PHYTOCHEMICAL AND ANTIVENOM EVALUATIONS OF METHANOL LEAF EXTRACT OF *CLERODENDRUM CAPITATUM* (WILLD) SCHUM. AND THONN. (VERBENACEAE)

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF MASTER OF SCIENCE IN PHARMACOGNOSY

DEPARTMENT OF PHARMACOGNOSY AND DRUG DEVELOPMENT,

FACULTY OF PHARMACEUTICAL SCIENCE,

AHMADU BELLO UNIVERSITY, ZARIA - NIGERIA

JUNE, 2014

DECLARATION

I declare that the work in this thesis entitled **Phytochemical and Antivenom Evaluations of Methanol Leaf Extract of *Clerodendrum capitatum* (Willd) Schum. and Thonn. (Verbenaceae)** has been carried out by me in the Department of Pharmacognosy and Drug Development, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria under the supervision of **Prof. M.S. Abubakar and Prof. K.Y. Musa**. The information derived from the literature has been duly acknowledged in the text and list of references provided. No part of this thesis has been previously presented for another higher degree or diploma at any University.

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Abdulrahman Adamu **Date**

CERTIFICATION

This Thesis entitled "**Phytochemical And Antivenom Evaluations of Methanol Leaf Extract of *Clerodendrum Capitatum* (Willd) Schum. And Thonn. (Verbenaceae)** by Abdulrahman Adamu meets the Regulations governing the award of the degree of Masters of Science in Pharmacognosy of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

To my wife Farida for her patience and to our three natural products Rumaysah, Muhsin and Miqdad.

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I acknowledge the sufficient grace of God that has sustained me through the difficult moments encountered during this course of work.

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Finally, to all who have helped in diverse ways I say ‘May God bless you abundantly and be with you always’.

ABSTRACT

Clerodendrum capitatum (Willd) Schumach and Thonn. is a common medicinal plant found mainly in the tropical and sub-tropical regions of the world where it is used in traditional medicine for the treatment of various conditions including erectile dysfunction, wounds, diabetes mellitus, hypertension. The investigations started with the qualitative microscopic and macroscopic evaluation of the leaf material and establishment of its quality parameters, including physicochemical and phytochemical evaluation as no detailed pharmacognostic study or establishment of quality parameters has been done on this plant to date. The powdered leaf was then macerated using 70% aqueous methanol to obtain a brownish gummy extract. The extract was partitioned into n-Hexane fraction (HF), Ethylacetate fraction (EF) and n-Butanol fraction (NF) and the EF was further subjected to phytochemical analysis. In addition, the extract was then evaluated for its acute toxicity and anti-venom properties. Prominent characters include numerous multicellular covering trichomes, anomocytic stomata, vascular bundles and prisms, sheath and cluster of calcium oxalate crystals were recorded. Various physicochemical parameters were also established. Phytochemical screening revealed the presence of many therapeutically important classes of phytoconstituents such as alkaloids, flavonoids, phenolics, sterols, triterpenoids, saponins, proteins and carbohydrates. Analysis of EF through column and thin layer chromatography led to the isolation and identification of the known compound Quercetin-3-O-α-arabinoside. The structure of this compound was determined by spectroscopic methods including 1D NMR as well as comparison with published data. The LD_{50} of 3.8 and 4.4 gKg^{-1} body weight for Lorke's and Karber's arithmetic methods via i.p and oral routes respectively reveals the relative safety of the extract. Investigations carried out on isolated frog rectus abdominis muscle showed that *C. capitatum* extract did not demonstrate any

contracting or blocking effects on frog rectus abdominis muscle when varying doses of 0.1 mg/ml, 1 mg/ml, 10 mg/ml were used. The optimum contraction of ACh and median inhibitory concentration (IC_{50}) for the venom is 0.0004 and 0.00083 $mgmL^{-1}$ organ bath concentrations respectively. In the in vitro neutralization studies, the extract at concentration of 10 $mgmL^{-1}$ and 20 $mgmL^{-1}$ showed 60% and 100% survival respectively. The extract at 20 $mgmL^{-1}$ showed significant level of detoxification where all the mice survived. The phospholipase A2 assay showed that the crude extract inhibited the enzyme (crude phospholipase) in a non competitive manner as revealed by the double reciprocal plots. The Km was calculated as 2.17 $mgmL^{-1}$ and *V*max as 1, 1.13 and 1.28 mg/mL. Such a study would serve as a useful tool in standardization of the leaf material, isolation of medicinally important phytoconstituents, performing pharmacological investigations and ensuring quality formulations. The activity of the extract and the presence of Quercetin-3-O-α-arabinoside in the extract may provide a scientific support to the use of the plant *C. capitatum* for the treatment of snake bites**.**

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LIST OF ABBREVIATIONS

CC	Column chromatography
°C	Degree Celsius
DMSO (d6)	Deuterated dimethyl sulphoxide
Fig.	Figure
g/gm	Gram
Hz	Hertz
J	Coupling constant
m	Multiplet
MeOD	Deuterated Methanol
Mg	Milligram
μg	Microgram
μL	Microliter
MHz	Mega Hertz
Na	Sodium
Ca	Calcium
K	Potassium
NMR	Nuclear Magnetic Resonance
ppm	Parts Per Million
R_f	Retention factor
s	Singlet
TLC	Thin Layer Chromatography
UV	Ultra Violet
v/v	Volume by Volume
w/w	Weight by Weight

α	Alpha
β	Beta
δ	Delta
C	Carbon
^{13}C NMR	Carbon Nuclear Magnetic Resonance
COSY	Correlation Spectroscopy
1D	One Dimensional Spectroscopy
2D	Two Dimensional Spectroscopy
d	doublet
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Coherence
^{1}H - NMR	Proton Nuclear Magnetic Resonance
EF	Ethylacetate Fraction
NF	*n*-Butanol Fraction.
LD_{50}	Median Lethal Dose
IC_{50}	Median Inhibitory Concentration
Ach	Acetylcholine
*V*max	Maximum Velocity
Km	Michealis Mentin's constant

CHAPTER ONE

1.0 INTRODUCTION

Plants are indispensable to the existence of man. The important necessities of life – food, clothing, shelter and a hosts of others are supplied by the plant kingdom. The biotic and abiotic elements of nature are all interdependent. It has provided a complete store – house of remedies to cure all ailments of mankind. The knowledge of drugs has accumulated over thousands of years as a result of man's inquisitive nature so that today we possess many effective means of ensuring health care (Kokate *et al.,* 2008).

In the 19th century, microscopy was introduced for the quality control of pharmaceutical preparations from plants. However, for too many years pharmacognosy remained with these methods, and with the rapidly decreasing number of herbal preparations in the pharmacy, the discipline had great difficulties in holding their prominent position in the pharmaceutical curriculum in the sixties and seventies. Fortunately, some visionary pharmacognosists were able to open up new directions (Kokate *et al.,* 2008).

The development of thin layer chromatography by the well known pharmacognosist Professor Egon Stahl is probably the best example. Also in the field of gas chromatography and high pressure liquid chromatography, pharmacognosists were among the pioneers in the analysis of plant materials. For studies of the active compounds in plants, these chromatographic methods became important tools. Moreover, in the seventies, the spectrometric methods such as MS and NMR became common place for the pharmacognosists in their search for new biologically active compounds in plants. But in that period developing new drugs from plants was not as easy as with synthetic drugs, as the pharmacological test systems, mainly in vivo animal experiments and in-vitro isolated organs, are not suited for bioassay-guided fractionation of active compounds from plant extracts (Verporte, 1998).

1.1 Definition and Scope of Pharmacognosy

Pharmacognosy may be defined as a branch of bioscience which treats in detail medicinal and related products of crude or primary type obtained from plant, animal and mineral origins (Kokate *et al.,* 2008). In short, it is the study of crude drug obtained from plants, animals and mineral kingdom and their constituents (Shah and Seth, 2010). It is an objective study of crude drugs from natural sources treated scientifically and it encompasses the knowledge of the history, distribution, cultivation, collection, processing for market and preservation, the study of sensory, physical, chemical and structural characters and it uses. It also includes the study of other materials used in pharmacy such as suspending, disintegrating and flavouring agents etc. In the 19th century, the term “materia medica” was used for the subject. It was in 1815 that the word “pharmacognosy” was coined by a German Scientist called Seydler in his book titled "Analecta Pharmacognostica." by combining two Greek words “Pharmakon” – (Drug) and “Gignosco”- (To acquire knowledge of). Though, recent historical research has found an earlier usage of the term by a physician, J.A Schmidt (Vienna) in his *Lehrbuch der material medica* in 1811 where he used it to describe the study of medicinal plants and their properties. Pharmacognosy has played a fundamental role in the development of different discipline of science. A pharmacognosist should posses a sound knowledge of the terms used to describe the vegetable and animal drugs as covered under botany and zoology respectively. In addition, the knowledge of plant taxonomy, plant breeding, plant pathology and plant genetics is helpful in the development of cultivation technology for medicinal and aromatic plants (Shah and Seth, 2010).

Plant chemistry (phytochemistry) has undergone significant development in recent years as a distinct discipline. The knowledge of chemotaxonomy, biogenetic pathways for the formation of medicinally active primary and secondary metabolite, plant tissue culture and other related fields is essential for the complete understanding of pharmacognosy (Kokate *et al.,* 2008).

Pharmacognosy is an essential link between pharmacology, medicinal chemistry, pharmaceutics and basic sciences. It is in a nutshell the road and rail network on which depend evolution of novel medicine.

1.2 Importance of Plants in Pharmacognosy

Plants have been an important source of medicines since the beginning of human life on earth. There is a growing demand for plant-based medicines, health products, pharmaceuticals, food supplements, cosmetics etc. People of all ages in both developing and developed countries use plants in an attempt to cure various diseases and to get relief from physical sufferings. Natural products are a source for bioactive compounds and have potential for developing some novel therapeutic agents (Kokate *et al.,* 2008).

Plants have the ability to synthesize a wide variety of chemical compounds that perform important biological functions, as defence substances against predators such as insects, fungi and herbivorous mammals. Many of these chemicals have beneficial effects on long-term health when consumed by humans, and can be used to effectively treat human diseases. At least 12,000 such compounds have been isolated so far; a number estimated to be less than 10% of the total (Tapsell, *et al.,* 2006; Lai and Roy, 2004).

1.3 Taxonomy of medicinal plants

Taxonomy is the study and description of the variation of organisms, the investigation of the causes and consequences of this variation and the manipulation of the data obtained to produce a system of classification. This definition was intentionally drawn up to coincide with the meaning of the term systematics which is synonymously used with taxonomy. However, some authors define taxonomy simply as the study of classification (Stace, 1991). Taxonomists would like to include all aspects of a plant's variation in their considerations but, in practice, many concentrate on a particular set of characters. Those concerned with fieldwork and identification are apt to stress the variation in morphological and ecological characters; those involved in plant breeding will often focus on chromosome numbers, sizes, and shape; those concerned with phylogeny currently focus on variation in gene sequences. That is because most of the preferred methods of numerical phylogenetic analysis are not well suited to examination of morphological characters (Heywood *et al.*, 2007).

The most fundamental step in the scientific study of medicinal plants is establishing their botanical identity. The omission makes positive identification impossible and hinders reproducibility. Taxonomy has two primary objectives. The first is practical; the identification of the diversity of life. The second is cognitive; understanding relationships among organisms. Both are relevant to medicinal plant studies. Unless a plant under investigation can be unambiguously identified, there is no certainty about reproducibility, the fundamental underpinning of science. Understanding evolutionary relationships among species also is germane to the study of plant medicines. Whereas primary metabolites are similar across broad taxonomic categories, secondary metabolites (more accurately plant natural products) exhibit significant qualitative and quantitative variation (Bradley *et al.*, 2008).

1.4 Chemistry and Biological Activity of Plant Extracts

Plant constituents are divided into primary metabolites such as sugars and fats, which are found in all plants; and secondary metabolites – compounds which are found in a smaller range of plants, serving a more specific function. For example, some secondary metabolites are toxins used to deter predation and others are pheromones used to attract insects for pollination. It is these secondary metabolites and pigments that can have therapeutic actions in humans and which can be refined to produce drugs. Examples are inulin from the roots of Dahlias, quinine from the Cinchona, morphine and codeine from the Poppy, and digoxin from the Foxglove (Meskin and Mark, 2002). Chemical compounds in plants mediate their effects on the human body through processes identical to those already well understood for the chemical compounds in conventional drugs; thus herbal medicines do not differ greatly from conventional drugs in terms of how they work. This enables herbal medicines to be as effective as conventional medicines, but also gives them the same potential to cause harmful side effects (Tapsell, *et al.*,2006; Lai and Roy, 2004).

Modern medicine now tends to use the active ingredients of plants rather than the whole plants. The phytochemicals may be synthesized, compounded or otherwise transformed to make pharmaceuticals. Examples of such derivatives include Digoxin, from digitalis; capsaicine, from chili; and aspirin, which is chemically related to the salicylic acid found in white willow. The opium poppy continues to be a major industrial source of opiates, including morphine. Few traditional remedies, however, have translated into modern drugs, although there is continuing research into the efficacy and possible adaptation of traditional herbal treatments (Meskin and Mark, 2002).

1.5 Justification of this study

Snakebite is a major health hazard that had led to high mortality rate in Nigeria. It has been reported that globally over one million humans are bitten annually by numerous snakes resulting in 70,000 deaths (Paramonte, 2007). The numerous side effects (Maya *et al.*,2002), the cost, availability and accessibility (Houghton, 1993) of antivenom immunotherapy, a specific treatment against snake venom envenomation, in the rural areas have been cause of worry and challenge. Many herbs have been used traditionally for the treatments of snakebites. Back in history, countless attempts have been made for the development of snake venom antagonists especially from plants sources since there are limitations in the development of anti sera. Extracts from plants have been used among traditional healers, especially in tropical areas where there are bountiful sources as therapy for snake bite. Therefore, there is a need to curtail the defects potentiated by these hazards and drawbacks and have a scientific basis for the folk and traditional herbal medicines, as an alternative therapy.

1.6 Hypothesis

The leaf extract of *Clerodendrum capitatum (*Willd) Schumach & Thonn. (Verbenaceae) contains constituents or compounds with antivenom activity.

1.7 Aim and Objectives of the Study

Aim of the study

To carry out chemical analysis and antivenom evaluation on the aqueous methanol extract of the leaves of *C. capitatum.*

Specific Objectives

1. To carry out some pharmacognostic studies on the leaves and leaf powder of *C. capitatum* with the aim of identifying the diagnostic properties of the plant.

2. To carry out chemical analysis on the aqueous methanol extract of *C. capitatum* leaves with the aim of isolating a compound.

3. To carry out biological evaluation on the extract of *C. capitatum* leaves with a view of establishing its safety and antivenom activity.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Introduction to the Plant Family Verbenaceae

The Verbenaceae family consists of trees, shrubs and herbs which are mainly flowering plants found in the tropical regions of the world (Rahmatullah *et al.,* 2011). The family consist of 34 genera and around 1,200 species (Franka and Atkin, 2009). The family is recently circumscribed to comprise of approximately 32 genera and 840 species (O'Leary *et al.,* 2012). The Verbenaceae family plants are well known for their use in traditional medicinal systems of various countries (Franka and Atkin, 2009).

Recent molecular phylogenetic studies identified the major lineages in the family and resolved generic relationships throughout most of the group. This resulted in a revised classification for Verbenaceae, dividing the family into eight tribes within which, tribes Verbeneae and Lantaneae represent relatively recent radiations with historically uncertain generic boundaries (O'Leary *et al.,* 2012).

In 1992, Watson, described this family as Plants that are non-succulent, self supporting, or climbing; the climbers stem twiners, or scrambling e.g *Clerodendrum* twining clockwise. The leaf form been non-laticiferous and without coloured juice; bearing essential oils. The leaves are well developed, or much reduced (occasionally). The Leaves are opposite (usually), or whorled, or alternate (rarely); petiolate to sessile; foetid (nauseating), or without marked odour, or aromatic; simple, or compound; pulvinate; when compound, ternate to pinnate (e.g.*Vitex*), or palmate. The Leaf anatomy reveals the mesophyll with sclerencymatous idioblasts, or without sclerenchymatous idioblasts and minor leaf veins without phloem transfer cells (Watson, 1992).

In the stem anatomy, young stems are tetragonal (often), or cylindrical, or oval in section. Cork cambium are present, initially deep-seated (rarely), or superficial and the nodes unilacunar. Internal phloem is absent and contains secondary thickening developing from a conventional cambial ring. The secondary phloems are not stratified. The Xylem contains fibriform fibres with vessels and the vessels end-walls are simple without vestured pits (Watson, 1992).

The plants are usually hermaphrodite and pollination is entomophilous (insect pollinated). The flowers are usually aggregated in 'inflorescences'; in cymes, in racemes, in spikes, in heads, and in verticils (circular arrangement). The ultimate inflorescence unit cymose, or racemose. Inflorescences terminal, or axillary; with involucral bracts (often, these commonly coloured), or without involucral bracts (Watson, 1992).

The p*erianth* has distinct calyx and corolla with two whorls which might be isomerous or anisomerous. The Calyx usually 2–8 in number having one whorl which are gamosepalous; entire, or lobulate, or blunt-lobed, or toothed. The Calyx is lobes markedly shorter than the tube to markedly longer than the tube. It is unequal but not bilabiate, or regular, or bilabiate (e.g. *Phyla*); persistent. The Corolla is usually 4–8 in number, single whorled and gamopetalous.It lobes are markedly shorter than the tube, or about the same length as the tube. Corolla imbricate are usually tubular, or rarely campanulate (bell shape) and unequal but not bilobiate (Watson, 1992).

The a*ndroecium* is usually 2–5 in number with its members adnate (to the corolla tube). It markedly unequal (usually), or all equal (rarely) and free from one another with a single whorl. Androecium exclusively of fertile stamens, or including staminodes. Stamens (2–) 4 (–5) (the posterior member usually, and sometimes the three upper members, reduced or missing); inserted near the base of the corolla tube, or midway down the corolla tube, or in the throat of the corolla tube; usually didynamous; reduced in number relative to the adjacent perianth (usually), or isomerous with the perianth; oppositisepalous; alternating with the corolla members. Anthers connivent (in pairs), or separate from one another; dorsifixed; dehiscing via longitudinal slits. Endothecium developing fibrous thickenings. The initial microspore tetrads tetrahedral, or isobilateral, or decussate. Anther wall initially with one middle layer; of the 'dicot' type, or of the 'monocot' type. Pollen grains aperturate; (2–)3(–5) aperturate, or 6 aperturate; colpate, or colporate, or rugate; 2-celled (in 9 genera), or 3-celled (in 3 genera) (Watson, 1992).

The Gynoecium is 2 carpelled (usually), or 4 carpelled, or 5 carpelled which reduced in number relative to the perianth, or isomerous with the perianth. The pistil 2–10 celled. Gynoecium are syncarpous; eu-syncarpous; superior. The Ovary has 2 locules, or 4–5 locular (but the original locules (usually two) early becoming divided by a 'false septum' in each — *Labiatae*). Locules secondarily divided by 'false septa' (usually), or without 'false septa'. The Gynoecium usually a median; stylate. Styles usually 1 (one) is attenuate from the ovary, or from a depression at the top of the ovary (but the ovary apex no more than slightly lobed); apical, or lateral. Stigmas 1(one); 1 lobed, or 2 lobed; papillate. Placentation is usually basal to axile. There are two (2) ovules per locule (i.e. in the true locules, one each in the locelli). The embryo-sac development is a Polygonal-type. The antipodal cells formed are usually three (3) not proliferating, ephemeral (usually), or persistent. The endosperm formation is cellular (Watson, 1992).

