the highest number followed by Taraba and then Plateau (Ademola-Majekodumi *et al.*, 2010)

2.1 Classification of Snakes

There are more than 2340 species of snakes living on the earth and more than 600 known species are venomous snakes - about a quarter of all snake species - They belong to polyphyletic group of Colubroidea, however, can be classified into several families: These include the *Colubridae* (colubrids), *Elapidae* (elapids), *Hydrophiidae* (sea-snakes), and *Viperidae* (true vipers), (Fry, 2005).

The family *Colubridae* is the largest of all families of snakes encompassing 80 to 85% of all living snakes. Many snakes in this family are well known and harmless; however, some are venomous (*Lawson et al., 2005*). Some common examples of colubrid snakes include Boomslang, and Black Rat Snake. The family *Elapidae* are mostly venomous; however, not all are dangerous to man (Mackessy 2002). Examples of Elapid snakes include Kraits, the Black Mamba, King Cobra, Green Mamba and the Indian Cobra. The family *Hydrophiidae* comprises the sea snakes and has evolved several adaptations to allow almost complete existence in the water. Only a small proportion of bites are fatal to man because the snake can control the amount of venom injected. They are usually found in the warm waters of the Indian and Pacific Oceans (*Peichoto et al., 2004*). Examples of *Hydrophiidae* snakes include False Coral Snake, and Grass Snake. The family *Viperidae* is the most diverse family of venomous snakes. The venom fangs are large, which permit deep penetration during envenomation of prey. *Viperidae* are found throughout Europe, Africa, Asia, and the Americas (*Mebs, 2002*). Examples include the Russell's viper, Saw-scaled viper and the Gaboon viper.

2.2 Overview of Snakes Found in Africa

It is generally accepted that modern day snakes (sub-order serpentes) arose from lizards in the early cretaceous period, about 130 million years ago (Mc Dowell, 1972). There is however no hard and fast fossil evidence to link the suborders. Since small lizards and snakes do not make good fossils, as the small, delicate bones tend to break or become scattered (Anon, 2009). Due to this incomplete fossil evidence, snake evolution is largely theoretical. The earliest known fossil creatures resembling snakes are from the cretaceous period some 130 million years ago. These were short and heavy and had a mixture of lizard and snake characteristics. Unfortunately, there is no intermediary evidence to link these creatures with modern snakes (Lee, 2009).

One of the most widely accepted theories is that all snakes evolved from burrowing lizards. Certain primitive lizards would have taken to burrowing into the substrata in order to escape predators and to hunt other subterranean creatures (as some modern day species still do) (Jonathan, 2015). In Africa, about 100 different species have been documented (Mahanta and Mukherjee, 2001). Out of the 100 known species in Africa, only 13% are known to be venomous (Hile, 2004).

2.3 Some Poisonous Snakes in Africa

Some distinctive features and common characteristics of this group of snakes include:

- The presence of venom glands anatomically makes the heads of all poisonous snakes more triangular than oval.
- The presence of second set of pits on the face of a snake, below the nostril, is an indication of a poisonous snake.
- The presence of a rattle is always a guarantee that the snake is venomous.

2.3.1 Carpet saw-scaled viper (*Echis ocellatus*):

The saw-scaled viper, *Echis ocellatus* is among the commonest cause of envenomation in West Africa, being responsible for approximately 95% of the reported cases in northern Nigeria causing several hundreds of deaths annually (Bharati *et al.*, 2003; Hasson *et al.*, 2003). Carpet viper is one of the most aggressive and feared venomous snakes in the world. It grows up to 35cm in length. Carpet viper moves around in a coiled shape when it is threatened. It is also known as saw-scaled viper because it has scales on the sides of its body that it rubs together to produce rasping sound, very similar to that of a saw, to ward off predators.

There are two species of the genus *Echis*: *Echis coloratus gunther* (Bourton's carpet viper) and *Echis ocellatus stemmler*. *Echis coloratus gunther* occurs in the Middle East, whereas *Echis ocellatus stemmler* is widely distributed (Stemmler, 1970). *Echis ocellatus stemmler* is the subspecies that is commonly found in Nigeria (Pugh and Theakston, 1980). *Echis ocellatus* belongs to the family *Viperidae* and is considered as the most dangerous snake in the world (Warrell *et al.*, 1977; Leonard; 2010) because of its wide distribution, abundance in farming areas, good camouflage, irritability, and its venom (Warrell *et al.*, 1974). It is strongly haemotoxic, affecting the vessels, blood, and heart muscle. Bites from *Echis ocellatus* is the most important cause of morbidity and mortality in human beings, biting and killing more people than any other species of snake in the world (Warrell *et al.*, 1974; Mustapha, 2003). The incidence of carpet viper bite in endemic areas is often associated with its prevalence; it implies that its bites are correspondingly uncommon where the snake species are rare. The endemic areas of *Echis ocellatus* bites in Nigeria are Kaltungo (Gombe State), Bambur (Taraba State), Zungeru (Niger State), Garkida- 9 Hong-Michika area

(Adamawa State), Langtang- Shendam (Plateau State), and Zaria (Kaduna State) (Anon, 2009; Lee, 2009).



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Plate i: Adult *Echis ocellatus* photographed from the wild in Kaltungo, Gombe State, Nigeria.

2.3.2 Red-necked spitting cobra (*Naja nigricollis*)

Naja nigricollis is a predominantly black snake with pinkish to reddish bands at the ventral surface of the neck region. It has long thin front fangs and gives an average venom yield of 2ml (1.0 – 3.1ml) per snake per day (William, 1998). A species of *Naja nigricollis* (spitting cobra) can eject venom from a distance of 6-12 feet. The venom is aimed at victim's eyes resulting in conjunctivitis and corneal ulceration (Warell *et al.*, 1977).



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Plate ii: Adult *Naja nigricollis* photographed in Abuja, Nigeria

2.3.3 Puff adder (*Bitis arietans*)

Bitis arietans is a brown coloured snake with black markings. It is stout and relatively large (flukeshaped). It is a slow (in terms of speed of locomotion), nocturnal, ground-dwelling snake with a somewhat triangular head. It has large hinge-like fangs and gives an average venom yield of 4.9 ml (4.1 – 5.4ml) per snake per day. It is found throughout the Savannah regions as well as the rainforest belt of Nigeria and is believed to be the most widely distributed snake in Africa. (William, 1998).



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Plate iii: Adult *Bitis arietans* from Zaria, Kaduna State

2.4 Incidence of Snakes in Nigeria

In Nigeria, snakes are found everywhere (Habib *et al.*, 2008). From the coastal regions of the tropical rain forest in the southern part of Nigeria to the Sahel savannah at the upper part of the North. The most prevalent venomous snakes reported in Nigeria based on hospital records of casualties with snake envenomation are *Echis ocellatus*, *Naja nigricollis*, *Bitis arietans*, *Naja katiensis*, *Naja haje*, *Atractaspis microlepidota*, and *Dendroaspis augusticeps* (EchiTAb, 2008). Other known but non-venomous snake species include *Causus maculatus*, *Telescopus variegatus*, different species of rat snakes, the green twig snakes, and the sand snake, royal python (*Python regeus*), Rock python (*Python sebe*) and some other species whose DNA is being studied for proper identification (Hile, 2004).). Generally, only 57 different species of snakes are documented in Nigeria (Abubakar *et al.*, 2000).

2.5 Biochemistry of Snake Venom

Snake venom is highly modified saliva containing zootoxins that facilitates the immobilization and digestion of prey and defends against a threat. It is injected by unique fangs after a bite but some species are also able to spit (Bauchot, 1994). The glands which secrete the zootoxin is a modification of the parotid salivary gland and are situated on each side of head below and behind the eye encapsulated in muscular sheath. The glands have large alveoli in which venom is stored before being conveyed by the duct to the tubular fangs, through which it is injected. Snake venom is a combination of many different proteins, peptides and enzymes and they are generally not dangerous when ingested. Snake venoms are complex mixture of enzymatic and toxic proteins, which include phospholipase A₂ (PLA₂s), myotoxins, hemorrhagic metalloproteases and other proteolytic enzymes, coagulant components, cardiotoxins, cytotoxins and neurotoxins (Margarita *et al.*, 2012).

It has acidic pH, specific gravity is 1.03 and is water soluble. Phospholipase A₂ causes haemolysis by lysing the cell membrane of red blood cells. Oxidases and proteases are used for digestion. Snake venom contains inorganic cations such as sodium, potassium, magnesium and small amount of zinc, nickel, cobalt, iron. Zinc is necessary for anticholinesterase activity. Calcium is required for activation of enzyme like phospholipase (Priyanka *et al.*, 2014).

Envenomings induced by snakebite is characterized by local tissue damage involving haemorrhage, blistering, myonecrosis and inflammation. The inflammatory response has relevance in the evolution of tissue damage; it is associated with oedema, pain, leukocyte infiltration and release of several mediators. Pathogenesis induced by snake venoms is multifactorial and complex; it is characterized by local and systemic alterations (Margarita *et al.*, 2012).

The induced symptoms after bites vary in humans, depending on the amount of inoculated venom, bite site, age, weight and response of each bitten patient. Several studies reported clinical symptoms in relationship with the biochemical variability of venom composition which leads to tissue damage causing failure of various vital organs. Death can occur few days or several weeks after snake bite (Fry and Wuster, 2004). Secreted proteins represent the major components of snake venoms, they are encoded by poly-adenylated mRNA of venomous glands (12S and 20S) (Stroka *et al.*, 2005). These proteins have diverse biological activities, most of them are hydrolytic enzymes which help snake in its digestion, and some others are able to induce metabolic dysfunctions of the prey and/or to kill it (Costa *et al.*, 2008). In addition to secreted proteins, other components are also found in the venoms, such as lipids, polysaccharides, nucleotides, nucleosides, free amino acids, riboflavin, serotonin and histamine. Pharmacologically active substances of the venoms are enzymes and low molecular weight peptides. Some of these enzymes contribute to the toxic activity of the venoms (Valeriano-Zapana *et al.*, 2012). Main targets of isolated enzymes from snake venoms are cell membranes, vascular wall and blood coagulation cascade (Laing *et al.*, 2005).

2.5.1 Composition of snake venom

Snake venom is a complex mixture of organic compounds. Many of these compounds produce a variety of pathophysiological effects including local tissue damage and/or systemic effects in the affected individual (Dubovskii *et al.*, 2013). The major types of biomolecules found in snake venom are proteins, most of which are enzymes whereas other proteins lack catalytic activity. The most potent toxins of snake venom, which are responsible for causing severe pathophysiological effects after envenomation are α -neurotoxins – non-enzymatic nAChRs binding proteins (Tsetlin *et al.*, 2004), β -

neurotoxins – pre-synaptic phospholipase A₂ (PLA₂) (Chaisakul *et al.*, 2013), cytotoxins non-neurotoxic PLA₂s (Gutiérrez and Lomonte, 2013), and Zn²⁺-dependent metalloproteinases (Konshina *et al.*, 2011).

2.5.2 Composition of venom of *Viperidae*

The *Viperidae* snakes venoms are toxic secretions produced by a pair of specialized exocrine glands connected to the fangs by ducts (Markland and Swenson, 2013). *Viperidae* venoms may contain well over 100 protein components. This heterogeneous nature of venom composition was evidenced since the earliest analytical studies, and hence associated with the wide variety of bioactivities, both *in vitro* and *in vivo*, that were observed clinically or experimentally (Angulo and Lomonte, 2009). However, majority compounds of the *Viperidae* snakes venom can be grouped into a few major protein families, including enzymes (serine-proteinases, Zn²⁺-dependent metalloproteases, and group II phospholipase A₂ isoenzymes) that interfere with the coagulation cascade, the normal homeostatic system and tissue repair, and proteins with no enzymatic activity such as C-type lectin-like proteins (Angulo and Lomonte, 2009; Calvete *et al.*, 2007). Human envenomations from *Viperidae* are often characterized by clotting disorders, hypofibrinogenemia, and local tissue necrosis (Ayvazyan and Ghazaryan, 2012).

2.5.3 Enzymes and proteins of snake venom

Snake venoms are complex mixtures composed of varied enzymatic and nonenzymatic toxins. Although a single snake venom sample may contain dozens of enzymatic toxins, these enzymes are generally grouped into a few classes by toxinologists. The most commonly quantified classes of snake venom enzymes include phospholipase A₂ (PLA₂), phosphodiesterase, phosphomonoesterase, L-amino

acid oxidase, specific endopeptidases, and nonspecific endopeptidases (Takeda *et al.*, 2012).

Qualitatively, snake venoms consist of a mixture of protein with or without catalytic activity such as phospholipases A₂ (PLA₂), proteases, hyaluronidases, L-amino acid oxidases (LAAOs), acetylcholinesterases, growth factors, protein C activators, lectins, and von Willebrand factor-binding proteins; peptides mainly comprising bradykinin potentiators and disintegrins; low molecular weight organic compounds such as carbohydrates, serotonin, histamine, citrate, and nucleosides; and inorganic ions such as calcium, cobalt, magnesium, copper, iron, and potassium, as well as enzymatic inhibitors (Ramos and Selistre-De-Araujo, 2006).

