ISOLATION AND CHARACTERIZATION OF SNAKE VENOM PROTEINS AND PEPTIDES FROM MEMBERS OF VIPERIDAE AND ELAPIDAE SNAKE FAMILIES FROM KILIFI COUNTY

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Isolation and Characterization of Snake Venom Proteins and Peptides from Members of Viperidae and Elapidae Snake Families from Kilifi County

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

Signature: ……………………… Date: ………………………..

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This thesis has been submitted for examination with our approval as University supervisors.

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DEDICATION

This work is dedicated to my parents, Mr.Gabriel Otieno and Mrs. Christine Otieno, my wife Mrs. Florence Onyango and our two daughters M/s. Michelle and M/s. Maureen for making sure that I had easy moment during my studies. I wish you many blessings from God Almighty.

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LIST OF ABBREVIATIONS AND ACRONYMS

1D	First dimension
2D	Second dimension
2-DE	Two dimensional gel electrophoresis
A (mA)	Ampere (milliampere)
AA	Amino acid
BCA	Bicin Choninic Acid
BJC	Bothrojaracin ,thrombin inhibitor venom from *Bothrops jararaca*
BSA	Bovine serum albumin
BPPs	Bradykinin potentiating peptides
Ca^{2+}	Calcium ions
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-propan-sulfonate
CM	Centimetre
CRD	Cysteine rich domain
CRISPs	Cysteine-rich secretory proteins
Da (kDa)	Dalton (kilodaltons)
DMSO	Dimethylsulfoxide
DTT	1, 4-dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylene diamine tetraacetic acid
ED_{50}	Effective dose
EMBL	European Molecular Biology Laboratory

EtOH	Ethanol
3FTXs	Three-Finger toxins
FXa	Coagulation Factor ten activated
FV	Coagulation Factor five
FVa	Coagulation Factor five activated
FVIII	Coagulation Factor eight
FIX	Coagulation Factor nine
FXIII	Coagulation Factor thirteen
G	gravity used in centrifugation
G	(mg, ng) gram (milligram, nanograms)
G1a	γ-carboxyglutamic acid
GRAVY	Grand average hydropathy value
H	Hour
ICU	Intensive care unit
IEF	Isoelectric focusing
IPG	Immobilized pH Gradient Gels
KGF	Lysine-Glycine-Phenylalanine
KTS	Lysine-Threonine-Serine
KWS	Kenya World life Service
l (mL, µL)	Liter (milliliter, microliter)
LD_{50}	Lethal dose
M (mM)	Molar (millimolar)
MALDI-	Matrix assisted laser desorption ionization-Quadrupole/ time of

QTOF	flight
MeOH	Methanol
Min	Minute
MtDNA	Mitochondrion DNA
MW	Molecular weight
NMK	National Museums of Kenya
No.	Number
°C	Degree Celsius
PARs	Protease activated receptors
PBS	Phosphate Buffered Saline
pI	Isoelectric point
PLs	Phospholipids
P-IV	Snake venom metalloproteinase class, Protein-type IV Class
PVDF	Polyvinylidenedifluoride
Q1	Selection cell in MALDI-QTOF-MS
Q2	Collision cell in MALDI-QTOF-MS
RGD	Arginine-Glycine-Aspartate
Rpm	Revolutions per minute
RVV-V	Russell‘s viper *(Daboia russelli)* Venom
SDS	Sodium dodecylsulfate
SDS-PAGE	Sodium dodecylsulfate-Polyacrylamide gel electrophoresis
Sec	Second

SEC	Size-Exclusion Chromatography
SECD	Serine-Glutamic acid-Cysteine-Aspartate
TCA	Trichloroacetic acid
TEMED	N, N, N,'N',-Tetramethylethylenediamine
TFA	Trifluoroacetic acid
TOF	Time-of-flight
Tris-base	Tris (hydroxyl methyl)-amoniomethane
Tris-HCL	Trizma hydrochloride
TLEs	Thrombin-like Enzymes
TVS-PA	*Trimeresurus stejnegeri* venom-Plasminogen activator
V (mV)	Volt (millivolt)
VNN	Venom protein from *Naja(naja) haje*
v/v	Volume per volume
w/v	Weight per volume.
WHO	World Health Organization
XXCD	X-X-Cysteine-Aspartate (X- means unknown amino acid residue)

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ABSTRACT

Snake venom comprises highly complex mixture of protein and peptides. Kenya is inhabitated by many species of venomous snakes from both Viperidae and Elapidae families. This study aimed to isolate and biochemically characterize snake toxins from members of Viperidae (*Bitis arietans*) as well as Elapidae (*Naja melanoleuca*, *Naja ashei* and *Dendroaspis angusticeps)* from Kilifi County. This cross sectional study was done on venom samples extracted from eight adult snake specimens, both male and female representing each species. Two-dimensional gel Electrophoresis method was used to analyze the samples and later stained by silver staining technique. Two-dimensional gels protein spots were analyzed using verified empirical isoelectric points and molecular weights generated in comparison with the incorporated protein standards and these data used as parameters in the protein identification program, TagIdent. This program allowed the identification of whole protein present in the 2D gel spot using its estimated pI, molecular masses, keywords and species (or group of species) of interest. The estimated isoelectric points and molecular weights data generated enabled further determination of the physical and biochemical characteristics of the venom protein toxins identified earlier. This study findings suggest that the venom protein toxins from Elapidae families contained very low molecular weights of approximately 6000 daltons to 14,400 daltons, while on the other hand, the Viperidae family had spotting at the molecular mass ranges of 14,400 daltons to 36,500 daltons. Overall, the venom toxin's isoelectric point ranged from pI 4 to pI 8 and molecular mass of 7515 daltons to 13473 daltons. This study identified the five snake venom proteins, four from *Naja (Boulengrina) subfulva* formerly *melanoleuca* and one from *Dendroaspis angusticeps* in a two-dimensional gel electrophoresis (2D) after an in-depth computer search from Swiss-Prot protein database. They were {P00599}- Basic phospholipases A2 I, with amino acid chain length of 1-118, pI=7.55, Mw:13473 daltons; {P00600}-Acidic phospholipases A2 DE-II, chain:1-119, pI=6.82, Mw: 13427 daltons; {P00601}–Acidic phospholipases A2 DE-III, chain:1-119, pI=6.27, Mw: 13360 daltons); {P01383}- Long chain neurotoxin 1 with 1-71 amino acid residues, 8056 daltons, pI=8.73 and {P81030}- Three-finger muscarinic toxin1 with 1-66 amino acids, 7518 daltons, pI=8.48. This study finding suggests that the five identified venom protein toxins are thermodynamically stable and highly hydrophilic molecules. However, five 2D-gel proteins spots turned out to be negative and/or undetermined which may represent some of the previously undescribed toxins that are yet to be deposited in protein databases and requires further in-gel enzymatic digestion, liquid chromatography mass spectrometry and western blot analysis to confirm their presence in the venom sample. Therefore, further studies are needed to confirm the presence and identities of these unknown proteins to enable better understanding of their structure—function relationships and application as vaccine immunotherapeutic agents aiming to address the public health problem of snakebite disabilities and improve human health in the affected populations.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Snake venoms are complex mixtures of bioactive components, predominantly proteins and polypeptides that play offensive, defensive and digestive roles (Doley & Kini, 2009; Wray *et al*., 2015). Previous research studies on snake venom proteins have shown that they contain biomolecules with various enzymatic activities and examples includes proteases, phospholipases, phosphodiesterases, nucleases, nucleotidases, hyaluronidases, L-amino acid oxidases, and acetylcholinesterases (Kang *et al*., 2011). Isolation and biochemical characterization of protein and peptide enzymes content of snake venoms has a number of potential benefits in the fields of basic research, clinical diagnosis, development of new research tools and drugs for potential clinical use and antivenom production strategies (Calvete *et al*., 2007).

The snake venoms are known to be rich sources of pharmacologically active polypeptides and proteins (Georgieva *et al*., 2008). It also represents the most studied of all other venomous lineages and has helped in the discovery of lead compounds targeting specific components of cardiovascular processes with relevance to hemostasis (Slagboom *et al*., 2017). The first blockbuster drug developed from a venom toxin was Captopril (Capoten). Captopril was developed from a Bradykinin potentiating peptides (BPPs), isolated from a Jararaca pit viper (*Bothrops jararaca*) venom. It inhibits the conversion of angiotensin I into angiotensin II, thereby reducing systemic blood pressure by preventing the production of a crucial vasoconstrictor (Ferreira *et al*., 1970; McCleary & Kini, 2013). Snake venom polypeptides are highly catalytic and may contribute to the digestive action and induce various pharmacological effects including neurotoxic, myotoxic, cardiotoxic, hemorrhagic, hemolytic, procoagulant and anticoagulant effects (Kini, 1997). Some polypeptides do not exhibit enzymatic activities and are thus described as non-enzymatic proteins such as neurotoxins, cardiotoxins, myotoxins, Ion channel inhibitors and anticoagulant proteins. Over 1000 non- enzymatic venom proteins are known and grouped into notable families such as:-Three finger toxins—neurotoxins and cardiotoxins; serine proteinase inhibitors; lectins; sarafotoxins; nerve growth

factors; disintegrins and cysteine-rich secretory proteins (CRISPs/helveprins) (McCleary & Kini, 2013).

Snake venom is a mixture of bioactive substance produced in a highly specialized venom gland which is predominantly proteins and polypeptides and delivered by an associated specialized apparatus that is deleterious to other organisms in a given dosage and is actively used in the subjugation and/or digestion of prey and/or defense and offense (Chahl & Kirk, 1975; Mebs, 2002). After their inoculation through a bite, these proteins and polypeptides function as toxins and attack various physiological systems, often leading to death and debilitation of the prey or victim. They are classified as enzymatic and non-enzymatic proteins. Enzymatic venom proteins contribute towards the lethal and debilitating effects of venom while non-enzymatic on the other hand acts by immobilizing the prey (McCleary & Kini, 2013). Non-enzymatic venom proteins bind to specific receptors, ion-channels or plasma proteins and interfere with the prey's physiological processes by acting as agonists or antagonists leading to neurotoxicity, cardiotoxicity and/or tissues necrosis (Doley & Kini, 2009). The non-enzymatic venom proteins have been classified based on their amino acid sequences and protein fold into several structural or functional protein families which includes:- (i) three-finger toxins, (ii) serine proteinase inhibitors, (iii) nerve growth factors, (iv) cystatin, (v) natriuretic peptides, (vi) cysteine-rich secretory proteins (CRISPS) or helveprins, (vii) Kunitz-type proteinase inhibitors, (viii) Snaclecs (C-type lectins & related proteins), (ix) bradykinin potentiating peptides, (x) sarafotoxins, (xi) cobra venom factor, (xii) vascular endothelial growth factors, (xiii) waprins, (xiv) vespryns, (xv) veficolins and (xvi) disintegrins (McCleary and Kini, 2013; Tasoulis and Isbister, 2017). The proteins within each family shares high similarities in their primary, secondary and tertiary structural arrangement but only differ in terms of pharmacological effects (McCleary & Kini, 2013). These functional differences have been associated with three-finger toxins (Kini & Doley, 2010), Snaclecs (Fry, 2015; Clemetson, 2010), Serine proteinase inhibitors (van Gent *et al.*, 2003).

Snakes are represented by approximately 3,070 species (Vidal *et al.*, 2007; Vonk *et al.*, 2011), which are divided into two main groups:- (i) the fosorial scolecophidians (blind snakes and thread snakes, ca.370 species) are small snakes with limited gape

size that feed on small prey (e.g., ants and termites) routinely and (ii) the alethinophidian (includes all other snakes, ca.2640 species) are ecologically diverse and feed on relatively large prey, primarily vertebrates, on an infrequent basis (Vidal & Hedges, 2009). Among alethinophidian, Caenophidians (advanced snakes, ca.2470 species) widely use venom in addition (or not) to constriction to subdue their prey, while the remaining alethinophidian snakes (ca.170 species) use constriction only (Vidal & Hedges, 2009). Medically important alethinophidian venomous snakes are classified into three families: Atractaspidae (Burrowing Asps, 69 species), Elapidae (Elapids, 360 species), and Viperidae (Vipers, 340 species). This last family is in turn divided into two subfamilies, Viperinae (True Vipers, 101 species), and Crotalidae (Pit Vipers, 239 species) (data taken from www.reptile-database.org). Venoms are secreted and stored in venom glands and snakes inject it into the victim via the sharp fangs and cause damage in several ways (Warrell, 2010).

Venom toxin component seems to be fairly common and related within each snake family. Neurotoxins are typical of Hydrophidae (e.g., Hydrophis spp, Aipysurus spp) and Elapidae (e.g., cobras, mambas, kraits, coral snakes, taipans) venoms while haemostatically active components are generally found in Viperidae, Crotalidae and Elapidae snakes (Lu *et al*, 2005).

Venoms affect the key elements of almost every animal physiological pathway. For example, neurotoxins affect the central nervous and muscular systems thereby blocking the nerve-muscle junction, while cytotoxins and phospholipases on the other hand induce muscle necrosis (Aird, 2002). Hematoxic snake venom proteins primarily affect the cardiovascular system and disrupt the endothelium (Persson, 2001).

In many snake venomics project, there have been major challenges concerning the eligibility criteria which are to be adopted when selecting an appropriate snake donor for venom extraction and later utilization in antivenom production. Furthermore, it is important to choose venoms which are rich in the toxin components of study interests. Lack of knowledge on the venom variations at all levels, including the inter-species and intra-species remains a major drawback when making decision for choosing appropriate antivenom to develop an efficient and effective treatment for snakebite envenomation. There is little information in the literature about the snake

venom components from the common venomous snakes in Kenya. There are also far fewer new cases of snakebites being reported in the Government health facilities as majority of the victims do not seek immediate medical attention as required, but instead prefer traditional healers or herbalists (Owour *et al*., 2005). The problem is confounded by the fact that there is lack of accurate data on snakebite incidences despite it a huge problem in places like Mwingi, Baringo and Kilifi counties (Coombs *et al*., 1997). In order to get a better understanding of these novel venom enzymes, a detailed and comprehensive knowledge of their toxin composition as well as biochemical properties of protein constituents should provide an opportunity for the design of new more effective immunotherapeutic agents for the treatment of hematoxic, myotoxic and central nervous system disorders, inflammation, cancer and consequences of snakebites to the victims. This study was carried out with the aim to isolate and biochemically characterize venom proteins from members of Viperidae and Elapidae snake's families using a Two-dimensional gel electrophoresis.

1.2 Problem statement

Snakebite envenomation constitutes a highly relevant public health issue on a global basis, although it has been systemically neglected by health authorities in many parts of the world (Gutierrez *et al*., 2007; Stock *et al*., 2007; Kasturiratne *et al*., 2008). Available epidemiological data on the incidence of snakebites estimates that there are over 5 million accidents per year. This has resulted in fatalities ranging from 20,000 to 125,000, with much higher incidences of disabilities. Snakebites results into neurological disorders and physical handicap to victims, from its necrotic effect which requires amputation, thereby greatly jeopardizes the quality of life (Kasturiratne *et al*., 2008; Gutierrez, 2014; Xiao *et al*., 2017). The highest burden of snakebite has been reported in South East Asia, Sub-Saharan Africa, Central and South America. Snakebites incidences in Kenya was reported with an overall frequency of 14 per 100000 populations per year (range 2-68) and Snake bite among the rural coastal population was 151 per 100 000 people per year (Snow *et al*., 1994) with Kilifi County accounting for 44 per 100000 populations per year (Coombs *et al*., 1997). Snake antivenom used in Kenya

originates from venom proteins of foreign snake population which brings us to the question whether they are useful for our local clinical use. The ubiquity of venom variation in snake populations poses special problems for the manufacture of antivenom and has undermined the commercial attractiveness of this class of therapeutic agent. In particular, it has been amply documented that both interspecies and intraspecies variation in venom constituents can affect the neutralization capacity of antivenoms (Fry *et al.*, 2003). This may be exacerbated by the selective use of tests to confirm venom toxicity and antivenom efficacy, such as the lethal dose LD50 and ED50, resulting in inadequate neutralization time, rather than dose dependent toxins, particularly enzymes involved in defibrinogenating, hemorrhagic and necrotizing venom activities. The clinical consequences of this can be reduced efficacy against some important venom activities or even complete treatment failure in critical envenomation (WHO, 2010). All these factors combined with the ongoing reduction in the number of antivenom manufacturer's world-wide and concomitant contraction in the range of available antivenoms, present significant challenges for the treatment of snakebite in the 21st century.

1.3 Hypothesis

1.3.1 Null hypothesis

Snake venom secreted from members of Viperidae and Elapidae snake families venom gland does not contain proteins and peptides of medical importance.

1.3.2 Alternative hypothesis

Snake venom secreted from members of Viperidae and Elapidae snake families venom gland contains proteins and peptides of medical importance.

1.4 Objectives

1.4.1 General objective

To isolate and characterize snake venom proteins and peptides from members of Viperidae and Elapidae families.