The fruits are fleshy, or non-fleshy; dehiscent (rarely), or indehiscent (mostly), or a schizocarp. Mericarps when schizocarpic, 4 (usually), or 8–10, or 2; comprising nutlets, or comprising drupelets. When fruits are non-schizocarpic a drupe (usually), or a capsule. Capsules valvular (with 2–4 valves). The drupes with separable pyrenes, or with one stone.

The germination of seedling are phanerocotylar, or cryptocotylar. Seeds are non-endospermic with two Cotyledons either expanded or flat (Watson, 1992).

2.2 The Verbenaceae Family Identification Characteristics

Phylogenetic studies of this family is rare so also the groups within the family. The plants in Verbenaceae can usually be recognized in the field by a combination of traits, including being woody trees or shrubs (a few genera, including *Verbena* and *Glandularia*, are herbaceous, and *Petrea* species are lianas) with opposite leaves and flowers with slightly bilateral corolla symmetry. Their fruits are fleshy or dry, generally with two or four seeds, often dividing into two or four segments (Hannah, *et al.*,2010).

The difficulty that led to confusion in distinguishing the Labiateae family which the Verbenaceae superficially resembles had to do with the degree of separation of the locules and the position of the style. However, the fundamental distinction has to do with where the ovules attach in relation to the false partitions that divide each carpel into two locules. In Verbenaceae, the ovules attach directly to the margins of the false carpel septa, whereas in Labiateae, the ovules attach to the sides of the inrolled carpel walls. There are also key differences distinguishing the two such as characters like racemose inflorescence, the included stamens, thickened stigma lobes with conspicuous stigmatic tissue, and Colporate pollen (Franca and Atkin, 2009).

In Summary, the key characters for the family includes opposite simple leaves, racemose inflorescence, usually in the form of a head or spike, Hermaphrodite flowers, Stamens 2 - 4 (5), calyx are tubular/campanulate, tubular flower with spreading limb and importantly the leaves are often aromatic. (Franca and Atkin, 2009).

2.3 The Classification of the family Verbenaceae

General taxonomic classification is

Kingdom – Planta

Sub-Kingdom – Tracheobionta

Division – Magnoliophyta

Class – Magnoliopsida

Sub-Class – Asteridae

Order – Lamiales

Family – Verbenaceae

Tribe - Teucrieae

Genus – *Clerodendrum*

Species – *capitatum*

From studies carried out by O'Leary *et al.*, 2012, the phylogeny shows that none of the traditional classifications reflect phylogenetic relationships very well. Eight clades are recognized as tribes (Casselieae, Citharexyleae, Duranteae, Lantaneae, Neospartoneae trib. nov., Petreeae, Priveae, and Verbeneae). Two genera, *Dipyrena* and *Rhaphithamnus*, remain unplaced in these larger clades. Petreeae, which consist of Neotropical lianas, are sister to the rest of the family. Lantaneae and Verbeneae together form a derived clade that comprises approximately two-thirds of the species in Verbenaceae (O'Leary *et al.*, 2012).

Fig. 2.1 Cladistic description of the Verbanaceae family (Hannah, *et al.*,2010).

Clades **Genera** **Species**

Casselieae

Casselia Parodianthus Tamonea 14 species

Citharexyleae

Baillonia Citharexylum Rehdera 130 species

Duranteae

Duranta, Recordia

Stachytarpheta, Bouchea, Chascanum

Lantaneae

Lantana (100 species)

Lippia (120 species)

Aloysia (30 species)

Nashia (7 species)

Acantholippia (6 species)

Phyla (5 species)

Coelocarpus (5 species)

Burroughsia (2 species)

Xeroaloysia (1 specie)

Neospartoneae trib. nov.

Neosparton

Lampaya

Diostea

5 species

Petreeae

Petrea (11 species)

Xolocotzia (9 species)

Priveae

Priva (20 species)

Pitraea (species)

Verbeneae.

Verbena

Glandularia

Junellia

Urbanea

Hierobotana

Other genera in addition to above include; *Acantholippia*, *Adelosa*, *Aegiphila*, *Aloysia*, *Amasonia*, *Archboldia*, *Asepalum*, *Baillonia*, *Bouchea*, *Burroughsia*, *Callicarpa*, *Caryopteris*, *Casselia*, *Chascanum*, *Citharexylum*, *Clerodendrum*, *Coelocarpum*, *Coelocarpum*, *Cornutea*, *Cyclocheilon*, *Dimetra*, *Diostea*, *Dipyrena*, *Duranta*, *Faradaya*, *Garrettia*, *Geunsia*, *Glandularia*, *Glossocarya*, *Gmelina*, *Hierobotana*, *Holmskioldia*, *Hosea*, *Huxleya*, *Hymenopyramis*, *Junellia*, *Karomia*, *Lampaya*, *Lantana*, *Lippia*, *Monochilus*, *Nashia*, *Neorapinia*, *Neosparton*, *Nesogenes*, *Oncinocalyx*, *Oxera*, *Paravitex*, *Parodianthus*, *Peronema*, *Petitia*, *Petraeovitex*, *Petraea*, *Phyla*, *Pitraea*, *Premna*, *Priva*, *Pseudocarpidium*, *Recordia*, *Rehdera*, *Rhaphithamnus*, *Schnabelia* (or *Labiatae*), *Stachytarpheta*, *Stylodon*, *Surfacea*, *Tamonea*, *Tectona*, *Teijsmanniodendron*, *Tetraclea* (or *Labiatae*), *Teucridium*, *Tsoongia*, *Ubochea*, *Urbania*, *Verbena*, *Verbenoxylum*, *Vitex*, *Viticipremna*, *Xeroaloysia*, *Xolocotzia* (Watson, *et al.*,1992).

2.4 Chemical Constituents and Biological Activity of Verbenaceae family

The Verbenaceae contain chemical constituents such as cyanogenic, alkaloids, iridoids (*Verbena*), arthraquinones (*Tectona*) derived from shikimic acid, verbascosides detected in 5 genera, excluding *Phyla* having cornoside. Ursolic acid and Saponins/sapogenins have been shown to be present. Sugars are transported as oligosaccharides and sucrose (Watson, *et al.*, 1992).

A number of the plants have been reported to contain bio-active metabolites with important pharmacological effects. The ethanolic extract of *Premna corymbosa* (Burm.f.) Rottl. & Willd. leaves reportedly suppressed the development of chronic arthritis in rats (Karthikeyan and Deepa, 2010). Tectone (3,8-dihydroxy-2-methyl anthraquinone), a new anthraquinone, which is known to posses anti-hyperglycemic properties has been isolated from leaves of *Tectona grandis* L.f (Shukla *et al.*,2010). The Ethanol extract of *Stachytarpheta jamaicensis* (L.) Vahl in animal models of nociception and inflammation, reportedly demonstrated anti-inflammatory properties (Sulaiman *et al.*,2009). Anti-oxidant and anti-fungal properties have been shown of various solvent extracts of leaves of *V. officinalis* (Casanova *et al.*,2008).

The essential oils obtained from leaves of *Lippia gracilis* Schauer reportedly possessed antinociceptive and anti-inflammatory activities (Mendes *et al.*,2010). While that obtained from *Lippia graveolens* Kunth reportedly inhibited the growth of *Giardia lamblia*, the causative agent for the diarrheal disease, giardiasis (Machado *et al.*,2010). The essential oil from leaves and fine stems of *Lippia schomburgkiana* Schauer (main constituents 1,8-cineole and α-terpineol) demonstrated to have anti-oxidant and cytotoxic activity (da Silva *et al.*,2009). Essential oils obtained from *Lippia turbinata* Griseb. and *Lippia polystachya* Griseb. reportedly demonstrated lethal effects against mosquito larvae of *Culex quinquefasciatus*, where ambulation speed and total time of ambulation was significantly decreased (Kembro *et al.*,2009). Anti-spasmodic activity has been reported for essential oil from *Lippia dulcis* Trev., a plant used in traditional medicine of Central America for cough, colds, bronchitis, asthma, and colic (Görnemann *et al.*,2008).

2.4.1 Economic uses of plants in the Verbenaceae family

Tectona grandis (teak) is a source of timber; some notable ornamentals, e.g. *Clerodendrum*, *Callicarpa*, *Vitex*, *Lantana*, *Verbena*; noxious, photosensitizing weeds (*Lantana*).

2.5 The Distribution and Description of *Clerodendrum* species

Clerodendrum is a very large and diverse genus with about 580 identified species. It is the largest genus of the tribe Teucrieae (Steane *et al.*,1999). It is widely distributed in the tropical and warm temperate regions of the world, with most of the species occurring in tropical Africa and Asia. It occurs in Northern Africa, in Egypt, and spreads through the remainder of Africa and Madagascar. The genus comprises small trees, shrubs and herbs and it is well known for its ornamental uses. The first description of the genus was given by Linnaeus in 1753, with the identification of *C. infortunatum* (Steane et al., 1999).

2.5.1 Botanical Description of *Clerodendrum* species

Species of this genus are usually small trees with most of them being climbers. They are characterized by simple decussate - opposite leaves with most of them being petiolated. There is a wide variability in the sizes of the leaves. *C. aculeatum*, for instance, has leaves ranging from 0.9-4 cm long and 0.3-1.4 cm wide whiles *C. paniculatum* has leaves that are 6-35 cm long and 6-30 cm wide. The leaf shape is also variable with *C. walichii* having a lanceolate shape but *C. japanicum* being ovately shaped. The inflorescences of members of this genus are both axilliary and terminal. The inflorescence may range from 1-39 cm long and 1-25 cm wide. They may be 7 cymes, panicles or solitary flowers which may be crowded or sparsely arranged. Almost all the species of *Clerodendrum* have foliaceous bracts and linear lanceolate bracteoles. The calyx is usually gamosepalus, commonly green but sometimes red or white. They are almost always campanulate, rarely elliptic, truncate, 5-lobed, glabrous or pubescent. The size of the corolla ranges from 0.6-4.0 cm long to 0.3-2.0 cm wide. The corolla may be white, red, pink or purple. The corolla is glabrous, pubescent or glandular-puberulent. The fruits are drupaceous, mostly subglobose, ovoid or glabrous usually separating at maturity. The exocarp is fleshy and black when mature (Rueda, 1993).

2.6 Ethnomedical and Biological Action of *Clerodendrum* species

The genus *Clerodendrum*, is reported to demonstrate biological activities such as antitumorgenic, Hypoglycemic, hypolipidemic, Hepatoprotective activity against CCl4 induced liver injury in rats, Anti- inflammatory, free radical scavenging activity, Anti-diarrhoeal, Antinociceptive and antipyretic effects, antibacterial, anthelmintic, anti fungal, antidiarrheal, and immune modulatory activities (Abdulwehab *et. al.*, 2012; Shirvastava

2010). These activities may be due to the presence of flavonoids, terpenoids, tannins, steroids in the plant extracts. Upon this different ethnobotanical claims have been made.

2.6.1 Ethnobiology of Clerodendrum species

A number of species in the genus have been documented to be used by various tribes in Asia and Africa. The Leaves of *C. buchholzii* are reported in some parts of Africa for the treatment of rheumatism, furunculosis, echymosis and gastritis (Burkill, 2000, Nyegue *et al.*,2005). In Ghana, the leaves of *C. splendens* are used in the form of poultice to treat burns and wounds (Mshana *et al.*,2000). The aqueous extract of *C. umbellatum* is used as an enema for the treatment of gonorrhoea and placental retention in some parts of Africa (Irvine, 1961). In southern Africa, the leaf of *C. glabrum* is taken as a remedy for cough, colic, intestinal parasites and as an insect repellant. It is also used to control convulsions in children by inducing sleep. The root infusion is taken by mouth for snake bite especially the mamba's (Burkill, 2000). In the Chinese system of medicine *C. bungei* is used for the treatment of headaches, dizziness, furuncles and hysteroptosis (Zhou *et al.*,1982; Yang *et al.*,2002). Cheng (2001) has reported the use of *C. cyrtophyllum* and *C. chinense* in the treatment of fever, jaundice, typhoid and syphilis in China. The roots of *C. serratum* have been used in the treatment of dyspepsia, seeds in dropsy and leaves as a febrifuge and in cephalagia and ophthalmia (Burkill, 2000). *C. calamitosum* is used for the treatment of kidney, gall and bladder stones. This plant is also reported to have diuretic and antibacterial properties (Cheng *et al.*, 2001). In China, the fruits of *C. petasites* are used as medicine for malaria while in India the plant is used to induce sterility (Hazekamp *et al.*, 2001; Panthong *et al.*, 2003).

2.6.2 Biological Action of *Clerodendrum* Species

In Nigeria, this plant is used to treat diabetes mellitus, obesity, and hypertension. The genus *Clerodendrum* is reported to demonstrate versatile biological activities such as antitumorgenic, hypoglycemic, hypolipidemic, hepatoprotective activity against CCl4-induced liver injury in rats, anti-inflammatory, radical-scavenging activity, antidiarrhoeal, antinociceptive, and antipyretic effects (Abdulwehab *et al.,* 2012). The plant posses various pharmacological activities like anti diarrheal, anti fungal, Immunomodulatory, Anthelmintic, Anti-inflammatory, and anti heptaotoxic activities (Shrivastava, 2010).

The leaves of *Clerodendrum trichotomum* Thunberg have been shown to have Anti-inflammatory effects as a result of phenylpropanoid glycosides present in the leaves (Kim *et al.*,2009). The plant, *Clerodendrum umbellatum* Poir is used in the traditional medicinal system of Cameroon for treatment of intestinal helminthiasis; aqueous extract of leaves have been shown to demonstrate schistosomicidal properties in *Schistosoma mansoni* mice model (Jatsa *et al.,* 2009). The aqueous leaf extract of *Clerodendron inerme* showed potent antilipidperoxidative effect and improved the antioxidant defence system in (7,12-dimethylbenzanthracene (DMBA)-induced hamster buccal pouch carcinogenesis (Manoharan, *et al.*,2006).

2.7 Reported Phytochemical Constituents of *Clerodendrum* Species

The different species of *Clerodendrum* contain chalcone glycoside, flavonoids, tannins, steroids, terpenes, phenolic compounds, phenyl propanoids, sucrose etc. The phytochemical analysis of crude extracts reveals the presence of flavonoids like pectolinarigenin, hispidulin, apigenin and luteolin, tannins like clerodin, clerodendrin, and steroids like clerosterol, β- sitosterol, γ- sitosterol and ceryl alcohol. Researchers have made great efforts to isolate and identify biologically active principles and other major constituents from various species of the genus. These include steroids, terpenoids, flavonoids and others. This is represented in a table below as culled from Boonsri, 2004:

Table 2.1: Compounds isolated from *Clerodendrum* Species

Steroids	Plant part	Plant Specie
***β*-sitosterol**	Root	*C. serratum*
	Root	*C. paniculatum*
	Root and Stem	C. *fragrans*
	Leaves	*C. inerme*
	Leaves	*C. infortunatum*
	Leaves	*C. nerifolium*
	leaves and aerial parts	*C. colebrookianum*
	aerial parts	*C. nutans*
	Stems	*C. fragrans*
	Stem	*C. indicum*
Clerosterol	Stem	*C. crytophyllum*
	Entire plant	*C. bungei*
	leaves and stem	*C. inerme*
	aerial parts	*C. infortunatum*
	Aerial parts	*C. inerme*
	Aerial parts	*C. nutans,*
	Root bark	*C. mandarinorium*
	Root	*C. phlomoides*
Colebrin A and B	Aerial parts	*C. colebrookianum*
Stigmasterol	Aerial, leaves and stem	*C. fragrans*
Cholesterol	Aerial, leaves and stem	*C. fragrans*
Poriferasterol	Aerial, leaves and stem	*C. fragrans*

22-Dehydroclerosterol	Aerial, leaves and stem	*C. fragrans*
Campesterol	Leaves	*C. neriifolium*
	Leaves and stem	*C. scadens*
	Leaves and stem	*C. inerme*
Terpeniods		
Triterpenes		
α-amyrin	roots and stem	*C. fragans*
	leaves and stem	*C. inerme*
β-amyrin	Roots	*C. colebrookianum*
	leaves and stem	*C. inerme*
	Roots	*C. paniculatum*
Friedelin	Stem	*C. crytophyllum*
	Stem	*C. inerme*
	Bark	*C. trichotomum*
Lupeol	Stem bark	*C. neriifolium*
Betulin	Leave and stem	*C. inerme*
Oleanolic acid	Leave and stem	*C. inerme*
Diterpenes		
Clerodendrin A	Roots	*C. phlomoides*
Clerodendrin B	Leaves	*C. inerme*
Clerodendrin C	Leaves	C. inerme
Clerodin	Flowers	*C. infortunatum*
Clerodin	Leaves	*C. brachyanthum*
Clerodin	Roots	*C. phlomoides*
Clerodinin A, B, C	Leaves	*C.brachyanthum*

Uncinatone	Stem	C. cryophyllum
Uncinatone	Stem	*C. bungei*
Uncinatone	Roots and root bark	*C. uncinatum*
Uncinatone	leaves and stem	*C. siphonanthus*
bungone A and B	stem	*C. bungei*
Uncinatone	stem	*C. bungei*
teuvincenone F	stem	*C. bungei*
Sugiol 12	stem	*C. bungei*
Royleanone	leaves and stem	*C. inerme*
Iridoid monoterpene		
Harpagide	Leaves	*C. tomentosum*
Ajugoside	Leaves	*C. thomsonae*
Flavonoids		
Scutellarein	Roots	*C. serratum*
Scutellarein	aerial parts	*C. indicum*
Flavones		
Cirsimaritin	leaves and stem	*C. fragans*
	root bark	*C. mandarinorum*
	Leaves	*C. neriifolium*
Apigenin	leaves and stem	*C. inerme*
	Flowers	*C. infortunatum*
	Leaves	*C. neriifolium*
	Flowers	*C. phlomoides*
	Roots	*C. serratum*
Hispidulin	Flower	*C. phlomoides*

	aerial parts	*C. indicum*
Luteolin	Flowers	*C. phlomoides*
	roots and leaves	*C. serratum*
Salvigenin	Leaves	*C. neriifolium*
	Leaves	*C. inerme*
7-Hydroxyflavanone	leaves and flowers	*C. phlomoides*
Cyanogenic glycosides		
Lucumin		*C. grayi*
Prunasin		*C. grayi*
Phenolic compounds		
Anisic acid	leaves and stem	*C. bungei*
Vanilic acid	leaves and stem	*C. bungei*
Lignin		
Eudesmin	Leaves	*C. brachyanthum*
Phenyl propanoid		
Verbacoside	Leaves	*C.inerme,C.capitatum buchananii*
Indole alkaloid		
Trichotomine	Fruits	*C. trichotomum*

Abietane diterpenoids having anti-microbial and anti-parasitic (anti-leishmanial, anti-malarial) properties have been isolated from the roots of *Clerodendrum eriophyllum* Gürke (Machumi *et al.*,2010). There are also other chemical constituents present which include volatile constituents such as 5-O-ethylcleroindicin D, linalol, benzyl acetate and benzyl benzoate, which have been isolated from *C. canescens*, *C. cyrtophyllum, C. inerme and C. philippinum* (Yang *et al.*,2002 and Nyegue *et al.*,2004). D-mannitol from *C. serratum* (Garg and Verma 2006). Carbohydrates like glucose, fructose, sucrose are been reported in *C. mandarinorum* and *C. inerme.*

Other constituents such as ribosome-inactivating protein, salidroside, jinoside-D, acetoside have been isolated from *C. inerme* (Olivieri *et al.*,1996), while trichotomoside, cytotoxic pheophorbides and cleromyrin-I have been isolated from *C. trichotomum*, *C . calamitosum* and *C. Cyrtophyllum* (Bashwira *et al.*,1989 and Chae *et al.*,2006).