2.5.3.1 Phospholipase A₂

Snake venoms from *Colubridae* (*sensulato*), *Elapidae*, and *Viperidae* families are rich sources of phospholipase A₂s (PLA₂s, phosphatidylcholine 2-acylhydrolases). These enzymes predominantly hydrolyze phospholipids containing unsaturated fatty acid tails at the *sn*-position which leads to the generation of lysophospholipids and unsaturated fatty acids (Kang *et al.*, 2011). These products of hydrolysis change the physical properties of cell membranes and activate downstream signal transduction pathways, which can produce widespread cellular pathology (Kini, 2003)). Structural studies on snake venom PLA₂s have identified highly conserved regions in these enzymes including a conserved structural scaffold (Gutiérrez and Lomonte, 2013). These enzymes display an impressive array of pharmacological and toxicological activities which likely originated through a process of ‘accelerated evolution’ that incorporated multiple amino acid substitutions of solvent exposed charged residues in these molecules (Kini, 2005) Interestingly, there seems to be no clear relationship between the amount of enzymatic activity (lipid hydrolysis) and pharmacological

action of PLA₂ s *in vivo* (Gutiérrez and Lomonte, 2013). Hence, this phenomenon has baffled toxinologists for decades from the view point that there is no clear correlation on structure to function or enzyme function to pharmacological action of snake venom PLA₂s.

2.5.3.2 Metalloproteases

Snake venom metalloproteinases (SVMPs) are major components of most *Crotalidae* and *Viperidae* venoms (Markland and Swenson, 2013). These enzymes - the key contributors to lethal toxicity in these venoms - are large multi-domain proteins that are classified as P-I, P-II, and P-III based on the presence or absence of non-catalytic ancillary domains that extend beyond the mature proteinase domain. Snake venom metalloproteinase induce local myonecrosis, skin damage, inflammatory reaction, arthritis and are responsible for causing paralysis (Markland and Swenson, 2013). The majority of SVMPs induce profuse hemorrhage, and the inactivation/activation of complement proteins (Berger *et al.*, 2008). SVMPs also possess diverse functions such as the disruption of homeostasis mediated by procoagulant or anticoagulant effects, platelet aggregation, and apoptotic or pro-inflammatory activities (Takeda *et al.*, 2012). Some of the studies indicated that the mechanisms of envenoming might be contributing to the accumulation of venom proteins at the bitten area causing capillary disruption. Microscopic studies also revealed that erythrocytes are leaked through widened inter-endothelial gaps when capillaries are exposed to venomous protein (Markland *et al.*, 2013).

2.5.3.3 L-Amino Acid Oxidases (LAAOs)

In snake venoms, LAAOs are found in high concentrations that vary according to each species of snakes, which may contribute to the toxicity of the venom. The

LAAOs exhibit catalytic specificity for long chain hydrophobic and aromatic amino acids and are active in a wide range of pHs and temperatures. Their structures, molecular masses, and isoelectric points are quite varied. They are able to induce changes in platelet function, which cause local effects on plasma clotting disorders among other things. They are also capable of inducing apoptosis in various cell lines and show antimicrobial and antiparasitic activity. The existence of LAAOs may be a means of protection against natural agents, parasites, and bacteria (Ande *et al.*, 2008).

2.5.3.4 *Hyaluronidases*

These enzymes from the snake venom break down the glycosaminoglycans of the extracellular matrix and connective tissues surrounding intact blood vessels and capillaries leading to haemorrhage and they also cause other enzymes to be absorbed more rapidly by the victim by attacking hyaluronic acid in mammalian cell membrane (Ed, 2001).

2.5.3.5 *Adenosine triphosphatases*

These enzymes are probably present in most snake venoms and they are one of the central agents resulting in the shock of victims and immobilizing small preys (Ed, 2001).

2.5.3.6 *Cholinesterases*

These are enzymes from snake venom that attack the nervous system by binding to the cholinergic receptors and relax muscles to the point where the victim has very little control (Ed, 2001).

2.6 Biochemical Basis of Snake Envenomation

2.6.1 Neurotoxic envenoming

Venom of snakes predominantly the Elapids and Hydrophids contain primarily neurotoxic components, which cause a presynaptic neuromuscular blockade, potentially causing respiratory paralysis (Chérifi *et al.*, 2013). This is characterized by moderate or absent local swelling, progressive descending paralysis starting with drooping eyelids (ptosis) and paralysis of eye movements causing double vision. There may be painful and tender enlargement of lymph glands draining the bite site (León *et al.*, 2011). The patient may vomit, the saliva may become profuse and stringy, and eventually there may be difficulties with swallowing and breathing. Once the neurotoxic venom effects manifest, they are difficult to reverse and may last 3 to 6 days (Montecucco *et al.*, 2008). Species involved include black and green mambas and non-spitting cobras. Neurotoxic venoms cause paralysis due to their effects on the nervous system.

There are two types of neurotoxins:

- Neurotoxins of hydrophids bind to post synaptic acetylcholine receptors resulting in paralysis. Respiratory paralysis is the primary cause of immediate death.
- Neurotoxins of Elapids (cobras and mambas) have pre-synaptic action which inhibits the release of acetylcholine at myoneural junction.

2.6.2 Myotoxic envenoming

Myonecrosis is due to the myotoxins that induce irreversible damage of skeletal muscle fibers. These molecules bind to the plasma membrane of muscle cells and alter

its permeability and integrity. The induced muscle tissue damage could be due to the penetration of myotoxins into muscle cells by endocytosis, probably through membrane receptors onto the surface of muscle cells or following hydrolysis of phospholipids causing membrane disruption (Montecucco *et al.*, 2008). These molecules enter into the cytosol, reach and alter the membrane of mitochondria and sarcoplasmic reticulum of muscle cells. The intracellular effect of these toxins occurs only after their initial action on the plasma membrane, which marks the onset of degenerative events (Hamza *et al.*, 2010a).

Tissue necrosis is a relevant local effect caused after snakebites, it is considered as a serious consequence in severe cases of envenomation. When myonecrosis appears tissues are altered leading to the gangrene and infections. This type of complication can be the cause of amputation. Indeed, myotoxins of snake venoms affect mainly the plasma membrane of muscle cells to which they bind through their cationic sequence (Falconi *et al.*, 2000).

2.6.3 Cytotoxic envenoming

This is characterized by painful and progressive swelling with blood-stained tissue fluid leaking from the bite wound, hypovolaemic shock, blistering and bruising. The victim will complain of severe pain at the bite site and throughout the affected limb, painful and tender enlargement of lymph glands draining the bite site resulting from cytolysis, ischaemia, blood extravasations and direct proteolytic activity, irreversible death of tissue may occur (necrosis/gangrene) (Fatima and Fatah, 2014).

2.6.4 Haemotoxic envenoming

Haemotoxic venom attacks the circulatory system and muscle tissue causing a breakdown or inflammation in the body. This venom basically destroys tissue and

blood cells. The venom breaks down protein in the region of the bite, making prey easier to digest. This is characterized by bleeding from the gums, gastro-intestinal and genito-urinary tracts, recent and partly healed wounds, vomiting of blood, haemoptysis, haematuria, persisted bleeding from the bite site and inflicted wound (Hamza *et al.*, 2010b).

Haemotoxic bites are the most painful as breathing hurts and tissues start to die.

Species involved include saw-scaled/carpet vipers, puff adders, boomslang, and vine snakes. The effect of haemotoxic snakes are manifested in different ways including:

2.6.4.1 Haemolytic effect:

This is believed to occur as a result of the presence of an enzyme known as haemolysin in *Viperidae* venoms. This enzyme continually lyse erythrocytes which are broken down rapidly resulting in anaemia, haemoglobinuria, pherocytosis, angioedema, blisters and in some cases icterus (Warrell, 1983; Ed, 2001).

2.6.4.2 Haemorrhagic effect:

This is the ability of venom to lyse intact blood vessels, especially capillaries through the activities of the enzymes, haemorrhagins and phospholipases (especially, phospholipase A₂) (Koh *et al.*, 2006). This is clinically manifested as gum bleeding, haematuria, haemachezia, melaena, haemoptosis, haematemesis, and spontaneous bleeding from the skin (Warrell, 1983; Marsh, 1994; EchiTab, 2006).

2.6.4.3 Coagulant effect:

This is the failure of blood to clot as a result of inhibition of clotting factors II and X, thus, resulting in excessive blood loss from wounds, fang marks, or bruises. This coagulant effect has been used to develop the 20 Minutes Whole Blood Clotting Test,

(WBCT) which is used clinically to diagnose carpet viper envenomation in endemic areas (Warrell, 1983; EchiTab, 2008).

2.7 Anti-Snake Venom Development

Antivenoms are the only effective specific treatments or antidotes for snakebite. They are raised in large domestic animals (usually horses, donkeys or sheep) by hyperimmunizing them against a single snake venom (producing a monovalent/monospecific antivenom) or against venoms of several species of snakes whose bites are common and frequently lead to severe envenoming in the geographical area where the particular antivenom is intended to be used (producing a polyvalent/polyspecific antivenom). Developing effective and cheap antivenoms (sometimes called "antivenins"), designing control assays, and recruiting the resources needed to validate them are an economic and ethic problem (Gutiérrez et al., 2011).

2.7.1 Plant as an antidote for snake envenomation

Plant extracts constitute rich sources of pharmacologically active compounds and have been utilized in folk medicine as antiophidians, and some of them have been reported to antagonize the activity of various crude venoms and purified toxins (Coe & Anderson, 2005). This activity has been attributed to secondary metabolites (chemical constituents) such as flavonoids, coumarins, alkaloids, sitosterol or glucoside, lupeol, gymnemagenin, pentacyclic triterpenes like oleanoic acid, ursolic, tannins, taraxasterol, α and β amyryl and other polyphenolic metabolites widely distributed in different plant families (Pithayanukul *et al.*, 2005). All these classes of chemical compounds are capable of interacting with macromolecular targets with enzymes or receptors and it can effectively inhibit the toxic effect of snake venoms *in*

vitro and *in vivo* (Costa *et al.*, 2010). An animal study has also been well documented in the case of immunity of the opossum (*Didelphis marsupials*) towards the venom of the *Jararaca* (*Bothrops jararaca*) snake (Biesalski, 2007).

2.7.2 Plants constituents with anti-venom activities

Several plants are used in traditional medicine as active agents against various effects induced by snakebite (Gomes *et al.*, 2009). There are numerous Folk medicines and its isolated constituents recommended as snake venom antidotes, for example, haemorrhagic effect of *Bothrops atrox* venom and their strong lethal enzymatic effect were neutralized by plants (Soares *et al.*, 2005)

The aqueous extract of *Pentaclethra maculosa* (EPema) exhibited full inhibition of hemorrhagic and nucleolytic activities induced by several snake venoms. *In vivo* tests showed that EPema is able to inhibit a *Bothrops jararacussu* metalloprotease. Additionally, partial inhibition of PLA₂s activities from snake venoms was also reported (Meenatchisundaram and Michael, 2009). Also, Di-n-octyl Phthalate, Isolated from *Ceiba pentandra* Leaves was effective in the neutralization of *Echis ocellatus* Venom (Ibrahim *et al.*, 2012).

Ethanol leaf extract of *Acalypha indica* potentially neutralized *Viper russelli russelli* (Russell's viper) venom and *Morus alba* leaf extract abolished the *in vitro* proteolytic and hyaluronolytic activities of *D. russelli russelli*' venom. Edema, hemorrhage and myonecrotic activities were also neutralized effectively (Fernandes *et al.*, 2011). Aqueous root extract of *Mimosa pudica* inhibited the hyaluronidase and protease activities of *Naja naja*, *Vipera russelli* and *Echis carinatus* venoms in a dose dependant manner (Vale *et al.*, 2008). The ethanol leaf extract and essential oil of *Nectandra angustifolia* were tested against the hemolytic and coagulant venom activities from coral snake. The ethanol leaf extract was the most active and inhibited

both venom activities (hemolytic and coagulant), while the oil was only active on the coagulant activity (Sanchez *et al.*, 2008).

2.7.3 The use of *Mangifera indica* seed kernel in the treatment of snake bites envenomation.

Mangifera indica L. (family: Anacardiaceae): Aqueous extract of stem bark had shown anti-myotoxicity of *D. russellii* venom. In vitro PLA₂ activity of the venom was totally inhibited by the extract. Several enzymes associated with envenomation were also inhibited significantly supporting its traditional antiophidian use (Dhananjaya *et al.*, 2011). Ethanol extract of seed kernel of Thai *Mangifera indica* (*Mangifera indica* L. cv. 'Fahlun') has shown to inhibit caseinolytic and fibrinogenolytic activities of Malayan pit viper and Thai cobra venoms in vitro. The phenolic constituent of the plant, pentagalloyl glucopyranose has also prevented enzymatic activities and necrotic effects of the snake venom in a dose dependent manner (Pithayanukul *et al.*, 2009). *Calloselasma rhodostoma* and *Naja Naja kaouthia* venoms were inhibited in vivo by the anti-hemorrhagic and anti-dermonecrotic properties of the ethanol extract of seed kernel of Thai *Mangifera indica*. Molecular docking studies were performed to explore the mechanism of action of the phenolic compound (Leanpolchareanchai *et al.*, 2009). Therefore, there is the need to also evaluate the potentials of the *Mangifera indica* seed kernel in the treatment and management of snake bite envenomation of *Echis ocellatus*.

