CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Snakes are animals found on every continent except Antarctica. Of the roughly 3,000 known species of snakes found worldwide, only 15% are considered dangerous to human (Kasturiratne*et al.*, 2008).There are more than 600 known species of venomous snakes (about a quarter of all snake species) classified into several families: *Elapidae, Viperidae, Crotalidae, Hydrophiidae, Actractaspididae*and *Colubridae*(Mebs, 2002; Fry and Wuster, 2004). Three species: black-necked spitting cobra (*Naja nigricollis*), carpet viper (*Echisocellatus*), and puff adder (*Bitisarietans*), belonging to the first two families, are the most important snakes associated with envenomation in Nigeria (Habib*et al.*, 2001). This is a problem to Nigeria, particularly the North-Eastern part of the country (Warrell, 1992; Chippaux, 1998; Theakston *et al.*, 2003). *Naja nigricollis* (also known as black-necked spitting cobra) is a long medium bodied snake with a moderately distinct head; the shape of which is primarily due to two large venom glands found on each side of the head. It is generally grey with a solid black hood and head. Its length ranges from 120 to over 280 cm. *Naja nigricollis* (family *Elapidae*) is found in Eastern Africa, Western and Eastern Kenya, widespread in Uganda, South-Western Africa, Tanzania, Rwanda, Burundi, Senegal, Namibia and Nigeria (Naja, 2008). The toxins of most importance in human envenoming include those that affect the nervous, cardiovascular, and haemostatic systems, and cause tissue necrosis. Snake venom neurotoxins block or excite peripheral neuromuscular junctions by acting at various sites (Freiber*et al.*,1984). However, a common symptom of

snakebite is drowsiness, suggesting the possibility of a central sedative action such as that associated with a small non-protein toxin that is found in king cobra (*Ophiophagushannah*) venom (Bevan *et al.*, 1983). Most venom neurotoxins bind to their receptors with high affinity, making reversal of paralysis by antivenoms implausible (Saha*et al*.,2006). However, rapid improvement in neurotoxicity has been noted when postsynaptic toxins were implicated—eg, after envenoming by Asian cobras and Australasian death adders (*Acanthophis*spp) (Bevan *et al*.,1983). The venom of *Naja nigricollis* retains the typical elapid neurotoxic properties while combining these with cytotoxic and significant anticoagulant effect. Haemotoxic features of the venom are responsible for bite symptoms which include severe external haemorrhaging and tissue necrosis around the bite area. Death generally occurs due to asphyxiation due to paralysis of the diaphragm. The murine LD50 of *Naja nigricollis* is 2mg/Kg subcutaneous and 0.03mg/Kg intravenous. The average venom yield of this snake is 150-350mg (Naja, 2008).Cardiotoxins act on smooth muscles. Cardiotoxins, also known as cytotoxins are found exclusively in the venom of cobras and ringhals (Jeyaseelan*et al*., 1998; Chang *et al*., 2000; Koh*et al*., 2006), and are direct lytic factors and membrane-active polypeptides.They are single-chain, highly hydrophobic, basic, short 60-65KDa polypeptides closely related to the α-neurotoxin that binds to acetylcholine receptor, but cardiotoxins do not show any significant affinity for the receptors (Dufton*et al*., 1991; Koh*et al*., 2006).Involvement of inflammatory process in the pathogenesis of snake envenomation was reported since 90s (Franceschi*et al*., 2000, Rivière*et al*.,1998, Lomonte*et al*.,1993). It is well known that snake venoms contain various activities able to activate several pathways. Increase of capillary permeability was reported after snake envenomation leading to the release of several mediators. Many components of snake venoms (PLA2s, bioamines and proteinases) contribute to the induced

inflammatory response which is initiated by an increase of vascular permeability followed by cell infiltration (Sebia-Amrane,2013).Several studies reported that Snake venom metalloproteinases such as Jararhagin from *Bothrops jararaca*, are involved in the inflammatory pathogenesis leading to an increase of pro-inflammatory cytokine production (Rucavado*et al*.,1995). The induced inflammatory response by snake venoms particularly those of *Viperidae*, is amplified by the presence of metalloproteinase, serine proteases, phospholipases A2b and other non-enzymatic proteins such as disinterring.

Figure 1: ***Naja nigricollis*** **(African spitting cobra).**

1.2 JUSTIFICATION

Even though there are a lot of research on the application of medicinal plant on the treatment of snakebite these extracts from medicinal plant are used to supplement the used of commercial prepared serum antiserum due to its adverse side effects. Non or major of these plant antivenoms properties are only apply without known concrete in neutralizing the venom activities, therefore there is need to screen the plant for possible mechnism toward

Inhibiting the venom toxic effects such as Bleed and Clotting Time, Defibrinogenating activities, Hemolytic activities, Hemorrhagic activities and Necrotizing activities.

1.3 AIM AND OBJECTIVES OF THE RESEARCH

The aim of this experiment is to determine the *In vivo* and *In vitro* Nuetralization of *Naja nigricollis* Venom Toxic Effects by *Azadirachta indica* Hexane and Ethyl-acetate Fractionated Leaves Extracts.

1.4 Objectives of the Study

- Extract and Fractionate the test *Azadirachta indica* leaves using 95% Methanol (as the crude), hexane and ethyl-acetate for the fractionation.
- Screen the protective effect of the hexane and ethyl-acetate Fractions on Bleed and Clotting Time, Defibrinogenating activities, Hemolytic activities, Hemorrhagic activities and Necrotizing activities.