Two new long chained feruloyl esters, Formadienoate-A and B have been isolated from *Clerodendrum formicarum* (Ali *et al.*,2010). Using Chromatographic separation of the roots of a Kenyan medicinal plant, *Clerodendrum eriophyllum*, led to the isolation of ten abietane diterpenoids (1-10), one of which (1) was isolated for the first time from a natural source (Machumi *et al.*,2010). A saponin (SN1) was isolated from *Clerodendrum infortunatum* leaves (Dilipkumar *et al.*,2009).

Plate I : ***C. capitatum*** **during flowering stage from Willd.**

Plate II: ***C. capitatum*** **during fruiting stage from Willd.**

2.8 Botanical Description of *Clerodendrum capitatum (*Willd*)* Schumach & Thonn.

The genus *Clerodendrum* (Verbenaceae) is widely distributed in tropical and subtropical regions of the world. More than five hundred species of the genus are identified till now, which includes small trees, shrubs and herbs. Ethno-medical importance of various species of *Clerodendrum* genus has been reported in various indigenous systems of medicines and as folk medicines. The genus are widely distributed in Asia, Australia, Africa and America. It is being used as medicines for the treatment of various life threatening diseases such as syphilis, typhoid, cancer, jaundice and hypertension. The genus *Clerodendron,* is reported to demonstrate biological activities such as antitumorgenic (Manoharan, *et al.,*2006 and Liu, *et al.,*2008), hypoglycemic, hypolipidemic (Adeneye *et al.,*2008, and Devi and Sharma, 2004), hepatoprotective activity against CCl_4 induced liver injury in rats (Vidya, *et al.,*2007 and Gopal, 2008), anti-inflammatory (Narayanan, 1999 and Panthong 2003), free radical scavenging activity, anti-diarrhoeal, antinociceptive and antipyretic effects (Chae, *et al.*,2006).

Few species of the genus like *Clerodendrum inerme, C. thomosonae, C. indicum,* and *C. speciosum* are ornamental and being cultivated for aesthetic purposes (Shirvastava and Patel, 2007).

Clerodendrum capitatum (Willd) Schumach & Thonn. (Verbenaceae), locally named "bambaro or maashayi" in Hausa and "korlejiga" in Fulfulde (Burkill, 2000), is an indigenous tropical African plant, which is growing fast, erect, well branched, having perennial under shrub and grows up to 0.5-2 m high (Adeneye *et al.,*2008). Its stem is quadrangular in transverse section and it is covered with soft hair. The plant leaf is simple, oval or elliptical, generally bent at the base, rigid, leaving an under thorn stab on the stem after the fall of the leaf. Its inflorescence is glomerular with globular terminal auxillage and flowers yearly. In Nigeria, The plant though reputed for bone healing in fractures and as such popularly called medicinal magical plant (Adeneye *et. al.*, 2008); used traditionally in the management of erectile dysfunction in male (Mahmoud, *et al.,* 1995). It is equally reputed for local management of diabetes mellitus, obesity and hypertension (Adeneye *et. al.*, 2008). The major chemical components reported from the genus are phenolics, steroids, di- and triterpenes, flavonoids, volatile oils, etc (Shrivastava and Patel, 2007).

2.9 Reported Phytochemical Consitutents of *C. capitatum*

Phytochemical results revealed the presence of saponins, flavonoids, alkaloids, tannin, glycosides and reducing sugars in the extract (Adeneye *et al.*, 2008). To the best of my knowledge, only verbascoside, a phenyl propaniod have been isolated from the leaves of this plant by Taoubi in 1992 as reported by Boonsri, 2004 in his review on *Clerodendrum*.

2.10 Biological Action of *C. capitatum*

The folkloric use of *Clerodendrum capitatum* in the treatment of suspected type 2 diabetics had a positive correlation with scientific data generated in the study carried by Adeneye *et al.*, 2008. The results of the study showed *C.capitatum* to cause significant dose dependent hypoglycemic and hypolipidemic effects in rats. From pharmacological studies carried out by (Abdelwahab *et al.*,2008), *C. capitatum* did not demonstrate any contracting, relaxant or blocking effects on frog rectus abdominus muscle and rat aortic strip preparations. The extract only produced a concentration-dependent decrease of the normal rhythmic contraction of rabbit jejunum and in addition, *C. capitatum* also produced a stimulant activity on rat uterus which was blocked by cyproheptadine (Abdelwahab *et al.*, 2008).

Furthermore, Abdelwahab *et al.*,2012, tested the relaxation effect of this plant on phenylephrine precontracted rabbit Corpus Cavernosum Smooth Muscle (CCSM) and on isolated Guinea pig atria alone, in the presence of calcium chloride (Ca^{2+} channel blocker), atropine (cholinergic blocker), and glibenclamide (ATP-sensitive K+ channel blocker). It was suggested that *C. capitatum* possesses a relaxant effect on CCSM, which was attributable to the inhibition of phosphodiesterase type 5 (PDE-5), but not mediated by the release of calcium, activation of adrenergic or cholinergic receptors, or the activation of potassium channels (Abdelwahab *et al.*,2012).

2.11 Thin layer chromatographic studies of extracts

Thin Layer Chromatography (TLC) studies are carried out on extract to confirm the presence of different phytoconstituents in the extract. TLC is a mode of liquid chromatography, in which, the extract is applied as a small spot or band at the origin of thin sorbent layer supported on a metal plate. The mobile phase migrates through the stationary phase by capillary action. The separation of solutes takes place due to their differential adsorption / partition coefficient with respect to both mobile and stationary phases. Each separated component has same migration time but different migration distance. The mobile phase consists of a mixture of solvents. Silica gel (type 60) is most commonly used adsorbent. The Retardation Factor (R_f) is calculated using following formula,

$$R_f = \frac{\text{Distance travelled by solute from the origin}}{\text{Distance travelled by solvent from the origin}}$$

(Harborne, 1991)

2.12 Venom Research

Snake venoms are complex mixtures of proteins, nucleotides and inorganic ions. These combinations confer a formidable array of toxic properties on the venom. Annually, about 2.5million people around the world are the victims to snake bites, of whom about 100,000 lose their lives (Koh *et al.*,2006).

From a medical perspective, the venomous snakes of Africa can be divided into five categories:

1) Snakes that bite frequently, and are associated with serious or life- threatening envenoming.

2) Snakes that bite frequently, but rarely cause serious or life threatening envenoming.

3) Snakes that bite rarely, but are capable of causing severe or life threatening envenoming.

4) Snakes that bite rarely, and have not caused significant envenoming.

5) Other potentially venomous snakes which have not caused documented bites (WHO, 2010).

2.12.1 Epidemiological Data on Snakebite in African Countries

Epidemiological data on snakebite in Africa are hard to obtain. There are probably more than 100,000 envenomings and 5,000 deaths in Africa each year. However, Chippaux (1998) has estimated five-fold higher figures of one million bites, 500,000 envenomings and 20,000 deaths (10,000 of which are reported) each year. Important species involved in these envenomings include *Echis ocellatus, E. leucogaster, E. pyramidum, Bitis arietans,* spitting cobras *(Naja nigricollis, N. katiensis, N. pallida, N. mossambica etc), N. haje, N. annulifera, N. nivea, Dendroaspis polylepis and D. angusticeps*. In Nigeria**,** 174 snakebites/100,000 population/year are reported. The saw-scaled or carpet viper (*Echis ocellatus*) is responsible for 90% of bites and 60% of deaths. In the Benue Valley of north-eastern Nigeria, the incidence of snakebite was found to be 497 per 100,000 population per year with a mortality of 12.2%. A community survey of snakebite by the black-necked spitting cobra (*Naja nigricollis*) in Malumfashi, northern Nigeria, found that in a population of 43,500 there were 15–20 bites/100,000/ year. Only 8.5% of the victims had visited a hospital. The case fatality was 5%, and 19% of survivors had persistent physical disability from the locally necrotic effects of the venom (WHO, 2010).

2.12.2 Snake venom

The primary function of snake venom is to help the snake immobilize and eventually digest its prey. Snake venoms are complex mixtures of numerous toxic and nontoxic components. More than 90% of the dry weight is protein. The most important venom components that lead to significant clinical effects after a bite are enzymes and polypeptide toxins. The amount of venom injected during a bite depends on various factors: species and size of the snake, mechanical efficiency of the bite, whether one or two fangs penetrated the skin, and whether there were repeated bites. Not all bites by venomous snakes lead to venom injection. On an average of 50% of occasions, no venom is injected; this is referred to as a “dry bite”. Even after several bites or after eating their prey, snakes do not exhaust their venom, and they remain just as venomous (WHO, 2010).

2.12.3 Snake venom composition

The most important venom components that cause serious clinical effects are pro-coagulant enzymes, cytolytic or necrotic toxins, haemolytic and myolytic phospholipases A2, pre-and post-synaptic neurotoxins, and haemorrhagins (WHO, 2010).

The Haemolytic and myolytic phospholipases A2 damage cell membranes, endothelium, skeletal muscle nerves and red blood cells. Phospholipases A2 are the most widespread and extensively studied of all venom enzymes. Under experimental conditions, they damage mitochondria, red blood cells, leucocytes, platelets, peripheral nerve endings, skeletal muscle, vascular endothelium and other membranes. They produce presynaptic neurotoxic activity, opiate-like sedative effects and the auto pharmacological release of histamine (WHO, 2010).

The Pre-synaptic neurotoxins are mainly found in elapid venoms and in some vipers. They are phospholipases A2 that damage nerve endings, initially releasing acetylcholine followed by interfering with its release and the Post-synaptic neurotoxins are mainly found in elapid venoms. They are polypeptides that compete with acetylcholine for binding at neuromuscular junction receptors and lead to curare-like paralysis (WHO, 2010).

2.12.4 Major clinical manifestations following envenomation by snakes

Major clinical manifestations following envenomation by snakes among others are a) Flaccid paralysis b) Systemic myolysis c) Coagulopathy and haemorrhage d) renal damage and failure e) cardiotoxicity and f) local tissue injury at the bite site. (Koh *et al.*,2006). These venomous snakes include all family members of Elapidae, Viperidae and some members of Atractaspidae and Colubridae (McCue, 2005). The genus *Naja* belongs to the Elapidae family with about 25 species (Perrys brigde, 2009).

Investigation on *Naja Nigricollis* have been carried out and some of it toxic effects were eliminated by plant extracts like the methanolic extracts of *Indigofera pulchra, Aristolochia albida, Sterculia setigera* (Abubakar, 2003) and *Guiera senegalensis* (Abubakar *et al.*,2000). Other genera reputed to be used against snake bite include *Amaranthus, Rauwolfia, Vernonia, Ipomoea, Acalypha, Euphorbia, Phyllanthus, leucas, Ocimum, Acacia, Cassia, Piper, Zanthoxylum, Clerodendrum, Lantana* (Houghton, 1993).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 List of Chemicals and Reagents

All solvents and reagents used in this work were of analytical grade. All references to water indicate distilled water.

3.1.1 List of chemicals

Ethanol (Qualikems fine chem., Nandesari, India)

Methanol (Sigma Aldrich St. Louis, MO, USA)

Ethyl acetate (Qualikems fine chem., Nandesari, India)

Chloroform (Qualikems fine chem., Nandesari, India)

N-butanol (Qualikems fine chem., Nandesari, India)

Acetone (Qualikems fine chem., Nandesari, India)

N-hexane (Qualikems fine chem., Nandesari, India)

Distilled Water

3.1.2 List of reagents

The following reagents were used for chromatographic studies.

3.1.2.1 Universal detecting reagent

10% Sulphuric Acid

Iodine vapour

3.1.2.2 Specific Detecting Reagent

* Dragendorff reagent for alkaloids and other nitrogen-containing compounds.
* Ferric chloride solution
* Aluminium chloride solution.
* Liebermann-Burchard reagent: Acetic anhydride - sulfuric acid for 5-3-sterols (cholesterol and esters), steroids and triterpene glycosides

3.2 List of Materials and Equipment

3.2.1 Chromatographic Materials

Silica gel was used for both the column and thin layer chromatographic (TLC) techniques. The types used are:

• Silica gel (60-120 mesh) for column chromatography (Merck Germany)

• Pre-coated thin layer chromatography (TLC) aluminium plates (Silica gel 60 F_{254}) for TLC analysis (Merck Germany).

3.2.2 Materials used in Antivenom Studies

1. Microdynamometer.
2. Syringes and needles (1 – 10 mL)
3. 20ml beakers
4. Scapel
5. Scissors

3.2.3 Animals used

Mice

Frogs

3.3 Plant Collection and Identification

This section involves the collection, identification, shade drying and comunition of the leave material.

3.3.1 Collection, Identification and Preparation of plant material

The leaves of *Clerodendrum capitatum* were collected around Samaru Zaria, Kaduna State, Nigeria in August, 2012. The plant material was identified and authenticated by the herbarium keepers of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria, and a specimen voucher number (301) was deposited for future reference. The leaves were cleaned, air dried for 5 days and grounded to powder using pestle and mortar then stored at room temperature in the laboratory until when needed.

3.4 Pharmacognostic Studies of the leaves of *C. capitatum*

Pharmacognostic study was carried out on the plant leaves to identify diagnostic features not in the literature as at this time. This included macroscopical, microscopical and chemomicroscopical examination of the leaves and leaf powder. In addition, preliminary phytochemical screening of the leave extract, determination of ash and extractable matter were carried out to develop standard for the leave of this plant. This is to serve as a basis for comparison with other species of this genus.

3.4.1 Macroscopical examination of the leaves of *C. capitatum*.

An initial macromorphological study was carried out on the plant leaves using mainly organoleptic methods. This is based on shape, size, colour, surface characteristics, and texture (WHO, 2011).

3.4.2 Microscopical examination of the leaves of *C. capitatum*.

Different anatomical sections of the leaves were examined and the results were recorded using the usual descriptive terms (WHO, 2011).

3.4.3 Examination of the powdered leaves of *C. capitatum*.

The leaves of *C. capitatum* were grounded in an electric grinder and sieved. The powder was examined for its colour, odour, taste, epidermal cells, stomata, trichomes, etc (WHO, 2011).

3.4.4 Determination of some physical constants of the powdered leaves of *C. capitatum*

3.4.4.1 Total ash of the leave of *C. capitatum*

Two gram portion of the grounded air dried powdered leaves was weighed into three platinum crucibles. It was ignited by gradually increasing the heat, until it became white. It was cooled in a dessicator and weighed. The content of total ash in mg per g of air – dried powdered was calculated (WHO, 2011).

3.4.4.2 Acid insoluble ash of the leaves of *C. capitatum*

To the three crucibles containing the total ash, 25ml of hydrochloric acid (~70g/L) was added and covered with a watch glass and boiled gently for 5 minutes. About 5 ml of hot water was used to rinse the cover glass into the crucible. The insoluble matter was collected on an ashless filter paper and washed with hot water until the filtrate was neutral. This was then transferred back to the crucible and dried on a hot plate and ignited to a constant weight. The residue was allowed to cool in a suitable dessicator for 30 minutes. It was then weighed without delay. The content of the acid insoluble ash in mg per g of air dried powder was calculated (WHO, 2011).

3.4.4.3 Water soluble Ash

To another three crucibles containing the total ash, 25ml of water was added and boiled for 5 minutes. The insoluble matter was collected in a sintered glass crucible. It was washed with hot water and ignited in a crucible for 15 minutes at 400°c. The weight of the residue in mg was subtracted from the weight of total ash. The content of water soluble ash in mg per g of air dried material was calculated (WHO, 2011).

3.4.5 Determination of solvent extractive values of the powdered leaf of *C. capitatum*

3.4.5.1 Alcohol – Soluble Extractive value

Powdered leaves of *C. capitatum* (5 g) was weighed and macerated with 100 ml of 90% ethanol in a stoppered flask for 24 hours shaking frequently during the first six hours. It was then filtered and evaporated to dryness in a tared shallow dish. It was dried to constant weight at 110^0C. The percentage of Alcohol – soluble extractive with reference to the air dried drug was calculated (Brain and Turner, 1975). This procedure was repeated three times and the average recorded.

3.4.5.2 Water – Soluble Extractive value

Powdered leaves of *C. capitatum* (5 g) was weighed and macerated with 100 ml of 0.25% chlolroform water (1: 400) in a stoppered flask for 24 hours shaking frequently during the first six hours. 20 ml was then filtered and evaporated to dryness in a tared shallow dish. It was dried to constant weight at 110^0C. The percentage of water – soluble extractive with reference to the air dried drug was calculated (Brain and Turner, 1975).

3.4.6 Moisture content (Loss on drying at 110°C)

An accurately weighed quantity of about 5 g of powdered drug was taken in a tared porcelain crucible. The powder was distributed evenly. The porcelain crucible was kept open in vacuum oven and the sample was dried at temperature of 110°C for 6 hours until constant weight was recorded. Then it was cooled in a dessicator to room temperature, weighed and recorded. This procedure was repeated three times. The percentage loss on drying was calculated using the following formular.

$$\% \text{ Loss on Drying} = \frac{\text{Loss in weight of sample}}{\text{Weight of sample}} \times 100$$ (Brain and Turner, 1975).

3.4.7 Preliminary phytochemical studies of the leave extract of *C. capitatum*

The extract was subjected to preliminary qualitative phytochemical investigation using test tubes and thin layer chromatography.

3.4.7.1 Preliminary phytochemical screening using spray reagents

The aqueous methanolic leaf extract of *C. capitatum* was subjected to thin layer chromatography to detect the various constituents present in it.

3.4.7.2 Procedure for the preparation of spray reagents used for TLC

1. **Ninhydrin for amino acids, amines and amino-sugars.**

0.3 g ninhydrin was dissolved in 100 ml 1-butanol and 3 ml glacial acetic acid was added. Spray solution: 0.2% ethanolic ninhydrin solution. After-treatment, it was heated at 110°C for maximal visualization of the spots (Stahl, 1988).

2. **Dragendorff reagent** for alkaloids and other nitrogen-containing compounds. Solution a: 0.85 g Bismuth(III) nitrate was dissolved in 10 ml glacial acetic acid and 40 ml water.

Solution b: 8 g potassium iodide was dissolved in 20 ml water.

Stock solution: Equal parts of a and b was mixed. Spray solution: 1 ml stock solution was mixed with 2 ml glacial acetic acid and 10 ml water before use (Stahl, 1988).

3. **Ferric chloride solution:** 2.7g of ferric chloride salt was dissolved in 100 ml of 2M hydrochloric acid and used as spray for detection of phenols and phenolic compounds (Stahl, 1988).

4. **Liebermann-Burchard reagent:** This reagent was prepared fresh. 5 ml Acetic anhydride is placed in an ice bath, to this is added 5 ml concentrated sulphuric acid. The mixture is added to 50 ml ice cold absolute ethanol. After spraying, it was heated for 10 min

at 110°C. The compounds produce spots visible under ultraviolet light (360 nm) Characteristic of sterols (cholesterol and esters), steroids and triterpene glycosides (Stahl, 1988).

5. **Sulfuric acid (general visualisation reagent)**: 10% sulfuric acid in ethanol was prepared. The chromatogram was sprayed with this reagent and allowed to dry for 10 minutes in the air and heated to 110°C for maximal visualisation of the spots (Stahl, 1988).

6. **Aluminium chloride for flavonoids:** 1% ethanolic solution of aluminium chloride was prepared and sprayed on the chromatogram. A yellow fluorescence in long-wave UV light is indicative of a flavonoid (Stahl, 1988).

7. Anisaldehyde - sulfuric acid for sugars, steroids, terpenes.

A solution of 0.5 ml anisaldehyde in 50 ml glacial acetic acid and 1 ml 97% sulfuric acid was prepared freshly before use. After-treatment, it was heated to 100-105°C until maximal visualisation of the spots were seen (Stahl, 1988).

