STUDIES ON PHOSPHOLIPASE A_2 (PLA_2) INHIBITOR FROM *Echis ocellatus* SERUM AND ITS EFFECT ON NEUROTOXIC PLA_2 AND NON TOXIC SECRETORY PLA_2

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DECLARATION

I hereby declare that the work in this thesis entitled *"Studies on Phospholipase A_2 Inhibitor from Echis ocellatus Serum and its effects on Neurotoxic PLA_2 and non toxic Secretory PLA_2"* has been carried out by me in the Department of Biochemistry, Ahmadu Bello University, under the supervision of Prof. A.J. Nok (MFR) and Prof. E. Onyike. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

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CERTIFICATION

This thesis entitled *"Studies on Phospholipase A_2 Inhibitor from Echis ocellatus Serum and its effect on Neurotoxic PLA_2 and non toxic Secretory PLA_2"* by Fatima Amin ADAMUDE, meets the regulations governing the Award of the Degree of Master of Science degree of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This research work is dedicated to Almighty Allah, *The Lord of The Magnificient Throne. Surely, You are The Best Disposer of affairs*

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ABSTRACT

The most effective and acceptable therapy for snakebite victims is the immediate administration of antivenin which is limited by problems of hypersensitivity reactions in some individuals and its inability to resolve the local effects of the venom. The aim of this study was to investigate the presence and specificity of Phospholipase A_2 Inhibitor from *Echis ocellatus* serum towards snake venom neurotoxic PLA_2 and mammalian secretory PLA_2. Phospholipase A_2 (EC.3.1.1.4) was isolated and partially purified from the venom of *Echis ocellatus* (carpet viper). A two step purification process on DEAE-cellulose and Sephadex G-75 column chromatography gave a specific activity of 53.17µmol/min/mg protein, 16.36 purification fold and 43.11% recovery. Initial velocity studies for the determination of kinetic constants using L-α- Lecithin as substrate revealed a K_M and V_{max} of 1.4mgml^{-1} and 4.5µmolmin^{-1} respectively. A protein (Phospholipase A_2 Inhibitor from *Echis ocellatus* Serum (PIES)) isolated and purified from *E. ocellatus* serum inhibited the carpet viper PLA_2 enzyme in a dose dependent manner. A two step purification process on Sephadex G-200 column and DEAE- cellulose chromatography gave 2 active fractions that inhibited the venom PLA_2 by 78%. The result from SDS-PAGE showed the inhibitor to be a 24.98kDa protein and its kinetic study revealed a mixed pattern of inhibition on the carpet viper PLA_2 with an estimated K_i values of 3.8%(v/v) to 7.3%(v/v). Membrane stabilizing and protective ability of PIES was recorded by its potential to reduce hemolysis due to venom PLA_2 from 81.20 % to 35.30 % *in vitro*. Coagulant potentials of PIES were also seen in its ability to restore plasma coagulation time to less than a minute. Interestingly, PIES does not affect the enzymatic activity of mammalian secretory PLA_2 but strongly inhibits PLA_2 activity of carpet viper in this study. In conclusion, the present study shows that PIES may be a promising candidate for future development of a novel antivenin drug.

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LIST OF ABBREVIATION, GLOSSARRIES AND SYMBOLS

Da = Dalton

^{0}C = Degree Celsius

DIC = Disseminated Intravascular Coagulation

hr = Hour

kDa = killo-Dalton

µl = Microlitre

mg = Milligram

mM = Millimolar

ml = milliliter

% = Percentage

PCR = Polymerase Chain Reaction

SDS- PAGE = Sodium Dodecyl Sulphate Polyacrilamide gel Electrophoresis

WBCT = Whole Blood clotting Test

CHAPTER ONE

INTRODUCTION

Out of the 2,700 known species of snakes worldwide, only about 300 are known to be venomous (Anon, 2004; 2009) with another 350 different species whose venomous status is doubtful. Thus, the number of known venomous species of snakes does not exceed 650 (Hile, 2004). About 100 different species have been reported in Africa. In Nigeria, 57 species have been documented of which only 10 are known to be fatally venomous (Abubakar, 2003; Theakston et al., 2003; Anon, 2004; Habib et al., 2008). Based on hospital records, the four most deadly snakes found in northern Nigeria are Echis ocellatus, Naja nigricollis, Bitis arietans and Naja katiensis (EchiTAb, 2008). Commercial antivenins available are Pan African (EchiTAb^{+}) which is polyspecific, EchiTab and SAIMR produced by United Kingdoms and South Africa, respectively, which are mono specific for Echis ocellatus. (EchiTAb, 2009). The market is infiltrated with in-efficacious antivenins and hence increase in mortality from snake envenomation (Warrell, 2008).

The incidence of snakebite is often associated with agricultural activities with the highest at the beginning of the rainy season. The other important factor responsible for the increase in snakebite cases is flood which drives out snakes from their burrows (Warrell et al., 1977; Finney, 2010).

The saw-scaled viper, Echis ocellatus has been reported to be the most medically important snake and 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). Echis ocellatus is commonly found in the Benue-Niger valley axis and the hilly north-eastern part of Nigeria (Warrell and Arnett, 1976; Mustapha 2003).

Snake venom, the most complex of all poisons is a mixture of enzymatic and non enzymatic toxic compounds as well as other nontoxic proteins, non proteins including carbohydrates and metals all stored in the poison gland (Antony et al., 2010a) . The pathophysiologic base for morbidity and mortality is the disruption of normal cellular functions by these enzymes and toxins. Some of these proteins include enzymes like phospholipase A_2, B, C, D, hydrolases, hyaluronidases, phosphatases, esterases, phosphodiesterase, neuclosidases and metalloproteases (Sallau et al., 2005).

Snake venom is one of the most abundant sources of secretory PLA_2 ($sPLA_2$), which are one of the potent molecules in snake venoms (Chakrabarty et al., 2000). Phospholipase A_2 (PLA_2) is a lipolytic enzyme that hydrolyses the fatty acyl ester bonds at the 2-sn position of membrane phospholipids producing equimolar amounts of free fatty acid (FFA) and lysophospholipid, mainly arachidonic acid (AA); these products then become available for conversion to potent pro inflammatory mediators, such as platelet-activating factor and eicosanoids (Fuly et al., 2002; Huang and Mackessy, 2004) respectively. The enzyme from snake venoms is primarily used for trophic and defence functions in

most species but show wide range of pharmacological activities such as neurotoxicity, myotoxicity, cardiotoxicity, but with a greater impact on platelet aggregation and blood coagulation (Bharati et al., 2003; Hasson et al., 2003; Shashidharamurthy and Kemparaju, 2006; Higuchi et al., 2007).

The treatment for snake bite is as variable as the bite itself. The only available treatment is the usage of antivenin against snake bite. Antivenin binds to and neutralises the venom, stopping further damage, but do not reverse the damage already done (Antony et al., 2010b). Some individuals may react to the antivenin with an immediate hypersensitivity reaction (Cannon et al., 2008). Other alternative treatment involves the usage of folk and traditional medicines in snake bite. Some of the plants reported to have been used in treatment of snake bite include: root extract of Abruspra catorius is used against krait bite, leaf paste of Azadirachta indica with rock salt is used against viper bites, leaves and bark of Casearia sylvestris (guacotonga) are used as a standard Ayurvedic drug to treat snake bite in Columbia and India etc. (Antony et al., 2010b).

Venomous snakes are resistant to their own venom and several natural neutralizing proteins have been identified in their plasma (Shao et al., 1993). In particular, PLA_2 inhibitors have been isolated from various snake sera and their primary structures have been determined (Biondo et al., 2005). However, information as regards the presence of these inhibitors in E. ocellatus is still scanty. Thus this study aims at investigating the presence of PLA_2 inhibitor in E. ocellatus serum and its specificity towards snake venom neurotoxic PLA_2s (β-neurotoxins) and nontoxic mammalian PLA_2s and its potential as therapeutic drug for snakes envenoming.

1.1 STATEMENT OF RESEARCH PROBLEM

1. The incidence of snake bite worldwide cannot be accurately estimated due to lack of immediate access to healthcare, influence of traditional beliefs and practices and poverty (Fadare and Afolabi, 2012). Snakebite is a global medical health problem especially in the rural areas of the tropics with about 40,000 deaths and 375,000 cases of permanent physical disabilities worldwide annually (Finney, 2010; Leonard, 2010). The annual snakebite incidence in northern Nigeria has been estimated to be 497 per 100,000 populations with 12.2% mainly due to the carpet viper, *Echis ocellatus* (Pugh and Theakston, 1980). In the recent past, studies indicate that the situation has not improved as the incidence of snakebite worldwide has been reported to be in excess of 300,000 per year with more than 150,000 deaths (Mustapha, 2003). In another report

the incidence of snakebite was estimated at 10,000 deaths per year (Mustapha, 2003) and the carpet viper remains the main culprit.

2. The most effective and acceptable therapy for snakebite victims is the immediate administration of antivenin following envenomation (Mahanta and Mukherjee, 2001). The orthodox medical treatment of snake venom poisoning so far is limited by the use of antivenin, which is prepared from equine sera. Although, the use of anti-snake venom for the treatment of snake venom poisoning is universally accepted, therapeutic benefits are limited by the problems of hypersensitivity reactions in sensitive individuals. Furthermore, the conventional anti-snake venoms have not always been able to resolve the local effects of the venom (Warrell, 1976) such as haemorrhage, local swelling, bacterial infections, fever, pain, and bleeding.

3. The main concern about the empirical use of antivenin is the limited efficacy against the local tissue damaging activities of venoms, its relative scarcity and cost factor (WHO, 2008; Ramar *et al*., 2012). Thus, specific inhibitors may alleviate the local effects of the venom, which is difficult to achieve with conventional antivenins (Ramar *et al*., 2012).

1.2 JUSTIFICATION OF STUDY

The search for new strategies for treatment of snake envenomation has prompted the discovery of proteins which neutralize the major toxic components of these venoms. Venomous snakes are resistant to their own venom and several natural neutralizing proteins have been identified in their plasma (Shao et al., 1993). Certain molecules like endogenous antitoxic serum proteins with neutralizing capacity against snake venoms have been previously reported (Ramar et al., 2010). In particular, PLA_2 inhibitors have been isolated from various snake sera (Agkistrodon, Trimeresurus, Bothrops, Crotalus, Naja, Laticaudataand and Elaphe) and their primary structures have been determined (Biondo et al., 2004; Ramar et al., 2012). However, information as regards the specificity

of these inhibitors is still scanty and yet to be fully elucidated. Furthermore, the potential of PLA_2 inhibitors may provide therapeutic molecular models with antisnake activity to supplement the conventional serum therapy against these multifunctional enzymes (Ramar et al., 2012).

1.3 AIM AND OBJECTIVES

The general aim of this study is to investigate the presence and specificity of a PLA_2 inhibitor from Echis ocellatus serum towards snake venom neurotoxic PLA_2 (β-neurotoxins) and nontoxic secretory PLA_2 and its potential as therapeutic drug for snakes envenoming. The specific objectives are to;

I. Isolate and partially purify PLA_2 from *Echis ocellatus* venom.

II. Isolate and partially purify PLA_2 neutralising protein (inhibitor) from *Echis ocellatus* serum.

III. Characterise the partially purified PLA_2 neutralising protein.

IV. Investigate the inhibitory effect of partially purified PLA_2 inhibitor on snake venom neurotoxic PLA_2 (β-neurotoxin) and mammalian nontoxic secretory PLA_2s.

CHAPTER TWO

LITERATURE REVIEW

2.1 BIOLOGY OF SNAKES

2.1.1 Description

Snake is a limbless scaly reptile with very long body. Snakes are closely related to lizards, differing from them in the complete absence of limbs, movable eyelids and external ear. Like other reptiles, snakes are cold-blooded animals (Russell, 1983). Although venomous snakes have well been described by Russell (1983), some distinctive features and common characteristics of this group of snakes are:

2.1.1.1 Snake identification: The presence of venom glands anatomically makes the heads of all poisonous snakes more triangular than oval.

2.1.1.2 Pit on the face: The presence of second set of pits on the face of a snake, below the nostril, is an indication of a poisonous snake.

2.1.1.3 Presence of a rattle: The presence of a rattle is always a guarantee that the snake is venomous.

2.1.2 Classification

2.1.2.1 Family: There are four major groups of venomous snakes:

i. Crotalidae (e.g. Pit viper)

ii. Elapidae (elapids) e.g. cobras and mambas

iii. Viperidae (vipers) e.g. Echis, and Bitis

iv. Hydrophiids (sea snakes).

2.1.2.2. Fang orientation: Fangs are teeth and are like hypodermic needles with two openings: one near their base connecting with the venom duct and the other near the tip for the injection of venom into the prey. There are three types of venomous snakes described on the basis of fang orientation.

a) Opisthoglyphs. These are the rear-fanged snakes. The fangs are enlarged rear teeth with a “groove” through which the venom flows down while the snake swallows its prey. The members of this group are mostly harmless or mildly venomous. Example of this group is the Boomslang (Dispholidus typus).

b) Proteroglyphs. These are the fixed-front fang snakes. They have small non-movable front fangs. Obvious examples of this group of snakes are the cobras (Naja), sea snakes (Hydrophidae) and mambas (Dendroaspis).

c) Solenoglyphs. These snakes have movable front fangs. The fangs fold back into the mouth until they are needed. The poison fangs have enclosed canals within the teeth that transmit the venom out of the body, very much like hypodermic needle. Examples of this group are puff adder (Bitis arietans) and carpet viper (Echis ocellatus).

2.1.3 Distribution

Carpet viper (Echis ocellatus) is widely distributed in Africa, Middle East, Pakistan, India, and Sri Lanka (Warrell et al., 1974). In Africa, it is found in the dry savannah or desert region. The spitting cobra is commonly found in the densely populated areas of the northern savannah region of Nigeria, whereas carpet viper and puff adder are the major causes of snakebite fatalities in the sparsely populated areas of the savannah region of northern Nigeria. Echis ocellatus is one of the most medically important venomous snakes in Nigeria (Warrell et al., 1977), and the commonest race found in Nigeria is Echis ocellatus. The snake species are widely distributed in the savannah region of northern Nigeria especially in the Benue- Niger valley and the hilly areas of North Eastern savannah.

2.2 OVERVIEW OF SNAKES FOUND IN NIGERIA

It is generally accepted that modern day snakes (sub-order serpentes) arose from lizards in the early cretaceous period, about 130 million years ago. 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). This subterranean existence for countless generations would mean that certain modifications to the body would be necessary for the animal to remain successful. In the dark, subterranean world, eyes were of little use so they gradually became absorbed and remain as vestiges that are used for detecting the difference between light and darkness. The method of burrowing meant that limbs also became dispensable and all snakes have lost their external limbs (although some have retained limb girdles).

External ear openings would also have been an encumbrance in a subterranean environment, so these were also lost (Lee, 2009).

Generally, there are about 2,700 known species of snakes in the world (Hile, 2004). In Africa, about 100 different species have been documented (Mahanta and Mukherjee, 2001). Abubakar, (2003) documented only 57 different species in Nigeria. Of the 2,700 known species globally, only 300 species (13% of the world total snake population) are known to be venomous (Hile, 2004).

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, Atractaspidis microlepidota, and Dendroaspis augusticep (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 been studied for proper identification (Hile, 2004).

2.2.1 Description of Common Nigerian Snakes

There are no clear-cut features used in describing snakes generally (Revault, 1996; Abubakar 2003, Keogh et al., 2003). Herpetologists have designed several means which could be reliably used to speciate snakes. They include:

2.2.1.1 Color: This feature is helpful in the identification of snakes especially those of the same genus e.g. Naja nigricollis is black and the adults have a red band on the ventrum of its neck while Naja katiensis is reddish brown. But this color is not reliable in describing snakes of different genera e.g. both Naja nigricollis and Atractaspidis microlepidota are black in color, and Echis ocellatus and Bitis arietans are both brown and spotted (Abubakar, 2003; Joseph et al., 2000).

2.2.1.2 Zoometric features: Those generally used include body length, mid circumference, and tail length. This may only be useful in adult snakes of the same sex since there are both species and sex variations in these features (Joseph et al., 2000; Donald et al., 2003; Keogh et al., 2003; EchiTab, 2008).

2.2.1.3 Scale count: This is a recent technique used to identify snakes. It is more reliable than the color and zoometric features. Its only setback is that not adequate work has been done in this area to have a data bank for easy reference in speciation. This takes into account the head shields, body scales, and ventral scales (Anon, 2009).

2.2.1.4 Head shape: The shape of the head can be used to differentiate snakes e.g. while a young Bitis could be easily mistaken for an adult Echis, the triangular shape of the head of Bitis may serve as a clear feature for defining the species (Anon, 2009).

2.2.1.5 Genetics: The use of gene typing is by far the most reliable modern technique for snake speciation. Different methods have been used including liver DNA analysis and blood typing (Anon, 2009).

2.2.1.6 Venom Chemistry: The venom component of most snakes had been fully analyzed using SDS–PAGE, PCR, and other modern sensitive techniques. The limitation of these techniques is that it is limited only to venomous snakes which make up only about 13% of the global population of snakes (Anon, 2009).

2.2.2 Description of Some Poisonous Snakes

2.2.2.1 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). Echis ocellatus is a small, brownish, nocturnal, ground-dwelling snake with a pear-shaped head, and somewhat triangular flank. It has an average length of 53.4cm (44.5 – 61.0cm), 6.1cm (5.4 – 7.2cm) in diameter, with an average tail length of 6.1cm (5.4 – 7.3cm) and gives an average venom yield of 0.325 ml (0.2 – 0.45ml) per snake per day (William, 1998). 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. The carpet viper (Echis ocellatus) belongs to the family Viperidae. There are two species of the genus Echis: Echis coloratus gunther (Bourton‟s carpet viper) and Echis ocellatus stemmler. The former occurs in the Middle East (Warrell and Arnett, 1976), whereas the later is widely distributed. Echis ocellatus stemmler is the subspecies that is commonly found in Nigeria (Pugh and Theakston, 1980). Echis ocellatus is considered as the most dangerous snake in the world (Warrell and Arnett, 1976; 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), is strongly haemotoxic, affecting the vessels, blood, and heart muscle. Bites from Echis ocellatus is the most important cause of morbidity and mortality in man 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 (Plate I). 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-

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



2.2.2.2 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 (plate II), it has an average length of 123cm (i.e. 109 - 136cm), 8.8cm (7.8 – 11cm) thick, a tail length of 8cm (7 – 9.4cm), and give 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 conjuctivitis and corneal ulceration (Warell et al., 1977).



