ISOLATION AND CHARACTERIZATION OF ANTI *ECHIS OCELLATUS* VENOM PRINCIPLES OF *CEIBA PENTANDRA*

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DECLARATION

I declare that the work in the dissertation entitled "Isolation and characterization of anti E*chis ocellatus* venom principles of *Ceiba pentandra "* has been perform by me in the department of biochemistry under the supervision of Dr S. Ibrahim,Prof J.A.Nok and Dr M.S. Abubakar.

The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this project dissertation was previously presented for another degree or diploma at any University.

Sarkiyayi Shehu Signature---------- Date----------

CERTIFICATION

This dissertation entitled "ISOLATION AND CHARACTERIZATION OF ANTI *ECHIS OCELLATUS* VENOM PRINCIPLES OF *CEIBA PENTANDRA* " by Sarkiyayi Shehu meets the regulations governing the award of the Degree of Doctor of Philosophy of Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

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ABSTRACT

The leaves extract of *Ceiba pentandra* plant was tested for anti venom activity against *Echis ocellatus* snake venom. Among parameters investigated include: LD_{50} of the *Echis ocellatus* snake venom, phospholipase A_2 activity, percentage hemolysis, plasma recalcification time, Invivo analysis of total protein content, white blood cells, packed cell volume and haemoglobin contents. The toxicological studies of *Ceiba pentandra* was also investigated. The result reveals that *Echis ocellatus* has an LD_{50} of 0.280mg/kg $\pm$ 0.06. Haemolysis due to venom was drastically reduced by the extract from 66% to 27.4% suggesting that the plant has Protective activity against the lethal action of the snake venom and is effective in reducing haemolysis in mice and prolonging the recalcification time . Phospholipase A_2 was purified from the venom of *Echis ocellatus* with a yield of 77% on Sephadex A-25 column and appeared as band in polyacrylamide gel electrophoresis (SDS-PAGE) with molecular weight of 15KDa. Phospholipase A_2 activity was drastically reduced in a dose dependent manner with Km of 0.8M, revealing that the purified phospholipase incubated with the extract had some anti venom effects against the phospholipase A_2 activity. The invivo studies reveals that there were significant ($P<0.05$) decrease in packed cell volume, total protein and haemoglobin contents for the venom group and there was only slight changes in the venom/extract and control groups, suggesting that the extract has some inhibitory effect on the venom activity. The IR analysis reveals that the bioactive compound in *Ceiba pentandra* leaves is an organic ester with carbonyl functional group. The GC/MS result showed that the organic ester compound (Di –n- octyl phthalate) has some inhibitory effects on PLA_2. The result has shown that *Ceiba pentandra* leaves extract possess potent snake venom neutralizing capacity. The plant leaves extract could be used as an antidote for snake bite envenomation.The toxicity profile of aqueous methanol extract of *Ceiba pentandra* leaves reveals that the LD_{50} was found to be over 5000 mg/kg. For chronic toxicity test, the mice were injected with graded doses (250 - 500 mg/kg) of the leaves extract daily for 21 days and the effects on body weight, haematological and serum biochemical parameters were determined. The results revealed that there was no significant change in body weight, meaning that the extract is relatively safe for oral medical use. In fact there were no variable changes in the body weight of the mice from day zero (D_0) to day twenty one (D_{21}) in all the groups. The parameters determined during chronic toxicity test reveals that the concentrations of urea, chloride ion sodium ion ,potassium ions and bicarbonate were 11.2, 83.2, 131.6, 8.44 and 38 mMol/L respectively for the test animals .The result showed that apart from urea, all other parameters were within physiological acceptable range. The results further indicated that ,there was a decrease in Creatinine (88 uMol/L) and Total Protein (57.4 g/L), while AST (19 IU/L), ALT (10 IU/L) and ALP (204 IU/L) concentrations has significantly increased .While there was no significant ($p<0.05$) change in PCV and bilirubin concentrations The finding reveals that *C. pentandra* has very low toxicity profile in all the tested animals and

the plant is relatively safe for herbal oral medication. The inhibitor (Di –n- octyl phthalate) isolated during this research might become a highly effective therapeutic agent for the reducing of snake bite envenomation. The relevance of these findings would be of importance in the area of development of anti snake bite drugs that are going effective, cheap, accessible and less allergic plant compound that can be scientifically explored.

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LIST OF ABBREVIATIONS

ALT	Alanine transaminase
ALP	Alkaline Phosphatase
AST	Aspartate transaminase
DEAE	Diethyl Amino Ethyl
GC/MS	Gas Chromatography/Mass Spectrometry
IR	Infrared Spectroscopy
PCV	Packed Cell Volume (PCV)
TP	Total Protein
PL A_2	Phospholipase A_2
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis
SEM	Standard Error of Mean
SGOT	Serum Glutamate Oxaloacetate Transaminase
SGPT	Serum Glutamate Pyruvate Transaminase
TLC	Thin Layer Chromatographic

CHAPTER ONE

1.1 Introduction

Snakes have always been subjects of special interest to mankind. This is primarily due to their mode of limbless progression. Snakes are feared to such an extent that the very mention of the word "snake", is sufficient to excite loud exclamations of disapproval and horror, snakes are most feared by those who have never had an encounter. Despite all this, there must be a large number of people who would like to learn something more about snakes.

Snakes are among the most widely distributed animals and for the past thirty years or so there has been an explosion of information on the chemistry, biochemistry and pharmacology of snake venom. This is mostly due to the usefulness of their toxins as tools and probes of many biological processes. A large and still increasing number of toxins have been isolated. In recent years there has been renewed interest in snake venoms principally to elucidate the mechanism of toxicity of these venoms.

It has been reported that globally over one million humans are bitten annually by numerous snakes resulting in 70,000 deaths (Paramonte, 2007). Further more, Nasidi (2007) reported that *Echis ocellatus*, the west African carpet viper, is the most common, it accounts, for 90% of bites and 60% of the fatalities in Nigeria. Paramonte (2007) reported that Taraba state had the highest number of cases (900) while Jigawa had the highest frequency (20/1000) of patients admitted due to venomous snake bite and cases fatality rate (31%). The studies conducted by

Paramonte (2007) at Kaltungo Hospital Gombe state revealed that snake bites rates increases over time and 75% of the majorities of the patients admitted were male.

Echis Ocellatus snake belongs to the Viperidae family and is one of the most poisonous snakes found in Nigeria (Nasidi,2007). Their envenomations are characterized by conspicuous local tissues damage and in severe cases by systemic bleeding, haemorrhagic and cardiovascular shock (Onuaguluchi, 1960). The venoms of viperidae snakes are rich sources of proteins with activity against various factors involved in coagulation and fibrinolysis (Faure, et al., 2000). Haemorrhagic activity is mostly due to the enzymatic action of metalloprotease on capillary basement membrane component. (Anima, 1967).

Phospholipase A_2 is a highly pathological enzyme found in the venoms of several species of snake worldwide. It is known to be toxic to cell membrane, causing local cell and tissue damage as well as systemic effects in snakebite victims (Lizano, *et. al.,* 2001). Phospholipase A_2 is a common enzyme found in snake venoms. Its central pathological role in the venom has heightened interest in search for potential inhibitors of the enzyme for drug development (Nok, *et.al.*, 2001). Besides, enzymatic activity, venoms induce various pharmacological and insulin stimulating effects (Gao, *et.al.,* 2001). Some components of snake venom have great potential for medical use because of the specific actions of such components. Nowhere is whole venom used as a medicine. However, specific components are extracted and

used (Bucherl, *et.al.,*1968). Snake venom components are also used in basic research in physiology, biochemistry and immunology. Venom components allow researchers to examine the operation of the process and to develop drugs to correct malfunctions due to diseases. For instance, venoms are currently being investigated for their potential use as antiviral and antibacterial agents (Petkovic, *et. al.,* 1991). Actions of different snake venom are broad and the understanding of their multiple poisoning processes is desirable in the formulation of a satisfactory antidote.

It is a fact that rural communities patronize traditional herbal plant products in the treatment of snakebite and may even prefer this to orthodox antivenom therapy. This perhaps may be due to ignorance, cost or accessibility to antivenom therapies. It is in the light of this that, it has become necessary to study the herbs that are used locally in snake bite treatment for possible drug development. Pereira, *et. al.,* (1994) reported that extracts of *Phylanthus klotzschianus*, *Casearia sylvestris* and *Apoleia leiocarpa* conferred 100% protection up to 48 hours after administration by oral treatment of mice against the lethal dose of *Bothrops jararaca* venom.

1.2. Economic uses of *Ceiba pentandra*

The plant *Ceiba pentandra:* Is an emergent tree of the tropical rainforests, and is often describes as majestic. It can grow to a height of 150 feet or more, towering over other trees in the rainforest. Originally a native to South America, it now has spread to the primary rainforests of West Africa, and the Indonesian archipelago (Catherine and WoodWard, 2006). In many places the straight trunks of the *Ceiba*

pentandra tree are used to make dugout canoes. The white, fluffy seed covering it is used in pillows and mattresses. Since it is buoyant and water resistant it is often used in flotation devices and padding. The seeds, leaves, bark and resin have been used to treat dysentery, fever, asthma, and kidney disease. In Mayan myths the ceiba pentendra tree was sacred. They believed the souls of the dead would climb up into the branches which reached into heaven (Catherine and Woodward, 2006)

The *Ceiba pentandra* plant is widely spread around the world and occupies an important niche in the ecosystem of a rainforest. Emergent trees like the *ceiba pentandra* rise above the canopy of the rainforest and provide a home for plants dependent on sunlight. Their branches provide a habitat for countless epiphytes, which provide food and shelter for many types of animals. They allow animals to move around the rainforest with Mexico through Central America and south to peru, Balivia and Brazil, as well as in West Africa. All other members of the genus only occur in the neotropics; *Ceiba trichistandra* is found on drier forests of the pacific Coast of Geudor and Peru. *Ceiba partandra* has been transported to other regions of the tropics by humans and is even planted in warm temperature zones as a novelty tree (Mc Dade *et. al.,* 1994). In some parts of Nigeria, Cameroon and Ghana, the plant seed is used as a source of oil for cooking. The cotton is used widely in manufacturing textiles and other materials for human use (Burkill, 1966).

1.3 Scope of Study

This study was designed to investigate the antivenom properties of C*eiba pentandra* in the prevention of *Echis Ocellatus snakebite* envenomation with a view of exploring its biological activity and evaluate possible toxicity of the plant.

1.4 Statement of Research Questions

1. How do constituents of C*eiba pentandra* modulate the activities of *Echis ocellatus* snake venom?

2. Under what circumstances does the plant extract become effective in preventing snake bite envenomation?

3. What are the chemical constituents in C*eiba pentandra* that possess anti venom acivity?

1.5 Significance and Justification of the Study

The empirical indigeneous knowledge on medicinal plants plays a vital role in the primary health care and has potential of the discovery of new herbal drugs, new sources of pharmaceuticals, contraception and for sustainable utilization of medicinal plants genetic resources and their conservation. Over the years, medicinal plants have been used in the treatment of snakebites, particularly in rural areas. However, the mode of action and the active components of these plants are almost unknown (Nok, *et. al.,* 2001). Furthermore, it has been found out that the traditional medicine practitioners use a particular herb from a tree called *Ceiba pentandra* linn for the treatment of cattle snakebite victims. As search for new drugs continue, there

is also a need to assess locally available herbs as source for antivenom drugs. It is in view of the importance of herbs currently used in the envenomation therapy by the traditional practitioners, that the current study was undertaken to investigate the role of C*eiba pentandra* in the prevention of *Echis ocellatus* snakebite envenomation and to evaluate possible toxicity of the plant extract and to identify the bioactive components of the plant extract for the purpose of drug development.

It would be of paramount importance to validate the scientific basis for the medicinal uses of *Ceiba pentandra* and assess whether the plant has some toxicological effects on mice. During the course of investigation of the toxicity profile, two major parameters were assessed. Vide the acute toxicity test and chronic toxicity test. This study was therefore undertaken in order to assess the toxicity profile of plant *Ceiba pentandra* that will provide information on the safety or otherwise of the use of *Ceiba pentandra* in medicine and its continual ethno medicinal applications.

1.6 Statement of the Null Hypothesis

It is hereby hypothesized that the C*eiba pentandra* leaves extract has no anti *Echis ocellatus* venom properties.

1.7 Objective of the Study

1.7.1 Statement of Objectives

The aim of the present research is:

To evaluate the anti venom activity of extract of C*eiba pentandra* on the venom of *Echis ocellatus* with the view of identifying the active constituent of the extract.

1.7.2 Specific Objectives of the Project

1. To evaluate the anti venom property of crude extract of *Ceiba pentandra* on the venom of *Echis ocellatus*.
2. To evaluate the biological and enzymatic activity of phospholipase A_2 enzyme of *Echis ocellatus* venom.
3. To carry out interactive studies between the extracts, crude venom and purified phospholipase A_2 enzyme of *Echis ocellatus*.
4. To conduct toxicological studies of the extract in a suitable mammalian host.
5. To identify the functional group of the phytochemical and illucidate the possible mechanism of action of inhibitory effects of phospholipase A_2.

CHAPTER TWO

2.0 Literature review

It has been reported that globally that over one million humans are bitten annually by numerous snakes resulting in 70,000 deaths (Paramonte, 2007). Therefore scientific effort must be directed towards the prevention of this tragedy. Furthermore, Nasidi (2007) reported that *Echis ocellatus*, the West African carpet viper is the most common, it accounts for 90% bites and 60% of the fatalities in Nigeria. Paramonte (2007) reported that in Nigeria Taraba state had the highest number of cases (900) while Jigawa had the highest frequency of patients admitted due to venomous snake bite and cases fatality rate (31%). In the same studies it was shown that at Kaltungo Hospital Gombe the majority of the patients admitted due to snake bite were males (75%).

2.1 Classification and Occurrence of Snakes

Snakes are one of the most widely distributed of all animals, and as they do not have mandibles they swallow their prey whole (Bucherl, *et. al.,* 1968). Snakes are feared to such an extent that, the very mention of the world "snake" is sufficient to excite loud exclamations of disapproval and horror, which reach climax amongst those who have never encountered a snake and are hardly likely to do so. Despite all this, there must be a large number of people who would like to learn something more about snake especially in the area of poison's mode of action in the body and the

possible mechanism of action of phytochemicals in snake bite evenomation treatment.

The teeth of poisonous snake, usually called fang, is curved in shape and attached to venom gland. Both the fang and skin are constantly shed and replaced. Snakes are believed to have poor eyesight, so snake is responsive to ground vibration. The life span ranges from 25 to 40 years (Bucherl , *et. al.,* 1968).

It is estimated that in West Africa there are over eighty species of snakes of which ten are most poisonous (Akubue, 1997). Examples of most poisonous snakes are as follows:

1. *Echis ocellatus* formally known as *Echis carinatus*
2. *Bitis arietans* (puffadder)
3. *Bitis gabonica* (Gaboon viper)
4. *Naja melanoleuca* (black cobra)
5. *Naja nigricollis* (spitting cobra)
6. *Dendroaspis viridus* (green mamba)
7. *Dendroaspis Jamesoni*
8. *Dendroaspis angusticeps*
9. *Causus maculatun* (night adder)

Snakes are cold blooded ectodermic animals, reptiles, and vertebrates, order squamata, which also include lizards, crocodiles, tortoises and turtles (Smith, *et. al.,*

1977). There are at least 4000 snake species worldwide most of which are non-venomous (Swaroop and Grab 1954). Some snakes have specialized glands that produce venom, mostly derived from salivary glands. Snakes can be divided into families and are grouped accordingly to the presence or absence of fangs (Smith, *et. al.,* 1977, Kodiva, 1987).

Families of snakes

1) Typhlopidae – small primitive snakes, specialized for burrowing. The snakes are uniform without any differentiation into dorsals, ventral and subcaudals. One genus and four species.
2) Anilidae – primitive burrowing snakes with scale differentiation poorly developed, the ventral being slightly enlarged. Single genus species.
3) Xenopeltidae – A family containing single known specie, *Xanopeltis unicolor*, a burrowing snake with the scales well differentiated.
4) Boidae – the pythons; large and primitive constricting snakes, scales well differentiated, one genus and twenty eight species.
5) Colubridae – A very large family containing by far the greater number of the known species of snakes with specialization for burrowing, climbing and swimming. The family is divided into a number of sub-families.There are thity four genera which are found to comprised eigty five species.

6) Elapidae – They form large terrestrial poisonous snakes. They have hollow poison fangs. The Elapidae includes some of the most dangerous poisonous snakes. There are four genera and nine species.

7) Hydrophidae – The sea snakes, a well defined family who's most obvious character is the flattened, fin like tail. They are front-tayed like the Elapidae and some are estremely venomous. Most of them are purely marine and incapable of progression on land. They have eight genera with seventeen species.

8) Viperidae – In the vipers the poison fangs reach their greatest development. Their fangs are very long and maxillary bones to which they are attached is short and movably attached to the skull and bear no other teeth.They have two genera with seven species.

2.2 Common African Snakes

Three main families of snakes of medical importance are found in Africa,Viperidae (Vipers and Adders), Elapidae (Cobras and Mambas) and Colubridae (Boomslang) . The vipers are relatively shorter, thicker snakes with long hinged front fangs. The main example in Nigeria is the saw-scaled or carpet viper (*Echis* spp) of which there are two main species in West Africa – *Echis ocellatus* and *Echis leucogaster*. Its world distribution stretches from Senegal to Bengal, a range perhaps second only to that of the European Viper, *Vipera berus (*Abdulrazaq, 2010*).* The snake is mainly nocturnal entering human dwellings only rarely unlike the spitting cobra. *Echis*

ocellatus may be confused with at least four other snakes in West Africa – *Dasypeltis scabra*, *Telescopus variegatus*, *Bitis arietans* (juvenile specimens) and *Causus maculatus* – the former two lack fangs while the latter two are also venomous.

2.3 Common African Poisonous Snakes

Africa has some of the most dangerous and venomous snakes in the world. The Black Mamba (*Dendroaspis polylepis*) is one of Africa's most dangerous snakes and feared in East, Central and Southern Africa. It is aggressive when cornered and will not hesitate to strike (Anouk, 2010). If a Black Mamba encounters prey it can strike up to 12 times, each time delivering enough neuro and cardio-toxic venom to kill a dozen men within 1 hour. Without anti-venom, the mortality rate is 100%. The Puff Adder (*Bitis arietans*) is considered to be Africa's deadliest snake because it is responsible for the most human fatalities. The *Puff Adder* has large fangs and its venom is powerful enough to kill a grown man with a single bite. Puff Adders rely on camouflage for protection and lie still if approached. Because of this, people tend to step on them and get bitten. Many fatalities occur because bites are not treated correctly, leading to infection and gangrene (Anouk, 2010.)

The Boomslang (*Dispholidus typus*) is an extraordinarily dangerous snake found in sub-Saharan Africa. Human fatalities are rare, since this snake is very timid, but spectacular. It's venom is *haemotoxic*, which means that it affects the body's natural blood clotting mechanism resulting in the bleeding of the internal organs.

Sometimes it can take as long as 24 hours before the symptoms of the venom can be felt or seen. Once it gets to work however, a person can bleed to death from every orifice. The Boomslang is a tree-dwelling snake (Boomslang means "tree snake" in Afrikaans). Females are brown, and males are light green with black highlights. The Boomslang reaches an average length of 5 feet. Its fangs are at the back of its head.

The Gaboon Viper (*Bitis gabonica*) has the longest fangs and the highest venom yield of any venomous snake in the world. Gaboon Vipers can be found in West, Central and parts of East Africa, they prefer forested areas. Adults reach an average length of around 5 feet. While the Gaboon Viper delivers a huge dose of venom, the venom is not as toxic as some of the other snakes on this list. A single bite could kill a man.

The Egyptian Cobra *(Naja haje*) is a deadly venomous snake most commonly found in Egypt but also in other parts of North Africa (Anouk, 2010). The Egyptian Cobra has glands located behind its eyes which produce deadly neurotoxic venom that's released through its large fangs. The venom of an Egyptian Cobra is so deadly it can kill a full grown Indian Elephant in three hours. In humans its venom causes paralysis and death due to respiratory failure.

The Saw-Scaled or Carpet Vipers are small, bad tempered, highly venomous snakes found north of the equator in Africa (Anouk, 2010). Their venom is hemotoxic and

very virulent. This means the venom causes spontaneous internal bleeding, sometimes days after the bite has occurred.

The Cape Cobra (*Naja nivea*) has powerful venom and is one of the deadliest snakes in Southern Africa. Cape Cobras grow to an average of around 4 feet. Cape Cobras are particularly dangerous because they tend to be nervous and aggressive. Without treatment, the mortality rate in humans is 60% and death normally occurs 2-5 hours after being bitten, usually as a result of respiratory failure due to the onset of paralysis. The Eastern Green Mamba (*Dendroaspis angusticeps*) is a venomous snake related to the highly dangerous Black Mamba.

The Green Mamba is less aggressive and smaller than the Black Mamba, they average around 6 feet. The Green Mamba's venom is also significantly less toxic, however a single bite could certainly be fatal to a human (Anouk, 2010).

2.4 Biochemistry of Snake Venoms

Abubakar (2006) reported that the biological action of snake venoms can be broadly divided into hemotoxic and neurotoxic action. Venom is referred to as hemotoxic when it is primarily affects the blood, destroy tissues and produces very painful symptoms. Neurotoxic venoms attack the nervous system and brain, these may cause almost no pain but shut down the respiratory systems and interfere with heart

functions. Huddson and Hudalson (1995) reported that they destroy capillary walls, causing hemorrhages near and distant from the bite sites, both local as well as systemic heamotological changes are observed in haemotoxin envenomation.

2.4.1 Composition of Venoms of Common Poisonous Snake in Africa

Snake venom is a complex mixture of organic and inorganic substances and one of the most complex toxic chemicals known. It has been revealed that most snake venoms contain about 60-80% water, 20-40% as residue and 80-90% of the residue is proteins which are mostly enzymes and polypeptides (Anima, 1967).

The protein components of snake venoms can be classified into three groups

i) Proteins with toxic properties

ii) Proteins with enzyme properties

iii) Proteins with unknown biological activities.

Some physical properties of the venom are given by Devi and sarker (1956), in table 2.1.

Table 2.1: Physical properties of dried snake venoms.

Properties	*Elapidae Naja Naja*	*Viperidae Russells Viper*	*Erotalidae Crotaius terrificus*
Solubility in distilled water	99.5%	79.5%	80%
Solubility in physiological Saline	99.5%	93%	95%
pH of the solution in distilled water	6.6 - 7.0	5.8-6.2	5.5
Relative viscosity (fresh)	1.5 - 2.5	1.5-2.0	2.0

Sourse: Devi and Sarker (1956)

Defination of LD_{50}

LD_{50} is the dosage of venom that kills 50% of the animals within 24 hours after injection. This dosage LD_{50} is measured in milligrams of venom per kilogram of body weight. For instance, the LD_{50} for puff adder was found' to be 0.l4mg/kg. The mouse-test estimates the strength of venom by injecting measured amounts into a large sample of mice and recording the dosage of venom that kills 50% of the mice within 24 hours. This dosage called the LD_{50} (LD standing for lethal dose) is measured in milligrams of venom per kilogram of mouse. The LD_{50} of some snakes are given in table 2.2.

T**able 2.2: Snakes and the LD_{50} of their venoms**

Snake	Amount venom injected per bite	LD_{50}
Russells viper	130-250mg	0.03mg/kg
Black mamba	50-100mg	0.05mg/kg
Puff adder	100-300mg	0.14mg/kg
Echis carinatus	5-48mg	0.24mg/kg
King cobra	350-500mg	0.90mg/kg

Source: Zug, *et. al.,* (1999).

2.4.2 Composition of Venoms of Viperidae and Elapids

Composition of Venoms of Viperidae and Elapids are given in table 2.3

Table 2.3: Venomous snakes and their toxins

Snake venom	**Compound present**	**Effect of the venom on normal body function**
Bothrops, crotalus	Peptide bradykinin potentiators	Enhances diffusion of venom into blood stream increasing bleeding.
Naja naja, Vipera Crotalus, Scutulates bungarus, laticauda	Polypeptide toxins	Causes heart or respiratory failure.
Several genera	Proteases	Catalyses the reactions that break muco polysaccharide links in connective tissues.
Vipers pitvipers	Proteases	Catalyse the reaction that disrupts protein peptide bonds in tissues damage blood vessels and resulting to hemorrhage
Elapidae Cobra		disrupt normal transmission of nerve impulses

Table 2.3cont'd

Agkistrodon both rops Crotalus naja, vipera	Phospholipase	Catalysis reaction that harm musculature and nerves.
vipers ,elapids but (vane)	Thrombin like enzymes	Inhibit blood clotting.
Agkistrodon crotalus	Nerve growth factor enzyme	Stimulates the growth of nerve cells.
Some vipers	Glycoproteins	Suppress normal immune response of tissue.
Trimersurus Agkistrodon crotalus	Biogenic amines	Disrupt normal transmission of nerve impulse.
Contain in several genera	Acetylcholine enzyme	Cause heart and respiratory failure.

Source: Bucherl, *et. al.,* 1968.

2.4.3 Composition of *Echis Species* Venom

Echis ocellatus is a saw-scaled viper found commonly in Nigeria and India. It attains a maximum size of 45cm. The viper is olive brown dorsally and has white pale brown wavy lines blends, with an irregular brown sport in the vertebral region. The head in addition to being triangle shaped, has a pale brown arrowhead mark plate, this is the most characteristic feature of this snake. Pale yellow brown marks are also found near the nasal and eye regions. The dorsal spots number from 25-31 and have a faint white border. The ventral scales have small black spots on their sides. The viper locomotes sideways, rubbing the scales causing a hissing type of noise. It can raise its head to an average height of about 25cm and attack while moving. There are two other snakes *Disposal triagnoate* and *Psammophis species* that resembles the viper but they do not locomote in this fashion nor are they poisonous. *Echis ocellatus* has venom output per snake between 5mg and 48mg and has $LD_{50\ of}$ 0.24mg/kg. The reaction to a bite is intense swelling and burning pain. There is continuous oozing from the puncture marks. There is bleeding from the gum, kidney or nose. Necrosis occurs at the site of the bite. If sufficient antivenom is not given, the secondary symptoms persist for days and the patient succumbs to them after sometime.



Plate 2.1: *Echis ocellatus*

2.4 Enzymes and Proteins of Snake Venoms

Snake venoms are composed of various collections of polypeptides. These polypeptides are toxins that are either enzymes or non-enzymatic polypeptides. The effects or actions of these toxins are,on the most part, either by a means of degradation of cells, tissues and/or intercellular bonds or by a competitive inhibition blocking transmission of acetylcholine. Exceptions to this is found in all species belonging to the genus Dendroapsis, snakes known as *mambas*. Other venom components have been identified including carbohydrates, lipids, nucleoside and some metals such magnesium, calcium, and zinc are the most prevalent metals, copper has been detected in some venoms. There are studies that suggest that some of these co-constituents may play an active role in the lethality of venoms (Moore, 2003).

Most snake venoms have enzymes that act as toxic constituents, and they are composed of several enzymes and polypeptides . More than 20 enzymes have been detected in snake venoms, and 12 are found in all venoms. The majority of toxic effects of viperid and crotalid (pit vipers, including rattle snakes moccasins, etc.) envenomations are due to enzymes collectively termed “proteases”. These are actually hydrolases that primarily act to breakdown proteins by hydrolysis and thus are proteolytic and serve a digestive role (Moore, 2003). They are also the cause of most of the local damage

following envenomation. . Based on the structure, activity and components, crude venoms are also classified into cardiotoxin, neurotoxin, cytotoxin and myotoxin (O'Shea ,2006). Viper venoms are also hemolytic and haemorrhagic **(**Paulchamy,2010).

2.5.1 Proteases and Esterases from Snake Venom

Venom of Crotalidae exhibit higher proteolytic activity than the venoms of the true vipers. Venom of elapids does not show any proteolytic activity (Bucherl *et. al.,* .1968). Venom that exhibits protease activity also exerts esterase activity. It has been reported that injection of the purified protease intramuscularly into guinea pigs cause significant myolysis with hemorrhage (Bucherl, *et. al.,* 1968). The proteolytic or esterase activity of venom is not inhibited by ovomucoid or by soybean trypsin inhibitor, although they can effectively prevent the action of trypsin on denatured hemoglobin. Venoms of *Bothrops jararaca, Bothrops cotiara* and *Bothrops terrificus* all contain at least one proteolytic enzyme very similar but not identical to trypsin. It is known that venoms of *pit vipers* enhance the clotting of blood. The venoms of some snakes coagulate blood by virtue of their ability to convert either prothrombin to thrombin as tissue thromboplastin does or fibrinogen to fibrin as thrombin does.Therefore, the presence of proteases in venoms of various snakes indicate that they might be involved in aggravating the cause of death in snake bitten animals. It has been clearly demonstrated that the venoms of *bothrops* and *Russell*

viper can indirectly cause death by blocking circulation through the formation of clots and necrosis. In general, most snake venoms contain proteases which catalyse the reaction in the body that break structural components of tissues and cause blood vessel wall damage resulting in hemorrhaging and muscle fibre deterioration (Bucherl *et. al.,*1968).

2.5.2 Phospholipases from Snake Venom

Phospholipases are group of enzymes found in most snake venoms, especially phospholipase A_2. Phospholipase A_2 is inactive at pH 9 and splits off fatty acids residue of phosphoglycans. Their toxicity is associated with their ability to hydrolyse lipid. This may have direct action on lipid membrane and also splits phospholipids in blood cell.

Snake venoms constitute a rich source of phospholipase A_2 (Tu, 1978). Though many of the phospholipases are not toxic, there is growing list of phospholipase A_2 which exerts toxin effect such as neurotoxicity, myotoxicity and anticoagulability. Phospholipase A_2 is thought to be toxic to cell membrane; they can cause local cell and tissue damage as well as systemic effects in snakebite victims (Nuchprayoon, *et. al.,* 2001). So far more than 150 variant phospholpase A_2 have been characterized (Valentin, 2001).Three dimensional structure of the class II anticoagulant phospholipase A_2 toxin from the venom of *Russell's viper* and the class II neurotoxin phospholipase A_2 notechus 11-5 from Australian tiger snake venom were

determined. Both enzymes were found to be monomeric and consisting of 121 and 119 residues of amino acids respectively (Carredatio, *et. al.,* 1998). Endogenous protein isolated from serum of snakes has been found to be natural inhibitors displaying antivenom activity. Some of these proteins inhibit PLA_2 activity. Faure (2000) purified and characteristised a natural PLA_2 which may be used to develop more effective therapeutic strategies for dealing with snake envenomation. Most venomous animals including snakes, bees and scorpions contain a variety of venom PLA_2 which participate in both digestion of pray and venom toxicity. Valentine and Lambeau (2001) reported that they all have a conserved fold with several disulphide bridges and can be catalytically active or not and several of them can display a tremendous array of toxic effects including neurotoxicity and myotoxicity.

Nearly all venoms hitherto tested for their ability to hydrolyse phospholipids particularly phosphotidylethanolamine and phosphotidylcholine, show invariably the presence of a very active enzyme capable of hydrolyzing L-α lecithin or cephaline at the beta or 2 position α-acyl-L-α-gIycerophosphorylcholine and lysolecithin and free fatty acids are formed.

High phospholipase activity is observed in venoms of S. *catenatus* and A. *piscivorus.* Thus lecithin in presence of these venoms is converted to lysolecithin which is the true hemolytic agent. Likewise cephalin can be converted to Iysolecithin and they can dissolve washed red blood cells. Lysolecithin produced by

the action of phospholipase A_2 on lecithin might attack capillary endothelium and thereby cause hemorrhage, edema and necrosis. Since phospholipase catalyses the production of lysolecithin which dissolves erythrocytes, it can also strongly inhibits respiration as well as oxidative phosphorylation through its action on mitochondrial membranes. Seliste-de-strajo *et. al.,* (2000) reported that snake venoms are rich sources of proteins that strongly affect the vascular system by promoting blood coagulation, haemorrhage and fibrinolysis. Haemorrhagic activity is mostly due to the enzymatic action of metalloprotease on capillary basement membrane component (Sarkar and Anima, 1964).

Phospholipase A_2 EC 3:1:1.4 – is an upstream regulator of many inflammatory processes. This particular phospholipase specifically recognizes the Sn-2-acyl bond of phospholipids and catalytically hydrolyses the bond releasing arachidonic acid and lysophospholipids. Upon downstream modification by cyclo oxygenases, arachidonic acid is modified into active compounds called eicosanoids. Eicosanoids includes prostaglandin and leukotrienes which are catagonised as inflammatory mediators (Dennis ,1994).

Phospholipase A_2 is commonly found in mammalian tissues as well as insects and snake venom (Nicholas and Jonathan, 2007). Localization of structural elements of Bee venom, PLA_2 involved in N-type receptor binding and neurotoxicity. Venom from both snakes and insects is largely composed of melittin which is a stimulated

of PLA_2. Due to the increased presence activity of PLA_2 resulting from a snake or insect bite, arachidonic acid is released from phospholipids membrane disproportionately. As a result, inflammation and pain occur at the site of the bite (Argiolas , 2008).

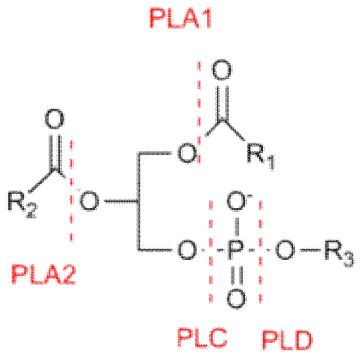


Plate 2.2: Phospholipase A_2 , A_1, C, and D cleavage sites

In normal brain cells , PLA_2 regulation accounts for a balance between arachidonic acid conversion into pro-inflammatory mediators and arachidonic acid re-incorporation into the membrane. In the absence of strict regulatory mediators,free fatty acids are produced which induced oxidative stress and neuroinflammatoion analogous to neurological diseases such as Alzheimer's diseases, epilepsy, multiple sclerosis, Ischemia and neuropsychiatric disorders (Dennis, 1994).

2.5.3 Amino Acid Oxidases from Snake Venom

L-Amino-acid oxidase is another enzyme found in all snake venoms. It causes the release of the powerful lytic chemical hydrogen peroxide. Its toxic effect is by oxidation of cells. ZeIIer (1944) found the presence of this enzyme in venoms of many different species of viperid. The enzyme is found in the liver, lungs kidney and erythrocytes of snakes. It had been reported that L-amino acid oxidase can act as anaerobic dehydrogenase and transfer hydrogen to ferricyanide in anaerobic condition in the absence of added carrier. Snake venoms have been widely used for the preparation of highly purified α-keto acids from corresponding amino acid analogues. An attempt to relate the toxicity of the venom with its L-amino acid oxidase activity did not succeed, but it had been reported that amino acid oxidases in snake venoms can activate proteases and peptidases (Sarkar and Anima, 1964). L-amino acid oxidases are widely found in snake venoms and are thought to contribute to the toxicity upon envenomation. The mechanism of these toxic effects and whether they result from the enzymatic activity are still uncertain although many papers describing the biological and pharmacological effects of L-amino acid oxidases have appeared recently, which provide more information about their action on platelets, induction of apoptosis, haemorrhagic effects, and cytotoxicity (Du and Clemetson, 2002). Since there is no catalase in the venom, the hydrogen peroxide produced decarboxylate the α keto acid oxidatively. A number of neutral amino acids and their analogues can be converted to their corresponding keto acids by the snake venom (Sarkar and Anima, 1964).

2.5.4 Nucleotidases from Snake Venoms

This enzyme is found in some snake venoms. All venoms tested were capable of hydrolyzing diphenylphosphate, but only a few of them would hydrolyse monophosphate. Some of these venoms were also capable of hydrolyzing nucleoside-5 monophosphate. The $5^{/}$ nucleotidase activity in venoms is higher than what has been reported for animal tissues, bull sperm, microorganisms. Venom $5^{/}$ nucleotidase remove other phosphate from adenosine 5' rnonophaphate (AMP) liberating adenosine as one of the reaction products. The enzyme has optimum pH of 8.5 to 8.6, and requires Mg^{++} and Zn^{++} for its activity. The $5^{/}$ Nucleotidase is most active among all the phosphatase such as ATPase, DPN-pyro phosphatase and alkaline phosphatases. There are other enzymes present in some snakes involved in the conversion of prothrombin to thrombin and fibrinogen to fibrin clot.