1.5 Literature Review

Tissue necrosis is a relevant local effect caused after snakebites, it is considered as a serious consequence in severe cases of envenomation. When myonecrosis appears tissues are altered leading to the gangrene and infections. This type of complication can be the cause of amputation. Indeed, myotoxins of snake venoms affect mainly theplasma membrane of muscle cells to which they bind through their cationic sequence (Falconi*et al*., 2000, Lomonte*et al*., 1999). Molecular mechanism by which they caused the muscle tissue damage is not yet fully elucidated. Myonecrosis is due to the myotoxins that induce irreversible damage of skeletal muscle fibers. These molecules bind to the plasma

membrane of muscle cells and alter its permeability and integrity. The induced muscle tissue damage could be due to the penetration of myotoxins into muscle cells by endocytosis, probably through membrane receptors on to the surface of muscle cells or following hydrolysis of phospholipids causing membrane disruption. These molecules enter into the cytosol, reach and alter the membrane of mitochondria and sarcoplasmic reticulum of muscle cells. (Montecucco *et al*., 2008, Hamza*et al*., 2010). *Vitisvinifera*it belongs to a family Vitaceae. Mahadeswaraswamy Y H*et al.* Studied methalolic extract ofgrapes (*Vitisvinifera*L.) against the Indian Daboia/Viperarusselli venom induced localeffects. The extract showed complete inhibition of proteolytic and hyaluronidase activities andalso neutralized the hemorrhage, edema-inducing and myonecrotic actions of venom. Inaddition, the extract showed partial inhibition of pro coagulant activity of the venom andcompletely abolished the degradation of alpha andβeta chains of human fibrinogen (Mahadeswaraswamy*et al.*, 2009).Hemorrhages: are zinc metalloproteinase that damage the endothelial lining of blood vessel walls causing spontaneous local and systemic hemorrhage. The chemical composition of snake venom could be classified mainly in to protein component and non-protein component. The non- protein component is further subdivided into organic and inorganic constituents (Meier, 1991). The extract of *S. nux vomica* (in low doses) effectively neutralized *Doboiaressulli* venom induce lethal, hemorrhage, defibrigenating PhospholipaseA_2 enzyme Activity and *Naja kaouthia* venom induce lethal, cardiotoxic, Neurotoxic PLA_2 enzyme Activity. The seed extract potentiate polyvalent snake venom antiserum actionwas significantly potentiate by the activitie compound. Spectral studies revealed it to be a small, straight chain compound containing methyl and amide radical (CHatterjee*et al*., 2004).The procoagalant activity induce by cobra and *krat* venom was studies using human citrate plasma and *Macunapruriens* seed

extract was found to be effective in the neutralization of procoagalant Activity. *Macunapruriens* seed extract was effectively antagonized the fibrinolytic Activity. So *Mucunapruriens*seed extract was effecvtive in neutralizing the main toxic effect of the cobra and krat venom (meenatchisundaram*et al*., 2010). The methanolic extract ofgrape (*Vitisvenifera L.*).Seed was studied against the India *Veprerusselli* venom-induced local effect. The extract obolished proteolytic and Hyaluronidase activity and also efficiently neutralized the hemorrhagic, edema-inducing and myonicrotic properties of the venom(Msahadeswaraswamy*et al*.,2009). Tissue necrosis is a relevant local effect caused after snakebites, it is considered as a serious consequence in severe cases of envenomation. When myonecrosis appears tissues are altered leading to the gangreneand infections. This type of complication can be the cause of amputation. Indeed, myotoxins of snake venoms affect mainly theplasma membrane of muscle cells to which they bind through their cationic sequence (Falconi*etal*.,2000, Lomonte*etal*., 1999). Molecular mechanism by which they caused the muscle tissue damage is not yet fully elucidated. Myonecrosis is due to the myotoxins that induce irreversible damage of skeletal muscle fibers. These molecules bind to the plasma membrane of muscle cells and alter its permeability and integrity. The induced muscle tissue damage could be due to the penetration of myotoxins into muscle cells by endocytosis, probably through membrane receptorsonto the surface of muscle cells or following hydrolysis of phospholipids causing membrane disruption. These molecules enter into the cytosol, reach and alter the membrane ofmitochondria and sarcoplasmic reticulum of muscle cells. Theintracellular effect of these toxins occurs only after their initial actionon the plasma membrane, which marks the onset of degenerativeevents (Montecuccoe*t al*.,2008,Hamza*etal*.,2010).Metalloproteinase are responsible of the induced local and systemic bleeding after bites;affecting various organs (heart, liver, lungs,

intestines and brain), so they are called hemorragins. They can also cause swelling, blisters and necrosis. These enzymes are, therefore, widely involved in the pathogenesis of tissue necrosis (Paine *et al*., 1992).*Hemidesmusindicus* root extract and methanolic leaf extractof *Azadirachta indica* have been proved to neutralize phospholipase A2 activity induced by Russell's viper venom.Snake venom phospholipases hyposaline induced hemolysis by stress in Human red blood cell membranes. It is due to the formation of transient resealing fissures in the Human red blood cell membranes during cell swelling process (Arias *et al*., 2010).*Tamarindus indica*it belongs to a family*Leguminosae*. Ushannandini S *et al.*used dried seed extract of *Tamarindus indica* to inhibit thepharmacological as well as enzymatic effects induced by *V.russelli*venom. The seed extract inhibited the PLA2, protease,hyaluronidase, L-amino acid oxidase and 5' nucleotidaseenzyme activities. The extract also neutralized the degradationof the beta chain of the human fibrinogen and indirect hemolysis caused by venom (Ushanandini*et al*., 2006).