PLATE II: Adult Naja nigricollis photographed in Zaria, Kaduna State

2.2.2.3 Puff adder (Bitis arientans)

Bitis arietans is a brown coloured snake with black markings. It is stout and relatively large (fluke-shaped). 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 is found throughout the Savannah regions as well as the rainforest belt of Nigeria. It is believed to be the most widely distributed snake in Africa (plate III). It has an average length of 110.6cm (98.2 – 118cm), 21.4cm (18.6 – 24.2cm) in diameter, with an average tail length of 7.7cm (7.0 – 11.0cm), and gives an average venom yield of 4.9 ml (4.1 – 5.4ml) per snake per day. (William, 1998).

PLATE III: Adult Bitis arietans from Zaria, Kaduna State



2.2.2.4 Naja katiensis

Naja katiensis is a long, brown colored ground-dwelling spitting cobra. Some authors prefer to call it Naja nigricollis katiensis as they consider it a subspecie of Naja nigrcollis. It is more aggressive than the black spitting cobra as it is known to pursue its victims before biting (plate IV). It has an average length of 71.7 cm (62 – 82 cm), 7.2 cm (6.6 – 8.0 cm), a tail length of 6.9cm (6.0 – 7.6 cm) and average venom yield of 1.2 ml (0.8 – 2.0 ml) per snake per day (William, 1998).



PLATE IV: Adult Naja katiensis from Bakori LGA, Katsina State

2.3 SNAKE VENOM PATHOLOGY

Snake bite is a worldwide environmental and occupational hazard 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 (Pugh and Theakston, 1980; Ahmed et al., 2008; Habib et al., 2008; Alirol et al., 2010). In Nigeria, it commonly affects the rural population of the savannah region of the country where farming and animal husbandry are the major occupation. Snake venoms are complex mixtures of predominantly proteinaceous and peptidyl toxins. In addition, small organic substances, such as citrate, nucleosides and acetylcholine may also be present (Aird, 2002). Snakebite results in subcutaneous/intramuscular injection of venom into the prey or human victims resulting in complicated pathology, comprising both local and systemic effects. Systemic toxicity may include pre or postsynaptic neurotoxicity, myotoxicity, pro- or anticoagulant activities and hypotensive or rarely, hypertensive effects. The local effects include oedema, haemorrhage, dermonecrosis and myonecrosis. (Shashidhara et al., 2002; Cher et al., 2005) .The extent of systemic effects depends on the concentrations of systemic toxins injected and on the rate at which these toxins diffuse into the circulation from the site of the bite. Basically, toxin diffusion depends upon the extent of local tissue destruction (Girish et al., 2004). Locally-acting enzymes/toxins include myotoxins (enzymic/non-enzymic), zinc containing haemorrhagic metalloproteases of the metzincin family and hyaluronidases. These degrade proteins and glycosaminoglycans of the extracellular matrix (ECM) and connective tissue surrounding blood vessels and capillaries. The loss in structural integrity facilitates toxin diffusion. However, these degradative enzymes vary in relative abundance in different venoms (Girish and Kemparaju 2005). Cobra venoms are rich in myotoxic phospholipase A_2 while viper venoms are rich in haemorrhagic metalloproteases (Shashidhara et al., 2002). Therapeutically, the knowledge of intra specific variability would allow for more efficacious treatment of bite victims, and symptoms in bite victims from specific localities may aid in the choice of appropriate antivenin. Thus the production of antivenin is reliant on the knowledge of the variability of venoms within and between specific localities (Chippaux et al., 1991).

2.4 CONSTITUENTS OF VENOMOUS SNAKES

Snake venom, the most complex of all poisons is a mixture of enzymatic and non enzymatic toxic compounds as well as other nontoxic proteins, non proteins including carbohydrates and metals all stored in the poison gland (Antony et al., 2010a) . The pathophysiologic basis for morbidity and mortality is the disruption of normal cellular functions by these enzymes and toxins. Some of these proteins include enzymes like phospholipase A_2, B, C, D, hydrolases, hyaluronidases, phosphatases, esterases, phosphodiesterase, neuclosidases and metalloproteases (Sallau et al., 2005). Snake venoms are composed of various collections of polypeptides (Lee, 2009) which are toxins that are enzymatic or non-enzymatic. Other components include carbohydrates, lipids, nucleosides, biogenic amines and metals; magnesium, calcium, and zinc (Abubakar, 2003; Koh et al., 2006). More than 20 different enzymes have been isolated from snake venoms (Koh et al., 2006; Lee, 2009). These enzymes determine the toxicity of the snake venom as to whether it is hemotoxic, cardiotoxic or neurotoxic but the venoms cannot be classified as being exclusively haemotoxic, cardiotoxic or neurotoxic. Although any venomous snake should be considered dangerous, snakebite does not always result in envenomation (Warrell, 2003). However, when there is a clear indication of systemic

envenomation, the condition of the patient becomes an emergency, which calls for urgent medical attention. All venoms are composed of several toxins and are capable of producing various effects in living tissues and systems (Russell, 1983). Usually snake venoms are classified based on their modes of action. The different types so far documented are characterized as hemotoxic, neurotoxic, necrotoxic or cardiotoxic (Theakston et al., 2003).

Table 2.1 Components of Snake Venom and their Pharmacological Effects

Class	Examples	Mode of action
Alpha-neurotoxins	Alpha-bungarotoxin, Alphatoxin, Erabutoxin And Cobrotoxin	Block neuromuscular transmission by binding (like curare) to the cholinergic receptors found on the skeletal muscles.
Kappa-toxins	Kappa-toxin	Block some of the central nervous system' cholinergic receptors.
Beta-neurotoxins	Notexin, ammochytoxin, β-bungarotoxin, Crotoxin, and Taipoxin	Block neuromuscular transmission by preventing nerve endings from librating acetylcholine. Could interact with potassium channel sensitive to voltage.
Dendrotoxins	Dendrotoxin, toxins I and K	Increase the amount of acetylcholine librated by nerve endings. Could interact with potassium channel sensitive to voltage.
Cardiotoxins	γ-toxin, Cardiotoxin, and Cytoxin.	Disturb the plasma membrane of some cells (excitable cells) and lead to their lyses. Lead to cardiac arrest.
Myotoxins	Myotoxin A and Cratamine	Lead to muscular degeneration by interacting with a sodium channel dependent on voltage.
	Phospholipase A_2 haemorrhagic toxins A, B, C ….. HT 1, HT2	Leads to very serious haemorrhages by altering the vascular walls.

(Anon, 2004)

Snake venom exerts different effects on different body tissues. The haemotoxic venom causes breakdown of blood cells and inflammation, and is usually the most painful (Anon, 2004). This effect manifests in several ways:

2.4.1 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 (Plate V). Blood picture show spherocytosis, decrease in phagocyte population, lymphocytopenia, anisocytosis, poikilocytosis, polychromacia, and leucocytopenia (Warrell, 1983; Ed, 2001).

2.4.2 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 A_2) (Furtado et al., 2003; Koh et al., 2006). This is clinically manifested as gum bleeding, haematuria, haemachezia, melaena, haemoptosis, haematemesis, and spontaneous bleeding from the skin (Plates VI and VII). (Warrell, 1983; Marsh, 1994; EchiTab, 2006).

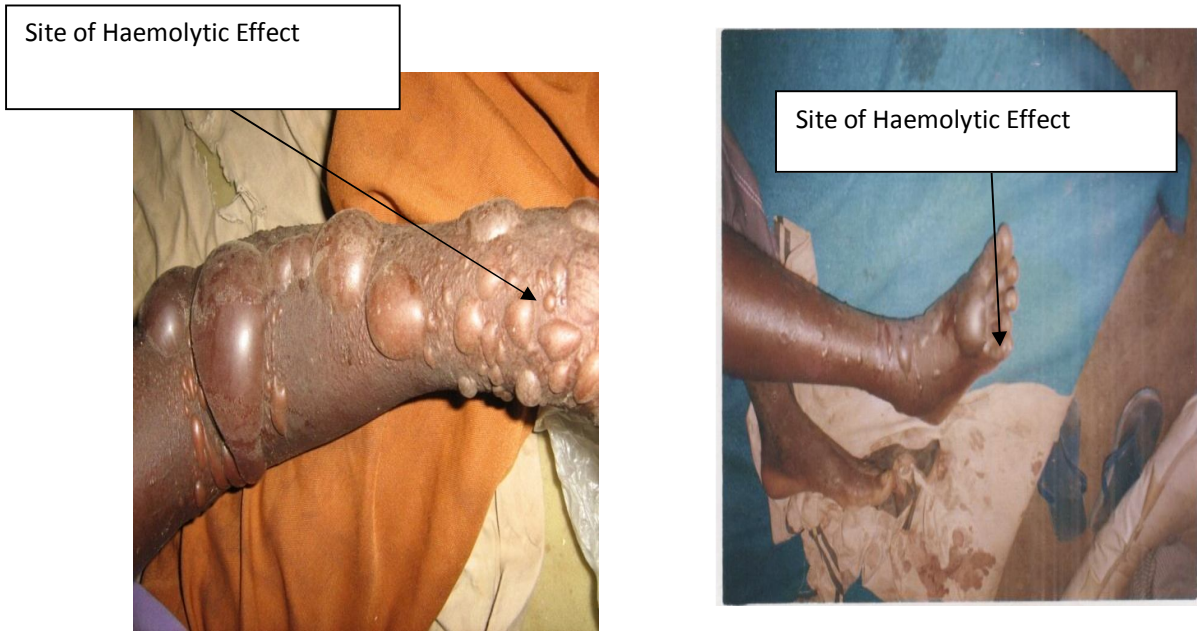
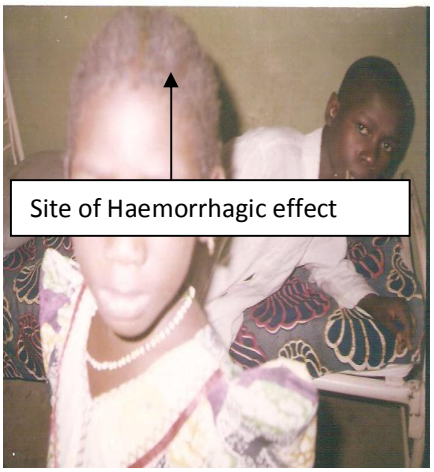


PLATE V: Haemolytic Effects of Snake Venom in a Victim from Kaltungo LGA, Gombe State



PLATEVI: Haemorrhagic Effects of Snake Venom in a Victim from Kaltungo LGA, Gombe State



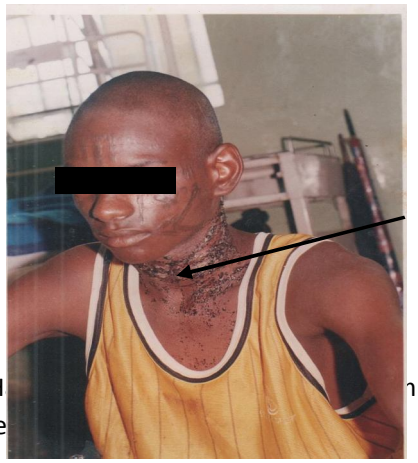


PLATE VII: H n from Kaltungo LGA, Gombe

2.4.3 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 (Theakston and Reid 1983; Warrell, 1983; EchiTab, 2008).

2.4.4 Neurotoxic Effect: Neurotoxins disable muscle contraction and paralyze the heart as well as hinder respiration (Hati et al., 1999; Mukunda and Leela, 2008).The neurotoxic venom attacks the nervous system leading to seizures and death (Anon, 2004). This is manifested clinically as ptosis, blespherospasm, tachycardia, arrhythmias, paralysis (usually flaccid), hallucinations, hysteria, hyperptyalism, difficulty in swallowing, bradycardia, coma and death (mostly due to respiratory arrest). This is a common feature of elapids envenomation (Theakston and Reid 1983; Warrell, 1983; EchiTab, 2008).

2.4.5 Necrotoxic Effect: The necrotoxic venom is usually associated with proteolysis by the enzymes of the proteinase and metalloproteinase groups. Another protein that has been associated with necrotoxicity is myotoxin. Myotoxins break down tissues and digest mammalian proteins especially those found in skeletal muscles leading to pains, swelling, pus formation, tissue necrosis and gangrene (plate VIII, IXa and IXb) (Theakston et al., 2003; EchiTab, 2008). This feature is seen in most classes of venomous snakes.

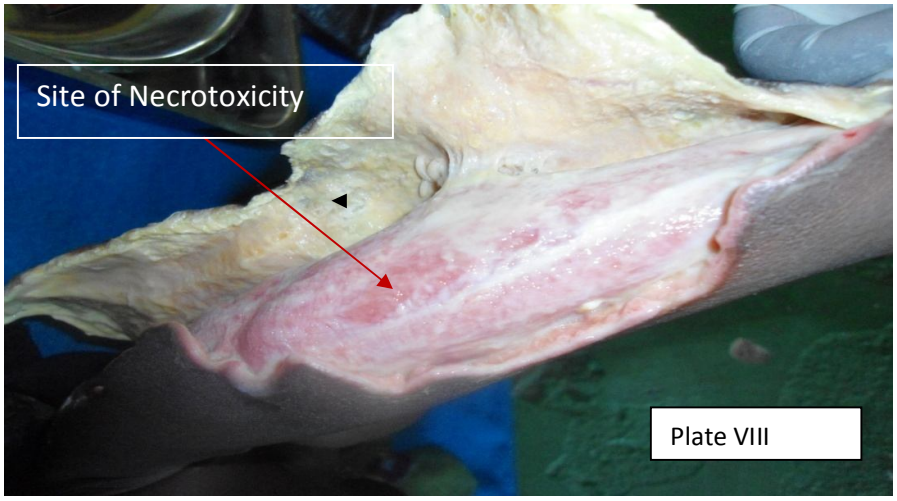
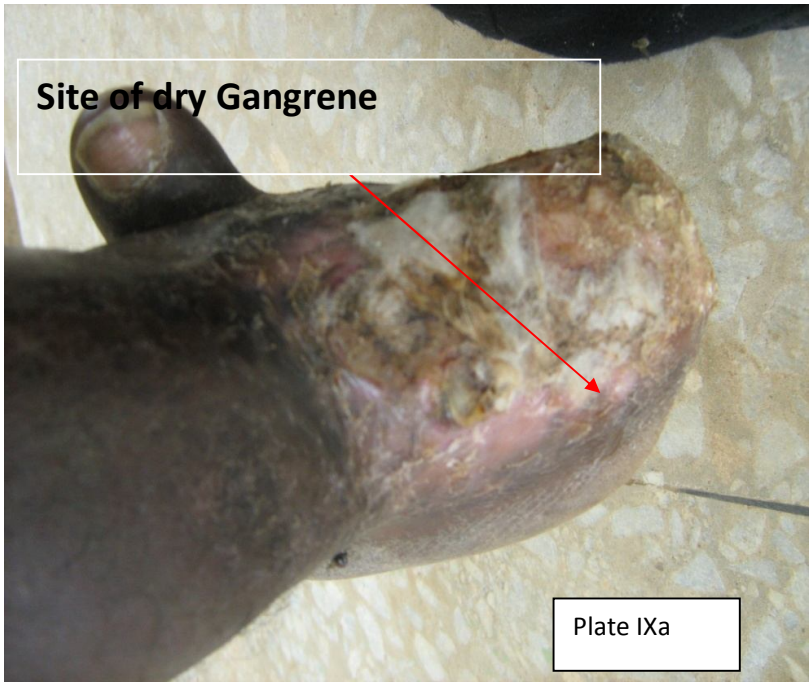


PLATE VIII: Necrotoxic Effect of Snake Venom on the Arm of a Victim in Kaltungo LGA, Gombe State.



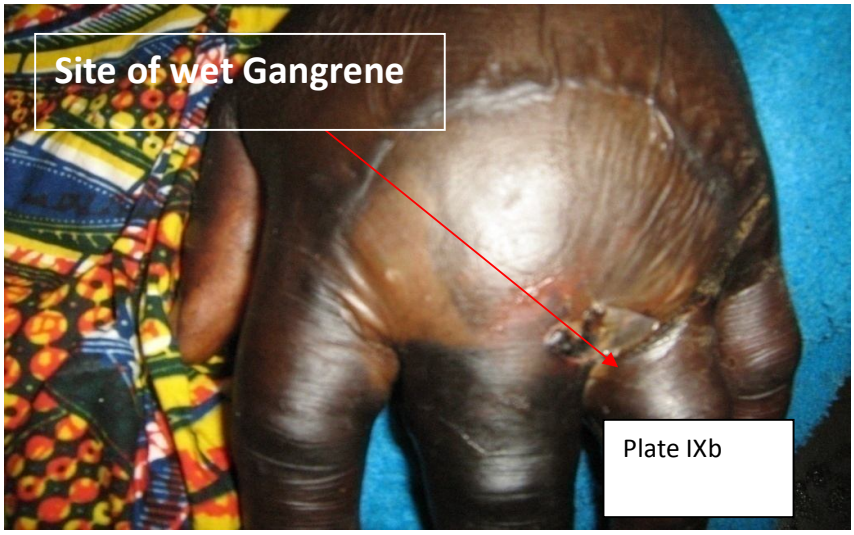


PLATE IXa and IXb: Gangrenous Effect of Snake Venom photographed from a victim in Kaltungo LGA, Gombe State

2.4.6 Cardiotoxic Effect: Cardiotoxins elicit specific toxicity to cardiac and muscle cells, causing irreversible depolarization of cell membranes (Hati et al., 1999; Mukunda and Leela, 2008). Cardiotoxic venom causes cardiac toxicity that is deduced at autopsy since its effect is usually masked by the neurotoxic effect. However, autopsy results have shown effects on the heart similar to that seen in skeletal muscles. The pacemaker region (sino-atrial node) of the heart has been shown to be the most affected (Philips, 1996).