2.5.5 Phosphodiesterases from Snake Venom

Snake venom also contains phosphodiesterase which is capable of hydrolyzing highly polymerized DNA. The phosphodiesterase hydrolyses stepwise, the nucleotide chains of nucleic acids from the end of the chain carrying 3' hydroxyl end group. *Cobra* venom and other venom of the *elapidae* family have been more widely used than any other venom for the study of the enzyme that hydrolyses ATP (Sarkar and Anima, 1964).

2.5.6 Nucleases from Snake Venoms

Deoxyribonuclease and ribonuclease (nuclease) are also present in snake venoms. The nuclease has the ability to hydrolyse nucleic acids (DNA and RNA). Nucleases in snake venom hydrolyse phosphodiester bond, in DNA and RNA. DNAase in venom has an optimum pH of 5.0 and does not require Mg^{++} for its activity. The venom DNAase of *B. Jararaca* is more stable to heat than the venom RNAase, since at 70^0C the latter loses all its activity, while only 20% of the DNAase activity is destroyed at this temperature (Sarkar and Anima, 1964).

2.6 Biochemical Basis of Snake Envenomation

The toxicity of venom may be due to the action of one or more of the component present. These components that are responsible for the toxicity may be neurotoxins, cardiotoxin, heamorrhagic, phospholipases, and direct Iytic factor. Moore (2003) reported that snake venom enzymes are proteolytic and hemorrhagic toxins. These cause hemorrhage by targeting fibrinogen converting it by digestion into an unclottable substance or by directly causing lysis of blood cells. Blood platelets and Prothrombin are also sometimes affected leading to coagulopathy of sorts and even heamorrhage.

2.6.1 Neurotoxins Activity of Snake Venom

Elapidae venoms are generally neurotoxic. The neurotoxic action is due to neuromuscular blocker and it produces paralysis and death within few hours. Some

of the early signs of neurotoxicity include inability to speak or swallow and convulsion. Moore (2003) reported that the lethality of some snake venoms is not attributed to enzymes, but rather to other polypeptides that are devoid of enzymatic activity. There are also polypeptide neurotoxins termed curare-mimetic toxins or postsynaptically acting neurotoxins that blocks acetylcholine to receptors at the neuromuscular junctions at the heart side of the synapse. However, the greatest effects of these toxins are generally exerted on respiratory muscles. They interfere with the reception of acetycholine on nicotinic receptors at the neuro muscular junctions.

It has been found that high concentration of erabutoxin (100-l50nM) from the venom of *Laticauda semifasciata* did not cause much fade in the rat diaphragm preparation. However, low concentration of toxin (5nM) caused severe fade which is similar to the effects of d-tubocurarine. The data suggest that fade may be due to toxin binding a high affinity site on the post-synaptic acetylcholine receptor (Bradley, *et. al.,* 1990).

2.6.2 Cardiotoxin Action of Snake Venoms

Cardiotoxin acts by depolarizing the cell membrane and it affects all types of muscle especially cardiac muscles leading to circulatory failure. Some of the signs of toxicity are not seen because the prevalent effect is due to neurotoxin, which causes death very quickly. The cardiotoxins are mostly alkaline with pH of about 12 and rich in cystine bonds. It has been revealed that phospholipase A_2 activity enhances

snake venom cardiotoxin - induced and general anesthetic (halothane) - induced hemolysis of red blood cells (Fletcher, *et. al.,* 1991). The phospholipid binding activity of cardiotoxin from *Naja naja atra* was found to exhibit pH dependence in binding to phosphotidyl choline membrane with an apparent Pka of 6.0 (Chiang, *et. al.,* 1996). Recently it was reported that cardiotoxin analogue III isolated from the venom of the Taiwan cobra, is a small molecular weight all beta sheet protein cross linked by four disulphide bridges (Chang, *et. al.,* 1998).

2.6.3 Haemotoxic Action of Snake Venoms

Haematological effects of snake venom was found to be due to the fact that lysolecithin which is developed from plasma lecithin by the action of the venom phospholipase A_2 become attached to the red blood cells and platelets are trapped in the clots that form secondary to haemorrhage. (Gitter & De Vries 1968).

2.6.3.1 Haemolytic Action of Snake Venoms

Haemolysis of red blood cells due to snake venoms is classified into direct haemolysis and indirect haemolysis. The directly lytic venoms, such as those of elapidae, Lyse washed red blood cells while indirectly lytic venoms such as those of the viperidae are able to lyse red blood cells membrane phospholipids (Gitter & De Vries ,1968). Jeng *et. al,.*(1997) reported that the haemolytic actions of snake venoms are mostly due to phospholipase enzymes which catelyses the hydrolysis of

erythrocyte membrane phospholipids leading to the release of free fatty acids and lysolecithin and causes extensive hemolysis and membrane solubilisation.

2.6.3.2 Haemorrhagic Action of Snake Venoms

These are a group of unrelated different chemical substances that are vasculotoxic, causing damage to blood vessels. Their primary target is blood vessels that supply the lungs. They are believed to destroy intercellular cement substances leading to disturbance of the clotting system. It is interesting to note that snake venom protein flavoridin; a polypeptide of 70 amino acid residues is a potent inhibitor of blood platelet aggregation (Senn and Klaus, 1993).

It has been found that albolabrin, a naturally occurring peptide from snake inhibits platelets aggregation by blocking the binding of fibrinogen to the glycoprotein, GPI 16-1 la on the surface of activated platelets (Jaseja, *et. al.,* 1993). A coagulant, okinaxobin 1 which was purified from *Trime rusrus okinavenis* snake venom released specifically fibrinopeptide from fibrinogen to form fibrin clots (Nose, *et. al.,* 1994). Local and systemic haemorrhages are common consequence of *erotanline* and *viperine* envenomation. Several studies carried out using purified toxins have indicated that local haemorrhage can be attributed to a distinct class of venom metalloprotease (Kamiguti, *et. al.,* 1996). Seliste-de-strajo , *et. al.,* (2000) reported that snake venom are rich sources of proteins that strongly affect the vascular system by promoting blood coagulation, haemorrhage and fibrinolysis. Haemorrhagic

activity is mostly due to the enzymatic action of metalloprotease on capillary basement membrane component.

2.7 Anti-Snake Venom Development

The snake venom proteins are of biological interest because of their diverse and selective pharmaological and physiological effects through their interaction with various molecular targets. These proteins are prototypes for: (i) therapeutic agents (ii) pharmacological probes for the diagnosis of several diseases and (iii) research tools to decipher physiological and pathological processes (de Lima, 2005). Captopril, an antihypertensive drug, is a classic example that was designed based on the peptide inhibitor of angiotensin-converting enzyme from *Bothrops jararaca* venom (Ferreira , *et. al.*,1970) . Peptide neurotoxins from snake venoms are used for the identification and characterization of membrane ion channels and receptors in vertebrate cells, including human neurons (Sher, *et. al.,* 2000). Snake venom metalloproteinases and peptide neurotoxins can be used to study myoblast fusion. Fertilization and matrix metalloproteinase cell interactions can be used for characterizing human cancers and small-cell carcinoma (de Lima , *et. al,* 2005). All of these studies demonstrate the significance of snake venom proteins as valuable tools for basic research, disease diagnosis and drug development. However, most snake venoms remain poorly characterized despite being a rich source of biologically active proteins with therapeutic potential. Hence, further studies are essential to identify and characterize novel venom proteins which can be used as

leads or structural templates for developing new therapeutic agents (Paulchamy ,2010.)

2.7.1 Conventional Anti-Snake Venom Development

Venom contains a protein that has the potential for new drug discovery and to advance understanding of disease mechanisms, according to researchers. Manjunatha and Nirthanan (2010) has discovered a protein called haditoxin. He said that haditoxin was structurally unique and therefore expected to have unique pharmacological properties. This toxin is like a conjoined twin. It is a relatively large complex made up of two identical protein molecules known as three-finger toxins linked together. Generally, toxins display diverse biological actions on the human nervous system, cardiovascular system and blood clotting. Some have directly led to the development of compounds with potent analgesic and blood pressure reducing properties ,so it is likely that haditoxin in its 'conjoined twin' state or as individual components will offer us more novel insights.

Manjunatha and Nirthanan (2010) revealed that many common drugs such as the widely prescribed blood pressure medication Captopril and anti-clotting drug Eptifibatide have been developed from snake and other animal venoms. Researchers have been studying *King cobra* venom for over 50 years and yet we are still identifying new compounds. It is a complex cocktail of biological molecules that can change composition depending on the environment, the season or even the

snake's diet. Primarily, the venom acts on neurotransmitter receptors which regulate communication between nerve cells or between nerves and muscles, resulting in symptoms such as paralysis and respiratory failure. We may be able to improve management of snakebite as we better understand the mechanism of action of these venoms. Because of the high specificity of these toxins, haditoxin may also be useful as a 'molecular probe' which will help us study neurotransmitter receptors and their role in diseases.

Antivenom is a serum that is commercially produced to neutralize the effects of envenomation by poisonous snakes or animals Antivenom is the only definitive treatment for effective bites by venom snakes. The spent antivenom and the neutralized venom are then excreted from the body. Some monovalent and polyvalent antivenom and species treated are given in table 2.4

Table 2.4: Antivenom and species treated

Antivenom	Species covered
Australian and New Guinea Polyvalent	Can be used for bites from any Australian or New Guinean elapid but monovalent venoms are more effective
Brown snake monovalent	All *Pseudonaja* (brown snakes) species
Black snake monovalent	*Pseudechis australis* (Mulga) and likely P. butleri (Butler's snake/Spotted Mulga)
Death adder monovalent	All *Acanthophis* (Death adder) species and possibly for *Echiopsis curta* (Bardick snake) bites
Sea snake monovalent	All members of the sea snake families Hydrophiidae and Laticaudidae
Taipan monovalent	All *Oxyuranus* (Taipan) species
Tiger snake monovalent	All species of *Austrelaps, Hoplocephalus* (banded/broad-headed/pale-headed snakes), *Notechis (*Tiger snakes), *Tropidechis carinatus* (Rough-scaled snakes) as well as all *Pseudechis* (Black snake) species.

2.7.2 Development of Alternative Anti-Snake Venoms

Since prehistoric times, man has always suffered from snake bite, and he has always been able to identify this disease by the objective relation of cause and effect. There is still a high incidence of snakebite in most areas of the world. Snake bite is a bite inflicted by a snake. Snakes often bite their prey when feeding, but occasionally they bite humans, people can avoid and treat snake bite by knowing their etiology, along with prevention tips and first aid and hospital treatment. Attention in recent years is focused on tropical medicine, for the treatment of snake bite. According to Rosenfeld, *et. al.,* (1965), about 30,000 to 40000 deaths caused by, the bites of venomous snakes occur annually not including the USSR, China and central Europe. Joann (2008) reported that some of these plants were used by Native Americans for the purpose of snake bite remedies.

2.8 Purification of Venom Component

2.8.1 Biochemical Basis for Purification of Venom Constituent

The separation of molecules from biological materials is an important part of biochemical work and often involves the isolation of one molecular species from a mixture of compounds with similar properties. Generally, extreme pH, temperature organic solvent, and the use of oxidizing and reducing agents have to be avoided when dealing with molecules separated from living matter otherwise there is a risk that any biological activity will be lost through denaturation. The common techniques use in separation of biochemical compounds from their mixture include

dialysis, gel filtration chromatography and electrophoresis (Renee and Joan ,1993). Since snake venoms are made up of protein and enzymes, these techniques are usefull in purification of venom constituents.

2.8.2 Methods of Purification of Venom Components

Gel Chromatography

Gel chromatography (or molecular-sieve chromatography, as it is sometimes called) is a special type of partition chromatography in which separation is based on molecular size. The basis of gel chromatography is quite simple. A column is prepared of tiny particles of an inert substance that contains small pores. If a solution containing molecules of various dimensions is passed through the column, molecules larger than the pores move in the space between the particles and hence are not retarded by the column material. However, molecules smaller than the pores diffuse in and out of particles with a probability that increases with decreasing molecular size.

Materials in Gel Chromatography

A gel is a three-dimensional network whose structure is usually random. The gels used as molecular sieves consist of cross-linked polymers that are generally inert, do not bind or react with the material being analyzed, and are unchanged. The space within the gel is filled with liquid and this liquid occupies most of the gel volume.The gels currently in use are of three types: dextran, agarose and polyacrylamide. Gel chromatography is basically separation procedure and has its

wide spread use in purifying enzymes and other proteins and in fractionation of snake venom and nucleic acids, assay of enzymes and isolation of organic plant natural product compounds.

Ion Exchange Chromatography

Ion exchange chromatograph is a separation method relying on charged molecules. The procedure is dependent on the formation of electronic linkages between the resin and the substance being separated. Both cationic and anionic resins are available, they consist of a polystyrene or cellulose metrix with an attached functional group, and they require activation by treatment with a buffer of suitable pH. The compounds being separated likewise must be charged by reacting specific groups at some designated pH before the mixture is put onto the column.

The technique is particularly useful for separating compounds of similar structure such as proteins and snake venom components. This can be continues gradient elution or stepwise elution. To separate a given protein effectively, it is necessary to take into account the size of the sample. Proteins have numerous functional groups that can have both positive and negative charges. Ion exchange chromatography separates proteins according to their net charge, which is dependent on the composition of the mobile phase. By manipulating the pH various protein molecules can be separated. For example, if a protein has a net positive charge at pH 7, then it will bind to a column of negatively-charged beads, whereas a negatively

charged protein would not. By changing the pH so that the net charge on the protein is negative, it too will be eluted.

Ion-exchange chromatography retains analyte molecules on the column based on ionic interactions. The stationary phase surface displays ionic functional groups (R-X) that interact with analyte ions of opposite charge. This type of chromatography is further subdivided into cation exchange chromatography and anion exchange chromatography. The ionic compound consisting of the cationic species M+ and the anionic species B- can be retained by the stationary phase. Cation exchange chromatography retains positively charged cations because the stationary phase displays a negatively charged functional group. Anion exchange chromatography retains anions using positively charged functional group:

Electrophoresis

Electrophoresis is a method use for separation of proteins, it is dependent on the charge carried by the molecules in solution to which a current is applied.For instance acrylamide gel electrophoresis is based on the movement of charged solutes in an applied electrical field. The movement of the charged particle is influenced not only by the charge but also by voltage, distance between the electrode, size and shape of the molecules, temperature and time (Maurer ,1971). This technique is very useful in determination or separation of biological molecules such as proteins and enzymes in snake venom according to their sizes such as molecular weight.

Thin Layer Chromatography

Thin layer chromatography (TLC) is a separation technique in which a uniform thin layer of an adsorbent material is coated onto a support such as a glass plate or a plastic sheet. The solution of compounds to be separated is spotted 2.0 to 2.5cm from the bottom of the plate but above the level of the solvent. The solvent moves through the adsorbent by capillary action. Many classes of compounds can be separated using this technique but dependent on proper selection of adsorbent and the running solvent system. The separation is generally dependent on polarity of the sample.

2.8.3 Biological and Medical Use of Some Venom Components

Snake venom components have several biological and medical uses . For instance venoms are used in the treatement of cardiovascular diseases, it is also use as analgesics and anticoagulants in hospitals.

2.9 Antidotes of Snake Envenomation

Envenomation is a systematic poisoning that occurs when a snake bites someone. This depends on a number of factors such as health status of individuals, immobility, types of snake and site of the bite. Envenomation by snakes of the family Viperidae usually causes local tissue damage, including edema, hemorrhageand myonecrosis, drastic effects that are only partially neutralized by conventional serotherapy. In severe cases, such effects may lead to permanent tissue loss, disability or amputation

(Gutierrez, 2002). Many plants are traditionally use for treatments of Snake Bites .Abubakar (2006) reported that some plants in Northern Nigeria are effective in treatement of poisonous snake bites as given in table 2.5. In a related development, Andreimar, *et. al.,* (2005) revealed that some plant species have anti venom properties and are usefull in the treatment of snake as shown in table 2.6

2.9.1 Medical Emergence of Snake Bites It is a well known fact that most envenomation occurs in rural zones where the level of the development is low and many fatal cases that occur could have been avoided, if people have access to adequate medical care. Whenever there is snakebite there are some general immediate measures to be taken before reaching the hospital. First and foremost, if possible the snake should be killed and taken to the hospital for identification. Before treatment, suction should be applied with mouth immediately at the site of the bite. Immobility of the patient has been advised in order to avoid the activation of circulation, which would favour venom dissemination. (Ameno, *et. al.,* 1990).Absorption of proteolytic and coagulant venoms is slow, due to the local reaction produced which keeps the venom at the site of the bite and the longer it remains there the greater the risk of necrosis. Antivenoms are viable to neutralize specifically the venoms. Rosenfeld, *et. al.,* (1965) observed that different snake venoms have their antivenoms. In the events that snake victim did not recognize the snake that bites him, only polyvalent instead of monovalent antivenom is administered. In general, the factors to be considered for successful antivenom

therapy are specificity of antivenom, early administration, sufficient dose and proper injection route.

Table 2.5: Plants used in poisonous snake bites in northern Nigeria

Plant	Plant part used	Method of preparation	Indication for bites vipers	Locations used in Northern Nigeria
Indigofera pulchra	Aerial part	Decoction is prepared from dried aerial part	Such Echis	Zaria, Malumfashi Kaltungo.
Aristolochia albida	Rhizomes	Decoction and maceration	For bites due to elapids, and vipers.	Katsina and Malumfashi area.
Guiera Senegalense	Leaves	Dried powdered leaves placed on incisions at bite site to retard absorption of the venom	For all snake bites	Throughout the North especially used by Fulani herdsmen.
Sterculia setigera	Bark	The dried powdered back is taken with milk.	It is used in prophylaxis of snake bites.	Through out the North especially used by Fulani herdsmen.

Source: Abubakar 2006.

Table 2.6: Bioactivity of Anti-snake venom plants

PLANT FAMILY	PLANT SPECIES	SNAKE SPECIES	ANTIOPHIDIAN ACTIVITY	PLANT SOURCE(S)
Malvaceae	*Althaea officinalis*		Anti-PLA_2	Leaves
Menispermaceae	*Abuta sp.*	Brazilian snakes	Anti-PLA_2, ethnobotany research	Stems
Euphorbiaceae	*Acalypha indica*	*V. russeli*	Ethnobotany research, anti-lethality, anti-hemorrhagic, anti-necrotizing, anti-cardiotoxic, anti-neurotoxic	Leaves
Araceae	*Alocasia sp.*	*N. naja, N. hanna, Bungarus multicintus, B. atrox*	Ethnobotany research, anti-hemorrhagic.	Rhizomes
Solanaceae	*Anisodus*		Treat snakebite	Compound

Table2.6cont'd

	Tanguticus			
Annonaceae	*Annona purpurea*	*B. asper*	Anti-hemorrhagic	
Fabaceae	*Apuleia leiocarpa*	*B. jararaca*	Anti-lethality	
Apocynaceae	*Asclepias curassavica*	*B. asper*	Anti-hemorrhagic	Leaves
Arecaceae	*Astrocaryum*		Anti-PLA_2	Fruits
Asteraceae	*Baccharis sp.*	*B. alternatus, B. moojeni, B. jararacussu, B. neuwiedi, B. pirajai*	Anti-hemorrhagic, anti-proteolytic, anti-PLA_2	Leaves
Arecaceae	*Bactris gasipaes*		Anti-PLA_2	Fruits
Asteraceae	*Bidens pilosa*	D. jamesoni, E. oceliatus	Ethnobotany research, anti-lethality	

Table 2.6cont'd Bixaceae	*Bixa orellana*	B. atrox, B. asper, L. muta, C. d. terrificus, M. mipartitus	Anti-lethality, anti-hemolytic, anti-edema, anti-clothing, defibrinating	Leaves
Fabaceae	*Bowdichia major*		Anti-PLA_2	Seeds
Polygalaceae	*Breddemeyera floribunda*	B. jararaca	Anti-lethality, ethnobotany research	Roots
Asteraceae	*Calendula officinalis*	B. alternates	Ethnobotany research, anti-edema, anti-hemorrhagic, anti-necrosis	Flowers
Salicaceae	*Casearia marriquitensis*	B. neuwiedi	Anti-coagulation	Leaves
Salicaceae	*Casearia sylvestris*	Brazilian snakes, B. asper	Ethnobotany, anti-hemorrhagic	Leaves

Table 2.6cont'd	*Cassia hirsute*		Anti-PLA_2	Leaves and
Fabaceae				Roots
Urticaceae	*Cecropia palmate*	Brazilian snakes	Ethnobotany research, anti-PLA_2	Leaves, latex
Meliaceae	*Cedrela tonduzii*	B. asper	Anti-hemorrhagic	Leaves, stems
Vitaceae	*Cissus sicyoides*		Anti-PLA_2	Creeper
Verbenaceae	*Citharexylum macrodenium*	B. asper	Anti-hemorrhagic	Leaves
Rutaceae	*Citrus limon*	b. atrox, B. asper, L. muta	Anti-lethality, anti-edema, anti-clothing, defibrinating	Ripe fruits
Asteraceae	*Clibadium Silvestre*	B. atrox	Ethnobotany research, anti-hemorrhagic	Whole plant
Euphorbiaceae	*Croton draco*	B. asper	Anti-hemorrhagic	Stems, compounds
Zingiberaceae	*Curcuma sp.*	C. d. terrificus, B. jararaca, B. **Table**	Anti-lethality, anti-hemorrhagic, anti-proteolytic, anti-neurotoxic, ant-	Roots, Rhizomes, compound

		2.6cont'd alternatus, N. naja diamensis	edema, anti-necrosis	
Asteraceae	*Cynara scolymus*	B. jararaca	Anti-lethality	
Ebenaceae	*Diospyros kaki*	T. flavoviridis, L. semifasciata	Ethnobotany research, anti-detoxification, anti-lethality	Fruits, seeds
Araceae	*Dracontium croatii*	B. atrox, B. asper	Anti-lethality, anti-edema, anti-clothing, defibrinating	Rhizomes
Asteraceae	*Eclipta prostate*	B. jararaca, B. jararacussu, C. d. terrificus, C. viridis viridis, A. contortrix	Anti-lethality, anti-myotoxicity, anti-hemorrhagic, ethnobotany research	Aerial parts, compounds
		Table		

		2.6cont'd		
		laticinctus, C. rhodostoma		
Boraginaceae	*Ethretia buxifolia*	E. carinatus	Ethnobotany research, anti-lethality	Roots
Euphorbiaceae	*Emblica officinalis*	V. russellii, N. kaouthia	Anti-Lethality, anti-hemorrhagic, anti-coagulant, defribrinogenerating, fibrinolytic, anti-edema, immunological interaction	Roots
Apiaceae	*Eryngium creticum*	Cerastes cerastes	Anti-hemolytic	Leaves, roots

Source:Andreimar , *et al.*, (2005)

2.9.2 Plants and Tradional Treatments of Snake bite

It is a fact that rural communities patronize traditional herbal plant products in the treatment of snake bite and may even prefer this to orthodox antivenom therapy. This perhaps may be due to either ignorance cost or accessibility to artivenom therapies Pereira, *et .al*., (1994) reported that extracts of *Phylanthus klotzschianus*, *Casearia sylvestris* and *Apoleia leiocarpa* conferred 100% protection up to 48 hours after administration by oral treatment of mice against the lethal dose of *Bothrops jararaca* venom. Melo, *et. al.* (2003) evaluated the antiophidian activity of two Brazilian plants known as "arnica". Extracts in dichloro methane and ethanol from *Lychnophora pinaster* leaves, as well as from *Solidago chilensis* aerial parts were not able to counteract the edema induced by *Bothrops alternatus* venom, thus showing that not all popularly utilized plants are indeed scientifically validated. Aqueous extracts from *Schumannophyton magnificum* skins and *Mucuna pruriens* var. *utilis*, *Strophanthus gratus* and *Strophanthus hispidus* leaves prolonged the clotting time of blood treated with *E. carinatus* venom (Houghton, *et. al* 1994). Aqueous extracts from rhizomes of *Curcuma* sp prevented the neuromuscular blockage caused by *Naja naja siamensis* neurotoxins. However, when the extract and venom were administered independently, there was no prolongation of the surviving time, showing that inactivation occurred *in vitro* (Cherdchu , *et. al.,* 1978). The aqueous extract of *Clinacanthus nutans* leaves, a herb reputed in Thailand and Malaysia to be "snake bite antidote", has been found to have no effect on the inhibition of neuromuscular transmission produced by purified *Naja naja siamensis*

neurotoxin. It was concluded that the extract of *C. nutans* cannot neutralise the action of cobra venom (Cherdchu, *et. al.,* 1978). Guerranti, *et al,* (2002) demonstrated that certain proteins from seed extract of *Mucuna pruriens* protected mice against the toxic effects of *Echis carinatus* venom. Protection was explained by an immunological mechanism based on a series of shared epitopes common to venom proteins. When *M. pruriens* proteins are injected into mice in such a way as to induce abundant antibody production, a polyclonal serum against epitopes present on one or more *Echis carinatus* venom proteins is obtained. These findings open new perspectives in the field of vaccination by natural products and may be useful in the therapy of snakebite and other coagulation disorders Guerranti, *et. al.,* (2002).

2.9.3 Plant Constituents with Anti-Venom Activity.

Plant compounds have guided the development of an impressive number of novel synthetic drugs. The structures of some vegetal antiophidian inhibitors already isolated and characterized are listed in table 2.7, together with their neutralizing activities.

Table 2.7 Chemical structures of inhibitors of snake bite

plant family	plant species	isolated compounds
Aristolochiaceae	*Aristolochia* sp.	Aristolochic acid (AA)
Asteraceae	*Baccharis trimera*	Clerodane diterpenoid
Leguminosae	*Brongniartia podalyrioides*	Edunol
Papilionaceae	*Derris urucu*	2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine
Combretaceae	*Guiera senegalensis*	Tannins
Apocynaceae	*Hemidesmus indicus*	2-hydroxy-4-methoxy benzoic acid
Asteraceae	*Mikania glomerata*	Coumarin
Sapindaceae	*Sapindus saponaria*	Flavonoids

Source: Andreimar, *et .al.*, (2005)

It is a known fact that Fulani rural communities use leaf extract of *Ceiba pentandra* linn for treating cattle snakebite victims. It is in view of this that this plant *Ceiba pentandra* linn became the central research material for the purpose validation and identification of the active ingredient responsible for curing the snakebite victims.

2.9.4 The Use of *Ceiba pentandra* in Treatment of Diseases and Snake Bites

The *Ceiba pentandra* plant belongs to the family of bombacaceae kapok tree. The plant is indigenous to Nigeria and it is popularly known as "Rimi" in Hausa language, "Igi Araba" in Yoruba language and "Akpu" in Igbo language. The plant is also found in Ghana and in most part of the tropical areas of the world. The plant is a gigantic tree and can grow up to 60m tall in tropical areas of Africa.

The picture of the plant *Ceiba pentandra* linn is given in plate 2.4.



Plate 2.3: The picture of the plant *Ceiba pentandra* linn

Ceiba pentandras' bark part is used for chest pains and as diuretic in Nicaragua (Coe and Anderson, 1996). It was reported that the dried bark is used in lndia for treatment of diabetes (Jam and Sharma, 1967). Coe and Anderson (1996) revealed that human adult use the dried bark orally for the treatment of respiratory and pulmonary disorders. In a related development the dried bark is used orally for treatment of diarrhea, fever, gonorrhea and parasitic infections (Noreen, *et. al.,* 1998). It has been reported that in Senegal it is used for abcesses, mouth sores and tooth ache as well as stomach ache (Le-Grand, 1989). It has been demonstrated that leave extract of *Ceiba pentandra* was used in Indonesia for treatment of fever (Grosvenor, *et. al.,* 1995). *Ceiba pentandra* (part not specified) is used in Cameroon for the treatment of hypertension (Kandem, *et. al.,* 1986). Research findings in Nigeria reveal, that the extract of *Ceiba pentandra* has anti-fungal activity (Sawhney, *et. al.,* 1978). In Congo, the dried bark of *Ceiba* has been shown to have antiameobic activity and antibacterial activity (Tona, *et. al.,* 1999). The biological activities of the extract of *Ceiba pentandra* have shown that it affects central nervous system generally in mouse. (Cox, *et. al*., 1989). In Nigeria it was also reported that fresh bark of the plant has antibacterial; activity (Odama, *et. al.,* 1997). Some biologically active compounds found in *Ceiba pentandra* are given in table 2.8.

Table 2.8. Some biologically active compounds found in *Ceiba pentandra.*

Plant part	Where discovered	Compound	Reference
Seed oil 7-hydroxyl Cadalen	Philippines	Sesquiterpene	Burkill (1966)
Root bark catechin	India	Flavonoid	Rao, et al., (1993)
Bark daurosterol	Samoa	Steroid	Noreen, et al., (1998)
Stem bark lionleicadid	Cameroon	Linid	Ngounou, et al., (2000).
Seed oil pentadrin	Philippines	Iso flavones	Bur kil (1966)
Stem bark protein	Cameroon	Proteid	Ngounou, et al., (2000).
Root bark naphthoquinone	India	Sesquiterpene	Rao, et al., (1993)
Entire plant	Costa rica	Tannins	Cespedes, et al., (1992)

2.9.5 Biological and Biochemical Basis for the Use of *Ceiba pentandra* in Snake Bites

It has been found out that the traditional medicine practitioners use *Ceiba pentandra* linn for the treatment of cattle snakebite victims. As search for new drugs continue, there is also a need to assess locally available herbs as source for antivenom drugs. It is in view of the importance of herbs currently used in the envenomation therapy by the traditional practitioners, that the current study was undertaken to investigate the role of C*eiba pentandra* in the prevention of *Echis Ocellatus snakebite* envenomation, to evaluate possible toxicity of the plant extract and to identify the bioactive components of the plant extract for the purpose of drug development.

CHAPTER THREE

3.0 Materials and Methods

3.1 Materials

3.1.1 Reagents and solvents

All reagents and solvents used were of analytical grade and supplied by Sigma chemicals Co. St. Louis or BDH chemical Company, England.

3.1.2 Equipment

Micro haematocrit centrifuge (Hawksley, England)

Colorimeter (Sher wood 257.U K)

pH meter (Chestsey Kent, England)

UV spectrophotometer (Jenway 6405 uv/vis U K)

Electrophoresis EPS6 (Amersham Pharmacia biotech sigma – Aldrich)

Centrifuge machine (BHG Hermle Z 230 ,Cambridge)

Sohxlet apparatus (Pyrex)

IR spectroscopy (8400S, Japan)

GC/MC spectroscopy (GCMS-QP2010 PLUS Shimadzu, Japan)

3.1.3 Experimental Animals

Adult albino mice (20-30g) of either sex were used for the research .They were procured from Animal house of faculty of Pharmaceutical Sciences Ahmadu Bello University, Zaria. The animals were housed , fed with normal animal feeds and maintained in Animal house of faculty of Pharmaceutical Sciences Ahmadu Bello University, Zaria .

Echis ocalletus snake venom was obtained from Pambegua in kaduna state, Nigeria. The snake was captured and identified at the Zoological garden in the Department of Biological Sciences Ahmadu Bello University, Zaria .

3.1.4 The Plant Sample

Fresh leaves of *Ceiba pentandra* were obtained from Samaru,Sabon Gari local government ,Zaria ,Kaduna state Nigeria during the month of June. The plant collected was brought to the Department of Biological Science of Ahmadu Bello University, Zaria and identified by officer in charge of the herbarium with voucher number 5079.

3.2 Methods

3.2.1 Collection and Preparation of Plant Material

The leaves removed from the plant were air dried in a shade at room temperature for a period of three weeks. The dried parts were powdered by pounding in mortar with pestle and sieved to fine powder. The fine powder was packed in air tight labeled container and stored in refragerator until required for use.

3.2.2 Capturing of *Echis ocellatus* and Milking of Venom

The snake was captured by snake charmer identified by officer in the Department of Biological Sciences Ahmadu Bello University, Zaria . The venom was collected in a standard way as described by Bucherl, *et.al.,*1968 shown in plate 3.1. Briefly, the snake was held by snake charmer. The mouth of the snake was opened and its poisonous fangs were placed on the inner edge of the fixed glass, and the milking of

the venom was facilitated by pressing on and off of the tail of the snake and venom drops into the glass at time intervals. The venom was collected, dried at room temperature and kept until needed.

3.2. 3 Preparation of Extracts of *Ceiba pentandra*

3.2.3.1 Extraction of the Leaves with Aqueous Methanol (60/40)

Two hundred grams portions of the dried powdered *Ceiba pentendra* leaves was defated with 60/80 petroleum ether using soxhlet extractor. The petroleum ether mack was dried, and then extracted with aqueous methanol (60/40 v/v) exhaustly .The extract was concentrated in rotary evaporator. The concentrate (extract) was kept under shade for four days and weighed and the percentage yield was calculated. Amount extracted using soxhlet apparatus = 210.65g. Amount of leaf powder used during extraction = 1000g

$$\% \text{ yield} = \frac{210.65}{1000} x100$$

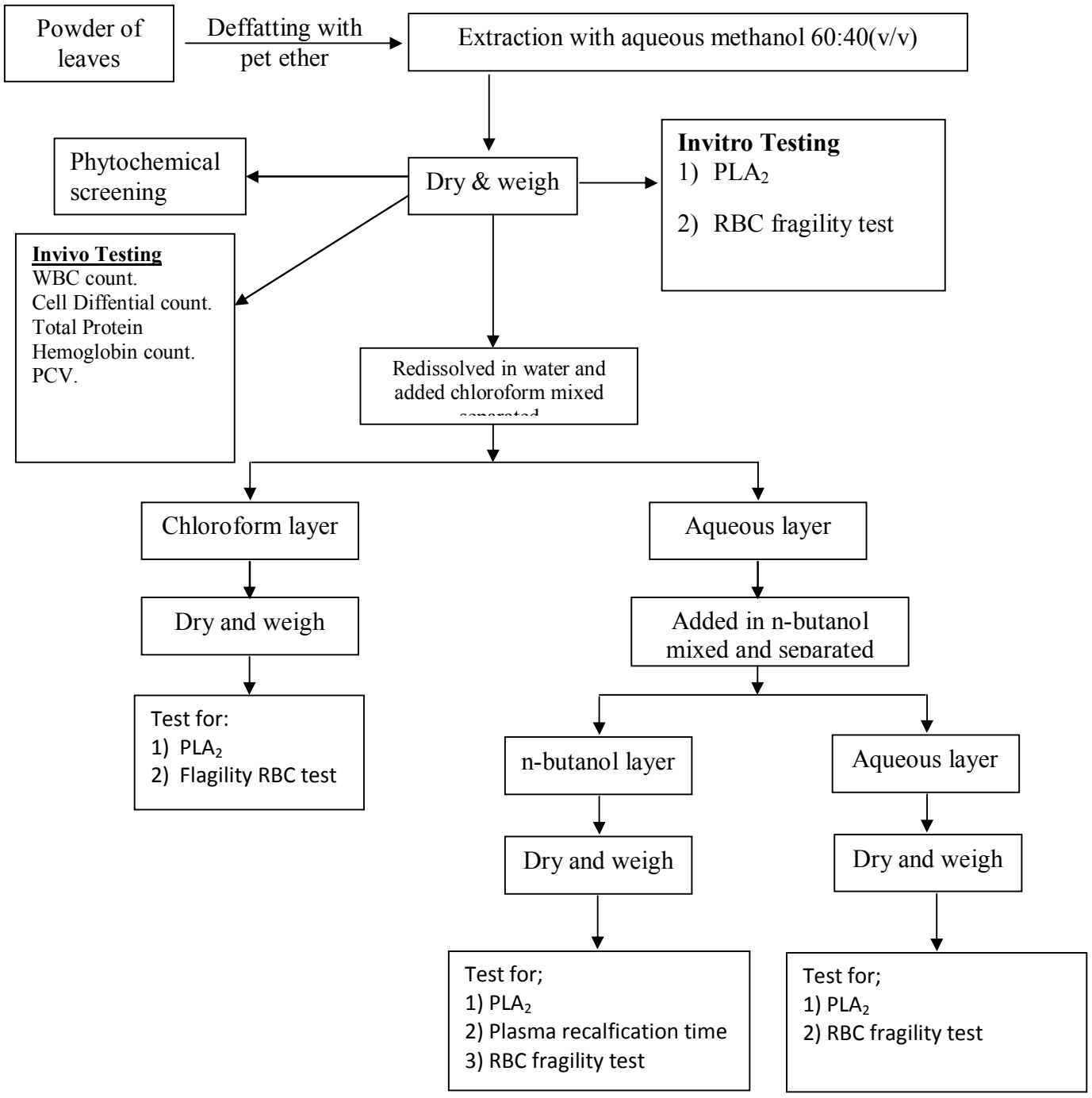
$$= \quad 21.07\%$$



Plate 3.1: Milking of *Echis ocellatus* venom

3.2.3.2 Experimental Flow Chart for Extraction of Leaves of *Ceiba pentandra* Anti venom Principle(s)

A



3.2.3.3 Fractionation of Extract of C*eiba pentandra* Leaves

Procedure

The aqueous methanol leaves extract was partitioned with different solvents as described by Abubakar (2006). Briefly, the powdered aqueous methanol extract 88.25g was ground and dissolved in 1.2 litres of distilled water. The solution was decanted and filtered to give a light reddish coloured solution. The solution was placed in a separating funnel, then two litres of chloroform were added bit by bit followed by shaking for a few minutes and then allowed to stand for 30 minutes.The denser light yellow chloroform layer was carefully removed. The chloroform extract was concentrated in a roteryevaporator dried and then weighted. To the aqueous layer in the funnel, 2.4 litres of n – butanol was added mixed thoroughly and allowed to stand for 40 minutes before the aqueous layer was removed and concentrated as above. The butanol layer was similarly concentrated. Both the aqueous extract and n–butanol extracts were air dried and weight. All the three fractions were kept in a cool and dry place until needed.