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Sample collection

3.1.1.1 Snake venom and *Mangifera indica* seeds

Freeze dried *Echis ocellatus* venom was obtained from the Department of Pharmacognosy and Drug Development, Ahmadu Bello University, Zaria. *Mangifera indica* seeds were obtained from Zaria metropolis, Kaduna State.

3.1.2 Reagents and solvents

Sephadex G-75, DEAE Sephadex, Acrylamide, Bovine Serum Albumin (BSA), Bis-Acrylamide, Ammonium Persulfate (APS), Sodium Dodecyl Sulfate (SDS), PageSilver™ Silver Staining kit, sodium phosphate monobasic and dibasic salts, NNNN-tetramethylethylenediamine (TEMED), ethylenediaminetetraacetic acid, 1,10-phenanthroline, Methanol, n-Butanol, Ethyl Acetate, Dimethyl Sulfoxide, glycerol, 2-Mercaptoethanol, hydrochloric acid, acetic acid, Sodium chloride, Sodium hydroxide, Trichloroacetic acid (TCA), Bromophenolblue,

3.1.3 Experimental animals

Adult albino mice (20-30 g) of both sexes were procured, fed with normal animal feeds and maintained in the Animal house of faculty of Pharmaceutical Sciences Ahmadu Bello University, Zaria.

3.2 Method

In vitro Assessment of Effect of *Mangifera indica* Seed Kernel

3.2.1 Preparation of extract and its fractionation

The kernel was removed from the seed coat, sun-dried and pulverized to powder. Extraction was carried out with methanol using soxhlet apparatus and the solvent was

removed by rotary evaporator under reduced pressure to obtain the crude extract. The crude methanol extract was suspended in aqueous : methanol (4:1) followed by an assay guided fractionation with ethyl acetate and n-butanol (saturated with water). Each solvent fraction was tested for activity by inhibiting the action of metalloprotease present in the snake venom and the most active fraction was recorded.

3.2.2 Partial purification of metalloprotease

The venom was diluted with phosphate buffered saline solution (PBS) at pH 7.4. The small amount of insoluble material was centrifuged and the clear supernatant was used for the partial purification of metalloprotease from *Echis ocellatus*.

3.2.2.1 Ion exchange chromatography on DEAE sephadex

DEAE-cellulose was prepared by dissolving 2g of the anion-exchanger in 20 ml of phosphate buffer, pH 7.4. The slurry was then poured into a 3.0 X 20 cm column. Crude *Echis ocellatus* venom (100 mg) was dissolved in 10ml phosphate buffer, pH 7.4 in a beaker. This was transferred to a centrifuge tube and the insoluble component was removed by centrifugation and 2ml of the recovered supernatant was loaded onto DEAE sephadex column (3.0 x 20 cm) pre-equilibrated with 0.2M phosphate buffer pH 7.4. Using a syringe with a thick needle, the sample was carefully applied directly onto the top of the gel and it was ensured that the sample was equally distributed across the surface of the gel. The column was then eluted stepwise with NaCl gradient (0.0 – 0.5 M) at a flow rate of 1ml per minute. Thirty fractions were collected and assayed for Metalloprotease activity and total protein. The fractions showing high specific activities were pooled together.

3.2.2.2 Gel filtration chromatography on sephadex G-75

The gel was prepared by dissolving 2g of sephadex G-75 in 20ml phosphate buffer, pH 7.4 for 24 hours at room temperature and mixed with a glass rod to make the swollen particles form slurry. The slurry was then poured into a 2 by 100cm column packed with glass wool at the bottom. The column was first equilibrated with phosphate buffer, pH 7.4, before the sample was applied. The pooled metalloprotease active fractions from the ion exchange chromatography were loaded onto Sephadex G-75 column equilibrated with phosphate buffer (pH 7.4). The column was eluted with the same buffer solution, maintaining a flow rate of 1ml/min. Twenty one fractions were collected and metalloprotease specific assay and total protein concentration were carried out.

3.2.3 Metalloprotease assay.

This was carried out by incubating 0.5 ml of 20mg/ml enzyme solution with 0.05 ml of 1mg/ml Casein solution in 0.2 M phosphate buffer pH 7.4 for 30 min at 50 °C. The reaction was stopped by adding 1ml of 10% TCA and the mixture allowed standing for 10 min at room temperature. Soluble peptides were separated by centrifugation for 10 min and the absorbance of the TCA-soluble peptides in the supernatant measured at 280 nm. A control assay, without the enzyme in the reaction mixture, was also carried out and used as the blank in all spectrophotometric measurements (Mohsen *et al.*, 2013).

3.2.4 Determination of protein concentration.

The protein concentration of partially purified metalloprotease from *Echis ocellatus* venom was determined spectrophotometrically by taking absorbance at 280nm and 260nm wavelength.

3.2.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)

Electrophoresis under denaturing conditions was performed in 12% Acrylamide gel according to the method of Laemmli (1970) using Tris-glycine buffer, pH 8.3. The electrophoresis was carried out on the active fraction from Sephadex G-75 column gel filtration chromatography and the proteins were separated according to their electrophoretic mobility, which is a function of the length of the polypeptide chain or molecular weight as well as higher order protein folding, post transcriptional modifications and other factors. The protein bands were located by staining with Silver stain.

3.2.5.1 Sample preparation for SDS-PAGE

The sample was prepared by mixing in a test tube, 100µl of sample, one drop of 2-mercaptoethanol, which is a reducing agent that denatures the protein by reducing the disulphide linkages; one drop of bromophenol blue serves as tracking dye, one drop of 2% SDS, 2mM EDTA, and 50µl of Tris-HCL buffer, pH 6.8. The mixture was incubated at 100°C for 5 minutes, after which 3 drops of 20% glycerol was added to increase the density. The mixture was ready for loading on the gel.

3.2.5.2 SDS Gel Preparation

From 30% acrylamide and Bis-acrilamide stock, gel of composition 12% acrylamide was prepared. The gel buffer stock (30ml) consists of 10% SDS (9.4ml), 1.5M Tris-HCL (7.5ml, pH 8.8) and distilled water (12.3ml). Polymerization was initiated by adding freshly prepared 10% ammonium persulphate (APS) (0.5 ml) to the mixture followed by TEMED (20µl). The solution was swirled to mix and the mixture was poured into the gel tube immediately before polymerization occurred.

3.2.5.3 Loading, running and staining of the sample

The prepared sample (50 μ l) was dispensed into the gel tubes that were fixed in the disc Shandon apparatus. The upper and lower parts of Shandon tubes were filled with 125 mM Tris-glycine buffer, pH 8.3 containing 4% SDS and the power switched on at 150 V and 25 mA. It was then switched off when the marker dye was a few milliliters away from the bottom. The gel was removed and placed in staining tray rinsed with deionized water, before adding 50ml of gel glycerol and agitated gently for 30 min. It was then washed twice (at 10 min intervals) with 50 ml of washing solution (PBS), before placing in pretreating solution for 1 min, and then rinsed briefly in 50ml deionized water three times. The gel was then stained using Silver staining kit and procedure was as provided in the kit's manual. The molecular weight markers for the SDS-PAGE include; trypsinogen (24kDa), Carbonic Anhydrase (29kDa), α -lactalbumin (14.2kDa), Trypsin Inhibitor (20.1kDa), Bovine Serum Albumin (45kDa), Albumin (66kDa) and Glyceraldehydes-3- phosphate Dehydrogenase (36kDa). The relative mobility (R_f) which is a function of the distance travelled by the protein divided by the distance travelled by the solvent front was calculated for each of the marker proteins by measuring the distance travelled by each protein in the gel and the molecular weight of partially purified metalloprotease was extrapolated from the plot of log of molecular weight of the marker proteins against their respective R_f values.

3.2.6 Kinetic Studies

Initial Velocity Studies of the partially purified metalloprotease was carried out with varying concentrations of the substrate (0.5 – 2.0mg/ml) of Casein. Initial velocity values obtained were used to obtain a double reciprocal plot to determine the kinetic properties of the partially purified metalloprotease.

3.2.7 Inhibition Studies

Specific volume of varying concentrations of the active ethyl acetate fraction preparations 0, 5, 10 and 20% (w/v) were used as a substrate to inhibit the partially purified metalloprotease. The initial velocity data obtained was used for double reciprocal plots and a secondary plot was obtained from the primary plot to determine the inhibition binding constant (K_i) of the extract.

In vivo assessment of effect of *Mangifera indica* seed kernel

3.2.6 Animal grouping

Wistar albino mice were randomly divided into ten (10) groups of three (3) mice:

Group 1: Control group that received water

Group 2: Control group that received normal saline

Group 3: Low dose (0.05mg/kg body weight) envenomed mice that did not receive any extract treatment

Group 4: High dose (0.1mg/kg body weight) envenomed mice that did not receive any extract treatment

Group 5: Mice that received low dose (20mg/kg body weight) of *Mangifera indica* seed extract

Group 6: Mice that received high dose (40mg/kg body weight) of *Mangifera indica* seed extract

Group 7: Low dose envenomed mice (0.05mg/kg body weight) treated with low dose (20mg/kg body weight) of *Mangifera indica* seed extract (EAF)

Group 8: Low dose (0.05mg/kg body weight) envenomed mice treated with high dose (40mg/kg body weight) of *Mangifera indica* seed extract (EAF)

Group 9: High dose (0.1mg/kg body weight) envenomed mice treated with low dose (20mg/kg body weight) of *Mangifera indica* seed extract (EAF)

Group 10: High dose (0.1mg/kg body weight) envenomed mice treated with high dose (40mg/kg body weight) of *Mangifera indica* seed extract (EAF)

3.2.9 Envenomation of mice

The *in vivo* study was carried out according to the method of Omale *et al.* (2013). The venom was administered intradermally at a low dose of 0.05mg/kg (0.005mg/ml) and a high dose of 0.1 mg/kg (0.01mg/ml) body weight of mice. After which, the extract was also administered intradermally at a low dose of 20mg/kg (2mg/ml) and a high dose of 40mg/kg (4mg/ml) body weight of mice. After envenomation, the mice were left for four hours then bleeding time and clotting time were measured. The animals were sacrificed and a 2ml blood was collected for the measurement of haematological properties.

3.2.10 Measurement of incoagulability in envenomed mice

3.2.10.1 Bleeding time:

For the determination of the bleeding time, modified procedure of Mohamed *et al.* (1969) was used. Four hours after the treatment of the animals, the tail of each rat was gently pierced with lancet and a piece of white filter paper was used to blot the blood gently from the punctured surface of the body. The readings were taken every 15 sec. The end result occurred when the paper was no longer stained with blood.

3.2.10.2 Clotting time:

For the determination of the clotting time, the modified method of Igboechi and Anuforo (1986) was used. Clotting time is the time required for a firm clot to be formed in fresh blood on glass slides. The blood sample was collected from the mice via tail bleeding and a drop was placed on a clean plain slide and every 15 sec, a tip of office pin was passed through the blood until a thread-like structure was observed between the drop of blood and tip of the pin. The thread-like structure was an indication of a fibrin clot. The time was recorded.

3.2.11 Measurement of hematological parameters

3.2.11.1 Packed cell volume (PCV) of mice

Procedure

Anticoagulated blood were placed in plain capillary tubes and one end of the tubes sealed with clay. The filled tubes were placed in a microhematocrit centrifuge and centrifuged for 5 minutes at 1000 RPMs. The centrifugal force layered the blood components according to weight. The heaviest components are the red blood cells and are pushed to the bottom of the tube. The total height of the column (cells + plasma) and the height of the packed red cell column are measured.

3.2.11.2 Haemoglobin Concentration

Principle

The cyanmethaemoglobin method described by Coles (1980). Haemoglobin is converted to cyanmethaemoglobin by the addition of potassium ferricyanide and sodium cyanide. The absorbance of the colour produced is directly proportional to the amount of haemoglobin present.

Procedure

Twenty microlitres (0.02ml) of blood were diluted with 5ml of the reagent. After 10 minutes, the absorbance was measured at 540nm with water as blank. The haemoglobin concentration was obtained from calibration curve using standards

3.2.11.3 White blood cell count

The leukocyte or WBC count is the determination of white cells per micro litre of blood (Schalm *et al.*, 1975).

Principles

The WBC involves diluting the blood 1:20, (selectively destroying RBC) and staining the white cells with gentian violet, which permits easy identification of white blood cell. The count was done with Haemocytometer (model Marienfield Jermamy66300103)

Procedure

Using Thoma white cell pipette, blood was drawn up to 0.5 mark, followed by the diluting fluid to the 11 marks. This gives 1 to 20 dilutions. The hemocytometer chamber was then filled and the white cells are allowed to settle for 1-3 minutes. The counting chamber was then placed on the microscope stage with a lower power objective $\times 10$ to ascertain the cells are evenly distributed and then $\times 40$ objectives was used for clearer viewing and counting.

3.2.11.4 Determination of total protein:

Refractometer model 7416 USA was used for protein determination.