2.4.7 Cytotoxic Effect: This has also been observed in most snake bites. This is a kind of localized effect which is manifested clinically as severe pains at the site of bite. The severity is dependent on the species of snake and the volume of the venom injected by the snake (Lee, 2009). Snake venom is 90% protein. The proteinous nature of snake venom was established by Napoleon Bonaparte's brother, Lucien in 1843 (Anon, 2004). These proteins are usually enzymes that include phospholipases, phosphodiesterases, phosphomonoesterases, 1- aminoacidoxydases, acetylcholinesterases, proteolytic enzymes of the serine proteinases and metalloproteinases classes, adenine diesterase's, 5-nucleotidases, hyaluronidases, nicotinamide adenine dinucleotide phosphatases and others which have not been clearly defined (Koh et al., 2006; Leny, 2009). Most snakes employ about 6 to 12 of these enzymes in their venoms. These enzymes act to achieve various objectives:

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).

Aminoacid Oxidases: These components of the snake venom play a part in tissue digestion and the triggering of other enzymes, and they are responsible for venoms' characteristic light yellowish colour (Ed, 2001).

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).

Proteinases: These enzymes in the snake venom play a major role in the digestive process, breaking down tissues at an accelerated rate and, thus, causing extensive tissue damage to mammalian tissues (Ed, 2001).

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 GEOGRAPHICAL DISTRIBUTION AND PATHOGENESIS OF ECHIS OCELLATUS (CARPET VIPER) ENVENOMATION

In northern Nigeria the incidence of bites, due predominantly to Echis ocellatus, peaks between March and June with the highest case loads occurring from May to June. This coincides with the surge in farming activity anticipating, and during, the early rainy season when human-snake contact is highest; the majority of patients are farmers bitten on the foot (Warrell and Arnett, 1976; Molesworth et al., 2003). Echis ocellatus favours semi-arid rocky terrain characterized by Guinea or sub-Sudan vegetation, cretaceous sediments and luvisol-type soils (Warrell and Amett, 1976; Molesworth et al., 2003). The species prefers high humidity, low population density and an altitude of less than 1000m, and avoids thickly wooded areas and rainforest. However, the precise boundaries of such areas are difficult to define and there is marked geographical variation in the seasonal incidence of snakebite within these regions, with mortality and morbidity varying considerably between closely located situations (Molesworth et al., 2003).

The carpet viper venom, like other snake venoms, is a multi component mixture, with more than 90% of the dry weight as protein in the form of enzymes, non-enzymatic polypeptides, toxic and non-toxic proteins (Reid and Theakston, 1983; Lee, 2009).

Echis ocellatus venom acts predominantly on the haematological system particularly on the capillary endothelium, which results in instant local swelling of the area (Samy et al.,2006). This is as a result of an increase in vascular permeability induced by proteases, phospholipases, membrane damaging polypeptide toxins, and endogenous acid oxidases released by the venom (Habib et al., 2001). Hyaluronidase promotes the spread of the venom (Kemparaju and Girish, 2006). Once an

envenomation has taken place, the primary target is the blood system. The blood of the victim becomes incoagulable (Warrell et al., 1974; Warrell and Arnett, 1976; Miyake, 1992) accompanied by prominent local irritation and symptoms of severe poisoning with burning pain, inflamed swelling, and formation of abscess, sudden fall in blood pressure and internal bleeding. Death ensues because the heart stops functioning (Fadare and Afolabi, 2012).

The venom contains a pro-coagulant enzyme, ecarin, which activates prothrombin to produce thrombin, which is resistant to anti coagulant heparin (Tu, 1977). The activity is responsible for incoagulability of blood of the victim. This is due to the rapid consumption of coagulation factors such as fibrinogen, prothrombin, Factors V and VIII, and of platelets (Tu, 1977). The reduction in the number of platelets in the blood also leads to spontaneous bruising and prolonged bleeding after injury. The pro-coagulatory action (direct activation of prothrombin) is the principal effect of the Echis ocellatus venom on blood coagulation in man. The venom also contains haemorrhagin, which causes direct endothelial damage of blood vessel wall that leads to spontaneous bleeding (Reid and Theakston, 1983). Consequently, there is spontaneous oozing of blood into vital organs, especially the brain, causing lethality through cerebral haemorrhage (Warrell and Arnett, 1976). The combination of defibrination, thrombocytopenia and vessel wall damage can result in massive bleeding, a common cause of death after bites by carpet viper. However, administration of anti-venin temporarily stops abnormal bleeding and restores clotting process to normal (Levy, 2003). Echis ocellatus venom also affects blood glucose level after envenomation. Tu, (1977) reported that the normal blood glucose in rabbit is 0.114%. However, on administration of snake venom to rabbit, the blood sugar increased significantly (Levy, 2003). In severe poisoning, blood glucose is also derived from the conversion of muscle glycogen to glucose. The increase in blood sugar is due to stimulation of the central nervous system, which controls carbohydrate metabolism, and also to an increase in epinephrine secretion in the adrenal gland leading to decrease in the glands but increased concentration in the blood (Russell, 1983). The activities of insulin are also affected by snake venom poisoning. The venom reduces insulin activity due to the inhibition of insulin release, thereby lowering glucose utilization resulting in an increase in blood glucose level (Levy 2003).

2.6 CLINICAL MANIFESTATION OF CARPET VIPER ENVENOMATION

Many people bitten by venomous snakes may develop no signs of poisoning because very little or no venom has been injected by the snake. The clinical manifestations of snake bites depend mainly on the specie responsible for envenomation and could affect the central nervous system, kidneys, blood coagulability and the cardiovascular system (Fadare and Afolabi, 2012). Amputation and disability (Abubakar et al., 2010), tetanus (Habib, 2003), gangrene (Abbas et al., 2009), cortical necrosis of the kidneys (Date and Shastry, 1981) etc are among the clinical manifestation of carpet viper bite. There are also local effects at the site surrounding the point of envenomation. The venom channel is recessed above the tip of the fang and the venom injected may be reduced by poor penetration or glancing blows, causing venom to be lost over skin and clothing surface. The volume of the venom available to a particular snake may also be reduced by previous bites (Warell et al., 1977; Girish and

Kemperaju, 2005). However, when adequate venom is injected, the victim of Echis ocellatus envenomation usually presents some clinical features which include local irritation and symptoms of severe blood poisoning with burning pain, inflamed swellings, and sudden drop in blood pressure, fever, spontaneous systemic bleeding, local tissue necrosis, and formation of local blistering (EchiTab, 2008; Fadare and Afolabi, 2012). Whether the snake is poisonous or non-poisonous and regardless of the venom injected, the commonest symptom following snakebite is 'fright' which may lead to a vasovagal episode (faint) (Ramar et al., 2010). In Nigeria, the main species responsible for envenomation, E. ocellatus has been found to contain a prothrombin activating procoagulant, haemorrhagin which causes bleeding, incoagulable blood, shock and local reactions or necrosis (Habib et al., 2001). The lethal dose of Echis ocellatus venom in animals causes convulsions and death within a few minutes (Lee, 2009). However, such effects are rare in humans because the doses of the venom usually injected by snake are much smaller in relation to body weight. Laboratory values reveal Disseminated Intravascular Coagulation (DIC) with thrombocytopenia as prominent feature, severely depleted fibrinogen, increased fibrin degradation products, depleted levels of clotting Factors V, VIII, II and XIII, and hyperglycaemia (Warrell et al., 1977).

2.6.1 Local Manifestations

After envenomation, local swelling starts within few minutes. Fang marks may be difficult to see. Local pain with radiation and tenderness and a small reddish wheal are first to develop, followed by oedema, swelling and the appearance of bullae, all of which can progress quite rapidly and extensively (Anthony et al., 2010a). In most viper bites, paraesthesia commences around the wound, and tingling and numbness over tongue, mouth and scalp can occur (Warrell et al., 1977; Fadare and Afolabi, 2012). The local bite may become necrosed and gangrenous (Abubakar et al., 2010). Secondary infection including tetanus and gas gangrene can also result (Habib, 2003). Since the venoms are largely absorbed by the lymphatics, lymphangitis may also appear early (Zamudio et al., 2000).

2.6.2 Systemic Manifestations

Vipers produce symptoms within a few minutes to several hours after the bite. In most cases, viper bites results in 75% envenomation; 35% mild, 15% severe cases (Hawgood and Hugh 1998).Viper venom can involve virtually every organ system. Nausea and vomiting are common and if presented early, suggests severe envenomation. Weakness, sweating, fever, chills, dizziness and syncope may occur (Fadare and Afolabi, 2012). Some patients complain of a minty, rubbery or metallic taste in their mouths with increased salivation (Hile, 2004). Tingling or numbness in the tongue, scalp, face and digits are indications of moderate to severe envenomation as are fasciculations of the face, neck, back or the bitten extremity (Hawgood and Hugh 1998.). Systemic anticoagulation can lead to gingival bleeding, epistaxis, hemoptysis, haematuria, haemetemesis and rectal bleeding or malena (EchiTab, 2008). Intra-abdominal or intracranial haemorrhages may occur. Visual disturbances may result from retinal haemorrhages, there may be tachycardia or bradycardia, often accompanied by hypotension (Ramar et al., 2010). Delayed shock may occur due to excessive blood loss and hemolysis (Fadare and Afolabi, 2012). Severe envenomation can result in pulmonary oedema as a result of destruction of the intimal lining of pulmonary blood vessels and pooling of pulmonary

blood. The venom and associated hypotension along with haemoglobin, myoglobin and fibrin deposition in renal tubules can contribute to nephrotoxicity (Hawgood and Hugh 1998).

2.7 SNAKE VENOM PHOSPHOLIPASE A_2

The super family of phospholipase A_2 enzymes has been classified into 15 groups and many subgroups that include five distinct types of enzymes, namely; the secreted PLA_2 ($sPLA_2$), the cytosolic PLA_2 ($cPLA_2$), the Ca^{2+} independent PLA_2s ($iPLA_2$), the platelet-activating factor acetyl-hydrolases (PAF-AH), lysosomal PLA_2s, and a recently identified adipose-specific PLA_2 (Duncan et al., 2008; Burke and Dennis 2009; Ramar et al., 2010).

Snake venom is one of the most abundant sources of secretory PLA_2 ($sPLA_2$), which are one of the potent molecules in snake venoms (Ritonja and Gubensek, 1985; Maung- Maung et al., 1995; Chakrabarty et al., 2000). $sPLA_2$ are low molecular weight proteins with molecular masses ranging from 13-19 kDa and generally requires Ca^{2+} for their activities (Kini, 1997; Valentin and Lambeau, 2000). Snake venom $sPLA_2$ are secreted enzymes belonging to only two groups that are based on their primary structure and disulfide bridge pattern (Six and Dennis, 2000; Rouault et al., 2003; Ramar et al., 2010).

Those of group I are the same as pancreatic $sPLA_2$ present in mammals and are found in venom of Elapidae snakes, while group II PLA_2s belong to the Viperidae and are similar to mammals' nonpancreatic, inflammatory $sPLA_2$s (Lambeau and Lazdunski, 1999; Dennis, 2000). Despite a high identity of their amino acid sequences, they exhibit distinct pharmacological effects including pre- or post-synaptic neurotoxicity, myonecrosis, cardiotoxicity, anticoagulant, antiplatelet aggregation, hemorrhagic, hemolytic, and cytolytic activities (Kini and Evans, 1988; Kasturi and Gowda, 1989; Stefansson et al., 1989; Maung Maung et al., 1995; Kole et al., 2000; Chakrabarty et al., 2002; Dong et al., 2003; Kini, 2003).

Phospholipase A_2 (PLA_2) are enzymes found to catalyze the hydrolysis of fatty acyl ester bonds in the 2-position of 3- sn-phospholipid to release fatty acid and lysophospholipid; the fatty acid so formed may act as either a second messenger or a precursor of eicosanoids (Fuly et al., 2002; Huang and Mackessy, 2004; Sallau et al., 2008). The enzyme (figure I) from snake venoms is primarily used for trophic and defense functions in most species but show wide range of pharmacological activities such as neurotoxicity, myotoxicity, cardiotoxicity, but with a greater impact on platelet aggregation and blood coagulation (Bharati et al., 2003; Hasson et al., 2003; Shashidharamurthy and Kemparaju, 2006; Higuchi et al., 2007). The enzyme is therefore a highly interesting molecule to venom researchers because in addition to digesting the prey, it mimics the pathological action of the whole venom poisoning (Shashidharamurthy and Kemparaju, 2006).

For protection from their own venom PLA_2, venomous snakes contain in their blood PLA_2 inhibitors (PLIs); and these inhibitors could be classified into three groups, i.e., PLIα, PLIβ, and PLIγ, according

to their fundamental structures (Lambeau and Lazdunski, 1999; Okumura et al., 2002; Kohji et al., 2003). PLIα is a trimer of the 20-kDa glycosylated subunit having a C-type lectin-like domain (CTLD), and it inhibits specifically group-II acidic PLA_2s (Inoue et al., 1991; Walter, 1999; Kohji et al., 2003). PLIβ selectively inhibits group II basic PLA_2s and has nine tandem leucine-rich repeats in its sequence (Okumura et al., 1999). In contrast to PLIα and PLIβ, PLIγ is a rather nonspecific inhibitor, inhibiting group-I, -II, and -III PLA_2s; and its primary structure is characterized by two tandem patterns of cysteine residues as are found in Ly-6-related proteins (Ohkura et al., 1994; Kohji et al., 2003).

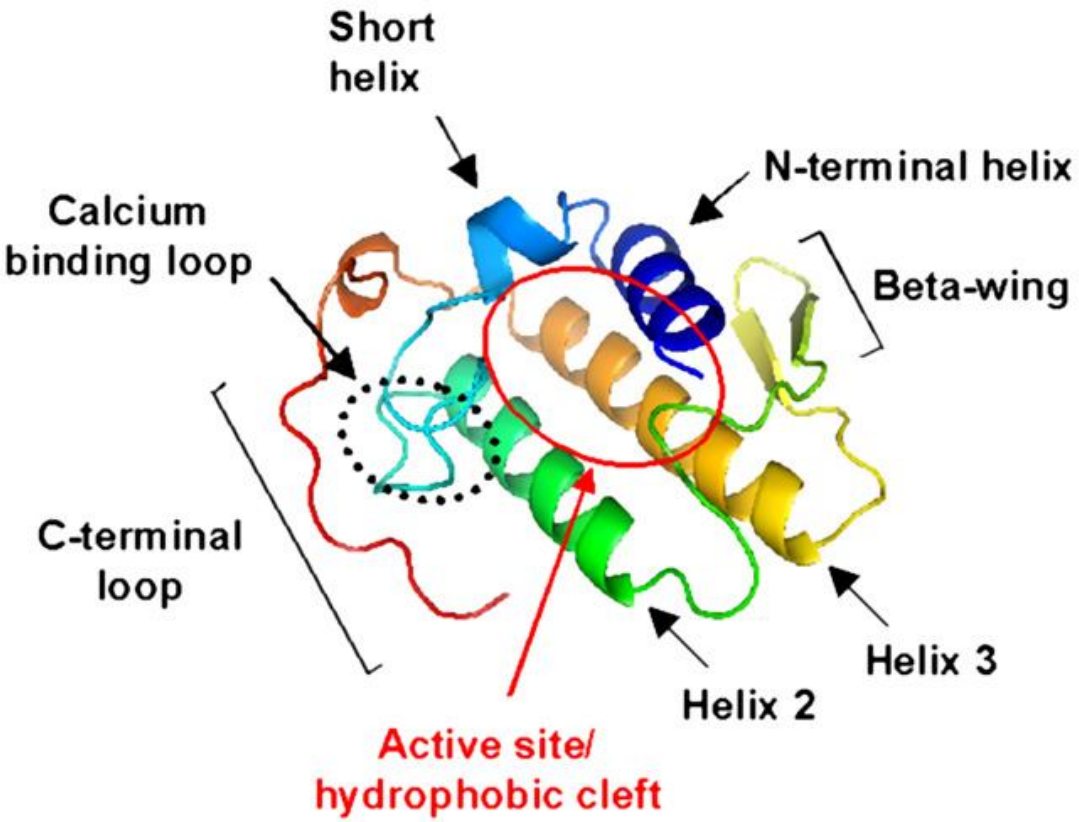


Figure 2.1: 3-D structure of PLA_2 (Duncan et al., 2008)

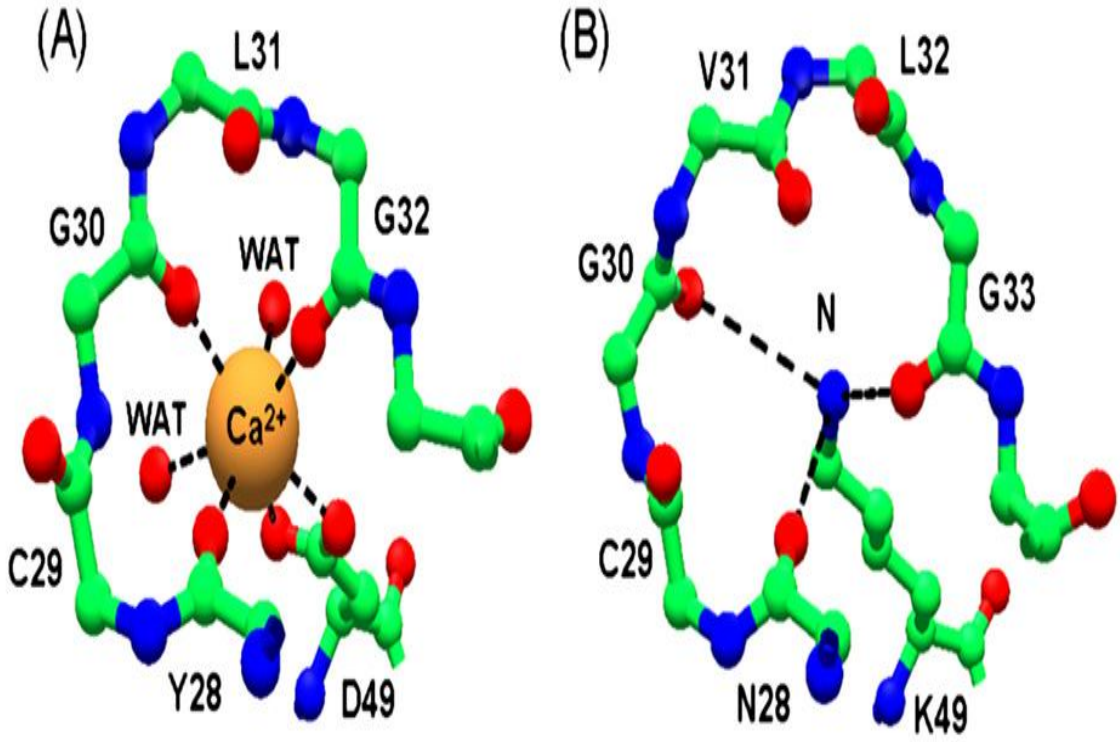


Figure 2.2: PLA_1 (A) and PLA_2 (B) structure showing active sites (Ohkura et al., 1994).