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Equipment

Table 1: List of Equipment Used

S/N	Equipments	Model	Manufacturer
1.	Water bath	HH-S stainless steel	Gallenkamp, London
2.	Refrigerator	HR-135A	Haier thermocool, USA
3.	Weighing Balance	Suntex, KONTES	Metler, london
4.	Rotary Evaporator		
5.	Spectrophotometer	6405 uv/v	Jenway Ltd. Dummo Essex Cm tested Dummo 63LB
6.	Centrifuge	90-1	Gallen Kamp England
7.	P^H Meter	800	Metrohm England
8.	Clock	Q-650	Quatz China
9.	Rotary evaporator	R-205D	NYC England
10.	Incubator	DHG-9101	Sanfa China

2.1.2 Apparatus

Table 2: List of Apparatus Used

S/N	Apparatus	Type	Manufacturer
1.	Beakers	Glass	Pyrex glass, England

2.	Pipette	Glass	Pyrex glass, England
3.	Measuring Cylinder	Glass	Pyrex glass, England
4.	Conical flasks	Glass	Pyrex glass, England
5.	Funnel	Glass	Pyrex glass, England
6.	Glass rod	Glass	Pyrex glass, England
7.	Test tubes	Glass	Pyrex glass, England
8.	Whatman Filter paper No.1	Paper	WhatmanInt.,Ltd, England
9.	Hand Gloves	Rubber	Chimex Int1,China
10.	Masking tape	Paper	Ndubest paper, Nigeria
11.	Muslin Cloth	Cotton	Asaba textile mill, Nigeria
12.	Micro Syringes	Plastic	
13.	Spatula	Metal	Pyrex glass, England
14.	Mortar	Wood	Local
15.	Pestle	Wood	Local
16.	Seperatory Funnel	Glass	
17.	Cannula	Rubber	
18.	Sample Bottles	Plastic	
19.	Glass Slides	Glass	
20.	Heparin Sample Bottles	Rubber	
21.	Aluminium foil	Sheet	Chimex Int1,China

Chemicals and Reagents

Table 3: List of Reagents Used

S/N	**Reagent**	**% Purity**	**Manufacturer**	**Concentration Used**
1.	Distilled Water		Biochemistry Lab, KSUSTA, Nigeria	
2.	Methanol	95% 33	Sure Chem product Ltd, England	95%
3.	Ethyl acetate	99%	Sure Chem product Ltd, England	

4.	n-Hexane	99%	Sure Chem product Ltd, England	
5.	Normal Saline			0.9%
6.	Phosphate Buffer			P^H 7.4
7.	Chloroform	99%	BDH Chemical, England	
8.	KH_2P0_4		Karmel, China	0.41g
9.	K_2HPO_4		Karmel, China	0.39g

2.1.4 Plant Sample

The leaves of *Azadirachta indica* ware Collected in the month of December, 2015 at Aliero, Kebbi State, Nigeria. The authentication wasdone at Biological Sciences Department, Kebbi State University of Science and Technology, Aliero, where a voucher Specimen (UDUTH/ANS/01155) was deposited.

2.1.5 Experimental Animals

Adult Wister albino rat of both sexes were used for the tests. They were purchased and kept under standard laboratory Conditions. The Animals were allowed free access to both food (Commercial rodents pellets) and water ad libitum. They were allowed to acclimatize for 2weeks. Weight of each rat was taken before the commencement of the experiment. All Animal experiments were conducted in accordance with WHO guidelines for the use of experimental Animals.

2.1.6 Naja nigricollis Snake Venom

The Venom used for this research work was purchased in a Lyophilized form from Faculty of Pharmaceutical Sciences, UsmanuDanfodio University, Sokoto, Nigeria.

2.2 METHODS

2.2.1 Preparation of Plant Extracts

The Collected *Azadirachta Indica* leaves were air-dried under shade then pulverized to small pieces using pestle and mortar. Five hundred gram (500g) was weighed and soaked in 1.2 liter of 95% methanol. The mixture was kept at room temperature for 72 hours and was filtered twice; initially with muslin cloth and later with Whatman filter paper No.1. The filtrate was evaporated to dryness at 45°C using rotary evaporator and %yield of the residue was calculated. The methanol residue of the leaves was dissolved in distilled water and fractionated with hexane, ethyl-acetate and butanol (saturated with water).

2.2.2 Fractionation of the Crude Metabolic Extract

The crude methanolic extract of the *Azadirachta indica* leaves was fractionated by liquid – liquid extraction using organic solvents in increasing order of polarity. Two hundred gram (200g) of the dry methanolic extract was reconstituted in 400ml of distilled water in 1L separatory funnel, then partitioned sequentially with equal volume of hexane andethyl-acetate to yield hexane and ethyl-acetate, (saturated with water) and aqueous fractions. Each fraction obtained was concentrated to dryness and the resulting residue was kept in a refrigirator in an air tight container for further analysis. Before used, each fraction was reconstituted in distilled water and expressed in terms of dry weight (mg/ml).

2.2.3 Inhibition Venom Toxic Effects by *Azadirachta indica* Hexane and Ethyl-acetate Fractionated Leaves Extracts.

2.2.3.1 Determination of Bleeding Time

For the determination of the bleeding time, modified procedure of Mohamed *et al*., (1969) was used. Six (6) rats were for each extract, three (3) rats were administered (*i.d*) only with ½LD_{50} dose of the venom, while, the other three (3) were administered (*i.d*) with ½LD50dose of the venom and immediate oral administration of the extract (100 mg/kg b.w).Two (2) hours after the animal treatment, the tail of each rat was gently pieced with lancet. A piece of white filter paper was used to blot the blood gently from the punctured surface of the body. The readings were taken every 15 sec.The end result occurs when the paper was no longer stained with blood.