3.2.4 Phytochemical Screening of Leaves Extract of *Ceiba pentandra*

3.2.4.1Test for Carbohydrates

(a) Mo1isch's test

A few drops of molisch reagent were added to a little quantity of extract in a test tube and a small quantity of concentrated Sulphuric acid was allowed to run down the side of the test tube to form a violet layer at the interface indicated the presence of carbohydrates (Trease and Evans 1983)

(b) Fehlings test

To two mililitres of extract, five mililitres of a mixture of Fehling's solution A and B in the ratio of 1:1 was added and the mixture boiled for few minutes. A brick-red precipitate indicated the presence of free reducing sugar (Trease and Evans 1983).

3.2.4.2 Test for glycosides

Five mililitres of concentrated Sulphuric acid was added to the extract and boiled for 15 minutes, this was then cooled and neutralized with 20% KOH and divided into two portions and used as below. Another part of extract was dissolved in distilled water, this was used as a control; no acid hydrolysis

(a) Fehling's solution test

Fehling's solution A and B (1:1) was added to one of the portions and boiled for few minutes. A brick red precipitate indicated the presenceof glycone as a result of hydrolysis of glycoside

(b) Ferric chloride test

Three drops of ferric chloride solution was added to the other portion. Green to black precipite indicated phenolic aglycone as a result of hydrolysis of glycoside (Trease and Evans 1983).

3.2.4.3Test for Anthraquinones Derivatives

(a)Test for Free Anthraquinones (Borntrager's test)

Small portion of the extract was mixed with ten mililitres of benzene and filtered. Then five mililitres of 10% of ammonia solution was added to the filtrate and stirred. The production of a pink-red or violet color indicated the presence of free anthraquinones

(b) Test for Combined Anthraquinones

Sample was boiled with five mililitres of 10% hydrochloric acid for 3 minutes. This would hydrolyze the glycosides to yield glycones which are soluble in hot water only. The solution was filtered hot. The filtrate was cooled and extracted with five mililitres of benzene. The benzene layer was filtered off and shaken gently with half its volume of 10% ammonia solution. A rose- pink or a cherry red colour indicated combined anthracene by presence of free anthraquinones

3.2.4.4 Test for Cardiac Glycosides

(a) Kella-Killiani Test

Extract was dissolved in glacial acetic acid containing traces of ferric chloride. The testube was held at an angle of 45^0 and one mililitre of concentrated Sulphuric acid was added down the side. Purple ring color at the interface indicated cardiac glycosides (Trease and Evans, 1983)

(b) Legal Test

Small quantity of the extract was evaporated to dryness in an evaporating dish. The residue was dissolved in a few drops of pyridine in the fume cupboard followed by a few drops of 2% sodium nitroprusside and a drop of sodium hydroxide. Production of a deep red color indicated the presence of cardenolides (reaction depends on five membered ring at c-17)

(c) **Kedde test**

One mililitre of 2% 3, 5-dinitrobenzoic acid in 95% alcohol was added to extract and the solution was made alkaline with 5% sodium hydroxide. Appearance of purple-blue color indicated the presence of cardenolides in the ring.

3.2.4.5 Test for saponins

(a) Frothing test

Small quantity of the extract was dissolved in ten mililitres of distilled water. This was then shaken vigorously for 30 seconds and was allowed to stand for 30 minutes.

A honey comb foam formed for more than 30 minutes indicated the presence of saponins.

(b) Hemolysis test

Two mi1ilitres of 1.8% aqueous sodium chloride solutions were taken in two test tubes. To one tube, two mililitres of the extract was added while same volume of distilled water was added to the other tube. Three drops of human blood was added to each tube. The content was observed for 30 minutes. Hemolysis in the tube containing the extract indicated the presence of saponins.

3.2.4.6 Test for Steroid and Triterpenes

(a) Lieberman-Burchards test

Equal volume of acetic anhydride was added to the extract. One mililitre of concentrated sulphuric acid was added down the side of the tube. The colour change was observed immediately and later. Red, pink or purple colour indicated the presence of triterpenes, while blue or blue-green indicated steroids (Trease and Evans 1983).

(b) Salkowosk test

To about 0.5g of extract, dissolved in two mililitres of chloroform, was added a few drops of concentrated sulphuric acid to form a lower layer. A reddish- brown colour at the interface indicated the presence of a steroidal ring. (Trease and Evans 1983)

3.2.4.7Tests for Flavonoids

(a) Shinoda Test

About 0.5g of extract was dissolved in two mililitres of 50% methanol in the tube. Metallic magnesium and four to five drops of conc. hydrocholic acid was added. A red or orange colour indicated the presences of flavanoic aglycones.

(b) Sodium hydroxide test

A few drops of aqueous NaOH were added to five mililitres of extract, a yellow colouration showed the presence of flavonoid. (Trease and Evans 1983)

3.2.4.8 Test for Tannins

(a) Lead sub-acetate test

Three drops of lead-sub acetate solution were added to a solution of the extract. A colored precipitate indicated that tannins are present.

(b) Ferric chloride test

About 0.5m1 of extract was dissolved in ten mililitres of distilled water, and then filtered. A few drops of ferric chloride solution were added to the filterate. Formation of a blue-black precipitate indicated the presence of hydrolysable tannins and green precipitate indicated that of condensed tannin.

(b) Bromine water test

Three drops of bromine water was added to a solution of the extract (in a fume cupboard). A buff colored precipitate indicated the presence of condensed tannins only. (Trease and Evans 1983).

3.2.4.9 Test for Alkaloids

(a) Meyer's Test

A few drops of the Meyers reagent was added to an aliquot of the extract in a test tube Cream precipitate indicated the presence of alkaloids.

(b) Dragendoffs test

A few drops of this reagent were added to the extract. A rose red precipitate indicated the presence of alkaloids.

(c) Wagners Test

Drops of this reagent were added to a small amount of the extract. A whitish precipitate indicated the presence of alkaloids.

(d) **Picric acid test**

A few drops of 1% picric acid solution were added to the extract and a yellow colored solution indicated the presence of alkaloids.

(e)Tannic acid test

Few drops of tannic acid were added to a sample of the extract. Black precipitate indicated the presence of alkaloids (Sofowora 1982).

3.2.5 Partitioning of the Aqueous Methanol (60:40) Extract of *Ceiba pentandra* Plant Extract with Different Solvents.

Column chromatography of the n -butanol fraction was conducted as described by Nok, *et. al*., (1993). A slurry was prepared by dissolving 30g silica gel in 100ml methanol: water (1:1) and packed in a column (1.5 x 30cm). The bioactive fraction was subjected to column packed with silica gel pre-equilibrated with N-butanol/water 4:5 and the samples were eluted, using running solvent system: n-butanol and water (4:5) .and ten fractions were collected, dried and the fractions were tested for antivenom activity.

The bioactive fractions were collected, dried and submitted to thin layer chromatographs analysis on silica gel (0.25mm) using different running solvents, chloroform methanol (2:98), chloroform: ethyl acetate (4:1) . The fractions were tested for antivenom.

3.2.9 Analytical Thin layer chromatographic Analysis of Fraction of the *Ceiba pentandra*.

Precoated analytical plate was used for the thin layer chromatography. Capillary tube was filled by dipping one end into the fraction to be examined and lightly applied to the thin layer plate. Ascending chromatography was conducted in a wide

chromatographic tank containing chloroform: ethyl acetate (4:1) as mobile phase.The chamber was saturated with the same solvent system before the loaded plates. After that ,the plates were dried spots were detected.The spots were scraped and the phytochemical was then eluted with suitable solvent. The eluates were tested for activity and active one used for structural analysis.

3.2.7 Preparative Thin Layer Chromatographic Analysis of Fraction of the *Ceiba pentandra*.

Preparative thin layer chromatographic analysis of the bioactive fraction of the *Ceiba pentandra* was also carried out and the spot region was detected. Spots were scrapped and eluted.The eluates were tested for activity and active one used for structural analysis.

3.2.8 Toxicological Studies of *Ceiba pentandra* Aqueous Methanol(60/40)Leaves Extract

3.2.8.1 Acute Toxicity Studies of Extract of *Ceiba pentandra*

The acute toxicity (LD_{50}) was estimated in mice following Lorke's method (1983) Briefly, nine groups of mice 3 in each group were used. Dose levels used range from 100mg/kg to 5000mg/kg, the number of death in each group of mice within 24 hours were monitored.

3.2.8.2 Chronic Toxicity Studies of Leaves Extract of *Ceiba pentandra*.

The chronic toxicity of *Ceiba pentandra* was determined according to method described by Aniagu, *et .al.,* (2004). Briefly mice (15) were randomly selected and then divided into three groups of five mice each group, the first group served as control, given only saline, while the remaining 2 groups, received 250 and 500mg/kg of *Ceiba pentandra* extract orally for 21 days as indicated in the method. The first day of dosing was taken as D_0 where as the day of sacrifice was designated as D_{21} and the body weight of each mouse was assessed once every seven days during the dosing period and on the day of sacrifice.

The following parameters were analyzed. Liver function, Haemotological parameters and Kidney to assess the acute toxicity of the extract as described below.

3.2.8.2.1 Effects of *Ceiba pentandra* on Body Weight of Mice

The body weight of each mouse was assessed using a sensitive balance during the acclimatization period, once before commencement of dosing, once every seven days and once on the day of sacrifice.

3.2.8.2.2 Determination of Serum Glutamate Oxaloacetate Transaminase (SGOT)(AST) Activity

Principle

Glutamic-Oxaloacetic Transsaminase is measured by monitoring the concentration of oxaloacetate hydrazone formed with 2, 4-dinitrophenylhydrazine.

α-Oxoglutarate + L-Aspartate → L-Glutamate + Oxaloacetate

Procedure

Serum glutamate oxaloacetate transaminase (SGOT)(AST) was determined using Randox reagent kits that based on the method described by Reitman and Frankel

(1957). Briefly, 0.1ml of test sample was mixed with solution one incubated for 30minutes at 37^0C, then 0.5ml of solution two was added and allowed to stand for 20minutes at 25^0C.Finally 5ml of sodium hydroxide was added to the reaction mixture and absorbances were taken at 546nm. The same procedure was repeated for sample blank. The absorbances of the sample against sample blank were recorded. The AST activity in the serum was obtained from the reagent's kits table.

3.2.8.2.3 Determination of Serum Glutamate Pyruvate Transaminase (SGPT)(ALT)

Principle

Glutamic-Pyruvic Transaminase is measured by monitoring the concentration of pyruvate hydrazone formed with 2, 4-dinitrophenylhydrazine.

α-Oxoglutarate + Alanine → L-Glutamate + Pyruvate

Procedure

Serum Glutamic-Pyruvic Transaminase (SGPT)(ALT) was determined using Randox reagent kits that based on the method described by Reitman and Frankel (1957). Briefly, 0.1ml of test sample was mixed with solution one incubated for 30minutes at 37^0C, then 0.5ml of solution two was added and allowed to stand for 20minutes at 25^0C. Finally 5ml of sodium hydroxide was added to the reaction mixture and absorbances were taken at 546nm. The same procedure was repeated for sample blank. The absorbances of the sample against sample blank were recorded. The ALT activity in the serum was obtained from the reagent's kits table.

3.2.8.2.4 Determination of Serum Alkaline Phosphatase (ALP) Activity

Principle

Phosphatases are enzymes that split off the phosphate moiety from various substrates. The method is based on the colorimetric determination of inorganic phosphate split off from ß – glycerophosphate with phosphatase of the blood serum in an alkaline medium.

Procedure

Serum Alkaline phosphatase activity was determined using randox reagent kits based on the method described by Stroeve and Makarova (1989). Briefly, one ml of distilled water was mixed with 1ml of the ALP substrate ,then 100µl of the randox standard reagent was added .The reaction mixture was incubated at 37^0C for 15 minutes and the absorbance was taken at 550nm. The same procedure was repeated by adding 100µl of serum sample instead of standard reagent and the concentration of alkaline phosphatase was calculated using the following formula.

Alkaline phosphatase = $\frac{Absorbance\ of\ Test}{Absorbance\ of Standard}\ x \frac{30}{1}$

3.2.8.2.5 Determination of Serum Billirubin

Principle

Direct billirubin react with diazotized sulphanilic acid in alkaline medium to form a blue coloured complex. Total billirubin is determined in the presence of caffeine which releases albumin bound billirubin by reacting with diazotized sulphanilic acid.

Procedure

Total serum billirubin was determined by using randox reagent kits and based on the method described by Gendrassik and Grof (1938). Briefly, 2ml of reagent 1, 0.5ml of reagent 2 and 1.0ml of reagent 3 were added followed by the addition of the test sample .The mixture was allowed to stand for 10 mins at 25^0C. Then 1.0ml of reagent 4 was added and allowed to stand for 20mins at 25^0C. The same procedure was repeated for blank sample but reagent 2 was not added, the absorbances were taken for the test sample against the sample blank at 758nm.

Total billirubin(mmMol) = 185 x Absorbance

3.2.8.2.6 Determination of Serum Protein

Principle

Cupric ions, in an alkaline medium, interact with protein peptide bonds resulting in the formation of a coloured complex, whose colour intensity is proportional to protein concentration in the medium.

Procedure

Serum protein was determination using Agappe diagnostic kits based on method described by Stroeve and Makarova (1989). Briefly, 0.1ml of serum was taken in a clean test tube and 0.1 ml of sodium chloride solution, in another test tube (control). Then 5 ml of biuret reagent was added to each test tube, mixed by shaking and set aside for 30 minutes. Absorbances were measure at 540nm.

3.2.8.2.7 Determination of Serum Packed Cell Volume (PCV)

Principle

Hematocrit is expressed as the percentage volume of blood that is occupied by red blood cells.

Procedure

Blood from all animals were used for determination of PCV by the micro heamatocrit method described by Coles, (1980). Blood was taken directly from the tail vein cuts in to heparinized capillary tubes and drawn up by capillary action to about three quarters of the tubes lengh.One end of the tubes was completely sealed and transferred in to hematocrit centrifuge. The tubes were centrifuged at 3000rpm for 10 minutes. After centrifugation, the capillary tubes were trasfered to a graphic reader and PCV was read directly.

3.2.8.2.8 Determination of Serum Blood Urea

Principle

Serum urea serum is hydrolysed to ammonia in the presence of urease. The ammonia is then measured photometrically.

Procedure

Serum urea was determined using Randox reagent kits based on the method described by Weatherbum (1967). Briefly to the sample standard (10µl), 100µl of each reagent 1 and 2 were added followed by addition of 2.5ml of reagent 3 mixed well and incubated for 15 minutes at 37^{o} C. The same procedure was repeated, but

10µl of test samples were added. Absorbances of the samples were measured against sample blank at 546 nm and the urea concentration was calculated.

Urea concentration $= \frac{\text{Absorbance } of \ sample \ test}{\text{Absorbance } of \ standard} \ x \ standard \ concentration$

3.2.8.2.9 Determination of Serum Potassium

Principle

The amount of potassium is determined by using sodium tetraphenylboron in a specifically prepared mixture to produce a colloidal suspension. The turbidity of which is proportional to potassium concentration.

Procedure

Potassium concentration was determined using Tecodiagnostics reagent kits and based on the method described byTerri and Sesin (1958).Briefly,one mililitre of potassium reagent was added to all tubes labeled standard, blank and the serum ,then followed by the addition of 10ul of samples to respective tubes, mixed and allowed to stand for three minutes at room temperature .The absorbance for all samples was read using spectrophotometer at a wavelength of 500nm and the potassium concentration was estimated.

Concentration of potassium $= \frac{\text{Absorbance } of \ sample \ test}{\text{Absorbance } of \ standard} \ x \ conc \ standard$

3.2.8.2.10 Determination of Serum Sodium

Principle

Sodium is precipitated with Mg-uranyl acetate; the uranyl ions remaining in suspension form a yellow-brown complex with thioglycolic acid. The difference between reagent blank (without precipitation of sodium) and analysis is proportional to the sodium concentration.

Procedure

Sodium concentration was determined using CE Human reagents kits and based on the method described by Henry (1974). Briefly, 50ul of sodium reagent was added to all tubes labeled standard and the serum ,then followed by the addition of 3000ul of precipitating reagents to respective tubes, mixed and allowed to stand for 30 minutes at room temperature, then centrifuged at 3000rpm speed for 5 minutes. To the supernatants, 50ul of color reagent were added and allowed to stand for 15 minutes.The absorbances for all samples were read using spectrophotometer at a wavelength of 410nm and the sodium concentration was estimated.

3.2.8.2.11 Determination of Serum Bicarbonate

Principle

A known amount of acid is added to the serum to reacts with the bicarbonate present and release carbon dioxide. The amount of acid remaining from which is calculated by carbonate present in the serum is determined by back titration with sodium hydroxide.

Procedure

Serum bicarbonate was determined using reagent kits based on the method described by Charles, *et .al.,* (1953). Briefly, 1ml of serum was taken in a test tube and 1ml of nitric acid was added and the solution was aerated, sodium hydroxide was added bit by bit until the colour of the sample matches that of the standard indicator and the amount of bicarbonate present in the serum was calculated.

3.2.8.2.12 Determination of Serum Chloride

Principle

In an acid medium, chloride ions reacts with mercury-II-thiocynate and form mercury chloride. These ions react with HNO_3 and Iron -III- ions and affect a red colour. The intensity of the colour is directly proportional to the concentration of chloride ions.

Procedure

The blood chloride was determined by using Agappe diagnostic kits and based on the method described Schonfeld and Lowellen (1964). Brief, three groups of test tubes were labeled as bank,standard,and test samples and 10ul of the respective solutions were added then ,to each of the test tubes ,1000ul of Chloride reagent was added,mixed and incubated for one minute.The absorbance were taken at 492nm and chloride concentration was calculated.

Concentration of chloride = $\frac{\text{Absorbance } of \ sample \ test}{\text{Absorbance } of \ standard} \ x \ 100$

3.2.8.2.13 Determination of Serum Creatinine

Principle

Creatinine reacts with picric acid to produce a colored (red) compound , creatinine picrate. The change in absorbance is proportional to the creatinine concentration.

Procedure

The creatinine concentration was determined by the use of Agappe diagnostic kits and based on the method described by Allen (1982). Briefly,two mililitres of picric reagent was put in all tubes labeled standard, blank and test samples ,then followed by the addition of 0.1 ml (100ul) of samples to respective tubes. Mixed and allowed to stand for one minute at room temperature .The optical density(T_1) was read and the second readings(T_2)were taken after 60 seconds ,for all samples the optical density were read at a wavelength of 500nm and the creatinine concentrations were calculated.

Concentration of creatinine = $\frac{\mathrm{T2-T1}of\ sample\ test}{\mathrm{T2-T1}\ of\ standard}\ x\ 2$

3.2.9 Venom Lethality and Purification of Phospholipase A_2 From Venom of *Echis ocellatus*

3.2.9.1 Lethality Assay for *Echis ocellatus* Venom (LD_{50} Determination)

The $LD_{50}$was determined by method described by Miller and Tainter (1944). Briefly, the mice were assembled in six groups of seven mice each, weighed and average weight was taken as reference point for LD_{50} determination. Each group was injected with the appropriate dose of the venom intraperitoneally as given

below.

Group 1: Injected with 0.1mg/kg of the venom

Group 2: Injected with 0.2mg/kg of the venom

Group 3: Injected with 0.3mg/kg of the venom

Group 4: Injected with 0.4mg/kg of the venom

Group 5: Injected with 0.8mg/kg of the venom

Group 6: Injected with 1mg/kg of the venom

The mice were left for 24 hours under observation and the number of death recorded which were converted to death percentage and a graph of probits against logdose of venom the various groups were plotted and LD_{50} determined by extrapolations.

3.2.9.2 Purification of Phospholipase A_2 From *Echis ocellatus*

3.2.9.2.1 Ion Exchange Chromatography Using DEAE-Cellulose

Ten miligram of the crude venom was suspended in sodium phosphate buffer, pH 7.2. It was then centrifuged for 5 minutes at $20.142x10^{-2}$xg. The supernatant was loaded on DEAE Cellulose chromatographic colunm 12 x 1.0cm which was equilibrated with same buffer. The column was eluted with a gradient of Sodium chloride 0–0.3M in 5mM phosphate buffer. Fractions of 5ml per tube were collected at the flow rate of 0.8ml/min. Absorbance was monitored at 280nm using UV spectrophotometer. The fractions were tested for phospholipase A_2 activity.Active fractions were pooled, dialysed and dried as shown in table 4.5.

3.2.9.2.2 Gel Filtration Using Sephadex G-150.

The pooled dialyzed phospholipase fraction SS1 was dissolved in 2ml 50mM acetate buffer, pH 5.0, and subjected to gel filtration on sephadex G -150 column (2cm x 60cm) pre equilibrated with 50mM acetate buffer, pH 5.0. The column was eluted with same buffer and a total of 12 fractions, of 5ml each were collected at the flow rate of 0.8ml/min. The fractions were assayed for protein by taking the absorbance at 280nm and 260nm. Fractions were freezed dried and phospholipase activity was determined as given in table 4.5.

3.2.9.2.3 Ion Exchange Chromatography Using Sephadex – A 25

The pooled phospholipase fractions of gel filteration was subjected to anion exchange chromatography on a Sephadex column (1cm x12cm) equilibrated with 50 mM Tris-HCI buffer, pH 8.0, and the column was eluted with gradient of Sodium chloride(0.0-0.3 M) in 50 mM Tris-HCI buffer, pH 8.0. Fractions were assayed for protein by taking absorbance at 280 nm and 260nm and PLA_2 activity determined as shown in table 4.5.

3.2.9.2.4 Estimation of Protein

The protein determination of venom fractions was done by measuring the absorbance at 280nm and 260nm using Janway 6405 uv – visible spectrophotometer with an optical path 1cm. Saline buffer(0.9%) was used as blank and the following formulas was applied for the estimation of total protein in the venom (Layne ,1957).

Protein conc. Mg/ml = 1.55 x A280 – 0.76 x A260.

3.2.9.2.5 Phospholipase A_2 Assay

Procedure

Phospholipase A_2 neutralization has been determined using the method described by Simuzu, *et al*., (1980). Thus 50µl of the crude venom was incubated with 50µl of egg yolk at 37^0C for 30 minutes. At the end of the incubation, the reaction was stopped by immersing in boiling water for five minutes. The reaction mixture was titrated using 20mM sodium hydroxide and phenolphthalein indicator. The same procedure was repeated using extract preincubated venom. The volume of sodium hydroxide used to neutralize free fatty acids was recorded and the activity of Phospholipase A_2 was calculated as detailed below.

Volume of 20mMNaOH that neutralized free fatty acid= yml

$\therefore$ 0.02 x y x 10^{-3} = k moles/30minutes

Enzyme activity = k moles/30minutes = Z moles/minute

3.2.9.2.6 SDS -PAGE Analysis of Purified Phospholipase A_2 Enzyme

The electrophoresis was carried out on 10% polyacrylamide gels in order to find out PLA_2 enzyme molecular weight according to the method described by Laemmlli, (1970). SDS -PAGE analysis of the purified phospholipase A_2 enzyme was carried out using 10% SDS polyacrylamide gel under control conditions. Briefly ,50µl of purified enzyme was mixed with 50 microlitres of mercapto ethanol and 50µl brumophenol blue ,then boiled for 5 minutes prior to application. The electrophoresis machine was assembled. The chamber was filled with electrolyte

buffer and 50µl of the prepared samples were loaded into wells. Ran for 45 minutes at control voltage 69 volts, 11 watts and 160 milliamphere. The gel was removed stained in coomassie blue for 30 minutes and destained with methanol (40%),acetic acid (10%)and distilled water (50%) for one hour and photographed.

3.2.10 In vivo Studies of Effect of *Ceiba pentandra* Leaves Extract on the Activity of Venom of *Echis ocellatus*

Procedure

For the in vivo tests, 15 mice were assembled and divided in to three groups of five mice each. Group one was injected with saline (0.02ml), group 2 with venom 0.1mg/ ml while group 3 injected with venom incubated with the extract (0.1mg/ ml) for 10 minutes at 37^{o}C. The mice were left for 24 hours and clinical symptoms were observed. All injections were done intraperitoneally . The following haemotological parameters were determined before and after injections.

Hemoglobin content method described by (Coles 1980)

White blood cell count (Schalm, *et. al*., 1975).

Differential count (Schalm ,*et .al* .,1975)

Total protein content

Packed cell volume (Coles 1980)

3.2.10.1 Hemoglobin Concentration

Principles

The cyanmethemoglobin method described by Coles (1980) was used .Hemoglobin is converted to cyanmethemoglobin by the addition of potassium ferricyanide and sodium cyanide. The absorbance of the color produced is directly proportional to the amount of hemoglobin present.

Procedure

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

3.2.10.2 White Blood Cell Count

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

Principles

The WBC count involves diluting the blood 1:20, (selectively destroying RBC) and staining the white cells with gentian violet, which permits easy identification of White blood cell.The count was done with Haemocytometer(model Marienfield ,Jermany 66300103).

Procedure – Using Thoma white cell pipette, blood was drawn up to 0.5 mark, followed by the diluting fluid to the 11 mark. This gives 1 to 20 dilution. The hemocytometer chamber was then filled and the white cells are allowed to settle for 1-3 minutes. The counting chamber was then placed on the microscope stage with a

lower power objective x10 to ascertain whether the cells are evenly distributed and then x40 objective was used for clearer viewing and counted.

3.2.10.3 Differential Count.

The blood smear was dried, fixed in alcohol for 3 minutes then stained in 10:1 Jiemsa for 20 minutes then washed with tap water, rinsed with buffer, pH 7.0 and aired dried.It was placed in oil emulsion andplaced under microscopic with 10 x magnification, various cells were searched and counted.

3.2.10.4 Determination of Protein

Refractometer model 7416 USA was used for protein determinantion.

Procedure .

The refractometer is advice use to measure the refractive index of a solution. Refractive index is defined as the degree that the light is bent when it is passed through a liquid, and is a function of the amount and type of solid material dissolved in the liquid. In clinical practice, this instrument is caliberated to give direct readings of total protein in serum samples. The refractometer was set to zero reading using distilled water, and the protein concentration was taken.

3.2.10.5 Effect of *Echis ocellatus* Venom on Packed Cell Volume (PCV)of Mice

procedure

The mice were grouped into three groups of five mice each, before treatment the PCV of the mice was determined. Group 1, which is the control group was injected with buffered saline (0.9%), group 2 mice were injected with venom (0.1mg/kg) and group 3 mice were injected with venom (0.1mg/kg) incubated with the extract (0.1g/ml) at 37^{o}C for ten minutes. All injections were done intraperitoneally. The PCV of the animals were determined after 24 hours of injection. Blood from the tails of all animals were used for determination of PCV by the micro heamatocrit method described by Coles, (1980).

3.2.11. In vitro Studies of Effect of *Ceiba pentandra* Leaves Partitioned Extracts on the Venom of *Echis ocellatus*

3.2.11.1 Red Blood Cell Fragility Test

principle

It is a measure of spontaneous red cell heamolysis after incubation for certain period of time. This test reveals subtle abnormality not detectable with standard osmotic fragility test (Doughterty, 1976).

Procedure

The tail tips of clean healthy mice were clipped and 200µl of blood were collected in heparinised capillary tubes and transferred to 5m1 of phosphate buffered saline P^{H} 7.2 and centrifuged. After centrifugation the cells were resuspended in 5ml of

the same buffer and one milliliter of the cell suspension was added a duplicate to test tube containing 4m1 of distilled water. This was repeated for the test samples. All samples were incubated at 37^0C for 6 hours then mixed and centrifuged. Percentage heamolysis was determined by comparing A_{540} of the supernatant of the control or test samples with A_{540} of completely heamolysed sample (distilled water treated sample).

3.2.11.2 Effect of the Partitioned *Ceiba pentandra* Leaves Extracts on Crude Venom Phospholipase A_2

Principle

Phospholipase catalyses the hydrolysis of the fatty acid ester bond in the position 2 of phospholipids. The substrate used was egg yolk phosphotidylcholine. The course of the enzymatic reaction is followed by titrating the fatty acid that is released with 20mM sodium hydroxide.

Procedure

Phospholipase A_2 neutralization has been determined using the method described by Simuzu, *et. al*., (1980). Thus 50µl of the crude venom(0.1mg/ml) was incubated with 50µl of egg yolk at 37^0C for 30 minutes. At the end of the incubation, the reaction was stopped by immersing in boiling water for five minutes. The reaction mixture was titrated using 20mM sodium hydroxide and phenolphthalein as indicator. The same procedure was repeated using extract preincubated venom. The

volume of sodium hydroxide used to neutralize free fatty acids was recorded from graduated micro-pipette and the activity of Phospholipase A_2 was calculated as shown in section 3.2.9.2.5.

3.2.11.3 Evaluation of the Isolated *Ceiba pentandra* Principle Incubated with *Echis ocellatus* Venom's Phospholipase A_2 on Red Blood Cell Fragility Test

Procedure

Evaluation of effects of isolated *Ceiba pentandra* principle incubated with *Echis ocellatus* venom's Phospholipase A_2 on red blood cell fragility test carried according to the method described by Doughterty (1976) earlier in section3.2.11.1

3.2.11.4 Effects of Isolated *Ceiba pentandra* Compound on Purified Phospholipase A_2 Activity.

Procedure

The procedure describe by Simuzu, *et. al*., (1980) earlier in section 3.2.11.2 was used. But various concentrations of the substrate (0.3mg/ml, 0.4mg/ml, 0.5mg/ml and 0.6mg/ml) were added to the reaction mixture used. The same procedure was used but the isolated inhibitor was added in the reaction mixture. A reciprocal graph of phospholipase A_2 actvity againt substrate concentration (egg yolk) was ploted.

3.2.11.5 Evaluation of Effects of Isolated *Ceiba pentandra* Principle on *Echis ocellatus* Venom's Phospholipase A_2

Phospholipase A_2 neutralization has been determined using the method described by Simuzu, *et. al*., (1980). This was described in section section 3.2.11.3. The same procedure was repeated except here we used purified (0.1mg/ml) Phospholipase A_2 and isolate from *Ceiba pentandra*. Different concentrations(0.2mg/ml, 0.3mg/ml ,0.4mg/ml ,0.5mg/ml 0.8mg/ml) were added to the reaction mixture.The volume of sodium hydroxide used to neutralize free fatty acids was recorded and compared.The activity of Phospholipase A_2 calculated.

3.2.11.6 Effect of *Ceiba pentandra* Leaves Extract on Anticoagulant Activity of *Echis ocellatus* Venom

Principles

The principles of recalcification time is to replace calcium in the plasma removed by anticoagulant during the collection procedure and the time for coagulation of plasma after additing calcium again represents the plasma recalcification time.

Procedure

This was done by determining the effect of the venom on recalcification time of citrated plasma using the method described by Theakston and Reich (1983). Citrated bovine plasma (200µl) was incubated in water bath at 37^0C. To each sample 10µl of crude venom (0.1mg/ml) was added, the mixture was then diluted with 100µl phosphate buffered saline (PBS), pH7.O. Finally 10µl of 25mM $CaCl_2$ was added

and the, coagulation activity was recorded. The effect was determined by replacing the phosphate buffered saline with 1mg/ml of the extract and the recalcification time was recorded.

3.2.12 Partial Identification of Active Constituents of Leaves Exract of *Ceiba pentandra*

3.2.12.1 Infrared Spectroscopy for Identification of Functional Groups in Isolated Active Principle

Principles

Infrared spectroscopy deals with the interaction of infrared light with matter. Chemical bonds in different environments will absorb varying intensities and at varying frequencies. Thus IR spectroscopy involves collecting absorption information and analyzing it in the form of a spectrum and the frequencies at which there are absorptions of IR radiation can be correlated directly to bonds within the compound in question.

Procedure

The IR spectroscopy analysis was carried out by using IR equipment (model 8400S,Japan)The purified *Ceiba pentandra* was dissolved in methanol placed on slice to allow the solvent to evaporate.The sample was placed in the sample vial, the machine was set on and the sample was scanned ten times. The print out of the

machine was obtained used to identify the functional groups present in the sample.The result was produced based on transmittance/frequency peak type.

3.2.12.2 Gas Chromatography/Mass Spectrometry (GC/MS)

Principle

The Gas Chromatography/Mass Spectrometry (GC/MS) instrument separates chemical mixtures (the GC component) and identifies the components at a molecular level (the MS component). It is one of the most accurate tools for analyzing organic compounds. The GC works on the principle that a mixture will separate into individual substances when heated. The heated gases are carried through a column with an inert gas (such as helium). As the separated substances emerge from the column opening, they flow into the MS.

Procedure

The peocedure was carried used using GCMS machine (QP 2010 plus shimad zu, Japan) The purified The purified *Ceiba pentandra* was dissolved in methanol and 2ml of the sample was transferred into sample vial placed in a chamber of the GCMS machine, the injection of the sample into the machine was programmed with syringe injection position at 250^0C temperature and 100.2kpa pressure and at 60% column oven. As the separated substances emerge from the column opening, they flow into the Mass spectrometry which identifies compounds by the mass of the analyte molecule. The machine is coupled to computer that has a “library” of known mass spectra, covering several thousand compounds which helps to identify the

likely compound that generates the data presented to.After that the print out of the result was obtained and used.

3.2.12.3 Data analysis

All data were expressed as mean ± standard deviation (SD)/SEM. Data analyses were processed with SPSS software (Version 15.0). The differece between experimental and control groups were compared by single-factor analysis of variance (One-way ANOVA) and independent sample *t* test. $P < 0.05$ was considered statistically significant.

CHAPTER FOUR

4.0 Results

4.1 Extraction and Fractionation of *Ceiba pentandra leaves*

The yield of the successive extraction using aqueous methanol (60/40), chloroform, and n-butanol and fractionation are given in Table 4.1. Using aqueous methanol (60/40) v/v extraction gave a yield of 21.07 % (w/w).The solvent was suitable, because this solvent system offered the best opportunity to extract both water soluble and other constituents present in *Ceiba pentandra* leaves .