7. **Iodine as general detection reagent.**

The chromatogram was placed into a chamber containing some crystals of iodine (Stahl, 1988).

3.4.7.3 Procedure for preliminary phytochemical screening using test tubes

1) Tests for Carbohydrates: Preparation of test solution:

The test solution was prepared by dissolving the extract with water. It was hydrolyzed with 1 volume of 2N HCl and subjected to following chemical tests.

a) Molisch's test (General test):

To 2-3 ml aqueous extract, few drops of α-naphthol solution in alcohol was added followed by concentrated H_2SO_4 which was added from the side of the test tube. It was then observed for violet ring at the junction of the two liquid.

b) Barfoed's test:

Equal volume of Barfoed's reagent and test solution were added in a test tube. Heated for 1 min, in a boiling water bath and cooled then observed for red precipitate.

2) **Tests for Proteins:**

The test solution was prepared by dissolving the extract in water.

a) Biuret test (General test):

To 3 ml of the test solution, 4% NaOH and 2 drops of 1% $CUSO_4$ solutions were added and observed for violet or pink colour.

b) Million's test (for proteins): 3 ml of the test solution was mixed with 5 ml Million's reagent, it was observed for white precipitate. Precipitate was then warmed to turn brick red or precipitate dissolves giving red colour.

c) Precipitation test: The test solution was observed for white colloidal precipitate with following reagents: absolute alcohol, 5% $HgCl_2$ solution, 5% $CUSO_4$ solution, 5% lead acetate, 5% ammonium sulphate.

3) Tests for Amino Acids:

a) Ninhydrin test (General test): 3 ml test sample and 3 drops 5% Ninhydrin solution were heated in boiling water bath for 10 min. It was observed for purple or bluish colour.

4) Tests for Alkaloids:-

a) **Dragendorff's test**: To 2-3 ml filtrate 5 drops of Dragendorff's reagent were added and observed for orange brown precipitate.

b) **Hager's test**: 2-3 ml filtrate with Hager's reagent observed for yellow precipitate.

c) **Wagner's test**: 2-3 ml filtrate with few drops of Wagner's reagent observed reddish brown precipitate.

5) Tests for Flavonoids:- The flavonoids are all structurally derived from the parent substance called flavone. The flavonoids occur in the free form as well as bound to sugars as glycosides. For this reason, when analyzing flavonoids it is usually better to examine the flavonoids in hydrolyzed plant extracts. Preparation of test solution:

i. To a small amount of extract, equal volume of 2M HCl was added and heated in a test tube for 30 minutes at 100°C

ii. The cooled extract was filtered, and extracted with ethyl acetate.

iii. The ethyl acetate extract was evaporated to dryness, and used to test for flavonoids.

a) **Shinoda test:** To dried extract, 5 ml 95% ethanol, few drops concentrated HCl and 0.5 g magnesium turnings were added. Pink colour shows the presence of flavoniods.

b) To small quantity of residue, **lead acetate solution** was added. A Yellow coloured precipitate shows presence of flavoniods.

c) Addition of increasing amount of **sodium hydroxide** to the residue will show a yellow colouration, which will decolourised on addition of acid.

d) **Ferric chloride test:**

Test solution with a 5 drops of ferric chloride solution were added. An intense green colour shows the presence of flavonoids.

6) **Tannins and phenol compounds:** To 2-3 ml of alcoholic or aqueous extract, few drops of following reagents were added in separate test tubes and observed.

a) 5% $FeCl_3$ solution: Deep blue- black colour.

b) Lead acetate solution: White precipitate.

c) Bromine water: Discoloration of bromine water.

d) Acetic acid solution: Red colour solution.

e) Dilute iodine solution: Transient red colour (Evans, 1996: 2002; Harborne, 1991 and Kokate *et al.*, 2008).

3.5 Preparation of extract and fractions from the leaves of *C. capitatum*

This section involves the preparation of extract using maceration with methanol - water (7:3) for 5 days. Subsequently, fractions were successively partitioned with different solvents in an increasing order of polarity.

3.5.1 Preparation of Extracts of *C. Capitatum*

The powdered leaves of *C. capitatum* (500g) was macerated with 2.5 L of 70% v/v aqueous methanol for five days after which, it was filtered. The filtrate was depigmented on activated charcoal and filtered. The filtrate was collected and concentrated in - vacuo and kept in a desiccator at room temperature for further analysis.

3.5.2 Fractionation of extracts of *C. capitatum*

The methanolic extract portion (50 g) was suspended in water (200ml). This mixture (solution) was then successively partitioned with (5 x 200ml) n – hexane, (7x200ml) Ethyl acetate, (5x200ml) n-butanol. The Ethylacetate and the N-butanol partitioned fractions were collected and concentrated at room temperature and using rotary evaporator and coded EF and NF respectively. The N- hexane (HF) was concentrated at room temperature likewise.

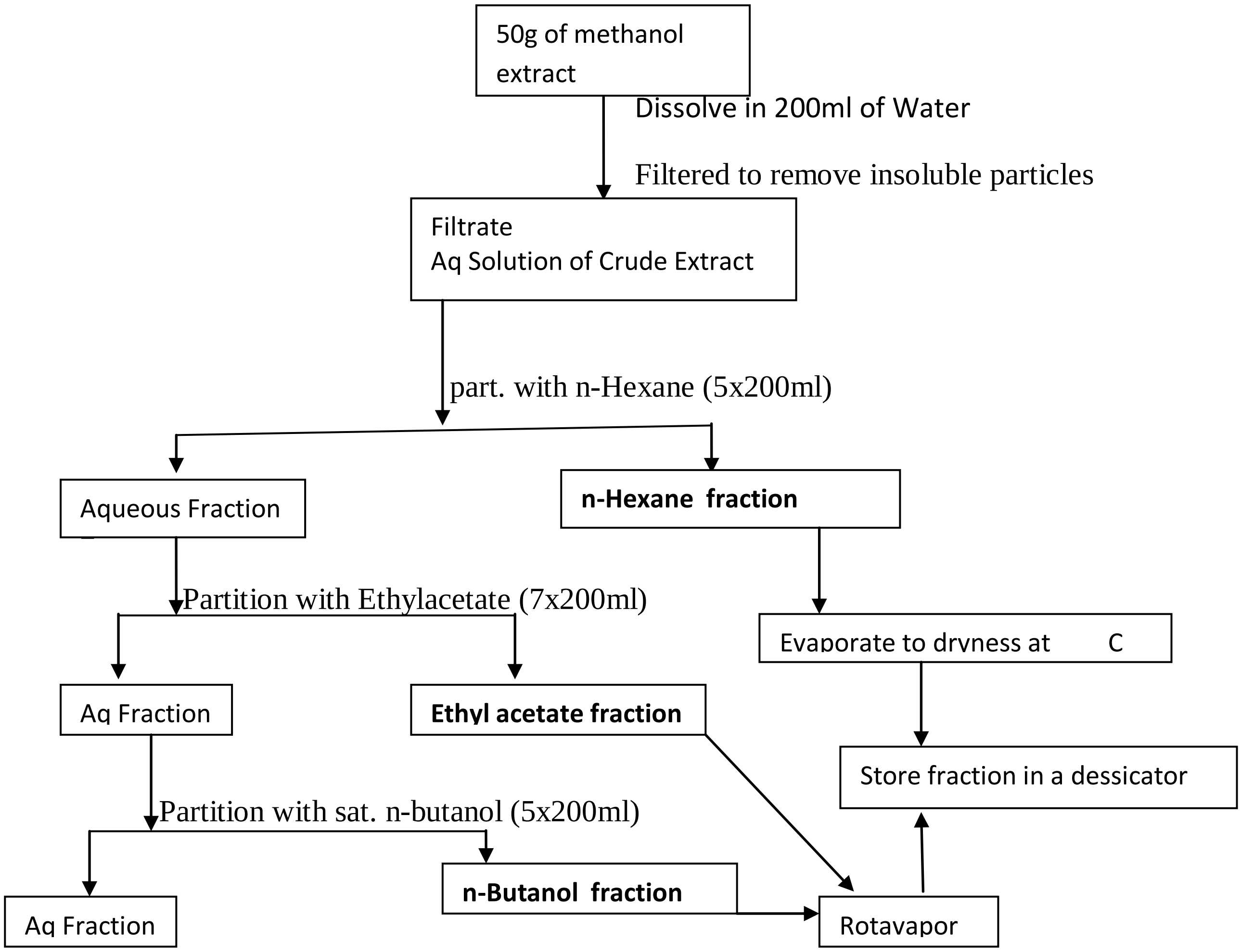


Fig 3.1: Fractionation Chart for *C. capitatum* aqueous methanol extract

3.6 Thin layer chromatographic studies of extract of *Clerodendrum*

The aqueous methanol leaf extract was subjected to thin layer chromatography to detect the various constituents present in it. The details of TLC plates used are as follows:-

Adsorbent: Silica gel GF_{254}

Solvent system: Ethylacetate-methanol-acetic acid (14:3:1)

Detecting Reagent: 10% sulphuric acid in ethanol.

3.6.1 Thin layer chromatography of fractions HF, EF and NF

The samples HF, EF and NF to be analysed by TLC were dissolved in methanol and applied on pre-coated silica gel TLC plates as spots with the aid of capillary tubes at one end of the plate in a straight line, about 0.5 cm above the bottom edge and 0.5 cm away from the sides. The spots were dried and the plates placed in a chromatank containing the mobile phase that has been prepared in the tank at least 30 minutes earlier. The mobile phase ran along the TLC plate in an ascending manner due to capillary action, carrying with it the components of the extract or the mixture. When the mobile phase reached the desired distance, the plate was removed, the solvent front marked and the plate dried. The separated compounds were located by observing the chromatogram under ultra-violet light for fluorescence. This was followed by spraying with 10% H_2SO_4 in ethanol and heating at 110°C. This method was used for all TLC analysis (Ghani, 1990).

A portion of each fraction solution was applied to the plate and developed. The spray of each chromatogram was done using reagents both universal (10% sulphuric acid) and specific (ferric chloride for phenolic compounds, drangedorff for alkaloids, ninhydrin for amino acids etc) to check for the presence or absence of various secondary metabolites. The separated components were detected visually and under a UV light at wavelengths of 254 and

366 nm before spraying. The solvent: (Ethyl acetate: methanol: acetic acid; 14:3:1) for EF gave an optimum resolution and was thus, used for further analysis.

3.6.2 Column chromatography of Ethyl acetate fraction of *C.capitatum*

The EF fraction which showed a good resolution on the TLC plate was then subjected to column chromatography. A slurry of the Silica gel was gently packed wet into a column. The fraction to be separated was adsorbed onto some amount of the silica gel and packed on top of it and a cotton wool placed on the packed column. The solvent to be used in eluting the chromatographic column was placed on top of the packed column. The eluates (20 ml) were collected into a 25 ml glass beakers. The solvent for elution include Ethyl acetate and methanol in a volume ratio gradients were used as means of monitoring the composition of eluates and aliquot.

3.6.2.1 Column chromatography properties

Adsorbent: Silica gel 60-120 mesh (Qualikems fine chem., Nandesari, India)
Column length: 3.5 cm × 40 cm
Sample loaded: 1 g
Mobile phase: 100% Ethylacetate (EA), 95% EA and 5% methanol (M), ...
Volume ratio gradients: 300 ml
flow rate: 20 drops/min.
Detection: sprayed with 10% v/v Sulphuric acid in ethanol.

3.7 Isolation of Compound from Fraction CF6

The bulked fractions were screened using TLC. Fractions **CF6** had about 3 spots. CF6 (137.0 mg) was then eluted and purified on a smaller column using 10 g of silica gel 60 – 120 mesh size. These solvent systems ethylacetate-methanol-acetic acid (14:3:1), Chloroform-methanol (4:1) and ethyl acetate-petroleum ether (7:3) were used to run and test the purity of the compound.

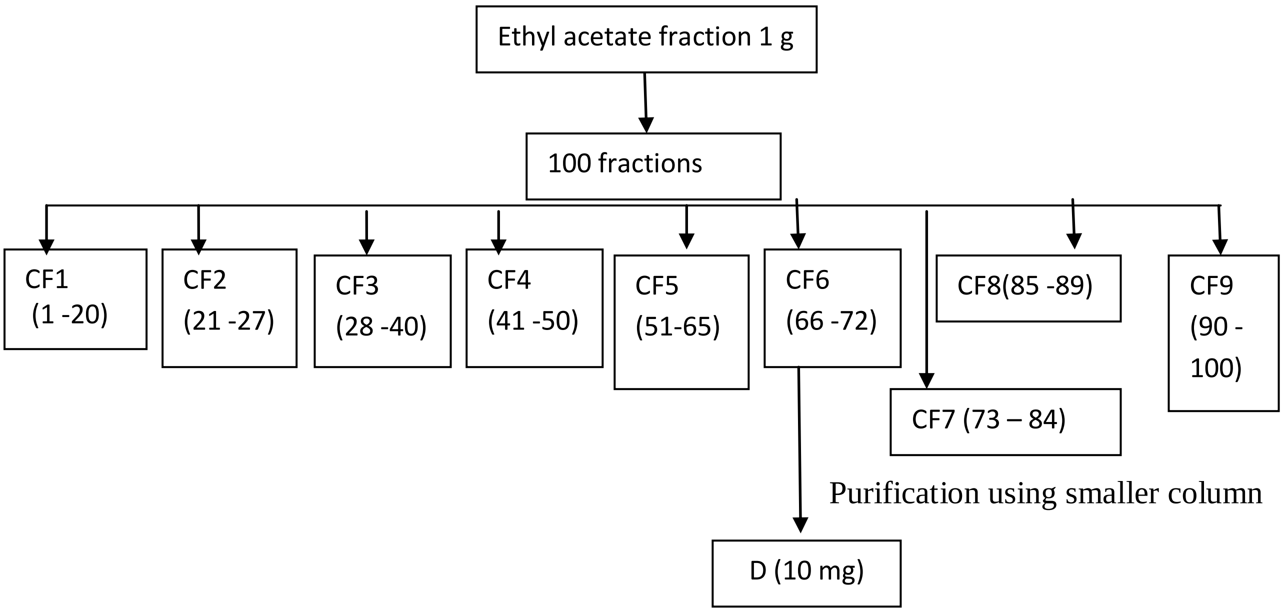


Fig 3.2: Isolation of compound D from the CF6 of Ethyl acetate fraction

3.8 Structure elucidation of compound D from *Clerodendrum capitatum*

3.8.1 Physicochemical evaluation of isolated compound D

The melting point of the pure compound was determined using Stuart Melting Point apparatus. About 2 mg of the sample was transferred into a capillary tube and inserted into the melting point apparatus. With the aid of a thermometer, the melting point was recorded.

3.8.2 Spectroscopic studies of isolated compound D

Nuclear magnetic resonance (NMR) spectroscopic experiments was carried out. The NMR spectra were obtained at 300 K on Top Spin 100 MHz and 400 MHz NMR spectrometer (Bruker, Germany) for ^{1}H-NMR and ^{13}C-NMR respectively at the University of Pretoria, Hatfield, South Africa. Chemical shift (δ – values) in ppm and multiplicities were recorded.

3.8.2.1 NMR sample preparation

The isolated compound was weighed (10mg) and dissolved in 1 ml of deuterated solvent (methanol). These solvent was used as solvent of choice because the compound was soluble in it. The solutions were pipette in NMR tubes (5 mm in diameter) using a clean Pasteur pipette and then subjected to 1D/ 2D NMR analyses.

3.9 Biological Studies of extracts of *C. capitatum* on venom of *N. nigricollis*

3.9.1 Source of Venom and preparation

The venom was kindly provided by Prof. M.S. Abubakar of Department of Pharmacognosy and Drug Development, Ahmadu Bello University, Zaria. It was preserved at 4^{0}C prior to experiment.

3.9.2 Median lethal dose determination (LD_{50}) of crude extract of *C. capitatum*

Acute toxicity studies were conducted to determine the median lethal dose (LD_{50}) of the aqueous methanol extract. The toxicity study was carried out according to Lorke's method (1983) and compared with the Arithmetic method of karber as modified by Aliu and Nwude (1982).

3.9.2.1 Acute toxicity testing using Lorke's Method

LD_{50} determination was conducted using the method of Lorke's (1983) using the intraperitoneal (i.p.) route in mice. The method was carried out in two phases. In the initial phase, 3 groups of three mice each were treated with the methanol leaf extract of the plant at doses of 10, 100 and 1000 mg/kg body weight i.p. and observed for signs of toxicity and death for 24 hours. In the second phase, 4 groups each containing one mouse was injected with four more specific doses of the extract based on the result in phase 1. The LD_{50} value was determined by calculating the square root of the geometric mean of the lowest dose that caused death and the highest dose for which the animal survived (0/1and 1/1).

3.9.2.2 Acute toxicity testing using Arithmetic method of Karber

The oral safety of the extract was tested using Arithmetic method of Karber as modified by Aliu and Nwude (1982). The animals were divided into six (6) groups (groups A to F) of 3 mices each. Groups A to E animals were the treatment groups, while group F served as untreated control animals. Animals in groups A, B, C, D and E were orally administered 300, 600, 1200, 2400, 4800 mg/kg doses, respectively of the crude extract, while the control group received an equivalent volume of distilled water. Animals were observed for signs of acute toxicity like behavioral changes and death over 48 hours and LD_{50} was determined.

3.9.3 Minimum Lethal Dose (MLD) LD_{99} of the crude venom of *Naja nigricollis*

The minimum lethal dose (MLD) that was reported by Abubakar et al., 2000 was used for this in vitro studies.

3.9.4 In vitro Studies of crude extract of *C. capitatum* against *Naja nigricollis* envenomation using Frog Rectus Abdominis Model

Frogs weighing 30-50 g were used in this study (n = 20). A frog was stunned and decapitated and the spinal cord was destroyed. The two recti muscles were localized, running each side of the midline from the base of the sternum to the fork. Then, the pair of rectus abdominus muscle were dissected out and transferred to a petri-dish containing frog-ringer solution (NaCl, KCl, $NaH_2PO_4.2H_2O$, Glucose, $NaHCO_3$ and $CaCl_2$) at ambient temperature. The muscle was mounted in a 10 mL organ bath filled with aerated Frog-Ringer solution and attached to isotonic transducer. The contractures of the muscle were recorded on a Ugo Basile Microdynamometer recorder. The tissue was allowed to equilibrate under 1 g tension for 1 h, before starting the administration of acetylcholine, venom and plant extract.

3.9.4.1 Preparation of Extract stock solution for Frog rectus abdominis studies

The extract (1 g) was dissolved in 10 ml of distilled water to give stock concentration of 100 mg/ml stock A. This was further diluted to a concentration of 10 mg/ml, 1 mg/ml, 0.1 mg/ml (B, C, and D respectively).

3.9.4.2 Preparation of Ach stock solution for Frog rectus abdominis studies

About 10 mg of the Ach was dissolved in 10 ml of distilled water to give concentration of 1 mg/ml. This was further diluted to a concentration of 0.1 mg/ml, 0.001 mg/ml, 0.0001 mg/ml and 0.000001 mg/ml. These were labelled stock A, B, C, D and E respectively.

3.9.4.3 Preparation of Venom sample stock solution for Frog rectus abdominis model

The crude venom (10 mg) was dissolved in 10 ml of distilled water to give concentration of 1 mg/ml (stock A). This was further diluted to a concentration of 0.1 mg/ml (Stock B), 0.001mg/ml (stock C), 0.00001 mg/ml (stock D), 0.000001 mg/ml (1ppm) as stock E.

3.9.4.4 Effects of *C. capitatum* on frog rectus abdominis muscle

The effect of *C. capitatum* crude extract was carried out on the frog rectus abdominis muscle using same experimental procedure reported above and the observations were recorded.