2.2.3.2 Determination of Clotting Time

For the determination of the clotting time, the modified method of Igboechi and Anuforo, (1986) was used, clotting time is the time required for a firm clot to be formed in fresh blood on glass slides. The rats used in section 3.13.3.1 above were also used for this test. Two(2) hours after the animal treatment blood sample were collected from the rats via tail bleeding and a drop was placed on a clean plain slide and every 15 sec, a tip of office pin was passed through the blood until a thread-like structure was observed between the drop of blood and tip of the pin. The thread-like structure was an indication of a fibrin clot. The time was recorded.

2.2.3.3 Inhibition of Venom Haemorrhagic Activity

To assess the antihaemorrhagic activity of the, six (6) rats were used for each extract, three (3) were administered (*i.d*) only with ½LD_{50} dose of the venom, while, the other three (3)rats were administered (*i.d*) with ½LD_{50} dose of the venom and immediate oral administration of the extract (100mg/kg b.w). Two (2) hours after the animal treatment, the coagulability of the blood was examined.

2.2.3.4 Inhibitions of Venom Defibrinogenating Activity

The defibrinogenating activity (DFA)ofvenom is defined as the effect of the venom which when injected (*i.v*)into rate causes' incoagulable blood 1 h later .To access the defrinogenating activity of the venom, six (6) were used for each extract, three (3) rat were administered (*i.d*) with½LD_{50} dose of the venom , while, the other three (3) were administered (*i.d*) with ½LD_{50}dose of the venom and immediate oral administration of the extract (100mg/kgb.w). One (1) hour after the animal treatment,the coagulabilityof the blood was examined.

2.2.3.5 Inhibition of Venom Hemolytic Activity.

The venom induced hemolysis and its neutralization by the extract fractions was carried out using method of Vijayabharathi*et al.*, (2005) with slight modifications. Blood were collected from healthy rats via the tail vein using heparin as anticoagulant. The blood was then subjected to centrifugation at 3000rpm for 10min. And packed cell were separated. The cells were washed thrice with buffer (0.15M, P^H 7.4) and then re-centrifuged to collect cell. Venom (100 μg/mL, 1mL), Phosphate buffer (0.15 M, pH 7.4, 1mL) and RBCs (1%

v/v, 1mL) were mixed and incubated at 37 ºC for 30 minutes. Subsequently, the mixture was centrifuged at 1000 rpm for 3 minutes. Absorbance of the supernatant (due to release of hemoglobin) was measured sepectrophotometricallyat 540 nm. For anti-hemolytic activity, snake venom was pre-incubated with 0.5 mg/ml of the extract at 37ºc for 30minutes.Solution of the saline and the solution of the venom and extract serve as control for hemolytic and anti-hemolytic assays respectively. All assays were conducted in triplicates. Finally percentage hemolysis and protection was calculated by using the following formula.

Percentage hemolysis = (Absorbance of treated sample / Absorbance of control) × 100 = Y

Percentage protection = 100 – Y = Z.

2.2.3.6 Inhibition of Venom Necrotizing activity

The minimum necrotizing dose (MND) of *Naja nigricollis* venom was determined by the method described by Theakston and Reid, (1983). The minimum necrotizing dose (MND) is defined as the amount of venom which when injected (*i.d*) in to rats. Result in necrotic lesion of 5mm diameter three (3)days later. To assess the anti- necrotizing effect, six (6) were used for each extract, three (3) rats were administered (*i.d)* only with ½LD_{50} of the venom(though shaved dorsal skin of the rats) while, the other three (3) were administered (*i.d*) with ½LD_{50} dose of the venom (also though shaved dorsal skin of the rats) and immediate oral administration of the extract (100mg/kg*b.w*).Three (3)days after the animal treatment, the necrotic lesion were measured and recorded.

2.2.4 Data Analysis

The data collected was subjected to statistical analysis. All result were expressed as means ± SEM. All assays were performed in triplicate.

CHAPTER THREE

3.0 Results

Table 4: Results

Parameter	Treatment			
	Normal Control	**Venom Control**	**n-Hexane fraction**	**Ethyl acetate fraction**
Bleeding Times (sec)	1.30± 0.00	1.35±0.05	1.40±0.10	1.83±0.33
Clotting Times (sec)	0.35±0.05	2.85± 1.45	0.50±0.00	0.60±0.40
Defibrinogenating Activity	–	–	–	–
Hemorrhagic Lesions (mm)	10.8±1.75	11.3±0.50	11.5±1.00	14.0±1.50
Necrotizing Lesions (mm)	4.50±3.50	11.50±0.50	4.50±1.00	4.00±2.00
Hemolytic (%) Activity	6.30±0.84	100±0.00	94.2±9.60	84.6±7.70

Values are Presented as means ± SEM, – = not observed.