The results of the fractionation of aqueous methanol (60/40), portion with chloroform, and n-butanol gave a yield of 0.80% and 18.50% respectively as shown in table 4.1.

4.2 Result of Phytochemical Screening of the Aqueous Methanol Extract of *Ceiba pentandra* Leaves.

From the results of phytochemical screening of the aqueous methanol extract of *Ceiba pentandra* leaves shown in table 4.2 it can be seen that unsaturated steroids and triterpenes as well as Anthraquinones were not detected in the aqueous methanol extract of *Ceiba pentandra* leaves but other phytochemicals were present.

Table 4.1. Extraction of *Ceiba pentandra* Leaves Using Different Solvents.

Extracting solvent	***Amount recovered (g)***	***% yield***
Crude aqueous methanol 60:40	210.65	21.07
Chloroform fraction of crude extract	0.75	0.80
n-butanol fraction of crude extract	16.32	18.50
Aqueous fraction of crude extract	24.33	27.58

Table 4.2 Phytochemical Constituents of the Aqueous Methanol (60:40) Extract of *Ceiba pentandra* Leaves.

Constituents	Test	Observation	Inference
Carbohyrates	Molisch's test	Purple coloration at interface	+ve
Free reducing sugar	Fehlings test	Brick red precipitate	+ve
Flavonoids	$FeCl_3$ test	Colour change to red	+ve
	NaOH test	Yellow colouration	
Tannins	Lead subacetate test	Brown precipitate Formed	+ve
	Ferric chloride test	Green black precipitate formed	+ve
	Bromine water test	Brown precipitate formed	+ve
Alkaloids	Mayer's test	Slightly white cream precipitate	+ve
	Wagner's test	colour change	+ve
	Dragendoff's	precipitate formed	+ve
Glycosides			
General test	Fehling's test $FeCl_3$ test	Brickred precipitate red	+ve
		Green ppt formed	
Saponins'	Frothing test	Froth occur for more than 15min	+ve
	Hemolysis test	Lysis of Blood occur in extract	+ve
		containing test tube	
Usaturated steroids	Lieberman Burchard test	No change	-ve
and triterpenes			
	Salkwoski's test Modified	No change	-ve
	test		
Anthraquinones	Bontrager's test Modified test	No colour change	-ve
Cardiac glycosides	Kellani- Killani test	Purple ring at interface	+ve
	Kadde's test Legal test	Purple blue colour	+ve
	Legal test	Deep red colour	+ve

4.3 Thin Layer Chromatography of the Purified Fraction of *Ceiba pentandra.*

The bioactive pale yellow fraction of *Ceiba pentandra* was found to contain one major spot and a minor spot. The major spot and the minor have R_f values of 0.112 and 0.866, respectively, as revealed in lanes 1 and 2 in plate 4.1 and the arrows showing spot of compound detected in plate

4.4 Large Scale Preparation of Purified Fraction of *Ceiba pentandra* by Preparative Thin Layer Chromatography.

The results in plate 4.2 revealed that there was only one spot detected using solvent system benzene, n-butanol and ethyl acetate as running solvent (8:5:2).

The results for preparative thin layer chromatography has shown that there was only one spot from leaves of *Ceiba pentandra* as indicated by arrow in plate 4.2.

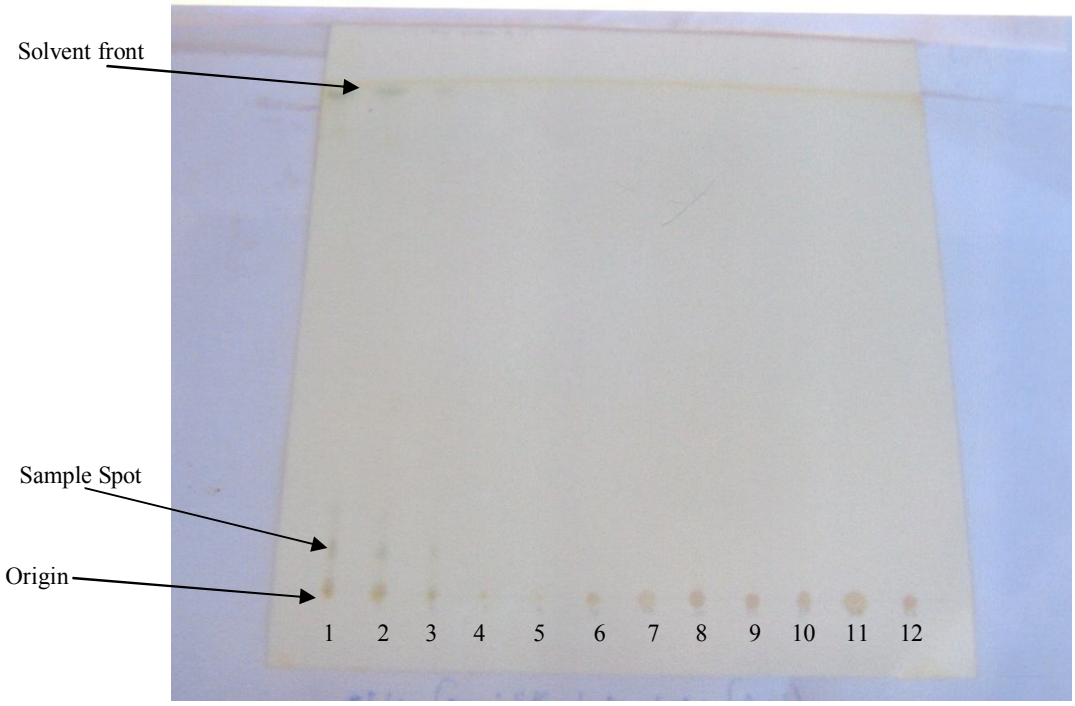


Plate 4.1: Thin layer chromatogram of isolated PLA_2 inhibitor from *Ceiba pentandra* leaves extract with arrows showing spot of compound detected on lanes 1,2 and 3 .

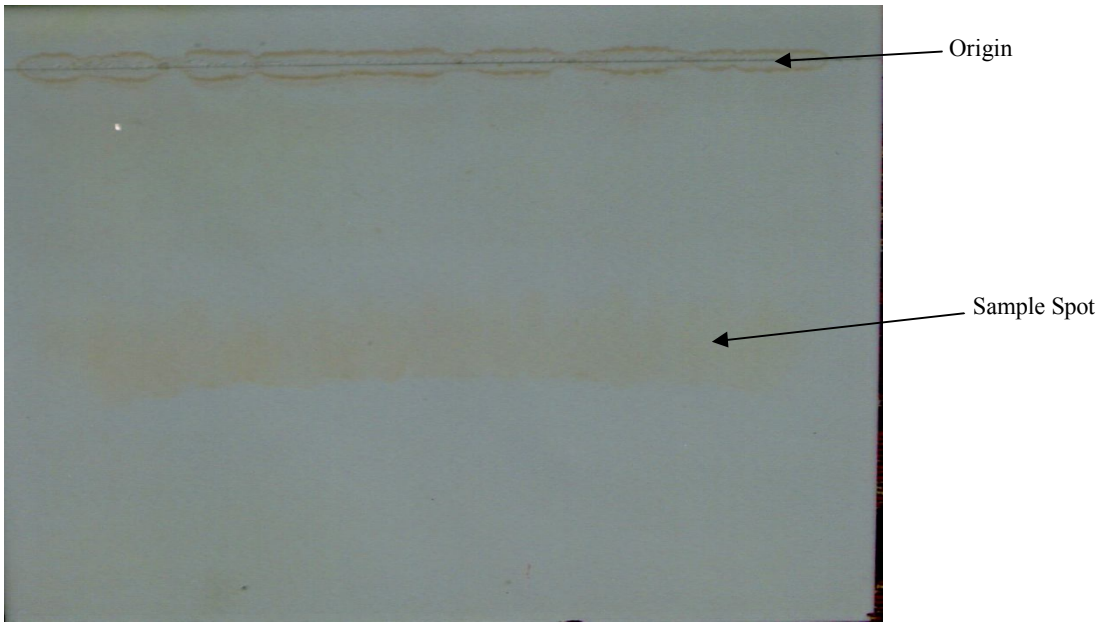


Plate 4.2 : Preparative thin layer chromatogram of isolated PLA_2 inhibitor from *Ceiba pentandra* leaves extract with arrow showing spot of compound detected.

4.5 Result of Toxicological Studies of *Ceiba pentandra* Leaves.

4.5.1. Acute Toxicity Studies

At the doses tested, there were no clinical signs observed in the treated mice. No mortality was observed in all the groups of mice that were given *Ceiba pentandra* orally after 24 hours. Therefore the LD_{50} value of *Ceiba pentandra* was estimated to be above 5000mg/kg body weight.

4.5.2. Chronic Toxicity Studies

Chronic toxicity test of the leaves extract *Ceiba pentandra* was determined and are given as follows.

4.5.2.1 Body Weight.

The animals were treated with 250mg/kg and 500mg/kg doses of *Ceiba pentandra* extract for groups 2 and 3, respectively, for a period of 21 days. The results shows that there were no significant changes in body weight as revealed in table 4.3.

4.5.2.2 Biochemical Indices of Hepatic Function

The results of serum biochemical changes in mice treated with aqueous methanol extract for a period of 21 days revealed that there were significant ($P<0.05$) decreases in creatinine, AST, and protein concentrations in the treated groups as shown in table 4.4. On the other hand, there were significant increases in concentrations of ALT and ALP. The findings also revealed that there were no significant changes in the bilirubin concentration and PVC of all the treated groups.

4.5.2.3 Biochemical Indices of Renal Function

Biochemical Indices of renal function showed that, there were significant ($P<0.05$) rises in bicabonate ,urea and chloride concentrations in the treated groups and there was decrease in concentration of potassium which was significant, but there was no significant change in the concentration of sodium ion as revealed in figure 4.1.

Table 4.3 Effects of *Ceiba pentandra* Leaf Aqueous Methanol Extract on Body Weight in Mice.

Group	Dosage	Mean body weight(g)			
		D_0	D_7	D_{14}	D_{21}
Group 1	Saline 0.9%	21.46±0.77	21.28±0.70	21.40±61.00	22.68±0.65
Group 2	250mg/kg	25.08±2.19	24.88±2.03	25.28±2.06	25.69±2.05
Group 3	500mg/kg	23.30±1.47	23.20±1.40	23.35±1.36	23.68±1.34

D_0 - D_{21} Represent days of treatment

Mean $\pm$ SEM for 5 determinations of body weight.

Group 1, 2 and 3 represent control, given 250mg and 500mg dosage of *Ceiba pentandra* extract for the period of 21 days.

The values are not significantly different ($P > 0.05$).

Table 4.4: Chronic Toxicity of *Ceiba pentandra* Leaves Aqueous Extract in Mice for Serum Biochemical Changes.

Group	Creatinine (µmol/l)	AST (IU/L)	ALT(IU/L)	ALP(IU/L)	Bilirubin(mMol/L)	T/Protein (g/L)	PCV (%)
Control	126.80±3.70	27.20±2.30	6.00±0.54	11.00±2.50	17.00	68.40±3.18	46.40±0.50
250mg/kg	117.60±15.50	25.60±20.00	8.80±2.40	14.00±8.04	17.00	71.60±0.67	47.40±0.74
500mg/kg	88.00±12.70	19.00±8.10	10.00±3.30	20.40±8.60	17.00	57.40±4.00	47.80±0.58

Mean $\pm$ SEM for 5 determinations of biochemical changes .

Group 1, 2 and 3 represent control, given 250mg and 500mg dosage of *Ceiba pentandra* extract for the period of 21 days .

The values are significantly different ($P< 0.05$).

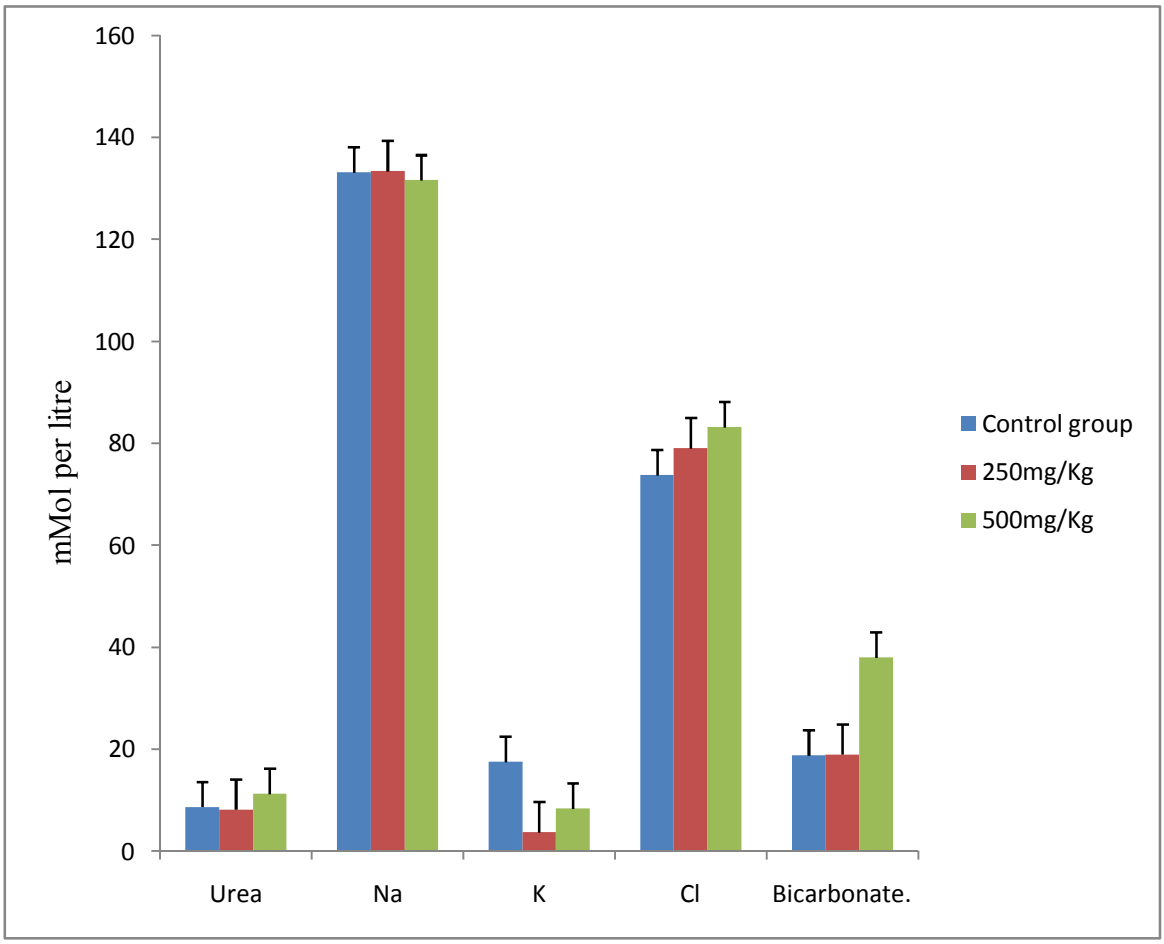


Figure 4.1 : Effects of *Ceiba pentandra* aqueous methanol leaves extract on renal functions of adult albino mice.

Animals were treated with aqueous methanol extract at above doses for 21 days.

The bars represent the mean ±SEM for five determinations and are significantly different $P<0.05$

4.6 Venom Lethality and Purification of Phospholipase A_2 From Venom of *Echis ocellatus*

4.6.1 Lethality in Mice

The acute toxicity study at the doses tested revealed that there were some clinical signs such as paralysis of the hind limbs, shivering and mortality rate were observed in different groups that were administered with 0.8mg/kg and 1mg/kg doses of the venom. The LD_{50} of *Echis oscllетus* venom was 0.280 ± 0.06mg/kg.

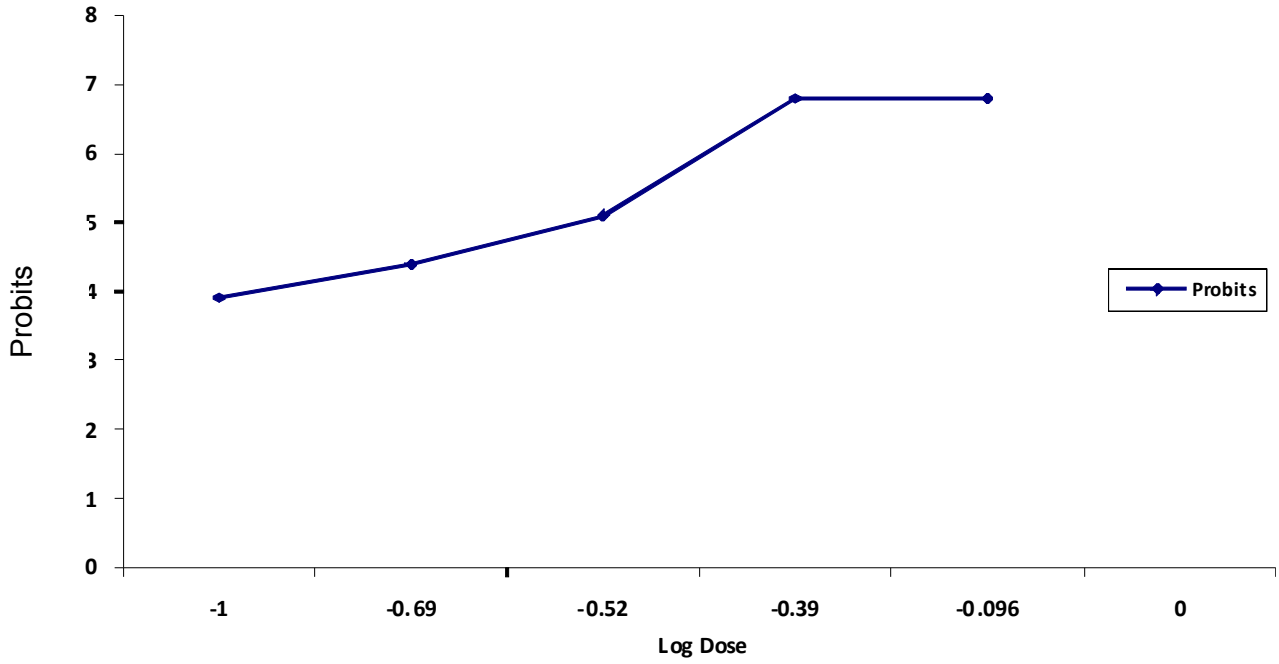


Figure 4.2: Acute toxicity of *Echis ocellatus* venom in albino mice.

4.6.2.1 Purification of Phospholipase A_2 Enyzme From *Echis ocellatus* Venom on Sephadex A 25

The elution profile of PLA_2 from Sephadex G 75 showed two prominent peaks as shown in figure 4.3 It reveals that fractions 7, and 10 had highest PLA_2 activity, and when the eluents were subjected to treatment with purified *Ceiba pentandra* leave extract, the PLA_2 activtity were reduced sighnificantly, suggesting that the *Ceiba pentandra* had some anti phospholipase A_2 venom properties(which is component of *Echis ocellatus* venom).

The purification profile shown in table 4.5 reveals that the PLA_2 specific enzyme activity for the crude venom has 688μmol/min/mg protein but when it had under gone the purification steps, the specific enzyme activity has increased to 4690μmolmin/mg protein with the yield of 77.00% .

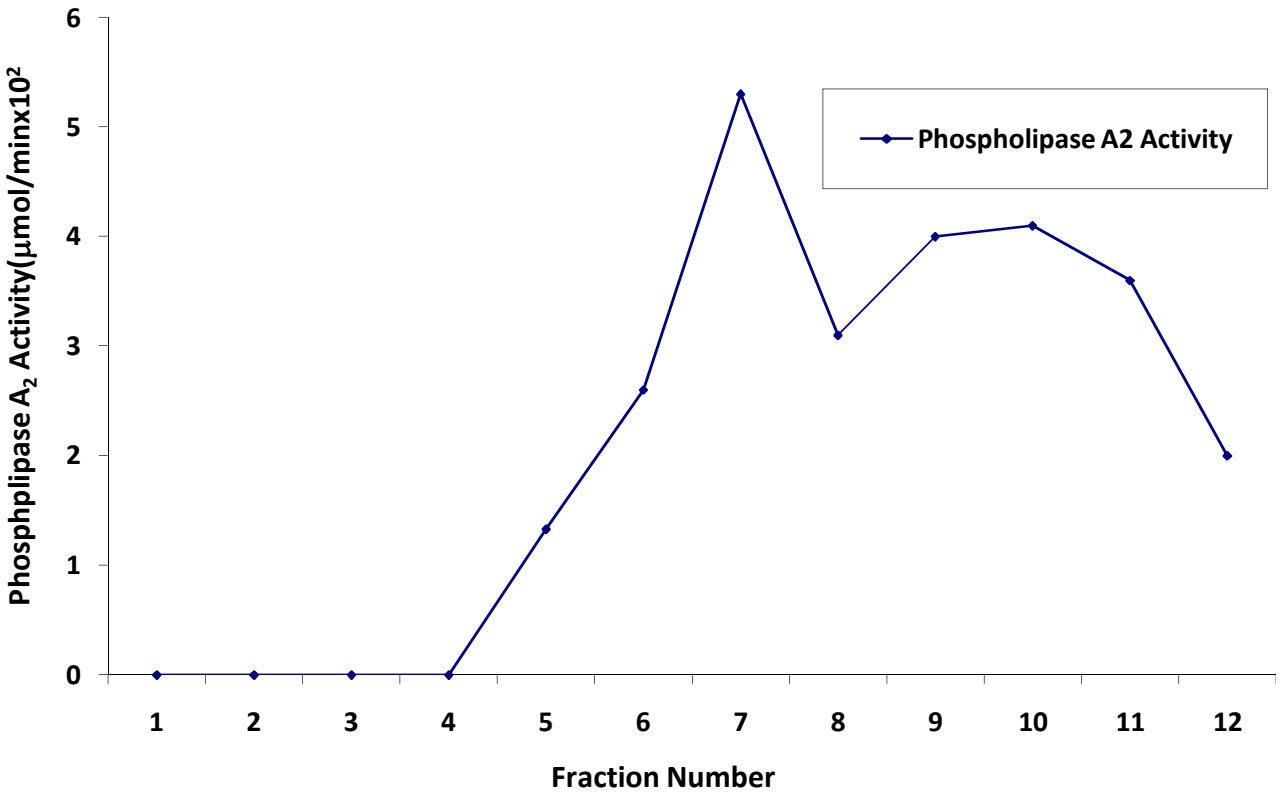


Figure 4.3 Elution profile of PL A_2 from *Echis ocellatus* venom on sephadex A 25

Table 4.5 Purification Table for Phospholipase A_2

Purification step	Protein(mg)	Total enzyme activity µMol/min	Specific enzyme activity µMol/min/mg protein	Purification fold	%yield
Crude venom	1.017	700	688	1	100
DEAE Cellulose	0.742	400	539	0.78	58.10
Sephadex G 150	0.405	600	1482	2.20	87.20
Sephadex A 25	0.113	530	4690	6.81	77.00

4.6.2.3. Results of SDS-PAGE for PhospholipaseA_2

Plate 4.3 shows Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis (SDS-PAGE) of phospholipase A_2 purified from venom of *Echis ocelletus* Compared to the molecular marker in lane 1,the purified enzyme has a molecular weight of 15KD (lanes 3 and 4).

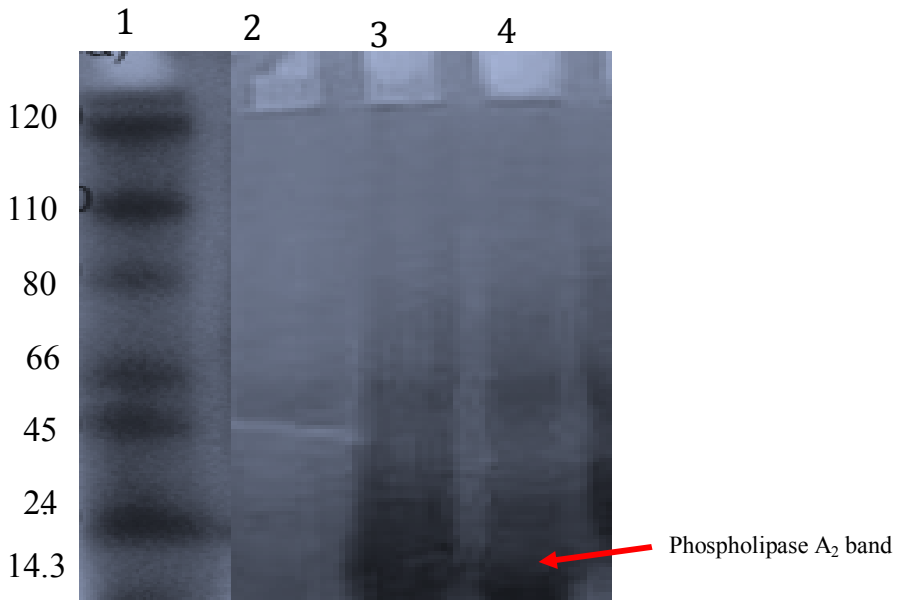


Plate 4.3 Results of SDS.PAGE for phospholipaseA_2 .Arrow showing band of phospholipaseA_2. Lane 1molecular weight marker,lanes 2, 3 and 4 are phospholipaseA_2 from the eluates of sephadex A 25 gel filtration steps

4.7 Effects of Venom and Aqueuos Methanol Extract of C*eiba pentandra* Leaves on Some Hematological Parameters

In vivo studies revealed that there were significant decreases ($P<0.05$) in PCV (24.32%) and hemoglobin concentration (17.15%) for the group treated with crude venom as revealed in table 4.6. This suggests that *Ceiba pentandra* possess some protective properties against snake bite envomation.

Table 4.6. Protective Effects of *Ceiba pentandra* Extract on Some Hematological Parameters.

GROUP	Hematological parameters.			
	PCV (%)	TP(%)	HB (%)	WBC (%)
Control	4.97 [a]	0	10.00 [a]	1.56 [a]
Venom only	24.32 [bi]	1.60	17.15 [bi]	0.57 [b]
venom/crude extract	9.12 [b]	8.56 [b]	4.79 [a]	0

Values in table 4.6 represents changes in PCV, total protein(TP), haemoglobin (HB)content and white blood cells(WBC).

Values with alphabetical superscripts (a) indicates increase in values of the parameters

Values with alphabetical superscripts (b and bi) indicates decrease in values of the parameters.Values with (bi) are significantly different ($P<0.05$) from the control.

Table 4.7 Effects of *Ceiba pentandra* Extract on Differential Counts of WBC

Group	Differential count of WBC					
	Eosophils	Basophils	B/neutrophils	S/neutrophils	Lymphocytes	Monocytes
Control	3.25±0.10	0.75±0.01	0	64.25±0.40	31±1.02	0.75±2.03
Venom	2.50±0.32	1.25±0.12	0	56.75±0.11	38.25±3.21	1.00±0.11
Venom/Extract of *Ceiba p*.	3.20±0.33	0.80±0.41	0	59.80±0.22	33.8±4.22	1.00±0.12

Values in table 4.7 represents changes in WBC average mean for five determinations.

4.8 In vitro Studies of the Effect of *Ceiba pentandra* Leaves Extract on the Venom of *Echis ocellatus*

4.8.1 Effect of Venom and/or Extract on Fragility of Red Blood Cell.

Aqueous methanol extract (0.1g/ml) and butanol extract incubated with 0.1mg/ml of venom caused a significant($P<0.05$) reduction in hemolysis due to venom as shown in table 4.8. The result revealed that the venom had caused 70 % haemolysis of RBC, whereas venom incubated with aqueous-methanol extract and butanol extract had reduced the hemolysis by 58.5 % and 28.5% ,respectively ,suggesting that the plant extract of *Ceiba pentardra* protected the red blood cells from being lysed by the snake venom.

4.8.2 Inhibitory Effect of Various *Ceiba pentantra* Extracts on Phospholipase A_2 Activity of *Echis ocellatus* Venom

The phospholipase A_2 activity was reduced significantly by *Ceiba pentandra* extract as shown in table 4.9. The inhibitory effect is an indication of anti-*Echis ocellatus* venom effect of the extract. The crude aqeous methanol exract ,the n-butanol fraction as well as water portions have significant inhibitory activity on phospholipase A_2 enzyme

Table 4.8 Inhibitory Effect of Various *Ceiba pentantra* Extracts on Hemolytic Property of *Echis ocellatus* Venom

Group	Absorbance	%hemolysis
Distilled water group(control)	1.23±.03	100±.03
Venom incubated with aquoeus methanol extract (0.1g/ml)	0.72±.01	58.50±.01[a]
Venom incubated with n-Butanol extract (0.1g/ml)	0.35±0.20	28.50±.02[b]
Venom incubated with aquoeus portion (0.1g/ml)	0.44±0.06	35.77±.06[c]
Venom only (0.1mg/ml)	0.86±.03	70 .00±.03[d]

The values are mean ±SD of five determinations.

Values with alphabetical superscripts are significantly different ($P<0.05$) from the control.

Table 4.9 Inhibitory Effect of Various *Ceiba pentantra* Extracts on Phospholispae A_2 Activity of *Echis ocellatus* Venom

Group	PLA_2 Activity (μMol/min)	% Inhibition
Venom only(0.1mg/ml)	430± 0.27	0.00
Aqueous –methanol exract (0.1g/ml)	186±0.38[a]	56.74
n-butanol fraction of the extract (0.1g/ml)	236±0.22[b]	45.12
Aqueous portion of the extract(0.1g/ml)	225±.025[c]	47.67

Mean ±SD for five determinations.

The Values with alphabetical superscripts significantly different ($P<0.05$) from the from PLA_2 activity of crude *Echis ocellatus* venom.

4.8.3 Inhibition of Purified *Echis ocellatus* Venom Hemolysis of Mice Blood by Purified *Ceiba pentandra* Extract

The inhibitory effects of the plant isolate on the purified phospholipase A_2 from *Echis ocellatus* venom showed that the purified *Ceiba pentandra* leaves fraction has potentials of reducing the hermolysis due to venom from 66.1% to 27.4% ,suggesting that the plant leaves fraction has inhibitory effect on phospholipase A_2 activity as shown in table 4.10.

4.8.4 Lineweaver-Burk Plot of the Inhibition of Purified Phospholipase A_2 Activity by Purified Fraction of *Ceiba pentandra*

The Inhibition of purified phospholipase A_2 activity by the purified fraction of *Ceiba pentandra* extract shown in figure 4.4 revealed that increase in the concentration of substrate incubated with 0.1mg/ml of purified extract on phospholipase activity has non competitively.some inhibitory effect with Km value of 0.8.

4.8.5 Inhibition of Purified *Echis ocellatus* Phospholipase A_2 Activity by the Purified Fraction of *Ceiba pentandra.*

Phospholipase A_2 purified from *Echis ocellatus* venom was inhibited by the principal active agent of the *Ceiba pentandra* leaves in dose dependent manner. Suggesting that isolated compound can neutralise the PLA_2 activity in the snake venom, in figure 4.5.

4.8.6 Anticoagulant Effects of Purified *Ceiba pentandra* on Purified PLA_2 Isolated From *Echi ocellatus* Venom

The results in table 4.11 showed that the recalcification of bovine plasma was 4.30±0.20 minutes for the venom group, and the recalcification time of bovine plasma was prolonged (7.90±0.10minutes) after incubation of the purified fraction of *Ceiba pentandra* with purified phospholipase A_2.

Table 4.10: Inhibition of Hemolysis due to Phospholipase A_2 by Purified *Ceiba pentandra*

Parameters	Absorbance	% heamolysis
Water and Blood only	1.24±0.20	100
Venom + Purified *Ceiba p.* + Blood	0.34±0.40	27.4±0.02[a]
Venom and Blood only	0.82±0.50	66.1±0.04[b]

Mean ±SD for five determinations.

Values with superscripts are significantly different at P<0.05 one way ANOVA.

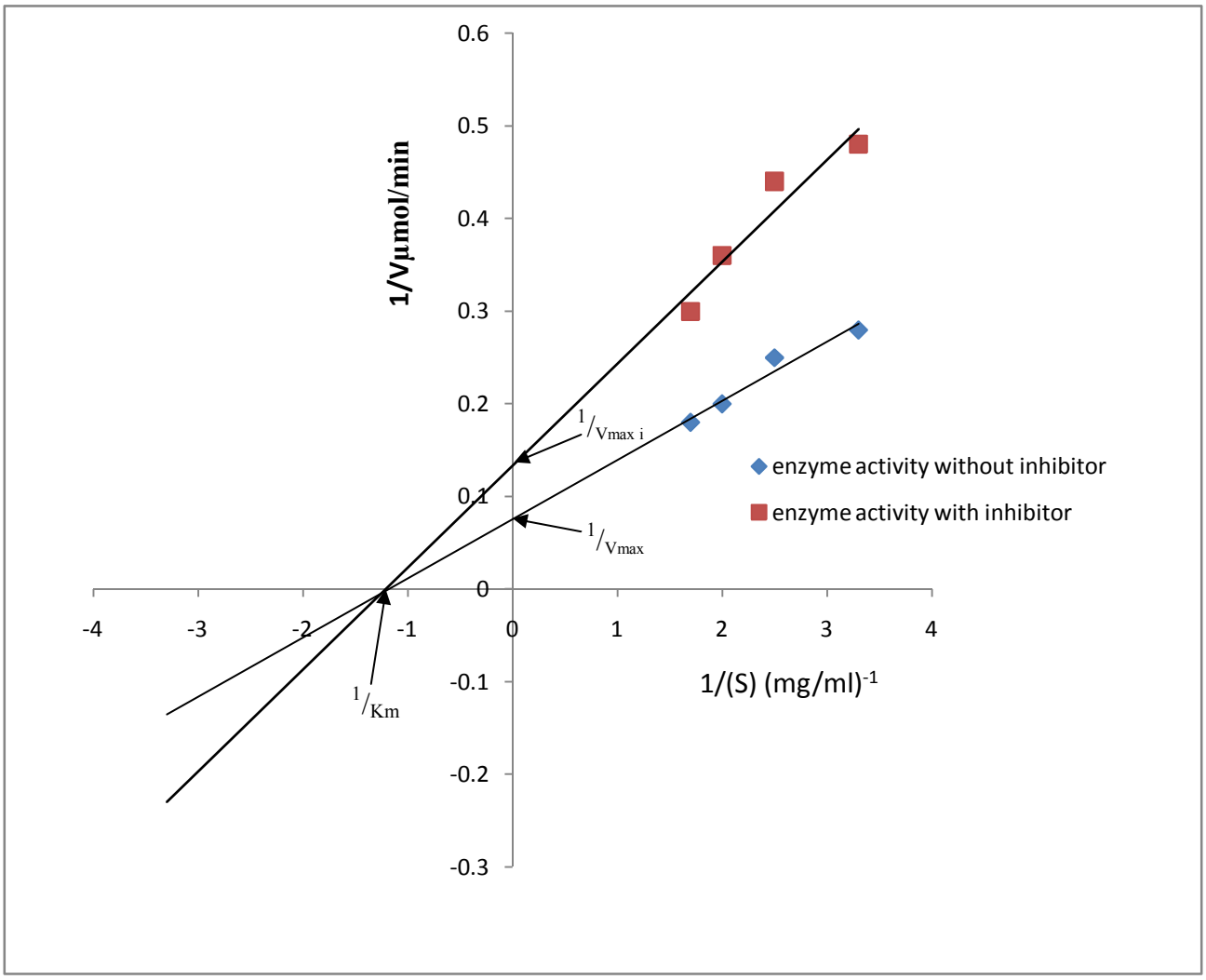


Figure 4.4: Lineweaver-Burk plot showing the effects of purified *Ceiba pentandra* on phospholipase A_2 activity.