Phospholipase A_2 (PLA_2) are abundant (~40%) in almost all snake venoms (Walter et al., 1999; Kini, 2005; Rouault et al., 2003; Murari et al., 2005). PLA_2 plays an important role in phospholipids digestion. The activation of PLA_2 releases free arachidonate from phospholipids. Arachidonate serves as the substrate for two alternate pathways catalyzed by lipoxygenase or cyclooxygenase resulting in prostaglandins and prostacyclins (Dunn and Broady, 2001; Balsinde et al., 2002; Sribar et al., 2003; Duncan et al., 2008) thus leading to inflammation. Various experimental studies of PLA_2 on lipid bilayers have shown that membrane surface properties, including membrane fluidity, curvature and membrane induced structural changes (Dennis, 1997; Jensen et al., 2003; Burke and Dennis, 2009) depend on membrane binding and extent of PLA_2 activation (Diaz et al., 2001; Tatulian, 2001). Utilizing phospholipids mixtures, the activity and specificity of cobra venom PLA_2 is shown to be dependent on the presence of other lipids and their organization in the erythrocyte membranes (Florin-Christensen et al., 2001).

Several snake venom PLA_2s evolve to become potent neurotoxins, developing selectivity for neuronal structures and recognizing specific protein acceptors. Neurotoxic PLA_2s from snake venom, also called β-neurotoxins, cause death by respiratory failure (Ramar et al., 2012). They act on the neuromuscular junction, primarily at a presynaptic level, inhibiting the release of neurotransmitter acetylcholine (ACh) (Strong, 1987; Harris, 1991; Dennis, 2000; Grazyna et al., 2000).

The β-neurotoxins differ in their quaternary structures and three classes have been distinguished on this basis (Hawgood and Bon, 1991).

The first class of β-neurotoxins comprises single chain polypeptides of 13±15 kDa. It includes monomeric Elapidae β -neurotoxins, belonging to PLA_2 group I, such as notexin (Ntx) from Notechis scutatus scutatus venom and monomeric Viperidae β -neurotoxins, belonging to PLA_2 group II, such as agkistrodotoxin (Agtx) from Agkistrodon blomhoffii brevicaudus and ammodytoxin (Atx) from Vipera ammodytes ammodytes (Grazyna et al., 2000).

The second class of β-neurotoxins includes multichain neurotoxic PLA_2 that are made of several non covalently linked polypeptide subunits, at least one of them being enzymatically active. This class includes multimeric Viperidae β -neurotoxins such as crotoxin from the venom of the South-American rattlesnake Crotalus durissus terrificus, Mojave toxin from Crotalus scutelatus scutelatus venom and CbICbII from Pseudocerastes fieldi venom, which belong to PLA_2 group II, and multimeric Elapidae β -neurotoxins such as taipoxin (Tpx) from Oxyuranus scutellatus scutellatus venom which belong to PLA_2 group I (Grazyna et al., 2000).

The third class of β-neurotoxins includes β -bungarotoxin (b-Btx) from Bungarus multicinctus venom which covalently associates a PLA_2 subunit (group I of PLA_2) with a nontoxic polypeptide, homologous to Kunitz type serine proteinase inhibitor or dendrotoxins (Grazyna et al., 2000).

Although β-neurotoxins belonging to different structural classes have similar electrophysiological effects on the neuromuscular junction (Hawgood and Bon, 1991).Their targets are different. High affinity binding proteins were identified in various neuronal tissues and several distinct types of PLA_2 acceptors have been proposed for neurotoxic PLA_2s (Ramar et al., 2010).

2.8 DEVELOPMENT OF ANTIVENIN

The treatment for snake bite is as variable as the bite itself. The only available treatment is the use of antivenin (formally, antivenom) against snake bite. The first antivenin (called an anti-ophidic serum) was developed by Albert Calmette, a French scientist of the Pasteur Institute in 1895, against the indian cobra (Naja naja). Initially only horses were used for this purpose, but with the increased hypersensitive reactions against horse serum, other animals like sheep, camels and rabbits were found suitable and are now been successfully used to raise antibodies (Ig) by injecting the snake venom into the animal and the serum collected (Chotwiwatthanakun et al, 2001; Theakston et al., 2003). Antivenin binds to and neutralises the venom, stopping further damage, but do not reverse the damage already done (Antony et al., 2010a). Some individuals may react to the antivenin with an immediate hypersensitivity reaction (Cannon et al., 2008). Alternative treatment involves the use of folk and traditional medicines in snake bite, for example, root extract of Abruspra catorius is used against krait bite, leaf paste of Azadirachta indica with rock salt is used against viper bites, leaves and bark of Casearia sylvestris (guacotonga) are used as a standard Ayurvedic drug to treat snake bite in Columbia and India etc. (Antony et al., 2010b).

In 1949, snake venom was shown to contain an enzyme which causes vaso-dilation resulting in production of a hypotensive bradykinin and leading to the production of drugs for the control of blood pressure (Hile, 2004). A natural component from snake venom is now being tried for the prevention of cancerous growth (Trikha and Nakada, 2002; Hile, 2004). An ingredient from the Malaysian pit viper has shown promise in breaking blood clots beneficial in treating stroke victims (Ed, 2001; Brian, 2003). Enzymes from cobra are today tried as the most reliable hope for Parkinson's and Alzheimer's diseases (Ed, 2001; Mulugeta et al, 2003; Ramar et al., 2012).

Administration of anti-venin should be selective and based on severity of clinical symptoms (Zamudio et al, 2000). The main concern about the empirical use of antivenin is the risk of allergic reactions in sensitive individuals, its relative scarcity and cost factor (Sanchez and Rodríguez-Acosta, 2008 and Ramar et al., 2012).

2.8.1 Pharmacology of Antivenin

Antivenin is only administered after envenomation has been established (Pereanez et al., 2011). This is because of the short biologic half life of the antivenin in blood. In the carpet viper, the 20 Minutes Whole Blood Clotting Test (20WBCT) is used (Theakston et al., 2003; EchiTAb, 2008). The dose is the same for all ages and sexes. The antivenin binds to the venom in the body in a simple antigen-

antibody reaction and is cleared by microsomal enzymes in the liver. Aspirin and other drugs that may affect blood clotting cascade (for viperids) or the central nervous system (for elapids) are avoided during therapy with antivenin (EchiTab, 2008).

2.8.2 Problems of Antivenin

The therapy for snakebites has been based on the intravenous administration of equine or ovine antivenins (Bon, 1996; Pereanez et al., 2009). However, it has been demonstrated that this therapy generally has a limited efficacy against the local tissue damaging activities of venoms (Gutiérrez et al., 1998; Pereanez et al., 2011). This may be due to the speed with which tissues are degraded or to the failure of systemically administered antivenin to reach the local tissue (Kemperaju and Girish, 2006). Therefore local tissue damage can continue even after the neutralization of systemic toxicity (Leon et al., 2000). First aid and care of the snakebite victims (Rushing, 2011; Saul et al., 2011) and treatment of paediatric victim (Cordasco et al., 2001) are among the important aspects of post snakebite measures. However, lack of medical infrastructure in the rural areas, ignorance, side effects of animal bases antivenins etc has necessitate the development of alternative therapy for snakebite (Abhijit and Jitendra, 2012). Due to higher risk of mortality in this medical exigency and the limitations associated with using conventional antivenin immunotherapy, a number of medicinal plants with anti snake venom activities have been investigated in order to achieve an alternative system of the antivenin therapy (Broadley, 2001; Abhijit and Jitendra, 2012; Ramar et al., 2012).

No doubt, the problem of non-availability of specific antivenin has contributed in no small measure to increasing mortality and morbidity from snake bites in the tropics (Lanh, 2010; Pereanez et al., 2011). In Nigeria, there are three types of antivenin therapy that are sometimes available for purchase: polyvalent non-specific antivenin produced in India, the Pasteur anti-snake venom and the EchiTab antivenin which was developed specifically for snake bites from the carpet viper, E. ocellatus (Habib et al., 2001).

Among non-specific antivenins, a variety of substances has been studied using PLA_2 (Kocholaty, 1966; Lindahl and Tagesson, 1997; Odell et al., 1998). In particular, the inhibitory effect of some polyphenols on venom PLA_2 obtained from Vipera russelli and Crotalus atrox snakes were investigated (Lindahl and Tagesson, 1997). It was found that polyphenols could inhibit PLA_2 and may be used for antivenin treatment. However, biotransformation of polyphenols reduces the efficacy of their inhibitory effect (Yao-Ching et al., 2004).

2.9 HERBS AGAINST SNAKEBITE

Use of herbs has always been a popular remedy against snakebite. These medicinal plants having antagonistic efficacy against snakebite have been evaluated pharmacologically and several active components like aristolochic acid, ellagic acid and vanillic acid having snake venom neutralisation capacity have been isolated (Ibrahim et al., 2011). The roots of the plant Ophiorrhiza mungo, Peristrophe bicalyculata, Gymnema sylvestre, stems of Gloriosa superba, Cucumis colosynthis, Alangium salvifolium and leaves of Enicostemma axillare, Calycopteris floribunda, Calotropis

gigantean and Aristolochia indica are used in Ayurvedic medicine. (Yang, 1994; Soares et al., 2005; Owour and Kisangau, 2006; Da Silva et al 2007; Sanchez and Rodriguez-Acosta, 2008; Nishijima et al., 2009; De- Paula et al., 2010; Gomes et al., 2010; Ibrahim et al., 2011; Abhijit and Jitendra, 2012).

2.9.1 Potential Antiophidians

Although many of the antiophidians have been investigated pharmacologically, a large number of medicinal plants traditionally used against snake bite are yet to be evaluated (Abhijit and Jitendra, 2012). Rauvolfia Serpentina (Dey and De, 2011a, 2011b), Achyranthes aspera (Dey, 2011a), Alstonia acholaris (Dey 2011b), Aristolochia tagala (Dey and De, 2011c) Amaranthus viridis, Acorus calamus, Calotropis Procera, Cassia fistula, Cissampelos pariera, Clitorea ternatea, Boerhaavia diffusa (Dey and De, 2011c), Azadirachta indica, Alangium salvifolium (Gomes et al., 2010) etc are among the very popular antiophidian ethnomedicinal plants particularly used worldwide. Although various reports are present on phytochemical constituents and pharmacological efficacy of the plants, these are yet to be fully evaluated for antivenin activity or constituents. Therefore, laboratory based evidence is required to analyse the scientific basis of the folk practice. Further studies on these botanicals may generate some novel compounds as candidates for natural plant antivenin complementing the conventional snakebite treatments (Abhijit and Jitendra, 2012).

2.9.2 Antiophidian Compounds

Vanillic acid (4- hydroxy-3-methoxy benzoic acid) Dhananjaya et al., 2006); Terpenoid saponins such as macrolobin-A and B(Da Silva et al., 2007); polyphenols (Leanpolchareanchai et al., 2009; Mahadeswarasmy et al., 2011); ellagic acid(Da Silva et al., 2008); Rosmarinic acid (Ticli et al., 2005); Aristolochic acid(8-methoxy-6-nitrophenantfro[3,4-d][1,3]dioxole-5-carboxylic acid) and quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromene-4-one) (Girish and Kemperaju, 2005); Apthalate (Sarkhel et al., 2011); Tannin (Ambikabothy et al., 2011); β-sitosterol, stigmasterol (Gomes et al., 2007) etc have been investigated for antiophidian properties.

Hydrocarbones, **(**figure 2.3**)** such as 2, 4 dimethyl hexane, 2 methylnonane, 2, 6 dimethyl heptanes obtained from traditional medicinal plants Tragia involucrata (Euphorbiaceae) member showed effective venom and PLA_2 neutralisation in mice (Ramar et al., 2012). PLA_2 inhibitors have also been isolated from plants such as Horsfieldia amygdaline (Miyake et al., 1992; Ramar et al., 2012). The active component was isolated and identified as rosmarinic acid (RA).

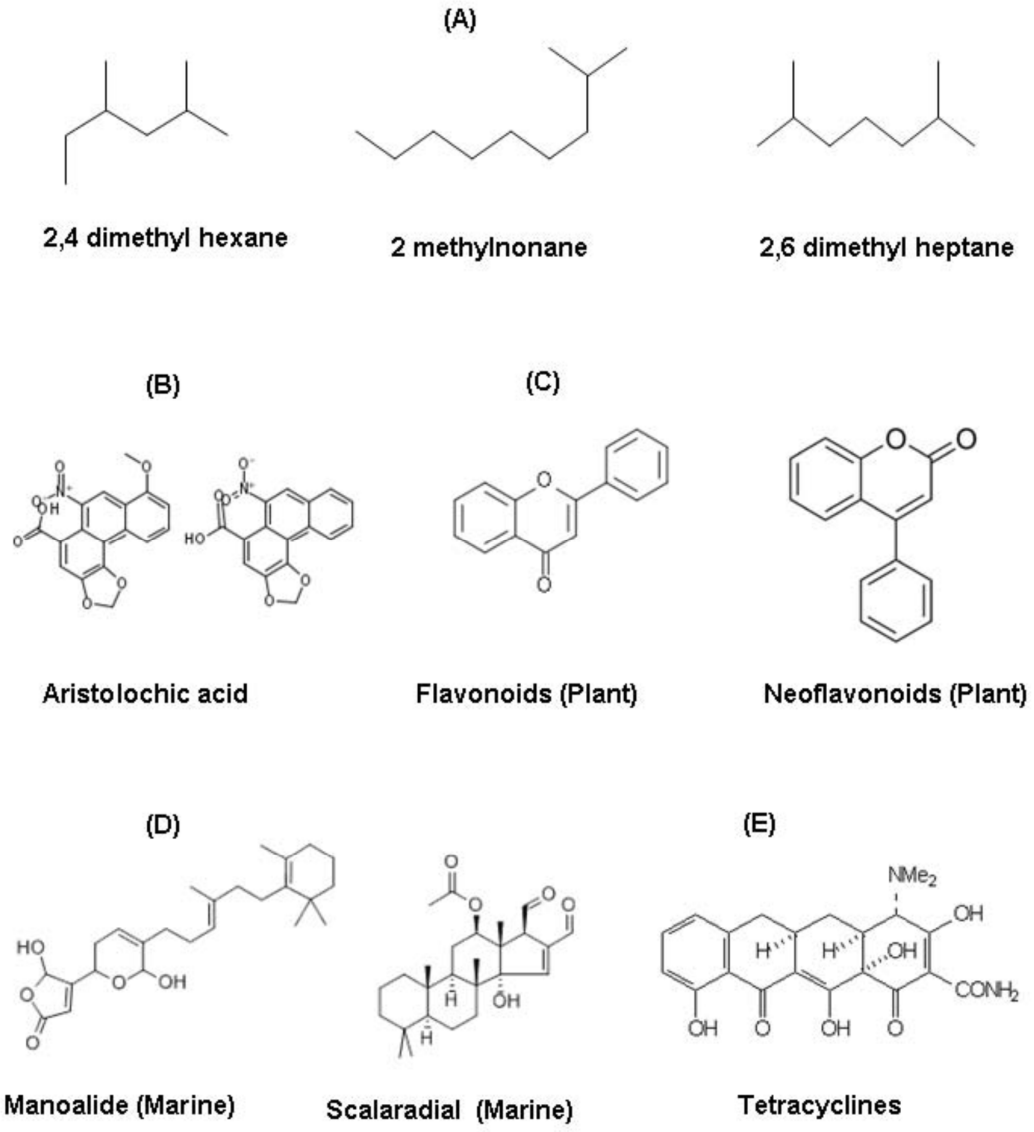


Figure 2.3: Structure of various bioactive molecules involved in the inhibitory potential of phospholipase A_2 (PLA_2) in snake venom. (A) Hydrocarbons like 2, 4 dimethyl hexane, 2 methylonane, 2,6 dimethyl heptanes obtained from traditional medicine plants Tragia involucrate (Euphorbiaceae) member (Samy et al, 2006,), (B) Aristolochic alkaloid, (C) Flavonoids and neoflavonoids from plants (D) Manolide and scalaradial from marine sources (E) tetracylne (antibiotics). (Ramar et al., 2012).

CHAPTER THREE

MATERIALS AND METHODS

3.1 REAGENTS

Sephadex G-75, Sephadex G-200 and DEAE- cellulose DE-52 were all purchased from Sigma Chemical Co. St. Louis, England. All other chemicals: sodium dihydrogen phosphate, Hydrogen disodium phosphate, sodium chloride, glycerol, Sodium hydroxide, Lecithin from egg yolk, 2-mecaptoethanol, Sodium dodecyl sulphate, protein markers, N N N N'-tetramethylethylenediamine (TEMED) were of analytical grade and obtained from reputable chemical companies.