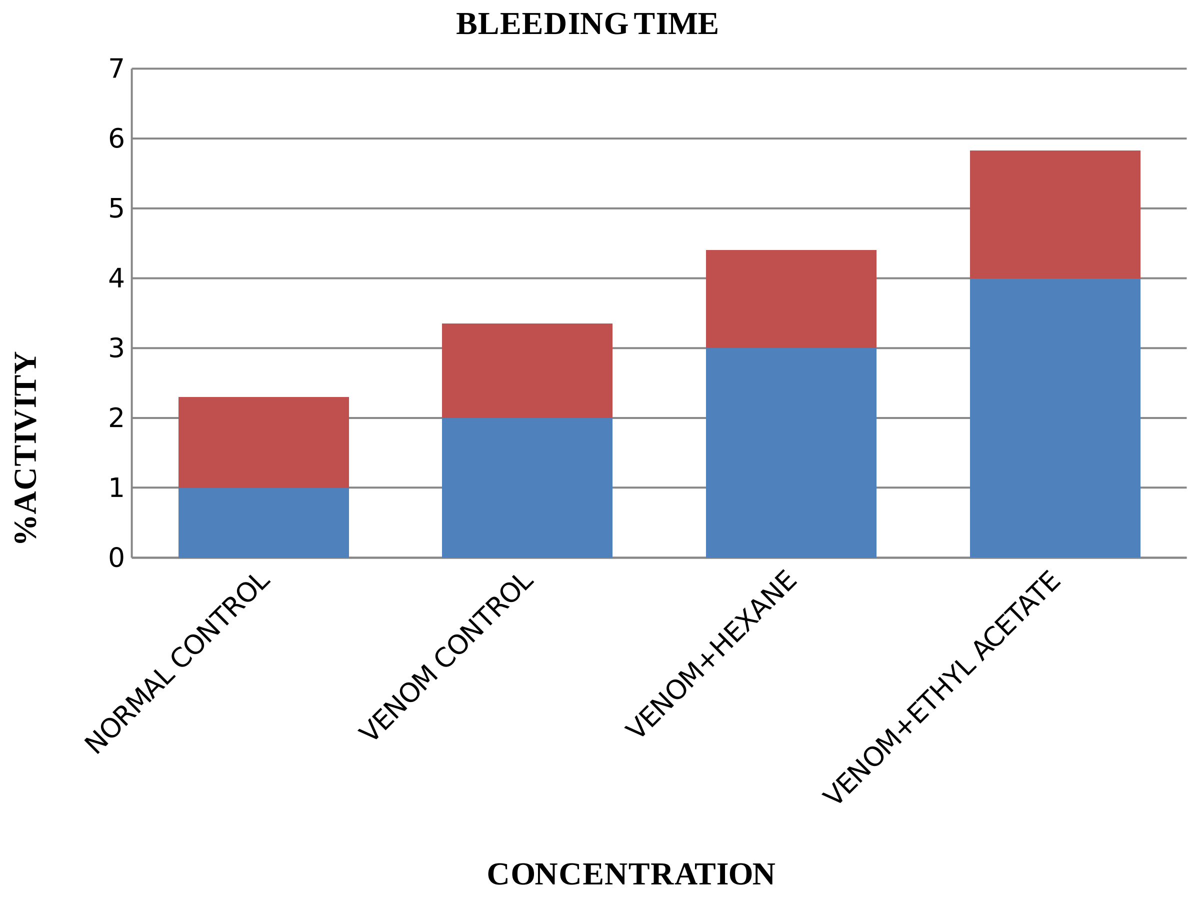

Figure 2: % Activity ofbleeding time.