Procedure

The refractometer is a device used to measure the refractive index of a solution. Refractive index is defined as the degree that the light is bent when it is passed through a liquid, and is a function of the amount and type of solid material dissolved

in the liquid. In clinical practice, this instrument is calibrated to give direct readings of total protein in serum samples. The refractometer was set to zero reading using distilled water and the protein concentration was taken.

3.2.12 Data analysis using statistical tools

The statistical significance of differences between groups was evaluated using one-way analysis of variance (ANOVA) and Duncan's Multiple Range Test. A p-value < 0.05 was considered significant.

4.0 RESULTS

4.1 The Yields of Methanol Extract of *Mangifera indica* Seed Kernel and Its Solvent Fractions

The yield of exhaustive soxhlet extraction with methanol and subsequent assay guided solvent fractionation with aqueous, ethyl acetate and n-butanol is given in Table 4.1. Extraction of the powdered *Mangifera indica* seed kernel gave a dark brown residue and weighed 10.10g. When fractionated, ethyl acetate fraction (EAF) gave 5.75g, n-butanol fraction (NBF) yielded 1.36g and that of aqueous fraction (AF) weighed 2.97g. The ethyl acetate fraction was further used in this work because it gave the highest inhibitory activity against metalloprotease present in the venom of *Echis ocellatus* as shown in Table 4.3.

Table 4.1: The yields of methanol extract of *Mangifera indica* Seed Kernel and its solvent fractions.

Extracting solvent	Amount recovered (g)	% yield
Methanol extract	11.10	10.10
Ethyl acetate	5.75	57.5
n-Butanol	1.36	13.6
Aqueous residue	2.88	28.8

4.2 Purification of Metalloprotease Enzyme from *Echis ocellatus* Venom

The results of the partial purification of metalloprotease from *Echis ocellatus* venom are summarized on table 4.2. The elution profile of MPs from Ion exchange chromatography on DEAE Cellulose showed four major peaks (fractions 14, 17, 20 and 26) which was from the column with 0.1 to 0.5M sodium chloride salt concentration, It was observed that these fractions showed the highest metalloprotease activity when the eluates were assayed for activity with casein and thus were pooled for the next purification step.

The elution profile of MPs on Sephadex G-75 pooled from DEAE Cellulose active fractions showed one prominent peak (figure 4.2) which gave the highest metalloprotease activity when assayed. The purification profile in table 4.2 shows that the metalloprotease specific enzyme activity for the crude venom was 9.25 $\mu\text{mol}/\text{min}/\text{mg}$ protein, but when subjected to the purification steps, the specific enzyme activity was increased to 19.00 $\mu\text{mol}/\text{min}/\text{mg}$ protein with a total yield of 71%.

4.3 Characterization of Partially Purified Metalloprotease

The double reciprocal plot (Figure 4.3) of partially purified MPs obtained from *Echis ocellatus* venom showed that the enzyme had an estimated K_m of 0.31mg/ml and V_{max} of 9.09 $\mu\text{mol}/\text{min}$. The purity and the molecular weight of the partially purified metalloproteases were determined by Sodium Dodecyl Sulphate Polyacrilamide Gel electrophoresis (SDS-PAGE). Plate 1 shows the electrophoretic pattern of the sample under denaturing conditions. A faint band of the protein sample was visualised against the standard marker proteins on the gel and the molecular weight of the partially purified metalloproteases (lane 3 and 4) was estimated to be 23kDa (Appendix 1).

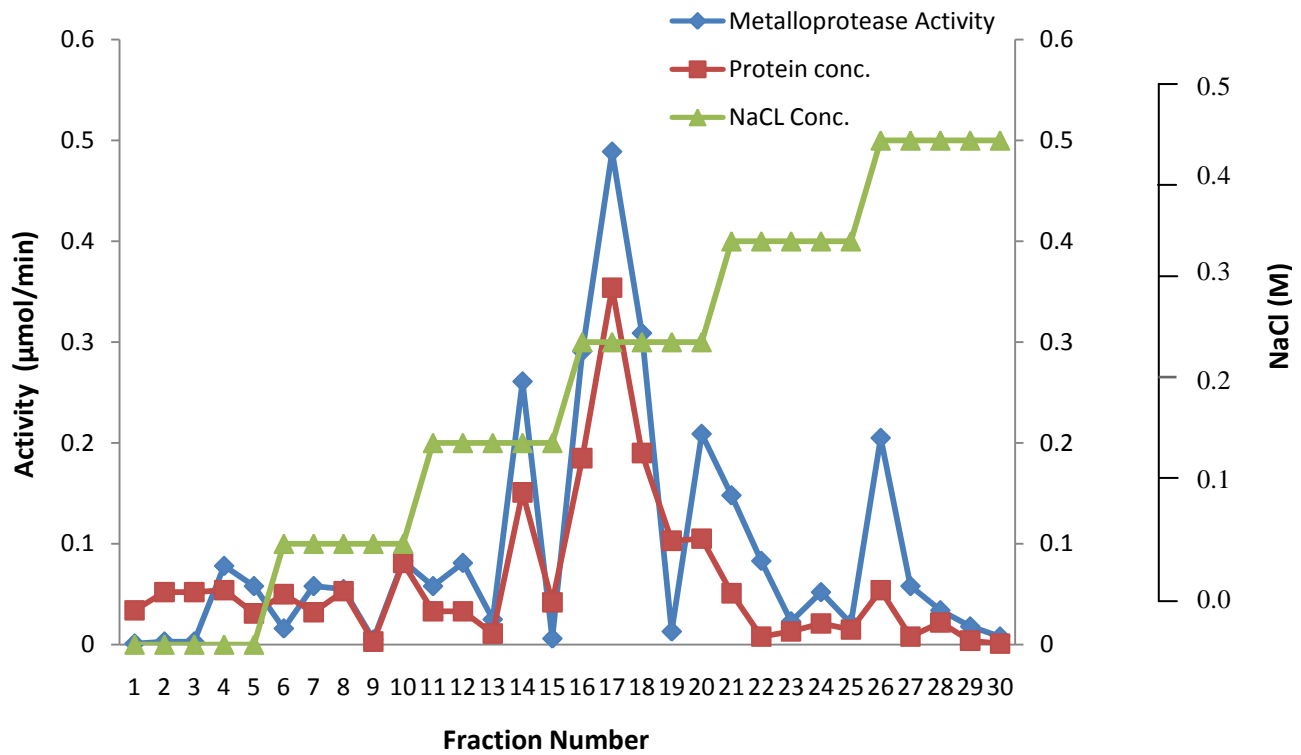


Figure 4.1 Elution profile of Metalloprotease from *Echis ocellatus* on DEAE Sephadex Ion exchange chromatography.

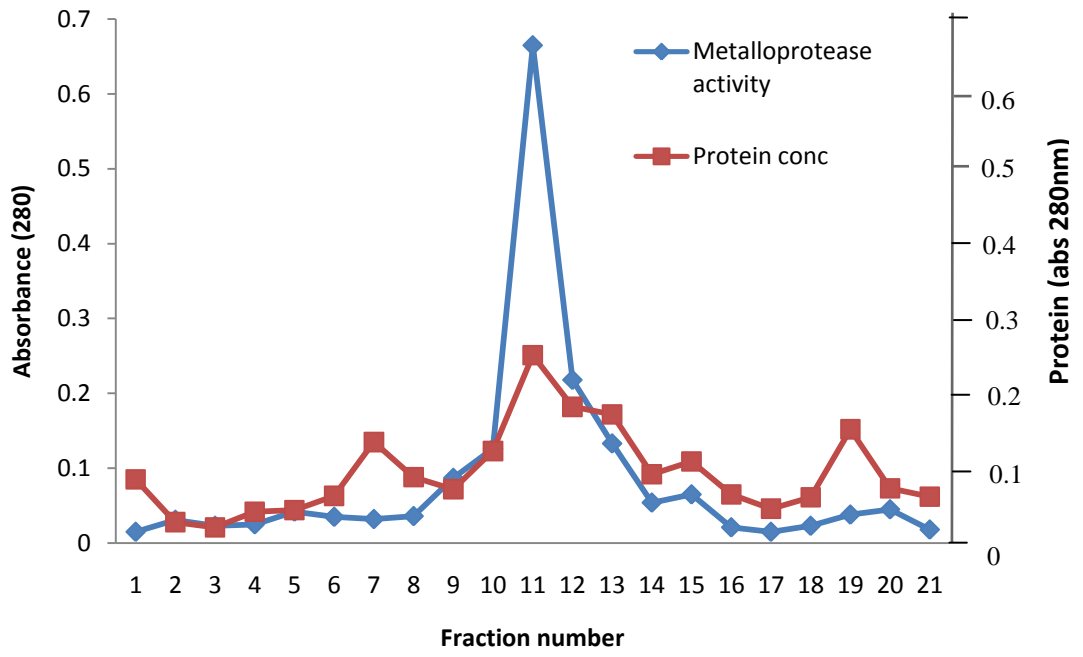


Figure 4.2 Elution profile of Metalloprotease from *Echis ocellatus* on Sephadex G-75 column chromatography.

Table 4.2 Partial purification for Metalloprotease from *Echis ocellatus* venom

Purification step	Protein (mg)	Total enzyme activity ($\mu\text{mol}/\text{min}$)	Specific enzyme activity ($\mu\text{mol}/\text{min}/\text{mg}$ protein)	Purification fold	% yield
Crude venom	1.10	10.17	9.25	1	100
DEAE Cellulose	0.45	5.27	11.71	1.27	52
Sephadex G-75	0.38	7.22	19.00	2.05	71

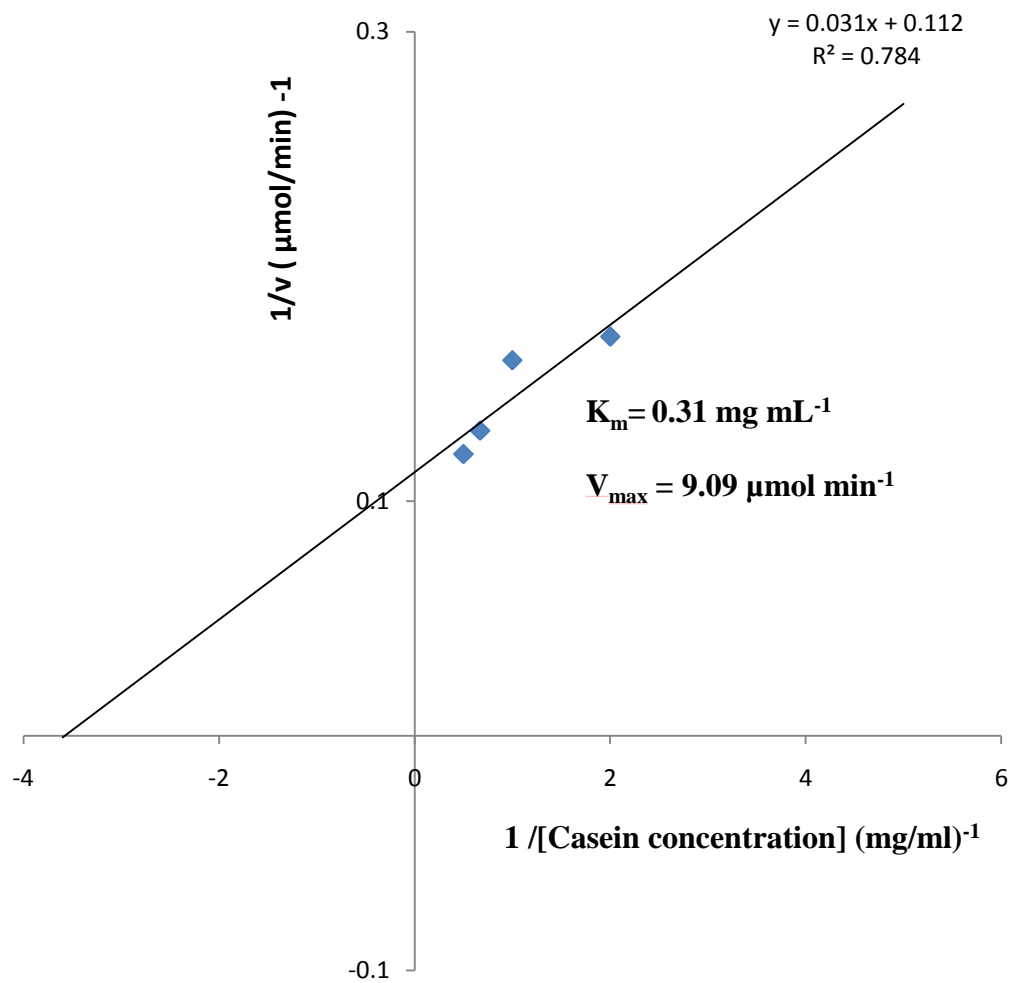


Figure 4.3: Double Reciprocal plot of partially purified metalloprotease from *Echis ocellatus* venom showing K_m and V_{max} .