1.4.2 Specific objectives

1.4.2.1 To isolate snake venom proteins from Viperidae and Elapidae families

1.4.2.2 To characterize snake venom proteins and peptides from Viperidae and Elapidae families

1.4.2.3 To identify the isolated snake venom proteins and peptides.

CHAPTER TWO

LITERATURE REVIEW

2.1 Venomous snakes and their distribution in Kenya

Kenya harbors many species of venomous snakes (Spawls *et al*., 1995; Coombs *et al*., 1997; WHO, 2010). Gaboon viper (*Bitis gabonica*) are found in Kakamega and Nandi Forest, Puff Adders (*Bitis arietans*) are widely distributed in Kenya, Carpet Viper (*Echis pyramidum*) is most commonly found in Northern Kenya in areas like Samburu, Garissa, Maralal, and Eastern Turkana. Egyptian Cobra (*Naja haje*) which is sporadically distributed across the country habitates various districts: — Kajiado, Machakos, Nairobi, Naivasha, Thika, Isiolo, Samburu, Bungoma and Elgon. Forest Cobras (*Naja melanoleuca*) habitates Mt. Elgon, Bungoma, Trans-Nzoia, Eldoret, Kakamega, Kericho, Elgeyo-Marakwet, Mau, Nakuru, Menengai, Rongai, Mt.Kenya, Galana and Arabuko Sokoke Forest in the coastal Kenya. Black-Necked Cobras (*Naja nigricollis*) species are widespread but rare in areas above 1200 meters except in Nairobi, Thika and Athi-River areas. Other Districts where it occurs include Machakos, Kajiado, and Kilifi. Meru, Nairobi, Naivasha, Nyanza, Isiolo. Black Mambas (*Dendroaspis polylepis*) species are also widespread in low woodland and shrub, along the Coast, Malindi, and Kilifi. Black mambas are also found in Mombasa, Voi, Tsavo, Amboseli, Kajiado, Kutus, Tharaka, Meru, Samburu, Maralal, Baringo, Kerio-Valley, Bungoma, Kitui, Mara, Mwingi, and Nandi Hills. Green mambas (*Dendroaspis angusticeps*) are common in coastal plains of Diani, Kilifi, Malindi, Mombasa, Mtwapa, Shimba Hills, Shimoni and Watamu, Tana River, Nyambene and Kibwezi Forest. Jameson's Mambas (*Dendroaspis jamesoni*) are found in the Kakamega Forest area. Mount Kenya Bush Vipers (*Atheris desaixi*) are found in Igembe in the northern Nyambene range, Chuka on the South East of Mt.Kenya (WHO, 2010).

2.1.1 Forest cobra (*Naja (Boulenrina) subfulva* formerly *melanoleuca*

The Forest cobra (*Naja (Boulengrina) subfulva* formerly *melanoleuca*, exhibits a highly polished body scales with a narrow head. It has the ability to climb trees with preference for thick vegetation in close proximity to water surfaces. This snake grows to an average length of 2 meters (but can grow up to 2.7 meters in length). It is

the largest Cobra in tropical Africa and prefers living in the lowland forest or coastal savanna thickets. The reptile is commonly found on trees and small shrubs (Wuster *et al*., 2018). It feeds on amphibians (frogs and toads), small animal (rodents), birds, other snakes and fish (Luiselli *et al*., 2002). The females are oviparous and lays12-60 eggs per year in summer. Adults have a lifespan of 20 years (up to 28 years). The venom it produces is highly dangerous and neurotoxic, affecting mainly the central nervous and muscular system of envenomed animals (https://sciencing.com/life-cycle-cobra-6622173.html); Accessed on 15/01/2015.

2.1.2 Green mamba (*Dendroaspis angusticeps*)

The main physical features of this species of mamba is its flat coffin shaped head, a completely green body and along thin tail. It also has an average length of 1.8 meters but may reach up to 2.5 meters. This species is strictly exhibits diurnal lifestyle. *Dendroaspis angusticeps* has been found along the East Coast of Africa especially Kenyan coast, with individuals being found near the coast of western South Africa stretching through all the way Mozambique, Swaziland Malawi and Tanzania (Spawls *et al*., 1995). The species habitates the lowland forest, moist savanna grassland, bamboo thickets and mango as well as tea plantations. This species never like to stay on the ground but spend most of its life on trees or shrubs. It has a lifespan of 12 years. It oviposites 6 to 17 eggs on hollow tree trunk amongst decaying vegetation. Its venom contains high levels of neurotoxins, targeting neuronal and muscular systems. Dendrotoxins binds to voltage-gated potassium channels at nerve endings, causing acetylcholine release, and Calcicludine another toxin, blocks the calcium channels. There are also two three-finger toxins that are unique to mamba venoms. One Fasciculins inhibits some acetylcholinesterases, causing persistent muscle fasciculation, while Calciseptine bind to calcium channels (Warrell *et al*., 2013). For example, Calcicludine is a 60 amino acid peptide toxin from Green mamba with a potential to block all type HVA (high voltage activated) calcium ion channels (L-, N-, and P- type) in a variety of excitable cells. Calcicludine targets more L-type calcium channels in cerebellar granular cells and cardiac myocytes (Stotz *et al*., 2000).

2.1.3 Large brown spitting cobra (*Naja ashei*)

The large brown spitting cobra (*Naja ashei*) can be differentiated from all other African spitting cobras in that it possesses a unique clade of mitochondrion DNA

(mtDNA) haplotypes (Wuster *et al*., 2007). *Naja ashei* differs morphologically from *Naja nigricollis* in a number of characteristic features as it exhibits various colour pattern and scalation. On its midbody and posterior ventral parts, the rest of the body colour is predominantly light, with dark pigment encroachment mostly from sides of the body. It lacks any red, orange or pink pigment under the throat (this is pronounced in *Naja nigricollis*) and the head is olive-brown as the rest of the body (Wuster *et al.*, 2007). *Naja ashei* exhibits a sympatric distribution with *Naja pallida* over a wide geographical area, most commonly in dry lowland regions of the northern and coastal Kenya, extending south along the coast at least Diani Beach and north into Southern Somalia (Wuster *et al*., 2007). Its venom has been shown to causes tender local swelling, blistering surrounded by demarcated pale or blackened anaesthetic area of severe tissue necrosis (Farrar *et al*., 2013).

2.1.4 Puff adder (*Bitis arietans*)

This species is commonly identified by its extremely stout, heavily built body with a broad flattened hornless triangular head covered by small scales with rounded snout and short tail. The eyes are of moderate size with vertical pupil. Its scales are strongly keeled. The body coloration is either yellow or brown (sometimes pale, dark orange or reddish) and grayish with a pattern of regular chevron-shaped bars on the back and tail, venter is yellowish or white. The adults can measure up to 90cm in length with some exceptions of 150cm and normally weighs 6 kilograms. Puff adder have the habit of resting on the surface of roads, paths during the night where they rest while hunting for prey or basking on the warm soil. The Puff adders (*Bitis arietans*) adults are normally sluggish in their movement and can be safety approached, but care must be taken as they are able to strike an intruder suddenly extremely fast. It feeds mainly on small terrestrial living ground mammals including rats, mice frogs and toads. The species is an ovoviparous, (lays eggs that hatches immediately into young ones) (WHO, 2010). Its venom is known to be one of the most toxic of all vipers with LD50 values ranging from 9 to 13µg per mouse. It interferes with the haemostatic and coagulation systems and its effects are felt immediately (Moura-da-Silva *et al*., 2016).

2.2 Snake's Housing

As a general rule, snakes require relatively little space because of their limited and non-exceptional activity. Generally speaking, the size of the enclosure can be made to allow inclusion of certain required items such as unprinted newsprint and provided the snakes with adequate space to stretch out and move about. Snakes use both the horizontal and vertical space within their enclosure if provisions were made for this activity. Aquaria or other similar glass or Plexiglas- lined enclosures are most suitable because they allow optimum visualization of and safety for the occupant(s), the curator and help to maintain desirable environmental temperatures and generally high relative humidity levels. Enclosures should have a secure top and escape-proof with hinges and locks secured to avoid escape (WHO, 2016).

2.2.1 Floor Coverings and Enclosure Items

Floor covering materials for snake‘s resting area such as unprinted newsprint, butcher paper; paper towels, terrycloth towels and indoor-outdoor carpeting are most suitable for covering the bottom of a snake's enclosures (WHO, 2016).

2.2.2 Visual Security

It is very important to provide some privacy for a captive snake. Many snakes will not feed without the privacy afforded by some degree of visual security. This requirement can be accomplished by providing a "hide box" into which the snakes retreated when feeding or at other times when privacy was desired. Visual security can be provided by the use and strategic placement of silk artificial plants. Silk plants are visually pleasing and easy to clean and disinfect. They require minimal maintenance, help to augment the relative humidity level of the enclosure if the foliage is frequently misted, and can complement a snake's ability to camouflage itself, thereby providing visual security (WHO, 2016).

2.2.3 Climatic Considerations

Tropical snakes kept in captivity (Elapids and Viperids) require relatively warm temperatures and high relative humidity. Daytime temperatures ranged between 80° F (26.7^{O}C) and 85° F (29.4^{O}C) and nighttime temperatures can fall between 70-75°F (21.1 ^{O}C - 23.9^{O}C) inside the enclosures (Chanhome *et al*., 2001).

2.3 Toxicological effects of snake venom on animals

The effects of venom and their severity are mediated by a number of factors; the toxicity of the venom is determined by its component enzymes and is usually denoted by an LD_{50} number (Zug & Ernst, 1999). LD_{50} as a measure is based on a test whereby large groups of mice are injected with varying amounts of venom and the dosage at which 50% of the mice die within 24hours is the LD_{50}.

The Viperidae families found in Kenya consist of front-fanged venomous snake which includes vipers and pit vipers for example Puff adders (*Bitis arietans*) and Saw-scaled vipers (*Echis [pyramidum] leakeyi*). They exhibit vertebrate dietary preferences and prey capture is usually considered as a major biological imperative driving the venom toxin selection process (Casewell *et al*., 2009). Their venom contains numerous proteins that interfere with coagulation cascade, the normal haemostatic system and tissue repair. Human envenomation is often characterized by clotting disorders, hypofibrinogenemia and local tissue necrosis (Watanabe *et al*., 2003; Fox & Serrano, 2005). The venom protein components from this family belong to a few major protein families, including enzymes (serine proteinases, Zn^{2+} - metalloproteinase, L-amino acid oxidases, group II- PLA_2) and proteins without enzymatic activities (Disintegrins, C-type lectin, Natriuretic peptides, myotoxins, CRISP toxins, nerve and vascular endothelium growth factors, Cystatin and Kunitz-type proteases inhibitors).

The Elapidae venomous snake families consist also of front-fanged snakes such as Cobras (*Naja (Boulengrina) subfulva* formerly *melanoleuca, Naja ashei,* and *Naja nigricollis*), Sea snakes (Genera: Laticauda), Mambas (*Dendroaspis polylepis*, *Dendroaspis angusticeps*) and Coral snakes (Genera: Micrurus). They produce highly toxic venoms with neurotoxic and cytotoxic activities when exposed onto mammalian cells, organs and systems.

Snake venoms generally consist of complex proteinacious components (circa 50-200), commonly referred to as toxins (Slagboom *et al*., 2017). Toxins have evolved from a number of non-toxic house-keeping genes by a variety of processes, notably gene duplication followed by neofunctionalization under the action of positive selection, alternative splicing, recombination and modification of expression (Lynch, 2007;

Casewell *et al*., 2011, Vonk *et al*., 2013). Variation in venom composition has been observed across all taxonomic levels of snakes; between families, genera and species, over the lifetime of individual snakes (Durban *et al*, 2013; Casewell *et al*., 2014). This diversity results in a variable pathological consequences and the clinical patterns can be broadly classified into three groups: neurotoxic, cytotoxic (myotoxic) and hematoxic (WHOA, 2010).

Neurotoxic envenoming is characterized by descending neuromuscular paralysis, beginning with the eyes (ptosis), facial muscles and other muscles innervated by the cranial nerves, before progressing to respiratory and generalized flaccid paralysis (Chippaux, 2010). The toxins predominantly responsible for neurotoxic venom effects are members of the diverse phospholipase A2 (PLA2) and three-finger toxins (3FTxs) families (Fry, 2003; Lynch, 2007).Theses toxins are capable of acting on the pre-synaptic and/or post-synaptic junction where they can have a multitude of actions, from blocking of the potassium or sodium ion channels, to acting as nicotinic or muscarinic receptor antagonists (Fry, 2003; Lynch, 2007; Casewell, 2013).

Cytotoxic snake envenoming are characterized by painful and progressive swelling at the bite site, developing into blistering and bruising that are sometimes coupled with systemic effects, which include hypovolemic shock (WHO, 2010). Hydrolytic enzymes such as snake venom metalloproteinases (SVMPs), phospolipases and non-enzymatic cytotoxic three-finger toxins are the common causative agents of cytotoxicity (Escalante *et al*., 2009; Rivel *et al*., 2016). Recently, it was also shown that the destruction of local tissue may be promoted by snake venom inducing the formation of neutrophil extracellular traps (NETs), which in turn blocks blood vessels and promotes cytotoxic pathology (Katkar *et al*., 2016).

Hematoxicity from snakebite envenoming is fairly common with and/or associated with Viperidae snakes (e, g., Bitis spp., Echis spp). Hematoxicity can have cardiovascular and/or haemostatic effects. Cardiovascular effects are best characterized by a dramatic fall in blood pressure, caused by a number of different venom toxins. For example, Snake venom metalloproteinases (SVMPs) indirectly contributes to hypotension by increasing vascular permeability through the degradation of capillary basement membranes, resulting into a leakage and reduction of in blood pressure (Gutierrez *et al*., 2016).

2.4 Snakebite management

2.4.1 Management of snake envenomation

Treatment of venomous snakebite varies depending on the particular snake and severity of the bite; however treatment maintains a common general procedure. First Aid measures involve getting the victim to lie flat and to avoid as much movement as possible (Davidson, 2000). The management of snakebite envenomation in human is controversial with advocates of differing protocols, but the following principles of treatment can be used with great outcome, which includes (i) allaying anxiety and fear (ii) prevention of the spread of venom (iii) use of antivenom and other antibiotics and general measures (Warrell, 2010).

2.4.2 Allaying anxiety and prevention of the venom spread

Victims should be reassured that not all snakes are poisonous, even venomous snakes are not fully charged with poison and also a poisonous snake fully charged with poison cannot always inject a venom containing lethal dose. Spread of the venom occurs mostly by diffusion and lymphatic circulation. Therefore, efforts to reduce lymph circulation are helpful. This can be achieved by; — Immobilization, application of tourniquet, cleaning the wound, incision and suction (Sutherland *et al*., 1979).

2.4.3 Immobilization

Activity increases the spread of venom, so the bitten part should be immobilized. This reduces the spread of the venom and eases the pain of snakebite. The Australian pressure–immobilization technique, in which the entire bitten limb is wrapped with elastic or crepe bandage and then splinted, is highly effective. The bandage is applied with the same snugness used for a sprained ankle. It restricts absorption and circulation of the snake venom. A Crepe bandage should be applied to slow the absorption of venom if possible the bitten limb put in a resting position below the level of the heart (Sutherland *et al*., 1979).

2.4.4 Application of tourniquet

This is possible if the bite is on a limb. In case of bite of face, neck, trunks, application of firm pressure, using rubber tube, gamucha (Scarf/Sash) is recommended. If the victim is more than 60 minutes from a medical care, a proximal lympho-occlusive constriction band may limit the spread of venom if

applied within 30 minutes of occurrence of the snakebite. The band should be wide and should occlude superficial venous and lymphatic circulation without impeding flow (typically with a pressure of 20mmHg). The bitten limb is splinted (if possible) and kept at approximately 5cm proximal to the bite site (WHO, 2010).

Normally, an additional tourniquet at a distance of 5cm to the first one is desirable. It should be released for a minute at 30 minute intervals or for 30 seconds every 15 minutes. The tourniquet may be applied for up to two hours. The tourniquet should be loose enough to insert a finger between the skin and the bandage (WHO, 2010).

2.4.5 Cleaning the wound

The wound should be cleaned with soap water and/or saline. Tetanus toxoid or tetanus immunoglobulin to be considered for under-immunized or none immunized victims (WHO, 2010).

2.4.6 Role of incision and suction

Local mechanical suction applied to the site within 3 to 5 minutes may remove a small percentage of deposited venom. The device extractor applies 1 atm. of negative pressure, and should be continued for at least 30 minutes. Mouth suction should be avoided. It is harmful for both the victim and rescuer (WHO, 2010).

2.5 Treatment of snakebite

Treatment in a Hospital Emergency Department or Intensive Care Unit (ICU) involves the administration of antivenin (Dart & Gomez, 1996). Antivenin also known as antivenom may be monovalent (snake specific) or polyvalent (incorporating antibodies for several species). Monovalent antivenin is preferable to polyvalent type since it is less hazardous to the patient and likely to be more effective in the treatment of the particular bite however a positive identification of the species of snake is required for the administration of snake specific antivenin (Thompson & Loadman, 1996). In many cases identification of the envenoming snake by the victim is difficult, impossible and/or when identity is at all uncertain then the polyvalent type should be used. Almost all antivenins are derived from horse serum. Horses are injected with progressively larger doses of venom until immunity is achieved (Roberts, 1987). Thus, antivenin neutralizes the effects of venom by giving antibodies to the patient (Immunoglobulins) (Dart & Gomez, 1996).