3.9.4.5 Determination of the Optimum Concentration (O.C) of acetyl Choline

The optimum concentration required to cause contraction of the isolated frog rectus abdominis muscle optimally was determined following the above procedure. Different concentration (0.1 mg/ml, 0.001 mg/ml, 0.0001 mg/ml and 0.000001 mg/ml) of Acetylcholine were used and the optimal was chosen.

3.9.4.6 Determination of the median inhibitory concentration IC_{50} of *Naja nigricollis* Venom against Acetylcholine induced contractions.

The median inhibitory concentration of *Naja nigricollis* venom against optimum concentration of acetylcholine induced contraction was determined using graphical method.

3.9.4.7 Effects of extract of *C. capitatum* on isolated frog rectus abdominis muscles.

The effect of crude extract of *C. capitatum* was studied on the isolated frog rectus abdominis muscles. The effects were recorded graphically after using varying concentrations of 0.1mg/ml, 1mg/ml, 10mg/ml, 100mg/ml.

3.9.4.8 Determination of the effects of extract of *C. capitatum* against Ach and *Naja Nigricollis*

The effects of crude extract of *C. capitatum* in varying doses of 0.1mg/ml, 1mg/ml, 10mg/ml, 100mg/ml were studied against the effects of the Ach(O.C) and *N. nigricollis* venom ($I.C_{50}$).

3.9.5 In vitro Venom detoxification by the extract of *C. capitatum*

Five groups of mice (*n=5*) were used in this protocol. The first group (positive control) received 0.2 ml of the MLD (LD_{99}, 9.55 mg/kg) of *N. nigricollis* venom only and

Group 5 (negative control) received 0.2 ml normal saline. Groups 2, 3, and 4 (serving as treatment groups) were given an equivalent of the MLD of the venom containing 2 mg, 5 mg and 10 mg extract, respectively. The venom and the extract were incubated at 37°C for 10 min and 0.2 ml of the incubated mixture was injected (i.p) into each animal in the treatment group. The signs of neurotoxicity and number of deaths were recorded within 48 hours.

3.9.6 Phospholipase Assay of extract of *C. capitatum*

This was carried out by method of Haberman and Neumann (1957) as modified by (Sallau et al., 2000). 0.5 ml of egg yolk suspension (2.5 mg/ml) was introduced into a clean test tube containing 50 μL of 1 mM $CaCl_2$. To this, 100 μL of 20 mg/ml venom solution and 50 μL of phosphate buffer (pH = 7.0) was then added and incubated at 37°C for 1 hour. The enzymes were inactivated by heating at 100^0C for 2 minutes. 1 – 2 drop of phenolphthalein indicator was added and titrated against 2mM NaOH solution to the end point. The same procedure was carried out in the absence of the enzyme in order to obtain titre value for the blank for adequate comparison to deduce effect of the enzyme on the yolk. The activity of phospholipase was defined as the amount of enzyme required to hydrolyse 1 mg of free fatty acid from the lecithin present in the egg yolk under the standard assay conditions.

3.9.6.1 Effects of extract of *C. capitatum on* Crude Phospholipase Enzyme.

This was carried out as above. The concentration of 500 μL of the substrate in different concentrations (2.5 mg/ml, 5 mg/ml and 10 mg/ml) in addition to 100 μL of 1.25 mg/ml, 2.5 mg/ml and 5 mg/ml of the extract was added to the reaction mixture. The initial velocity data obtained were used for the double reciprocal plots. This was used to calculate the Km and Vmax. Since the direct measurement of the numeric value of *V*max and therefore the calculation of *K*m often requires impractically high concentrations of substrate to achieve saturating conditions. A linear form of the Michaelis-Menten's equation which circumvents this difficulty and permits *V*max and *K*m to be extrapolated from initial velocity (Vi) data obtained at less than saturating concentrations of substrate was used. Starting with equation (Michealis Menten's equation);

$$Vi = \frac{Vmax\,[S]}{Km + [S]}$$ after inverting, factoring and simplifying,

$$\frac{1}{Vi} = \frac{Kmax + [S]}{Vmax\,[S]}$$ inverting,

$$\frac{1}{Vi} = \frac{Km}{Vmax\,[S]} + \frac{[S]}{Vmax\,[S]}$$ Factor,

Therefore,

$$\frac{1}{Vi} = \frac{Km}{Vmax}\frac{1}{[S]} + \frac{1}{Vmax}$$ simply,

The equation for a straight line is $y = ax + b$, where $y = 1/Vi$ and $x = 1/[S]$. A plot of 1/Vi as y as a function of 1/[S] as x therefore gives a straight line whose y intercept is 1/*V*max and whose slope is *K*m/*V*max. Such a plot is called a **double reciprocal** or **Lineweaver-Burk plot**. Setting the y term of equation equal to zero and solving for x reveals that the x intercept is −1/*K*m.

3.9.7 Statistical Analysis: The data were expressed as mean±SEM. Student t – test was used to compare the observations and results with $P<0.05$ or less was regarded as significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Plant Collection and Identification

The leaves of *Clerodendrum capitatum* (Willd) Schumach & Thonn. were collected around Samaru Zaria, Kaduna state Nigeria in August, 2012. The plant material was identified and authenticated by the herbarium keepers of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria, and a specimen voucher number (301) has been deposited for future reference. The leaves (1 kg) were cleaned, air dried for 5 days and grounded to powder using pestle and mortar then stored at room temperature in the laboratory until when needed.

4.2 Pharmacognostic Studies of *C. capitatum* Leaves

4.2.1 Macroscopical examination of C. capitatum leaves

Plate III: Whole leaf of *C. capitatum* showing petiole and lamina

a) Whole leaves

Type	:	Simple
Form	:	entire
Condition	:	Fresh and Dried

b) Lamina

Shape		:	Elliptic
Size	(Length)	:	12.5 – (11.5) – 10.5 cm
	(Width)	:	8.5 – (7.4) – 6.3 cm
Colour		:	Dark green
Venation		:	Reticulate
Margin		:	Entire
Apex		:	Mucronate
Base		:	Symmetrical
Surface		:	Hairy (not glabrous), pubescent (covered with short, soft straight hairs).

c) Petiole

Size	:	3.5 – (3.1) – 2.7 cm
Surface	:	Dark green and Grooved

d) Sensory Characters

Colour	:	Green
Odour	:	Characteristic smell
Taste	:	Bland
Texture	:	Papery, thin and pliable

4.2.2 Microscopical Examination of *C. capitatum* leaves (Appendix I)

a) Description of Leaf Upper Epidermal characteristic

Epidermal Cells	:	polygonal in shape
Anticlinal walls	:	slightly wavy
Stomata	:	present
Types	:	anomocytic

Frequency	:	many and evenly distributed
Trichome	:	present
Types	:	multicellular, Covering trichome 3 – 9 celled
Frequency	:	Many

b) Description of Leaf Lower Epidermal characteristic

Epidermal Cells	:	polygonal in shape
Anticlinal walls	:	Straight
Stomata	:	present
Types	:	anomocytic
Frequency	:	few
Trichome	:	present
Types	:	multicellular, Covering trichome 3 – 9 celled
Frequency	:	Many

c) Transverse and Longitudinal Sections of *C. capitatum* leaf

The transverse sections of the plant leaf through the midrib tissue and upper and lower surfaces were observed. (Appendix I)

4.2.3 Leaf powder examination

Colour:	pale Green
Odour:	Characteristic smell
Taste:	Bland

4.2.4 Determination of Ash values, Extractives and Moisture Content

The determination of ash and moisture content of the powdered leave of *C. capitatum* was carried out and the following observations were obtained. (Appendix II - VII)

Table 4.1: The Ash values, extractives and Moisture Content

s/n	Physico-chemical parameter	Inference/values (%w/w)
1	Total Ash Value	12
2	Acid Insoluble Ash Value	4.83
3	Water Soluble Ash Value	2.5
4	Alcohol soluble extractive	5.0
5	Water soluble extractives	27.4
6	Moisture Content (loss on Drying)	13

4.2.5 Preliminary Phytochemical Studies

The phytochemical studies carried out reveals the presence of carbohydrate, protein, alkaloids, amino acid, flavonoids, other phenolic compounds (appendix VIII).

4.2.5.1 Preliminary Phytochemical studies using Thin Layer Chromatography

The preliminary phytochemical screening conducted on TLC plate revealed the presence of spots of the following phytoconstituents: amino acids, amines and amino-sugars, alkaloids and other nitrogen-containing compound, phenols and phenolic compounds, sterols (cholesterol and esters), steroids and triterpene glycosides, and flavonoids. (see Appendix IX for plates).

4.3 Phytochemistry of aqueous methanol extract of *C. capitatum* leaves

This section entails results of extract and fraction preparation, TLC, column chromatography, isolation and structural characterization.

4.3.1 Extraction yield of *C. capitatum* leaves with aqueous Methanol

The aqueous methanol extract was dried to a brown gummy residue after maceration with 70 : 30 methanol:water and evaporated to obtain the following results.

Weight of powdered leave = 500 g

Weight of total extract = 97 g

Percentage yield = $\frac{97 \times 100}{500}$ = 19.4% w/w

4.3.2 Fractions of the aqueous methanol extract of *C. capitatum* leaves

The methanol extract portion (50 g) after partitioning successively with n – hexane, ethylacetate and n-butanol, the ethylacetate and the n-butanol partitioned fractions were collected and concentrated at room temperature and using rotator evaporator respectively.

These were coded EF (1.2 g) and NF (39.5 g) respectively. The N- hexane (HF; 4.7 g) was concentrated at room temperature likewise.

Table 4.2 Fractions of aqueous Methanol extract of *C. capitatum* leaves

S/N	Fractions	Nature of Fractions	Colour of Fraction	Weight	% yield (%w/w)
1.	NF	Solid Sticky	Greenish Brown	39.5 g	79.0
2.	EF	Solid	Yellowish brown	1.2 g	2.4
3.	HF	Semi-Solid	Dark brown	4.7 g	9.4

4.3.3 Chromatograms of EF of *C. capitatum*

Chromatographic analysis of the ethyl acetate fraction that was carried out on TLC plate (Silica gel) using Ethylacetate-Methanol-Acetic acid (14:3:1) and sprayed with the following spray reagents. The chromatograms are presented on plates IV and V.

(IV)  (a)

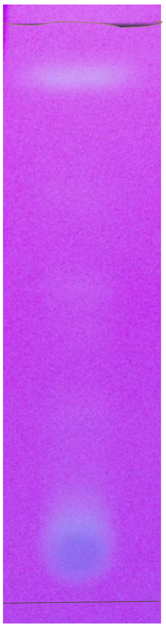
(V)  b)

Plate IV and V: TLC profile of *C. capitatum* ethyl acetate fraction (EF) with Solvent System Ethylacetate-Methanol-Acetic acid (14:3:1) and detected with a) 10% sulphuric acid in ethanol and b) UV lamp at 366 nm.

4.3.4 Column Chromatography of Ethyl Acetate Fraction (EF)

Fractionation of the Ethyl acetate fraction by column provided 100 Fractions of 20ml aliquots which were collected and concentrated in small volumes and rescreened using TLC. Similar fractions were pooled together, labelled CF1 – CF9. These were labelled CF1 (1 – 20), CF2 (21 – 27), CF3 (28 – 40), CF4 (41 – 50), CF5 (51 – 65), CF6 (66 – 72), CF7 (73 – 84), CF8 (85 – 89), and CF9 (90 – 100).

Table 4.3 Column Chromatography of ethylacetate fraction (EF)

S/N	Column fractions	Solvent ratio for elution (EA:M)	Approximate volume gradient
1.	CF1 (1 – 20)	100: 0	300 ml
2.	CF2 (21 – 27)	95: 5	300 ml
3.	CF3 (28 – 40)	90 : 10	300 ml
4.	CF4 (41 – 50)	85 : 15	300 ml
5.	CF5 (51 – 65)	80 : 20	300 ml
6.	CF6 (66 – 72)	75 : 25	300 ml
7.	CF7 (73 – 84)	70: 30	300 ml
8.	CF8 (85 – 89)	65 : 35	300 ml
9.	CF9 (90 – 100)	60 : 40	300 ml

4.3.5 Isolation and chromatograms of compound D from Ethyl Acetate Fraction (EF)

From the bulk fractions above, **CF6** (137 mg) was further eluted and purified on a smaller column using 10 g of silica gel 60 – 120 mesh size to afford a single spot, compound D (10 mg) with R_f value of 0.6 in Ethylacetate-methanol-acetic acid (14:3:1). Chromatographic analysis of the Compound D was carried out on TLC plate (Silica gel) using different solvent systems and spray reagents. It gave a yellow coloured spot on spraying with 10% sulphuric acid in ethanol followed by heating in an oven for few seconds and a blue black colour when sprayed with ferric chloride solution. Subsequent fractions gave mixtures and multiple spots of minor constituents and were not further investigated. The chromatograms for compound D are presented on plates VI and VII.

(VI) a)

(VII) b)

Plate VI and VII: TLC profile of Compound D from CF6 with Solvent System Ethyl acetate- Methanol - Acetic acid (14:3:1) and detected with (a) 10% sulphuric acid in ethanol and (b) Ferric chloride solution.

δδ δδ δ

4.4 Spectroscopic Analysis

4.4.1 Structure elucidation

Table 4.4: Physical properties of isolated compound

Compound	Weight (mg)	Melting Point	Physical Appearance
D	10 mg	>300	Yellow amorphous powder

4.4.2 Nuclear Magnetic Resonance Spectroscopy (NMR)

The interpretation of the NMR spectra of the compound was done by comparison with references obtained from literature.

4.4.3 Proton NMR of Compound D

Characteristic signals in the ^{1}H-NMR of Compound D include 6.19 (d, H-6), 6.38 (d, H2-8), 6.86 (d, H5; 116.2) and 8.0 (d, 8.9, H-6; 122.8 ppm). The spectrum showed signal 5.4 (1H, d) which is suggestive of a monoglycoside moiety with an alpha linked sugar. Other signals are seen in Fig. 4.1 and Table 4.5 (Ahmadu *et al.,* 2007).

4.4.4 Carbon NMR of Compound D

Several signals were observed in the spectrum. They include (116.2, 122.8, 179.6, 18.3, 105.8 ppm). Other signals were observed between (66.7- 77.1 ppm) which are suggestive of a pentose sugar. This is presented in Fig. 4.2 and Table 4.5 (Ahmadu *et al.,* 2007).