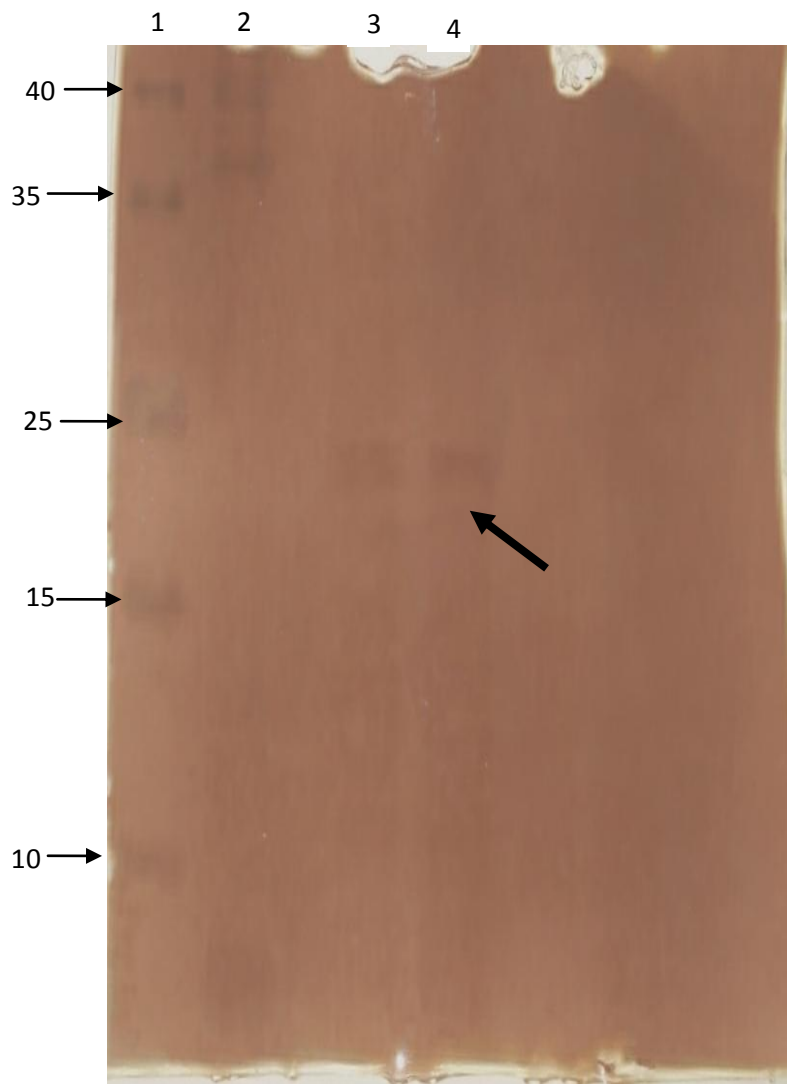


Plate IV: Electrophoretic pattern of Partially Purified Metalloprotease of *Echis ocellatus* on SDS-PAGE

Key: Lane 1=molecular weight marker, Lane 3 and 4 = partially purified metalloprotease (23kDa)

Table 4.3 The inhibitory effects of the methanol extract of *Mangifera indica* seed kernel and its solvent fractions on the Metalloprotease activity of *Echis ocellatus* venom.

Group (0.1mg/ml)	Metalloprotease Activity ($\mu\text{mol}/\text{min}$)	Relative Enzyme activity (%)	%inhibition
Blank (Buffer only)	2.76 \pm 0.02	0	0
Venom +Buffer	10.17 \pm 0.01	100	0
Venom+Methanol Extract	4.31 \pm 0.01	42	58
Venom+ Ethyl acetate fraction	3.74 \pm 0.02	37	63
Venom+n-Butanol fraction	8.64 \pm 0.01	85	15
Aqueous fraction	8.71 \pm 0.02	86	14

Mean \pm SD for 3 determinations.

4.4: Lineweaver-Burk and the Secondary Plot of the Inhibition of Partially Purified Metalloprotease Activity by the Ethyl Acetate Fraction.

The Lineweaver-Burk (double reciprocal) plot of the inhibition of metalloprotease activity by the most active fraction of the *Mangifera indica* seed kernel methanol extract in the presence of the substrate suggests a non competitive inhibitory effect with K_m values of 0.56 and 1.11 mg mL⁻¹ and V_{max} values of 6.67 and 4.17 μmol min⁻¹ for 5% and 20% inhibitor concentrations respectively whereas 0% inhibition shows a K_m of 0.31 mg mL⁻¹ and a V_{max} of 9.09 μmol min⁻¹. The secondary plot of the Lineweaver-Burk plot to determine the K_i (inhibition binding constant) showed an estimated K_i value of 0.168 mg mL⁻¹.

4.5 *In Vivo* Effect of *Mangifera Indica* Seed Extract on the Action of *Echis ocellatus* Venom

4.5.1 Physical signs of envenomation on mice

The physical signs of envenomation observed on the mice that were treated with the venom alone as compared with the mice that were treated with the venom and extract together and the mice that were treated with water and normal saline alone 4 hours after the introduction of the venom are all summarized in Table 4.4.

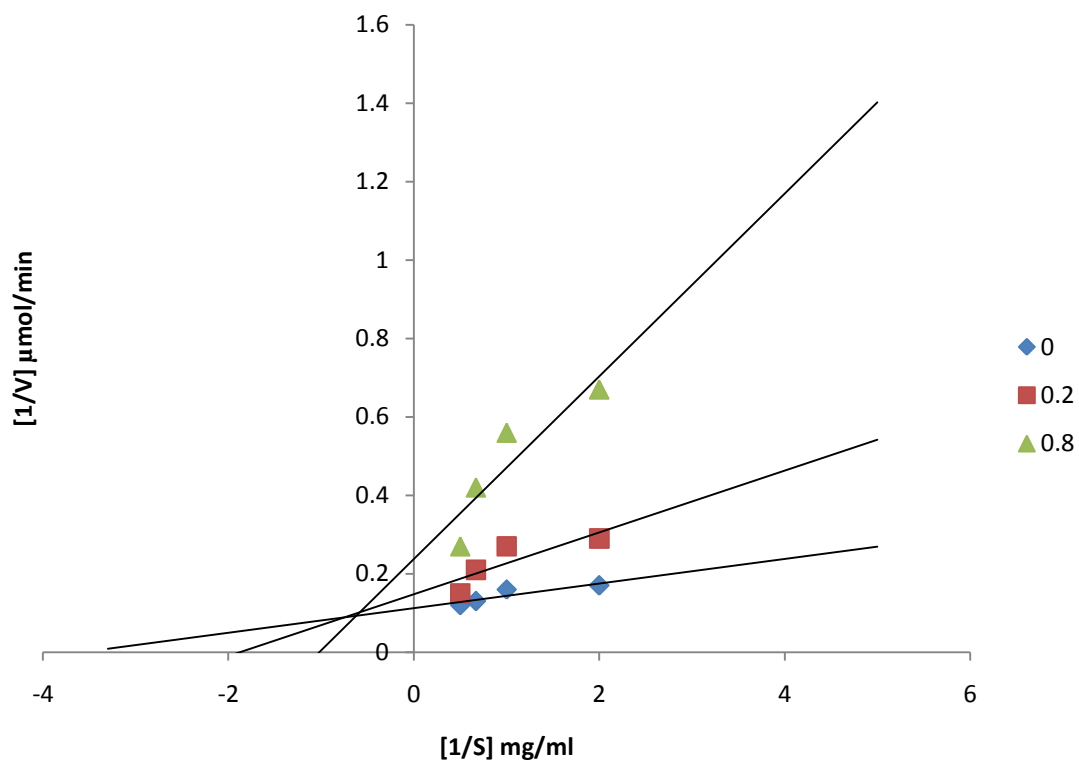


Figure 4.4: Double reciprocal plot showing the effects of different concentrations of the ethyl acetate fraction of *Mangifera indica* seed kernel with most inhibitory effect on partially purified Metalloprotease activity from *Echis ocellatus*

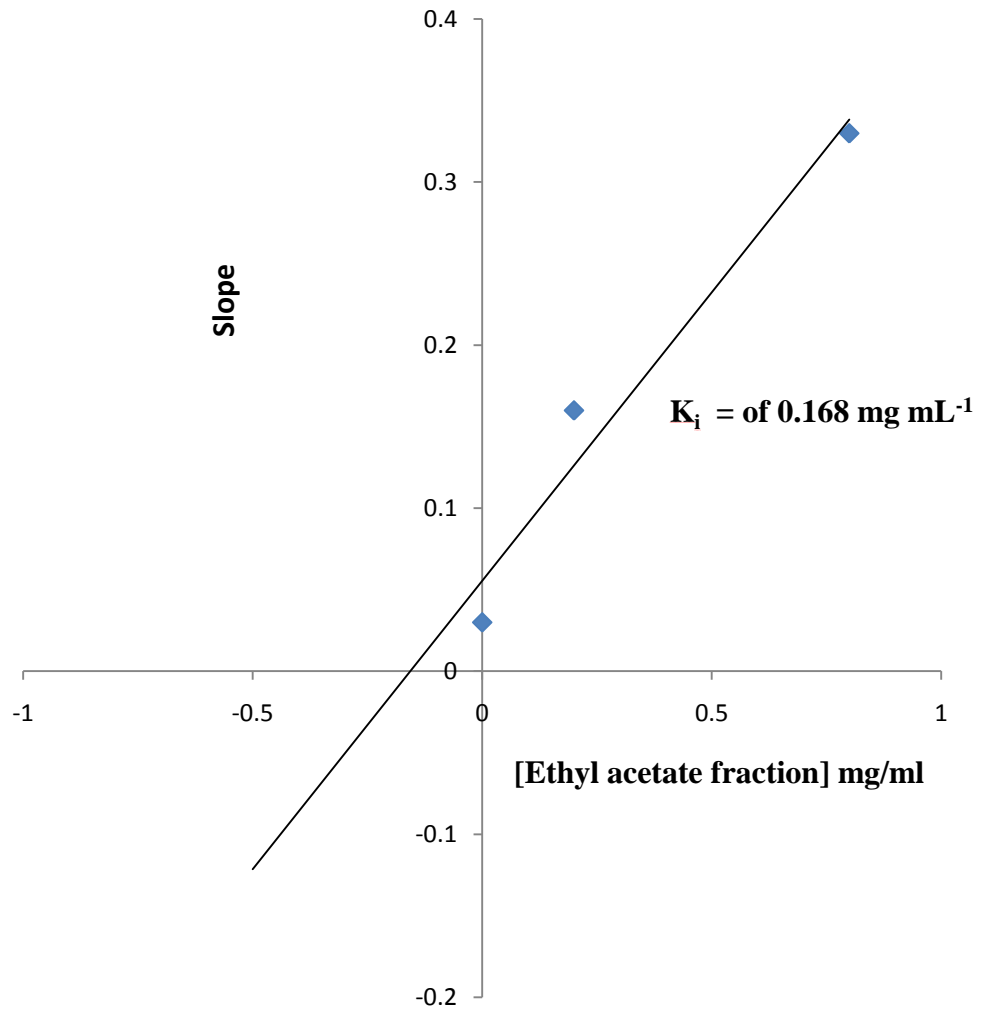


Figure 4.5: Secondary Plot of slope against Inhibitor concentration showing the inhibition binding constant (K_i)

Table 4.4: Physical signs of envenomation observed within four hours after envenomation in Mice

GROUPS	TREATMENT	OBSERVATIONS
1	Water	No signs of envenomation
2	Normal saline	No signs of envenomation
3	Low dose venom	Severe swelling and echymosis with palpitation and weakness
4	High dose venom	Severe swelling and echymosis with palpitation and weakness
5	Low dose extract	No signs of envenomation
6	High dose extract	No signs of envenomation
7	Low dose venom + low dose extract	Mild swelling and echymosis
8	Low dose venom + high dose extract	Mild swelling and echymosis
9	High dose venom + low dose extract	Slightly increased swelling and echymosis
10	High dose venom + high dose extract	Slightly increased swelling and echymosis

4.5.2: The effect of the ethyl acetate fraction on the blood coagulation system (Bleeding time and Clotting time) in Mice.

The effect of envenomation on the bleeding time and clotting time in mice as presented in Table 4.5 and 4.6 respectively, showed a significant increase in the bleeding time and clotting time of the group (3 and 4) that were treated with venom alone. This indicates the venom from *Echis ocellatus* has a deleterious effect on the blood coagulation system which is one of the indications of envenomation from *Viperidae* snake. Meanwhile, in the group treated with venom and extract together (group 7, 8, 9 and 10), there were significant reductions in the bleeding time and clotting time suggesting that the extract has an inhibitory effect on the action of the snake venom component responsible for the deleterious effect of *Echis ocellatus* on blood coagulation system.

4.5.3: The effect of the ethyl acetate fraction on haematological parameters

Hematological parameters were significantly ($p < 0.05$) reduced in group 3 and 4 (envenomed mice) when compared with the extract treated groups (7, 8, 9 and 10) (Table 4.4). The WBC was slightly elevated in the mice that received high dose venom though there was no significant difference when compared to the control and the group that received venom and extract. This therefore means that the extract neutralized the biological effect induced by the venom in the extract treated group that had increased PCV, Hb, WBC, and TP.