2.6 Venom proteins acting on blood coagulation factors

Venom proteins affecting coagulation factors may be classified as acting on coagulant factors including FV activators, FX activators, Prothrombin activators and thrombin-like enzymes (TLEs), Anticoagulant factors including FIX/X binding proteins, protein C activators, Thrombin inhibitors and phospholipase-A2 and those acting on fibrinolysis such as Fibrinolytic enzymes and Plasminogen activators.

2.6.1 The FV activators

Factor five (FV) is a multi-functional 330kDa glycoprotein with an important role in both procoagulation and anticoagulation. Thrombin activates FV by cleaving at 709, 1018 and 1545 to form FVa. The FVa is a heterodimers consisting of a 105kDa heavy chain and a 72/74kDa light chain doublet. It acts as a cofactor in FXa catalyzed by Prothrombin activation and it enhances thrombin generation >1000-fold. Its cofactor activity is down-regulated by activated Protein C– mediated FVIII inactivation (Mann & Kalafatis, 2003). Several FV activators have been described from *Bothrops attrox*, *Vipera russelli*, *Vipera lebetina*, *Vipera ursine*, *Naja* (*naja*) *oxiana* and *Naja* (*nigricollis*) *nigricollis* venoms (Rosing *et al*., 2001). RVV-V from *Vipera russelli* is a 236 amino acid single –chain serine protease. This enzyme activates FV by cleavage of a single peptide bond at Arg 1545 (Kalafatis *et al*., 2003). FV is also cleaved by RVV-V at Arg1018 to give two fragments of about 1051kDa (1-1018 and 1019-1545, the latter highly glycosylated). A protease (NN) from *Naja (nigricollis) nigricollis* cleaves FV at Asp697, Asp1509 and Asp1514 to form a heavy chain of 100kDa and a light chain doublet of 80kDa (VNN) (Tokunaga *et al*., 1988). VNN binds FXa eight times more weakly than FVa. Incubation of NN with thrombin activated FV (FVIIa) gives 80% loss of the clotting activity of FVIIa because removal of the last 13 residues (679-709) from the C-terminal of the heavy chain reduces prothrombin binding. A protease from *Naja* (*naja*) *oxiana* venom behaves similarly (Tokunaga *et al*., 1988).

2.6.2 The FX activators

FX activators have been isolated from many Viperidae and Crotalidae venoms as well as from a few Elapid Species. The FX activators are either metalloproteinases or serine protease (Tans & Rosing, 2001). The most important FX activator is RVV-X found in Vipera russelli. RVV-X is a disulfide-linked three chain

metalloproteinase, a 58kDa heavy chain with 19.4kDa and 16.4kDa light chains. The heavy chain has metalloproteinase, disintegrin-like and Cysteine-rich domains (Takeya *et al.*, 1992).

It recognizes and binds to the γ-carboxyglutamic acid (Gla) domain of FX via the two C-type lectin chains of RVV-X and the heavy chain of RVV-X cleaves the target Arg52-Ile53 bond in the heavy chain of FX. Metalloproteinase FX activators from other venoms have similar structures to RVV-X and probably similar catalytic mechanisms. RVV-X also activates FIX by cleaving a single peptide bond without affecting molecular mass (Lindquist *et al.*, 1978).

2.6.3 Prothrombin activators

A large number of snake venoms contain prothrombin activators, which convert prothrombin to thrombin (Rosing & Tans, 1992). Based on their structures functional characteristics and cofactor requirements they are classified into four groups. Group A prothrombin activators are metalloproteinases and activate prothrombin efficiently without cofactors, such as Ca2+, phospholipids (PLs) or Factor five activated (FVa). Group B prothrombin activators are Ca2+- dependent. They contain two subunits linked non-covalently: a metalloproteinase and C-type lectin- like disulfide-linked dimer. Group C prothrombin activators are serine protease found in Australian elapids requiring Ca2+ and negatively charged PLs, but not FVa for maximal activity. Proexosite 1 is a co-factor-dependent recognition site for them as well as for prothrombin. Group D- Prothrombin activators are serine proteases and are strongly dependent on Ca2+, negatively charged PLs and FVa (Rosing & Tans, 1992).

2.6.4 Thrombin-Like Enzymes (TLEs)

Snake venom TLEs are widely distributed in several pit viper genera (Agkistrodon, Bothrops, Lachesis and Trimeresurus) in some true Vipers Bitis, Cerastes and the Colubridae, *Dispholidus typus* (Pirkle, 1998). Sequences of more than thirty (30) Thrombin-like Enzymes (TLEs) have already been determined, with a high level of similarities. The catalytic domain, including the catalytic triad residues His57, Asp102 and Ser195 and the S1 (Asp189) and S2 (Gly216) specificity sites, are the main conserved regions. Like thrombin, Thrombin-Like Enzymes specifically catalyze limited cleavage of fibrinogen and lead to clotting of fibrinogen. Based on the rates of release of fibrinopeptides A and B, Thrombin-Like Enzymes can be

classified into three groups; Venombin A, Venombin B and Venombin AB (Markland, 1998). TLEs are inhibited by most of the serine protease inhibitors, but most are unaffected by thrombin inhibitors like anti-thrombin III and Hirudin. Unlike thrombin, most TLEs do not activate FXIII and protease activated receptors (PARs) on platelets. Consequently, the fibrin formed by TLEs is easily removed from the circulation allowing their clinical use as defibrinogenating agents (Markland, 1998).

2.6.5 The Factor IX/X inhibitors

Many anti-coagulants C-type lectin–like proteins interacting with FIX and/or FX have been isolated from various snake species (Morita, 2004). Based on their ligand recognition differences, these proteins can be classified as; blood coagulation FIX/FX-binding proteins (FIX/FX-BPs), which do not interact with FX; FX-BINDING PROTEINS (FX-BPs), binding predominantly to FX. They are heterodimers with highly similar structures. The gamma carboxyglutamic acid (Gla) domain of coagulation factors is responsible for Ca2+-dependent phospholipids (PLs) membrane binding. Snake venom FIX/FX binding proteins bind specifically to the gamma carboxyglutamic acid (Gla) domain of factor FX or FIX (Morita, 2004).

2.6.6 Protein C activators

Protein C is a vitamin K-dependent, two chain Zymogen activated by thrombin. Activated Protein C degrades FVa and FVIIIa and is therefore an anticoagulant. Most protein C activators have been purified from Agkistrodon venoms. Others have been purified from Bothrops; Trimeresurus and/or Cerastes venoms. Most venom toxins containing protein C activators have sequences with high similarity to other venom serine proteinases. Unlike thrombin-catalyzed protein C activation, requiring thrombomodin as a co-factor, venom activators directly converts protein C into the active form. The fast acting protein C activator Protac® from *Agkistrodon contortrix contortrix* venom is widely used to diagnose protein C Pathway disorders (Gempeler-Messina *et al*., 2001).

2.6.7 Thrombin Inhibitors

Bothrojaracin (BJC) is a 27kDa C-type lectin-like thrombin inhibitor, 13kDa and 15 kDa subunits linked by disulfide bridges (Zingali *et al*., 1993). BJC is highly resistant to Urea or Dithiothreitol (DTT), requiring both agents to denature if fully. BJC has two independent mechanisms for anticoagulation: - first it binds

strongly to exosite I and II to form a non-covalent equimolar complex, and second, it inhibits thrombin-induced platelet aggregation and secretion but does not interact with the catalytic site of thrombin. It prolongs fibrinogen-clotting time by inhibiting competitively the binding of thrombin to fibrinogen and it inhibits thrombin binding to thrombomodulin and decreases the rate of Protein C activation (Arocas *et al*., 1996). Secondly it inhibits prothrombin activation by interacting with Proexosite I. In the absence of PLs, BJC strongly inhibits the Zymogen activation by Factor five activated (FXa) in the presence but not in the absence of Factor five activated (FVa). Proexosite I blockage decreases cleavage of prothrombin by FXa-FVa complex or Prothrombinase complex (Zingali *et al*., 2001).

2.6.8 Phospholipase A 2

Snake venom phospholipase A_2 (PLA_2) are an extremely important and diverse group of proteins affecting hemostasis. They can be divided into two groups, Asp49 PLA_2S with high catalytic activity, and Lys49 PLA_2s with very low or no enzymatic activity (Gutierrez & Lomonte, 1995). Asp49 PLA_2s as part of the calcium-binding site which is very critical for its catalytic activity. PLA2s display a wide range of pharmacological effects. It has been proposed that their unique ability to target a specific organ or tissue is because of high affinity binding to specific receptors independent of the catalytic site. Upon binding to the target, the PLA2 causes pharmacological effects either via its catalytic activity or independently (Kini, 2003). Anticoagulant PLA_2S (mainly Asp49 PLA_2) have been described in all major snake groups. As PLA_2 cleaves phospholipids (PLs), it was first suggested that Venom PLA_2 inhibited blood coagulation by destroying Procoagulant phospholipids (PLs). Some PLA_2S have a higher affinity for anionic phospholipids (PLs) (Kini, 2003). Phospholipase A2 (PLA2) snake venom enzymes as small, stable calcium-dependent, disulfide- rich biomolecules hydrolyzes the stereospecifically numbered 2 (sn-2) ester bonds of the membrane glycerophopholipids, releasing free fatty acids and lysophospholipids (Murakami *et al.,* 2011). These noble enzymes can also be grouped into 16 according to their structure (GI to GXVI) and in a non-structure basis as: - Secretory phospholipase A2 (sPLA2s), Cytosolic (cPLA2s),

Ca2+-Independent (iPLA2s), Platelet-activating factor acetylhydrolases (PAF-AH) and Lysosomal PLA2s (Ritonja & Gubenek, 1985; Kini, 2003).

2.6.9 Fibrinolytic enzymes

Fibrin (ogen) lytic activity has been described in the venoms of members of the Crotalidae, Viperidae and Elapidae families. These Fibrinolytic enzymes are divided into metalloproteinases and serine proteases (Matsui *et al.*, 2000). Most of the first group of enzymes were characterized as Zinc metalloproteinases and degraded the A α-chain of fibrinogen preferentially. The second groups are serine proteases and most have specific activity toward the B β-chain of fibrinogen. However, there are exceptions to this generation, and specificity for the α- or β-chains is not absolute, as there is substantial degradation of the alternate chain with time. Most of the metalloproteinases are Fibrinolytic and the serine proteinases are both fibrinogenolytic and Fibrinolytic (Braud *et al.*, 2000). Fibrin (ogen) lytic metalloproteinase enzymes cleave amino- terminal to hydrophobic amino-acids, while serine fibrin (ogen) lytic enzymes cleave carboxy- terminal to basic amino-acids. Members of metalloproteinases family are metal ion containing proteinases (mainly zinc), having a common methionine turn below and carboxy-terminal to a helical segment containing two of the three histidine residues involved in the zinc-binding site (Jia *et al.*, 1996). The fibrin (ogen) olytic serine proteases share extensive sequence homology with other venom serine proteases but subtle differences determine their substrate specificities (Braud *et al.*, 2000).

2.6.10 Plasminogen activator

A direct acting Plasminogen activator in snake venom toxin was described from *Trimeresurus stejnegeri* venom (Zhang *et al.*, 1995). Plasminogen activators have also been reported from *Lachesis* (*muta*) *muta* and *Agkistrodon halys* venoms (Sanchez *et al.*, 2000). TSV-PA is a 33kDa single chain serine protease that activates Plasminogen by cleaving the Arg561-Val562 bond like the physiological activators u-PA and t-PA. However, unlike t-PA, fibrin fragments do not stimulate activation of TSV-PA. TSV-PA does not activate FX or Protein C and it does not degrade fibrin or fibrinogen in the absence of Plasminogen. The DNA sequence of TSV-PA shares more than 60% similarity with other snake venom serine proteases but only share approximately 20% with t-PA or u-PA. It contains only the catalytic

domain of the mammalian Plasminogen activators. TSV-PA lacks the sequence responsible for the interaction of t-PA (KHRR) and u-PA (RRHR) with Plasminogen activator inhibitor type1 (Zhang *et al*., 1995).

2.7 Venom proteins acting on the vessel walls

2.7.1 Metalloproteinases

Crotalidae and Viperidae contains many metalloproteinases which act synergistically to degrade the blood vessel extracellular matrix (ECM) and thus affect hemostasis based on the domain structure, the venom metalloproteinase are classified into four major groups (Hati *et al*., 1999; Hite *et al*.,1994). The protein-type1 (P-I) class has a single metalloproteinase domain. The protein-type 2 (P-II) class has a metalloproteinase, and a disintegrin domain that is separated post-translationally. The protein-type 3 (P-III) class has a metalloproteinase, disintegrin-like containing a disulfide-linked X-X-Cysteine-Aspartate (XXCD), mostly Serine-Glutamic acid-Cysteine-Aspartate (SECD) in place of Arginine-Glycine-Aspartate (RGD) and Cysteine-rich domains. The protein-type 4 (P-IV) class contains an additional disulfide-linked C-type lectin– like domain, compared with the P-III class. The hemorrhagic metalloproteinases are stored as inactive zymogens and activated by Cysteine switch-like mechanisms (Grams *et al*., 1993). They cleave key peptide bonds of the cellular basement membrane thereby affecting the interaction between endothelial cells. These cells then undergo morphological and functional alterations in vivo, associated with hemodynamic factors such as shear stress and eventually gaps form between endothelial cells allowing extravasations (Gutierrez & Rucavado, 2000). Disintegrins-like and Cysteine-rich domains of PI-III class hemorrhagic toxins make them more active than PI-I or PI-II by directing the enzymes to critical locations in capillary blood vessels and modulating the Catalytic specificity of the enzyme domain on extracellular matrix (ECM) substrates (Gutierrez & Rucavado, 2000).

2.7.2 Disintegrins

Disintegrins are low molecular weight, Cysteine-rich and Arg-Gly-Asp (RGD) containing peptides, which inhibit platelet aggregation by blocking fibrinogen binding to the platelet GPIIb/IIIa ($\alpha IIb\beta 3$) (Braud *et al*., 2000; McLane *et al*., 1998; Schlondorff & Blobel, 1999). They also prevent human umbilical vein endothelial

cell adhesion to fibrin or to immobilize extracellular matrix by inhibiting integrins, which play an important role in angiogenesis. Rhodostomin inhibits distinct steps in angiogenesis elicited by basic fibroblasts growth factor through integrins αvβ3. Moreover it induces endothelial cell apoptosis. Obtustatin, a KTS rather than RGD containing disintegrin, selectively inhibits α1β1 integrins (Marcinkiewicz *et al*., 2003). It has been demonstrated that it potently inhibits angiogenesis in vivo in the chicken chorioallantoic membrane assay; Contortrostin inhibits migration and invasion, and alters matrix gel–induced tube formation of human umbilical vein endothelial cells, but does not affect cell viability, possibly by blocking αvβ3 (Golubkov *et al*., 2003).

Generally, disintegrin inhibit integrins of the β1 and β3 subfamilies including the fibrinogen receptor GPIIb/IIIa (αIIbβ3), the Vitronectin receptor (αvβ3) and the fibronectin receptor (α5β1). More than 50 disintegrins have been purified from various venoms from the Viper or Pity viper families (Braud *et al*., 2000). GPIIb/IIIa antagonists inhibit aggregation caused by agonists including ADP, Thrombin, Collagen and Arachidonic acid. They consist of either single chain molecule of 40-80 amino acid residues and multimeric. Disintegrins contain an RGD or KGF sequence in the carboxyl-terminal half of the molecule which is essential for blocking integrins interactions with ligand (McLane *et al*., 1998).

Disintegrins structures are characterized by irregular turns and loops that form a rigid core and the RGD sequences are stabilized by two disulfide bonds. The RGD sequence is highly mobile allowing rapid binding to the integrins-binding site within GPIIIa residues 217-302 (Fujii *et al*., 2003). The width and shape of the RGD loop may be an important structural feature to fit into the binding pockets of integrins αIIbβ3 and αvβ3. Additional structural features may be important for the selectivity and affinity of disintegrins (Hantgan *et al*., 2004). The structure of a homodimeric disintegrin indicates that the N-terminal anchored two chains of the dimer diverge at the C-termini exposing the RGD motif in opposite directions to enhance binding efficiency for integrins (Bilgrami *et al*., 2004).

2.7.3 L-Amino acid oxidases

Snake venom L-amino acid oxidases (LAAOs) are homodimeric flavoenzymes that catalyze oxidative deamination of a LAAOs substrate to α-keto acid along with

ammonia and hydrogen peroxide. They are widely distributed in Viperidae, Crotalidae and Elapidae (Du &Clemetson., 2002). Each subunit has three domains; a FAD binding domain, a substrate-binding domain and a helical domain (Pawelek *et al*., 2000). The reported effects of LAAOs on platelet function are quite controversial. LAAO from *Echis colorata* inhibits ADP-induced platelet aggregation (Nathan *et al*., 1982). *Agkistrodon* (*halys*) *blomhoffi* and *Naja* (*naja*) *kaouthia* LAAOs inhibits agonists or shear stress-induced platelet aggregation (Takatsuki *et al*., 2001; Sakurai *et al*., 2001). It has been suggested that the interaction between activated platelet integrins GPIIb/IIIa and fibrinogen is inhibited by the continuous generation of hydrogen peroxide (H2O2). LAAOs from *Eristocophis macmahon*i; *Ophiaphagus Hannah*; *Bothrops alternatus* and *Trimeresurus jerdonii* induce human platelet aggregation through formation of hydrogen peroxide (H2O2) (Du *et al*., 2002; Lu *et al*., 2002; Stabeli *et al*., 2004).