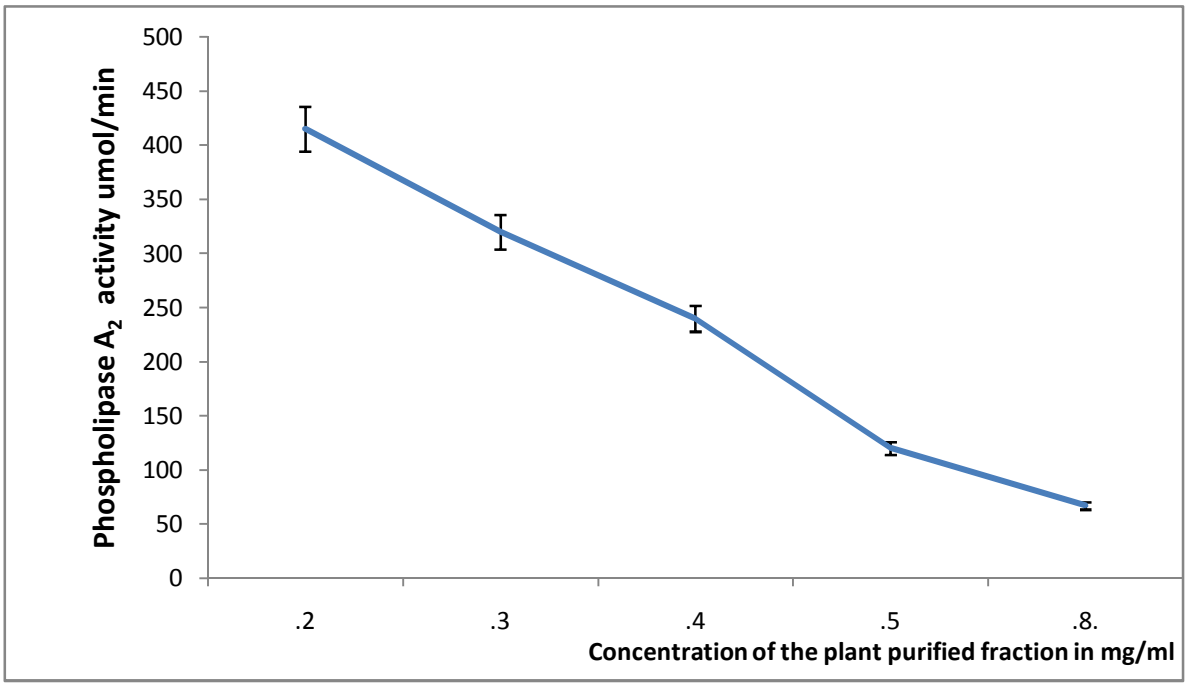


Figure 4.5 Inhibition of purified *Echis ocellatus* phospholipase A_2 activity by the purified fraction of *Ceiba pentandra.*

The values are means of five determinations, significantly different $P<0.05$.

Table 4.11: Clotting time of bovine plasma previously incubated with purified phospholipase A_2 from *Echis ocellatus* venom and the *Ceiba pentandra* extract.

Group	Plasma recalcification time in (minutes)			
Venom only	2.00	2.75±0.15	3.20±0.02	4.30±0.20
Venom/purified extract	2.20±.05	3.30±0.10	4.40±0.05	7.90±0.10

Values are mean ±SD for five determinations.

Delay in clothing time of plasma incubated with venom, and venom/purified extract, the clothing time was significantly different at $P<0.05$.

4.9 Identification of Active Constituents of Leaves exract of *Ceiba pentandra*

4.9.1 Infrared Spectrum of Purified Anti Venom Principle of *Ceiba pentandra*

The IR spectrum of *Ceiba pentandra* is shown in figure 4.6.The diagnostic band for ester carbonyl group 1648.23cm^1 can be observed indicating the presence of this functional group.The other prominent peaks are seen at C-O stretching of O-CH_2 (1020.16cm^1) and less than 928cm^1 out of plane bending (aromatic),C-H stretching at 3431.48cm^1.The characteristic frequencies for various types of bonds in organic compounds is found to absorb in a particular region for instance ,the purified compound found to absorb in the region 1400-1750cm^1 can be deduced to contain the carbonyl functional group.

4.9.2 GC/MS Spectrum of Purified Anti *Echis ocellatus* Venom of *Ceiba pentandra*

The chromatogram of *Ceiba pentandra* given in figure 4.7 indicated only one major peak at 3110525 intensity and retention time of 32 minutes, suggesting that the sample is a pure compound.

The mass spectrum of sample in fig. 4.8 indicated that the ($M+H^+$) ion at m/z 391 and the base peak at m/z 149. The other prominent peaks are those at m/z 57, 71, 167 and 279. The mass fragmentation pattern of the compound isolated revealed that the major ions m/z 167, 279 and 149 are base peaks. The results revealed that the predominant compound present in *Ceiba pentandra* that has inhibitory effects on PLA_2 is Di –n- octyl phthalate with molecular mass 391 as shown in figure 4.8

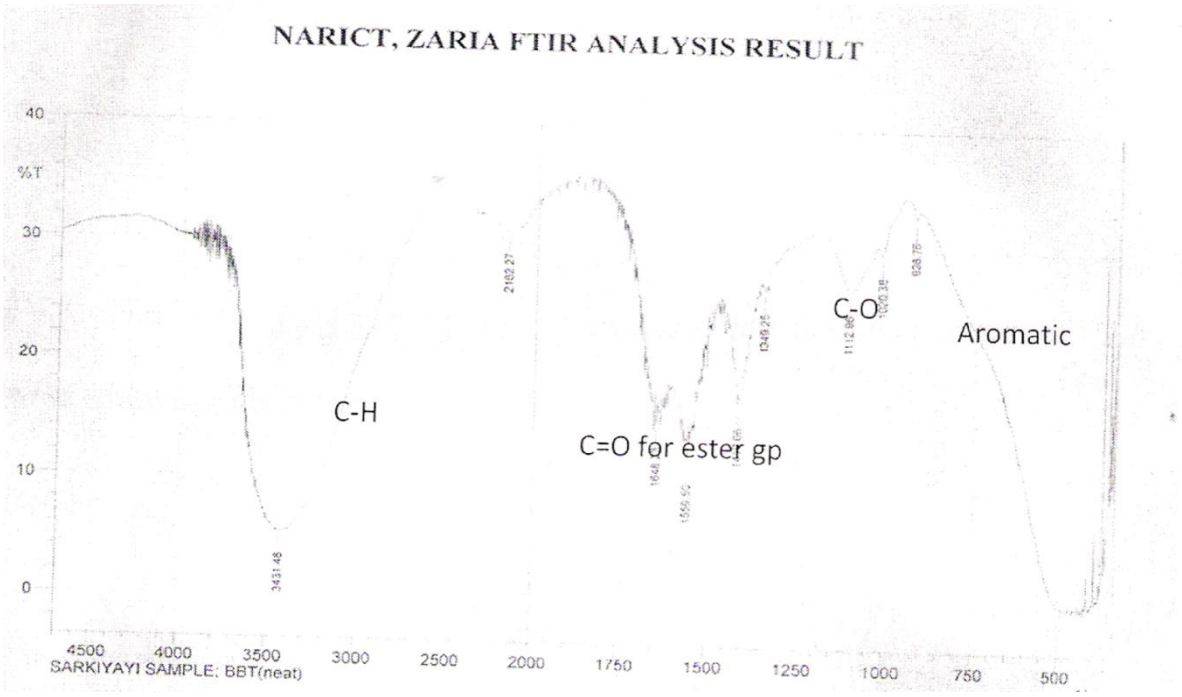


Figure 4.6: IR Spectrum of the Purified Anti *Echis ocellatus* Principle of *Ceiba pentandra* Leaves

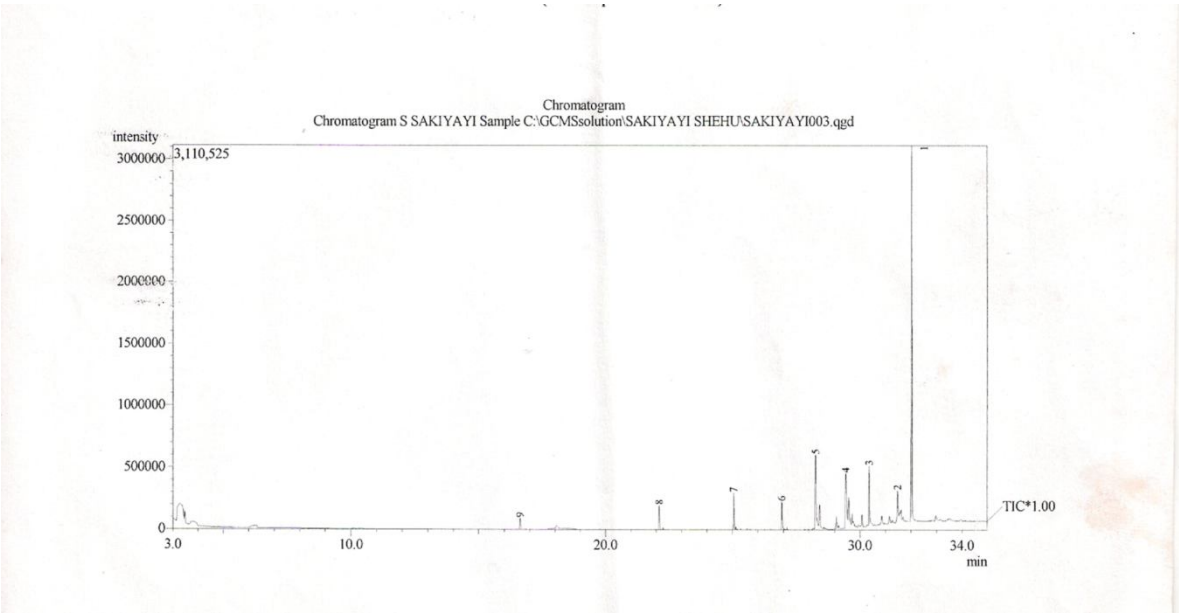


Figure 4.7: The GC/MS spectrum of purified anti *Echis ocellatus* venom of *Ceiba pentandra* showing fragments peaks

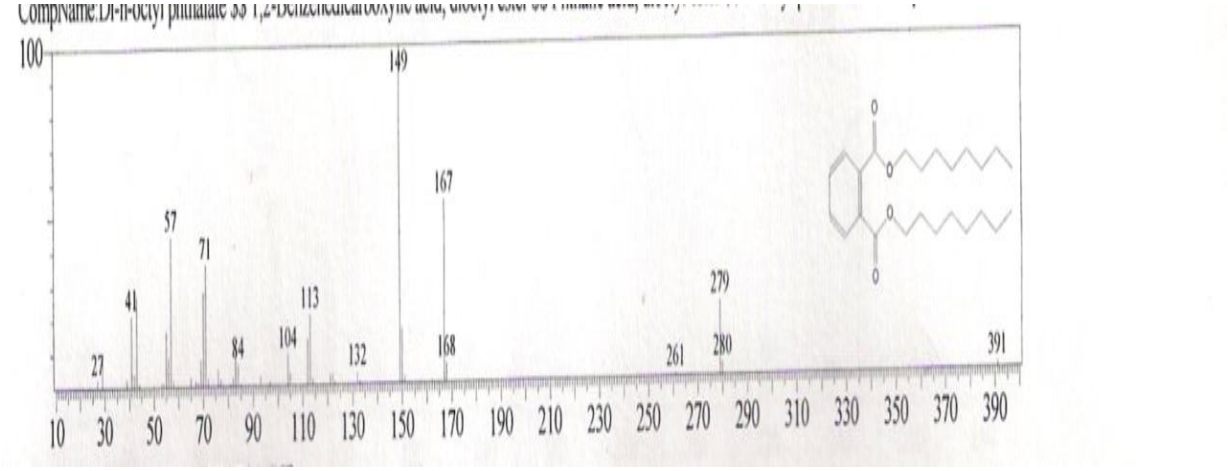


Figure 4.8. GC/MS showing spectrum showing possible identity of purified anti *Echis ocellatus* venom principle of *Ceiba pentandra* extract with likely compound is Di –n- octyl phthalate.

CHAPTER FIVE

5.0 Discussion and Conclusion

5.1 Introduction

Snake venoms are toxic but the deadliness of venom vary with prey and what influences the seriousness of the bite in human includes the individual's health status, site of the bite and psychological state of the victim (Burcherl, *et. al.*, 1968). The empirical indigeneous knowledge on medicinal plants plays a vital role in the primary health care and has potential of the discovery of new herbal drugs, new sources of pharmaceuticals, contraception etc. Kambaska *et. al.*, (2007) reported that the exploration of ethno medicinal data may serve as a useful source of information to the chemist and pharmacologists. Pankaj (2003) reported that *Medhuca longifolia* is used for treatment of snake bite. He further reported that in India, neem leaves are used as first-aid remedy to the victims of the bite and are advised to take more and more neem leaves in order to nullify the lethal effects of venom.In this work,the active principle of *Ceiba pentandra* responsible for its anti venom activity was studied against the venom of *Echis ocellatus* especially against the purified PLA_2 components of the venom.

5.2 Toxicity in *Ceiba pentandra*

The present research has provided first hand information on acute and chronic toxicity studies on aqueous-methanol *Ceiba pentandra* leaves extract. The acute toxicity studies revealed that the *Ceiba pentandra* leaves extract has LD_{50} of

5000mg/kg in mice, indicating that the plant is relatively non toxic to the experimental animals. The result of the chronic toxicity revealed that there was no significant change in body weight of mice fed for 21 days with 250mg/kg – 500mg/kg of *Ceiba pentandra* leaves extract, suggesting that the plant leaves extract is not toxic to mice as shown in table 4.3. Klaassen (2001) reported that lost of weight in animals is due to disturbance in carbohydrate, protein and lipid metabolism.

The indices of kidney function during chronic toxicity studies revealed that blood potassium, urea ,chloride and bicarbonate ions have been slightly significantly ($P<0.05$) increased. The extract produced a significant ($P < 0.05$) dose-dependent alteration in the serum enzymes and urea analyzed. Alterations in these parameters may be due to the pharmacological activity of the plant extract (Wannang ,*et. al*., 2005). The increase in the serum chloride and bicarbonate levels may not pose a great danger to the mice treated with *Ceiba pentandra* aqueous methanol extract because the values are still within the acceptable physiological range. In some situation, liver cell damage is characterized by a rise in plasma enzymes, AST, ALT and ALP. From our findings ALP concentration was consistently higher, but AST, ALT and Creatinine are within physiological acceptable range as given in table 4.4 and figure 4.1. This slightly contradicts another work by Ladeji, *et. al.*,(2003) who reported that the extract of *Ceiba pentandra* administered to rats for 28 days appeared non-toxic as evidenced by normal serum levels of AST, ALT, ALP and

bilirubin. A rise in plasma Alkaline phospholipase level is usually a characteristic finding in obstructive hepatobiliary disease as found in cholestrolic liver diseases (Kanaeko, 1989). The AST concentration is higher than ALT concentration which is expected, since body cells contain more AST than ALT (Mayne,1996). The present research findings also revealed that there was no significant ($P>0.05$) change in the PCV and bilirubin, but there was significant reduction of total protein levels in the tested animals. The toxicity profile studies shows that, the aqueous methanol leaves extract of *Ceiba pentandra* has very low toxicity profile in all the tested animals and the plant is therefore considered relatively safe for herbal medication in treatment of diseases.

5.3 The lethality in Mice due to Venom

Although, use of plants against the effects of snakes bite has been long recognized, more scientific attention has been given in the last 20 years (Santosh, *et.al*., 2004). It is very necessary to understand the pharmacological action of snake venom in order to device a rational treatment for snakebite. An important initial step in finding antivenom potential of plant extracts is the testing using In vivo and In vitro methods to assess the neutralizing potential of the antivenom (plant extracts) against the venom effects. The neutralization ability of snake antivenoms is still assessed by the traditional in vivo lethality assay (minimum effective dose ED_{50}), comparable to those used for bacterial antitoxins, usually performed in mice (WHO, 1981). In our present study we have tried to check the antivenom potential of *Ceiba pentandra*

plant extract against *Echis ocellatus* venom.

The lethality test in mice revealed that *Echis ocellatus* venom has an LD_{50} of 0.280 $\pm$ 0.056mg/kg . This lethal dose is comparable to 0.24mg/kg being the reported LD_{50} of *Echis ocellatus* (Zug, *et.al.*, 1999). In a related development Meenatchisundaram, *et. al.*,(2009) reported that 12µg of *Echis carinnatus* venom was found to be LD_{50} for 18g of mice. It is known that the amount of venom injected per bite for *Echis ocellatus* is 5-48mg, assuming that mice and human have similar susceptibilities to the venom of each species, then 5-48mg per bite for an average weight of 70kg means, that the amount of venom injected per bite in man will rationally kill him within 24 hours after bite, suggesting that *Echis ocellatus* is one of the most poisonous snakes. Therefore the lethal dose for man of 70kg will be 16.8mg. This means that injecting 48mg of the venom per bite into man may cause acute toxicity and death, if untreated. Also observed in mice during acute toxicity while determining the LD_{50} were some behavioral symptoms noted which include excitation, rigid paralysis of the hind limb and apparent insensitivity to loud noise. These behavioural changes in response to snake venom has been reported by Onuaguluchi and Obiekuwe, (1977).

5.4 Purification of *Echis ocellatus* Venom's Phospholipase A_2

The results in Figure 4.3 have shown that fraction 7 had highest phospholipase A_2 activity after purified finally on A-25 sephadex. Phospholipase A_2 isolated from the

venom of *Echis ocellatus* was also purified 6.81-fold with a yield of 77% on Sephadex A-25 column and appeared as a band in polyacrylamide gel electrophoresis (SDS-PAGE) with molecular weight of 15Kdalton. The specific enzyme activity of the purification profile was 4690μM/mins as given in table 4.10. In a related development Marunak, *et. al.*, (2007) reported that the PLA_2 isolated from snake has molecular mass 15.6 kds, similar to the one isolated from *B. Jararacussu* from Brazil 13.7 kda and Ketelhut *et. al.*,et al,.(2003) revealed that PLA_2 isolated has 15 Kda molecular mass.

5.5 Effect of *Ceiba pentandra* Extract and Venom on Some Hematological Parameters

The result of In vivo studies had shown that , the mice injected with venom only, one died, whereas the rest of the mice in the group exhihibited some clinical abnormalities such as shievernig, paralysis of the hind limbs.Where as the mice treated with the venom/extract combination showed little abnormalities and later recovered. The findings suggest that the extract has some protective properties on the mice against the venom. The result in table 4.6 for the Invivo studies on PCV, total protein and white blood cells count revealed that, there was significant decrease in total protein, pack cell volume (PCV) for the venom group and there was no significant change in white blood cells count, PCV, total protein for the

venom/extract group revealing that the extract has some inhibitory properties against snake venom.

Our findings showed that haemolysis of blood due to venom was 66% ,where as for that of venom incubated with extract, the haemolysis was reduced to 27.4% as given in table 4.10 for the purified sample. The findings suggest that the plant extract may have anti hemolytic factor or it is capable of blocking the venom-induced haemolysis. The result in table 4.5 reveals that the percentage heamolysis in the venom group was 70.70% whereas the venom incubated with extract of n-butanol portion has reduced it to 28.5%.

5.6 Effect of *Ceiba pentandra* Extract on Crude Phospholipase A_2.

The result reveals that the plant extract was effective in the inhibition of phospholipase A_2. The result in table 4.9 has shown that PLA_2 activity in the presence of equeuos mentanol and n-butanol extracts, the PLA_2 activity reduced by 45.12% and 47.67% due the extracts respectively, suggesting that the plant extract has inhibitory effect on PLA_2. Alcoholic extract of dried *Annona cassiflora* seed was shown to have anti venom propertiesbacking its use in treating snake bite (Weinberg, *et. al.*, 1993). The lineweaver plot showed on varying concentration of the extract/substrate, the results indicated that, the extract inhibit the phospholipase A_2 activityin a non competitive manner with a K_m value of 0.84M as shown in fig.4.4. In a related work, Sallau, *et .al*, (2008) reported the fairly low Km value for

the natural substrate of the enzyme is an indication of moderately high affinity of the enzyme for phospholipids which further substaintiates the observed toxicities in viperid snakes as a result of PLA_2 activities Consequently, the decrease in Vmax due to inclusion of the plant extract in the substrate mixture suggests that the plant leaves extract has some inhibitory effects on PLA_2. Abubakar, *et. al.,(2006)* reported that extracts of *Indigofera pulchra* and *Aristolochia albida* gave 33.3% and 44.4% protection to mice treated with minimum lethal dose of venom; some gross pathologic symptoms of envenomation were alleviated. However, minimal activities were shown by *Guiera senegalense* and *Sterculia setigera.* Again both *Indigofera pulchra* and *Aristolochia albida* were found to neutralize the anticoagulant, hemolytic, and phospholipase activity of crude venom (Abubakar, *et. al.,,2006).* Fifteen compounds isolated from plants were reported as snake venom antidote.They were shown to protect mice to a significant degree against the lethal action of the venom of *Bothrops jararaca* snakes (Pereira, *et. al.,* 1994). Zingali *et. al.,* (1990) reported that crude venom from *Bothrops jararaca* has pro-coagulant platelet aggregating and phospholipase A_2 activities. Meenatchisundaram , *et. al.,*(2009) showed that *Echis carinatus* venoms have the enzymes (PLA_2) that has the ability to lyses sheep RBC's. *Andrographis paniculata* and *Aristolochia indica* Plant extracts were capable of inhibiting PLA_2 dependent hemolysis of sheep RBC's induced by *Echis carinatus* venom in a dose dependent manner. The result reveals that the plant extract was effective in the inhibition of phospholipase A_2. This inhibitory effect may be responsible for the effect of *Annona cassifiora* as a remedy

against snake venom (Weinberg, *et. al.,* 1993). The extract of the leaves of *Guiera senegalensis* was found to detoxify (*in vitro*) venom from two common northern Nigerian snake species, *Echis carinatus* and *Naja nigricollis*, in separate experiments. There was a remarkable reduction in the mortality of albino mice after intra-peritoneal (i.p.) administration of reconstituted venom incubated with the extract, when compared to those challenged with the venom only. The survival of the animals exposed to the venom incubated with the different concentrations of the extract was used as the *in vitro* detoxification parameter (Abubakar, *et. al*., 2000)

5.7 Effect of the Purified *Ceiba pentandra* Extract and Venom on Plasma Recalcification Time

We further analyzed the effect of venom sample on coagulation of plasma and the results showed that low concentration. (0.2mg/ml) has a recalcification time of 2.2 $\pm$0.05min indicating a high pro-coagulant activity. On the other hand when venom is incubated with the plant extract the recalcification time was prolonged (7.9 $\pm$ 0.10min), as shown in figure 4.5, suggesting that the plant extract has some protective effect against the pro-coagulant effect of the venom. A similar research was conducted by Petkovic, *et. al.,* (1991) who reported that when antivenom was injected intraperitoneally in mice after 2-3 hours of venom injection, the antivenom neutralized the effect of the venom and the antivenom can be useful in the treatment of *vipers ammodytes* venom poisoning.).

It has been revealed that an organic extract isolated from roots of an Indian

medicinal plant *Sarsaparilla hemidesmus indicus* possessed viper venom inhibitory activity (Alam, *et. al.,* 1994). It was revealed that viper venoms cause coagulation disorders. One of the serious complications of viperidae bites is renal failure; the renal abnormality is correlated well with the degree of coagulation abnormality when left untreated due to late arrival at the hospital (Vijeth, *et. al.,*1997) Meenatchisundaram, *et. al.,*(2009) has found that 2.1mg of *Andrographis paniculata* and 1.8mg of *Aristolochia indica* plant extracts were able to completely neutralize coagulant activity. High dose of venom caused rapid clotting that required very high dose of antivenom to neutralize. Aqueous extract of the bark of *Schymanniophyton magnitinum* and the leaves of *Macuna pruriens , Strophasthus gratus* and *Strophanthus hispidus* show a dose related ability to prolong the time taken to clot blood treated, with a standardized dose of the venom of *Echis carinatus, Strophanthus hispidus* was shown to be the most potent of the plants extract tested and the dose response profile obtained showed that the increased clotting time was due to different processes for the different plant extract (Houghton and Skari, 1994).

5.8 Modulatory Constituent of *Ceiba pentandra* Against Snake Venom

The use of medicinal plants has been in practice through out human history .It is important to point out that plants have contributed to the discovering of many drugs and other substances with beneficial use.

These research findings identified that the leaves of *Ceiba pentandra* linn contains many constituents of organic nature. The IR analysis reveals that the bioactive (anti

venom) compound in *Ceiba pentandra* leaves is an aromatic organic ester with carbonyl functional group.The GC/MS result showed that the aromatic organic ester compound is Di octyl phthalate as given in figure 4.8. The result has shown that *Ceiba pentandra* leaves extract possess potent snake venom neutralizing compound. Studies on the interactions of this plant extract with snake venom PLA_2 provide an additional dimension to understanding of the mechanism of action of the compound on PLA_2 toxicity.These interactions occur at specific sites and hence are capable of recognizing subtle structures that are responsible for its biological activities. These investigations support the hypothesis that PLA_2 (toxin) have a specific site responsible for its enzymatic and pharmacological action observed. This isolated inhibitor is likely to interact with the active site of PLA_2 to non competitively inhibits its action on its natural substrate. From the structural analysis conducted the isolated inhibitor is Di –n- octyl phthalate wtith molecular mass 391 as shown in figure 4.8.

5.9 Possible Mechanism of Action of Di –n- octyl phthalate

The possible mechanism of inhibitory effect of Di –n- octyl phthalate is that: the carbonyl group of the compound can react with imino group of histidine 48 residue at the active site of the PLA_2 forming complex rendering the enzyme inactive or the carboxylic acid functional group can chelate calcium that is required for PLA_2 activity. This Di –n- octyl phthalate is capable of inhibiting PLA_2 and hence used as an anti venom. This observation is supported by earlier work by Rosalind, *et. al.*, (1988) using mono(2-methyl) phthalate (MEHP) to inhibits PLA_2. In a related

study, Labow(1988)revealed that the platelet was used to show that MEHP inhibits phospholipase A_2 (PLA_2). In a related development Alam, *et. al*., 1994 reported that an organic acid, isolated and purified from the root extract of an Indian medicinal plant sarsaparilla *Hemidesmus indicus* R. Br, possessed viper venom inhibitory activity.

Similar studies was conducted by Ushanandini ,*et. al*., (2006) which indicated that *Tamarind* seed extract inhibited the PLA_2 (Phospholipase A_2), protease, hyaluronidase, l-amino acid oxidase and 5'-nucleotidase enzyme activities of venom in a dose-dependent manner. Another compound with neutralizing ability against the myotoxic action of PLA_2s from the venoms of Viperidae snakes is fucoidan, a sulphated polysaccharide extracted from a variety of seaweeds, such as *Fucus vesiculosus* (Angulo and Lomonte, 2003). No structural or inhibitory spectrum information on these active principles is available to date.However, it is important to note that the presence of bioactive compounds opens the possibility of their application for therapeutic purposes.

5.10 Conclusion.

The aqeous methanol extract of *Ceiba pentandra* was biologically active in inhibiting the phospholipase A_2 which is one of the toxic agents in *Echis ocellatus* venom. Antivenom properties in the plant has been identified, therefore this study gave first hand information on the antivenom properties of *Ceiba pentandra.* The compound responsible for the pharmacological action has been shown to be **Di –n-octyl phthalate** which inhibits the venom's phospholipase A_2.

This leads support to the traditional use of *Ceiba pentandra* in treating snake bites.The identification of the active constituents responsible for anti PL A_2 activity may help in developing drug in future. .Finally, the relevance of these findings would be of importance in the area of drugs of medical interest with a view to actualize conventional snake bite therapy options with effective, cheap, accessible and less allergic plant compound and that can be scientifically explored.

5.11 Recommendations

1. There is the need for formulating antivenom drug using the purified Di –n-octyl phthalate and carrying out further clinical trials would be of significant importance.
2. With the growing knowledge on the biochemistry and pharmacology, efforts should be geared towards identification of natural inhibitors of venom toxins in varieties of plants origin.

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Appendix A

Probits and Logdose for LD_{50} determination

Probits	**Log dose**
3.92	-1
4.4	-0.69
5.1	-0.52
6.1	-0.39
6.79	-0.096
6.79	0

Appendix B

Result of Logdose for LD_{50} determination.

Group	Number that died	% of death	Dose mg/kg	Log dose	Probits	Corrected probits	Corrected death %
1	1	14.3%	0.1	-1	3.92	3.92	14.0
2	2	28.5	0.2	-0.69	4.41	4.4	29.0
3	4	57.1	0.3	-0.52	5.12	5.12	57
4	6	85.7	0.4	-0.39	6.03	6.1	96.0
5	7	100	0.8	0.096	8.09	6.79	96.4
6	7	100	1.0	0	8.09	6.79	96.4

Appendix C

Effects varing the concentration of purified Fraction of the Extract on purified phospholipase A2 activity.

Substrate conc(ml)	1/S	V for venom, mMol/min	1/Vfor venom	V,.mMol/min for v/extract	1/V v/extract
0.3	3.3	3.6	0.28	2.1	0.48
0.4	2.5	4	0.25	2.3	0.44
0.5	2	5	0.2	2.8	0.36
0.6	1.7	5.4	0.18	3.3	0.3

Appendix D

Effects of *Ceiba pentandra* aqueoes methano*l* leaves extract on renal functions.

Group	**Urea** **mMol/L**	**Na** **mMol/L**	**K** **mMol/L**	**Cl** **mMol/L**	**Bicarbonate** **mMol/L**
Control	8.7±0.58	133.2±1.8	17.6±0.67	73.8±11.10	18.8±4.00
250mg/kg	8.2±1.36	133.4±2.10	3.8±.44	79.2±15.09	19±2.77
500mg/kg	11.3±6.13	131.6±2.10	8.4±1.4	83.2±14.50	38±14.56

Appendix E

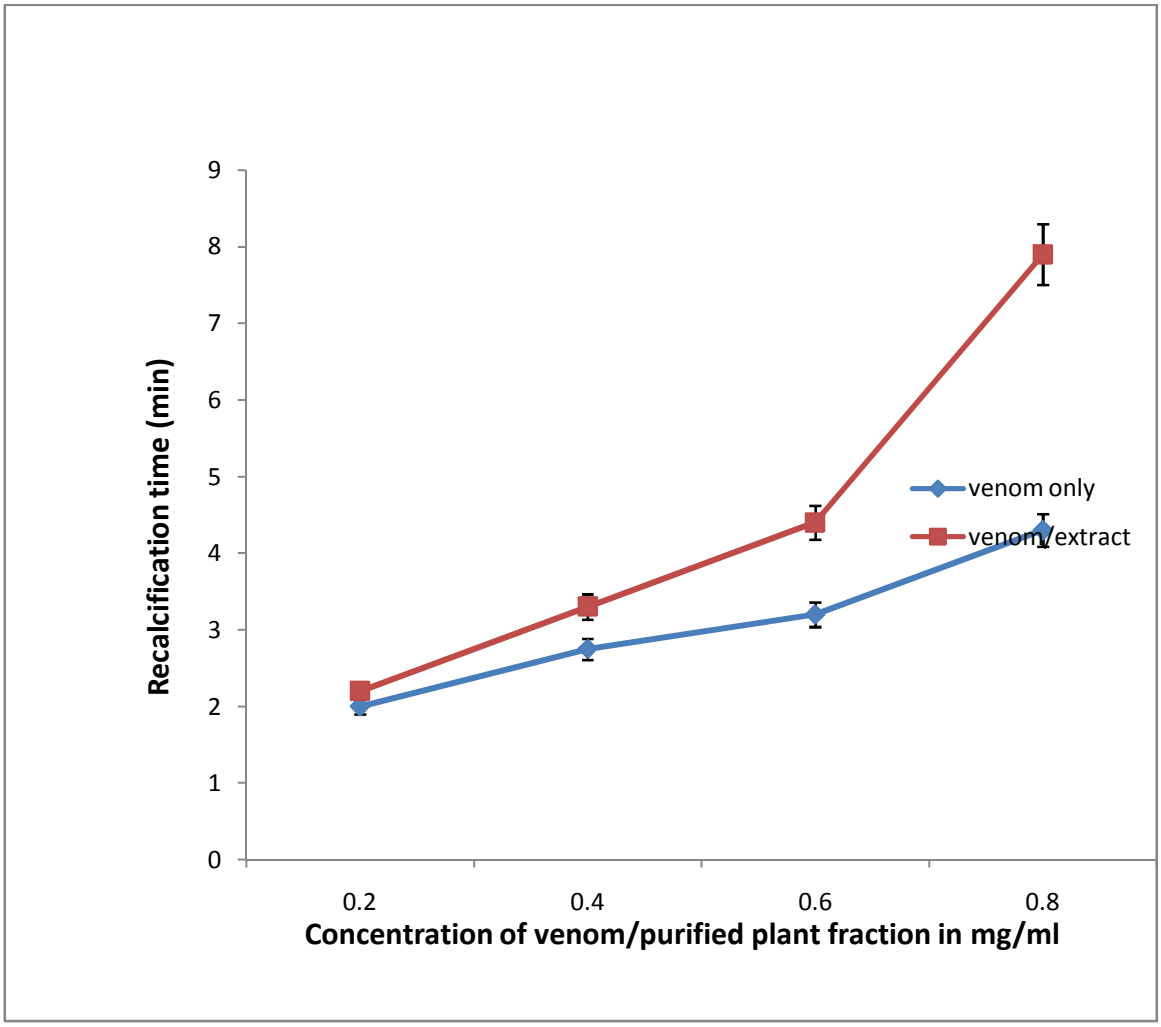
Inhibition of purified *Echis ocellatus* phospholipase A2 activity by the purified fraction of *Ceiba pentandra.*

Conc of fraction (mg)	0.2	0.3	0.4	0.5	0.8
phospholipase A2 activity μMol/min	415±.05	320±.1	240±.12	120±.05	67±.1

The values are mean ±SD for five determinations at different concentrations($P<0.05$)

Appendix F

PLasma recalcification time of fraction 1



Appendix G

Effectsof venom/extract on hemotological parameters

GROUP	PEREMETERS							
	PCV(before treatment)	PCV(after treatment)	TP(before treatment)	TP(after treatment)	HB(before treatment)	HB (after treatment)	WBC(before treatment)	WBC(after treatment)
Control	58.3±3.40	61.2±22	5.3±0.21	5.3±0.20	17.4±1.20	19.14±0.90	5.6±0.20	5.67±0.70
Venom only	52.2±2.20	39.5±2.10a	6.2±0.32	6.1±0.40	17.38±0.70	14.4±1.80a	5.3±0.25	5.27±0.60
venom/Extract	52.6±3.70	48.0±4.58	6.54±0.33	5.98±0.30	16.7±1.20	15.9±1.50	5.9±0.30	5.9±0.47

Appendix H

Reagents and Buffers

I. Buffers** and **Solutions

Phosphate buffer pH 7.1

Sodium di-hydrogen phosphate monohydrate(NaH_2PO_4) 6.0g^{-1}

Di-Sodium hydrogen phosphate (Na_2HPO_4) 7.0gl^{-1}

The solutions are mixed and the pH adjusted using a pH meter and then adjusted to the desired molality(50mM).

Dissolve in 100ml of distilled and ionised water. Usually a 10 X dilution is prepared and stored, to be diluted when required.

***Saline- Physiological or Normal** saline,*

Sodium chloride - - - - - - 0.9g

Tris-hydrochioric acid Buffer, pH 8.0 0./50mM

Tris(hydroxymethyl)aminomethane 0.3M - - - 6.05gl^{-1}

Hydrochloric acid, 1N

Dissolve 6.1g tris in II of water. And titrate 500m1 portions of the tris solution to pH 8.0 with IN HCI and final volume adjusted to I L. Any desired molality is prepared by adjusting with distilled water.

SDS-PAGE Electrophoresis reagents

Below is a list of' solutions for preparing polyacrylamide gels starting from scratch. They are taken rum a well- known procedure for SDS-polyacrylamide gel

electrophoresis. I have included the details for future reference. for this procedure polyacrylamide gels consisting of a 10% separating gel and a 4% stacking gel ‘all be discussed. The different solutions listed below were used the electrophoresis.