3.2 ANIMAL TREATMENT AND SAMPLE COLLECTION

Twelve (12) adult Echis ocellatus snakes (carpet viper) of both sexes obtained from local snake handlers in Kaltungo, Gombe state, Nigeria were identified at the Department of Biological Sciences, Ahmadu Bello University Zaria. The snakes were kept in wooden boxes, given water ad libitum and fed raw meat fortnightly. Blood was obtained from the snakes by bleeding through the caudal vein, allowed to clot at room temperature and serum obtained after centrifugation at 3000/xg for 5 mins. The sera were kept in the refrigerator at -4^{o}C until required (Perales et al., 1995).

Blood samples (10ml) were collected each from five (5) Bovine, Ovine and Caprine from Zangon Shanu abattoir in Zaria, Kaduna State. The blood samples were allowed to stand for 30 minutes at room temperature and thereafter centrifuged at 3000/xg for 5 mins to collect the serum.The sera obtained from these animals were regarded as the non toxic secretory $PLA_{2.}$

3.3 COLLECTION AND PREPARATION OF VENOM.

Venom was collected by milking method as described by Markfarlane (1967). Briefly silica gel was activated at 80^{o}C for 2 hours in an oven, placed in a dessicator and kept in a refrigerator. The snakes were restrained at the position of the joint between the last cervical vertebrae and the skull, using the thumb and the fore-finger, care was taken to avoid strangulation. A cellophane piece was used to cover a beaker and held in position using rubber band. The second hand was used to hold the beaker and brought to make contact with the mouth of the snake, such that as the snake bites through the cellophane using its upper fangs, venom was ejected into the beaker. The venom from all the E. ocellatus snakes were pooled and immediately placed in a desiccator with activated silica and allowed to crystallize at 4 ^{o}C. The crystallized venom was then transferred into eppendorf tubes, labelled properly and kept in a deep freezer at – 18 ^{o}C. These were referred to as crude venom.

3.4 PARTIAL PURIFICATION OF PHOSPHOLIPASE A_2 FROM CRUDE VENOM.

Partial purification of the crude venom was carried out by the method described by Sallau et al (2008). Briefly, two (2) ml of 10 mg/ml of crude E. ocellatus venom was loaded onto DEAE cellulose column (1.5 x 50 cm) pre-equilibrated with 50mM phosphate buffer (pH 6.8). The column was eluted stepwise with NaCl gradient (0.01 – 0.1 M) at a flow rate of 0.2 ml/min. Twenty, 2 ml fractions were collected and assayed for phospholipase A_2 activity and total protein. The PLA_2 active fractions were

pooled together and loaded on sephadex G-75 column equilibrated with phosphate buffer (pH 6.5). The column was eluted with the same buffer, maintaining a flow rate of 1ml/min. Twenty, 2ml fractions were again collected and assayed for PLA_2 and total protein concentration determined (Bhat and Gowda, 1989).

3.4.1 PLA_2 Assay

The PLA_2 activity was assayed as described by Bhat and Gowda, (1989). Briefly, 25 µl of 1 mg/ml L-α-lecithin substrate was incubated with 10µl of the partially purified enzyme for 10 min at 37^{o}C. The reaction was terminated by immersing the tube in a boiling water bath for 2 min and the amount of released free fatty acid measured titrimetrically at pH 8.0 using 20 mM sodium hydroxide and phenolphthalein indicator. The activity of phospholipase A_2 was defined as the amount of enzyme that hydrolyses 1 µmole of fatty acids from L-α -Lecithin per minute under standard conditions.

(L-α-LECITHIN $\xrightarrow{PLA_2}$ FREE FATTY ACID (FFA) +LYSOLECITHIN)

The phospholipase A_2 activity was given as thus,

Volume of 20 mM NaOH that neutralized free fatty acid = ymL

$0.02 \times y \times 10^{-3}$ = k moles/10 min

Enzyme activity = k moles/10 min = Z moles/min.

3.4.2 Determination of Protein Concentration.

The protein concentration of partially purified E. ocellatus venom was determined spectrophotometrically by taking absorbance at 280nm wavelength.

3.5 PURIFICATION OF PLA_2 INHIBITOR FROM ECHIS OCELLATUS SERUM

PLA_2 Inhibitor from Echis Serum (PIES) was purified by a modified method described by Perales et al., (1995). Briefly, 2 ml of the serum was directly fractionated at 4°C on a Sephadex G-200 column, pre equilibrated with 0.05M Tris Hcl buffer (pH 8.1), containing 0.15M NaCl. The column was eluted with the same buffer at a flow rate of 3 ml/hr. Twenty, 2 ml fractions were collected and inhibition studies (assay for residual PLA_2 activity) were carried out against the snake PLA_2. Fractions with inhibitory activities against the partially purified PLA_2 from E. ocellatus venom were pooled together and directly loaded on a DEAE cellulose column pre equilibrated with 0.05M phosphate buffer (pH 6.8). The column was eluted with a stepwise linear gradient of sodium chloride (0.01- 0.1M NaCl) at a flow rate of 5 min/ml. Fifty, 2 ml fractions collected were assayed for residual Phospholipase A_2 activity and total protein concentrations. Fractions with inhibitory activities against E. ocellatus PLA_2 were pooled together, dialysed and stored at -4^{o}C until required.

3.5.1 Inhibition Studies

To analyze the effect of PIES on partially purified Echis PLA_2 and non-toxic secretory (mammalian serum) PLA_2 activity, 10 µl of partially purified PLA_2 from E. ocellatus venom, 25 µl of 1 mg/ml L-α-lecithin substrate was incubated for 10 min at 37^{o}C with 25 µl of various concentrations of PIES (10%, 30%, 40% and 50%v/v). The reaction was then terminated by immersing the tube in a boiling water bath for 2 min and the amount of released free fatty acid measured titrimetrically at pH 8.0 using 20 mM sodium hydroxide and phenolphthalein indicator. The inhibition of phospholipase A_2 activity by PIES otherwise referred to as residual PLA_2 activity was defined as the amount of residual enzyme that hydrolyzes 1 µmole of fatty acids from L-α -Lecithin per minute in the presence of PIES under standard conditions (Bhat and Gowda 1989).

3.5.2 Initial Velocity Studies

The effect of partially purified PIES on partially purified PLA_2 activity was carried out with varying concentrations of the substrate (2 -5mg/ml) at 0%, 5%, 10% and 20% of PIES. Initial velocity values obtained were used to draw Double Reciprocal plot to determine the inhibition binding constant (K_i) and to ascertain the kind of inhibition (Sallau et al., 2008).

3.6 EFFECT OF PIES ON THE ENZYMATIC ACTIVITY OF NON TOXIC SECRETORY PLA_2S

3.6.1 Effect of PIES on Bovine, Ovine and Caprine Sera

The effect of partially purified PIES on Bovine, Ovine and Caprine sera were determined independently after measuring activities of the following groups:

Group 1: Bovine, Ovine and Caprine Sera PLA_2 + Substrate.

Group 2: Tannic acid + Bovine, Ovine and Caprine Sera PLA_2 + Substrate.

Group 3: Bovine, Ovine and Caprine Sera PLA_2 + partially purified PIES (10%) + Substrate.

Group 4: Bovine, Ovine and Caprine Sera PLA_2 + partially purified PIES (10%) + Echis PLA_2 + Substrate.

3.6.2 Effect of PIES on partially purified E. ocellatus PLA_2 Activity.

Group 1: Partially purified Echis PLA_2 + Substrate

Group 2: Tannic acid + partially purified Echis PLA_2 + Substrate (standard control)

Group 3: Partially purified Echis PLA_2 + partially purified PIES (10 %) + Subtrate

Group 4: Partially purified Echis PLA_2 + partially purified PIES (30 %) + Subtrate

Group 5: Partially purified Echis PLA_2 + partially purified PIES (40 %) + Subtrate

Group 6: Partially purified Echis PLA_2 + partially purified PIES (50 %) + Subtrate

3.7 IN VITRO STUDIES OF EFFECT OF PIES ON ECHIS OCELLATUS VENOM

3.7.1 Red blood cell fragility test

Membrane stabilizing activity of the partially purified PIES was assessed using hypotonic solution-induced rat erythrocyte hemolysis as described by Shinde et al., (1999). Briefly, the tail tips of 3 clean and apparently healthy Albino rats were clipped and 200 µL of blood collected in heparinised capillary tubes was washed in 5 ml of phosphate buffered saline (pH 7.4) by centrifuging at 3000/xg for 10 mins. The washing was repeated by decanting the supernatant until the supernatant was clear. The test sample consisted of stock erythrocyte (RBC) suspension (0.50 ml) mixed with 10 µl of partially purified PLA_2 from Echis venom, 4 ml of 10 mM sodium phosphate buffered saline (pH 7.4) and 25 µl of the partially purified PIES (5, 10 and 20%). The control sample consisted of 0.5ml of RBC mixed with 4ml hypotonic -buffered saline solution alone. The mixtures were incubated for 2 hours at room temperature and centrifuged at 3000/xg for 10 min and the absorbance of the supernatant was measured at 540 nm. The percentage inhibition of hemolysis or membrane stabilization was calculated thus:

% Inhibition of hemolysis = 100 x {OD1-OD2/OD1}

Where:

OD1 = Optical density of hypotonic saline solution alone

OD2 **=** Optical density of test sample.

3.7.2 Determination of Anticoagulant Activity

Anticoagulant activity was determined as described by Reid and Theakston (1983) with modification. Briefly, citrated bovine plasma was prepared by adding 1% w/v of sodium citrate to 2 ml of blood. The blood was then centrifuged at 3000/xg for 5 mins to obtain the plasma. The test sample consisted of 200 µl of plasma, 100 µl of crude E. ocellatus venom and 25 µl of 5%, 10% and 20% of PIES each. While the control samples consisted of 200 µl of plasma for the normal and 200 µl of plasma and 100 µl of crude venom for the positive controls respectively. The samples were incubated in a water bath at 37^{o}C for 60 seconds and a final 100 µl of 25 mM $CaCl_2$ was added, and the coagulation time (Recalcification time) recorded.

3.8 SODIUM DODECYL SULPHATE-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 crude serum from Echis ocellatus and the pooled active fractions from DEAE cellulose column

chromatography. The protein bands were located by staining with Coomasie brilliant blue. 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.

3.8.1 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^{o}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.

3.8.2 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 N, N, N', N'-tetramethylethylenediamine (20µl). The solution was swirled to mix and the mixture was poured into the gel tube immediately before polymerization occured.

3.8.3 Loading, Running and Staining of the Sample

The prepared sample (50 µl) 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. 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 deionised 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 pre-treating solution for 1 min, and then rinsed briefly in 50ml deionised water three times. The gel was then stained in Coomasie staining solution for 10 min, thereafter destained briefly two times in 50ml of deionised water. The protein bands were visualized by placing the gel in 50 ml of developing solution until the desired resolution was obtained. The reaction was terminated by placing the gel in 50 ml of stop solution.

3.8.4 Molecular Weight Determination of PIES

The molecular weight of partially purified PIES was estimated by SDS-Polyacrylamide Gel Electrophoresis as described by Laemmli (1970). The molecular weight markers for the SDS-PAGE include; trypsinogen (24kDa), Carbonic Anhydrase (29kDa), α-lactabulmin (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 PIES was extrapolated from the plot of log of molecular weight of the marker proteins against their respective R_f values.

3.8 SATISTICAL ANALYSIS

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 < 0.001 and p-value < 0.05 was considered significant.

CHAPTER FOUR

RESULTS

4.1 PURIFICATION OF PHOSPHOLIPASE A_2 FROM ECHIS OCELLATUS VENOM

The results of the purification of PLA_2 from E. ocellatus venom are summarised on table 4.1. The crude extract contained about 2.28 mg of protein with a total activity and specific activity of 7.40 µmol/min and 3.25 µmol/min/mg of protein respectively. Fractionation of the crude venom on DEAE cellulose chromatography gave a specific activity of 21.5 µmol/min/mg. Subsequent gel filtration on Sephadex G-75 chromatography gave an active peak (figure 4.1) with a specific activity of 53.17 µmol/min/mg of protein, 16.36 purification fold and 43.11% recovery.

Figure 4.1 shows the elution profile of Echis PLA_2 after ion exchange chromatography on DEAE cellulose column. An active peak eluted in fraction 13 of the void volume, had a protein concentration of 0.2 mg/ml and a corresponding enzyme activity of 4.3 µmol/min eluted by 80 mM Nacl.

From the elution profile of Echis PLA_2 after gel filtration on Sephadex G-75 column (Figure 4.2), an active peak (fraction 12) was found to contain about 0.06 mg/ml protein and a corresponding PLA_2 activity of 3.19 µmol/min.

The double reciprocal plot (Figure 4.3) of partially purified PLA_2 obtained from E. ocellatus venom showed that the enzyme had an estimated K_M of 1.4 mg/ml and Vmax of 4.5 µmol/min.

Table 4.1: Purification profile of partially purified phospholipase A_2 from E. ocellatus venom

Purification Step	Total Protein (mg)	Total Activity (µmol/min)	Specific Activity (µmol/min/mg protein)	Purification Fold	Percentage Yield (%)
Crude	2.28	7.40	3.25	1.00	100
Ion exchange Chromatography on DEAE Cellulose	0.20	4.30	21.5	6.62	58.11
Gel Filtration on Sephadex G-75	0.06	3.19	53.17	16.36	43.11

*One unit of Phospholipase A_2 was defined as the amount of enzyme that hydrolyses 1 µmole of fatty acids from L-α - Lecithin per minute under standard conditions.

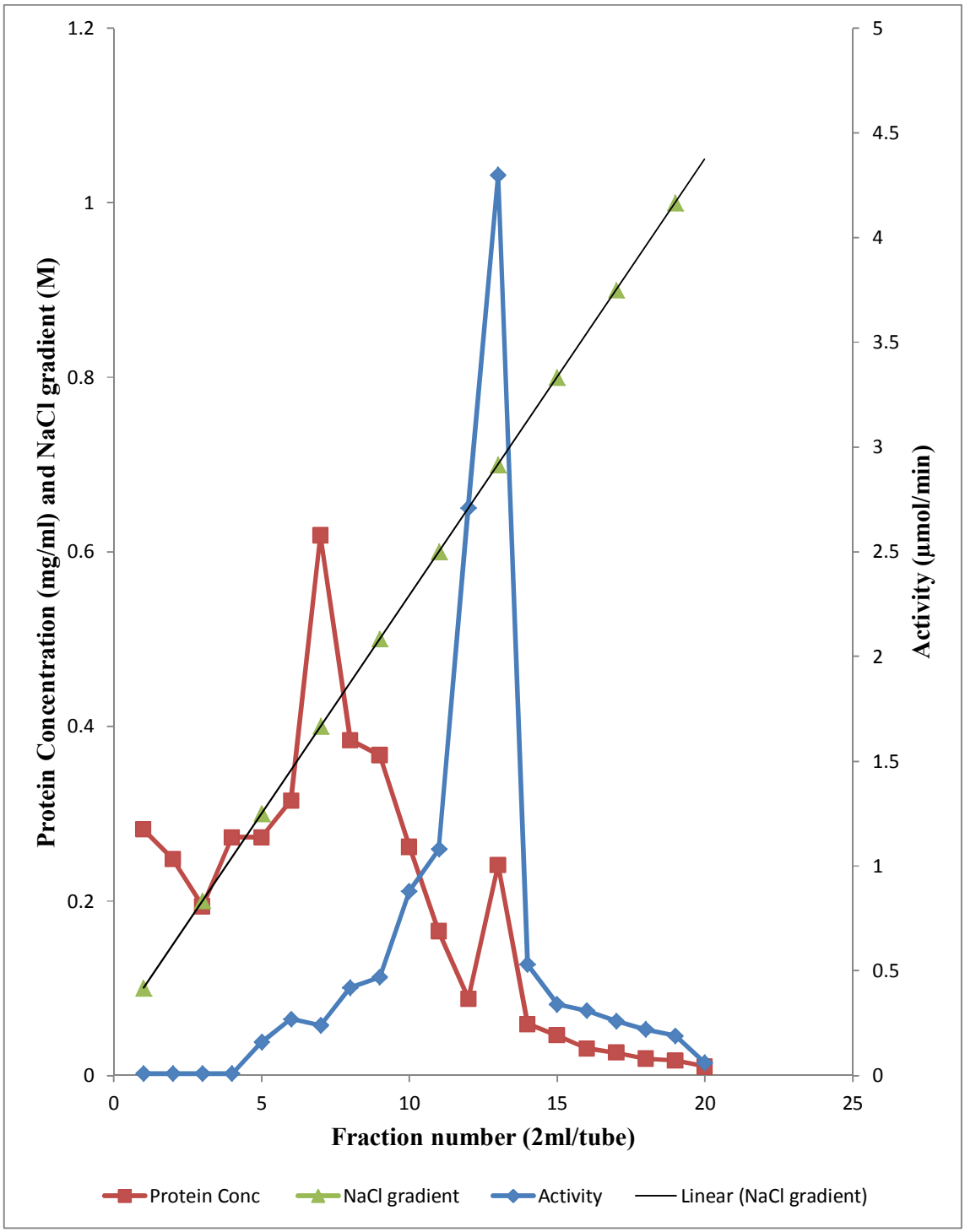


Figure 4.1: Elution profile of partially purified phospholipase A_2 from E. ocellatus venom on DEAE-cellulose column chromatography. (1.6 x 50cm)