2.7.4 The 5′-Nucleotidases

The 5′-Nucleotidase activity is widely distributed in many Vipers and Pit Viper Venoms (Nget-Hong & Ponnudrai, 1992). *Trimeresurus gramireus* venom contains a 74kDa thermostable, single chain 5′- Nucleotidase. Its activity has been shown to be inhibited by Ethylene diamine tetraacetic acid (EDTA) but supported by Zn2+ or CO2+. In rabbit platelet–rich plasma, 5′-Nucleotidase completely inhibits platelet aggregation induced by Adenosine diphosphate (ADP), Sodium arachidonate or collagen, most likely by ADP and possibly by generation of adenosine (Ouyang & Huang, 1983).

2.8 Methods used for venom protein isolation and purification

The analysis of snake venom proteins has involved many different analytical techniques and approaches. Chromatography, gel electrophoresis and dialysis all have been used as preparative methods. Venom proteins can be literally created in the laboratory from venom gland tissue using cloning technology (Recombinant DNA Technology). The primary structure of venom protein can be determined by Edman sequencing and mass spectrometry (Edman & Begg, 1967; Shuting *et al*., 2004).

2.8.1 Chromatography

Chromatography relies upon an analyte existing in equilibrium between a stationary and a mobile phase. The stationary phase can be a solid, or it can be a solid coated

with a liquid, the mobile phase can be a liquid, a gas or a supercritical fluid (Goetz *et al*., 2004).

2.8.2 Ion–exchange chromatography

Ion-exchange columns are packed with charged groups covalently attached to a stationary support, such as Agarose or cellulose. This allows the separation of ions or polar groups based on their charge orientation. An anion-exchange support will retain positively charged species. If the charge on the covalently bound group of the column is influenced by the environment, then the column is said to be a weak ion-exchanger. However, on the hand if the group has a permanent charge irrespective of the ionic conditions, the column is said to be a strong ion-exchanger (Goetz *et al*., 2004). Typically a sample will be introduced onto the column in a buffer of low ionic strength, thus allowing the analyte ions to bind to the charged groups of the column. Bound analyte species are eluted by altering the pH or the salt concentration of the mobile phase, this either cause the ionic species in the mobile phase to compete with the analyte molecule for the binding site or opposes the charge on the stationary phase. The analyte is eventually released from the ionic group and eluted from the column. Some difficulties can arise when using high ionic strength elution conditions which suppress electrospray ionization and potentially corroding the hardware. However, ion exchange chromatography is suitable for separating complex mixtures of proteins on the basis of their differing ionic properties (Goetz *et al*., 2004).

2.8.3 Reverse-phase high performance liquid chromatography (RP-HPLC)

Chromatography is a powerful separation of complex mixtures of macromolecules. It has a high resolving power, reproducibility and compatibility with Electrospray mass spectrometry. Reverse Phase-High Performance Liquid Chromatography has become the most widely used of all types of chromatographic procedures (Rey-Suarez *et al*., 2011). In Reverse phase high performance liquid chromatography (RP-HPLC), the stationary phase is non-polar, often a hydrocarbon (such as C4 or C18 chains), covalently bounded to the support particles of the packing. The mobile phase is relatively polar, usually a mixture of water and organic solvent. A reverse-phase separation is started from an aqueous solution containing low concentration of such solvents as Methanol, Acetonitrile, or Tetrahydrofuran and the

most polar component is eluted out first. As the organic component of the mobile phase is increased, the polarity is decreased and the less polar molecules are flushed out subsequently. In practice, C4 columns are used for protein separation while peptides are fractionated with C18 columns (Rey-Suarez *et al.*, 2011).

2.8.4 Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE still remains the most current standard method for protein separation using denaturing agent, Sodium dodecylsulfate Polyacrylamide gel electrophoresis. Sodium dodecylsulfate (SDS) is an ionic detergent with a net negative charge. It binds to most soluble protein molecules in aqueous solutions over a wide pH range. The amount of bound Sodium dodecylsulfate is proportional to the size of the molecules (Shapiro *et al.*, 1967). The sodium dodecylsulfate eliminates most of complex secondary, tertiary or quaternary structure of proteins, which is one requirement for protein sizing by SDS-PAGE (Schneider *et al.*, 2008).

It is necessary to reduce protein disulphide bridges before the proteins adopt the random-coil configuration necessary for separation by size. This is achieved with reducing agents such as Mercaptoethanol or Dithiothreitol. In addition, sodium dodecylsulfate also confers a negative charge to the polypeptide, which is proportional to its length. This negative charge is utilized to separate the protein in an electric field within polyacrylamide gels. The polyacrylamide gel with a certain acrylamide concentration restrains larger molecules from migrating as fast as smaller molecules. Because of the charge-to-mass ratio is nearly the same among Sodium dodecylsulfate denatured polypeptides, the final separation of proteins is dependent almost entirely on the differences in molecular weight of polypeptides. In a gel of uniform pore size the relative migration distance of a protein is negatively proportional to the logarithm of its molecular weight (Meleth *et al.*, 2005). If proteins of known molecular weight are run simultaneously with the unknowns, the relationship between its relative migration distance and molecular weight can be plotted, and the molecular weight of unknown proteins determined (Shapiro *et al.*, 1967).

2.8.5 Two-dimensional gel electrophoresis

Electrophoresis is based on the migration of charged proteins in an electric field. Two- dimensional gel electrophoresis is the most widely used method for protein separation, which combines two types of electrophoresis: Isoelectric focusing (IEF) and Polyacrylamide gel electrophoresis (PAGE) (Gorg *et al*., 2000). IEF is the procedure used to separate proteins according to their Isoelectric Points (pI). This is the characteristic pH value at which the net charge of protein is zero. A pH gradient is established by the distribution of Ampholytes, low molecular weight organic acids and bases, across the gel. After the protein mixture is applied, each molecule migrates in the electric field until it reaches the pH matching its isoelectric point (pI) (O'Farrell, 1975).

The introduction of immobilized pH gradient (IPG) gels eliminated the poor reproducibility associated with Ampholytes (Gorg *et al*., 2004). In an IPG gel, the pH gradient is covalently bounded to the Acrylamide gel matrix, resulting in stable gradient at all the pH values. The precast IPG gels are commercially available in a variety of narrow and broad pH ranges with the resolution as high as 0.01 pH unit. The second dimension is called SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis). In this step the proteins are separated based on their molecular weight. SDS-PAGE is carried out in gels made of cross- linked Polyacrylamide. Sodium dodecylsulfate is a detergent that binds to proteins, so that the proteins are denatured and the intrinsic charges of proteins eliminated. When an electrical field is applied smaller proteins migrate faster in the gel matrix and larger molecules move slowly (O'Farrell, 1975). The protein spots in the gel can be stained by various methods, including silver staining, Coomassie blue staining and SYPRO-Ruby fluorescence stains to aid in visualization (Neuhoff *et al*., 1988; Shevchenko *et al*., 1996; Berggren *et al*., 2002). The protein spots can then be excised for an in-gel protein digestion. The resulting peptides are extracted and subjected to mass spectrometry for protein identification in the readily available protein and peptide sequence data bases (Aebersold & Mann, 2003).

2.9 Gel staining and visualization

The protein staining process by silver and Coomassie stains are based on the fact that they are sensitive and compatible with mass spectrometry. Silver stain has

outstanding detection limits (spots with 1-10 ng of protein can be observed) and takes about 1-2 hours for the entire process. It also has the advantage that no destaining step is needed (Heukeshoven & Dernick, 1988).Coomassie blue is not quite as sensitive (detection limits of 50-100 ng) and takes longer time, generally 1 hour to stain and overnight to destain. Furthermore, with Coomassie blue stain, the gel pieces must be thoroughly washed before mass spectrometry analysis, or adducts from the stain can occur on peptides. The development of colloidal Coomassie blue stain led to improved detection limits (8-10 ng), due to decreased background (Neuhoff *et al*., 1998).

Due to the complexity of the protein patterns, it is difficult for the human eye to compare two gels. Thus, 2D-PAGE gels usually are scanned by using a computer-assisted digital camera, and the picture is converted to a digital image. This allows for precise determination of spot location, intensity and for computer assisted comparison of different gels. Protein spots are quantified by their visual intensities. This can then serve as a differential display of two different organism growth types or mutants. (Aebersold & Mann, 2003).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

The study area was Kilifi County situated along the northern coastal region of Kenya, where four pairs of male and female snake specimens were captured and/or rescued from Gongoni (*Dendroaspis angusticeps*), Gede-Malindi (*Naja ashei*), Arabuko Sokoke Forest (*Naja melanoleuca*) and Bamba village (*Bitis arietans*) for venom extraction purposes only (Figure 1).

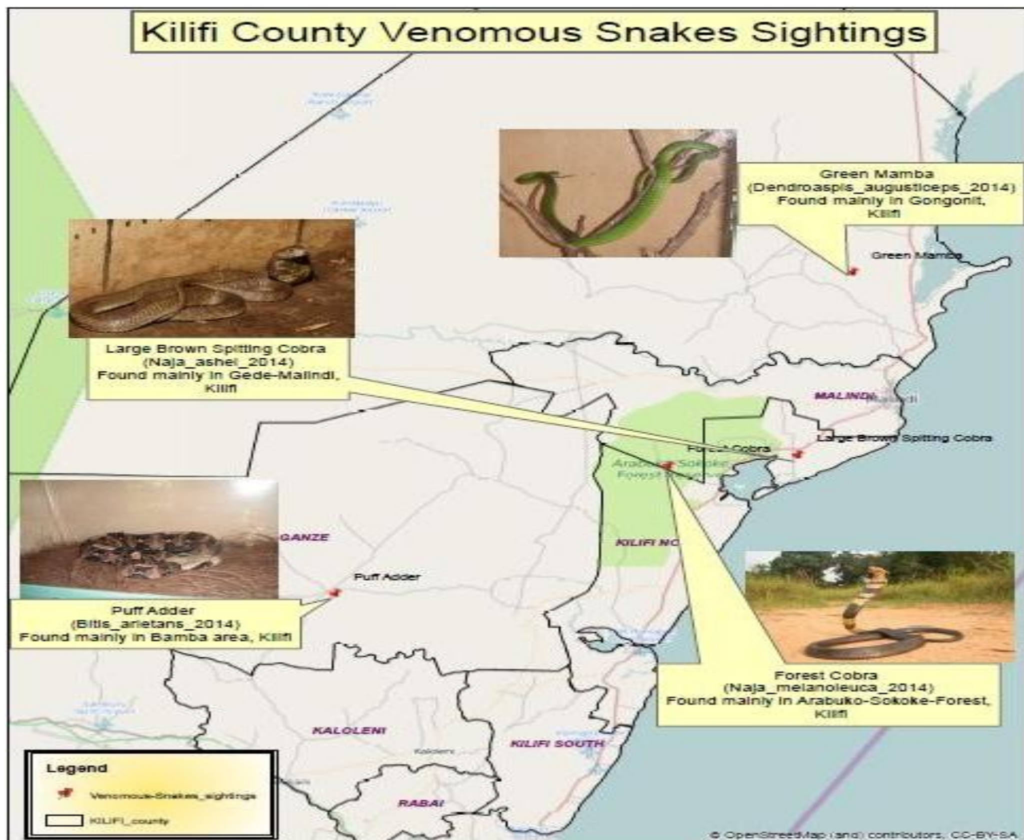

Figure 3.1: The Map of Kilifi County, Showing the locations of the study area

3.2 Study design

This was a cross sectional study conducted on snake venom samples. Data was collected between August 11th 2014 to August 10th 2015.

3.3 Study population

The study populations were four pairs of mature male and female snakes (Elapidae: —*Dendroaspis angusticeps*, *Naja ashei*, *Naja (Boulengrina) subfulva* formerly *melanoleuca* and one Viperidae family member: — *Bitis arietans*) captured and/rescued from the wild with the help of a snake catcher. These four represent venomous species of medical importance in Kenya (WHO, 2010). Their venom samples were extracted and transported in liquid nitrogen to the research Laboratory for experimental analysis. After the completion of the experiment, the animals were released back into the wild with the help of the snake handling expert within a month.

3.4 Inclusion and Exclusion criteria

3.4.1 Inclusion criteria

Mature venomous snakes were sampled for their venom toxin proteins.

The health statuses of the snake specimen were determined by a snake expert.

3.4.2 Exclusion criteria

Pre-mature, non-venomous and unhealthy snakes were excluded.

3.5 Ethical Clearance

The animals and research protocols used in this study were approved by Scientific steering (SSC), (Appendix: I) and Ethical review committees (ERC), (Appendix: II) of Kenya Medical Research Institute and Kenya Wildlife Service (KWS), (Appendix: III) following the regulations and guides for the animal care and use committee, Kenya protocol Number: **SSC No: 2716** and international laws and policies. All efforts were made to minimize the number of animals used and their suffering.

3.6 Laboratory Chemicals and Apparatus

Chemical used in this experiment were high quality ultra-pure molecular biology grade reagents supplied commercially as follows: Ammonium persulfate, Nonidet P-40, Tris-base, Sodium dodecyl sulfate (Fisher Scientific Co.UK); Ultrapure Urea, Un protein standards (2.5kDa-200kDa), Dithiothreitol, Iodoacetamide (bioWORLD,

Tuller Road, Suite 228Dublin, Ohio, USA); Acrylamide, N, N'-methylene bisacrylamide, Ampholytes pH3-10 (GE, Healthcare); Bromphenol Blue dye, N,N,N,'N,'-Tetramethylethylenediamine (TEMED) (Serva, Heidelberg, FRG, Germany); Deionized used was prepared by Veolia Laboratory water purification system with purity of 18MΩ, 5ppb total organic content (TOC). The Apparatus included; Water bath at 37°C, Thermometers, pH meters, Spectrophotometers, Timers, Parafilms, Hoefer Electrophoresis System, SE 600/400 16cm Unit (Hoefer, San Francisco, USA), Gel Drier, Analytical balance, Weighing boats, Biosafety Class II Cabinet, Fume Hood, Bench top centrifuge, Microcentrifuge (12,000 to 15,000 rpm), Refrigerator (0°C to 8°C), Ultra Deep Freezer (-80°C), Deep Freezer (-20°C), Ultra sonicator, Vortexer, Repeater pipettes, Micropipettes, Pipette tips (20µL, 200µL , 1000µL), Vacuum pump with cold trap, 1.5mL Screw-cap Eppendorf microcentrifuge tubes, 0.75mm Teflon comb. PowerPAC (3000V), Ice maker, Blunt-ended spatulas, 25µL or 100µL Syringe with flat-tipped needle, Side- arm glass flask (Erlenmeyer or Florence), Forceps, Diamond scribe pencil, 0.45µM filters, Single- edge razor blades, paper towels, modeling clay, Glass tubes for Gel (capillary IEF), Fractionation collector, Gel documentation system, Tube racks, UV transilluminator, PPE (Face masks, powder free hand gloves, Laboratory coat, safety eye glasses, UV shields), Computer, Experimental laboratory workbook, ball pens and permanent marker pens.

3.7 Field and Laboratory Procedures

3.7.1 Field Procedures

3.7.1.1 Safety precaution when handling venomous snakes and venom samples

All the venomous snake specimens were accurately identified by a designated Herpetologist via distance and physical examinations as well as verification of their health status. The animals were handled by experienced snake handlers and/or trained reptile veterinarians. Each and every member of the research team was sensitized to observe all the necessary universal standard precautions as described (Appendix: IV). Fortunately, no injury occurred during venom extraction procedures. During the laboratory experiments, the snake venom samples were handled as poisonous biological material and all the necessary laboratory standard universal precautions were followed including the use of personal protection equipment,

handling the sample in appropriate biological safety cabinet and storing them in lockable deep freezer rooms with limited access to any unauthorized persons. The chain of custody for the venom samples were properly followed and documented in the laboratory experimental work book and research was carried out as per the approved protocol SCC No.2716.

3.7.1.2 Snake's General Welfare

- Any caught snake specimen had priority for transportation to the holding facility.
- Only snakes' specimen in good health as were determined by the hired herpetological expert was transported for venom extraction.
- Sedatives were not used during the transport.
- Any snake specimen that became sick or injured during the capture and transport received veterinary treatment as soon as possible and records of the occurrence were recorded in the field/Laboratory experiment work book.

3.7.1.3 Packing for transport

- The captured snake specimens were placed in suitable bags sealed and labeled as ***"POISONOUS" REPTILES***. Foam chippings, newspaper wastes were used to pack around the reptile and the bags were then firmly attached to the transport containers.

3.7.1.4 Labeling and documentations

- All labeling were done using permanent marker pens that are durable and waterproof.
- All transport containers were labeled as:- "**LIVE REPTILES- DO NOT TIP**," "**POISONOUS**" or "**NON-POISONOUS**", as appropriate, on all sides and top. **"THIS SIDE UP",** with arrows indicating the top, on all sides.
- The **Consignor's** and **Consignee's"** Name, Address, telephone number, cell phone number were indicated on the sides.
- Detailed list of contents; number of reptiles; Scientific and common names.
- Temperature range required during transportation and the date on which the reptiles were packed for transport.