Solution 1

10% total acrylamide, 2.67% cross-linking

Combine 14.6g acrylamide and 0.4 g N’N’-bis-methylene-acrylamide. Dilute to 50 mL with distilled \\ater. Filter and store at 4 °C in the dark. Caution: solid acrylamide and acrylamide solutions are toxic. As old contact with skin and breathing of dust.

Solution 2

1.5 M Tris-HCl, pH 8.0

Dissolve 27.23 g Tris base in approximately 80 mL distilled water. Adjust the pH to 8.8 with 1 N HCI. Prepare to 150mL with distilled water and store at 4°C.

Solution 3

0.5 M Tris-1-ICl, pH 6.8

Dissolve 6.0g Tris base with approximately 60 mL distilled water. Adjust to pH 6.8 with 1 N HCI. Prepare to 100 mL with distilled water.

Solution 4

10% SDS (sodium dodecyl sulfate) Dissolve 10g SDS in distilled and bring to 100mL vim distilled water. Store at room temperature.

Solution 5

5X electrode buffer Combine 9.0g Tris base, 43.2g glycine, and 3g SDS. Bring to 600ml. with distilled water. Store at 4 °C. For one electrophoresis run, dilute 120mL of 5X stock with 480mL distilled water.

Solution 6

10% ammonium persulfate

Dissolve 100 mg smmonium persulfate in 1mL distilled water. Make fresh daily.

Solution 7 0.1% Coornassie Blue stain

Combine 200mL methanol, 50mL acetic acid, 250mL distilled water, and 0.5g Coomassie Blue. Filter solution before using.

Solution 8

Destainer

Mix 40% methanol, 10% acetic acid, and 50% distilled water.

Solution 9

2X Laemmli buffer

Combine 4mL distilled water, 1mL solution 3, 0.8mL glycerol, 1.6mL solution 4. 0.4mL 2-mercaptoethanol. and 0.2mL of 0.5% Bromophenol blue.

Preparation of 10% Separating Gel.

Combine 4.0mL distilled water 3.33mL solution 1 2.5mL solution 2 100-microliter solution 4. Degas for at least 15 mm at room temperature.

Add 50-µL solutions 6

Add 5 microliter TEMED

Swirl solution gently and fill the glass tubes immediately.

Carefully layer glycerol on the top of the gel, being careful not to disturb the gel a1lov undisturbed for 20-30 mm while the gels solidify.

P**reparation of the 4% stacking gel**

Combine 4.0mL distilled water 3.33mL solution 1 2.5mL solution 2 100 microliter solution 4Degas for at least 15 mm at room temperature.

Pour off the water from the top of the separating gel in the sandwiches.

Insert the combs into the glass sandwiches.

Add 50 microliters of solution 6

Add 10 microliters of TEMED to the solution.

Swirl the solution gently and fill the sandwich to the tops of the combs. NOTE: do not allow air bubbles to form under the combs.

Let stand for 20-30 mm.

Preparation and application of samples

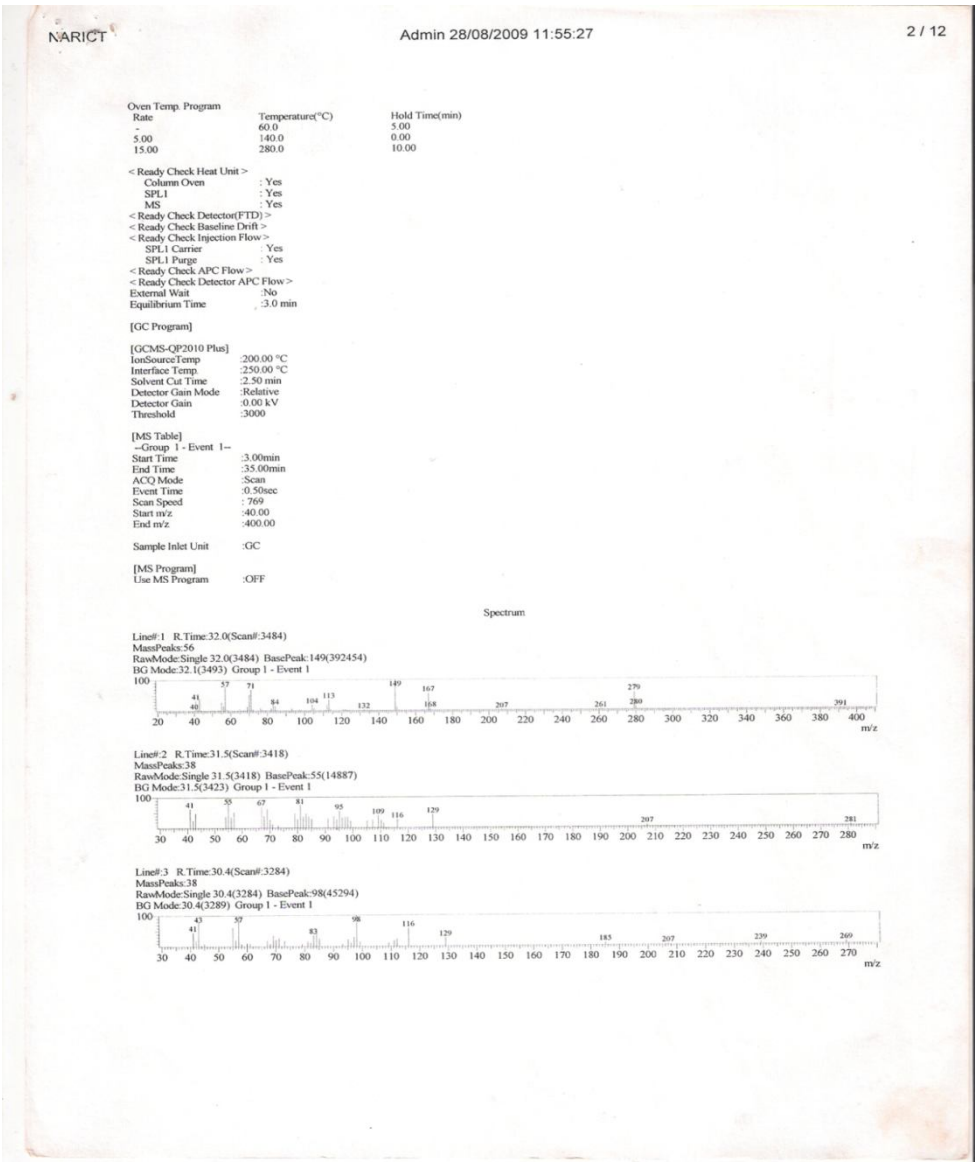
Add each sample (50μL) into separate microfuge tubes and mix with an equal volume of 2X Laemmli buffer (solution 9) mercaptoethanol (50μL), (and bromophenol blue dye (50μL).

Cover the tubes, mix them thoroughly and put them in a boiling water bath for 5 min immediately prior to application. Assemble the electrophoresis apparatus according to instructions in the manual. Fill the inner chamber with electrode buffer (solution 5).

Fill the outer chamber with the remaining buffer (solution 5).

Appendix I

GC/MS and IR Spectra



NARICT Admin 28/08/2009 11:55:27 2 / 12

Oven Temp. Program

Rate	Temperature(°C)	Hold Time(min)
--	60.0	5.00
5.00	140.0	0.00
15.00	280.0	10.00

< Ready Check Heat Unit >
Column Oven : Yes
SPL1 : Yes
MS : Yes
< Ready Check Detector(FTD) >
< Ready Check Baseline Drift >
< Ready Check Injection Flow >
SPL1 Carrier : Yes
SPL1 Purge : Yes
< Ready Check APC Flow >
< Ready Check Detector APC Flow >
External Wait :No
Equilibrium Time :3.0 min

[GC Program]

[GCMS-QP2010 Plus]
IonSourceTemp :200.00 °C
Interface Temp. :250.00 °C
Solvent Cut Time :2.50 min
Detector Gain Mode :Relative
Detector Gain :0.00 kV
Threshold :3000

[MS Table]
--Group 1 - Event 1--
Start Time :3.00min
End Time :35.00min
ACQ Mode :Scan
Event Time :0.50sec
Scan Speed : 769
Start m/z :40.00
End m/z :400.00

Sample Inlet Unit :GC

[MS Program]
Use MS Program :OFF

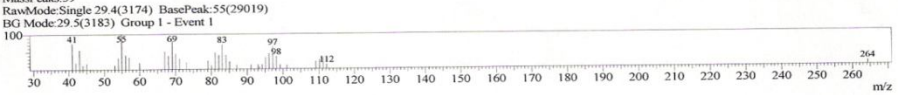
Spectrum

Line#:1 R.Time:32.0(Scan#:3484)
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RawMode:Single 32.0(3484) BasePeak:149(392454)
BG Mode:32.1(3493) Group 1 - Event 1

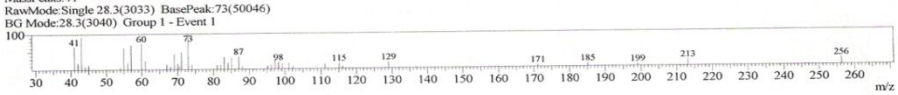
Line#:2 R.Time:31.5(Scan#:3418)
MassPeaks:38
RawMode:Single 31.5(3418) BasePeak:55(14887)
BG Mode:31.5(3423) Group 1 - Event 1

Line#:3 R.Time:30.4(Scan#:3284)
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BG Mode:30.4(3289) Group 1 - Event 1

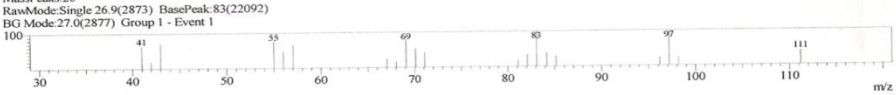
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RawMode:Single 29.4(3174) BasePeak:55(29019)
BG Mode:29.5(3183) Group 1 - Event 1



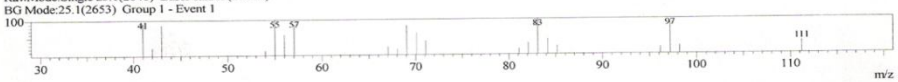
Line#:5 R.Time:28.3(Scan#:3033)
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BG Mode:28.3(3040) Group 1 - Event 1



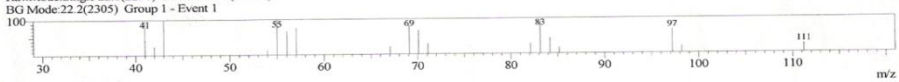
Line#:6 R.Time:26.9(Scan#:2873)
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BG Mode:27.0(2877) Group 1 - Event 1



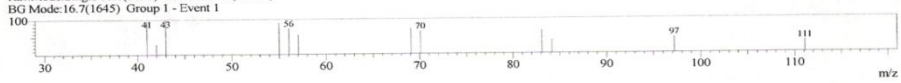
Line#:7 R.Time:25.1(Scan#:2648)
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RawMode:Single 25.1(2648) BasePeak:83(27668)
BG Mode:25.1(2653) Group 1 - Event 1



Line#:8 R.Time:22.1(Scan#:2296)
MassPeaks:18
RawMode:Single 22.1(2296) BasePeak:55(20268)
BG Mode:22.2(2305) Group 1 - Event 1



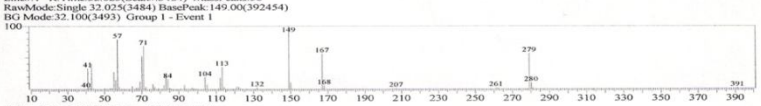
Line#:9 R.Time:16.6(Scan#:1636)
MassPeaks:12
RawMode:Single 16.6(1636) BasePeak:43(11924)
BG Mode:16.7(1645) Group 1 - Event 1



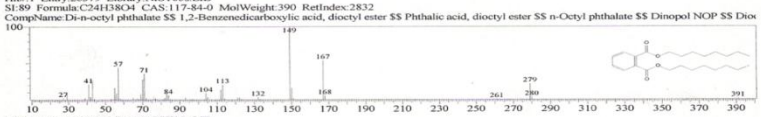
Spectrum Comparison

Library

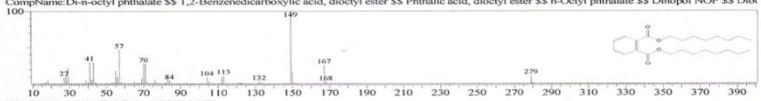
<< Target >>
Line#:1 R.Time:32.025(Scan#:3484) MassPeaks:56
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BG Mode:32.100(3493) Group 1 - Event 1



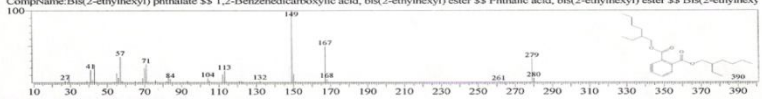
Hit#:1 Entry:26379 Library:NIST05s.LIB
SI:89 Formula:C24H38O4 CAS:117-84-0 MolWeight:390 RetIndex:2832
CompName:Di-n-octyl phthalate $$ 1,2-Benzenedicarboxylic acid, dioctyl ester $$ Phthalic acid, dioctyl ester $$ n-Octyl phthalate $$ Dinopol NOP $$ Dio



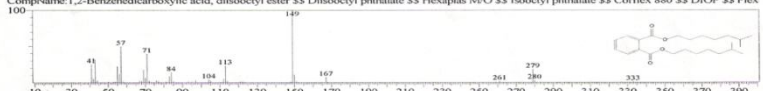
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SI:86 Formula:C24H38O4 CAS:117-84-0 MolWeight:390 RetIndex:2832
CompName:Di-n-octyl phthalate $$ 1,2-Benzenedicarboxylic acid, dioctyl ester $$ Phthalic acid, dioctyl ester $$ n-Octyl phthalate $$ Dinopol NOP $$ Dio



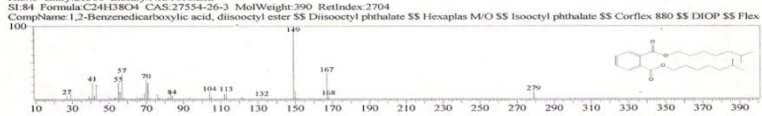
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SI:85 Formula:C24H38O4 CAS:117-81-7 MolWeight:390 RetIndex:2704
CompName:Bis(2-ethylhexyl) phthalate $$ 1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester $$ Phthalic acid, bis(2-ethylhexyl) ester $$ Bis(2-ethylhexy



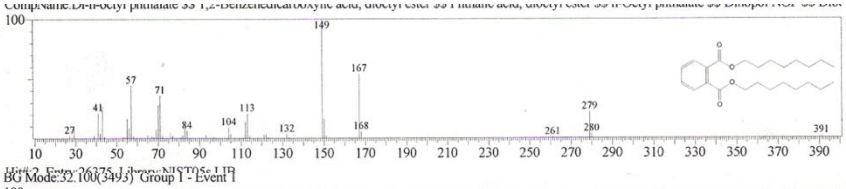
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SI:85 Formula:C24H38O4 CAS:27554-26-3 MolWeight:390 RetIndex:2704
CompName:1,2-Benzenedicarboxylic acid, diisooctyl ester $$ Diisooctyl phthalate $$ Hexaplas M/O $$ Isooctyl phthalate $$ Corflex 880 $$ DIOP $$ Flex



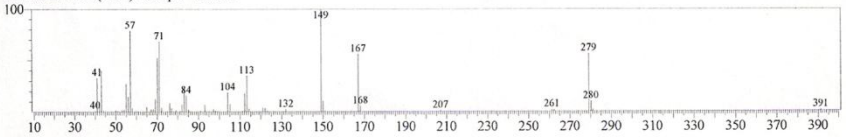
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CompName:1,2-Benzenedicarboxylic acid, diisooctyl ester $$ Diisooctyl phthalate $$ Hexaplas M/O $$ Isooctyl phthalate $$ Corflex 880 $$ DIOP $$ Flex



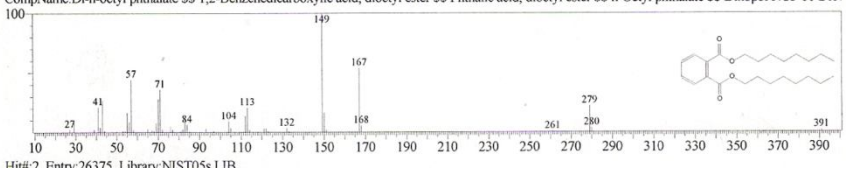
CompName:Di-n-octyl phthalate $$ 1,2-Benzenedicarboxylic acid, dioctyl ester $$ Phthalic acid, dioctyl ester $$ n-Octyl phthalate $$ Dinopol NOP $$ Dio



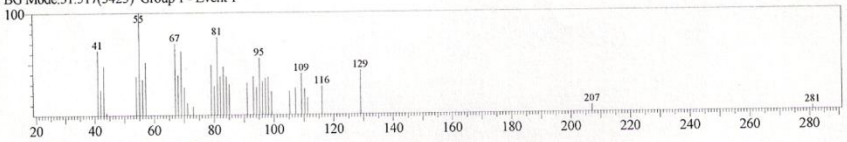
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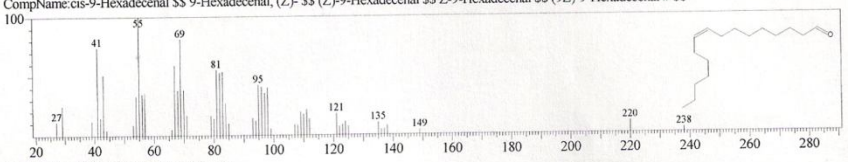
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CompName:Di-n-octyl phthalate $$ 1,2-Benzenedicarboxylic acid, dioctyl ester $$ Phthalic acid, dioctyl ester $$ n-Octyl phthalate $$ Dinopol NOP $$ Dio



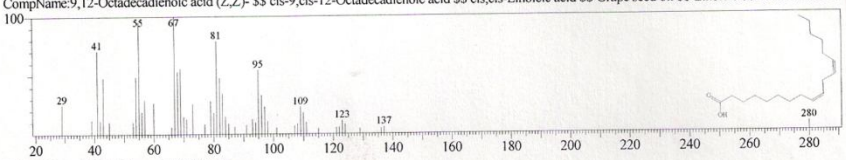
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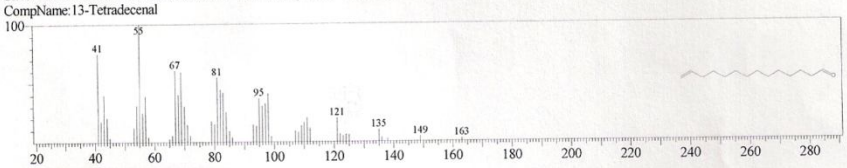
Hit#:1 Entry:64130 Library:NIST05.LIB
SI:83 Formula:C16H30O CAS:56219-04-6 MolWeight:238 RetIndex:1808
CompName:cis-9-Hexadecenal $$ 9-Hexadecenal, (Z)- $$ (Z)-9-Hexadecenal $$ Z-9-Hexadecenal $$ (9Z)-9-Hexadecenal # $$



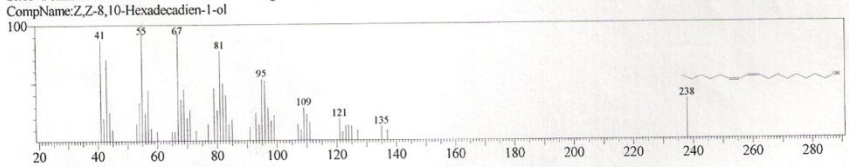
Hit#:2 Entry:22757 Library:NIST05s.LIB
SI:83 Formula:C18H32O2 CAS:60-33-3 MolWeight:280 RetIndex:2183
CompName:9,12-Octadecadienoic acid (Z,Z)- $$ cis-9,cis-12-Octadecadienoic acid $$ cis,cis-Linoleic acid $$ Grape seed oil $$ Linoleic $$ Linoleic acid $$



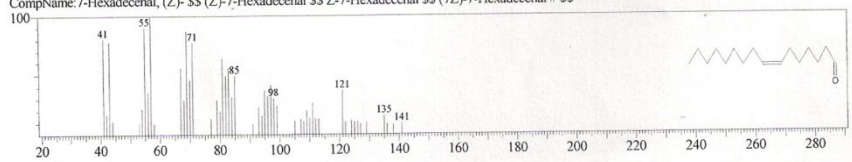
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SI:83 Formula:C14H26O CAS:85896-31-7 MolWeight:210 RetIndex:1591
CompName:13-Tetradecenal



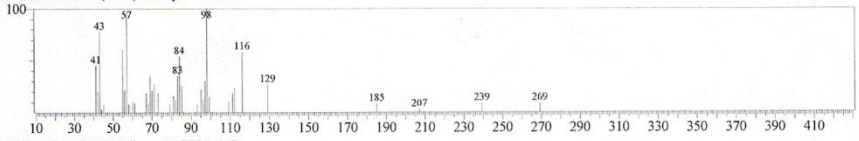
Hit#:4 Entry:64129 Library:NIST05.LIB
SI:83 Formula:C16H30O CAS:0-00-0 MolWeight:238 RetIndex:1870
CompName:Z,Z-8,10-Hexadecadien-1-ol



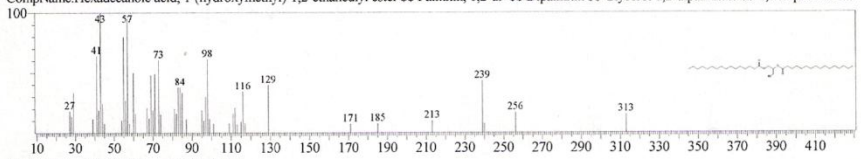
Hit#:5 Entry:20038 Library:NIST05s.LIB
SI:82 Formula:C16H30O CAS:56797-40-1 MolWeight:238 RetIndex:1808
CompName:7-Hexadecenal, (Z)- $$ (Z)-7-Hexadecenal $$ Z-7-Hexadecenal $$ (7Z)-7-Hexadecenal # $$



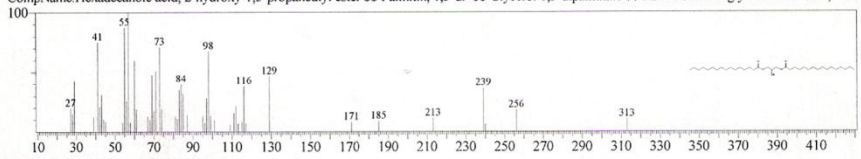
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Line#:3 R.Time:30.358(Scan#:3284) MassPeaks:38
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BG Mode:30.400(3289) Group 1 - Event 1



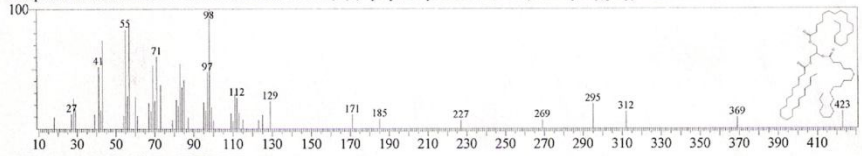
Hit#:1 Entry:27447 Library:NIST05s.LIB
SI:85 Formula:C35H68O5 CAS:761-35-3 MolWeight:568 RetIndex:4013
CompName:Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester $$ Palmitin, 1,2-di- $$ Dipalmitin $$ Glycerol 1,2-dipalmitate $$ 1,2-Dipalmitin $$



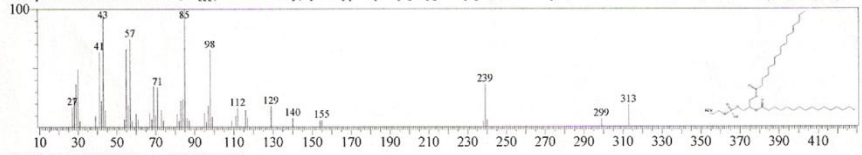
Hit#:2 Entry:160166 Library:NIST05.LIB
SI:83 Formula:C35H68O5 CAS:502-52-3 MolWeight:568 RetIndex:3997
CompName:Hexadecanoic acid, 2-hydroxy-1,3-propanediyl ester $$ Palmitin, 1,3-di- $$ Glycerol 1,3-dipalmitate $$ Palmitic acid diglycerin ester $$ 1,3-Di



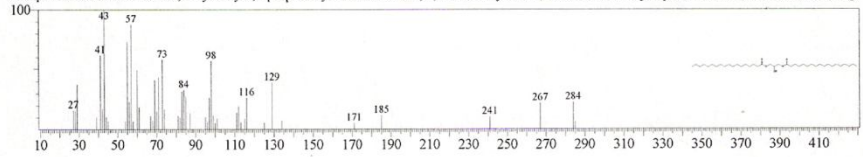
Hit#:3 Entry:163124 Library:NIST05.LIB
SI:83 Formula:C63H122O6 CAS:620-64-4 MolWeight:974 RetIndex:6721
CompName:Triarachine $$ Triarachidin $$ Eicosanoic acid, 1,2,3-propanetriyl ester $$ 2,3-Bis(icosanoyloxy)propyl icosanoate # $$



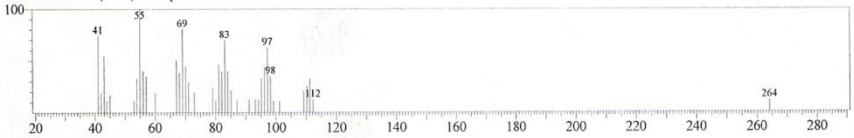
Hit#:4 Entry:162295 Library:NIST05.LIB
SI:83 Formula:C37H74NO8P CAS:3026-45-7 MolWeight:691 RetIndex:0
CompName:Hexadecanoic acid, 1-[[[(2-aminoethoxy)hydroxyphosphinyl]oxy]methyl]-1,2-ethanediyl ester $$ Palmitin, 1,2-di-, 2-aminoethyl hydrogen phos



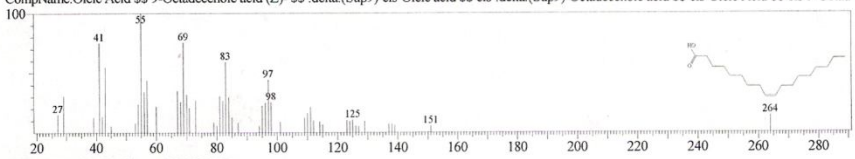
Hit#:5 Entry:27524 Library:NIST05s.LIB
SI:83 Formula:C39H76O5 CAS:504-40-5 MolWeight:624 RetIndex:4395
CompName:Octadecanoic acid, 2-hydroxy-1,3-propanediyl ester $$ Stearin, 1,3-di- $$ Glycerin 1,3-distearate $$ Glyceryl 1,3-distearate $$ Stearic acid digl



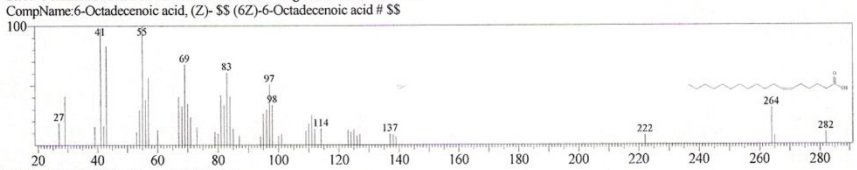
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Line#:4 R.Time:29.442(Scan#:3174) MassPeaks:39
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BG Mode:29.517(3183) Group 1 - Event 1



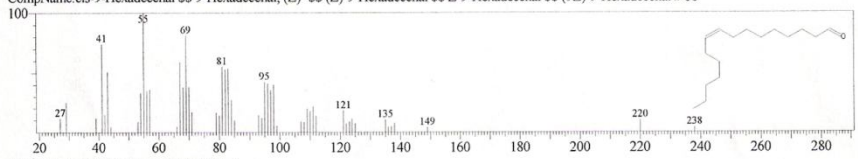
Hit#:1 Entry:90577 Library:NIST05.LIB
SI:88 Formula:C18H34O2 CAS:112-80-1 MolWeight:282 RetIndex:2175
CompName:Oleic Acid $$ 9-Octadecenoic acid (Z)- $$ .delta.(Sup9)-cis-Oleic acid $$ cis-.delta.(Sup9)-Octadecenoic acid $$ cis-Oleic Acid $$ cis-9-Octad



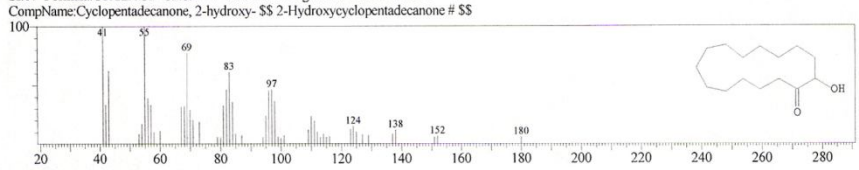
Hit#:2 Entry:90568 Library:NIST05.LIB
SI:87 Formula:C18H34O2 CAS:593-39-5 MolWeight:282 RetIndex:2175
CompName:6-Octadecenoic acid, (Z)- $$ (6Z)-6-Octadecenoic acid # $$



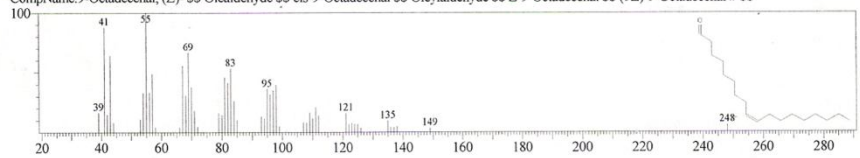
Hit#:3 Entry:64130 Library:NIST05.LIB
SI:87 Formula:C16H30O CAS:56219-04-6 MolWeight:238 RetIndex:1808
CompName:cis-9-Hexadecenal $$ 9-Hexadecenal, (Z)- $$ (Z)-9-Hexadecenal $$ Z-9-Hexadecenal $$ (9Z)-9-Hexadecenal # $$

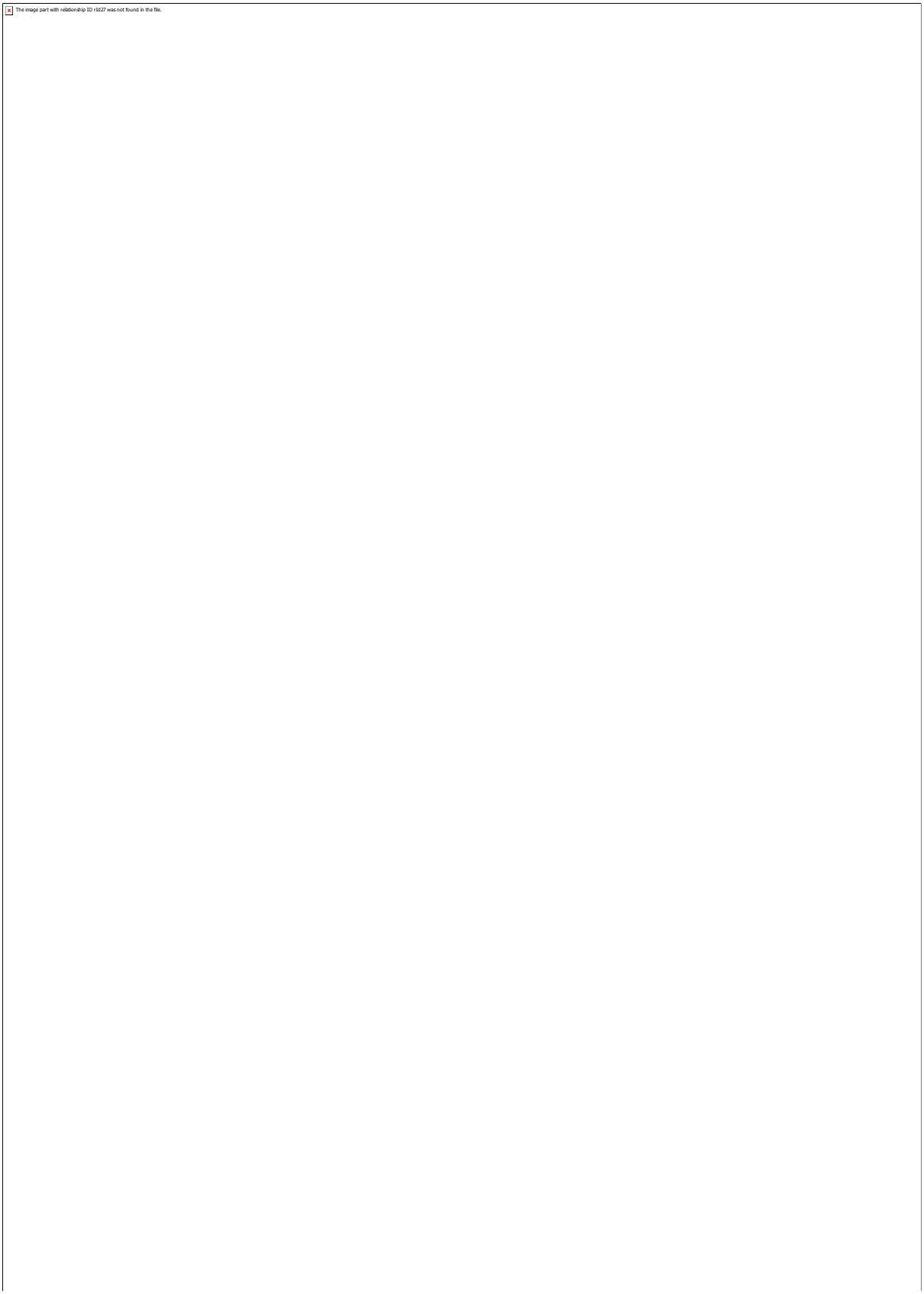


Hit#:4 Entry:65295 Library:NIST05.LIB
SI:87 Formula:C15H28O2 CAS:4727-18-8 MolWeight:240 RetIndex:2158
CompName:Cyclopentadecanone, 2-hydroxy- $$ 2-Hydroxycyclopentadecanone # $$

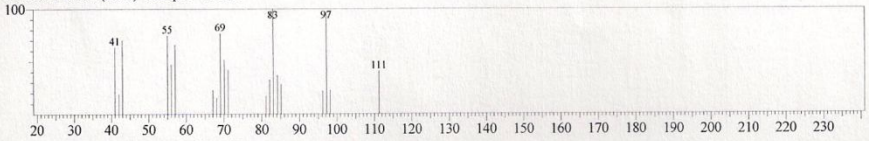


Hit#:5 Entry:80935 Library:NIST05.LIB
SI:86 Formula:C18H34O CAS:2423-10-1 MolWeight:266 RetIndex:2007
CompName:9-Octadecenal, (Z)- $$ Olealdehyde $$ cis-9-Octadecenal $$ Oleylaldehyde $$ Z-9-Octadecenal $$ (9Z)-9-Octadecenal # $$

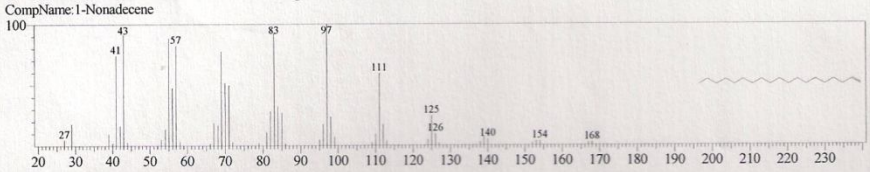



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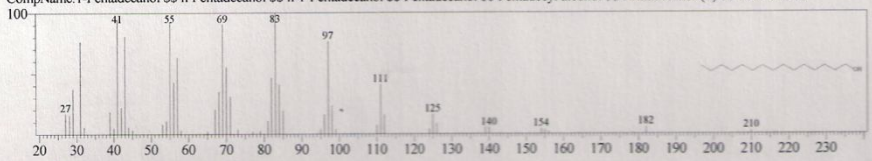
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BG Mode:26.967(2877) Group 1 - Event 1