Table 4.5: Effect of ethyl acetate fractions of *Mangifera indica* seed kernel methanol extract on bleeding time after envenomation

Groups	Treatment groups	Bleeding time (sec)
1	Water	80±8.66 ^a
2	Normal saline	85±8.66 ^{ab}
3	Low dose venom	200±22.91 ^e
4	High dose venom	275±8.66 ^f
5	Low dose extract	85±8.66 ^{ab}
6	High dose extract	85±8.66 ^{ab}
7	Low dose venom + low dose extract	105±15.00 ^{bc}
8	Low dose venom + high dose extract	110±8.66 ^c
9	High dose venom + low dose extract	125±7.32 ^{cd}
10	High dose venom + high dose extract	140±8.66 ^d

Values are mean ± S.E.M (n = 3)

Values with the same superscript are considered statistically significant (p<0.05) when compared with the control, using analysis of variance, IBM SPSS statistics 20.

Table 4.6: Effect of ethyl acetate fractions of *Mangifera indica* seed kernel on clotting time after envenomation

Groups	Treatment Administered	Clotting time (sec)
1	Water	255±25.96 ^c
2	Normal saline	260±8.66 ^c
3	Low dose venom	125±8.66 ^a
4	High dose venom	125±31.22 ^a
5	Low dose extract	255±15.00 ^c
6	High dose extract	255±15.00 ^c
7	Low dose venom + low dose extract	210±15.00 ^b
8	Low dose venom + high dose extract	205±8.66 ^b
9	High dose venom + low dose extract	215±8.66 ^b
10	High dose venom + high dose extract	205±7.32 ^b

Values are mean ± S.E.M (n = 3)

Values with the same superscript are considered statistically significant (p<0.05) when compared with the control, using analysis of variance, IBM SPSS statistics 20.

Table 4.7: Effect of ethyl acetate fractions of *Mangifera indica* seed kernel on some hematological parameters on mice

Groups	PCV (%)	Hgb (g/dl)	WBC ($\times 10^9/L$)	TP (g/dl)
1	47.33 \pm 2.52 ^d	15.13 \pm 0.42 ^{de}	6.87 \pm 0.96 ^a	6.60 \pm 0.53 ^a
2	46.33 \pm 3.51 ^d	15.87 \pm 0.81 ^e	6.73 \pm 0.25 ^a	6.50 \pm 0.46 ^a
3	25.33 \pm 1.53 ^a	8.73 \pm 2.73 ^b	7.67 \pm 0.85 ^a	3.80 \pm 0.20 ^b
4	22.33 \pm 2.52 ^a	5.13 \pm 0.42 ^a	9.77 \pm 1.88 ^a	3.53 \pm 0.31 ^{bc}
5	43.00 \pm 2.65 ^{bcd}	14.53 \pm 0.50 ^{cde}	7.33 \pm 0.58 ^a	6.33 \pm 0.58 ^{abd}
6	44.67 \pm 3.21 ^{bcd}	14.60 \pm 1.00 ^{cde}	7.43 \pm 0.40 ^a	6.00 \pm 0.00 ^{ad}
7	41.67 \pm 4.73 ^{bc}	12.97 \pm 0.65 ^c	7.30 \pm 0.61 ^a	5.13 \pm 0.23 ^{ad}
8	41.33 \pm 0.58 ^{bc}	13.83 \pm 0.40 ^{cd}	7.43 \pm 0.72 ^a	5.47 \pm 0.42 ^{abd}
9	40.67 \pm 2.08 ^b	13.63 \pm 0.65 ^{cd}	7.77 \pm 0.68 ^a	5.60 \pm 0.58 ^{abcd}
10	40.00 \pm 2.00 ^b	13.40 \pm 0.72 ^{cd}	7.93 \pm 0.11 ^a	5.67 \pm 0.83 ^{adcd}

Values are mean \pm S.E.M (n = 3)

Values in the same column with the same superscript are considered statistically significant ($p < 0.05$) when compared with the control, using analysis of variance, IBM SPSS statistics 20.

Groups 1 to 10 represent treatments with water, normal saline, low dose venom, high dose venom, low dose extract, high dose extract, low dose venom + low dose extract, low dose venom + high dose extract, high dose venom + low dose extract and High dose venom + high dose extract respectively.

5.0 DISCUSSION

The global incidence of snakebite, envenomings, and its associated mortality are overwhelming and majority of these incidences occur in rural areas of resource-poor countries and many victims do not seek hospital treatment but prefer traditional remedies (Gutierrez *et al.*, 2006). Plant extracts have been traditionally used as a folk medicine in the treatment of snake bite envenomations all over the world. This is of particular importance especially in resource-poor countries where antivenin is not readily available as a result; efforts are being made by research groups to identify more traditionally available plant extracts that are potent for curbing the menace of snake bite envenomation. As a part of that effort, this study has been carried out to evaluate the potency of *Mangifera indica* seed kernel methanol extract and its different solvent fractions in ameliorating the menace of snake envenomation.

Metalloprotease from *Echis ocellatus* venom was partially purified in a two step purification process using ion exchange chromatography and gel filtration. From the results, there was an increase in purification fold from 1.27 to 2.63 (Table 4.2); while the specific activity also increased from 11.71 $\mu\text{mol}/\text{min}/\text{mg}$ protein to 19.00 $\mu\text{mol}/\text{min}/\text{mg}$ protein. These findings are similar to those of Sallau, et al. (2008), who demonstrated that an increase in purification fold and specific activity of the crude venom metalloprotease after the two purification steps could be attributed to the removal of other synergistically interacting components of the venom. Similar to the findings of Sallau et al. (2008) and Ibrahim et al. (2012), a low K_m value of 0.31 mg/ml and a high V_{max} value of 9.09 $\mu\text{mol}/\text{min}$ was obtained (Figure 4.3) from the kinetics characterization of the partially purified enzyme and this is an indication of high affinity of the enzyme for its substrate which further substantiates the observed toxicities in *Viperidae* snakes as a result of the activities of metalloproteases. The

partially purified metalloprotease showed a faint band on sodium dodecyl sulphate - poly acrylamide gel electrophoresis (SDS-PAGE) with an estimated molecular weight of 23kDa. This result is in line with the work of Gomes *et al.* (2009) who purified a metalloprotease (BthMP) from *Bothrops moojeni* with a molecular weight of 23.5kDa. Also, Lingott *et al.* (2009) purified a metalloprotease with a molecular mass of approx. 23 kDa from the venom of *Bothrops asper* snake, which is similar to other previously identified metalloproteases such as *B. asper* hemorrhagins BH2 and BaP1 with molecular mass of 26 and 24 kDa, respectively (Borkow *et al.*, 1993).

The lineweaver-Burke plot of varying concentrations of the ethyl acetate fraction of *Mangifera indica* seed kernel methanol extract indicated that the extract inhibited the partially purified enzyme in a non-competitive manner with an increasing K_m and a decreasing V_{max} (figure 4.3). In a related study, Sallau *et al.* (2005) reported a non-competitive pattern of inhibition of metalloprotease activity of *Echis carinatus* by the aqueous extract of *Geuiera senegalensis* leaves which is also in consonance with the work of Ibrahim *et al.* (2012). The secondary plot of the lineweaver-Burke plot showed a low K_i (inhibition binding constant) value of 0.168 mg mL^{-1} obtained for the extracts towards the partially purified metalloprotease shows a high affinity of the extracts towards the enzyme when compared with the enzymes' substrate, therefore, it is an indication that it could serve as a good source of antidote against the activities of metalloproteases present in the venom of *Echis ocellatus* and could as well adequately help in designing a novel drug to be used as an antivenin.

Many compounds identified from plants having different chemical structure were reported to be capable of interacting with peptides and proteins (enzymes) of snake venom. The mechanism of action of the plant extracts/plant compounds are still not

clear and they may be attributed to the blocking of receptors-structure prone to chemical attack, and may block the active site of the snake venom. Other mechanism of action of the plant compounds are inhibition of enzymes (especially those requiring co-factors) present in the snake venom. This is due to the metal chelator substances present in the plant extracts/plant compounds (Alam, 2014). It was observed from this study that the ethyl acetate fraction from the crude methanol extract has the highest inhibitory activity against the action of metalloprotease present in the venom of *Echis ocellatus*. This enzyme is believed to be the major component of snake venom from the *Viperidae* family responsible for the haemorrhagic effect which is the primary cause of mortality in snake bite victims.

The result in table 4.5 reveals a inhibition in the activity of the enzyme by 58% when treated with the crude extract while treatment with the active fraction showed further inhibition by 63%. This suggests that the ethyl acetate fraction has extracted more active constituent from the methanol extract which is more effective in the inhibition of metalloprotease activities of snake venom of *Echis ocellatus*. In another study, the methanol extract of the seed kernel of *M. indica* was reported to have an inhibitory effect on the phospholipase A₂ activity of *Naja nigricolis* (Humphery *et al.*, 2014), showing the potency of both the crude methanol extract and its ethyl acetate fraction in the inhibition of snake bite envenomation.

Mangifera indica seed kernel extract appears to be a promising chemical agent for use as first hand treatment, or in combination with antiserum in the management of snake bite victims. This was evident in the protective effect it conferred on mice after envenomation as observed in this study. A noteworthy observation was that a rapidly progressive swelling (edema which exceeded the point of venom introduction),

echymosis, palpitation and weakness which has been consistently associated with systemic envenomation from *Viperidae* were observed in the mice that received venom alone within four hours following envenomation, whereas, in the mice that received venom and extract together, there were mild swelling and echymosis which were only confined at the site of venom introduction. This indicates that the extract was effective to subdue the effect of the venom thereby preventing further spreading of the earlier observed signs of envenomation. Meanwhile, the mice that received water, normal saline and the extract alone did not show any signs of envenomation.

Spontaneous bleeding and coagulation disturbances are some of the haematological effects of *Echis ocellatus* venom in victims (Gomes *et al.*, 2009). The increase in clotting time and bleeding time levels observed in the group that received venom alone (Table 4.6 and Table 4.7) showed the anticoagulant effect of venom from *Echis ocellatus* which was significantly reduced in the group that received venom and extract together showing the ability of the extract to neutralize the effect of the venom. This agrees with the work of Joseph and Afolabi, (2012) who reported the neutralization of the anticoagulant effect of venom of *Naja nigricollis* by *Uvaria chamae* plant extract. The increase in bleeding time and clotting time observed could be as a result of the inhibition of platelet aggregation or the activation of prothrombin.

The Packed Cell Volume (PCV) of the envenomed mice were reduced significantly ($p < 0.05$), when compare with non-envenomed Mice. This is in consonance with the report of Mwangi *et al.* (1995) and could be as a result of systemic bleeding which characterizes envenomation by *Echis ocellatus*. Also, the decrease in haemoglobin and total protein observed in the group that received venom alone as compared to the

group that received the venom and extract together may be due to hemolysis which causes dilution of the blood (Karthikeyan *et al.*, 2007).

White blood cells (WBC) are effectors of the immune system. The count of WBC was slightly increased after four hours in the group that received high dose venom alone and this observation is consistent with the observations of Amin *et al.* (2008) who reported that Bangladesh snake venom caused slight leucocytosis after a period of injection. Lifshitz *et al.* (2003) hypothesized that a sympathetic effect, as a result of stress (due to venom) experienced by the victims could release temporarily WBCs from the marginal pools. Whereas, the group that received the venom and extract together did not show any increase in the WBC. This suggests that the plant extract may have antagonized the venom directly without cells of the immune system producing effector cells.

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The ethyl acetate fraction gave the highest yield and was found to possess the most active inhibitory activity toward the partially purified Metalloprotease from *Echis ocellatus* venom. Metalloprotease was partially purified from *Echis ocellatus* venom with a yield of 71% and a specific activity of 19 μ mol/min/mg which appeared as a faint band on SDS-PAGE with a molecular weight of 23kDa. The inhibition study revealed a mixed non-competitive pattern of inhibition with an estimated K_i value of 0.168 mg mL⁻¹ demonstrating that the extract has a high affinity for the partially purified enzyme.

The ethyl acetate fraction showed a significant Inhibitory activity on the metalloprotease induced toxic effect on blood coagulation system (bleeding and clotting time) and some hematological parameters (PCV, Hgb, WBC and TP) when evaluated *in vivo* and also reduced the signs of envenomation in Mice. *Mangifera indica* (*Mangifera indica*) Seed Kernel may therefore provide a less sensitive, inexpensive and readily available alternative to sheep serum in the management of snake bite envenomation. This study shows a beneficial relationship between the *in vitro* anti-enzymatic activities and *in vivo* anti-hemorrhagic effect of *Mangifera indica* seed kernel methanol extract and its ethyl acetate fraction against *Echis ocellatus* venom.

6.2 Recommendations

There is the need to isolate and characterize the active component responsible for the inhibitory activity of the ethyl acetate fraction, and to determine their efficacy, safety and the antiophidian mechanism of action which could possibly lead to the development of pharmaceutical formulations for treating snake bite victims.