3.8 Extraction and storage of venom

Adult *Dendroaspis angusticeps*, *Naja ashei*, *Naja (Boulengrina)subfulva* formerly *melanoleuca* and *Bitis arietans* (Wild caught) of both sexes (***N*=8**), measuring 1.5 to 2.7 metres, were identified by a hired Herpetologist from BioKen Snake Farm-Watamu, handled and kept as was described before (Breno *et al.,* 1990).

Before the experiment, in order to maintain the animals in the same feeding and venom production status, the snakes were fed with two mice each and water was available *ad libitum*. For manual venom extraction (Belluomini, 1967), the snakes were anaesthetized using subcutaneous sodium pentobarbital injection (20 mgkg-1, Cristália, Brazil).

Extraction of fresh snake venom was performed by a highly trained snake expert after signing research participant‘s Informed Consent form either in English and/or Kiswahili language versions (Appendices V & VI). Briefly, using special snake handling grab stick, the head of each snake specimen were seized and the posterior part held firmly between the fore finger and the thumb of the left hand. The right hand finger was used to hold the neck against the palm of the hand. The body of the snake was then placed under the left axilla in order to prevent the animal from pulling free. The mouth of the snake was opened and its fangs placed in the inner edge of a sterilized plastic vial. The venom was extracted by pressing the fangs very gently on the edge of the vial. Snake specimens, their habitats and crude venom yields are shown below (Table 3.1). Freshly extracted crude venom was labeled with a unique identification number and frozen immediately at -80°C until time of use.

Table 3.1: Venomous snakes, their habitat and venom yields

No.	Species	Common Name	Habitat	Venom Yields (mL)
1	*Dendroaspis angusticeps*	Green mamba	Gongoni	3.0
2	*Bitis arietans*	Puff Adder	Bamba	4.0
3	*Naja (Boulengrina) subfulva* formerly *melanoleuca*	Forest cobra	Arabuko-Sokoke Forest	3.5
4	*Naja ashei*	Spitting Cobra	Watamu-Malindi	3.0

3.9 Laboratory experimental procedures

3.9.1 Snake venom sample processing and quantification

Just prior to the protein quantification, crude snake venom samples were precipitated with a precooled 10% Trichloroacetic acid/Acetone. The precipitated samples were quantified using the BCA Protein Assay Kit (bioWORLD, Dublin, Ohio, USA) following the manufacturer‘s instructions. Briefly, 100µL of Blank (Diluent), each standards (range 50µg-2000µg), venom samples, positive control (Bovine serum albumin, BSA) and negative control (deionized water only) were added in appropriately labeled tubes. 2 mL of the BCA working reagent solution was added into each tube and mixed gently. The tubes were then covered, incubated in a water bath at 37°C for 30 minutes. Absorbance of all standards, samples and controls were measured at 562nM within 10 minutes, using a spectrophotometer (BioSpec Mini Spectrophotometer, Shimadzu- Scientific and Inc. USA). A standard curve was obtained by plotting the average blank corrected 562nM optical density measurements for each bovine serum albumin standard versus its concentration in micrograms per milliliter. The curve was used to determine the protein concentration

in each snake venom sample (bioWORLD, Dublin-Ohio, USA) and the data was generated automatically from the spectrophotometer machine.

3.9.2 Preparation snake venom samples

Samples of two hundred milligrams (200mg) of lyophilized crude venom were dissolved at room temperature (25°C) in 15mL (Eppendorf centrifuge tubes) of the following buffer cocktails: 10mM Tris-HCL, pH 7.4 containing 150µL, 100mM PMSF and 60µL of 0.5M Disodium ethylene diamine tetraacetic acid solution, Serine proteinase inhibitors and metalloproteinase inhibitors respectively. The samples were then centrifuged at 13,000xg for 10 minutes at 4°C in an Eppendorf centrifuge to pellet non-soluble materials. The samples were freeze dried and kept at -80°C until time of use (Shuting *et al.*, 2004).

3.9.3 Snake venom protein solubilization

The freeze dried venom samples; 100mg were resuspended in 370µL sample solubilization buffer/Lysis buffer containing (9.5M Urea, 57.057g; NP-40, 2% w/v, 2g; Carrier Ampholytes pH 3-10, 2%, 200µL; Dithiothreitol 10% (w/v), 60mM, 0.0925g) all dissolved in 100mL deionized water) and then sonicated on ice for 30 seconds. The solubilized samples were either loaded directly onto the Isoelectric focusing gels or stored in 100µL aliquots at -80°C until time of use.

3.9.4 Isoelectric focusing (IEF)

Isoelectric focusing was performed with the tube cell Model 175 (Bio-Rad, Hercules's) using glass capillary tubes (measuring 1 mm internal diameter, 140 mm long). A stock solution of Acrylamide: 30% (w/v), Acrylamide, 0.8% (w/v), Bisacrylamide dissolved in deionized water. This solution was prepared and stored in the dark at 4°C for 1 month. Cathodic electrode buffer was made of 20mM NaOH dissolved in deionized water. The solution was made fresh each time. The Anodic electrode buffer was made of 6mM H_3PO_4, (0.085% w/v) ,dissolved in deionized water, made fresh each time for use. Ammonium persulfate stock solution was 10% (w/v) dissolved in deionized water, prepared and stored at 4°C, protected from light and always made fresh each time every two weeks. Sodium dodecyl sulfate stock solution was made of 20% (w/v). This solution was stored at room temperature. Bromophenol blue solution was made of 0.05% (w/v) dissolved in deionized water, stored at room temperature. The capillary gel equilibration buffer solution, pH 8.8,

was made from 8mL of 1.5M Tris-base stock; 2mL of 1.5M Tris-HCL stock; 6mL of 20% SDS stock; 24 mL of deionized water and 0.309g of Dithiothreitol. This solution yielded a solution of 3% SDS, 0.375M Tris.Cl, pH 8.8 and 50mM Dithiothreitol. This solution was made fresh each time just before use (O'Farrell, 1975).

3.9.4.1 Casting Isoelectric Focusing Capillary Gels

A line was drawn at 12.5cm using a fine marker pen on clean dry glass capillary tubes (Capillary glass tubes were previously cleaned with sulfochromic acid to eliminate any protein deposits). The remaining 1.5cm of the capillary tubes was used to load the sample. The necessary aliquots of Isoelectric focusing gels (Containing —UREA, 5.71g; Acrylamide stock, 1.33mL; Nonidet P-40, 2mL (10%, w/v); deionized water 1.97mL; Ampholytes pH3-10, 500µL (2%) were thawed at 37°C and 10µL of 10% Ammonium persulfate added to each 1.4mL of the solution. The IEF Gel mixture was degassed under vacuum for about 1 minute. To this solution, 7µL of TEMED was added. Very quickly, using a 1mL Hamilton microlitre syringe (Hamilton Bonaduz AG, Switzerland), 800µL of IEF gel mixture was pipette into the capillary glass gel tube, (along the side wall in order to prevent the formation of air bubbles in the solution—done within 3 minutes). The capillary tube was filled up to the marked height of 12.5cm, (Prior to gel addition, the bottom of each capillary glass was sealed with parafilm to support and stabilize the gels inside capillary tubes) (O'Farrell, 1975).

3.9.4.2 Application of the sample and isoelectric focusing

The lower chamber was filled with the Anodic buffer solution containing 0.085% (0.01M H_3PO_4). The external surfaces of the capillary tubes were wetted with deionized water and inserted in the Isoelectric focusing chamber. Using a Hamilton Syringe, 40 µL of final diluted venom samples in duplicates (100µg) were loaded onto the top of the capillary cathode side (Negative electrode). This was done carefully to avoid introduction of air bubbles between the samples and the gel. The cathodic buffer solution (20mM NaOH) was laid onto the top of the sample in the capillary tube and then the upper chamber was filled with this buffer up to three quarters full. The upper chamber was connected to a cathodic electrode and the lower

chamber connected to an anodic electrode. The isoelectric focusing was carried out at 200V for 2 hours, then 500V for 5 hours and finally at 1000V for 17 hours (Total of 19,900 Vhrs) at 20°C overnight. After IEF, the capillary gels were removed from the electrophoresis tank and forced out gently using a 1mL hypodermic syringe that was connected to flexible tubing and filled with deionized water (O'Farrell, 1975).

3.9.4.3 Isoelectric focusing gel equilibration

The extruded capillary gels were placed on higher glass plate of the polyacrylamide gel. Residual water around the capillary gel were wiped out or soaked up with a filter paper. Approximately, 140µL of IEF equilibration buffer (1.5M Tris-base, 8mL; 1.5M Tris-HCL, 2mL; 20% SDS Stock-6mL; Deionized water-24mL; Dithiothreitol- 309mg; gave a 3% SDS, 0.375M Tris.Cl, 50mM Dithiothreitol with a pH of 8.8) was poured onto IEF capillary gel in a clean Petri-dish and left for 15 minutes (O'Farrell, 1975).

3.9.4.4 SDS-PAGE analysis of snake venom proteins

SDS-PAGE of the crude snake venom was carried out as was described before (Laemmli, 1970), using 12% SDS-PAGE with some little modifications. Briefly, clean glass- plate sandwich of the electrophoresis apparatus, the Second dimension, Hoefer SE 900 Vertical Slab Gel Electrophoresis Unit (Hoefer, Inc.953 Indiana Street, Francisco, CA 94107, USA)—Using Hoefer PS300-B, Power Supply, Voltage 10-300V; Current : 4-500mAh; Power: 90W Max; Timer: 0-999 minutes, was assembled according to the manufacturer's instructions. The sandwich was locked to the casting stand and appropriate amount of separating gel (To make 360mL of 12% SDS-PAGE, 144mL of 30% Acrylamide/0.8% Bisacrylamide stock, 72mL of 1.5M Tris-base stock, 18mL of 1.5M Tris-HCL stock, 1.8mL of 20% SDS stock and 12.2 mL deionized water) was poured using a gradient gel former, (Model.395 Gradient Former—BioRad, Hercules, CA. USA) and peristaltic pump. 1.2mL of 10% Ammonium persulfate and TEMED was added, stirred gently to mix. Immediately after casting, gels were overlaid with water-saturated 2-butanol solution using a 1mL Hamilton microlitre syringe fitted with a needle (Hamilton Bonaduz AG, CH-7402; Bonaduz, Switzerland). The casted gels were allowed to polymerize for 1hr: 30 minutes at room temperature (Laemmli, 1970).

3.9.4.5 Loading the first-dimensional gel onto second-dimension gels

Prior to the second-dimension separation, the top of the slab gels were rinsed with deionized water to remove 2-butanol. Excess water was removed by suction using a Hamilton microlitre syringe. The IEF gels were removed from the equilibration buffer solution and pushed immediately between the glass plates of the polyacrylamide gel using a small spatula. The Cathodic side (basic-end) of the capillary gel was placed at the right side of the polyacrylamide gel (Care was taken to avoid the entrapment of air bubbles between the capillary and the polyacrylamide gels) (O'Farrell 1975).

3.10 SDS-PAGE Procedure

The unstained protein standards for accurate molecular weight estimation (2.5kDa-200kDa) (Invitrogen, Ltd. Inchinnan Business Park, 3 Fountain Drive, UK), was added onto already made side well. Duplicate glass plates for protein slab gel cells with casted gels were mounted onto the Hoefer SE 900 Vertical Slab Gel Electrophoresis Unit for the same sample. The upper chamber and the lower chamber were filled with the 1 x Electrophoresis buffer solution, pH 8.3 (5X SDS Electrophoresis Buffer Stock contained Tri-base 15.1g; Glycine 72g; SDS 5g and Deionized water 1000mL). Electrophoresis was carried out at a constant current of 40mAh per gel at 20°C until the dye front reached the end of the gel. The 2DE-gel containing 100µg of venom sample was silver stained and submitted for image analysis (Gorg *et al.*, 2004).

3.11 Silver Nitrate Staining

The 2DE-gels were stained with Silver stain as was described before (Shevchenko *et al.*, 1996), with some little modifications. Briefly, the gels were fixed with 50% Methanol/5% Acetic Acid for 20 minutes. After several changes of 50% methanol and water, the gels were sensitized by incubating them in 0.02% Sodium thiosulphate followed by several changes of water. Gels were then incubated in 0.1% Silver Nitrate for 20 minutes followed by thorough wash in water. The gels were developed in 0.04% Formalin with a 2% Sodium carbonate for 8 minutes and the reactions were stopped using 5% Acetic Acid. All gels were stored at 4°C immersed in a fresh 1% Acetic-Acid solution before the venom protein toxins spots on the 2DE-gels were captured with a digital camera.

3.12 Data Management and Analysis

The 2DE-gel electrophoresis protein spot images were captured using a digital camera and stored as Adobe Photoshop (.TIFF) files and later analyzed empirically to determine toxin's physical and chemical characteristics. The approximate molecular weights and isoelectric points were determined based on the protein molecular weight standard markers incorporated during the protein separation procedures. The venom protein toxin physical and chemical properties (estimated isoelectric points and molecular weights) were used as parameters to search the Swiss-Prot protein database and determine the identities of unknown isolated protein toxins (Wilkins *et al*., 1998). Briefly, given the availability of some snake venom toxin's amino acid sequence information in protein databases, unknown proteins in a sample was identified quite simply and efficiently. TagIdent protein identification program at ExPASy allowed identification of a whole protein separated on 2DE-gels using theoretical and experimental data from its estimated pI and molecular mass, Keywords and species (or a group of species) of interest (Wilkins *et al*.,1996). This program was available on the world-wide web at: https://web.expasy.org/tagident/. Accessed on 04/04/2016.

CHAPTER FOUR

RESULTS

4.1. Snake venom proteins two-dimensional electrophoresis profiles

Snake venom protein toxins electrophoresis profiles are shown in the figures 4.1, Fig 4.2, Fig 4.3, and Fig 4.4 below. These figures shows the distribution of various venom protein toxin's molecular weight (MW) differences and isoelectric points (pI) among members of snake families studied as was observed in the two dimensional gel electrophoresis. Figures 4.1 and 4.2, below shows the Two-dimensional gel electrophoresis images of protein toxins isolated from *Naja (Boulengrina) subfulva* formerly *melanoleuca* and *Bitis arietans* venoms respectively.

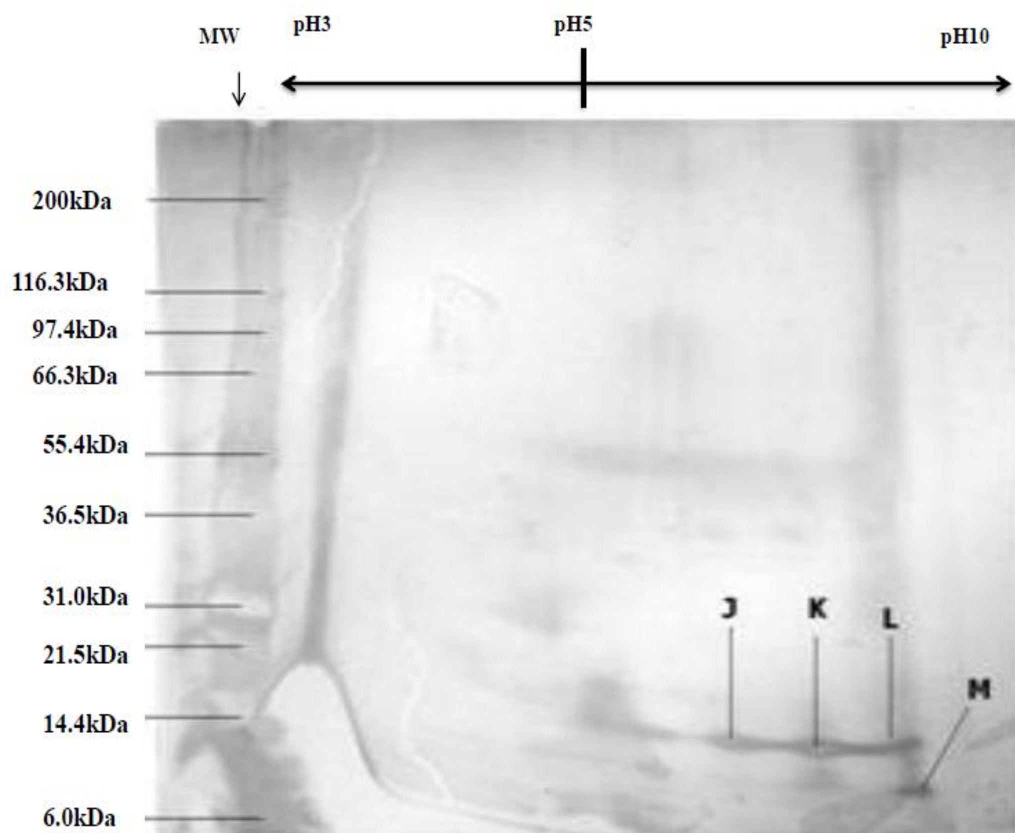

Figure 4.1: ***Naja (Boulengrina) subfulva***

Figure 4.1: Two-dimensional gel electrophoresis of venom protein toxin from *Naja Boulengrina) subfulva*; 100µg of venom sample was run on a 2DE- gel and stained by Silver stain. (MW: Protein standards with known molecular weights; kDa: kilodaltons).