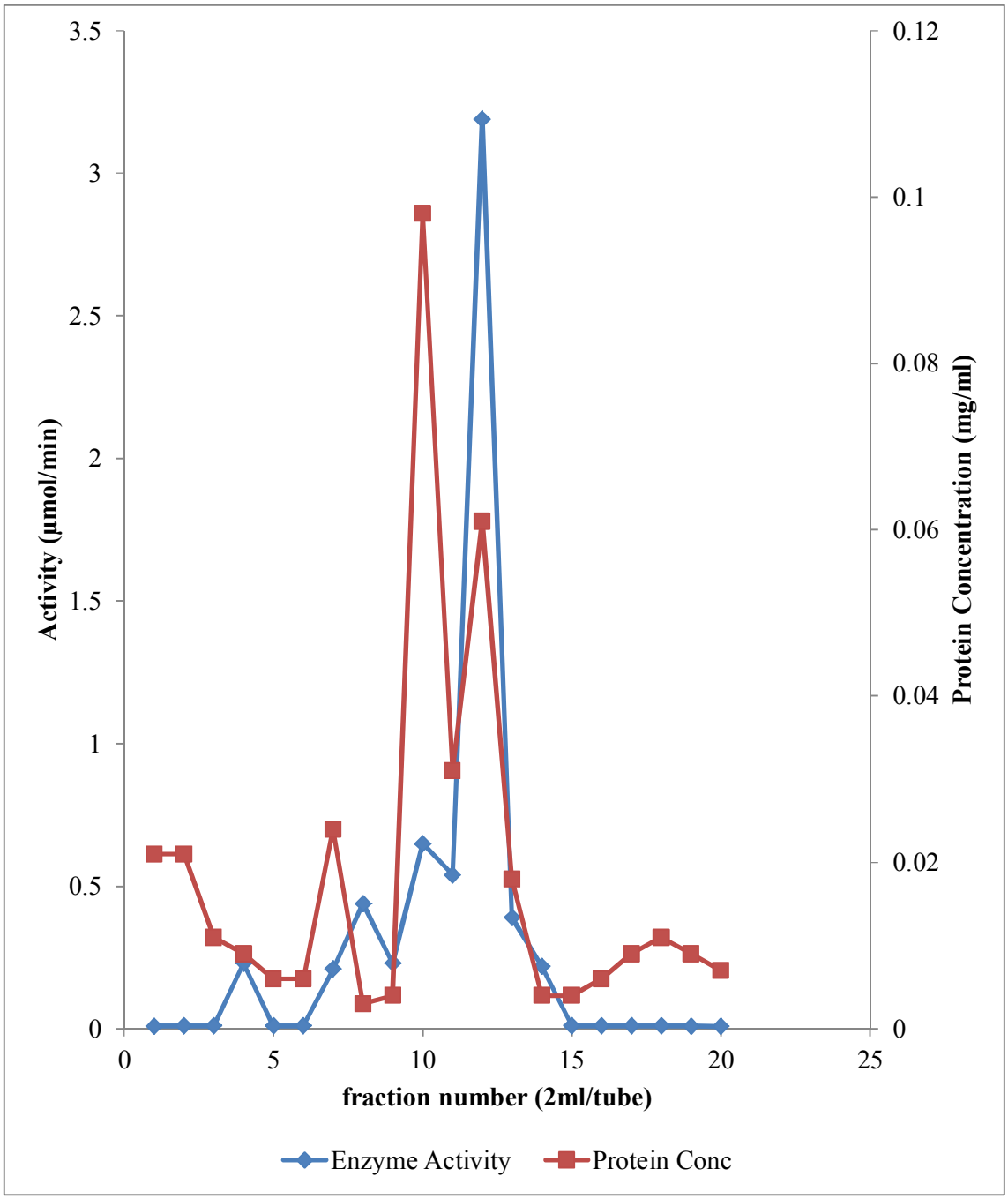


Figure 4.2: Elution profile of partially purified phospholipase A_2 from E. ocellatus venom on sephadex G-75 column chromatography (1.6 x 90cm).

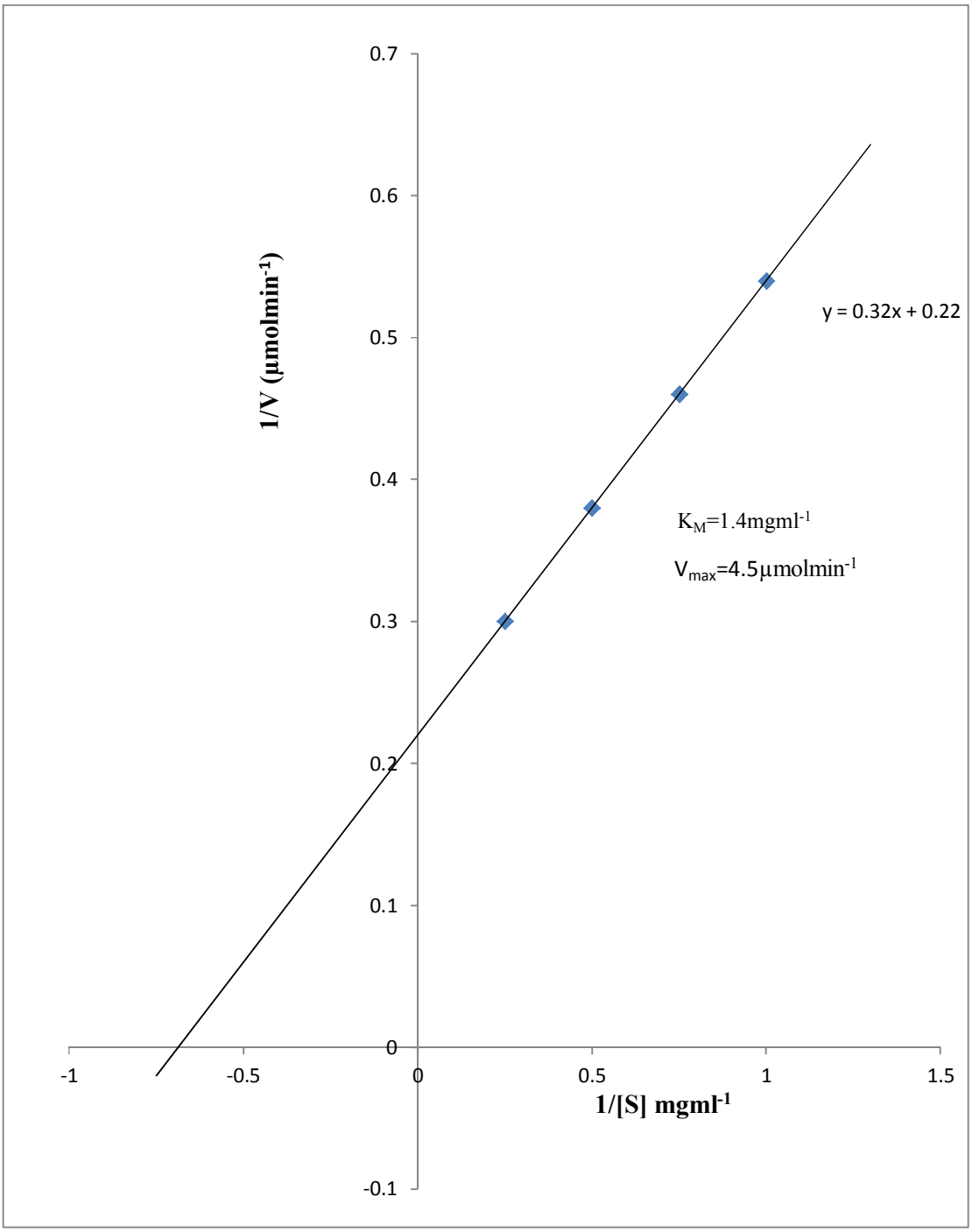


Figure 4.3: Double Reciprocal plot of partially purified phospholipase A_2 from Echis ocellatus venom showing K_M and $V_{max.}$

4.2 PURIFICATION OF PHOSPHOLIPASE A_2 INHIBITOR FROM ECHIS OCELLATUS SERUM.

The results of partial purification of phospholipase A_2 Inhibitor from Echis ocellatus serum (PIES) are shown in Figures 4.4 and 4.5. From the results (Figure 4.4), the elution of PIES on sephadex G-200 column chromatography produced an active peak (fraction 15) with a protein concentration of about 0.022 mg/ml and a relative inhibition of about 70% against the partially purified E. ocellatus PLA_2. Further purification on DEAE cellulose column (Figure 4.5) gave an active peak (fraction 37) with a relative inhibition of 0.778 (77.8%) and about 0.26 mg/ml of protein.

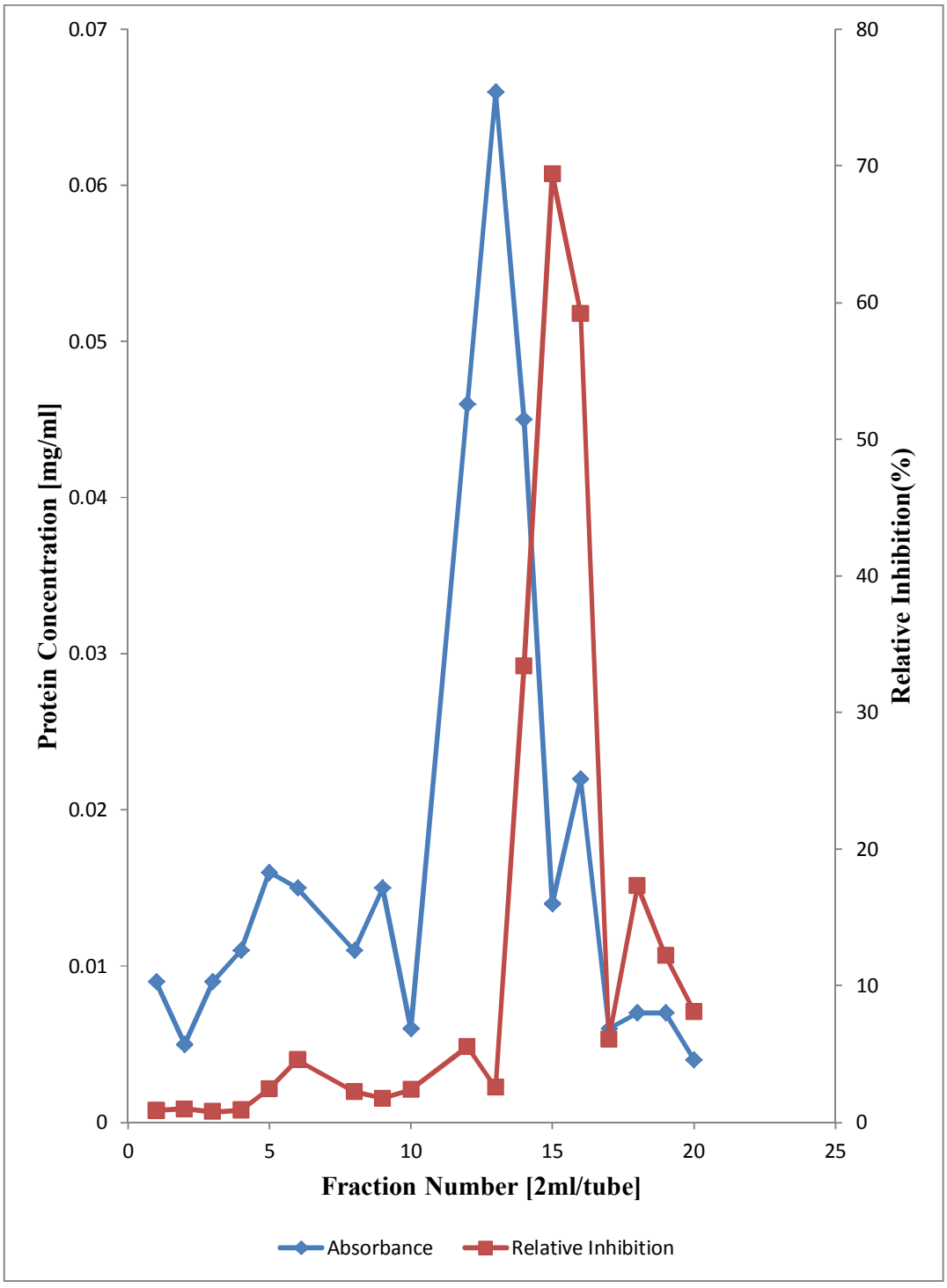


Figure 4.4: Elution Profile of phospholipase A_2 inhibitor from E. ocellatus serum (PIES) on sephadex G- 200 column showing relative inhibition (%) and total protein at 280nm.

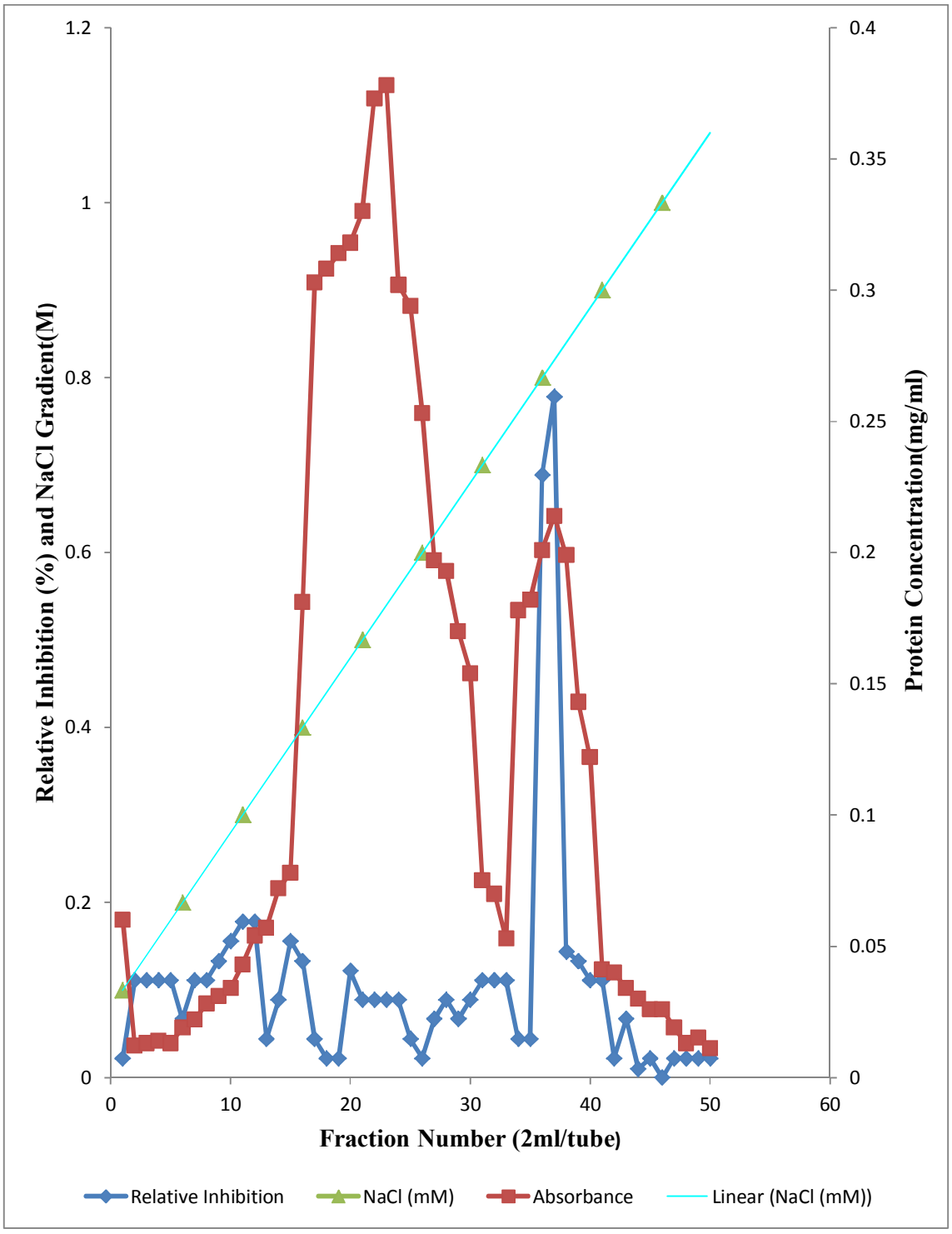


Figure 4.5: Elution profile of phospholipase A_2 inhibitor from E. ocellatus serum on DEAE- cellulose showing relative inhibition (%), total protein at 280nm and NaCl gradient (M).

4.3 PURITY AND MOLECULAR WEIGHT OF PHOSPHOLIPASE A_2 INHIBITOR FROM ECHIS OCELLATUS SERUM (PIES).

The purity and the molecular weight of the partially purified inhibitory protein were determined by Sodium Dodecyl Sulphate Polyacrilamide Gel electrophoresis (SDS-PAGE). Plate X shows the electrophoretic pattern of the sample under denaturing conditions. A distinct band of the protein sample was visualised against the standard marker proteins on the gel and the molecular weight of the partially purified PIES (Band A) was estimated to be 24,986.19Da. From the plot of log of molecular weight of the marker proteins against their respective relative mobility (R_f), the molecular weight of the partially purified PIES was found to be 24,986.19Da. (Figure 4.6)



Band A represent PIES with an estimated molecular weight of **24.986kDa.**

Plate X: Electrophoregram of phospholipase A_2 inhibitor from E. ocellatus serum (PIES) on polyacrilamide gel using Coomasie brilliant blue staining.