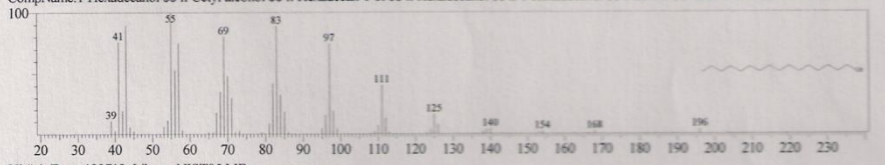
Hit#:1 Entry:21960 Library:NIST05s.LIB
SI:90 Formula:C19H38 CAS:18435-45-5 MolWeight:266 RetIndex:1900
CompName:1-Nonadecene



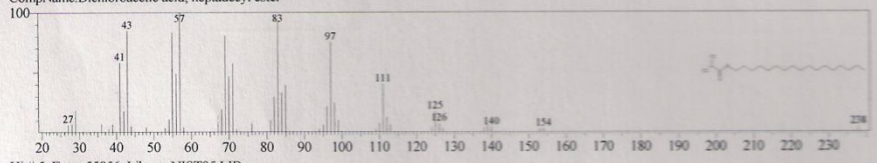
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SI:90 Formula:C15H32O CAS:629-76-5 MolWeight:228 RetIndex:1755
CompName:1-Pentadecanol $$ n-Pentadecanol $$ n-1-Pentadecanol $$ Pentadecanol $$ Pentadecyl alcohol $$ Pentadecanol-(1) $$ Neodol 5 $$



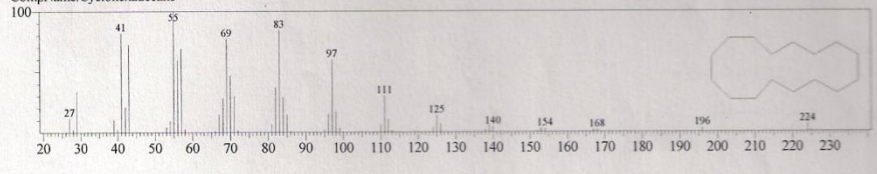
Hit#:3 Entry:20391 Library:NIST05s.LIB
SI:90 Formula:C16H34O CAS:36653-82-4 MolWeight:242 RetIndex:1854
CompName:1-Hexadecanol $$ n-Cetyl alcohol $$ n-Hexadecan-1-ol $$ n-Hexadecanol $$ n-1-Hexadecanol $$ Adol 52 $$ Adol 52 NF $$ Adol 54 $$ Aldc



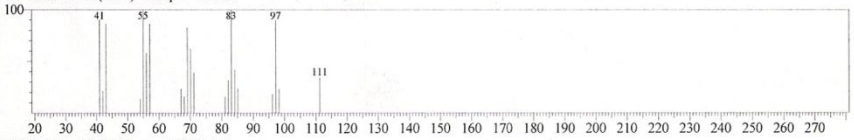
Hit#:4 Entry:133713 Library:NIST05.LIB
SI:89 Formula:C19H36Cl2O2 CAS:0-00-0 MolWeight:366 RetIndex:2423
CompName:Dichloroacetic acid, heptadecyl ester



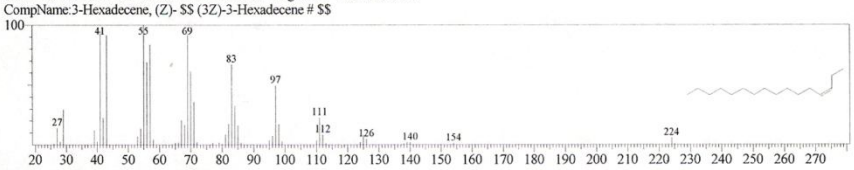
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SI:89 Formula:C16H32 CAS:295-65-8 MolWeight:224 RetIndex:1918
CompName:Cyclohexadecane



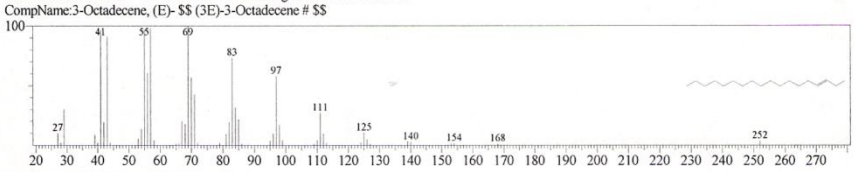
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Line#:7 R.Time:25.058(Scan#:2648) MassPeaks:21
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BG Mode:25.100(2653) Group 1 - Event 1



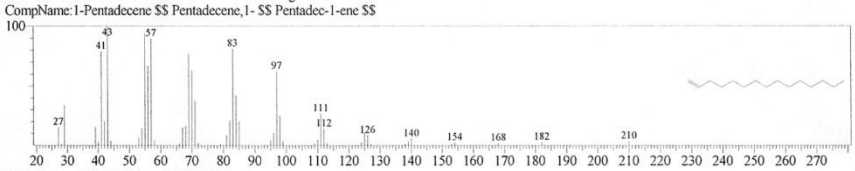
Hit#:1 Entry:55854 Library:NIST05.LIB
SI:92 Formula:C16H32 CAS:34303-81-6 MolWeight:224 RetIndex:1620
CompName:3-Hexadecene, (Z)- $$ (3Z)-3-Hexadecene # $$



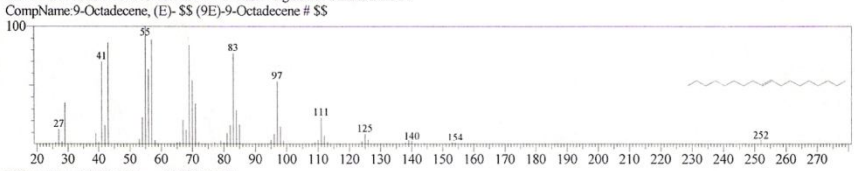
Hit#:2 Entry:72615 Library:NIST05.LIB
SI:92 Formula:C18H36 CAS:7206-19-1 MolWeight:252 RetIndex:1818
CompName:3-Octadecene, (E)- $$ (3E)-3-Octadecene # $$



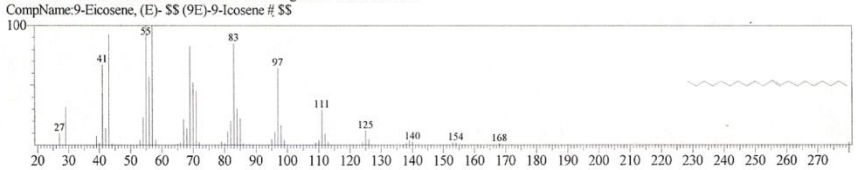
Hit#:3 Entry:17440 Library:NIST05s.LIB
SI:92 Formula:C15H30 CAS:13360-61-7 MolWeight:210 RetIndex:1502
CompName:1-Pentadecene $$ Pentadecene,1- $$ Pentadec-1-ene $$



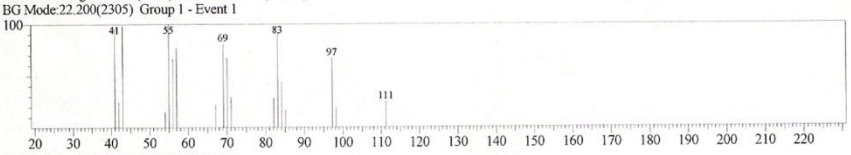
Hit#:4 Entry:72607 Library:NIST05.LIB
SI:92 Formula:C18H36 CAS:7206-25-9 MolWeight:252 RetIndex:1818
CompName:9-Octadecene, (E)- $$ (9E)-9-Octadecene # $$



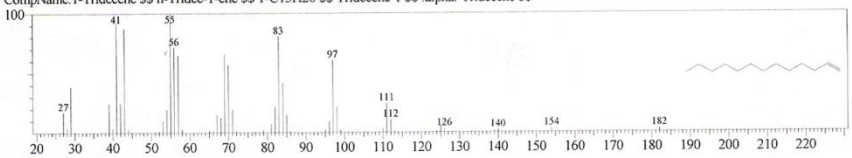
Hit#:5 Entry:89386 Library:NIST05.LIB
SI:92 Formula:C20H40 CAS:74685-29-3 MolWeight:280 RetIndex:2017
CompName:9-Eicosene, (E)- $$ (9E)-9-Icosene # $$



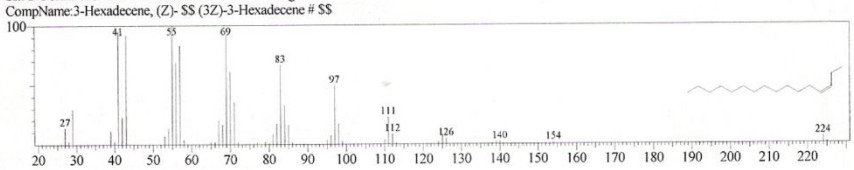
<< Target >>
Line#:8 R.Time:22.125(Scan#:2296) MassPeaks:18
RawMode:Single 22.125(2296) BasePeak:55.00(20268)
BG Mode:22.200(2305) Group 1 - Event 1



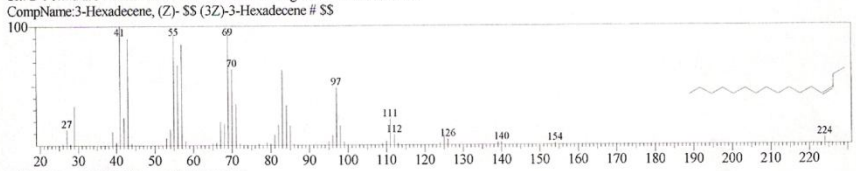
Hit#:1 Entry:13797 Library:NIST05s.LIB
SI:92 Formula:C13H26 CAS:2437-56-1 MolWeight:182 RetIndex:1304
CompName:1-Tridecene $$ n-Tridec-1-ene $$ 1-C13H26 $$ Tridecene-1 $$ .alpha.-Tridecene $$



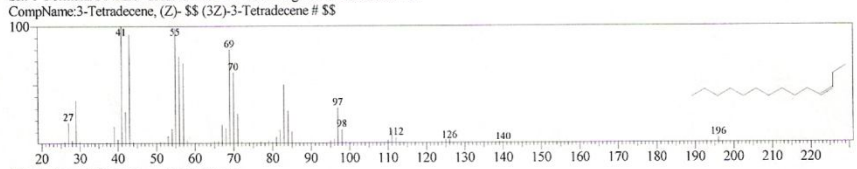
Hit#:2 Entry:55854 Library:NIST05.LIB
SI:92 Formula:C16H32 CAS:34303-81-6 MolWeight:224 RetIndex:1620
CompName:3-Hexadecene, (Z)- $$ (3Z)-3-Hexadecene # $$



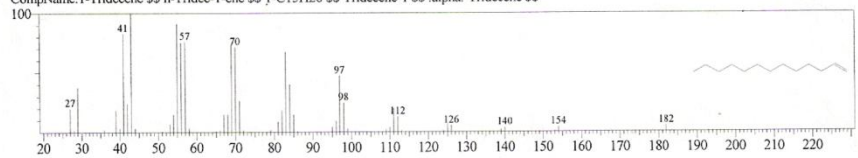
Hit#:3 Entry:18878 Library:NIST05s.LIB
SI:92 Formula:C16H32 CAS:34303-81-6 MolWeight:224 RetIndex:1620
CompName:3-Hexadecene, (Z)- $$ (3Z)-3-Hexadecene # $$



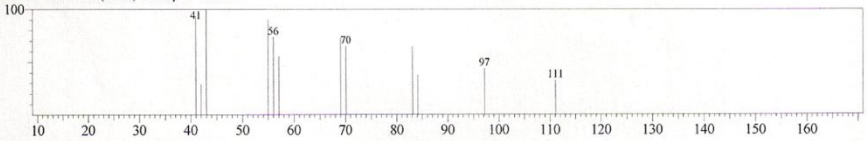
Hit#:4 Entry:39067 Library:NIST05.LIB
SI:91 Formula:C14H28 CAS:41446-67-7 MolWeight:196 RetIndex:1421
CompName:3-Tetradecene, (Z)- $$ (3Z)-3-Tetradecene # $$



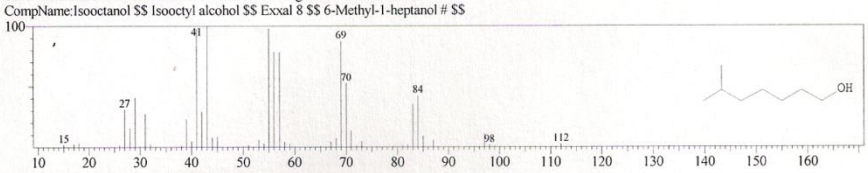
Hit#:5 Entry:31316 Library:NIST05.LIB
SI:91 Formula:C13H26 CAS:2437-56-1 MolWeight:182 RetIndex:1304
CompName:1-Tridecene $$ n-Tridec-1-ene $$ 1-C13H26 $$ Tridecene-1 $$ .alpha.-Tridecene $$



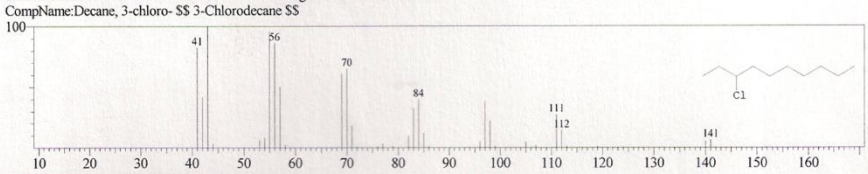
<< Target >>
Line#:9 R.Time:16.625(Scan#:1636) MassPeaks:12
RawMode:Single 16.625(1636) BasePeak:43.00(11924)
BG Mode:16.700(1645) Group 1 - Event 1



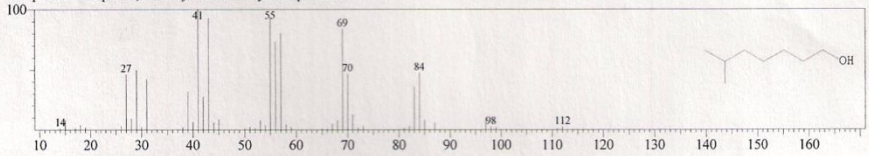
Hit#:1 Entry:7845 Library:NIST05.LIB
SI:89 Formula:C8H18O CAS:26952-21-6 MolWeight:130 RetIndex:995
CompName:Isooctanol $$ Isooctyl alcohol $$ Exxal 8 $$ 6-Methyl-1-heptanol # $$



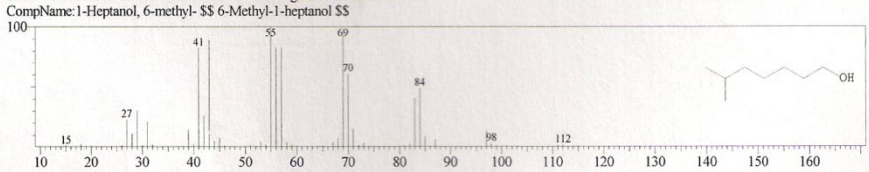
Hit#:2 Entry:27713 Library:NIST05.LIB
SI:89 Formula:C10H21Cl CAS:1002-11-5 MolWeight:176 RetIndex:1156
CompName:Decane, 3-chloro- $$ 3-Chlorodecane $$



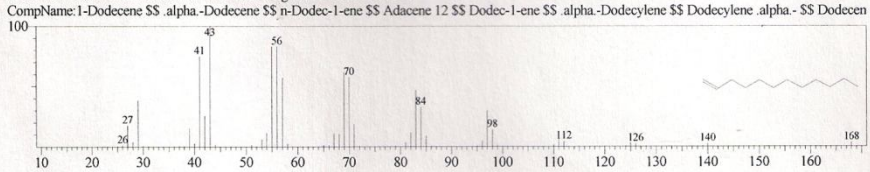
Hit#:3 Entry:7843 Library:NIST05.LIB
SI:88 Formula:C8H18O CAS:1653-40-3 MolWeight:130 RetIndex:995
CompName:1-Heptanol, 6-methyl- $$ 6-Methyl-1-heptanol $$

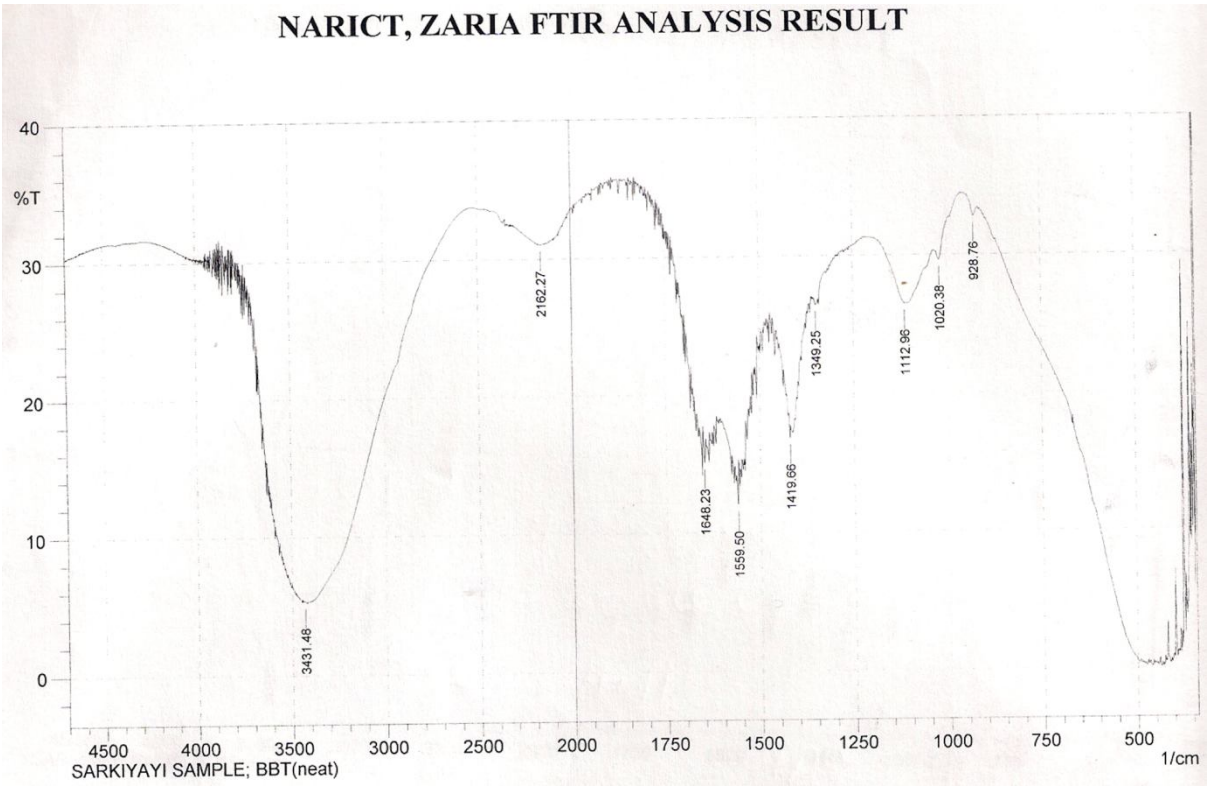


Hit#:4 Entry:5346 Library:NIST05s.LIB
SI:88 Formula:C8H18O CAS:1653-40-3 MolWeight:130 RetIndex:995
CompName:1-Heptanol, 6-methyl- $$ 6-Methyl-1-heptanol $$



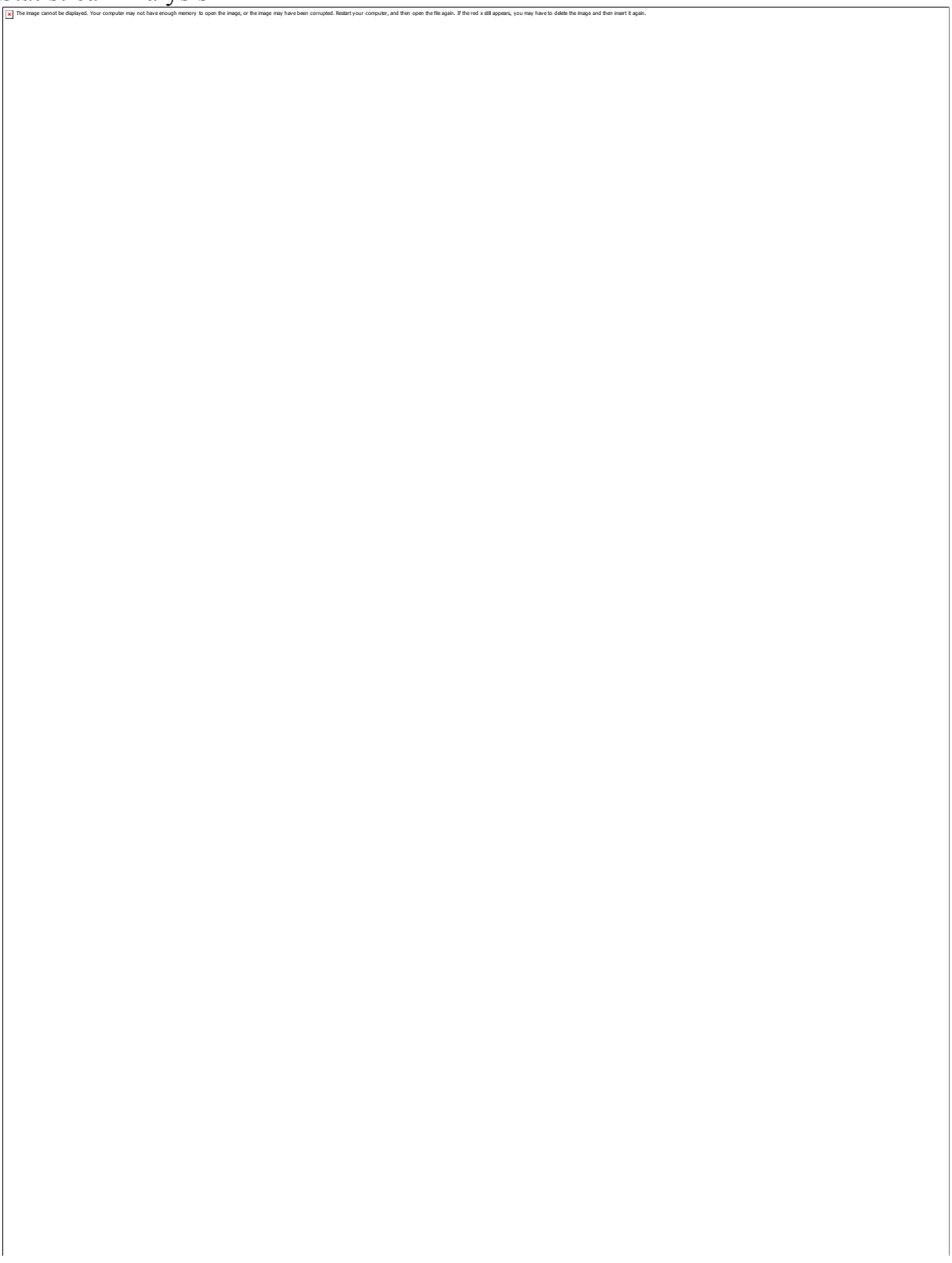
Hit#:5 Entry:11674 Library:NIST05s.LIB
SI:88 Formula:C12H24 CAS:112-41-4 MolWeight:168 RetIndex:1204
CompName:1-Dodecene $$ .alpha.-Dodecene $$ n-Dodec-1-ene $$ Adecene 12 $$ Dodec-1-ene $$ .alpha.-Dodecylene $$ Dodecylene .alpha.- $$ Dodecen




NARICT, ZARIA FTIR ANALYSIS RESULT
40
%T
30
20
10
0
3431.48
2162.27
1648.23
1559.50
1419.66
1349.25
1112.96
1020.38
928.76
4500
4000
3500
3000
2500
2000
1750
1500
1250
1000
750
500
1/cm
SARKIYAYI SAMPLE; BBT(neat)

Appendix J

Statistical Analysis



VAR00004	33.162	4	.000	21.68000	19.8649	23.4951

19.50	19.90	20.10	20.20	.	.
21.50	20.90	20.90	21.00	.	.
20.00	20.00	20.80	20.80	.	.
22.80	21.90	22.00	22.70	.	.
23.50	23.70	23.60	23.70	.	.CONTROL GROUP

BODY WT ENDS

Descriptive Statistics250MG/KG

	N	Minimum	Maximum	Mean		Std. Deviation	Variance
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Statistic
UREA	5	3.00	11.00	8.2400	1.36843	3.05990	9.363
Na	5	129.00	141.00	133.4000	2.11187	4.72229	22.300
K	5	3.00	5.40	3.8200	.44542	.99599	.992
CI	5	19.00	97.00	79.2000	15.09768	33.75944	1139.700
B/Carbonate	5	10.00	24.00	19.0000	2.77489	6.20484	38.500
creatinine	5	58.00	145.00	117.6000	15.59679	34.87549	1216.300
SGOT	5	19.00	31.00	25.6000	2.08806	4.66905	21.800
SGPT	5	5.00	18.00	8.8000	2.41661	5.40370	29.200
ALK/P	5	3.00	46.00	14.0000	8.04363	17.98611	323.500
TOTAL BILIRUBIN	5	17.00	17.00	17.0000	.00000	.00000	.000
CONJ BILI	5	17.00	17.00	17.0000	.00000	.00000	.000
TOTAL PROTEIN	5	70.00	73.00	71.6000	.67823	1.51658	2.300
ALBUMIN	5	33.00	38.00	36.2000	1.11355	2.48998	6.200
PCV	5	45.00	49.00	46.4000	.74833	1.67332	2.800
Valid N (listwise)	5						

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
UREA	5	8.2400	3.05990	1.36843
Na	5	133.4000	4.72229	2.11187
K	5	3.8200	.99599	.44542
CI	5	79.2000	33.75944	15.09768
B/Carbonate	5	19.0000	6.20484	2.77489
creatinine	5	117.6000	34.87549	15.59679
SGOT	5	25.6000	4.66905	2.08806
SGPT	5	8.8000	5.40370	2.41661
ALK/P	5	14.0000	17.98611	8.04363
TOTAL BILIRUBIN	5	17.0000	.00000(a)	.00000

SGOT	5	132.8000	4.38178	1.95959
SGPT	5	17.4000	1.51658	.67823
ALK/P	5	66.6000	30.69691	13.72807
TOTAL BILIRUBIN	5	14.8000	6.72309	3.00666
CONJ BILI	5	130.8000	9.12140	4.07922
TOTAL PROTEIN	5	25.2000	4.43847	1.98494
ALBUMIN	5	6.2000	.83666	.37417
PCV	5	7.0000	1.00000	.44721

a t cannot be computed because the sum of caseweights is less than or equal 1.
b t cannot be computed. There are no valid cases for this analysis because all caseweights are not positive.

One-Sample Test

	Test Value = 0					
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
	Lower	Upper	Lower	Upper	Lower	Upper
creatinine	12.402	4	.000	9.36000	7.2646	11.4554
SGOT	67.769	4	.000	132.80000	127.3593	138.2407
SGPT	25.655	4	.000	17.40000	15.5169	19.2831
ALK/P	4.851	4	.008	66.60000	28.4848	104.7152
TOTAL BILIRUBIN	4.922	4	.008	14.80000	6.4522	23.1478
CONJ BILI	32.065	4	.000	130.80000	119.4743	142.1257
TOTAL PROTEIN	12.696	4	.000	25.20000	19.6889	30.7111
ALBUMIN	16.570	4	.000	6.20000	5.1611	7.2389
PCV	15.652	4	.000	7.00000	5.7583	8.2417

10.00 130.00 19.00 33.00 10.00 140.00 30.00 5.00 6.00 17.00 17.00 72.00 37.00 48.00
8.80 140.00 16.00 90.00 20.00 120.00 21.00 7.00 8.00 17.00 17.00 72.00 38.00 44.00
8.00 130.00 19.00 33.00 24.00 124.00 22.00 6.00 8.00 17.00 17.00 70.00 36.00 46.00
8.00 130.00 17.00 87.00 10.00 130.00 23.00 6.00 7.00 17.00 17.00 70.00 34.00 49.00
12.00 134.00 16.00 90.00 10.00 140.00 30.00 7.00 6.00 17.00 17.00 78.00 38.00 42.00

Descriptive Statistics

	N	Minimum	Maximum	Mean		Std. Deviation	Variance
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Statistic
PCV B4 V/E	5	39.00	60.00	52.6000	3.57211	7.98749	63.800
TP B4	5	5.70	7.60	6.5400	.33556	.75033	.563
HB B4	5	13.00	20.00	16.7200	1.20764	2.70037	7.292
PCV AFT V/E	5	30.00	54.00	48.0000	4.56070	10.19804	104.000
TP AFT	5	5.00	7.00	5.9800	.32619	.72938	.532
HB AFT	5	10.00	18.00	15.9600	1.51545	3.38866	11.483
PCV B4 V ONLY	5	48.00	60.00	52.2000	2.20000	4.91935	24.200
TP B4	5	5.50	7.40	6.2800	.32156	.71903	.517

HB B4	5	16.00	20.00	17.3800	.73919	1.65288	2.732
PCV AFT	4	35.00	45.00	39.5000	2.10159	4.20317	17.667
TP AFT	4	5.00	7.00	6.1250	.42696	.85391	.729
HB AFT	4	11.70	20.00	14.4250	1.88740	3.77481	14.249
PCV B4 CONTROL	5	45.00	65.00	58.0000	3.39116	7.58288	57.500
TB B4	5	5.40	6.50	5.6600	.21119	.47223	.223
HB B4	5	15.00	20.00	17.4000	1.12250	2.50998	6.300
PCV AFT	5	53.00	66.00	61.2000	2.22261	4.96991	24.700
TP AFT	5	4.70	5.80	5.3000	.20000	.44721	.200
HB AFT	5	17.00	22.00	19.1400	.98975	2.21314	4.898
WBCAFT V ONLY	4	3.90	6.70	5.2750	.60052	1.20104	1.443
WBC AFT V/E	5	4.80	7.20	5.9200	.47476	1.06160	1.127
WBC AFT V/E Control	5	4.10	7.50	5.6200	.71652	1.60219	2.567
Valid N (listwise)	4						

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
PCV B4 V/E	5	52.6000	7.98749	3.57211
TP B4	5	6.5400	.75033	.33556
HB B4	5	16.7200	2.70037	1.20764
PCV AFT V/E	5	48.0000	10.19804	4.56070
TP AFT	5	5.9800	.72938	.32619
HB AFT	5	15.9600	3.38866	1.51545
PCV B4 V ONLY	5	52.2000	4.91935	2.20000
TP B4	5	6.2800	.71903	.32156
HB B4	5	17.3800	1.65288	.73919
PCV AFT	4	39.5000	4.20317	2.10159
TP AFT	4	6.1250	.85391	.42696
HB AFT	4	14.4250	3.77481	1.88740
PCV B4 CONTROL	5	58.0000	7.58288	3.39116
TB B4	5	5.6600	.47223	.21119
HB B4	5	17.4000	2.50998	1.12250
PCV AFT	5	61.2000	4.96991	2.22261
TP AFT	5	5.3000	.44721	.20000
HB AFT	5	19.1400	2.21314	.98975
WBCAFT V ONLY	4	5.2750	1.20104	.60052
WBC AFT V/E	5	5.9200	1.06160	.47476
WBC AFT V/E Control	5	5.6200	1.60219	.71652

One-Sample Test

	Test Value = 0					
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
	Lower	Upper	Lower	Upper	Lower	Upper

PCV B4 V/E	14.725	4	.000	52.60000	42.6822	62.5178
TP B4	19.490	4	.000	6.54000	5.6083	7.4717
HB B4	13.845	4	.000	16.72000	13.3670	20.0730
PCV AFT V/E	10.525	4	.000	48.00000	35.3375	60.6625
TP AFT	18.333	4	.000	5.98000	5.0744	6.8856
HB AFT	10.531	4	.000	15.96000	11.7524	20.1676
PCV B4 V ONLY	23.727	4	.000	52.20000	46.0918	58.3082
TP B4	19.530	4	.000	6.28000	5.3872	7.1728
HB B4	23.512	4	.000	17.38000	15.3277	19.4323
PCV AFT	18.795	3	.000	39.50000	32.8118	46.1882
TP AFT	14.346	3	.001	6.12500	4.7662	7.4838
HB AFT	7.643	3	.005	14.42500	8.4184	20.4316
PCV B4 CONTROL	17.103	4	.000	58.00000	48.5846	67.4154
TB B4	26.801	4	.000	5.66000	5.0737	6.2463
HB B4	15.501	4	.000	17.40000	14.2834	20.5166
PCV AFT	27.535	4	.000	61.20000	55.0290	67.3710
TP AFT	26.500	4	.000	5.30000	4.7447	5.8553
HB AFT	19.338	4	.000	19.14000	16.3920	21.8880
WBCAFT V ONLY	8.784	3	.003	5.27500	3.3639	7.1861
WBC AFT V/E	12.469	4	.000	5.92000	4.6018	7.2382
WBC AFT V/E	7.843	4	.001	5.62000	3.6306	7.6094

5.00	18.00		50.00		6.00	16.60		45.00		6.00	20.00
60.00	6.50	20.00		66.00		5.00	22.00		5.70	6.90	4.10
6.20	18.00		49.00		7.40	16.30		40.00		6.50	13.30
45.00	5.40	15.00		53.00		4.70	17.70		6.70	7.20	4.80
6.00	10.00		60.00		5.50	20.00		38.00		7.00	12.70
60.00	5.50	20.00		61.00		5.80	21.00		4.80	5.40	7.50
5.70	17.30		54.00		6.00	18.00		35.00		5.00	11.70
65.00	5.50	17.00		64.00		5.40	18.00		3.90	4.80	7.20
7.00	16.50		48.00		6.50	16.00	.	.	.		60.00
5.40	15.00		62.00		5.60	17.00	.		5.30	4.50	

PLA2 ACTIVITY

Descriptive Statistics

	N	Minimum	Maximum	Mean		Std. Deviation	Variance
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Statistic
PLA2Venomonly	5	3.66	5.20	4.3320	.27427	.61329	.376
PLA2 Venom/eq methanol extract	5	1.00	3.10	1.8600	.38288	.85615	.733
PLA2 Venom/n-butanol extract	5	1.80	3.10	2.3600	.22494	.50299	.253
PLA2 Venom/eq layer	5	1.70	2.90	2.2580	.25492	.57002	.325
Valid N (listwise)	5						

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
PLA2Venomonly	5	4.3320	.61329	.27427
PLA2 Venom/eq methanol extract	5	1.8600	.85615	.38288
PLA2 Venom/n-butanol extract	5	2.3600	.50299	.22494
PLA2 Venom/eq layer	5	2.2580	.57002	.25492

One-Sample Test

	Test Value = 0					
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
	Lower	Upper	Lower	Upper	Lower	Upper
PLA2Venomonly	15.795	4	.000	4.33200	3.5705	5.0935
PLA2 Venom/eq methanol extract	4.858	4	.008	1.86000	.7969	2.9231
PLA2 Venom/n-butanol extract	10.491	4	.000	2.36000	1.7355	2.9845
PLA2 Venom/eq layer	8.858	4	.001	2.25800	1.5502	2.9658

4.00	1.70	2.50	2.90
4.70	1.20	1.80	2.83
5.20	2.30	2.40	2.06
4.10	3.10	2.00	1.80
3.66	1.00	3.10	1.70

Descriptive Statistics

	N	Minimum	Maximum	Mean		Std. Deviation	Variance
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Statistic
hemolysis water gp	4	.83	.88	.8563	.01248	.02496	.001
hem ven/methanol extract	4	.70	.75	.7275	.01031	.02062	.000

venom only	4	.80	.87	.8255	.01539	.03078	.001
venom/n-butanol extract	4	.32	.39	.3500	.01581	.03162	.001
water only	4	1.15	1.30	1.2325	.03198	.06397	.004
venom/eq layer extract	4	.30	.59	.4400	.05958	.11916	.014
Valid N (listwise)	4						

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
hemolysis water gp	4	.8563	.02496	.01248
hem ven/methanol extract	4	.7275	.02062	.01031
venom only	4	.8255	.03078	.01539
venom/n-butanol extract	4	.3500	.03162	.01581
water only	4	1.2325	.06397	.03198
venom/eq layer extract	4	.4400	.11916	.05958

One-Sample Test

	Test Value = 0					
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
	Lower	Upper	Lower	Upper	Lower	Upper
hemolysis water gp	68.614	3	.000	.85625	.8165	.8960
hem ven/methanol extract	70.578	3	.000	.72750	.6947	.7603
venom only	53.631	3	.000	.82550	.7765	.8745
venom/n-butanol extract	22.136	3	.000	.35000	.2997	.4003
water only	38.536	3	.000	1.23250	1.1307	1.3343
venom/eq layer extract	7.385	3	.005	.44000	.2504	.6296

Inhibition due to extract

Descriptive Statistics

	N	Minimum	Maximum	Mean		Std. Deviation	Variance
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Statistic
PLA2 DEU FRACTION 1	2	4.10	4.20	4.1500	.05000	.07071	.005
PLA2 DEU FRACTION 1	2	3.00	3.20	3.1000	.10000	.14142	.020
PLA2 DEU FRACTION 1	2	2.20	2.40	2.3000	.10000	.14142	.020
PLA2 DEU FRACTION 1	2	1.10	1.20	1.1500	.05000	.07071	.005
VAR00005	2	.60	.75	.6750	.07500	.10607	.011
Valid N (listwise)	2						

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
PLA2 DEU FRACTION 1	2	4.1500	.07071	.05000
PLA2 DEU FRACTION 1	2	3.1000	.14142	.10000

PLA2 DEU FRACTION 1	2	2.3000	.14142	.10000
PLA2 DEU FRACTION 1	2	1.1500	.07071	.05000
VAR00005	2	.6750	.10607	.07500

One-Sample Test

	Test Value = 0					
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
	Lower	Upper	Lower	Upper	Lower	Upper
PLA2 DEU FRACTION 1	83.000	1	.008	4.15000	3.5147	4.7853
PLA2 DEU FRACTION 1	31.000	1	.021	3.10000	1.8294	4.3706
PLA2 DEU FRACTION 1	23.000	1	.028	2.30000	1.0294	3.5706
PLA2 DEU FRACTION 1	23.000	1	.028	1.15000	.5147	1.7853
VAR00005	9.000	1	.070	.67500	-.2780	1.6280

PLASMA RECALCIFICATION TIME FRACTION 1 **Descriptive Statistics**

	N	Minimum	Maximum	Mean		Std. Deviation	Variance
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Statistic
Recalcification time venom	2	2.00	2.00	2.0000	.00000	.00000	.000
Recalcification time venom	2	2.60	2.90	2.7500	.15000	.21213	.045
Recalcification time venom	2	3.00	3.40	3.2000	.20000	.28284	.080
Recalcification time venom	2	4.10	4.50	4.3000	.20000	.28284	.080
Recalcification time venom/ext	2	2.20	2.30	2.2500	.05000	.07071	.005
Recalcification time venom/ext	2	3.20	3.40	3.3000	.10000	.14142	.020
Recalcification time venom/ext	2	4.40	4.50	4.4500	.05000	.07071	.005
Recalcification time venom/ext	2	7.80	8.00	7.9000	.10000	.14142	.020
Valid N (listwise)	2						

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
Recalcification time venom	2	2.0000	.00000(a)	.00000
Recalcification time venom	2	2.7500	.21213	.15000
Recalcification time venom	2	3.2000	.28284	.20000

Recalcification time venom	2	4.3000	.28284	.20000
Recalcification time venom/ext	2	2.2500	.07071	.05000
Recalcification time venom/ext	2	3.3000	.14142	.10000
Recalcification time venom/ext	2	4.4500	.07071	.05000
Recalcification time venom/ext	2	7.9000	.14142	.10000

a t cannot be computed because the standard deviation is 0.