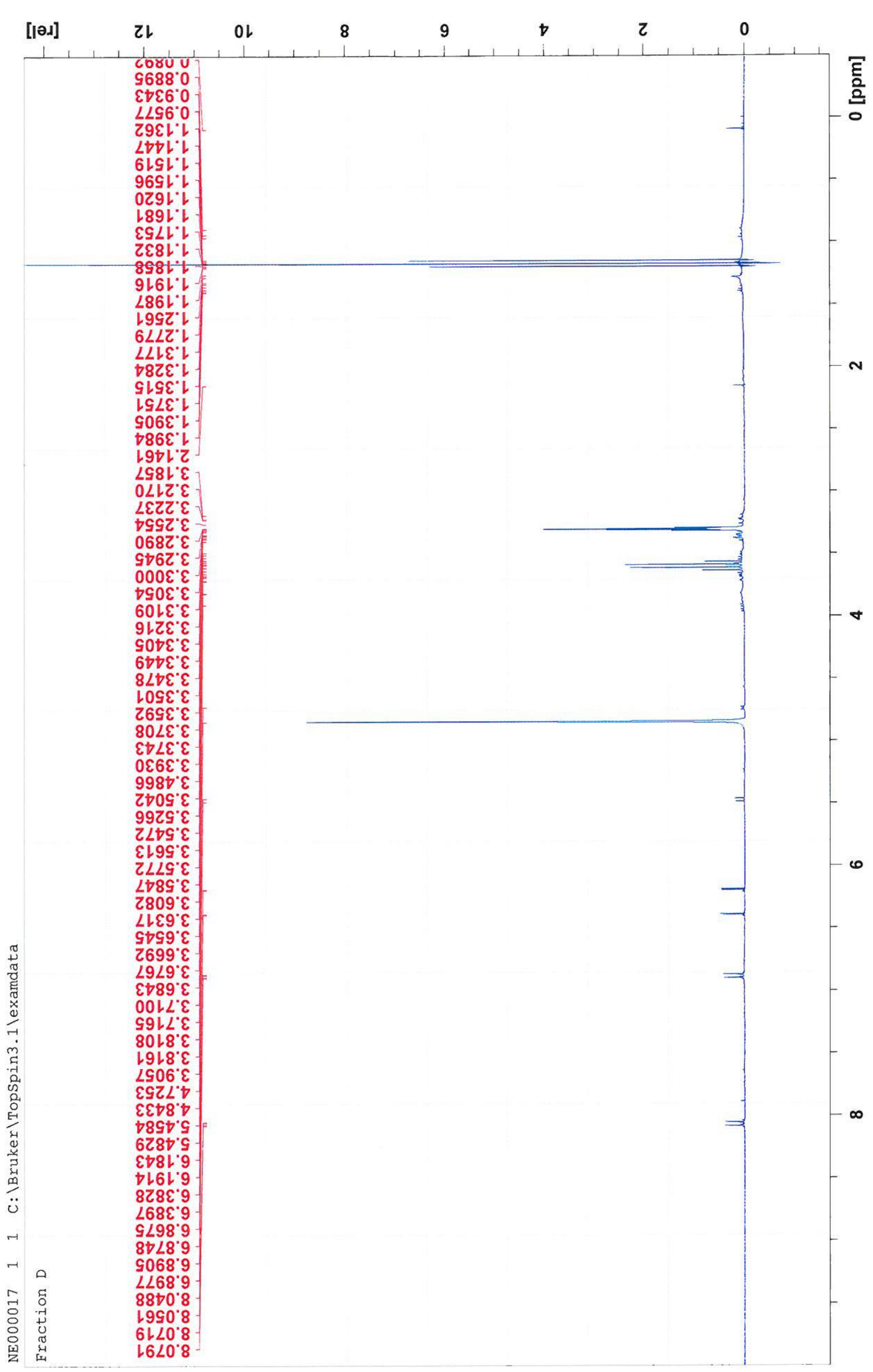


Fig. 4.1: ^{1}H Spectrum of Compound D in CD_3OD

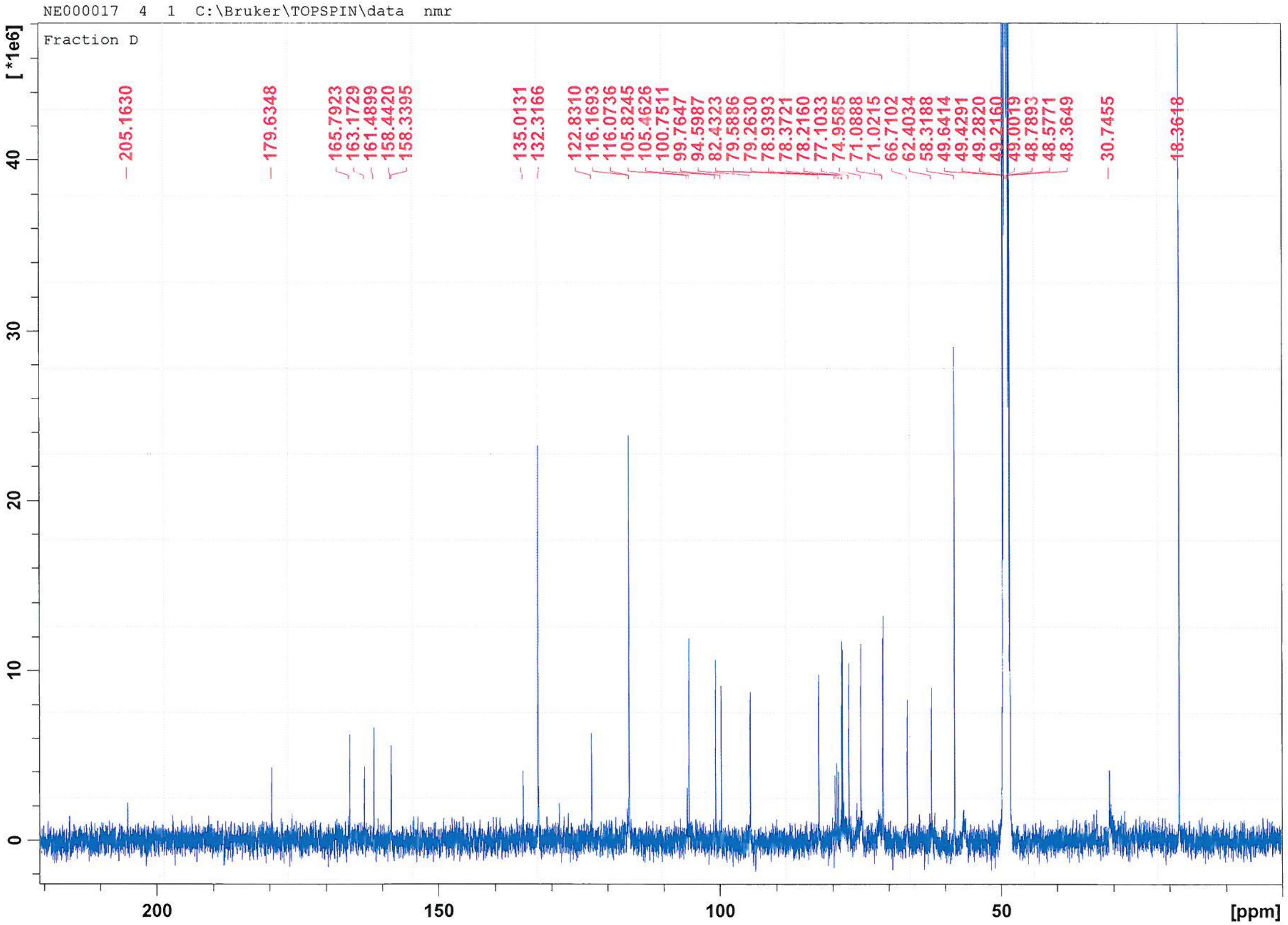


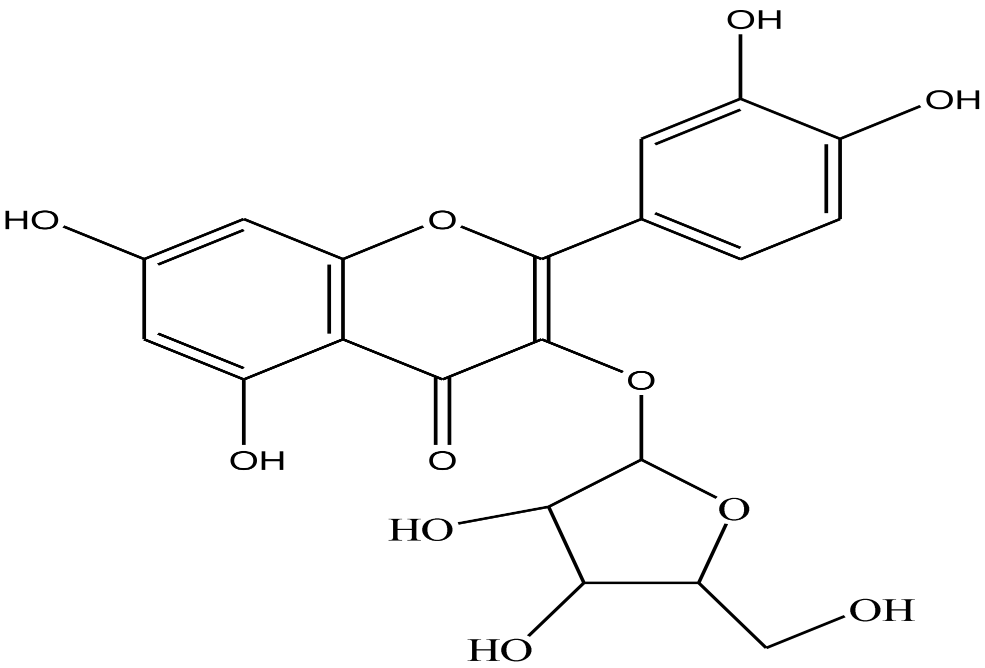
Fig. 4.2: ^{13}C **Spectrum of Compound D in** CD_3OD **100MHz**

Table 4.5: ^{1}H-NMR and ^{13}C-NMR Chemical shifts of compound D

Position C/H	δ^{a}_{H}	δ^{a}_{C}	$\delta_{H\ (Compound\ D)}$	$\delta_{C\ (Compound\ D)}$
2		158.7		158.4
3		135.7		135.0
4		179.5		179.6
5		163.2		163.2
6	6.20(d, 1.6Hz)	100.1	6.19(d)	100.7
7		166.8		165.8
8	6.39(d, 1.2Hz)	94.9	6.39 (d)	94.6
9		158.6		158.4
10		105.7		105.8
1'		123.0		122.8
2'	7.74(d, 1.5Hz)	117.6	8.04 (d)	116.2
3'		146.1		135.0
4'		150.1		158.3
5'	6.88(d, 8.5Hz)	116.3	6.88(d)	116.2
6'	7.58(d,d, 2.0Hz, 8.0Hz)	123.2	8.02 (d.d)	122.8
α-arabinoside	5.17 (d, 3.5Hz)	104.8	5.45 (d)	105.8
1"		73.0		74.9
2"		69.3		66.7
3"	3.62–3.65(d,d, 1.5, 7Hz)	74.3	3.62–3.65(d,d)	74.9
4"	3.46–3.48 (d, J = 7.0Hz)	67.1	3.46–3.48 (d)	66.7
5"				

(Lozoya *et al.,1994 and Ahmadu et al.,* 2007)

4.4.5 Proposed Structure of Compound D



$C_{20}H_{18}O_{11}$
Mol. Wt.: 434.35

Fig 4.3: Structure of Quercetin 3-O-α-arabinoside:

3-(3,4-Dihydroxy-5-hydroxymethyl-tetrahydro-furan-2-yloxy)-2-(3,4-dihydroxy-phenyl)-5,7-dihydroxy-chromen-4-one.

4.4.5.1 Proposed Structure of Compound D (numbered)

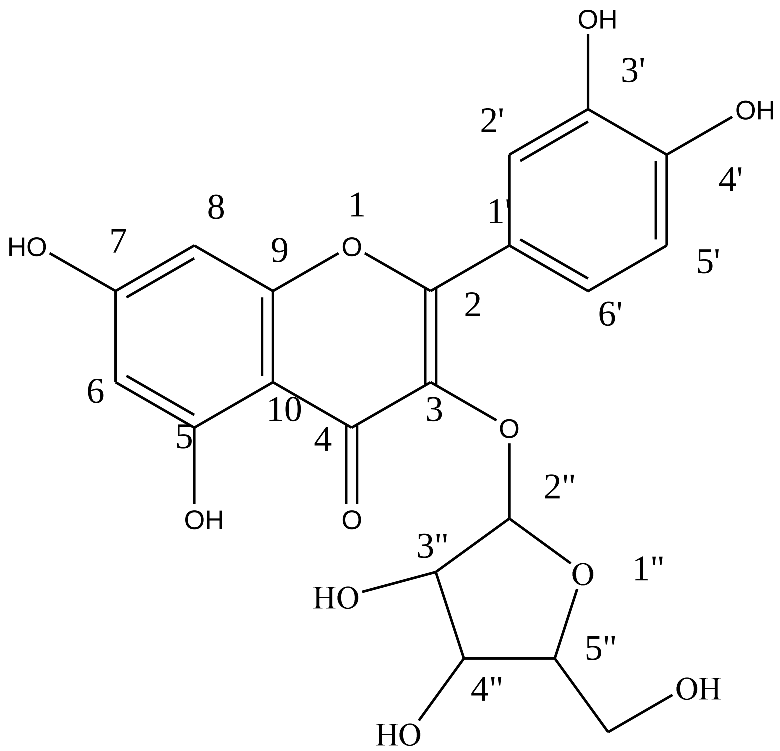


Fig. 4.4: Proposed structure of compound D

3-(3”,4”-Dihydroxy-5”-hydroxymethyl-tetrahydro-furan-2”-yloxy)-2-(3’,4’-dihydroxy-phenyl)-5,7-dihydroxy-chromen-4-one.

4.5 Biological studies of the aqueous Methanol extract

4.5.1 Median lethal dose determination (LD_{50}) of extract of *C. capitatum*

This involves acute toxicity testing using two different routes: Intraperitoneal (i.p) and oral. The (i.p) route was used in Lorke's method and oral in Karber's arithmetic method.

4.5.1.1 Acute toxicity testing of using Lorke's Method

The acute toxicity test showed no death in the initial phase where 3 groups of three mice each were treated with the leaf extract at a log dose of 10, 100 and 1000 mg/kg body weight i.p. but mild signs of toxicity like excitement, restlessness, lack of appetite and later reduced activity during the first 6-7 hours of extract administration. These subsided subsequently. In the second phase, dose of 1200, 1600, 2900 and 5000 mg/kg body weight i.p were injected and only one mouse died at 5000 mg/kg body weight. The LD_{50} was calculated as 3807 mg/kg body weight. See appendix X for table of result and formular.

4.5.1.2 Acute toxicity testing using Arithmetic method of karber

There was no death in all the treatment groups except in group E (4800 mg/kg) of which one mouse died out of the three. The LD_{50} was calculated to be 4400 mg/kg. This was higher than the Lorke's. See appendix XI for formular and table of results.

4.5.2 Minimum Lethal Dose (MLD) LD99 of the crude venoms of *Naja nigricollis*

The Minimum Lethal Dose (MLD) for crude venom of *Naja nigricollis* that was used is 9.55mg/kg as reported by Abubakar *et al.,* (2000).

4.6 Invitro Studies of the effects of extracts of *Clerodendrum* in the treatment of *Naja nigricollis* envenomation using Frog rectus abdominis model

The study carried out using frog rectus abdominis muscle model reveal the following results.

4.6.1 Determination of the Optimum Concentration (O.C) of acetylcholine

The optimum concentration required to cause contraction of the isolated frog rectus abdominis muscle optimally was chosen as 0.004 mg/ml. (i.e 0.4 ml of 0.01 mg/ml Ach and equivalent to 0.0004 mg/ml organ bath concentration). This was 3 cm $\pm$ 0.05 from the base line as a result of three trials. The responses are represented in the figure below.

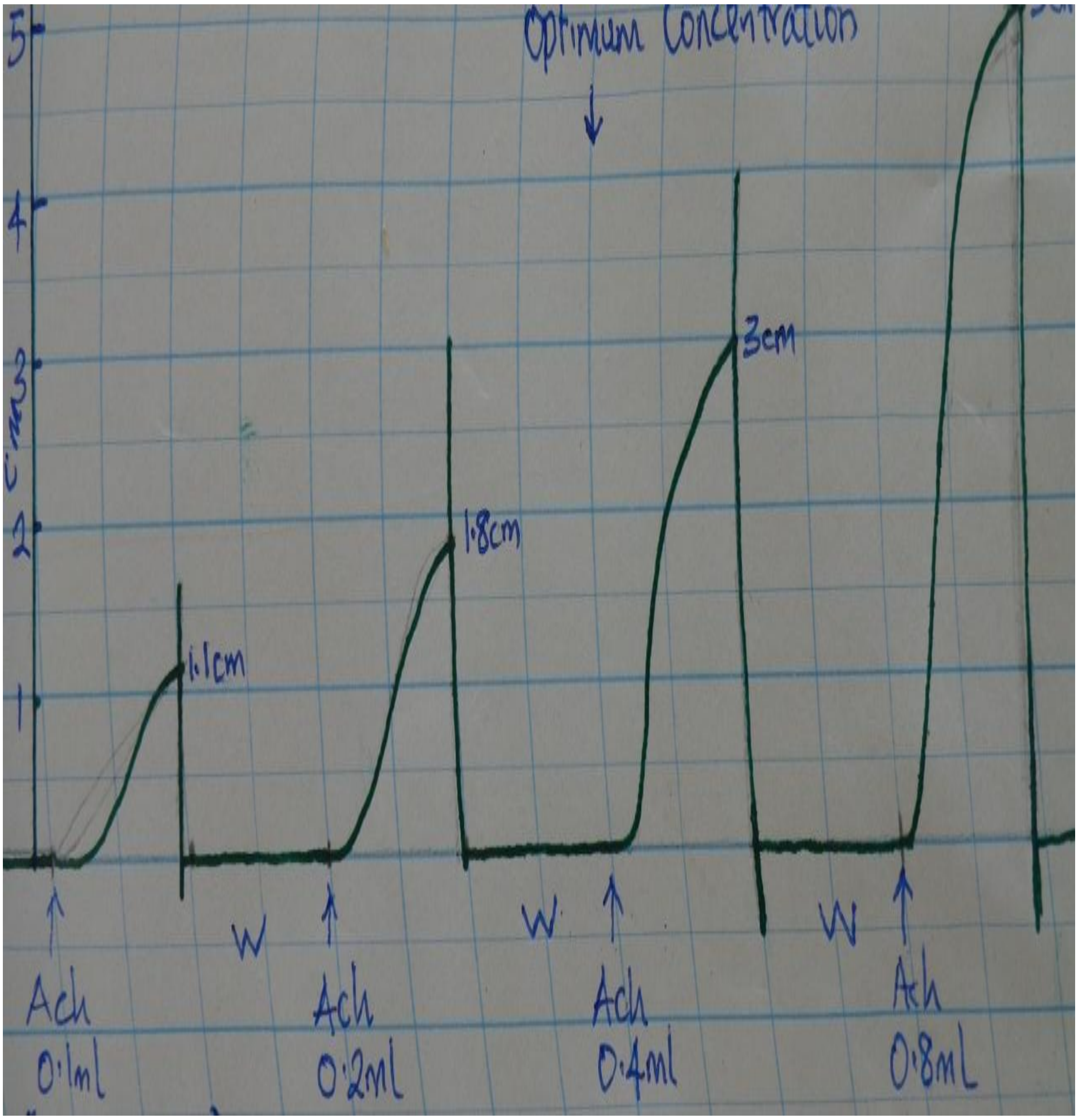


Fig. 4.5 Response of acetylcholine to frog rectus abdominis muscles.

4.6.2 Determination of the median inhibitory concentration IC_{50} of *Naja nigricollis* Venom against acetylcholine induced contractions.

The median inhibitory concentration (IC_{50}) which is a measure of the effectiveness of the snake venom in inhibiting acetylcholine induced contraction in frog rectus abdominis muscle by half was determined based on the dose response data generated. After several trial with different stock concentration of venom, 0.001 mg/ml gave a better representation of the inhibition from this the IC_{50} was calculated. A linear regression plot (fig. 4.7) was made with the % inhibition (length in cm) on the y-axis and the concentration of the venom (0.001 mg/ml) on the x-axis. The point that gave the 50% inhibition was extrapolated to the x-axis thus giving the IC_{50} (0.0084 mg/mg). Below (fig 4.6) are the responses from the effect of *N. nigricollis* venom against acetylcholine induced contractions.

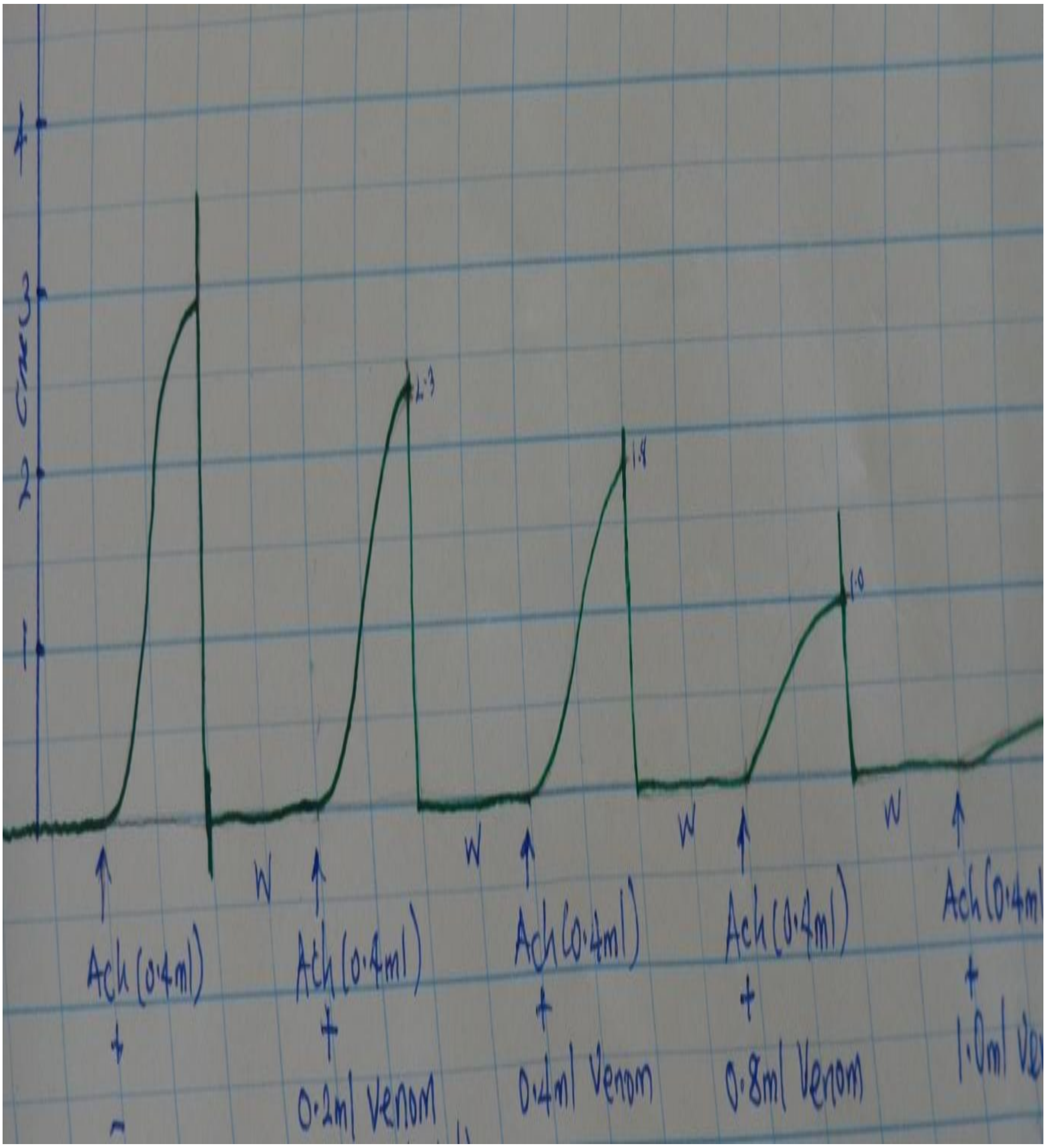


Fig 4.6: Effects of *N. nigricollis* venom on the contraction of isolated frog rectus abdominal muscles by acetylcholine

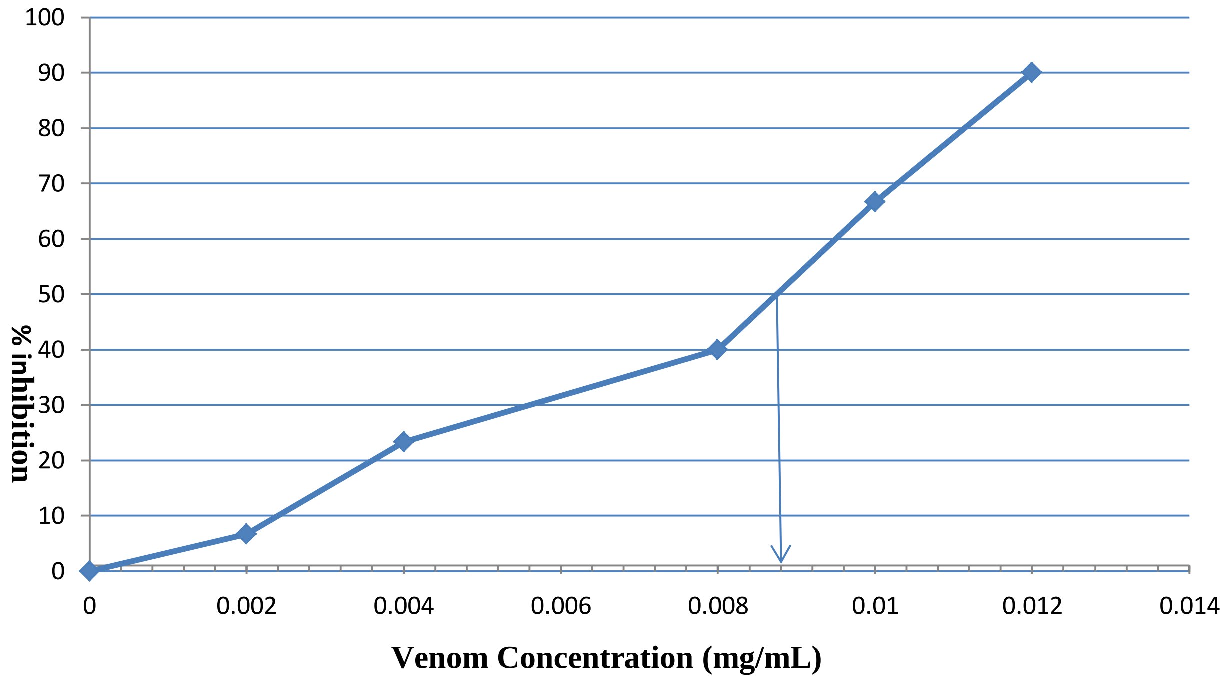


Fig. 4.7: **Median inhibitory concentration IC_{50} of *Naja nigricollis* venom against acetylcholine induced contractions (Appendix XII for table of values)**

4.6.3 Effects of *C. capitatum* on frog rectus abdominis muscle

The extract of *C. capitatum* did not show any significant change or response on the isolated muscles when varying concentrations of 0.1 mg/ml, 1 mg/ml, 10 mg/ml were used.