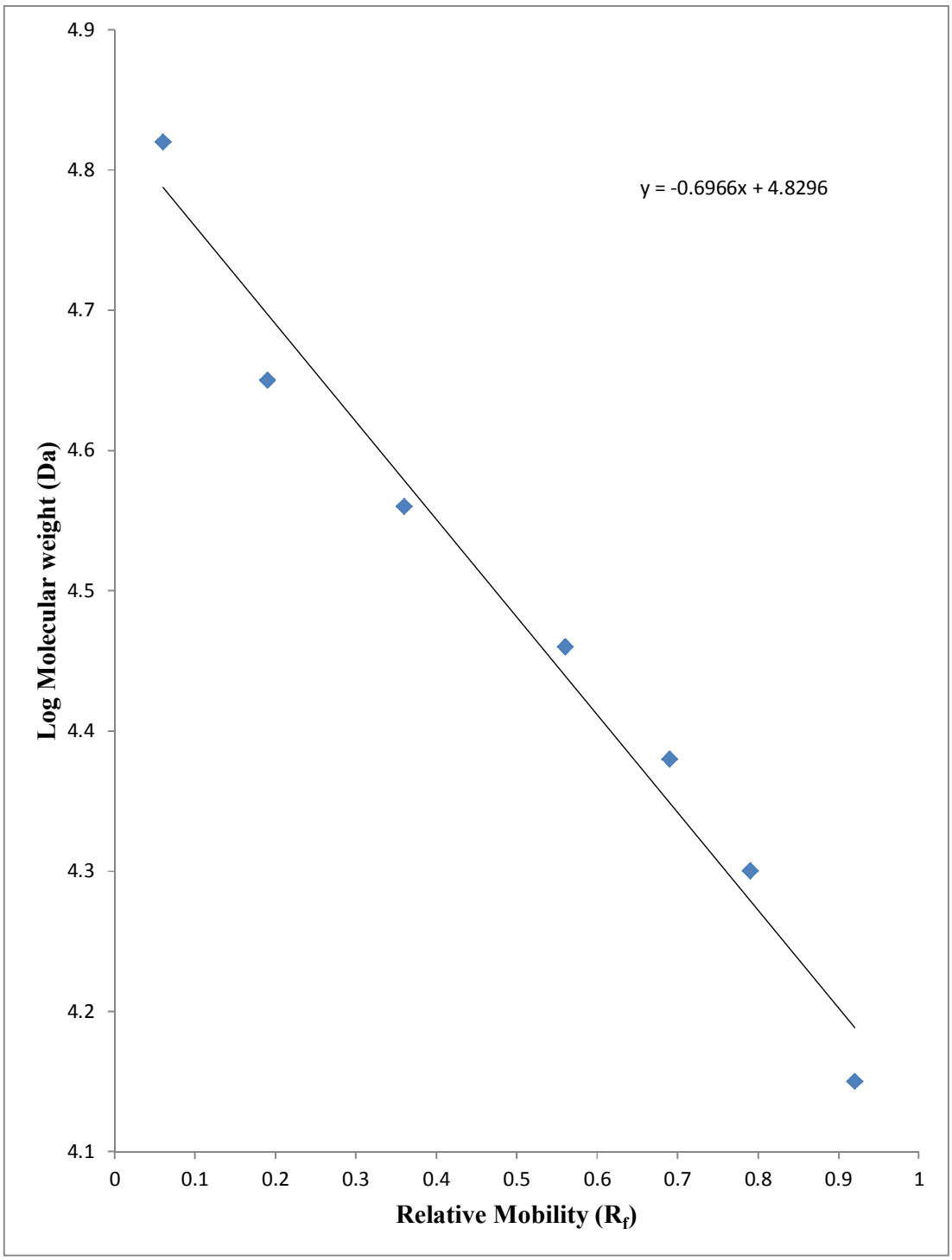


Figure 4.6: A plot showing the Log of molecular weight against the R_f of the marker protein bands which was used to estimate the molecular weight of partially purified PIES (R_f=0.62cm).

4.4 CHARACTERISATION OF PARTIALLY PURIFIED PHOSPHOLIPASE A_2 INHIBITOR FROM ECHIS OCELLATUS SERUM (PIES)

A study of the inhibition kinetics of partially purified PIES on partially purified PLA_2 revealed that PIES exhibited a mixed type of inhibition (Figure 4.7) with K_M decreasing from 4.54 mg/ml without inhibitor to 3.27 mg/ml, 3.27 mg/ml and 3.12 mg/ml at 0%, 5%, 10% and 20% concentration of PIES respectively. The K_i and K_{ii} values were 3.89% (v/v) and 7.28% (v/v) (Figure 4.8 and 4.9), respectively.

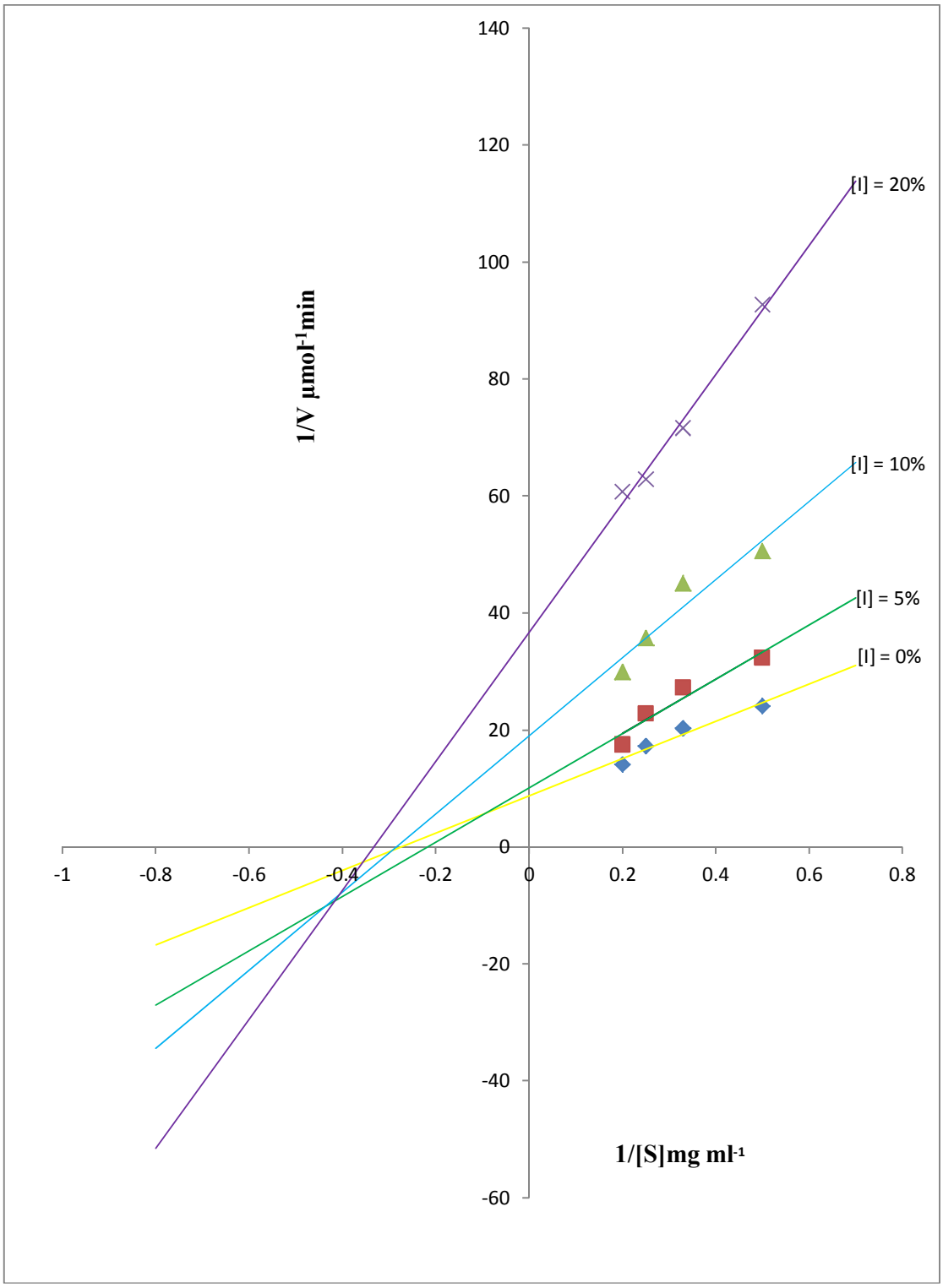


Figure 4.7: Double Reciprocal plot showing the effect of partially purified PIES on partially purified PLA_2 activity.

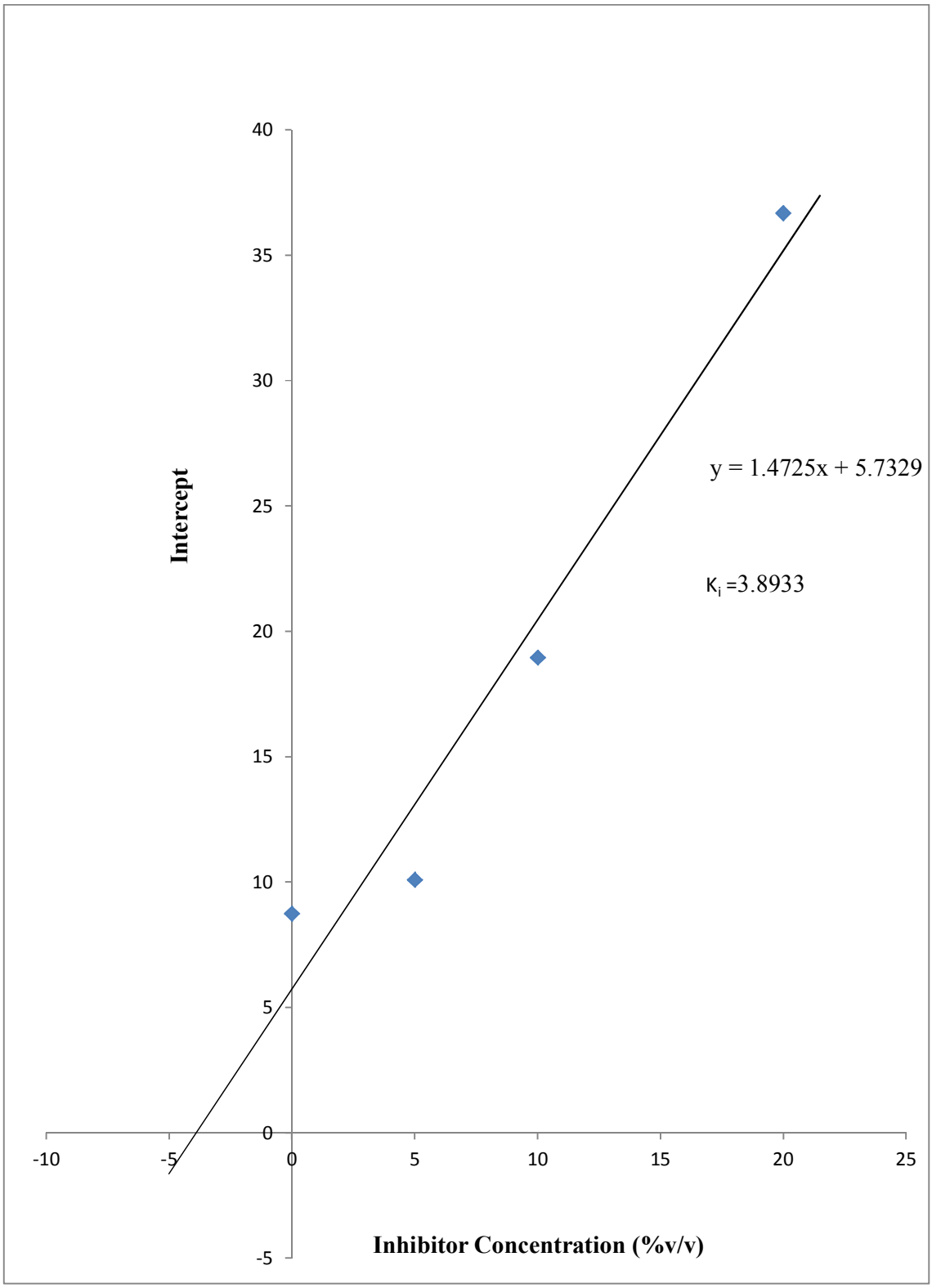


Figure 4.8: Secondary Plot of Intercept against Inhibitor concentration showing K_i.

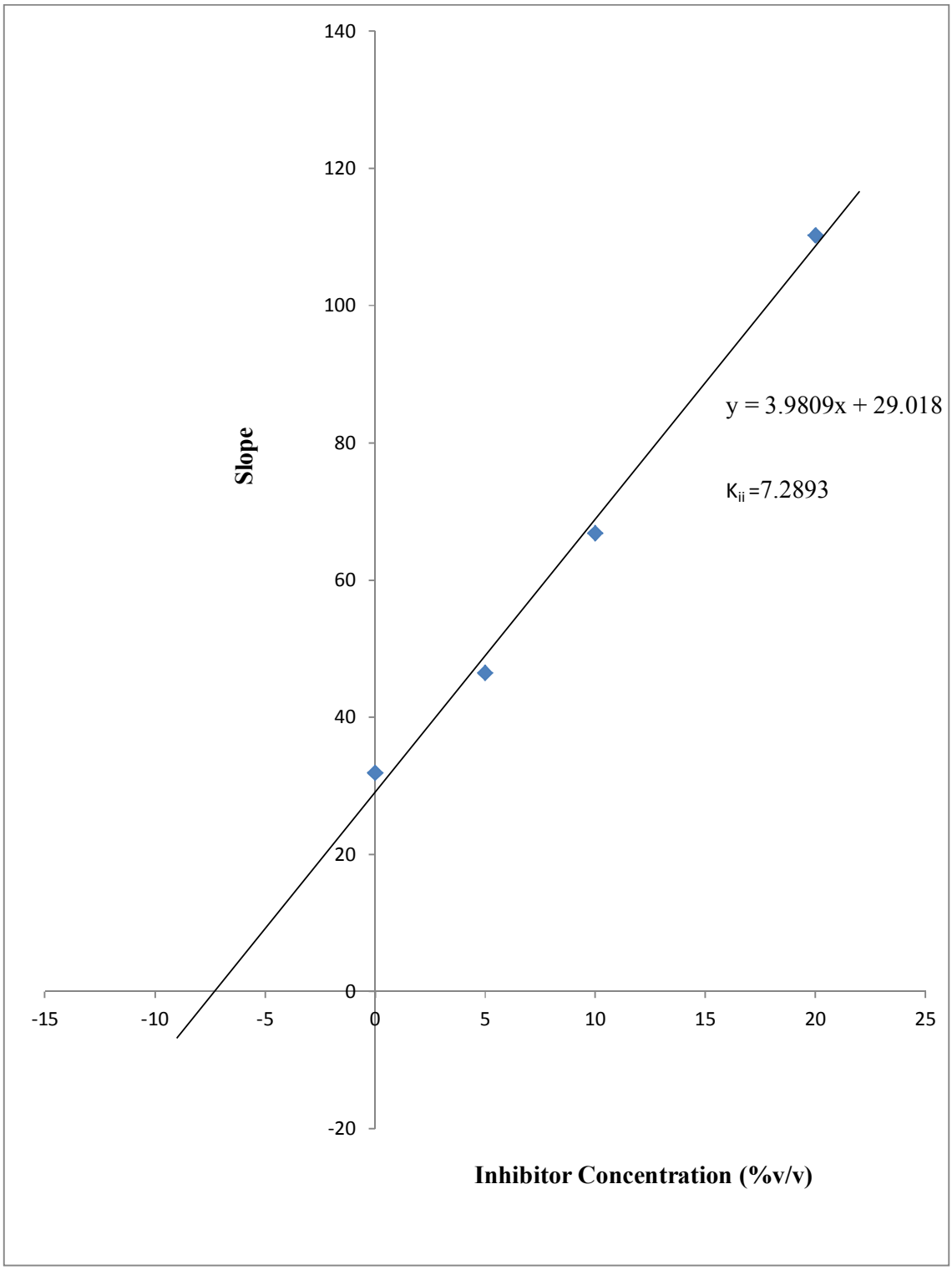


Figure 4.9: Secondary plot of Slope against Inhibitor concentration showing K_{ii}.

4.5 IN VITRO STUDIES OF THE EFFECT OF PARTIALLY PURIFIED PLA_2 INHIBITOR FROM ECHIS OCELLATUS SERUM (PIES) ON ECHIS OCELLATUS PLA_2

The in vitro hemolytic effect of Echis PLA_2 significantly ($p<0.001$) reduced from 81.2±0.010 in blood samples without PIES, to 64.3±0.019% and 35.3±0.017% in blood samples treated with 10% and 20% PIES respectively. However, the percentage observed in untreated blood samples (81.2±0.010) was not significantly different from samples treated with 5% PIES (83.6±0.024), showing a dose dependent effect (Table 4.2).

The anticoagulant activity of E. ocellatus venom in the presence and absence of PIES was given as the average recalcification time (in minutes) of citrated bovine plasma; and from the results (Table 4.3), it decreased significantly from 4.32 minutes in plasma without the inhibitor to 0.54 minutes on addition of 20% PIES. The recalcification time was found to be dose-dependent as it decreased with increasing concentration of PIES at 5% (3.56minutes), and 10% (1.23minutes).

Table 4.2: Inhibition of Hemolysis due to Phospholipase A_2 from Echis Venom by Partially Purified Phospholipase A_2 Inhibitor from Echis ocellatus Serum (PIES)

Parameter	Hemolysis (%)
Hypotonic solution + blood only	100
Echis Venom PLA_2 + blood only	81.2±0.010[a]
Echis Venom PLA_2 + 5%PIES + blood	83.6±0.024[a]
Echis Venom PLA_2 + 10%PIES + blood	64.3±0.019[b]
Echis Venom PLA_2 + 20%PIES + blood	35.3±0.017[c]

Mean ± SD for three determinations; values with different superscripts are significantly different at $p<0.001$.

Table 4.3: Effects of Partially Purified PIES on Anticoagulant Activity of E. ocellatus Venom

Sample	Recalcification Time (min)
Plasma only	4.23
Crude venom	***
Venom+plasma+5% PIES	3.56
Venom+plasma+10% PIES	1.23
Venom+plasma+20% PIES	0.54

***No clotting after 30 mins.

4.6 EFFECT OF PARTIALLY PURIFIED PHOSPHOLIPASE A_2 INHIBITOR FROM ECHIS OCELLATUS SERUM (PIES) ON NON-TOXIC SECRETORY PHOSPHOLIPASE A_2.

Table 4.4 shows the effect of partially purified PIES on non-toxic secretory PLA_2 from Bovine, Ovine and Caprine. Tannic acid completely inhibited non-toxic PLA_2 (0.200±0.000 µmol/min) and was significantly lower ($p< 0.05$) than values obtained in Bovine, Ovine and Caprine samples.

In Bovine, samples without treatment (6.880 ± 0.759 µmol/min) were not significantly different ($p< 0.05$) when compared with those treated with 10% PIES (5.720 ± 0.668 µmol/min). However, samples treated with 10% and partially purified PLA_2 from E. occelatus (4.965 ± 0.487 µmol/min) had values lower than the treated and untreated with partially purified PIES.