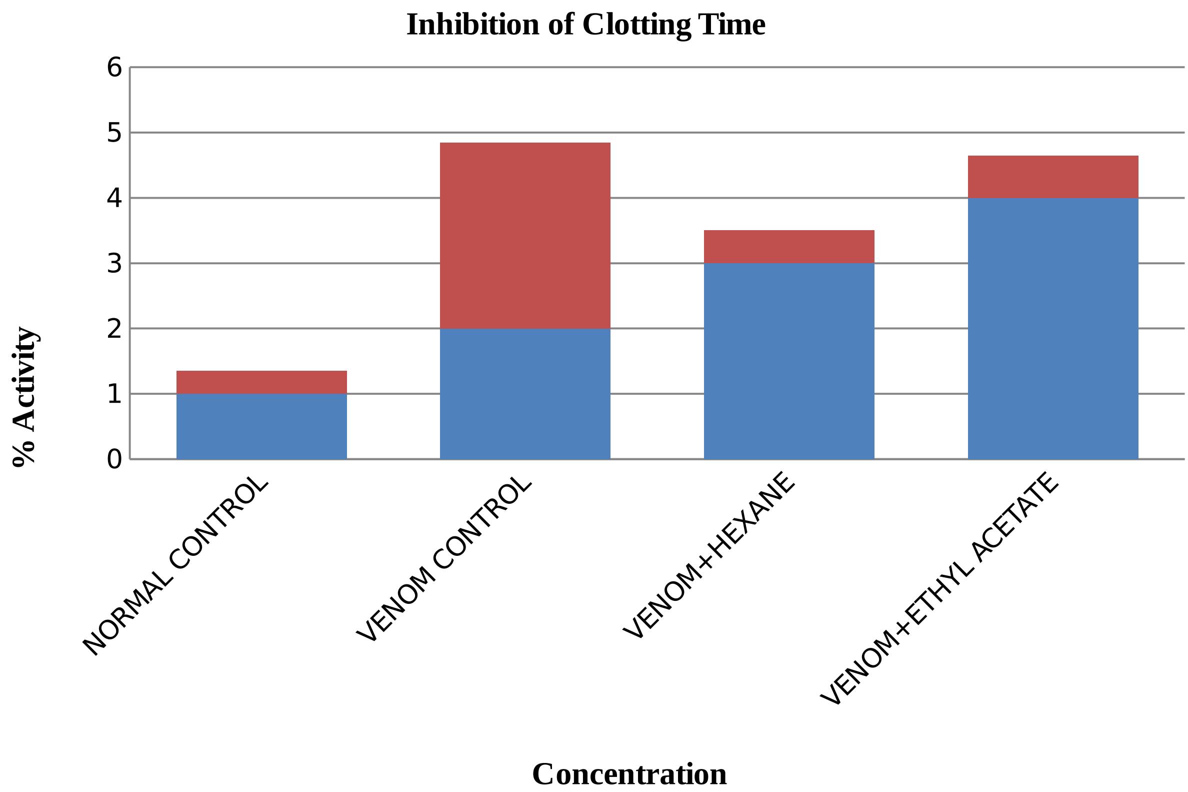

Figure 3: % % Activity ofClotting time

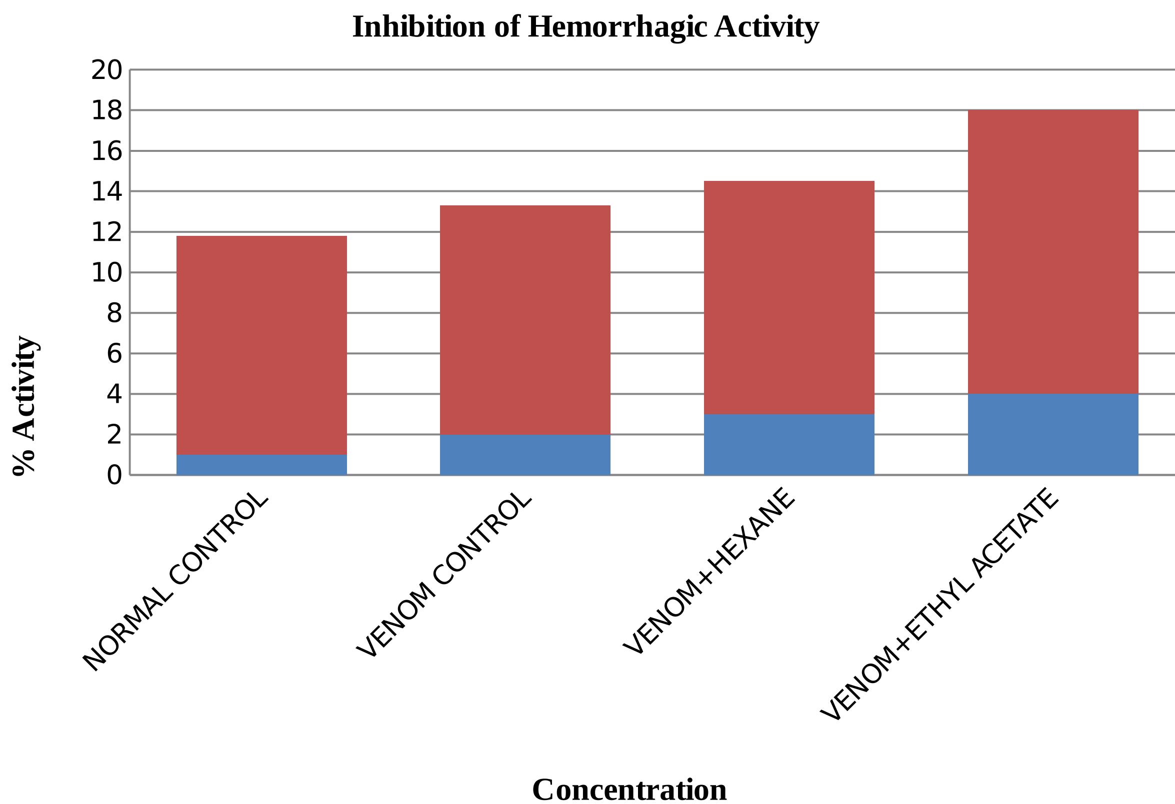

Figure 4: % Activity ofHemorrhagic Activity

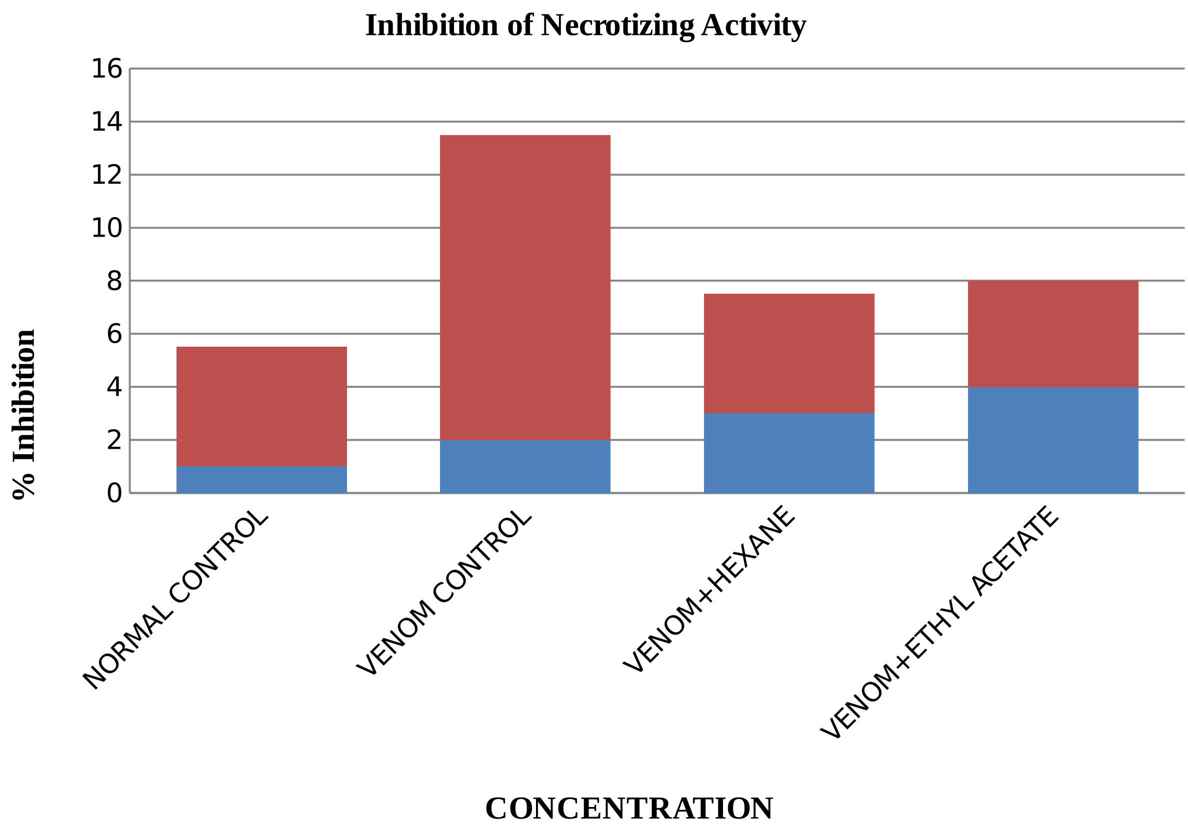

Figure 5:% Activity ofNecrotizing Activity

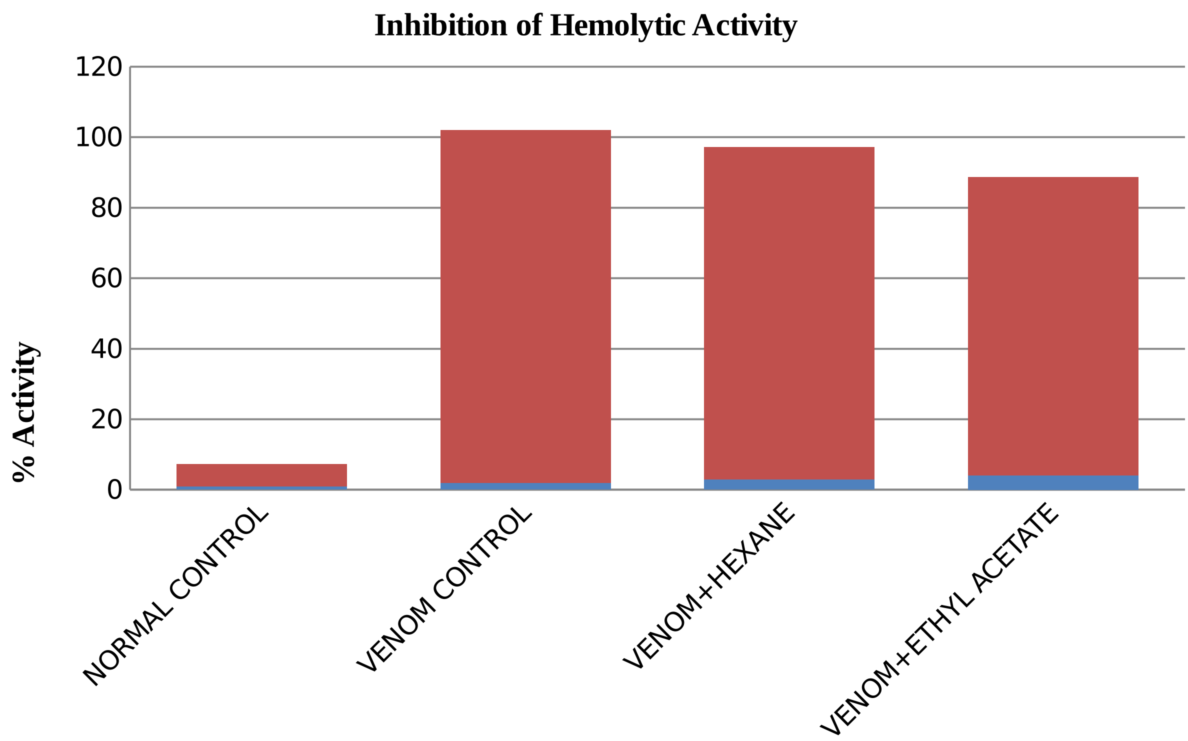

Figure 6: % Activity ofHemolytic Activity

CHAPTER FOUR

4.0 DISCUSSI0N, CONCLUTION AND RECOMMENDATION

4.1 DISCUSSION

Snake bite is an important cause of morbidity and mortality and is one of the major health problems in Nigeria. More than hundreds of plants have been used in folk medicine throughout the world for snakebites (Houghton, 1993and Sammy *et al.*,2008) .Till date few plants/plant materials have been evaluated in well controlled assays and about forty of them have been found to be effective against *Naja nigricollis* envenomation (Alam, 2003 and Martz, 1992). In this research, it has been revealed that the ethyl acetate and hexane fractions of the*Azadirachta indica*, possessed potent anti-snake venom activity. In this study it's also seen that *Azadirachta indica* neutralized some toxic effects induced by *Naja nigricollis* venom including various parameters such as blood clotting time, bleeding time, Defibrinogenating Activity, Hemorrhagic Activity, Necrotizing Activity, and Hemolytic Activity which were measured. The measurement of these parameters in plasma is of importance in the assessment of the pathophysiological state of snake bite victims.

The study showed that the extract of *Azadirachta indica* was effective in neutralizing the lethality and the effects of *Naja nigricollis* venom in animals (Tab: 4). In the contrasts of Bleeding Times the hexane fraction of crude methanolic extract were significantly neutralized the venom in the rat unlike Ethyl acetate fraction which has no significant different when compared with the normal control and venom control (Tab: 4). Bleeding time is associated with integrity of blood vessels and is known to cause pathological disturbances leading to coagulability of blood. The level of bleeding time increased

significantly ($p<0.05$) in the venom animals as in venom control and in the extract treated animals its decreases significantly as shown in table 4. The increase in bleeding time in this group revealed the blood incoagulability state (Denson *et al*., 1992).