4.6.4 Determination of the effects of crude extracts of *C. capitatum* against Ach and *Naja Nigricollis*

There was no significant stimulation or restoration of the muscle contraction after the inhibitory effect of the snake venom (IC_{50}) was used against varying concentrations of the crude extract of *C. capitatum* 0.1mg/ml, 1mg/ml, 10mg/ml.

4.6.5 Invitro Venom detoxification by the extract of *C. capitatum*

All mice injected with *N. nigricollis* venom (9.55 mg/kg) alone died. However, mice injected with the venom and plant extract at concentration of 5 mg/ml (20%), 10 mg/ml (60%), 20 mg/ml (100%) survived. Showing there was venom detoxification by the extract of *C. capitatum* in a dose dependent manner. Table 4.6 below shows the effect of administration (i.p.) of incubated mixture of venom of *N. nigricollis* and extract of *C. capitatum* in adult albino mice.

Table 4.6: Effect of administration (i.p.) of incubated mixture of venom of *N. nigricollis* and extract of *C. capitatum* (T_5= 5 mg, T_{10}= 10 mg, and T_{20}=20 mg of extract) in adult albino mice

Group	Treatment	No. of deaths/no. of mice used	Aver. Time of death within 24hrs (hr)	Survival % (within 24 h)	Signs of neurotoxicity over 48 h
1	LD_{99}	5/5	0.5 $\pm$ 0.14	0	+++
2	$LD_{99}+T_5$	4/5	3.1 $\pm$ 0.93	20	++
3	$LD_{99}+T_{10}$	2/5	5.4 $\pm$ 3.49	60	+
4	$LD_{99}+T_{20}$	0/5	---	100	-
5	Distill water	0/5	---	100	-

The results were expressed as mean $\pm$ SEM of 5 animals (n=5). -, no signs of neurotoxicity, +, Difficulty in breathing; ++, difficulty in breathing and impaired movement; +++, severe respiratory impairment or coma.

4.6.6 Phospholipase A2 Assay of *C. capitatum*

4.6.6.1 Effects of extract of *C. capitatum* on Crude Phospholipase Enzyme.

The addition of the extract in varying concentration of 1.25 mg/ml, 2.5 mg/ml and 5 mg/ml to the reaction mixture showed a decrease in the Vmax of 1.28, 1.13, and 1.0 respectively. The Km for the reaction was calculated from the graph as 2.78 mg/mL (Fig. 4.8).

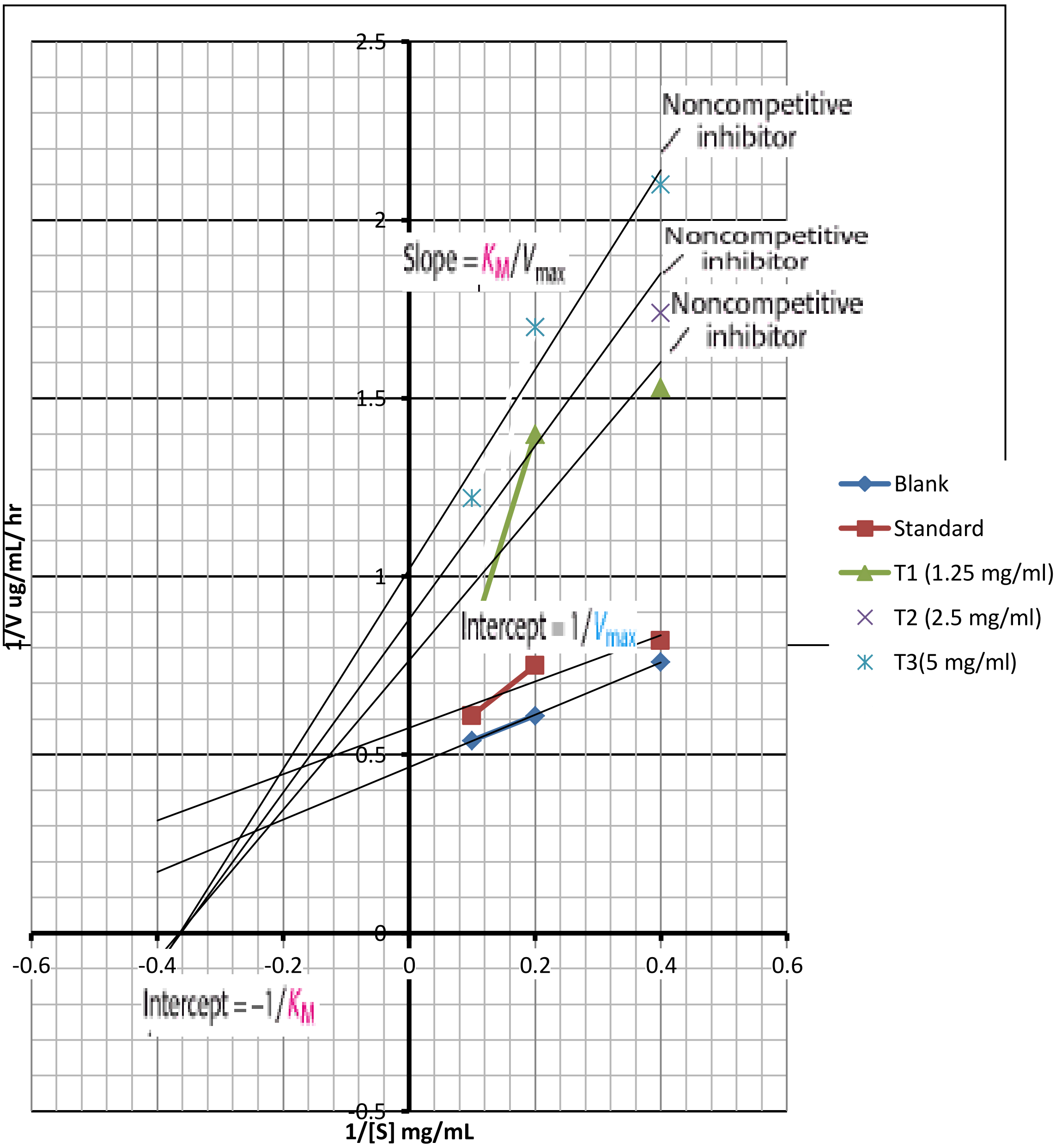


Fig. 4.8: **Double recprocal plot showing the effects of aqueous extract of *C. capitatum* on crude phospholipase enzyme (Appendix XIII).**

CHAPTER FIVE

5.0 DISCUSSION

5.1 Pharmacognostic Studies of the leaves of *C. capitatum*.

Herbal materials are categorized according to sensory, macroscopical and microscopic characteristics. An examination to determine these characteristics is the first step towards establishing the identity and the degree of purity of such materials and should be carried out before any further tests are undertaken. Wherever possible, authentic specimens of the material in question and samples of pharmacopoeial quality should be available to serve as a reference (WHO, 2011).

The result of the pharmacognostic studies on the plant leaves shows that the leaves are elliptic (shape of the ellipse; the leaf is long and almost equally expanded all through its length) with recticulate venation like the majority of dicotylendonous plant leaves.

The leaf margin is entire with a mucronate apex (abruptly tipped with a short point), hairy surface not glaborous and symmetrical base attached to a slender grooved dark green petiole. The leaves powder is pale green with a characteristic odour and bland taste.

The microscopical study of the leaf surface preparation reveal that the epidermal cells are polygonal with wavy anticlinal walls and smooth cuticle. The stomata is anomocytic and present both in the upper and lower epidermis. The presence of numerous trichomes were also observed in the upper and lower epidermal cell. There were few bicellular and many multicellular (3 – 9 celled), covering trichomes. These are strong diagnostic feature present in the leaf of *C. capitatum*.

The total ash of the leaf was found to be 12.0%, of which, acid insoluble ash was 4.83% and water soluble ash was 2.5%. The extractive values were found to be 5.0% and 27.4% for alcohol and water respectively. The moisture content was found to be 13%.

When a plant material is subjected to a high temperature, the organic matter is destroyed and the inorganic residue known as the ash or total ash is all that is left. The total ash represents the physiological ash and this consist of carbonates, chlorides, and silicates (African Pharmacopeia, 1985). The total ash obtained for *Dyschoriste perrottetii* leaf is 17.5% w/w (Musa, 2004), *Cannabis sativa* 15%, *Zingiber officinale* 5% and *Atropa belladonna* leaf 16% (African pharmacopeia, 1985).

The acid insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid and igniting the washed insoluble matter left on ash-less filter paper. The value of 4.83% w/w for *C. capitatum* leaf may be due to the presence of silica and siliceous earth materials in the plant drug. Some reported official acid insoluble ash value are; Capsicum not more than 0.15% w/w, euphorbia whole herb 3.0% w/w, cannabis leaf 5.0%w/w, Belladonna 3.0% w/w, Clove 0.75% w/w (African Pharmacopiea, 1985).

The water soluble ash was determined by calculating the differences between the total ash obtained and residue remaining after treatment of the total ash with water. The value obtained for some official drugs are: *Zingiber officinale* 1.7% w/w, *C. jamus* 4.23% w/w (Musa, 2004).

Extractive values of crude drugs are important for their evaluation. The determination is to measure the amount of constituents which are extractable by the solvent under specified condition. The result above for water and alcohol extractives 27.4% w/w and 5.0% w/w respectively shows that the water soluble is more than the alcohol. Thus, the use of water for this herb will be more beneficial with regards to its high extractive value. This is also used to check whether a crude drug have been used before.

Loss on drying is the loss of mass expressed as per cent w/w. The test for loss on drying determines both water and volatile matter in the crude drug. Moisture is an inevitable component of crude, which must be eliminated as far as possible. The determination of the moisture content is equally very important especially during storage. High percentage may activate enzymes that can catalyse the breakdown of medicinally active chemical compounds. In addition, also susceptibility to microorganisms and insect pest can be encouraged by high moisture content (Musa, 2004). Studies on physico-chemical constants can serve as a valuable source of information and provide suitable standards to determine the quality of this plant.

The preliminary phytochemical screening using test tube and thin layer chromatography showed the presence of flavonoids, phenols, saponins, alkaloids, and carbohydrates. These are preliminary test conducted to ascertain the presence of these phytoconstituents which can serve as a guide in isolation and purification.

5.2 Phytochemical and spectroscopical studies

5.2.1 Characterization of compound isolated from *C. capitatum* leaf

Many flavonoid aglycones, in particular isoflavone and highly methylated flavones are soluble in the commonly used solvent deuterated chloroform. However, most naturally occurring flavonoids including flavonoid glycosides have low solubility in deuterated chloroform (Mabry, 1969). The low solubility of compound D probably indicates that it is a glycoside. The H6 and H8 protons of flavones, flavonols and isoflavones which contain 5,7-dihydroxy substitution pattern give rise to doublets in the range of 6-0 – 6.5 ppm (H6 consistently occurred at a higher field than the H8) signal (Mabry, 1969) compared with flavonone with signal at 5.95 ppm. The spectrum for Compound D showed proton signals at H6 (6.19ppm) and H8 (6.39 ppm) a range for flavones, flavonols and flavones glycoside (Mabry, *et al.,* 1970). For flavonones and dihydroxy flavonol with 5,7 dihydroxy substitutions the signals for the A ring appeared at a higher field than corresponding flavones and flavonols (Mabry, 1969). Thus, Compound D is likely to be a flavonol or its glycoside when compared with literature cited.

In ^{13}C NMR, the electron density influences the chemical shift values of flavonoid nuclear carbon. Thus, carbon with relatively low electron density such as carbonyl and oxygenated aromatic carbon resonate at downfield (130 – 185 ppm) whereas hydrocarbons and other aliphatic carbon are generally represented by signal in the range of 0 – 110 ppm. The signal assignment for the carbon atoms of the aglycone moiety of compound D was based on previous works of (Ahmadu *et al.,* 2007; Lozoya *et al.*, 1994; Markham, 1978).

Compound D was isolated from the ethyl acetate fraction as a yellow amorphous powder by repeated gravitational coloumn chromatography on silica gel stationary phases. The chemical shifts were referenced to TMS at 0 for both proton and carbon. The solvent residual peak was 3.31 ppm for proton and 49.0 ppm for carbon. The ^{1}H NMR spectrum in deuterated methanol (CD_3OD) showed signals for quercetin, δ_H 6.19 (d, H-6), 6.39 (d, H-8), 6.88 (d, H-5'), 8.02 (d, H-6') and 8.04 (d, H-2').

The ^{13}C NMR 100MHz, in ppm, (methanol –d4) shows signals, δ_C ppm 122.8 (C-1'), 116.2 (C-2'), 116.2 (C-5'), 122.8 (C-6'), 158.4 (C-2), 135.0 (C-3), 179.6 (C-4), 163.2 (C-5), 100.7 (C-6), 165.8 (C-7), 94.6 (C-8), 158.4 (C-9) and 105.8 (C-10) correlated with literature for 3,5,7,3'4'- pentahydroxyflavone (Ahmadu *et al.,* 2007).

The ^{1}H NMR (CD_3OD) 5.45 (1H, d) suggest a monoglycoside moiety with an alpha linked sugar. The presence of 5 carbons in the region of the ^{13}C NMR spectra (66 – 77 ppm) suggest a pentose sugar probably arabinoside (Young *et al.*, 1996 and Ahmadu *et al.*, 2007).

The ^{1}H-NMR δ_H (CD_3OD) 6.19 and 6.39 are due to metacoupled protons of A ring (H-6 and H-8) of a flavonoid nucleus; signals at δ_H 6.88 (d), 8.04 (d) and 8.02 (d) are assigned to H-5',H-2'and H-6' of ring B. These suggest a quercetin nucleus (Mabry *et al.*, 1970; Markham *et al*, 1978 and Ahmadu *et al.,* 2007).

The ^{13}C NMR spectra (fig. 4.2) revealed 20 carbon signals typical of flavonoids monoglycoside nucleus (Ahmadu et al., 2007). However, other signals may be impurities. The low field signals at δC 179.6 ppm was due to the carbonyl group at C-4. The anomeric carbon of arabinoside appeared at δC 105.8. The spectra compares well with that reported in literature (Ahmadu *et al.,* 2007). Thus compound D was suggested to be Quercetin – 3- O-α-arabinoside. This is being reported for the first time from this plant. Abhijit and Jitendra (2012) have reported quercetin to have antivenom activity and (Fattepur and Gawade, 2007) reported that flavonoids are also responsible for antivenom activity. Quercetin and several of its glycosides are the most often encountered flavonoids in anti-snake venom plants where *Albizia lebbeck, Achillea millefolium, Euphorbia hirta, Camellia sinensis,* and *Casearia sylvestris* are some examples (Fattepur and Gawade, 2007).

5.3 Biological studies of extract of *C. capitatum*

5.3.1 Median lethal dose determination (LD_{50}) of crude extract of *C. capitatum*

Acute toxicity test gives clues on the range of doses that could be toxic to the animal; it could also be used to estimate the therapeutic index (LD_{50}/ED_{50}) of drugs and xenobiotics (Rang *et al.,* 2001).

The results of the acute toxicity of the methanolic extract of the leaves of *C. capitatum* showed an LD_{50} of 3,807 mg/Kg body weight in mice using Lorkes method via the intraperitoneal route and 4,400 $mgKg^{-1}$ body weight in mice using Karber's method via the oral route. The extract can therefore, be considered to be relatively safe (Lorke, 1983). According to the toxicity scale of Hodge and Sterner (1943), any compound with an oral LD_{50} of above 2000 mg/kg should be considered practically non toxic.

The result for the LD_{50} for the Karber's method is higher than that of the Lorke's method. This could be attributed to the fact that orally administered drugs and compounds usually undergo some events that potentially decrease the amount reaching systemic circulation for pharmacological effects (Brander *et al.*, 1991).

The results also indicate that the mice showed signs of toxicity such as restlessness, lack of appetite and later reduced activity, depression and unsteady movement. The manifestation of depression and lack of appetite observed in the mice may be linked to some chemical constituents present in the extract (Toma *et al.*, 2009).

5.3.2 Invitro studies of the effects of crude extract of *C. capitatum* in the treatment of *Naja nigricollis* envenomation using Frog rectus abdominis model

An isolated frog rectus abdominal muscles was placed in 10 mL glucose-Ringer solution (6.43 g of NaCl, 0.30 g of KCl, 0.17 g of $CaCl_2$, 0.10 g of $NaHCO_3$ and 0.71 g of glucose in 1.0 L of water), which was continuously bubbled with air. The contraction of the muscle was recorded by a transducer on a microdynamometer. In fig 4.5, the point marked Ach 0.4 ml, acetylcholine solution (0.4 mL) was added to the medium Ringer solution to give final concentrations 0.00004 mg/ml. At every interval between the curves, the muscle was washed three times with 10 mL of the Ringer solution and placed in 10 mL of fresh Ringer solution. The right and left muscles of a single individual frog were used as a pair. In fig 4.7, shows the inhibition by the crude venom from which the the IC_{50} was calculated.