In Ovine, there was no significant difference ($p=0.105$) between samples without treatment (7.260 ± 0.263 µmol/min), samples treated with partially purified 10% PIES (6.480 ± 0.580 µmol/min) and

samples treated with 10% PIES and partially purified PLA_2 from E. ocellatus. (6.800 ± 0370 µmol/min).

In Caprine, samples without treatment (6.580 ± 0.944 µmol/min) were not significantly different ($p< 0.05$) when compared to those treated with 10% partially purified PIES (6.425 ± 0.286 µmol/min), whereas, samples treated with partially purified 10% PIES and partially purified Echis PLA_2 (5.342 ± 0.628 µmol/min) were lower than values obtained for the untreated samples and samples treated with 10% PIES.

4.7 EFFECT OF PARTIALLY PURIFIED PIES ON E. OCELLATUS PLA_2 ENZYME ACTIVITY

The partially purified PIES significantly ($p< 0.001$) reduced the activity of partially purified PLA_2 from E. ocellatus venom. Treatment of E. ocellatus PLA_2 with tannic acid (0.200±0.000 µmol/min) significantly ($p< 0.001$) reduced the Echis PLA_2 activity when compared with untreated samples in group 1 (5.531 ± 0.11µmol/min) and samples treated with 10% (3.281 ± 0.28 µmol/min), 30% (2.277 ± 0.87µmol/min), 40% (2.114 ± 0.71 µmol/min) and 50% (1.587 ± 0.09 µmol/min) PIES. Also the PLA_2 activity of samples treated with 40% and 50% PIES were significantly ($p< 0.001$) lower than the untreated samples in group 1 (5.531 ± 0.1 µmol/min), but were not significantly ($p< 0.001$) different from samples treated with 10%and30%PIES.

. Table 4.4 Effect of Partially Purified PIES on Non Toxic Secretory PLA_2 Activity

Group	**Bovine**	**Ovine**	**Caprine**
1	6.880 ± 0.759^{a}	7.260 ± 0.263^{a}	6.580 ± 0.944^{a}
2	0.200 ± 0.000^{b}	0.200 ± 0.000^{b}	0.200 ± 0.000^{b}
3	5.720 ± 0.668^{a}	6.800 ± 0.370^{a}	6.425 ± 0.286^{a}
4	4.965 ± 0.487^{ab}	6.480 ± 0.580^{a}	5.342 ± 0.628^{ab}
	F=0.239; p=0.791	F=2.741; p=0.105	F=2.132; p=0.365

Statistically significant difference exists at $p<0.05$. Mean values were separated by Duncan's Multiple Range test.

All values are expressed as phospholipase A_2 activity in µmol/min

Group 1: L-α-lecithin + Bovine, Ovine and Caprine serum

Group 2: L-α-lecithin + tannic acid + Bovine, Ovine and Caprine serum

Group 3: 10% PIES + L-α-lecithin + Bovine, Ovine and Caprine serum

Group 4: 10% PIES + Echis PLA_2 + L-α-lecithin + Bovine, Ovine and Caprine serum

Table 4.5 Effect of Partially Purified PIES on E. ocellatus PLA_2

Group	E. ocellatus PLA_2
1	5.531 ± 0.11^{a}
2	0.200 ± 0.00^{c}
3	3.281 ± 0.28^{ab}
4	2.277 ± 0.87^{ab}
5	2.114 ± 0.71^{b}
6	1.587 ± 0.09^{b}
	$F=9.476;\ p< 0.001$*

a, b, c = statistically significant difference exists at $p<0.01$. Mean ± SD for three determinations separated by ANOVA.

All values are expressed as phospholipase A_2 activity in µmol/min.

Group 1: partially purified Echis PLA_2 + Substrate

Group 2: Tannic acid + partially purified Echis PLA_2 + Substrate (standard control)

Group 3: Partially purified Echis PLA_2 + partially purified PIES (10 %) + Substrate

Group 4: Partially purified Echis PLA_2 + partially purified PIES (30 %) + Substrate

Group 5: Partially purified Echis PLA_2 + partially purified PIES (40 %) + Substrate

Group 6: Partially purified Echis PLA_2 + partially purified PIES (50 %) + Substrate

CHAPTER FIVE

DISCUSSION

Toxicological properties of snake bite are thought to be associated with enzymes especially Phospholipase A_2 (PLA_2), which is found to be its most toxic component (Mahanta and Murkherjee, 2001). Considering the role of PLA_2 in envenomation, understanding the characteristics of the enzyme from snake venom has raised concern for venom researchers, as it would help in the production of effective therapeutic antivenins (Scott and Sigler, 1994; Yang, 1994). In this study, PLA_2 from E. ocellatus venom which belongs to the Class II PLA_2 predominantly found in Viperidae snakes was isolated and partially purified .The PLA_2 active fraction was eluted in the void volume from ion exchange chromatography on DEAE-cellulose column (Figure 4.1). After gel filteration on sephadex G-75 column, an active peak was eluted (Figure 4.2). From the results, there was an increase in purification fold from 6.62 to 16.36 (Table 4.1); while the specific activity also increased from 21.5 µmol/min/mg protein to 53.17 µmol/min/mg protein. Our 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 PLA_2 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, the fairly low K_M value of 1.4 mg/ml (Figure 4.3) is an indication of moderately high affinity of the enzyme for phospholipids which further substantiates the observed toxicities in Viperidae snakes as a result of PLA_2. Furthermore, the V_{max} obtained implies that at the end of 1min. post E. ocellatus envenomation, at least 4.5µmoles of free fatty acids would have been excised from the victim's red blood cells (RBCs) resulting in hemolysis of RBCs observed after envenomation (Rosenberg, 1979). It could also lead to liberation of free fatty acids from other membrane phospholipids making the internal environment highly acidic with the lysophospholipids generated exhibiting a detergent-like action that leads to degeneration of muscle fibres (myotoxicity) and/or inhibiting the release of neurotransmitters (neurotoxicity), both of which are major forms of Viperidae PLA_2 toxicity (Caratsch, et al., 1985, Sallau, et al., 2008).

Venomous snakes are resistant to their own venom and certain molecules like endogenous antitoxic serum proteins with neutralising capacity against snake venom have been previously reported (Perales, et al., 1995 and Soares, et al., 2005). In particular, PLA_2 inhibitors have been isolated from various snake sera and their primary structures have been determined (Nobuhisa et al., 1997). In the present study, a protein PLA_2 Inhibitor from E. ocellatus Serum (PIES) was identified in a carpet viper snake. PIES was purified from blood serum of E. ocellatus snake in a two step purification process on sephadex G-200, (Figure 4.4) and ion-exchange chromatography on DEAE-cellulose (Figure 4.5). An active peak eluted at 80mM NaCl inhibited the partially purified PLA_2 from the snake by 77.8%. The inhibitory effect of PIES may be attributed to its ability to chelate Ca^{2+}, an important co-factor of PLA_2 (Nok et al., 2002). The formation of enzyme-inhibitor or toxin-inhibitor complexes has been previously shown to be responsible for the inhibition of the PLA_2 enzyme from T. flavoviridis (Kogaki, et al., 1989) and for neutralization of the major toxins from Naja naja atra venom (Shao, et al., 1993). Ovadia et al., (1977) also reported that an anti-neurotoxic factor isolated from the serum of Vipera

palaestinae forms a complex with an acidic component of the venom which participates synergistically in the neurotoxic action of this venom.

The purity of partially purified PIES was checked by SDS-PAGE as shown in Plate X. The inhibitor appeared as a single sharp band with an estimated molecular mass of about 25kDa. This is similar to the molecular weight of PLA_2 inhibitor isolated from the serum of Crotalus d. terrificus snake reported by Perales et al., (1995). Fortes-Dias et al., (1991) also reported the isolation of a 23.6-kDa antivenin factor from Crotalus d. terrifcus plasma. However, proteins with higher molecular weight possessing antivenin activity have also been previously reported, for example, PLA_2 inhibitors isolated from the plasma of T**.** flavoviridis (Kogaki, et al., 1989**)** and Agkistrodon b. siniticus (Ohkura, et al., 1993) were found to b 100kDa and 75kDa proteins, respectively.

Kinetic study of this research reveals that PIES exerts a mixed pattern (non competitive and uncompetitive) of inhibition on Echis PLA_2 (Figure 4.8). This is suggestive that a site other than the active site could be involved in the inhibition since incubation of the Echis PLA_2 with PIES in ice for several hours did not reverse the inhibition. The result of this study is consistent with the findings of Ibrahim et al., 2012 who reported that the leaves extract of Ceiba pentandra exerts a mixed pattern of inhibition on Echis PLA_2. This may also likely suggest that the PIES-PLA_2 interaction involved strong covalent forces affecting the structure (Nok et al., 1993). The K_i values (3.8933-7.2893%) from this study are indicative of a relatively high affinity of the inhibitor for the enzyme. (Figure 4.8 and 4.9).

The partially purified E. ocellatus PLA_2 also catalysed the extensive hemolysis of RBCs (Table 4.2). About 80% hemolysis was achieved after 2 hours post incubation. However, in the presence of 20% (v/v) PIES, there was a significant decrease ($p<0.001$) in the hemolysis of RBCs to about 35.3 ± 0.017%. It can be construed that the hemolytic activity may be linked to hydrolysis of membrane lecithin since the hydrolysis was followed as a function of released free fatty acids (Nok et al., 2002). More so, the hydrolysis of lecithin generates free fatty acid and lysolecithin, while the former leads to acidosis, the later is a powerful hemolyzing agent with detergent-like effects (Nok et al., 1993). In a related finding, Meenatchisundaram et al., (2009) demonstrated that Andrographis paniculata and Aristolochia indica extracts were capable of inhibiting PLA_2 dependent hemolysis of Ovine RBCs induced by Echis venom in a dose dependent manner. PLA_2 causes a complete degradation of glycerol phospholipids in the cell membrane as they attack lecithin-converting 2/3 of the phospholipids into lysoderivative (Nok et al., 1993). The destruction of cell membrane integrity may in turn lead to phospholipase activation and release of arachidonate followed by the biosynthesis and release of eicosanoids to surrounding tissue, which, by themselves cause considerable membrane damage (Condrea et al., 1980; Kannagi et al., 1981; Mukherjee et al., 1998). In this context, an inhibition of PLA_2 enzyme plays a significant role in preventing membrane damage. The suppression of hemolysis by PIES suggests that PIES- PLA_2 interaction may protect the cell from hemolytic anaemia.

The anticoagulant action of venoms due to Viperidae, Crotalidae and Elapidae bites is mainly due to PLA_2 enzymes, though, there are quite a number of non PLA_2 anticoagulant from the same source. (Mukherjee, 2007; Pereanez et al., 2009; Garcia et al., 2010). Zingali et al., (1990) reported that crude venom from Bothrops jararaca snakes has anticoagulant and PLA_2 activities. Table 4.3 shows that E. ocellatus venom is capable of preventing coagulation of citrated bovine plasma. Treatment with partially purified PIES reduced the recalcification time from over 30 minutes to less than 60seconds. Our results conforms with the findings of Abubakar et al., (2006), who reported that both Indigofera pulchra nad Aristolochia albida were found to neutralise the anticoagulant, hemolytic and PLA_2 activities of crude venom from viperidae snakes. Similarly, Khunsap et al., (2011) also reported that PLA_2 purified from Daboia russeli siamensis venom significantly ($p<0.05$) shortened the rate of coagulation activity of human citrated plasma and this may be due to the binding of clotting factor Xa (FXa) to inhibit prothrombinase complex, which is the target protein for anticoagulant PLA_2s as previously reported (Yang, 1994 and Kerns et al., 1999).

From our results, treatment of Bovine, Ovine and Caprine sera with partially purified PIES did not affect the PLA_2 in the serum samples tested (Table 4.4); but significantly ($p<0.001$) decreased PLA_2 activity in partially purified Echis PLA_2 (Table 4.5). Our findings are similar to those of Grazyna, et al., 2000, who reported that a PLA_2 inhibitor isolated from Crotalus durissus terrificus snake serum; Crotoxin Inhibitor from Crotalus Serum (CICS) inhibits the PLA_2 activity and neutralizes the pharmacological action of crotoxin, the main neurotoxin from Crotalus durissus terrificus venom. It also inhibits and neutralizes other PLA_2 β-neurotoxins from Viperidae snake venom, but does not act on PLA_2 β-neurotoxins from Elapidae venom or the toxic or nontoxic PLA_2s from other sources. In a related study as reported by Abubakar et al., 2000, extract of Guiera senegalense and Sterculia setigera gave 33.4% and 44.3% protection to mice treated with a minimal lethal dose of Viperidae venom but offered no protection to mice treated with minimal lethal dose of Elapidae venom. Thus, our findings suggest that the carpet viper contain in its blood a PLA_2 inhibitor that inhibits the snake venom PLA_2 but does not act on the non-toxic secretory PLA_2 from mammals, indicating its specificity.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

From this study, it is concluded that E. ocellatus Serum contains Phospholipase A_2 (PLA_2) Inhibitor with biochemical characters similar to other Viperidea PLA_2 Inhibitors reported.

It is also envisaged that PLA_2 Inhibitor from E. ocellatus Serum (PIES) might prove to be useful as an adjunct to antivenin treatment or, more importantly, might prove useful in the treatment of the various diseases in which venom PLA_2 enzymes have been implicated like erythrocyte hemolysis.

The chelation of ca^{2+} by PIES or the formation of PLA_2-PIES complexes may be responsible for the inhibition of PLA_2 activity and for the protective effect of PIES against RBCs hemolysis.

Phospholipase A_2 Inhibitor from Echis Serum (PIES) might be used as an alternative to horse antisera in the antivenin treatment of human envenomations by Echis ocellatus. Because of its specificity, PIES might be therefore useful in the treatment of envenomations by the Viperidae snakes. In this context, it is important to note that PIES has no effect on the mammalian secretory PLA_2s, indicating that it should not interfere with the normal biological function of these enzymes.

6.2 RECOMMENDATIONS

Sequencing and structural elucidation of PIES can be carried out. Also further studies can be carried out with PIES to ascertain its effects on the lethal dose of E. ocellatus venom in vivo. Also the biotechnology and vaccine potential of PIES can be explored to provide therapeutic molecular models with anti-ophidian activity to supplement the conventional serum therapy against the multifunctional venom PLA_2 enzymes.

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APPENDIX 1.0

CALCULATION FORMULA FOR PURIFICATION PROFILE

$$\text{Total Protein} = \frac{\text{Total Activity}}{\text{Specific Activity}}$$

Total Activity = Specific Activity * Total Protein

$$\text{Specific Activity} = \frac{\text{Enzyme Activity (Total Activity)}}{\text{Total Protein}}$$

$$\text{Purification Fold} = \frac{\text{Specific Activity of Protein at a Purification Step}}{\text{Original Specific Activity (Crude)}}$$

$$\text{Percentage Yield} = \frac{\text{Total Activity of protein at a step}}{\text{Total Activity of Original Purification (Crude)}} \times 100$$

$$\text{Enzyme Activity } (\mu\text{mol/min}) = \frac{0.02 \times (\text{volume of NaOH in ml}) \times 0.001 \times 10^6}{\text{Incubation time}}$$

$$\text{Relative Inhibition (\%)} = 1 - \frac{\text{Residual Activity}}{\text{Enzyme Activity}}$$

APPENDIX 2.0

PREPARATION OF STANDARD OF REAGENTS

2.1 Preparation of SDS-PAGE Reagents

- **1.5 M Tris pH 8.8** exactly 181.65 grams of tris salt was weighed and dissolved in one litre of deionized water

- **0.5 M Tris pH 6.8** was prepared by weighing 60.5 grams of tris and dissolved in one litre of deionized water

- **10% SDS** was prepared by dissolving 10 grams of SDS in 100ml of deionized water

- **30% Bis-Acrylamide Solution** exactly 10 grams of Acrylamide and 2 grams of Bis were weighed and dissolved in 100ml of deionized water

- **Running buffer working solution** was prepared by weighing 1 gram SDS (1% SDS), 14.4 grams glycine (0.192 M glycine), and 3.03 grams tris (0.025M Tris pH 8.3) dissolved in one liter of deionized water

- **Loading buffer (×5)** this constitute the following; 2.5ml of 1 M Tris pH 6.8, 4ml of glycerol, 0.8 gram of SDS, 2ml of β-mercaptoethanol, Bromophenolblue and distilled water to final 10ml

- **Separating gel (12%)** was prepared by mixing the following: 3.35ml of deionized water, 2.5ml of 1.5 M tris pH 8.8, 0.1ml of 10% SDS, 4ml of 30% Bis-acylamide solution, 50µl of 10% Ammoniumpersulphate (APS) (0.1g/ml; made fresh) and 15µl of TEMED to a total volume of 10ml

- **Staking gel (4%)** this was prepared by mixing the following in a test tube; 3ml of deionized water, 1.25ml of 0.5 M Tris pH 6.8, 50µl of 10% SDS, 665µl of 30% Acrylamide, 25µl of 10% APS and 10µl of TEMED to a total volume of 5.0ml

2.2 Preparation of Phosphate Buffered Saline

The 10mM phosphate buffer (pH 8.0) used for this work was prepared dissolving 1.09 grams of Disodium hydrogen phosphate (Na_2HPO_4), 0.32 grams of Sodium Di-hydrogen Phosphate (NaH_2PO_4) and 9 grams of Sodium Chloride (NaCl) in one liter of distilled water.

2.3 Preparation of Glycerol Gradient

Each gradient system was prepared to a total volume of 10ml thus:

- 10% glycerol gradient (10/100*10) = 1ml glycerol + 9ml phosphate buffered saline
- 20% glycerol gradient (20/100*10) = 2ml glycerol + 8ml phosphate buffered saline
- 30% glycerol gradient (30/100*10) = 3ml glycerol + 7ml phosphate buffered saline
- 40% glycerol gradient (40/100*10) = 4ml glycerol + 6ml phosphate buffered saline
- 50% glycerol gradient (50/100*10) = 5ml glycerol + 5ml phosphate buffered saline