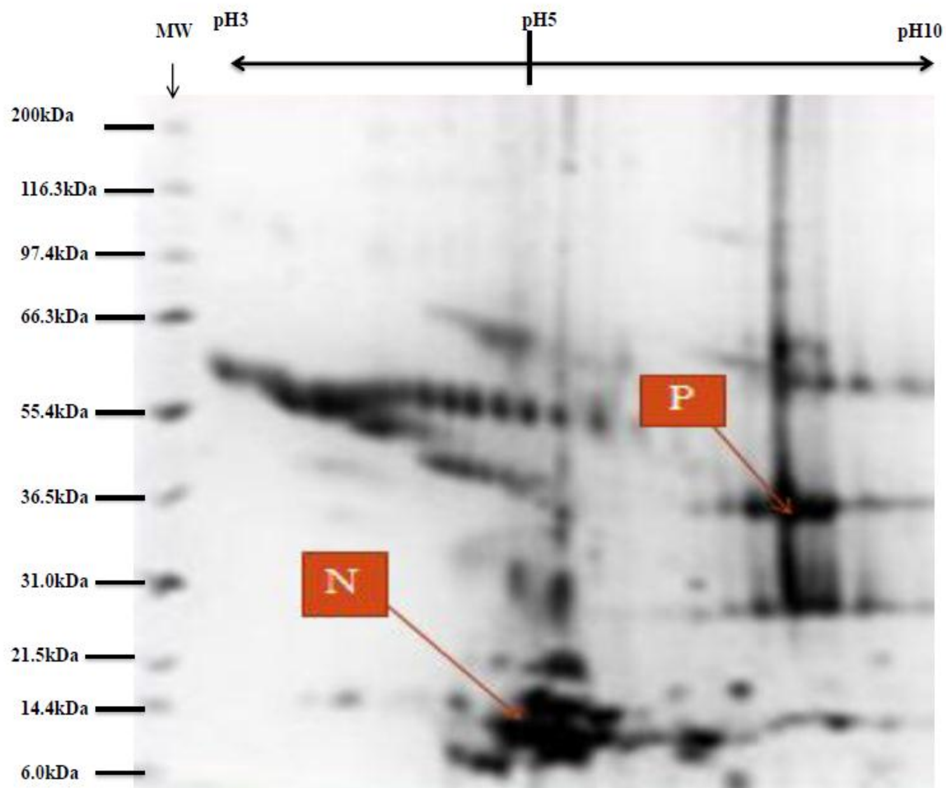

Figure 4.2: ***Bitis arietans***

Fig 4.2: Two-dimensional gel electrophoresis of venom protein toxin from *Bitis arietans*. 100 µg of venom sample was run on a 2DE- gel and stained by Silver stain. (MW: Protein standards with known molecular weights; kDa: kilodaltons).

Figures 4.3 and Fig 4.4 below shows Two-dimensional gel electrophoresis images of venom protein toxins isolated from *Dendroaspis angusticeps* and *Naja ashei* venoms respectively.

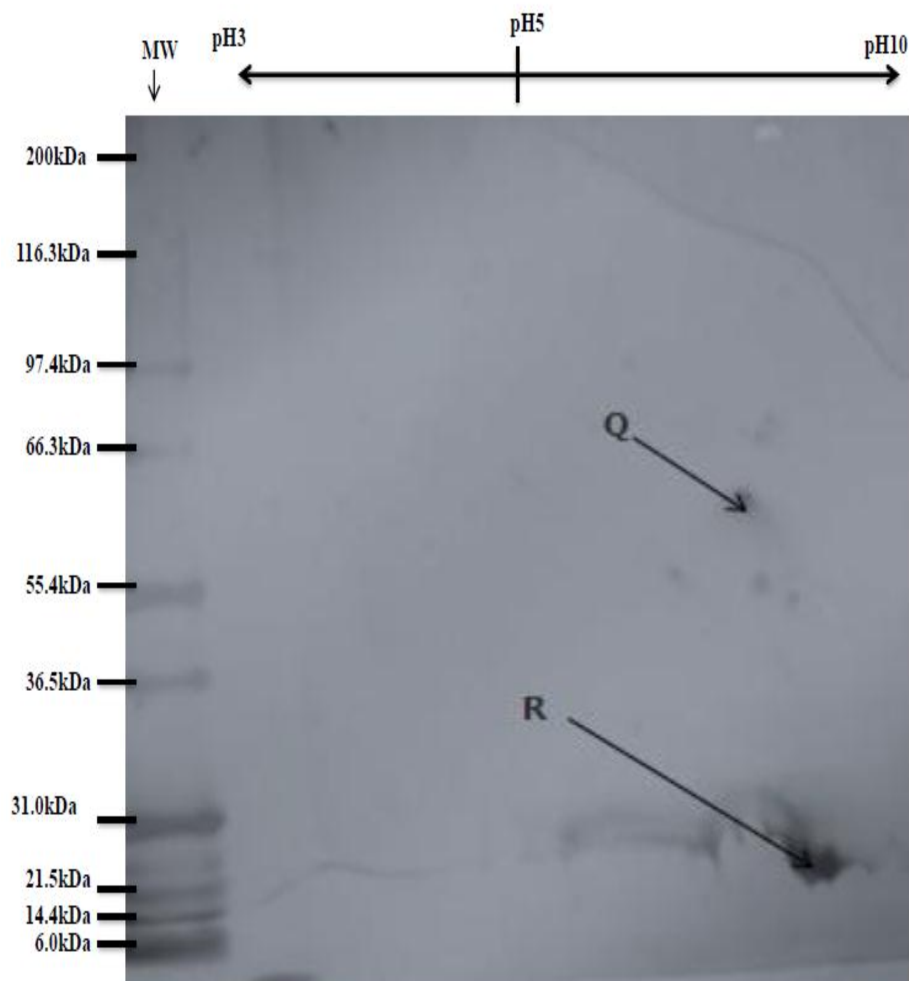

Figure 4.3: ***Dendroaspis angusticeps***

Figure 4.3: Two-dimensional gel electrophoresis of venom protein toxin from *Dendroaspis angusticeps*; 100µg of venom sample was run on a 2DE- gel and stained by Silver stain. (MW: Protein standards with known molecular weights; kDa: kilodaltons).

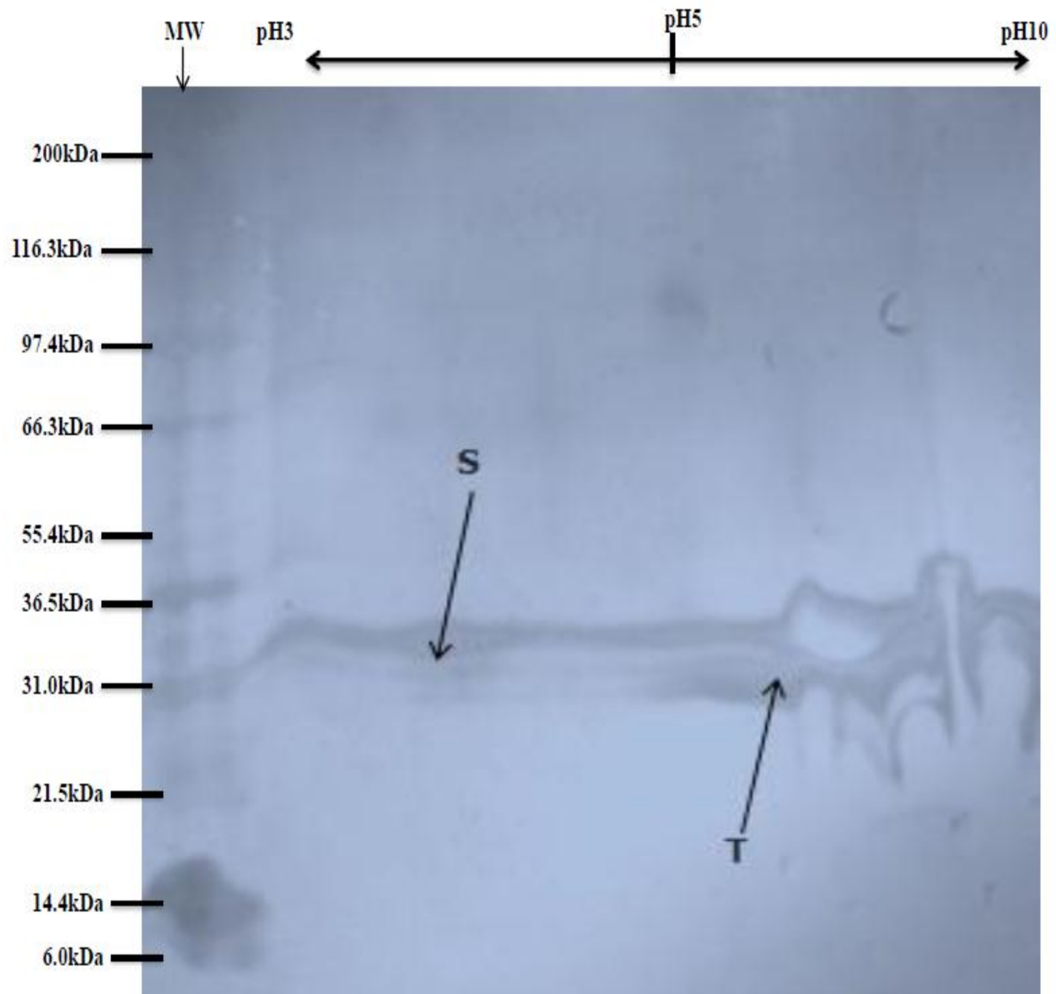

Figure 4.4*: Naja ashei*

Fig 4.4: Two-dimensional gel electrophoresis of venom protein toxin from *Naja ashei*. 100 µg of venom sample was run on a 2DE- gel and stained by Silver stain. (MW: Protein standards with known molecular weights; kDa: kilodaltons).

The various venom protein toxins' physical and chemical characteristics are shown (Table 4.1).

Table 4.1: Snake species Venom Toxins and their Physicochemical Properties

Snake species	2DE-SPOT	MW.STD (kDa)	Approx.MW(kDa) (Range)	Approx. pI (Range)
Naja(Boulengrina) subfulva formerly *melanoleuca*	J	14.4	(6.0-21.5)	6 (5-7)
N. (Boulengrina) subfulva	K	13.0	(6.0-21.5)	6 (5-7)
N. (Boulengrina) subfulva	L	13.0	(6.0-21.5)	7 (6-8)
N. (Boulengrina) subfulva	M	8.0	(6.0-14.4)	8 (7-9)
Bitis arietans	N	31.0	(21.5-36.5)	5 (4-6)
B. arietans	P	31.0	(21.5-36.5)	8 (7-9)
Dendroaspis angusticeps	Q	66.3	(55.4-97.4)	7.0 (6-8)
D. angusticeps	R	6.0	(6.0-14.4)	8.0 (7-9)
Naja ashei	S	31.0	(21.5-36.5)	7.0 (6-8)
N. ashei	T	31.0	(21.5-36.5)	7.0 (6-8)

The snake venom toxin proteins were identified from the UniProtKB/Swiss-Prot protein database using TagIdent, bioinformatics online tool. The Swiss-Prot database was accessed via the website: http://web.expasy.org/.Accessed on 4-Apr-2016, UniProtKB/Swiss-Prot Release 2016_03 of 16-Mar-16: 550740 entries (Table 4.2).

Table 4.2: Snake Venom Protein toxins identified by TagIdent Tool Website (https://web.expasy.org/tagident/)

Source Organism	2DE-SPOT	Access/ No.	Protein Name
Naja (Boulengerina) subfulva (formely, *melanoleuca*)	J	P00601	Acidic Phospholipase A2 DE-III
N. (Boulengrina)subfulva	K	P00600	Acidic Phospholipase A2 DE-II
N. (Boulengrina)subfulva	L	P00599	Basic Phospholipase A2 1
N.(Boulengrina)subfulva	M	P01383	Long chain neurotoxin 1
Bitis arietans	N	NONE	Unknown/Undetermined
B. arietans	P	NONE	Unknown/Undetermined
Dendroaspis angusticeps	Q	NONE	Unknown/Undetermined
D. angusticeps	R	P81030	Three-finger muscarinic toxin 1
Naja ashei	S	NONE	Unknown/Undetermined
N. ashei	T	NONE	Unknown/Undetermined

The venom protein toxins' amino acid sequences in FASTA format were retrieved from SWISS- PROT protein database using accession number as an input data type and used to compute the amino acid compositions (%), the number of positively and negatively charged residues, extinction coefficient, instability and aliphatic index, Grand Average of hydropathy (GRAVY) in protparam tool (http://web.expasy.org/protparam/) Accessed on 4-Apr-2016 (Table 4.3).

Table 4.3: Amino acid composition profiles (%) from *N. (Boulengrina) subfulva* and *D. angusticeps* venom toxin proteins

Acc/No.	Ala	Arg	Asn	Lys	Asp	Glu	Cys	Gln	Gly	His	Ile	Leu	Met	Phe	Pro	Ser	Thr	Trp	Tyr	Val
P00601	9.2	5.0	9.2	3.4	7.6	1.7	11.8	3.4	7.6	2.5	5.0	2.5	0.8	3.4	3.4	5.0	5.0	2.5	7.6	3.4
P00600	8.4	5.0	10.1	4.2	6.7	2.5	11.8	4.2	7.6	1.7	4.2	2.5	0.8	3.4	2.5	5.0	5.9	2.5	7.6	3.4
P00599	7.6	5.1	7.6	6.8	8.5	2.5	11.9	3.4	7.6	2.5	4.2	2.5	0.8	3.4	3.4	2.5	5.9	2.5	7.6	3.4
P01383	2.8	4.5	2.8	12.7	5.6	4.2	14.1	2.8	4.2	1.4	2.8	4.2	1.4	0.0	11.3	4.2	9.9	2.8	4.2	4.2
P81030	3.0	4.5	6.1	7.6	4.5	6.1	12.1	1.5	4.5	0.0	7.6	3.0	0.0	3.0	6.1	4.5	13.6	3.0	4.5	4.5

The snake venom toxin protein molecular characteristics such as the amino acid sequence chain length, molecular weights, isoelectric points, negatively charged Asp & Glu residues, positively charged Arg & Lys residues, Instability Index, Aliphatic Index and Grand average hydropathy value (GRAVY) are shown below as described (Table 4.4).

Table 4.4: Physical and chemical properties of venom toxin proteins from *N. (Boulengrina) subfulva* and *Dendroaspis angusticeps*

Acc/No.	2DE-SPOT	AA-Chain	MW (Da)	pI	R^-	R^+	II	AI	GRAVY
P00601	J	1-119	13360	6.28	11	10	27.98	48.49	-0.450
P00600	K	1-119	13427	6.82	11	11	24.30	44.37	-0.560
P00599	L	1-118	13473	7.55	13	14	21.50	43.90	-0.640
P01383	M	1-71	8056	8.73	7	12	30.06	42.54	-0.758
P81030	R	1-66	7515	8.48	7	8	3.69	57.58	-0.380

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

Snake venoms are complex mixtures of components with diverse array of actions both on prey and mammalian victims that are generally rich resources of water soluble enzymes as well as polypeptides. This study obtained ten 2DE-gel protein spots from four snake venom samples. Out of ten 2DE-gel venom protein toxins spots isolated, five different snake venom protein toxins were identified as:- Basic phospholipase A2; Acidic phospholipase A2 DE-II, Acidic phospholipase A2 DE-III, Long chain neurotoxin 1 and three-finger muscarinic toxin 1. The first four proteins were obtained from *N. (Boulengrina) subfulva* formerly *melanoleuca* and the latter were from *D. angusticeps* venom samples respectively. It was observed that the molecular weights of the isolated venom protein toxins from these two Elapidae snake families ranged between 7515 daltons to 13,473 daltons with an isoelectric point ranging between pI 6.23 to pI 8.73 while on the other hand the Viperidae (*B. arietans*) and an Elapidae (*N. ashei*) had spotting at molecular weight ranges of 10,000 daltons to 36,500 daltons as well as isoelectric points ranging from pI 4 to pI 8.

Further database analysis to determine the identities of the remaining protein toxin's spots represented by alphabetical letters (N, P, Q, S, & T) yielded negative results for venom protein toxins from *B. arietans, D.angusticeps* and *N. ashei* samples. Thus, these venom protein toxin components isolated with unknown identities are most likely to represent previously undescribed snake venom proteins—which remains the focus of our future research work.

Similar venom protein toxins had been previously isolated and purified from a South African Forest cobra (*Naja (Boulengrina) subfulva* formerly *melanoleuca*) by a combination of gel filtration on Sephadex G-50 and chromatography on DEAE-Cellulose and were found to be homologues by various physical and chemical criteria (Joubert & van der Walt, 1975). They found out that these enzymes were cross-linked by seven disulfide bridges and had molecular masses closer to 13000 daltons. However, results from their study did not provide the exact

molecular weights and isoelectric points of these enzymes. In this study we have provided an advancement of this knowledge and described the physicochemical properties of these novel snake venom toxin proteins by confirming their molecular weights, isoelectric points and amino acid composition profiles.