One-Sample Test

	Test Value = 0					
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
	Lower	Upper	Lower	Upper	Lower	Upper
Recalcification time venom	18.333	1	.035	2.75000	.8441	4.6559
Recalcification time venom	16.000	1	.040	3.20000	.6588	5.7412
Recalcification time venom	21.500	1	.030	4.30000	1.7588	6.8412
Recalcification time venom/ext	45.000	1	.014	2.25000	1.6147	2.8853
Recalcification time venom/ext	33.000	1	.019	3.30000	2.0294	4.5706
Recalcification time venom/ext	89.000	1	.007	4.45000	3.8147	5.0853
Recalcification time venom/ext	79.000	1	.008	7.90000	6.6294	9.1706

Descriptive Statistics250MG/KG

	N	Minimum	Maximum	Mean		Std. Deviation	Variance
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Statistic
urea	5	3.00	11.00	8.2000	1.36565	3.05369	9.325
Na	5	129.00	141.00	133.4000	2.11187	4.72229	22.300
K	5	3.00	5.40	3.8200	.44542	.99599	.992
Cl	5	19.00	97.00	79.2000	15.09768	33.75944	1139.700
bicarbonate	5	10.00	24.00	19.0000	2.77489	6.20484	38.500
creatinine	5	58.00	145.00	117.6000	15.59679	34.87549	1216.300
SGOT	5	19.00	31.00	25.6000	2.08806	4.66905	21.800
SGPT	5	5.00	18.00	8.8000	2.41661	5.40370	29.200
ALK/P	5	3.00	46.00	14.0000	8.04363	17.98611	323.500
Bilirubin	5	17.00	17.00	17.0000	.00000	.00000	.000
t/protein	5	70.00	73.00	71.6000	.67823	1.51658	2.300
albumin	5	33.00	38.00	36.2000	1.11355	2.48998	6.200
PCV	5	45.00	49.00	46.4000	.74833	1.67332	2.800
Valid N (listwise)	5						

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
urea	5	8.2000	3.05369	1.36565
Na	5	133.4000	4.72229	2.11187
K	5	3.8200	.99599	.44542
Cl	5	79.2000	33.75944	15.09768
bicarbonate	5	19.0000	6.20484	2.77489
creatinine	5	117.6000	34.87549	15.59679
SGOT	5	25.6000	4.66905	2.08806
SGPT	5	8.8000	5.40370	2.41661
ALK/P	5	14.0000	17.98611	8.04363
Bilirubin	5	17.0000	.00000(a)	.00000
t/protein	5	71.6000	1.51658	.67823
albumin	5	36.2000	2.48998	1.11355
PCV	5	46.4000	1.67332	.74833

a t cannot be computed because the standard deviation is 0.

One-Sample Test

	Test Value = 0				
	t	df	Sig. (2-	Mean	95%

			tailed)	Difference	Confidence Interval of the Difference	
	Lower	Upper	Lower	Upper	Lower	Upper
urea	6.004	4	.004	8.20000	4.4083	11.9917
Na	63.167	4	.000	133.40000	127.5365	139.2635
K	8.576	4	.001	3.82000	2.5833	5.0567
Cl	5.246	4	.006	79.20000	37.2821	121.1179
bicarbonate	6.847	4	.002	19.00000	11.2957	26.7043
creatinine	7.540	4	.002	117.60000	74.2964	160.9036
SGOT	12.260	4	.000	25.60000	19.8026	31.3974
SGPT	3.641	4	.022	8.80000	2.0904	15.5096
ALK/P	1.741	4	.157	14.00000	-8.3327	36.3327
t/protein	105.568	4	.000	71.60000	69.7169	73.4831
albumin	32.509	4	.000	36.20000	33.1083	39.2917
PCV	62.005	4	.000	46.40000	44.3223	48.4777

			tailed)	Difference	Confidence Interval of the Difference	
	Lower	Upper	Lower	Upper	Lower	Upper
urea	6.004	4	.004	8.20000	4.4083	11.9917
Na	63.167	4	.000	133.40000	127.5365	139.2635
K	8.576	4	.001	3.82000	2.5833	5.0567
Cl	5.246	4	.006	79.20000	37.2821	121.1179
bicarbonate	6.847	4	.002	19.00000	11.2957	26.7043
creatinine	7.540	4	.002	117.60000	74.2964	160.9036
SGOT	12.260	4	.000	25.60000	19.8026	31.3974
SGPT	3.641	4	.022	8.80000	2.0904	15.5096
ALK/P	1.741	4	.157	14.00000	-8.3327	36.3327
t/protein	105.568	4	.000	71.60000	69.7169	73.4831
albumin	32.509	4	.000	36.20000	33.1083	39.2917
PCV	62.005	4	.000	46.40000	44.3223	48.4777

	N	Mean	Std. Deviation	Std. Error Mean
urea	5	11.3400	13.72308	6.13715
Na	5	131.6000	4.72229	2.11187
K	5	8.4400	3.15642	1.41159
Cl	5	83.2000	32.53767	14.55129
bicarbonate	5	38.0000	32.56532	14.56365
creatinine	5	88.0000	28.60944	12.79453
SGOT	5	19.0000	18.17966	8.13019
SGPT	5	10.0000	7.38241	3.30151
ALK/P	5	20.4000	19.32097	8.64060
Bilirubin	5	17.0000	.00000(a)	.00000
t/protein	5	57.4000	9.09945	4.06940
albumin	5	33.4000	1.67332	.74833
PCV	5	47.8000	1.30384	.58310

a t cannot be computed because the standard deviation is 0.

One-Sample Test

	Test Value = 0					
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
	Lower	Upper	Lower	Upper	Lower	Upper

urea	1.848	4	.138	11.34000	-5.6995	28.3795
Na	62.314	4	.000	131.60000	125.7365	137.4635
K	5.979	4	.004	8.44000	4.5208	12.3592
Cl	5.718	4	.005	83.20000	42.7991	123.6009
bicarbonate	2.609	4	.059	38.00000	-2.4352	78.4352
creatinine	6.878	4	.002	88.00000	52.4767	123.5233
SGOT	2.337	4	.080	19.00000	-3.5730	41.5730
SGPT	3.029	4	.039	10.00000	.8335	19.1665
ALK/P	2.361	4	.078	20.40000	-3.5902	44.3902
t/protein	14.105	4	.000	57.40000	46.1015	68.6985
albumin	44.633	4	.000	33.40000	31.3223	35.4777
PCV	81.976	4	.000	47.80000	46.1811	49.4189

Descriptive StatisticsCONTROL

	N	Minimum	Maximum	Mean		Std. Deviation	Variance
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Statistic
urea	5	7.00	10.00	8.7600	.58103	1.29923	1.688
Na	5	130.00	140.00	133.2000	1.85472	4.14729	17.200
K	5	16.00	19.00	17.6000	.67823	1.51658	2.300
Cl	5	33.00	90.00	73.8000	11.10585	24.83345	616.700
bicarbonate	5	10.00	32.00	18.8000	4.07922	9.12140	83.200
creatinine	5	120.00	140.00	126.8000	3.77359	8.43801	71.200
SGOT	5	21.00	34.00	27.2000	2.35372	5.26308	27.700
SGPT	5	5.00	8.00	6.0000	.54772	1.22474	1.500
ALK/P	5	6.00	18.00	11.0000	2.52982	5.65685	32.000
Bilirubin	5	17.00	17.00	17.0000	.00000	.00000	.000
t/protein	5	56.00	74.00	68.4000	3.18748	7.12741	50.800
albumin	5	32.00	39.00	35.2000	1.39284	3.11448	9.700
PCV	5	45.00	48.00	46.4000	.50990	1.14018	1.300
Valid N (listwise)	5						

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
urea	5	8.7600	1.29923	.58103
Na	5	133.2000	4.14729	1.85472
K	5	17.6000	1.51658	.67823
Cl	5	73.8000	24.83345	11.10585
bicarbonate	5	18.8000	9.12140	4.07922
creatinine	5	126.8000	8.43801	3.77359
SGOT	5	27.2000	5.26308	2.35372
SGPT	5	6.0000	1.22474	.54772
ALK/P	5	11.0000	5.65685	2.52982
Bilirubin	5	17.0000	.00000(a)	.00000
t/protein	5	68.4000	7.12741	3.18748
albumin	5	35.2000	3.11448	1.39284
PCV	5	46.4000	1.14018	.50990

a t cannot be computed because the standard deviation is 0.

PCV B4 V/E	14.725	4	.000	52.60000	42.6822	62.5178
TP B4	19.490	4	.000	6.54000	5.6083	7.4717
HB B4	13.845	4	.000	16.72000	13.3670	20.0730
PCV AFT V/E	10.525	4	.000	48.00000	35.3375	60.6625
TP AFT	18.333	4	.000	5.98000	5.0744	6.8856
HB AFT	10.531	4	.000	15.96000	11.7524	20.1676
PCV B4 V ONLY	23.727	4	.000	52.20000	46.0918	58.3082
TP B4	19.530	4	.000	6.28000	5.3872	7.1728
HB B4	23.512	4	.000	17.38000	15.3277	19.4323
PCV AFT	18.795	3	.000	39.50000	32.8118	46.1882
TP AFT	14.346	3	.001	6.12500	4.7662	7.4838
HB AFT	7.643	3	.005	14.42500	8.4184	20.4316
PCV B4 CONTROL	17.103	4	.000	58.00000	48.5846	67.4154
TB B4	26.801	4	.000	5.66000	5.0737	6.2463
HB B4	15.501	4	.000	17.40000	14.2834	20.5166
PCV AFT	27.535	4	.000	61.20000	55.0290	67.3710
TP AFT	26.500	4	.000	5.30000	4.7447	5.8553
HB AFT	19.338	4	.000	19.14000	16.3920	21.8880
WBCAFT V ONLY	8.784	3	.003	5.27500	3.3639	7.1861
WBC AFT V/E	12.469	4	.000	5.92000	4.6018	7.2382
WBC AFT V/E	7.843	4	.001	5.62000	3.6306	7.6094

5.00 18.00 50.00 6.00 16.60 45.00 6.00 20.00 60.00 6.50 20.00 66.00 5.00
22.00 5.70 6.90 4.10

6.20 18.00 49.00 7.40 16.30 40.00 6.50 13.30 45.00 5.40 15.00 53.00 4.70
17.70 6.70 7.20 4.80

6.00 10.00 60.00 5.50 20.00 38.00 7.00 12.70 60.00 5.50 20.00 61.00 5.80
21.00 4.80 5.40 7.50

5.70 17.30 54.00 6.00 18.00 35.00 5.00 11.70 65.00 5.50 17.00 64.00 5.40
18.00 3.90 4.80 7.20

7.00 16.50 48.00 6.50 16.00 . . . 60.00 5.40 15.00 62.00 5.60
17.00 . 5.30 4.50

HB B4	5	16.00	20.00	17.3800	.73919	1.65288	2.732
PCV AFT	4	35.00	45.00	39.5000	2.10159	4.20317	17.667
TP AFT	4	5.00	7.00	6.1250	.42696	.85391	.729
HB AFT	4	11.70	20.00	14.4250	1.88740	3.77481	14.249
PCV B4 CONTROL	5	45.00	65.00	58.0000	3.39116	7.58288	57.500
TB B4	5	5.40	6.50	5.6600	.21119	.47223	.223
HB B4	5	15.00	20.00	17.4000	1.12250	2.50998	6.300
PCV AFT	5	53.00	66.00	61.2000	2.22261	4.96991	24.700
TP AFT	5	4.70	5.80	5.3000	.20000	.44721	.200
HB AFT	5	17.00	22.00	19.1400	.98975	2.21314	4.898
WBCAFT V ONLY	4	3.90	6.70	5.2750	.60052	1.20104	1.443
WBC AFT V/E	5	4.80	7.20	5.9200	.47476	1.06160	1.127
WBC AFT V/E	5	4.10	7.50	5.6200	.71652	1.60219	2.567
Valid N (listwise)	4						

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
PCV B4 V/E	5	52.6000	7.98749	3.57211
TP B4	5	6.5400	.75033	.33556
HB B4	5	16.7200	2.70037	1.20764
PCV AFT V/E	5	48.0000	10.19804	4.56070
TP AFT	5	5.9800	.72938	.32619
HB AFT	5	15.9600	3.38866	1.51545
PCV B4 V ONLY	5	52.2000	4.91935	2.20000
TP B4	5	6.2800	.71903	.32156
HB B4	5	17.3800	1.65288	.73919
PCV AFT	4	39.5000	4.20317	2.10159
TP AFT	4	6.1250	.85391	.42696
HB AFT	4	14.4250	3.77481	1.88740
PCV B4 CONTROL	5	58.0000	7.58288	3.39116
TB B4	5	5.6600	.47223	.21119
HB B4	5	17.4000	2.50998	1.12250
PCV AFT	5	61.2000	4.96991	2.22261
TP AFT	5	5.3000	.44721	.20000
HB AFT	5	19.1400	2.21314	.98975
WBCAFT V ONLY	4	5.2750	1.20104	.60052
WBC AFT V/E	5	5.9200	1.06160	.47476
WBC AFT V/E	5	5.6200	1.60219	.71652

One-Sample Test

	Test Value = 0					
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
	Lower	Upper	Lower	Upper	Lower	Upper

$SQRT	5	132.8000	4.38178	1.95959
	5	17.4000	1.51658	67823

		132.8000	4.38178	1.95959
SGPT	5	17.4000	1.51658	.67823
ALK/P	5	66.6000	30.69691	13.72807
TOTAL BILIRUBIN	5	14.8000	6.72309	3.00666
CONJ BILI	5	130.8000	9.12140	4.07922
TOTAL PROTEIN	5	25.2000	4.43847	1.98494
ALBUMIN	5	6.2000	.83666	.37417
PCV	5	7.0000	1.00000	.44721

a t cannot be computed because the sum of caseweights is less than or equal 1.
b t cannot be computed. There are no valid cases for this analysis because all caseweights are not positive.

One-Sample Test

	Test Value = 0					
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
	Lower	Upper	Lower	Upper	Lower	Upper
creatinine	12.402	4	.000	9.36000	7.2646	11.4554
SGOT	67.769	4	.000	132.80000	127.3593	138.2407
SGPT	25.655	4	.000	17.40000	15.5169	19.2831
ALK/P	4.851	4	.008	66.60000	28.4848	104.7152
TOTAL BILIRUBIN	4.922	4	.008	14.80000	6.4522	23.1478
CONJ BILI	32.065	4	.000	130.80000	119.4743	142.1257
TOTAL PROTEIN	12.696	4	.000	25.20000	19.6889	30.7111
ALBUMIN	16.570	4	.000	6.20000	5.1611	7.2389
PCV	15.652	4	.000	7.00000	5.7583	8.2417

10.00 130.00 19.00 33.00 10.00 140.00 30.00 5.00 6.00 17.00 17.00 72.00 37.00 48.00
8.80 140.00 16.00 90.00 20.00 120.00 21.00 7.00 8.00 17.00 17.00 72.00 38.00 44.00
8.00 130.00 19.00 33.00 24.00 124.00 22.00 6.00 8.00 17.00 17.00 70.00 36.00 46.00
8.00 130.00 17.00 87.00 10.00 130.00 23.00 6.00 7.00 17.00 17.00 70.00 34.00 49.00
12.00 134.00 16.00 90.00 10.00 140.00 30.00 7.00 6.00 17.00 17.00 78.00 38.00 42.00

Descriptive Statistics

	N	Minimum	Maximum	Mean		Std. Deviation	Variance
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Statistic
PCV B4 V/E	5	39.00	60.00	52.6000	3.57211	7.98749	63.800
TP B4	5	5.70	7.60	6.5400	.33556	.75033	.563
HB B4	5	13.00	20.00	16.7200	1.20764	2.70037	7.292
PCV AFT V/E	5	30.00	54.00	48.0000	4.56070	10.19804	104.000
TP AFT	5	5.00	7.00	5.9800	.32619	.72938	.532
HB AFT	5	10.00	18.00	15.9600	1.51545	3.38866	11.483
PCV B4 V ONLY	5	48.00	60.00	52.2000	2.20000	4.91935	24.200
TP B4	5	5.50	7.40	6.2800	.32156	.71903	.517

PCV	5	4.00	53.00	20.4000	8.64060	19.32097	373.300					
Valid N (listwise)	0											

2.10 138.00 2.90 98.00 20.00 40.00 45.00 18.00 53.00 17.00 17.00 48.00 32.00 47.00

5.50 132.00 10.60 98.00 21.00 83.00 10.00 3.00 8.00 17.00 17.00 58.00 34.00 49.00

35.50 133.00 8.90 98.00 27.00 108.00 31.00 18.00 4.00 17.00 17.00 72.00 46.00 49.00

8.00 130.00 9.80 25.00 96.00 104.00 4.00 5.00 19.00 17.00 17.00 57.00 33.00 46.00

5.50 125.00 10.00 97.00 26.00 105.00 5.00 6.00 18.00 17.00 17.00 52.00 32.00 48.00

Descriptive Statistics(CONTROL)

	N	Minimum	Maximum	Mean		Std. Deviation	Variance
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Statistic
UREA	0						
Na	0						
K	0						
CI	0						
B/Carbonate	0						
creatinine	5	8.00	12.00	9.3600	.75472	1.68760	2.848
SGOT	5	130.00	140.00	132.8000	1.95959	4.38178	19.200
SGPT	5	16.00	19.00	17.4000	.67823	1.51658	2.300
ALK/P	5	33.00	90.00	66.6000	13.72807	30.69691	942.300
TOTAL BILIRUBIN	5	10.00	24.00	14.8000	3.00666	6.72309	45.200
CONJ BILI	5	120.00	140.00	130.8000	4.07922	9.12140	83.200
TOTAL PROTEIN	5	21.00	30.00	25.2000	1.98494	4.43847	19.700
ALBUMIN	5	5.00	7.00	6.2000	.37417	.83666	.700
PCV	5	6.00	8.00	7.0000	.44721	1.00000	1.000
Valid N (listwise)	0						

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
UREA	0(a,b)	.	.	.
Na	0(a,b)	.	.	.
K	0(a,b)	.	.	.
CI	0(a,b)	.	.	.
B/Carbonate	0(a,b)	.	.	.
creatinine	5	9.3600	1.68760	.75472

Cl	0(a,b)	.	.	.
B/Carbonate	0(a,b)	.	.	.
creatinine	5	11.3200	13.67889	6.11739
SGOT	5	131.6000	4.72229	2.11187
SGPT	5	8.4400	3.15642	1.41159
ALK/P	5	83.2000	32.53767	14.55129
TOTAL BILIRUBIN	5	38.0000	32.56532	14.56365
CONJ BILI	5	88.0000	28.60944	12.79453
TOTAL PROTEIN	5	19.0000	18.17966	8.13019
ALBUMIN	5	10.0000	7.38241	3.30151
PCV	5	20.4000	19.32097	8.64060

a t cannot be computed because the sum of caseweights is less than or equal 1.
b t cannot be computed. There are no valid cases for this analysis because all caseweights are not positive.

One-Sample Test

	Test Value = 0					
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
	Lower	Upper	Lower	Upper	Lower	Upper
creatinine	1.850	4	.138	11.32000	-5.6646	28.3046
SGOT	62.314	4	.000	131.60000	125.7365	137.4635
SGPT	5.979	4	.004	8.44000	4.5208	12.3592
ALK/P	5.718	4	.005	83.20000	42.7991	123.6009
TOTAL BILIRUBIN	2.609	4	.059	38.00000	-2.4352	78.4352
CONJ BILI	6.878	4	.002	88.00000	52.4767	123.5233
TOTAL PROTEIN	2.337	4	.080	19.00000	-3.5730	41.5730
ALBUMIN	3.029	4	.039	10.00000	.8335	19.1665
PCV	2.361	4	.078	20.40000	-3.5902	44.3902

	N	Minimum	Maximum	Mean		Std. Deviation	Variance
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Statistic
UREA	0						
Na	0						
K	0						
Cl	0						
B/Carbonate	0						
creatinine	5	2.10	35.50	11.3200	6.11739	13.67889	187.112
SGOT	5	125.00	138.00	131.6000	2.11187	4.72229	22.300
SGPT	5	2.90	10.60	8.4400	1.41159	3.15642	9.963
ALK/P	5	25.00	98.00	83.2000	14.55129	32.53767	1058.700
TOTAL BILIRUBIN	5	20.00	96.00	38.0000	14.56365	32.56532	1060.500
CONJ BILI	5	40.00	108.00	88.0000	12.79453	28.60944	818.500
TOTAL PROTEIN	5	4.00	45.00	19.0000	8.13019	18.17966	330.500
ALBUMIN	5	3.00	18.00	10.0000	3.30151	7.38241	54.500

CONJ BILI	5	17.0000	.00000(a)	.00000
TOTAL PROTEIN	5	71.6000	1.51658	.67823
ALBUMIN	5	36.2000	2.48998	1.11355
PCV	5	46.4000	1.67332	.74833

a t cannot be computed because the standard deviation is 0.

One-Sample Test

	Test Value = 0					
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
	Lower	Upper	Lower	Upper	Lower	Upper
UREA	6.022	4	.004	8.24000	4.4406	12.0394
Na	63.167	4	.000	133.40000	127.5365	139.2635
K	8.576	4	.001	3.82000	2.5833	5.0567
CI	5.246	4	.006	79.20000	37.2821	121.1179
B/Carbonate	6.847	4	.002	19.00000	11.2957	26.7043
creatinine	7.540	4	.002	117.60000	74.2964	160.9036
SGOT	12.260	4	.000	25.60000	19.8026	31.3974
SGPT	3.641	4	.022	8.80000	2.0904	15.5096
ALK/P	1.741	4	.157	14.00000	-8.3327	36.3327
TOTAL PROTEIN	105.568	4	.000	71.60000	69.7169	73.4831
ALBUMIN	32.509	4	.000	36.20000	33.1083	39.2917
PCV	62.005	4	.000	46.40000	44.3223	48.4777

11.00 134.00 3.00 19.00 10.00 145.00 19.00 5.00 6.00 17.00 17.00 72.00 38.00 45.00

9.50 129.00 5.40 96.00 23.00 125.00 31.00 5.00 3.00 17.00 17.00 70.00 34.00 46.00

3.00 133.00 3.60 94.00 24.00 58.00 23.00 18.00 46.00 17.00 17.00 73.00 38.00 45.00

9.00 130.00 3.00 90.00 23.00 120.00 27.00 7.00 7.00 17.00 17.00 70.00 33.00 47.00

8.70 141.00 4.10 97.00 15.00 140.00 28.00 9.00 8.00 17.00 17.00 73.00 38.00 49.00

Descriptive Statistics500MG/KG One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
UREA	0(a,b)	.	.	.
Na	0(a,b)	.	.	.
K	0(a,b)	.	.	.

VAR00004		33.162	4	.000	21.68000	19.8649	23.4951

19.50 19.90 20.10 20.20 . .

21.50 20.90 20.90 21.00 . .

20.00 20.00 20.80 20.80 . .

22.80 21.90 22.00 22.70 . .

23.50 23.70 23.60 23.70 . .CONTROL GROUP

BODY WT ENDS

Descriptive Statistics250MG/KG

	N	Minimum	Maximum	Mean		Std. Deviation	Variance
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Statistic
UREA	5	3.00	11.00	8.2400	1.36843	3.05990	9.363
Na	5	129.00	141.00	133.4000	2.11187	4.72229	22.300
K	5	3.00	5.40	3.8200	.44542	.99599	.992
CI	5	19.00	97.00	79.2000	15.09768	33.75944	1139.700
B/Carbonate	5	10.00	24.00	19.0000	2.77489	6.20484	38.500
creatinine	5	58.00	145.00	117.6000	15.59679	34.87549	1216.300
SGOT	5	19.00	31.00	25.6000	2.08806	4.66905	21.800
SGPT	5	5.00	18.00	8.8000	2.41661	5.40370	29.200
ALK/P	5	3.00	46.00	14.0000	8.04363	17.98611	323.500
TOTAL BILIRUBIN	5	17.00	17.00	17.0000	.00000	.00000	.000
CONJ BILI	5	17.00	17.00	17.0000	.00000	.00000	.000
TOTAL PROTEIN	5	70.00	73.00	71.6000	.67823	1.51658	2.300
ALBUMIN	5	33.00	38.00	36.2000	1.11355	2.48998	6.200
PCV	5	45.00	49.00	46.4000	.74833	1.67332	2.800
Valid N (listwise)	5						

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
UREA	5	8.2400	3.05990	1.36843
Na	5	133.4000	4.72229	2.11187
K	5	3.8200	.99599	.44542
CI	5	79.2000	33.75944	15.09768
B/Carbonate	5	19.0000	6.20484	2.77489
creatinine	5	117.6000	34.87549	15.59679
SGOT	5	25.6000	4.66905	2.08806
SGPT	5	8.8000	5.40370	2.41661
ALK/P	5	14.0000	17.98611	8.04363
TOTAL BILIRUBIN	5	17.0000	.00000(a)	.00000

	Test Value = 0					
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
	Lower	Upper	Lower	Upper	Lower	Upper
VAR00001	15.766	4	.000	23.30000	19.1969	27.4031
VAR00002	16.455	4	.000	23.04000	19.1524	26.9276
VAR00003	17.258	4	.000	23.52000	19.7361	27.3039
VAR00004	17.668	4	.000	23.68000	19.9587	27.4013

18.00 18.10 18.50 18.70

27.00 26.00 26.20 26.30

24.20 25.00 25.00 25.10

23.00 22.00 22.90 23.20

24.30 24.10 25.00 25.10 (500MG/KG) **Descriptive Statistics**

	N	Minimum	Maximum	Mean		Std. Deviation	Variance
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Statistic
VAR00001	5	19.50	23.50	21.4600	.77240	1.72714	2.983
VAR00002	5	19.90	23.70	21.2800	.70456	1.57544	2.482
VAR00003	5	20.10	23.60	21.4800	.61106	1.36638	1.867
VAR00004	5	20.20	23.70	21.6800	.65376	1.46185	2.137
Valid N (listwise)	5						

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	5	21.4600	1.72714	.77240
VAR00002	5	21.2800	1.57544	.70456
VAR00003	5	21.4800	1.36638	.61106
VAR00004	5	21.6800	1.46185	.65376

One-Sample Test

	Test Value = 0					
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
	Lower	Upper	Lower	Upper	Lower	Upper
VAR00001	27.784	4	.000	21.46000	19.3155	23.6045
VAR00002	30.203	4	.000	21.28000	19.3238	23.2362
VAR00003	35.152	4	.000	21.48000	19.7834	23.1766

21.00	21.20	22.00	22.10	-.83149	-.81671
	-.70950	-.75185			
29.40	29.00	30.20	30.10	.88041	.89574
	1.06425	.98654			
27.30	26.70	27.20	27.30	.45243	.39079
	.41532	.37810			
29.00	28.70	28.00	28.90	.79889	.82988
	.58836	.72578			
18.70	19.00	19.00	19.40	-1.30023	-1.29970
	-1.35843	-1.33856			

Descriptive Statistics

	N	Minimum	Maximum	Mean		Std. Deviation	Variance
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Statistic
VAR00001	5	18.70	29.40	25.0800	2.19440	4.90683	24.077
VAR00002	5	19.00	29.00	24.9200	2.03701	4.55489	20.747
VAR00003	5	19.00	30.20	25.2800	2.06746	4.62299	21.372
VAR00004	5	19.40	30.10	25.5600	2.05806	4.60196	21.178
Valid N (listwise)	5						

250mg/kg

Descriptive Statistics

	N	Minimum	Maximum	Mean		Std. Deviation	Variance
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Statistic
VAR00001	5	18.70	29.40	25.0800	2.19440	4.90683	24.077
VAR00002	5	19.00	29.00	24.9200	2.03701	4.55489	20.747
VAR00003	5	19.00	30.20	25.2800	2.06746	4.62299	21.372
VAR00004	5	19.40	30.10	25.5600	2.05806	4.60196	21.178
Valid N (listwise)	5						

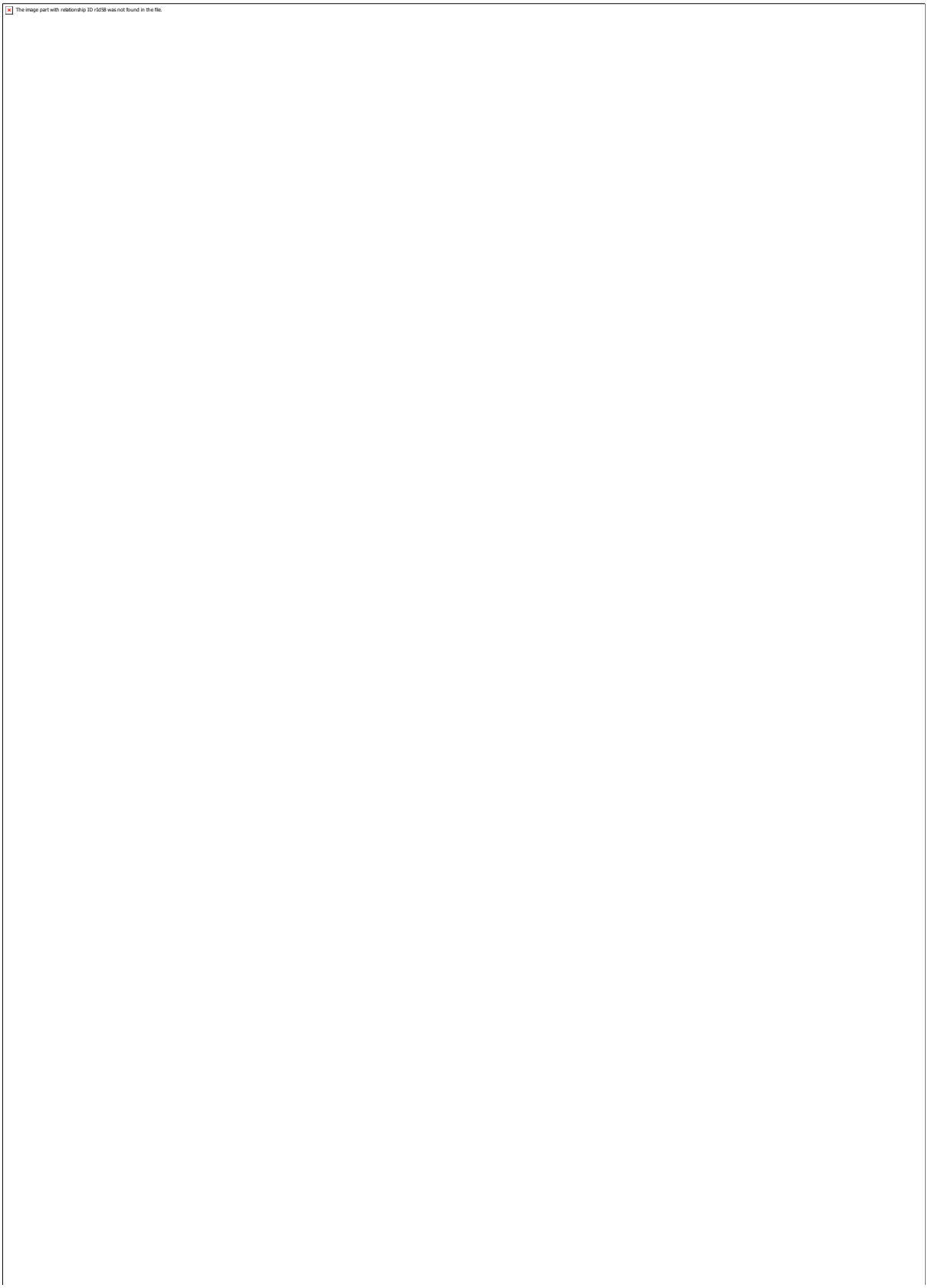
Descriptive Statistics

	N	Minimum	Maximum	Mean		Std. Deviation	Variance
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Statistic
VAR00001	5	18.00	27.00	23.3000	1.47784	3.30454	10.920
VAR00002	5	18.10	26.00	23.0400	1.40021	3.13097	9.803
VAR00003	5	18.50	26.20	23.5200	1.36286	3.04746	9.287
VAR00004	5	18.70	26.30	23.6800	1.34030	2.99700	8.982
Valid N (listwise)	5						

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
VAR00001	5	23.3000	3.30454	1.47784
VAR00002	5	23.0400	3.13097	1.40021
VAR00003	5	23.5200	3.04746	1.36286
VAR00004	5	23.6800	2.99700	1.34030

One-Sample Test


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