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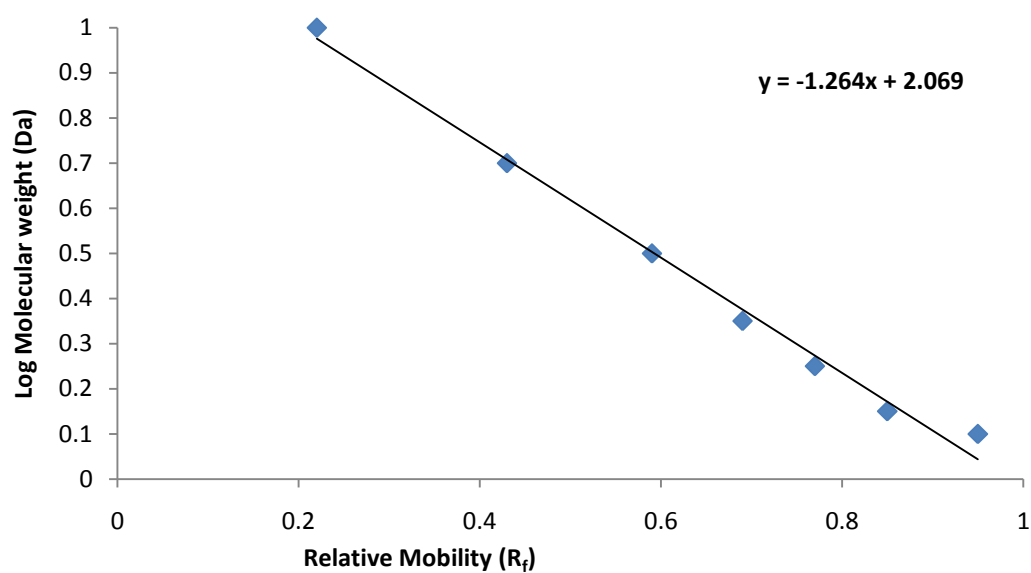
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APPENDICES

APPENDICES I



A plot showing the Log of molecular weight against the R_f of the marker protein bands used to estimate the molecular weight of partially purified metalloprotease (R_f = 0.56cm)

APPENDICE II

ONEWAY Bleeding Clotting PCV Hgb WBC TP BY Grouping/ STATISTICS HOMOGENEITY

Oneway

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Bleeding	1.401	9	20	.253
Clotting	2.021	9	20	.091
PCV	1.464	9	20	.228
Hgb	4.583	9	20	.002
WBC	4.240	9	20	.003
TP	2.792	9	20	.027

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Bleeding	Between Groups	106920.000	9	11880.000	75.429	.000
	Within Groups	3150.000	20	157.500		
	Total	110070.000	29			
Clotting	Between Groups	75630.000	9	8403.333	24.182	.000
	Within Groups	6950.000	20	347.500		
	Total	82580.000	29			
PCV	Between Groups	1954.533	9	217.170	28.701	.000
	Within Groups	151.333	20	7.567		
	Total	2105.867	29			
Hgb	Between Groups	295.668	9	32.852	29.236	.000
	Within Groups	22.473	20	1.124		
	Total	318.142	29			
WBC	Between Groups	19.014	9	2.113	2.967	.021
	Within Groups	14.240	20	.712		
	Total	33.254	29			
TP	Between Groups	30.216	9	3.357	15.567	.000
	Within Groups	4.313	20	.216		
	Total	34.530	29			

ONEWAY Bleeding Clotting PCV BY Grouping /STATISTICS DESCRIPTIVES
HOMOGENEITY /MISSING ANALYSIS /POSTHOC=DUNCAN ALPHA(0.05).

Descriptives								
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		M	
					Lower Bound	Upper Bound		
Bleeding	group 1	3	80.0000	8.66025	5.00000	58.4867	101.5133	
	group 2	3	85.0000	8.66025	5.00000	63.4867	106.5133	
	group 3	3	200.0000	22.91288	13.22876	143.0813	256.9187	
	group 4	3	275.0000	8.66025	5.00000	253.4867	296.5133	
	group 5	3	85.0000	8.66025	5.00000	63.4867	106.5133	
	group 6	3	85.0000	8.66025	5.00000	63.4867	106.5133	
	group 7	3	105.0000	15.00000	8.66025	67.7379	142.2621	
	group 8	3	110.0000	8.66025	5.00000	88.4867	131.5133	
	group 9	3	125.0000	17.32051	10.00000	81.9735	168.0265	
	group 10	3	140.0000	8.66025	5.00000	118.4867	161.5133	
	Total	30	129.0000	61.60777	11.24799	105.9953	152.0047	
Clotting	group 1	3	210.0000	25.98076	15.00000	145.4602	274.5398	
	group 2	3	215.0000	8.66025	5.00000	193.4867	236.5133	
	group 3	3	320.0000	20.00000	11.54701	270.3172	369.6828	
	group 4	3	360.0000	34.64102	20.00000	273.9469	446.0531	
	group 5	3	210.0000	15.00000	8.66025	172.7379	247.2621	
	group 6	3	210.0000	15.00000	8.66025	172.7379	247.2621	
	group 7	3	225.0000	15.00000	8.66025	187.7379	262.2621	
	group 8	3	220.0000	8.66025	5.00000	198.4867	241.5133	
	group 9	3	230.0000	8.66025	5.00000	208.4867	251.5133	
	group 10	3	220.0000	17.32051	10.00000	176.9735	263.0265	
	Total	30	242.0000	53.36278	9.74267	222.0740	261.9260	
PCV	group 1	3	47.3333	2.51661	1.45297	41.0817	53.5849	
	group 2	3	46.3333	3.51188	2.02759	37.6093	55.0573	
	group 3	3	25.3333	1.52753	.88192	21.5388	29.1279	
	group 4	3	22.3333	2.51661	1.45297	16.0817	28.5849	
	group 5	3	43.0000	2.64575	1.52753	36.4276	49.5724	
	group 6	3	44.6667	3.21455	1.85592	36.6813	52.6521	
	group 7	3	41.6667	4.72582	2.72845	29.9271	53.4062	
	group 8	3	41.3333	.57735	.33333	39.8991	42.7676	
	group 9	3	40.6667	2.08167	1.20185	35.4955	45.8378	
	group 10	3	40.0000	2.00000	1.15470	35.0317	44.9683	
	Total	30	39.2667	8.52151	1.55581	36.0847	42.4486	

Bleeding

Duncan

Grouping	N	Subset for alpha = 0.05					
		1	2	3	4	5	6
group 1	3	80.0000					
group 2	3	85.0000	85.0000				
group 5	3	85.0000	85.0000				
group 6	3	85.0000	85.0000				
group 7	3		105.0000	105.0000			
group 8	3			110.0000			
group 9	3			125.0000	125.0000		
group 10	3				140.0000		
group 3	3					200.0000	
group 4	3						275.0000
Sig.		.661	.087	.078	.159	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Clotting

Duncan

Grouping	N	Subset for alpha = 0.05		
		1	2	3
group 1	3	210.0000		
group 5	3	210.0000		
group 6	3	210.0000		
group 2	3	215.0000		
group 8	3	220.0000		
group 10	3	220.0000		
group 7	3	225.0000		
group 9	3	230.0000		
group 3	3		320.0000	
group 4	3			360.0000
Sig.		.264	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

PCV

Duncan

Grouping	N	Subset for alpha = 0.05			
		1	2	3	4
group 4	3	22.3333			
group 3	3	25.3333			
group 10	3		40.0000		
group 9	3		40.6667		
group 8	3		41.3333	41.3333	
group 7	3		41.6667	41.6667	
group 5	3		43.0000	43.0000	43.0000
group 6	3		44.6667	44.6667	44.6667
group 2	3			46.3333	46.3333
group 1	3				47.3333
Sig.		.197	.079	.057	.091

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

ONEWAY Hgb WBC TP BY Grouping /STATISTICS DESCRIPTIVES HOMOGENEITY
 /MISSING ANALYSIS /POSTHOC=T3 ALPHA(0.05) .

		Descriptives						
		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum
						Lower Bound	Upper Bound	
Hgb	group 1	3	15.1333	.41633	.24037	14.0991	16.1676	14
	group 2	3	15.8667	.80829	.46667	13.8588	17.8746	15
	group 3	3	8.7333	2.73008	1.57621	1.9514	15.5152	5
	group 4	3	5.1333	.41633	.24037	4.0991	6.1676	4
	group 5	3	14.5333	.50332	.29059	13.2830	15.7837	14
	group 6	3	14.6000	1.00000	.57735	12.1159	17.0841	13
	group 7	3	12.9667	.65064	.37565	11.3504	14.5829	12
	group 8	3	13.8333	.40415	.23333	12.8294	14.8373	13
	group 9	3	13.6333	.65064	.37565	12.0171	15.2496	13
	group 10	3	13.4000	.72111	.41633	11.6087	15.1913	12
	Total	30	12.7833	3.31216	.60471	11.5466	14.0201	4
WBC	group 1	3	6.8667	.96090	.55478	4.4797	9.2537	6
	group 2	3	6.7333	.25166	.14530	6.1082	7.3585	6
	group 3	3	7.6667	.85049	.49103	5.5539	9.7794	6
	group 4	3	9.7667	1.88237	1.08679	5.0906	14.4427	7
	group 5	3	7.3333	.57735	.33333	5.8991	8.7676	7
	group 6	3	7.4333	.40415	.23333	6.4294	8.4373	7
	group 7	3	7.3000	.60828	.35119	5.7890	8.8110	6
	group 8	3	7.4333	.72342	.41767	5.6363	9.2304	6
	group 9	3	7.7667	.68069	.39299	6.0757	9.4576	7
	group 10	3	7.9333	.11547	.06667	7.6465	8.2202	7
	Total	30	7.6233	1.07083	.19551	7.2235	8.0232	6
TP	group 1	3	6.6000	.52915	.30551	5.2855	7.9145	6
	group 2	3	6.5000	.45826	.26458	5.3616	7.6384	6
	group 3	3	3.8000	.20000	.11547	3.3032	4.2968	3
	group 4	3	3.5333	.30551	.17638	2.7744	4.2922	3
	group 5	3	6.3333	.57735	.33333	4.8991	7.7676	6
	group 6	3	6.0000	.00000	.00000	6.0000	6.0000	6
	group 7	3	5.1333	.23094	.13333	4.5596	5.7070	5
	group 8	3	5.4667	.41633	.24037	4.4324	6.5009	5
	group 9	3	5.6000	.52915	.30551	4.2855	6.9145	5
	group 10	3	5.6667	.83267	.48074	3.5982	7.7351	5
	Total	30	5.4633	1.09118	.19922	5.0559	5.8708	3

Post Hoc Tests

Multiple Comparisons

Dunnett T3

Dependent Variable	(I) Grouping	(J) Grouping	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval		
						Lower Bound	Upper Bound	
Hgb	group 1	group 2	-.73333	.52493	.953	-4.6250		
		group 3	6.40000	1.59443	.308	-10.3063		
		group 4	10.00000*	.33993	.000	7.9381		
		group 5	.60000	.37712	.918	-1.7358		
		group 6	.53333	.62539	.999	-4.5666		
		group 7	2.16667	.44597	.130	-.8361		
		group 8	1.30000	.33500	.206	-.7330		
		group 9	1.50000	.44597	.328	-1.5027		
		group 10	1.73333	.48074	.290	-1.6502		
		group 1	.73333	.52493	.953	-3.1584		
	group 2	group 3	7.13333	1.64384	.246	-8.0529		
		group 4	10.73333*	.52493	.003	6.8416		
		group 5	1.33333	.54975	.605	-2.4104		
		group 6	1.26667	.74237	.882	-3.3554		
		group 7	2.90000	.59907	.113	-.8336		
		group 8	2.03333	.52175	.258	-1.8879		
		group 9	2.23333	.59907	.238	-1.5003		
		group 10	2.46667	.62539	.199	-1.3558		
		group 3	group 1	-6.40000	1.59443	.308	-23.1063	
			group 2	-7.13333	1.64384	.246	-22.3195	
	group 4		3.60000	1.59443	.682	-13.1063		
	group 5		-5.80000	1.60278	.361	-22.2065		
	group 6		-5.86667	1.67862	.352	-20.2848		
	group 7		-4.23333	1.62036	.573	-20.0690		
	group 8		-5.10000	1.59339	.440	-21.8453		
	group 9		-4.90000	1.62036	.468	-20.7357		
	group 10		-4.66667	1.63027	.504	-20.2137		
	group 4		group 1	-10.00000*	.33993	.000	-12.0619	
		group 2	-10.73333*	.52493	.003	-14.6250		
		group 3	-3.60000	1.59443	.682	-20.3063		
		group 5	-9.40000*	.37712	.000	-11.7358		
		group 6	-9.46667*	.62539	.010	-14.5666		

	group 7	-7.83333 ⁺	.44597	.002	-10.8361
	group 8	-8.70000 ⁺	.33500	.000	-10.7330
	group 9	-8.50000 ⁺	.44597	.002	-11.5027
	group 10	-8.26667 ⁺	.48074	.003	-11.6502
	group 1	-.60000	.37712	.918	-2.9358
	group 2	-1.33333	.54975	.605	-5.0771
	group 3	5.80000	1.60278	.361	-10.6065
	group 4	9.40000 ⁺	.37712	.000	7.0642
group 5	group 6	-.06667	.64636	1.000	-4.9097
	group 7	1.56667	.47493	.326	-1.4242
	group 8	.70000	.37268	.820	-1.6241
	group 9	.90000	.47493	.813	-2.0908
	group 10	1.13333	.50772	.677	-2.1718
	group 1	-.53333	.62539	.999	-5.6333
	group 2	-1.26667	.74237	.882	-5.8887
	group 3	5.86667	1.67862	.352	-8.5514
	group 4	9.46667 ⁺	.62539	.010	4.3667
group 6	group 5	.06667	.64636	1.000	-4.7763
	group 7	1.63333	.68880	.624	-2.9730
	group 8	.76667	.62272	.976	-4.3755
	group 9	.96667	.68880	.957	-3.6396
	group 10	1.20000	.71181	.886	-3.3812
	group 1	-2.16667	.44597	.130	-5.1694
	group 2	-2.90000	.59907	.113	-6.6336
	group 3	4.23333	1.62036	.573	-11.6024
	group 4	7.83333 ⁺	.44597	.002	4.8306
group 7	group 5	-1.56667	.47493	.326	-4.5575
	group 6	-1.63333	.68880	.624	-6.2396
	group 8	-.86667	.44222	.784	-3.8813
	group 9	-.66667	.53125	.984	-3.8889
	group 10	-.43333	.56075	1.000	-3.8559
	group 1	-1.30000	.33500	.206	-3.3330
	group 2	-2.03333	.52175	.258	-5.9546
	group 3	5.10000	1.59339	.440	-11.6453
group 8	group 4	8.70000 ⁺	.33500	.000	6.6670
	group 5	-.70000	.37268	.820	-3.0241
	group 6	-.76667	.62272	.976	-5.9088
	group 7	.86667	.44222	.784	-2.1480