The computed number of amino acids, molecular weights, Isoelectric points, negatively charged Asparagine and Glutamic acid residues, positively charged Arginine and Lysine residues, Instability Indices (II), Aliphatic indices (AI) as well as Grand average hydropathy values are now well described in this study. The data obtained from this study suggests that majority of snake venom toxic protein from Elapidae families have molecular weight ranges between 7000 daltons to 14,000 daltons with isoelectric point (pI) of 6 to 8. The most significant findings of this study were that of the physical and chemical properties of these venom toxins with the data showing the Aliphatic index (the relative volume of a protein occupied by aliphatic side chains) values for all the five toxins isolated to be very high. This demonstrates that these biomolecules are thermodynamically stable with Instability Index of more than 27 and aliphatic index values ranging from 42.54 to 57.58. In addition, these toxic proteins also had negative hydrophilic GRAVY value which means that they are hydrophilic (water-friendly) and so their proper separation can be enhanced using matrices such as α-Cyano-4-hydroxycinnamic acid (HCCA) in mass spectrometric procedure.

Earlier research studies have shown that Elapids (e.g., *Naja (Boulengrina) subfulva* formerly *melanoleuca*) venoms cocktails contain proteins with higher concentration of phospholipase enzymes (Kocholaty *et al*., 1971), results which is similar to observations made in this study. The presence of abundant phospholipase A2 from snake venom as multiple iso-enzymes has been a common observation by various groups (Angulo & Lomonte, 2009). Phospholipases A2, neurotoxins and three-Finger toxins (3FTXs) are highly abundant components in Elapidae venoms, for example, a high throughput sequencing of an American Elapid *Micrurus fulvius* was shown to be dominated by three-finger toxins and phospholipase A2 (PLA2s) enzymes (Vergara *et al*., 2014 ; Margres *et al*., 2013). These iso-enzymes as well as nonenzymatic ones are involved in prey digestion; exhibit a wide variety of pharmacological and/or toxic effects by interfering with the normal physiological

function of the prey or victims. This study isolated and characterized secretory phospholipase A2, both basic and acidic isoforms as well as three finger neurotoxins and muscarinic toxins. The most toxic effect of the PLA_2 has been believed to be indirectly mediated by the catalytic active acidic PLA_2 which consequently enhances cellular or subcellular (mitochondrial) membrane damage through phospholipid hydrolysis and generation of pharmacological mediators including platelet activating factors and eicosanoids causing hemolysis, hemorrhage, edema and loss of organ functions. Phospholipases A2 enzymes have also been shown to cause prolonged blood coagulation, dermo-/myo-necrosis and cardiotoxicity.

Snake venom neurotoxins are classified into distinct types: - postsynaptic and presynaptic neurotoxins in relation to the neuromuscular junction. The presynaptically active neurotoxins (e.g., β –neurotoxins mostly PLA2s) have been shown to bind the motor nerve terminals, leading to depletion of synaptic acetylcholine vesicles, impairs the release of acetylcholine and later degeneration of the motor nerve terminal (Dixon & Harris, 1999; Harris & Goonetilleke, 2004; Ranawaka *et al*., 2013). They produce neuromuscular block that occurs in three phases: an immediate depression of acetylcholine release, followed by a period of enhanced acetylcholine release, and then complete inhibition of neuromuscular junction (NMJ) transmission (Aird, 2002). Pre-synaptic toxicity induced by snake venom phospholipase A2 (PLA2s) is still not completely understood. However, recent studies have shown that PLA2 from snake venom neurotoxins produce complex effects on the pre-synaptic membrane, morphological changes in such as nerve terminal bulging, changes in mitochondrial morphology and permeability increase in cytosolic calcium levels, changes in expression and interaction of SNARE (Soluble N-ethylmaleimide-sensitive factor Attachment Receptor) proteins, increased vesicle, neurotransmitter release and impaired vesicle recycling (Rigoni *et al*., 2008 ; Tedesco *et al*., 2009; Ranawaka *et al*., 2013).

This study also isolated and characterized two venom protein's three finger neurotoxins toxins: - Long chain neurotoxin 1 and muscarinic toxins 1. Depending on their amino acid chain length and the number of disulfide bonds, these nonenzymatic proteins can be categorized into two groups; (i) short-chain three finger toxins (four

disulfide bridges, 60-62 amino acid residues) with cysteine connectivity of 1-3, 2-4, 5-6, 7-8 , and (ii) long-chain three finger toxins (with five disulfide bridges, 66-75 amino acid residues) with cysteine connectivity of 1-3, 2-4, 5-6, 7-8, and 9-10 (Tsetlin *et al*, 1999; Fry *et al*, 1999).

Long chain neurotoxin 1 had been isolated from the venom of *D.angusticeps* and its primary structure determined (Shipolint *et al*., 1974). They found out that it's a basic protein containing 71 amino acid residues. This study suggests a similar finding which shows that this non-enzymatic snake venom protein toxin contain 71 amino acid residues, with a molecular weight of 8056 daltons and quite basic molecule of pI 8.73, highly positively charged stable toxin. It has been suggested that it binds to muscle (α1) nicotinic acetylcholine receptor (mAChR) as well as neuronal (α7) acetylcholine receptor (nAChR) and inhibits acetylcholine from binding to its receptor, thereby impairing the neuromuscular transmissions (Changeux, 1990).

Previous studies have shown that post-synaptic neurotoxins are the main toxic proteins constituents of cobra snake venom proteins which are implicated in the blockage of neuromuscular transmission and cause animals death by respiratory collapse. Postsynaptic neurotoxins bind specifically to nicotinic acetylcholine receptor (nAChR) at the motor end plate and produce a non-depolarizing blockage of the neuromuscular transmission while on the other hand presynaptic neurotoxins block the release of acetylcholine from the presynaptic motor nerve terminals.

Snake venom neurotoxins generally block the acetylcholine receptor on the plasma membranes of the skeletal muscle of envenomed animals and prevent the binding of chemical neurotransmitter acetylcholine and excite the muscles. This blockage at the neuromuscular junction leads to flaccid paralysis (Yang, 1996).

Snake venom three-finger toxins (3FTxs) form one of the largest families of bioactive molecules commonly found in the venom of snakes particularly those from the Elapidae family (Utkin *et al*., 2015; Utkin, 2013; Sunagar *et al*., 2013; Kini & Doley, 2010).This study also isolated and characterized a low molecular weight three-finger muscarinic toxin 1 from the venom of *Dendroaspis angusticeps.* This study finding suggests that the toxin is a basic protein containing 66 amino acid residues and highly stable molecule with molecular weight of 7515 daltons. Muscarinic toxins from Elapidae venoms has been previously purified from the venom of African green

mamba (*D.angusticeps*) and characterized for their ability to inhibit the binding of selective muscarinic ligands (Adem *et al*., 1988). The study (Adem *et al*., 1988) found out that this muscarinic toxin 1 had 64 amino acid residues, four disulfide bonds and molecular weight of 7200 daltons, a result which is highly similar to the findings of this study. Generally these non-enzymatic venom proteins are a small, β-structural protein (60-75 amino acids) which consists of three loops ('fingers') protruding from the compact globular core ('head') stabilized by four variant disulfide bonds (Tsetlin & Hucho, 2004). Separate groups of three-finger' toxins act on numerous targets (Kini & Doley, 2010), including two classes of acetylcholine receptors: - nicotinic acetylcholine receptors (nAChRs) belong to ligand-gated ion channels (Papke & Grosman, 2014) and muscarinic acetylcholine receptors (mAChRs) of the G-Protein coupled receptor (GPCR) family (Kruse et al., 2014). Previous work has shown that this enzyme non-competitively interacts with adrenergic and muscarinic receptors and binds to alpha-2b (ADRA2B), alpha-1a (ADRA1A), alpha-1b (ADRA1B), and alpha-2c (ADRA2C) adrenergic receptors (Max *et al*., 1993). Further studies have also shown that it reversibly binds M1 (CHRM1) Muscarinic acetylcholine receptors by interacting with orthosteric sites (Karlsson *et al*., 1994).

5.2 Conclusion

Kenya is inhabited by many species of venomous snakes from both Viperidae and Elapidae families. Four species of venomous snakes found in Kilifi County were studied. Five snake venom proteins belonging to two major protein families were isolated, characterized and identified as: - Phospholipase A2 and three finger toxins. These venom protein toxins were found to be of low molecular weight (7 to 13 kDa), but were highly stable, slightly acidic (pI 6.28 to 6.82) and basic protein molecules (pI 7.55 to 8.73). These venom toxins had been previously described and this study interrogated further their physical and chemical properties. This study found out that they are thermodynamically stable molecules with Instability index (II) of more than 27 and aliphatic index (AI) ranging from 42.54 to 57.58. The remaining five venom protein toxins with unknown identities are most likely a representative of some previously undescribed snake venom toxins which remains the focus of our future research studies. Any immunotherapeutic intervention against these

venomous species should be directed towards neutralization these identified protein toxin families. Further look at their structures and function relationships will provide us with new knowledge on their mechanism of action and how they cause injuries to the bite victims.

5.3 Recommendation

- ❖ Venom fractionation in combination with in-gel enzyme digestion is highly recommended as a complementary process in cases where the two-dimensional gel electrophoresis protein spot results are undetermined and/or proteins unknown.
- ❖ Venom proteins from the Viperidae (e.g., *Bitis arietans*) are quite unstable, therefore absolute caution must be considered when analyzing them.
- ❖ Further studies are necessary to focus on transcriptomics, genomics, advanced microcopy, molecular physiology and epidemiology in combination with bioinformatics to complement on venomics studies.

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APPENDICES

Appendix I: Research project ethical approval letter from the Animal Care and Use Committee

KENYA MEDICAL RESEARCH INSTITUTE

Centre for Virus Research, P.O.Box 54628 - 00200 NAIROBI - Kenya
Tel: (254) (020) 2722541, 254 02 2713349, 0722-205901, 0733-400003 Fax (254) (020) 2726115
Email: cvr@kemri.org

KEMRI/ACUC/ 01.07.14 **16th July, 2014**

Otieno, Kepher Onyango
TM305-0218/2009
ITROMID

Kepher Onyango,

RE: Animal use approval for SSC 2716(revised) - "Isolation and characterization of snake venom proteins and peptides from Viperidae (*Bitis arietans*, and Elapidae (*Naja melanoleuca, Naja ashei, Dendroaspis angusticeps*) families in Kilifi County" protocol

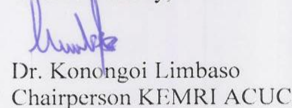
The KEMRI animal care and use committee acknowledges the resubmission of the above mentioned protocol addressing the issues raised earlier.

It has also been confirmed that the study has been sanctioned by the Kenya Wildlife service as stated in the letter **KWS/BRM/5001** and the PI is required to work in conjunction with the senior KWS scientist based in the coastal conservation area.

Approval is granted for a period of one year starting from when the final ethical approval will be obtained. The committee expects you to adhere to all the reptile handling procedures as described in the protocol.

The committee wishes you all the best in your work.

Yours sincerely,



Dr. Konongoi Limbaso
Chairperson KEMRI ACUC

Appendix II: Research project ethical approval letter from the Ethics Review Committee

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1 **August 11, 2014**

TO: **KEPHER OTIENO,**
PRINCIPAL INVESTIGATOR

THROUGH: **DR. KIMANI GACHUHI,**
DIRECTOR, CBRD,
NAIROBI

Dear Sir,

RE: SSC PROTOCOL NO. 2716 (*RESUBMISSION*): ISOLATION AND CHARACTERIZATION OF SNAKE VENOM PROTEINS AND PEPTIDES FROM VIPERIDAE (*BITIS ARIETANS*), AND ELAPIDAE(*NAJA MELANOLEUCA, NAJA ASHEI, DENDROASPIS ANGUSTICEPS*) FAMILIES IN KILIFI COUNTY

Reference is made to your letter dated 14th July, 2014. The ERC Secretariat acknowledges receipt of the revised protocol on July 21, 2014.

This is to inform you that the Ethics Review Committee (ERC) reviewed the documents submitted and is satisfied that the issues raised at the 223rd meeting of the KEMRI ERC on 21st January, 2014 have been adequately addressed.

The study is granted approval for implementation effective this **11th August, 2014**. Please note that authorization to conduct this study will automatically expire on **August 10, 2015.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **June 29, 2015**.

Any unanticipated problems resulting from the implementation of this protocol should be brought to the attention of the ERC. You are also required to submit any proposed changes to this protocol to the SSC and ERC prior to initiation and advise the ERC when the study is completed or discontinued.

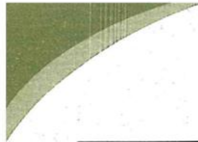
You may embark on the study.

Yours faithfully,

PROF. ELIZABETH BUKUSI,
ACTING SECRETARY,
KEMRI/ETHICS REVIEW COMMITTEE

In Search of Better Health

Appendix III: Letter of permission to conduct research in Kilifi County from the Kenya Wildlife Service



ISO 9001:2008 Certified

KWS/BRM/5001

3 March 2014

Mr. Otieno Kepher Onyango
C/O JKUAT/ITROMID
P.O.Box 62000-00200
NAIROBI
e-mail: kotieno@nphls.0r.ke
mobile: 0722591528

Dear Onyango,

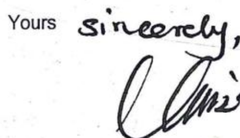
PERMISSION TO CONDUCT RESEARCH IN KILIFI COUNTY

We acknowledge receipt of your letter dated 3 March 2014 requesting for permission to conduct research on a project titled: '*Isolation and Characterization of Snake Venom Proteins and Peptides from Viparidae (Bitis arientans) and Elapidae (Naja melanoleuca, Naja ashei, Dendraospis angusticeps) Familiies in Kilifi County*'. The study will generate data and information to enhance the conservation of the herpato-faunal species in Kilifi County.

You have been granted permission to conduct the study from **March 2014 to February 2015** upon payment to KWS of academic research fees of **Ksh 5000**. However, you will abide by the set KWS regulations and guidelines regarding the conduct of research in and outside protected areas. You will also be required to work closely with our Senior Scientist in-charge of Coast Conservation Area (CCA), whom you will give a copy of the research proposal and progress report on the study.

You will submit a copy of your MSC thesis to the KWS Deputy Director, Biodiversity Research and Monitoring on completion of the study.

Yours sincerely,



SAMUEL M. KASIKI, PhD, OGW
DEPUTY DIRECTOR
BIODIVERSITY RESEARCH AND MONITORING

Copy to:
- AD, CCA
- Senior Scientist, SCA

P.O Box 40241-00100, Nairobi, Kenya. Tel: +254-20-6000800, 6002345. ISDN: +254-020-3992000/1000
Wireless: +254-020-2379407/8/9. Mobile: +254-735 663 421, +254-726 610 508. Fax: +254-020-6003792
Email: kws@kws.go.ke Website:www.kws.go.ke

Appendix IV: Universal standard precautions when handling poisonous reptiles

- Know how to identify poisonous snakes common in your area.
- Be alert for snakes in unusual places. They may be found in or around homes, barns, outside the buildings, driftwood, leaves, dikes, dams, stalled automobiles, piles of debris, building materials, trash or any type of rubble or shelter.
- Before beginning any cleanup or rescue operations, search the premises thoroughly for snakes.
- Snake may be under or near any protective cover.
- In rescue or cleanup operations, wear heavy leather or rubber high-topped boots and heavy gloves. Wear trouser legs outside boots. Be extremely careful around debris.

Use rakes, pry bars or other long-handled tools when removing debris. Never expose your hands, feet or other parts of your body in a place where a snake might be.

- After dark, carry a strong spot light.
- Explain to children the dangers of snakes under storm or flood conditions and the precautions they should follow.
- Do not allow children to play around debris.
- If you kill a venomous snake, use a stick; rake other long–handheld tool to carry the snake away for disposal.
- Do not use your hands to handle dead snake because they might bite even when they appear completely dead.
- If you realize that you are near a snake, avoid sudden movement. Sudden movements may cause the snake to strike.

- If you remain still, the snake may leave. If the snake doesn't move away from you after a few minutes. Slowly back away from it.
- If someone is bitten by a venomous snake, call an emergency number 911 immediately.

Controlling Snakes

- Get rid of snakes in buildings and to prevent others from entering.
- Remove snakes' food supply. Eliminating rats and mice from an area will often discourage snakes.
- Remove snakes 'hiding places. Get rid of lumber piles, trash piles, high weeds and grasses and debris.
- Block openings where snakes might enter buildings. Snakes can pass through extremely small openings and usually enter near or below ground level.
- Be sure doors, windows and screens fit tightly.
- Search walls and floors for holes or crevices.
- Inspect the masonry of foundations, fireplaces and chimneys; plug or cement cracks.
- Plug spaces around pipes that go through outside walls.
- Fasten galvanized screen over drains or ventilators or over large areas of loose construction.

(Adapted from "**The Disaster Handbook-**National Edition Safety Precautions with Snakes; Institute of Food and Agriculture Sciences {IFAS} Publication-DPR-0424-2007. University of Florida-Chapter Four

Appendix V: Research Participant's Informed Consent Form

PARTICIPANT IDENTITY NUMBER: ________________________________

Research Participant Informed Consent Form

You are being asked to participate in a research project. Researchers are required to provide an informed consent form to inform you about the study, to convey that participation is voluntary, to explain risks and benefits of participation, and to empower you to make an informed decision. You should feel free to ask the researchers any questions you may have.