As presented in (Tab: 4), Clotting Times measurement, there is no significant differentWhen compered between the Hexane and ethyl acetate fraction of the crude methanolic extract but there is significant different in the venom control and Normal control. This showed that venom has potent activity to induced the blood clotting when injected in the rat. The decrease in clotting time level observed after treatment of animals with extract/venom mixture inhibits the blood coagulability. In the Hemorrhagic activity the Venom induced the hemorrhagic lesion in the rat but *Azadirachta Indica* extract both Hexane and ethyl acetate fraction does not showed chemical potent activity against protection of hemorrhagic lesion as showed in (Tab: 4) but there is no significant difference between venom control and Hexane fraction and there is highly significant different between ethyl acetate when compared with venom control and hexane fraction of the extract. Hemorrhagic activity of venoms is due to the action of metalloproteases and phospholipases A2 (PLA2). Several symptoms of envenomation by snakes such as coagulation and hemorrhage are associated with such enzymes, and they are one of the major groups of enzymes that cause the deleterious effects of venoms in victims (Oliveira*et al*., 2010,). As neurotoxins are compounds which adversely affect the nervous system, a number of mechanisms through which they function are through the inhibition of neuron cellular processes. The results of the Necrotizing Activity on the effects of *Azadirachta indica* extract in rats after *Naja nigricollis* envenomation is as presented in the (tab: 4) showed that the venom has higher potent activity to induced necrotic lesion.

Inhibition of hemolytic activity: Minimum hemolytic dose was found to be 15µg. Hexane fraction of crude methanolic extract of.*Azadirachta indica* showed excellent inhibition of hemolysis (94.5%) while ethyl acetate fraction showed only moderate activity (84.6%). *Azadirachta indica* extract was totally effective in neutralizing the venom induced hemolysis. Among toxic proteins Phospholipase A2 (PLA2) are the most abundant and one of the contributing factors towards hemolysis. Phospholipases enzymes bind with cell membrane lipids by complex formation via covalent, non-covalent or disulfide bonds. After binding PLA2 enzyme caused hydrolysis of intact phospholipids and released free fatty acids and lysophospholipids. As a result human red blood cell membranes destroyed and change the environment of target protein(s) therefore resulted in agonistic, antagonistic effects and additionally posed interference in binding with physiologic ligands. All these aspects are contributing factors for venom induced hemolysis of human red blood cell (Kini, 2003; Condrea*et al*., 1964). In contrast to snake venom phospholipases hyposaline induced hemolysis by stress in human red blood cell membranes. It is due to the formation of transient resealing fissures in the human red blood cell during cell swelling process (Arias *et al*.,2010).

4.2 Conclusion

The present experimental results indicate that *Azadirachta indica* extract was effective in neutralizing the toxic effects of *Naja nigricollis* venom and or has an alternative or complementary treatment strategy of envenomation by *Naja nigricollis*.

4.3 Recommendation

- Further research may be done on the whole extract of this plant to compare and establish its inferiority or superiority with respect to various fractions.

- Future research should also encompass the characterization of these fractions with the help of modern chromatographic and spectroscopic techniques.

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APPENDICES

Appendix 1: Preparation of Reagents

95% Methanol

1140ml of 100% methanol was diluted with 60ml of distill water to make 1.2 liter of 95% methanol where 500g of plant sample was dissolved.

0.15M, Phosphate buffer (pH 7.4)

0.39g of (K_2HPO_4) and 0.41g of (KH_2PO_4) was weighed and dissolved to make up to 20ml with distilled water. The pH of the mixture was adjusted to 7.4 and make up to volume (20ml) with distilled water.

Appendix ii: Calculation

Percentage hemolysis = (Absorbance of treated sample / Absorbance of control) × 100 = Y

Percentage protection = 100 – Y = Z.

Normal control

1. %Hemolysis= 0.028/0.52×100=5.4%

2. %Hemolysis=0.037/0.52×100=7.11%

Therefore mean = 5.4+7.11/2=6.30%

Standard deviation=7.11-5.4/2=0.84

Percentage protection= 100-Y=Z

100-6.30=93.70%

Venom control

%Hemolysis=0.52/0.52×100=100%

Standard deviation=0.00

Hexane fraction

1. %Hemolysis=0.54/0.52×100=103.8%   2. %Hemolysis=0.44/0.52×100=84.6%

Therefore mean =103.8+84.6/2=94.2%

Standard deviation=103.8-84.6/2=9.60

Percentage protection= 100-Y=Z

100-94.2=5.8%92.3+76.9/2

Ethyl acetate fraction

1.%Hemolysis=0.48/0.52×100=92.3%   2. %Hemolysis=0.40/0.52=76.9%

Therefore mean=92.3+76.9/2=84.6%

Standard deviation=92.3-76.9/2=7.7

Percentage protection= 100-Y=Z

100-84.6=15.4%

Appendixiii:Table of the works

Hexane and Ethyl acetate fraction *Azadirachta Indica*PlantExtract. (LD_{50} = 0.05mg/ml)

Groups	Rats S/NO	Weight (g)	Administered Substance		Biochemical Analysis					
			Venom	Plant Extract	Bleeding Times	Clotting Times	DFA Activity	MHD Activity	Necrotizing Activity	Hemolytic
Group1	1	111.8	½LD_{50}	Haxane	1.50	0.50	_	12.5	5.50	0.54
Group2	2	110.9	½LD_{50}		1.30	0.50	_	10.5	3.50	0.44
Group1	1	117.1	½LD_{50}	Ethyl acetate	2.15	1.00	_	16.0	6.00	0.48
Group2	2	116.2	½LD_{50}		1.50	0.20	_	13.0	2.00	0.40
Group1	1	95.4	½LD_{50}	Normal Control	1.30	0.40	_	21.5	8.00	0.028
Group2	2	93.2	½LD_{50}		0.00	0.30	_	18.0	4.50	0.037
Group1	1	87.8	½LD_{50}	Venom control	1.30	4.30	_	11.5	12.00	0.027
Grouzp2	2	86.1	½LD_{50}		1.41	1.40	_	11.0	11.00	0.025

- = Not observed