WBC		group 9	.20000	.44222	1.000	-2.8146	
		group 10	.43333	.47726	.999	-2.9709	
		group 1	-1.50000	.44597	.328	-4.5027	
		group 2	-2.23333	.59907	.238	-5.9669	
		group 3	4.90000	1.62036	.468	-10.9357	
		group 4	8.50000*	.44597	.002	5.4973	
		group 9	group 5	-.90000	.47493	.813	-3.8908
			group 6	-.96667	.68880	.957	-5.5730
			group 7	.66667	.53125	.984	-2.5556
			group 8	-.20000	.44222	1.000	-3.2146
			group 10	.23333	.56075	1.000	-3.1892
			group 1	-1.73333	.48074	.290	-5.1169
			group 2	-2.46667	.62539	.199	-6.2892
			group 3	4.66667	1.63027	.504	-10.8803
			group 4	8.26667*	.48074	.003	4.8831
		group 10	group 5	-1.13333	.50772	.677	-4.4385
			group 6	-1.20000	.71181	.886	-5.7812
			group 7	.43333	.56075	1.000	-2.9892
			group 8	-.43333	.47726	.999	-3.8376
			group 9	-.23333	.56075	1.000	-3.6559
			group 2	.13333	.57349	1.000	-5.3476
			group 3	-.80000	.74087	.996	-5.3333
			group 4	-2.90000	1.22020	.628	-11.9836
			group 5	-.46667	.64722	1.000	-4.9424
		group 1	group 6	-.56667	.60185	.998	-5.4534
			group 7	-.43333	.65659	1.000	-4.8739
			group 8	-.56667	.69442	1.000	-4.9746
			group 9	-.90000	.67987	.973	-5.3026
			group 10	-1.06667	.55877	.793	-7.0393
			group 1	-.13333	.57349	1.000	-5.6143
			group 3	-.93333	.51208	.823	-5.6648
			group 4	-3.03333	1.09646	.539	-14.6616
			group 5	-.60000	.36362	.884	-3.5071
		group 2	group 6	-.70000	.27487	.561	-2.5719
			group 7	-.56667	.38006	.927	-3.6743
			group 8	-.70000	.44222	.899	-4.5722
			group 9	-1.03333	.41899	.607	-4.6193
			group 10	-1.20000	.15986	.056	-2.4493
			group 1	.80000	.74087	.996	-3.7333
		group 3	group 2	.93333	.51208	.823	-3.7981
		group 4	-2.10000	1.19257	.850	-11.4843	

	group 5	.33333	.59348	1.000	-3.5702
	group 6	.23333	.54365	1.000	-3.9486
	group 7	.36667	.60369	1.000	-3.5296
	group 8	.23333	.64464	1.000	-3.7370
	group 9	-.10000	.62893	1.000	-4.0252
	group 10	-.26667	.49554	1.000	-5.5153
	group 1	2.90000	1.22020	.628	-6.1836
	group 2	3.03333	1.09646	.539	-8.5950
	group 3	2.10000	1.19257	.850	-7.2843
	group 5	2.43333	1.13676	.717	-7.9549
group 4	group 6	2.33333	1.11156	.731	-8.7626
	group 7	2.46667	1.14212	.710	-7.7952
	group 8	2.33333	1.16428	.764	-7.4805
	group 9	2.00000	1.15566	.855	-7.9750
	group 10	1.83333	1.08883	.862	-10.1016
	group 1	.46667	.64722	1.000	-4.0090
	group 2	.60000	.36362	.884	-2.3071
	group 3	-.33333	.59348	1.000	-4.2368
	group 4	-2.43333	1.13676	.717	-12.8216
group 5	group 6	-.10000	.40689	1.000	-2.7460
	group 7	.03333	.48419	1.000	-2.9083
	group 8	-.10000	.53437	1.000	-3.4374
	group 9	-.43333	.51532	1.000	-3.6089
	group 10	-.60000	.33993	.839	-4.0360
	group 1	.56667	.60185	.998	-4.3200
	group 2	.70000	.27487	.561	-1.1719
	group 3	-.23333	.54365	1.000	-4.4153
	group 4	-2.33333	1.11156	.731	-13.4293
group 6	group 5	.10000	.40689	1.000	-2.5460
	group 7	.13333	.42164	1.000	-2.6630
	group 8	.00000	.47842	1.000	-3.4175
	group 9	-.33333	.45704	1.000	-3.5104
	group 10	-.50000	.24267	.744	-2.7654
	group 1	.43333	.65659	1.000	-4.0072
	group 2	.56667	.38006	.927	-2.5409
	group 3	-.36667	.60369	1.000	-4.2630
	group 4	-2.46667	1.14212	.710	-12.7286
group 7	group 5	-.03333	.48419	1.000	-2.9749
	group 6	-.13333	.42164	1.000	-2.9296
	group 8	-.13333	.54569	1.000	-3.5017
	group 9	-.46667	.52705	1.000	-3.6874
	group 10	-.63333	.35746	.837	-4.2766
group 8	group 1	.56667	.69442	1.000	-3.8412

		group 2	.70000	.44222	.899	-3.1722
		group 3	-.23333	.64464	1.000	-4.2036
		group 4	-2.33333	1.16428	.764	-12.1472
		group 5	.10000	.53437	1.000	-3.2374
		group 6	.00000	.47842	1.000	-3.4175
		group 7	.13333	.54569	1.000	-3.2350
		group 9	-.33333	.57349	1.000	-3.8195
		group 10	-.50000	.42295	.976	-4.9099
		group 1	.90000	.67987	.973	-3.5026
		group 2	1.03333	.41899	.607	-2.5526
		group 3	.10000	.62893	1.000	-3.8252
		group 4	-2.00000	1.15566	.855	-11.9750
	group 9	group 5	.43333	.51532	1.000	-2.7422
		group 6	.33333	.45704	1.000	-2.8438
		group 7	.46667	.52705	1.000	-2.7540
		group 8	.33333	.57349	1.000	-3.1528
		group 10	-.16667	.39861	1.000	-4.2929
		group 1	1.06667	.55877	.793	-4.9060
		group 2	1.20000	.15986	.056	-.0493
		group 3	.26667	.49554	1.000	-4.9820
		group 4	-1.83333	1.08883	.862	-13.7682
	group 10	group 5	.60000	.33993	.839	-2.8360
		group 6	.50000	.24267	.744	-1.7654
		group 7	.63333	.35746	.837	-3.0099
		group 8	.50000	.42295	.976	-3.9099
		group 9	.16667	.39861	1.000	-3.9596
		group 2	.10000	.40415	1.000	-2.3813
		group 3	2.80000 [*]	.32660	.049	.0288
		group 4	3.06667 [*]	.35277	.027	.5838
		group 5	.26667	.45216	1.000	-2.4882
	group 1	group 6	.60000	.30551	.775	-2.7794
		group 7	1.46667	.33333	.210	-1.1968
		group 8	1.13333	.38873	.427	-1.3034
		group 9	1.00000	.43205	.641	-1.6206
		group 10	.93333	.56960	.898	-2.9127
		group 1	-.10000	.40415	1.000	-2.5813
		group 3	2.70000 [*]	.28868	.033	.3934
	group 2	group 4	2.96667 [*]	.31798	.017	.8606
		group 5	.16667	.42557	1.000	-2.4949
		group 6	.50000	.26458	.798	-2.4267
		group 7	1.36667	.29627	.175	-.8520

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	group 8	1.03333	.35746	.430	-1.1467
	group 9	.90000	.40415	.678	-1.5813
	group 10	.83333	.54874	.927	-3.1132
	group 1	-2.80000*	.32660	.049	-5.5712
	group 2	-2.70000*	.28868	.033	-5.0066
	group 4	.26667	.21082	.979	-1.1397
	group 5	-2.53333	.35277	.079	-5.6270
group 3	group 6	-2.20000*	.11547	.017	-3.4773
	group 7	-1.33333*	.17638	.024	-2.4162
	group 8	-1.66667	.26667	.085	-3.7078
	group 9	-1.80000	.32660	.137	-4.5712
	group 10	-1.86667	.49441	.329	-6.6902
	group 1	-3.06667*	.35277	.027	-5.5495
	group 2	-2.96667*	.31798	.017	-5.0727
	group 3	-.26667	.21082	.979	-1.6730
	group 5	-2.80000*	.37712	.048	-5.5615
group 4	group 6	-2.46667*	.17638	.032	-4.4178
	group 7	-1.60000*	.22111	.032	-3.0018
	group 8	-1.93333*	.29814	.048	-3.8411
	group 9	-2.06667	.35277	.084	-4.5495
	group 10	-2.13333	.51208	.252	-6.5289
	group 1	-.26667	.45216	1.000	-3.0216
	group 2	-.16667	.42557	1.000	-2.8283
	group 3	2.53333	.35277	.079	-.5604
	group 4	2.80000*	.37712	.048	.0385
group 5	group 6	.33333	.33333	.992	-3.3539
	group 7	1.20000	.35901	.375	-1.7773
	group 8	.86667	.41096	.727	-1.7783
	group 9	.73333	.45216	.910	-2.0216
	group 10	.66667	.58500	.992	-3.1508
	group 1	-.60000	.30551	.775	-3.9794
	group 2	-.50000	.26458	.798	-3.4267
	group 3	2.20000*	.11547	.017	.9227
group 6	group 4	2.46667*	.17638	.032	.5156
	group 5	-.33333	.33333	.992	-4.0206
	group 7	.86667	.13333	.137	-.6082
	group 8	.53333	.24037	.695	-2.1256
	group 9	.40000	.30551	.954	-2.9794

	group 10	.33333	.48074	1.000	-4.9845
	group 1	-1.46667	.33333	.210	-4.1301
	group 2	-1.36667	.29627	.175	-3.5853
	group 3	1.33333*	.17638	.024	.2504
	group 4	1.60000*	.22111	.032	.1982
group 7	group 5	-1.20000	.35901	.375	-4.1773
	group 6	-.86667	.13333	.137	-2.3416
	group 8	-.33333	.27487	.983	-2.3032
	group 9	-.46667	.33333	.949	-3.1301
	group 10	-.53333	.49889	.990	-5.2296
	group 1	-1.13333	.38873	.427	-3.5701
	group 2	-1.03333	.35746	.430	-3.2133
	group 3	1.66667	.26667	.085	-.3745
	group 4	1.93333*	.29814	.048	.0255
group 8	group 5	-.86667	.41096	.727	-3.5116
	group 6	-.53333	.24037	.695	-3.1922
	group 7	.33333	.27487	.983	-1.6365
	group 9	-.13333	.38873	1.000	-2.5701
	group 10	-.20000	.53748	1.000	-4.2395
	group 1	-1.00000	.43205	.641	-3.6206
	group 2	-.90000	.40415	.678	-3.3813
	group 3	1.80000	.32660	.137	-.9712
	group 4	2.06667	.35277	.084	-.4162
group 9	group 5	-.73333	.45216	.910	-3.4882
	group 6	-.40000	.30551	.954	-3.7794
	group 7	.46667	.33333	.949	-2.1968
	group 8	.13333	.38873	1.000	-2.3034
	group 10	-.06667	.56960	1.000	-3.9127
	group 1	-.93333	.56960	.898	-4.7794
	group 2	-.83333	.54874	.927	-4.7799
	group 3	1.86667	.49441	.329	-2.9569
	group 4	2.13333	.51208	.252	-2.2622
group 10	group 5	-.66667	.58500	.992	-4.4841
	group 6	-.33333	.48074	1.000	-5.6512
	group 7	.53333	.49889	.990	-4.1629
	group 8	.20000	.53748	1.000	-3.8395
	group 9	.06667	.56960	1.000	-3.7794

*. The mean difference is significant at the 0.05 level.