Protocol Title: Isolation and Characterization of Snake Venom Proteins and Peptides from Viperidae (*Bitis arietans*), and Elapidae (*Naja melanoleuca, Naja ashei, Dendroaspis angusticeps*) families in Kilifi County

Protocol Application No: SSC No.2716

Principal Investigator: OTIENO, Kepher Onyango

Sponsor: Jomo Kenyatta University of Agriculture and Technology,

Department of Medical Laboratory Sciences, College of Health Sciences,

Contact Address: P.O. Box 62000-00200, Nairobi-Kenya,

Telephone: (067) 52711, FAX (067) 52164.

E-mail: kotieno@nphls.or.ke

THE PURPOSE OF THE STUDY

We would like to invite you to participate in a research project which aims at catching live snakes for later extraction of their venom to determine the presence of proteins and peptides from Kilifi County. We are interested in the isolation and characterization of the snake venom components to aid us better understand the role protein plays in envenomation and how to utilize that knowledge to discover new drugs for clinical applications.

The study will have three phases:

(a) The first phase will involve the field trapping to rescue live snakes and later transfer to the Bio-Ken research laboratory holding Facility.

(b) The second phase will involve the extraction of venom from the captured live snake specimen within 2 weeks.

(c) The third will involve the wet-laboratory experiment processes at ICIPE Laboratory of Molecular Biology and Bioinformatics Unit.

THE STUDY PROCEDURES

This study will involve a lot of travel in the field around Kilifi County, whereby you are expected to trap and catch live snakes, identify then carefully and restraint the animals, place them in the right transportation bags and restraint equipment ready for transport to the holding facility. You will be required to observe all the safety standard precautions when dealing with all types of reptiles and amphibians. You will be provided with personal protection equipment during field work and the safety standard precautions so that you familiarize yourself with them.

POTENTIAL BENEFITS

The potential benefits to you in participating in this study include the opportunity to sharpen your skills as snake catcher and handler geared towards your accumulation of more points for continued professional development.

POTENTIAL RISKS AND HOW THEY WILL BE MITIGATED

Your participation in this study may expose you to the danger of snakebite envenomation and other risks in the forest. We want to reassure you that just in case of such eventuality, proper rescue and ambulatory services will be on the ground to respond and provide First Aid services and enough antivenom will be available at all times which will be provided by highly qualified and fully dedicated team of medical experts as part of the research members.

PRIVACY AND CONFIDENTIALITY

The data for this project will be kept confidential until published or presented in a professional forum or meeting. Only project investigators and reviewers will have access to the data. All files will be stored in locked files when not in use by the investigators. All electronic files will be stored in private external hard drives dedicated to this project and no information shared with other participants during the specimen and data collection process.

YOUR RIGHTS TO PARTICIPATE, SAY NO, OR WITHDRAW

Your participation in this study is completely voluntary, and the study is not connected to an evaluation of your work. You have the right to say NO.

Should you decide to participate in the study, you may choose to withdraw from the study at any time, for any reason. To withdraw any personal information, please notify the principal investigator.

COSTS AND COMPENSATIONS FOR BEING IN THE STUDY

Phase one of the study will involve travelling several kilometers deep down in Arabuko-Sokoke forest as well as Bamba area of Kilifi County. You will spend most of your time searching for the snake specimen early in the morning hours and late in the evening. This means you will not have enough time to spend with your family. To this end the project management will compensate you for the days you are in the field as per your local field work payment regulations.

Phase two of the project will involve the extraction and milking of the snake specimen for venom in the main facility and there will be no monetary compensation for this.

Phase three will involve laboratory experiment and you will not be involved in this work.

CONTACT INFORMATION FOR QUESTIONS AND CONCERNS

If you have any questions about this study or your participation in it please contact the following researchers directly:

(a) Mr. Kepher Otieno,

Ministry of Health-NPHLS,
P. O. Box 103945-00101, NAIROBI,
Mobile: 0722591528; E-mail: kotieno@nphls.or.ke

(b) Dr. Joseph Gikunju,

Jomo Kenyatta University of Agriculture and Technology,
P.o.Box 62000-00200, NAIROBI.
Mobile: 0722 808671; E-mail: joekangangi2@yahoo.com

If you have any questions about your role and rights as a research participant, or would like to register a complaint about this study, you may contact anonymously if you wish, the Director, Dr. Kimani Gachuhi, KEMRI-CBRD at Telephone: 0722 864834, E-mail: KGachuhi@kemri.org; KEMRI HQs, Off Mbagathi Road, Nairobi-Kenya.

DOCUMENTATION OF INFORMED CONSENT

Your signature below means that you have voluntarily agreed to participate in this research study.

I agree to participate in this research study as a snake catcher/Herpetologist (Tick the one that applies)

Yes **No** **Initials**

Signature of participant **Date**

Signature of person obtaining consent **Date**

Signature of witness to consent procedure **Date**

Appendix VI: Kiswahili Translation of the Research Participant's Informed Consent Form

Mshiriki Kitambulisho Nambari:..

Utafiti wa Mshiriki fomu ya ridhaa

Wewe unaulizwa kushiriki katika mradi wa utafiti. Watafiti wanatakiwa kutoa fomu ya ridhaa kutoa taarifa juu ya utafiti, kufikisha kwamba ushiriki ni hiari, kuelezea hatari na faida ya ushiriki, na kwa kuwawezesha wewe kufanya maamuzi sahihi. Unatakiwa kujisikia huru kuuliza watafiti maswali yoyote unaweza kuwa nayo.

Itifaki ya Utafiti: Kutengwa na Tabia ya nyoka sumu Protini na Peptides kutoka Viperidae (*Bitis arietans*), na Elapidae (*Naja melanoleuca, Naja ashei, Dendroaspis angusticeps*) Familia katika Kilifi Kauti.

Itifaki ya Matumizi Nambari: **SSC No.2716**

Mpelelezi Mkuu: Otieno, Kepher Onyango

Mdhamini: Jomo Kenyatta Chuo Kikuu cha Kilimo na Teknolojia,
Idara ya Sayansi Kwa Mahabara ya Afya, Chuo cha Sayansi ya Afya,
Kuwasiliana mitaani: Sanduku La Posta 62000-00200,
Nairobi-Kenya, Simu: (067)52711, FAX (067)52,164
BaruaPepe:kotieno@nphls.or.ke

1. Madhumuni Ya Utafiti

Tungependa kuwakaribisha kushiriki katika mradi wa utafiti ambayo inalenga katika kuambukizwa na sumu ya nyoka hai kwa ajili ya baadaye ili tuchimbe kwa undani kuamua kama kuna protini na peptides kutoka Kilifi Kauti. Sisi tunania ya kutengwa na kuangalia tabia ya sumu ya nyoka ili utasaidie kuwelewa kwa undani majukumu yao katika envenomation na jinsi ya kutumia maarifa kugundua dawa mapya kwa kliniki.

Utafiti huo itakuwa na awamu tatu:

(**A**) Awamu ya kwanza utahusisha utegaji na usafirishaji wa nyoka hai kutoka porini na kuelekeza kwa Kituo cha Mahabara ya Bio-Ken, Watamu.

(B) Awamu ya pili itahusisha uchimbaji wa sumu kutoka kwa nyoka aliyetekwa ndani ya wiki ya pili.

(C) Awamu ya tatu itahusisha kazi ya maabara ya mchakato wa majaribio katika ICIPE, Maabara ya Masi ya Biolojia na Bioinformatics Unit.

2. Mafunzo Taratibu Ya Utafiti

Utafiti huu utahusisha usafiri mara kwa mara katika viwanja na msitu, Kilifi Kauti, ambapo wewe utatakikana utege na kukamata nyoka hai, kuzitambua kisha kwa makini na kisha kuwa kuwazuia hao wanyama, alafu kuwaweka katika mifuko ya usafiri na kuzuia wanyama tayari kwa ajili ya usafiri kuelekea kwa kituo cha utafiti na mahabara Bio-Ken. Utahitajika kuyashika yote tahadhari iliyowekwa ya usalama wakati wowote ukishughulika na kila aina ya nyoka na vyura. Utapewa vifaa vya kinga binafsi wakati wa kufanya kazi shambani na tahadhari kiwango usalama ili familiarize mwenyewe kwa hayo.

3. Faida Kwako Kushirika Kwa Utafiti Hii

Kushiriki kwako kwa utafiti huu utakupatia nafasi ya kuimarisha ujuzi wako kama msaanii mwenye ujuzi saidi kwa kushika nyoka, ambayo na maendeleo yako kwa kuendelea kitaaluma.

4. Uwezekano Wa Hatari Na Namna Ya Hawatapunguziwa

Ushiriki wako katika utafiti huu inaweza kuweka wewe kwa hatari ya kuhumua na nyoka na hatari nyingine katika msitu. Tunataka kuwahakikishia kwamba kama kutatokea kesi kama hayo, kutakuwa na huduma ya Ambulance ya kuwaokoa sahiyo hiyo. Huduma ya Ambulance itakuwa tayari kila wakati karibu na pahali ya kazi. Pia kutakuwa na huduma ya kwanza na madawa ya kutosha itakuwa inapatikana wakati wote ambayo yatatolewa na timu yenye sifa na kujitolea kabisa ya wataalam wa afya.

5. Faragha Na Uaminifu

Data kutoka kwa mradi huu itakuwa siri mpaka kuchapishwa au kuwasilishwa katika kongamano la mtaalamu au mkutano. Tu mradi wapelelezi na reviewers watakuwa na fursa ya kuangalia data. Mafaili yote itakuwa kuhifadhiwa katika faili na kufungiwa wakati si katika matumizi na wapelelezi. Mafaili yote ya umeme itahifadhiwa katika sehemu binafsi nje ya sehemu ya utafiti na hakutakuwa na rusa kwa wale ambao

wamejitolea kushiriki kwa mradi kusiangalia na pia hakuna taarifa ya pamoja na washiriki wengine wakati wa kuchukuwa sumu na ukusanyaji wa data mchakato.

6. Haki Yako Kushiriki, Sema La, Au Kujitoa

Ushiriki wako katika utafiti huu ni kwa hiari kabisa, na utafiti si zimeunganishwa na tathmini ya kazi yako. Una haki ya kusema la. Lazima kuamua kushiriki katika utafiti, unaweza kuchagua kuondoka kutoka utafiti wakati wowote, kwa sababu yoyote. Kuondoa taarifa zozote za kibinafsi, tafadhali muarifu mpelelezi mkuu.

7. Gharama Na Fidia Kwa Kuwa Katika Utafiti

Awamu ya kwanza ya utafiti huo utahusisha kusafiri kilomita kadhaa kirefu chini katika Arabuko-Sokoke na sehemu ya Bamba katika eneo la Kilifi Kauti. Utatumia muda wako mengi kwa ajili ya kutafuta hao nyoka mapema katika masaa ya asubuhi na jioni. Hii ina maana huwezi kuwa na muda wa kutosha kutumia na familia yako. Kwa maana hii, wasimamizi wa mradi huo wameonelea kukupatia fidia aina ya pesa kwa siku sile ambayo wewe utakuwa katika kwa harakati ya kufanya kazi kule kwa uwanja na porini kulingana na kanuni za malipo ya kazi ya shamba.

Awamu ya pili ya mradi huo utahusu uchimbaji na kukamua sumu ya nyoka katika kituo kuu na hakutakuwa na fidia ya aina ya fedha kwa ajili hii.

Awamu ya tatu itahusisha majaribio ya maabara na autaweza kushiriki katika kazi hiyo.

8. Maelezo Ya Kuwasiliana Kwa Maswali Na Wasiwasi

Kama una maswali yoyote kuhusu utafiti huu au ushiriki wako katika tafadhali wasiliana na watafiti zifuatazo moja kwa moja:

(A) Mheshimiwa. Kepher Otieno,
Wizara Ya Afya-NPHLS,
S.L.P#103945-00101, Nairobi-Kenya.
Simu: 0722 591528; E-mail: kotieno@nphls.or.ke

(B) Daktari,Joseph Gikunju, Ph.D.
Jomo Kenyatta Chuo Kikuu cha Kilimo na Teknolojia,
S.L.P #62000-00200 Nairobi-Kenya.
Simu: 0722 808671; Barua pepe: joekangangi2@yahoo.com

(C) Kama una maswali yoyote kuhusu jukumu lako na haki kama mshiriki utafiti, au ungependa kujiandikisha kwa malalamiko kuhusu utafiti huu, unaweza

kuwasiliana na anonymously kama unataka,

Kwa: Daktari, Kimani Gachuhi, Ph.D.

Mkurugenzi wa KEMRI-CBRD, Katika Simu: 0722 864834,

Barua pepe: KGachuhi@kemri.org,

KEMRI HQS, Off Mbagathi Road, Nairobi-Kenya.

9. Nyaraka Za Ridhaa

Sahihi yako chini ina maana kwamba kwa hiari alikubali kushiriki katika utafiti hii.

• Mimi kukubali kushiriki katika utafiti huu utafiti kama mweny kushika nyoka,

Catcher / Herpetologist (Jibu moja ambayo inatumika)

Ndiyo: ☐ Hapana: ☐ Vifupi: ☐

Sahihi ya mshiriki: Tarehe:

Sahihi ya mtu kupata kibali: Tarehe:

Sahihi ya shahidi kwa idhini ya utaratibu Tarehe:

Appendix VII: Manuscript publication letter

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/AJHS/CORRESP/2016 3rd November 2016

Kepher Otieno, Bsc,
P.O BOX 103945-00101,
Nairobi
Email: kepher.otieno98@gmail.com

Dear Kepher Otieno,

REF: AJHS/2015/434 'ISOLATION AND CHARACTERIZATION OF SNAKE VENOM PROTEINS AND PEPTIDES ISOLATED FROM FOREST (NAJA MELANOLEUCA) IN KILIFI KENYA'- BY KEPHER OTIENO ET AL.

We are pleased to inform you that your above titled manuscript has been accepted for publication in the African Journal of Health Sciences.

Thank you for taking interest in the AJHS.

Kind Regards,

Jane M Rintari (Miss), B.A (Hons) Degree in Sociology & Gov't (UON), MPSM (AU),
Principal Administrative Officer/Head of Administration (AJHS),
For: Editor-in-Chief, AJHS,
KENYA MEDICAL RESEARCH INSTITUTE (KEMRI).

In Search of Better Health

Appendix VIII: Published Manuscript

ISOLATION AND BIOCHEMICAL CHARACTERIZATION OF SNAKE VENOM PROTEINSAND PEPTIDES FROM FOREST COBRA (*NAJA MELANOLEUCA*) IN KILIFI-KENYA

Otieno K[1], Ochola F[4], Mungai G[2], Gachuhi K[3], Gikunju J[2]

SUMMARY

Background: Forest cobras (*Naja melanoleuca*) belong to the family: Elapidae, highly venomous snakes with wide distribution along the Coast of East and Southern Africa. The family attracts much research attention due to their scientific and medical importance. Snake bite is still a neglected tropical condition affecting people living in rural areas of the developing world. Approximately 98% of the World's snake bites occur in Africa, Asia and Latin America. It is estimated that 2.5 million people are affected, with some 421,000 envenomation and 20,000 deaths yearly. Snake bite is a public health problem in Kenya, therefore this study was aimed to isolate and biochemically characterize venom protein toxins.

Methods: Snake venom toxic enzymes were isolated and characterized by determining their molecular masses, isoelectric points, charged residues, instability and aliphatic indices. Silver staining was used to visualize the locality of the toxins.

Results: The phospholipases; Basic phospholipase A2 1, Acidic phospholipase A2 DE-II / DE-III isoforms and a three-finger toxin; Long neurotoxin 1 presented molecular masses of 13,473kDa, 13,427kDa, 13,360kDa, and 8,056kDa respectively. Results suggest that these novel enzymes are low molecular weight and thermally stable molecules based on their 2D gel migratory features as well as their theoretical instability and aliphatic indices.

Conclusion: The present study successfully isolated and characterized four venom toxins from *Naja melanoleuca* with very low molecular weights. These enzymes will be useful in the future research studies to elucidate venom protein toxin structure–function relationships and find ways of neutralizing their toxicity to prevent snakebite disabilities.

Key Words: Venomous snakes; Elapidae; *Naja melanoleuca;* Phospholipase A2; Cobra neurotoxins; Isolation; Characterization; Chromatography; Kenya.

INTRODUCTION

Forest cobra (*Naja melanoleuca*) belongs to the family: Elapidae, and is commonly found along coastal lowland forests and/or savanna shrubs in East and Southern Africa. It is among many species of this family considered as of great scientific and medical importance due to unreported deadly snakebites affecting small scale farming communities along the coastal Kenya. Approximately 98% of the world snake bites occur in Asia, Africa and Latin America resulting in 421,000 envenomation and 20,000 deaths per year. However, when considering the under-reporting that happens in these regions, these numbers are as high as 1,841,000 envenomation and 94,000 deaths per year (1). The mortality caused by snakebites is higher than other neglected tropical diseases such as Schistosomiasis, Leishmaniasis, Dengue Hemorrhagic fever and Chagas disease (2). The World Health Organization now recognizes Snake bites as an important neglected tropical disease.