Toxicity of *N. nigricollis* was demonstrated with isolated frog muscle. Acetylcholine-induced contracture (Fig. 4.5, optimal contraction) of frog rectus abdominal muscle (3 cm) was inhibited by the toxin (Fig. 4.6). These results clearly demonstrated that the toxin (crude venom) interfered with the binding of acetylcholine to the acetylcholine receptor (AChR) on the post synaptic membrane in a manner similar to that of curare, because they blocked neuromuscular transmission, and inhibited the muscle contraction induced by acetylcholine. The toxin was suggested to exert toxicity by binding at neuromuscular junction receptors and lead to curare- like paralysis thus blocking respiration.

The methanol leaf extract of *C. capitatum* in a concentration of 0.01 $mgmL^{-1}$, 0.1 $mgmL^{-1}$, 1 $mgmL^{-1}$ organ bath concentration, did not produce cholinergic effect and fail to block acetylcholine on the isolated muscles when it was interacted. In addition, the extract of *C. capitatum* did not show any contracting, relaxant or blocking effects when interacted with the combination of the optimal contraction induced by the Ach and the IC_{50} of the venom. This result is similar to previous studies where the methanol root extract of this plant was reported not to have this activity but shown to act via 5-hydroxytryptamine receptors on rabbit small intestine and rat uterus (Abdelwahab, *et al.*, 2008).

5.3.3 In vitro Venom detoxification by the extract of *C. capitatum*

The toxins of *Naja nigricollis* venom are composed of neurotoxin, cardiotoxin, enzymes and proteins. The victim may die of respiratory paralysis which is the major cause of death. The results of the in vitro venom detoxification showed that *C. capitatum* methanol leaf extract posses the ability to neutralize *N. nigricolis* venom with protection (60% and 100%) recorded in mices injected with 10 $mgmL^{-1}$ and 20 $mgmL^{-1}$ respectively. Many plant extracts have been reported to possess detoxifying effect on snake venoms (Haruna and Choudhury, 1995 and Abubakar *et al.*, 2000). In some of these plant extracts, the active constituents responsible for the antivenin effects have been identified and mechanisms of action suggested. The extract was observed to provide protection against the development of toxic signs and lesions due to envenomation.

It can be observed that it is not possible to be due to the extract acting through a mechanistic involvement rather a direct physical interaction with the venom in vitro. This is similar to the mode of action of many polyphenolic compounds found in plant extracts (Abubakar *et al.*, 2000). This claim was confirmed partially by the "protective" effects of plant extract when they are pre incubated with *N. nigricollis* venom before administration.

Therefore, to validate the above speculation(s) future work is necessary to isolate and examine such component(s) (Abubakar *et al*., 2000).

These findings reflect that the extract of *C. capitatum* plant may contain an endogenous inhibitor of venom. This obviously would need further investigations. A large number of antiophidian plants have been investigated in the search of new biologically active substances able to inhibit snake venom effects. Many studies however, were not sufficient to elucidate the mechanisms involved with this neutralizing activity. The active components, named secondary metabolites, generally act as enzymatic inhibitors, chemical inactivators or immunomodulators able to interact with the venom target macromolecules. The therapeutic potential of medicinal plants is traditionally attributed to classes of active constituents, including flavonoids, alkaloids, sesquiterpenes, lignans and others. These natural inhibitors can represent an additional aid in the traditional serum therapy and can be useful as molecular models for new drugs at a lower price, with lower side effects and easily distributed to communities far from medical access (Abubakar, *et al.*, 2000).

5.3.4 Phospholipases A2 assay of extract of *C. capitatum*

Phospholipases constitute a diverse subgroup of lipolytic enzymes that share the ability to hydrolyse one or more ester linkages in phospholipids, with phosphodiesterase as well as acyl hydrolase activity (Taghrid *et al.*, 2006).

The classification of phospholipases is based on their site of attack on the phospholipid. Qualifying letters, such as A, B, C and D, are used to differentiate the phospholipases and to indicate the specific bond targeted in the phospholipid molecule. Phospholipases A are acyl hydrolases classified according to their site of action upon the hydrolysis of the acyl ester bond. Phospholipase A1 (PLA_1) hydrolyses the fatty acyl ester bond at the sn-1 position of the glycerol moiety, while phospholipase A_2 (PLA_2) removes the fatty acid at the sn-2 position of this molecule. The action of PLA_1 and PLA_2 results in the accumulation of free fatty acids and 2-acyl lysophospholipid or 1-acyl lysophospholipid, respectively (Taghrid *et al.,* 2006).

The line weaver plot showed on varying concentration of the substrate, the results indicated that, the extract inhibited the phospholipase A_2 activity in a non competitive manner with a Km values of 2.7 $mgmL^{-1}$ and Vmax of 1.0 $mgmL^{-1}$, 1.13 and 1.28 $mgmL^{-1}$ for 5, 2.5 and 1.25 $mgmL^{-1}$ extract concentrations respectively as shown in (Fig. 4.8). Consequently, the decrease in Vmax may be due to inclusion of the plant extract in the substrate mixture suggesting that the plant leaves extract has some inhibitory effects on PLA_2.

In non competitive inhibition, binding of the inhibitor does not affect binding of substrate. Formation of both enzyme – inhibitor (EI) and enzyme – inhibitor - substrate (EIS) complexes is therefore possible. However, while the enzyme-inhibitor complex can still bind substrate, its efficiency at transforming substrate to product, reflected by *V*max, is decreased. Non-competitive inhibitors bind enzymes at sites distinct from the substrate- binding site and generally bear little or no structural resemblance to the substrate. For simple non-competitive inhibition, E and EI possess identical affinity for substrate, and the EIS complex generates product at a negligible rate (Robert, *et al.,* 2003).

Marcussi *et al.,* 2007 reported an interesting results that *Casearia sylvestris,* significantly inhibited the activity of PLA_2s isolated from *Bothrops*, *Crotalus* and *Micrurus* snake venoms. These plant species also showed inhibitory effects against the hemorrhagic activity induced by several *Bothrops* venoms. Belonging to the same genus, the aqueous extract from *Casearia mariquitensis* also showed neutralizing activity on hemorrhagic and hemostatic effects induced by neuwiedase, a metalloprotease isolated from *B. neuwiedi*.

Abubakar *et al*. (2006) reported that extracts of *Indigofera pulchra* and *Aristolochia albida* gave protection to mice treated with minimum lethal dose of venom with some gross pathologic symptoms of envenomation lessened. These plants were found to neutralize the anticoagulant, hemolytic and phospholipase activity of crude venom (Abubakar *et al*., 2006).

These investigations support the hypothesis that PLA_2 (from crude venom) have a specific site responsible for its enzymatic and pharmacological action observed. This inhibitor is likely to interact with the active site of PLA_2 to non-competitively inhibits its action on its natural substrate.

CHAPTER SIX

CONCLUSION, SUMMARY AND RECOMMENDATION

6.1 Conclusion

The pharmacognostic studies carried out revealed diagnostic features that could be used as first step towards establishing the identity and the degree of purity of *C. capitatum.* Fractionation of the methanol extract of *C. capitatum* led to the isolation of a known compound quercetin-3-O-α-arabinoside which was determined by spectroscopic methods as well as comparison with published data.

The acute toxicity testings carried out via the intraperitoneal route using Lorke's method and the oral route using Karber's arithmetic methods showed that the aqueous methanol leaf extract of *C. capitatum* is relatively safe.

The studies carried out using frog rectus abdominis muscle demonstrated that the extract does not have any cholinergic activity and failed to block acetylcholine effect. However, in the detoxification studies, the extract posses the ability to neutralize *N. nigricollis* venom with a 60% and 100% protection. This is likely due to the direct physical interaction with the venom toxins.

The phospholipase A_2 assay revealed that the extract upon varying concentrations of the substrate (egg yolk), inhibited the Phospholipase (crude venom of *Naja nigricollis*) in a non competitive manner. This might have been due to the binding of the extract molecules at specific sites on the phospholipase enzyme, thus, modifying the enzyme conformation.

The therapeutic potential of *C. capitatum* can be attributed to classes of active constituents, including flavonoids, alkaloids, tannins, phenolics and others. These natural inhibitors can represent an additional aid in the traditional serum therapy and can be useful as molecular models for new drugs at a lower price, with lower side effects and easily distributed to communities far from medical access.

6.2 Summary of Findings

C. capitatum is a tropical and sub-tropical plants used in traditional medical practice for the treatment of various disease conditions including snakebite poisoning. A flavonoid glycoside, quercetin-3-O-α-arabinoside was isolated and identified from the plant for the first time. The effects of *C. capitatum* extract on *Naja nigricollis* venom were investigated in animals and on isolated tissue. Findings from phytochemical screening indicate that the aqueous methanol extract of *C. capitatum* leaf contains carbohydrates, saponins, steroids, tannins and flavonoids. The leaf extract given to mice, shows that it is relatively safe and significantly reduced mortality in *Naja nigricollis* venom treated animals in detoxification studies. The extracts of the plants also exhibited phospholipase A_2 activities. The extract, however, did not exhibit Cholinergic nor any acetylcholine blocking effect in frog rectus abdominis muscle studies. The evidence that the extract of the plant can reduce morbidity and mortality in venom treated animals, suggests that the medicinal plants contain active principles that can be useful in the treatment of venom toxic effects and lethality arising from *Naja nigricollis* bite. The results of this study have therefore provided some scientific support for their use in the traditional management of snakebite patients.

6.3 Recommendations

It is undeniable that plants have played very crucial roles in the lives of humans for centuries. *C. capitatum* is used traditionally as aphrodisiac, antihypertensive, anti diabetic, and antioxidant. This research was conducted on the leaves of *C. capitatum* to authenticate these folkloric uses and to isolate the chemical constituents responsible for these actions. From the outcome of the study, the following recommendations are made to promote the use of *C. capitatum* as a medicinal plant.

In traditional medicine, the leaves of *C. capitatum is* used for the treatment of diabetes and this activity of the plant has been validated by Adeneye *et al*. (2008). In addition, this study has established the antivenom activity of methanol leaf extract of *C. capitatum.* Therefore, further studies should be done on the standardization and subsequent preparation of the plant extracts into appropriate and acceptable dosage forms that will aid the use of *C. capitatum* as an antivenom.

To the best of my knowledge, only one chemical constituent a phenyl propanoid (verbascoside) have been isolated from *C. capitatum*. Until now and for the first time quercetin-3-O-α-arabinoside has been identified from the leaves of *C. capitatum.* There is therefore the need for further investigations into the chemical constituents of the plant. This will unearth the medicinal potential of the plant and also provide a basis for its standardization as a drug.

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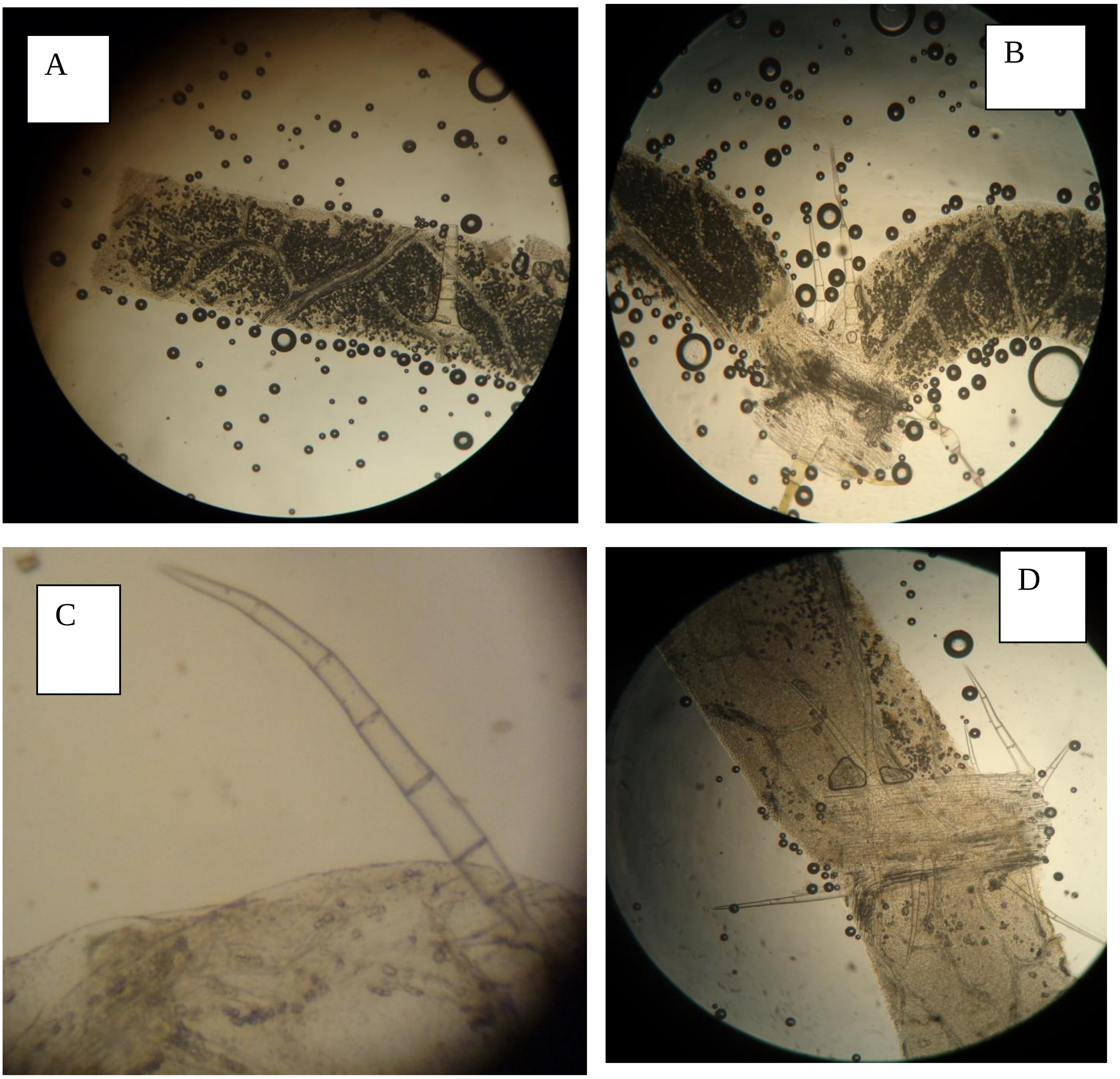
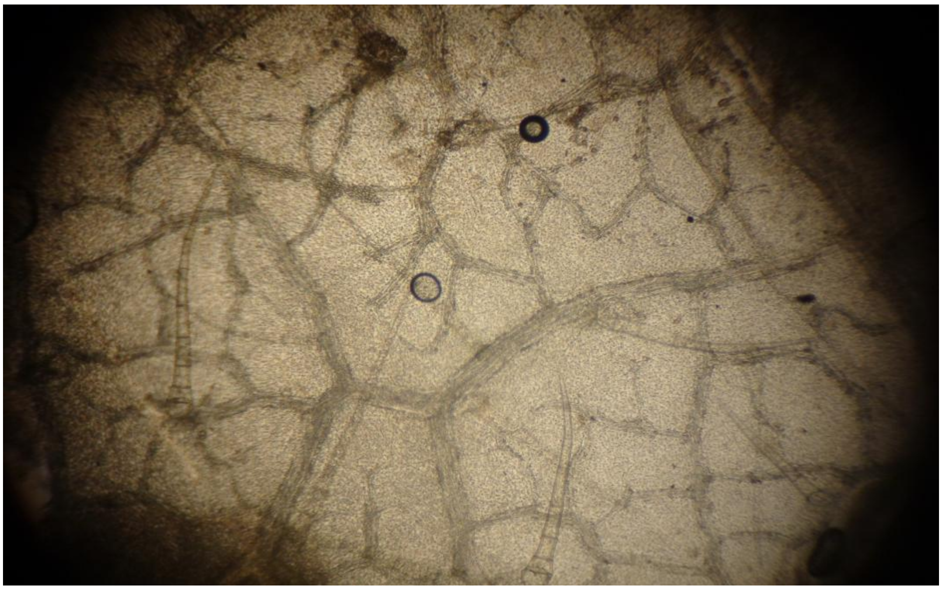
APPENDIX I





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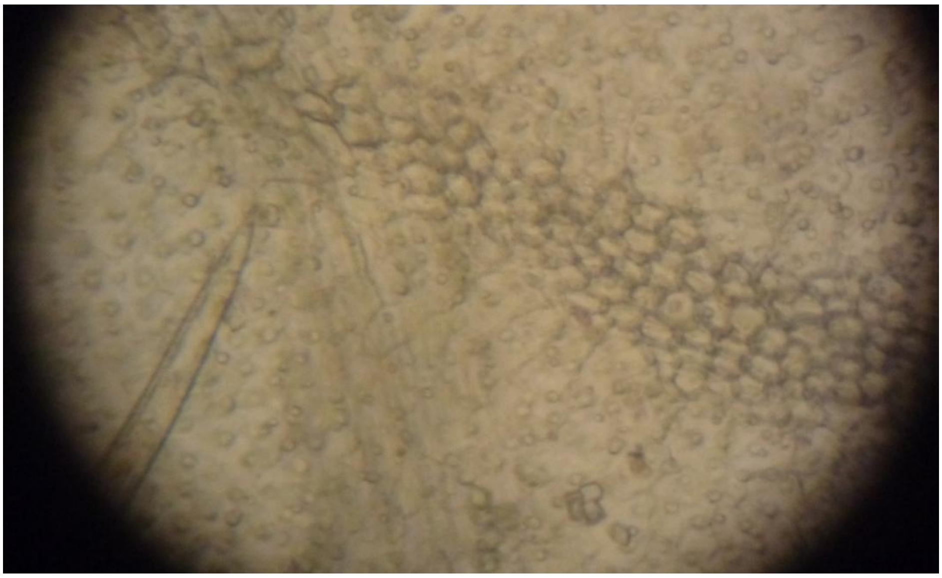

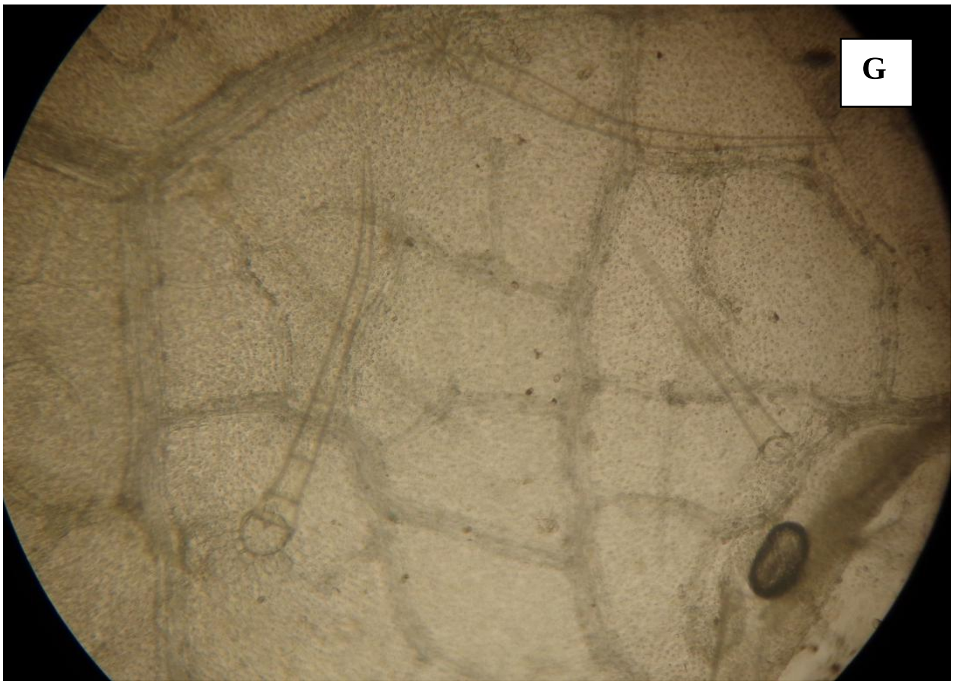

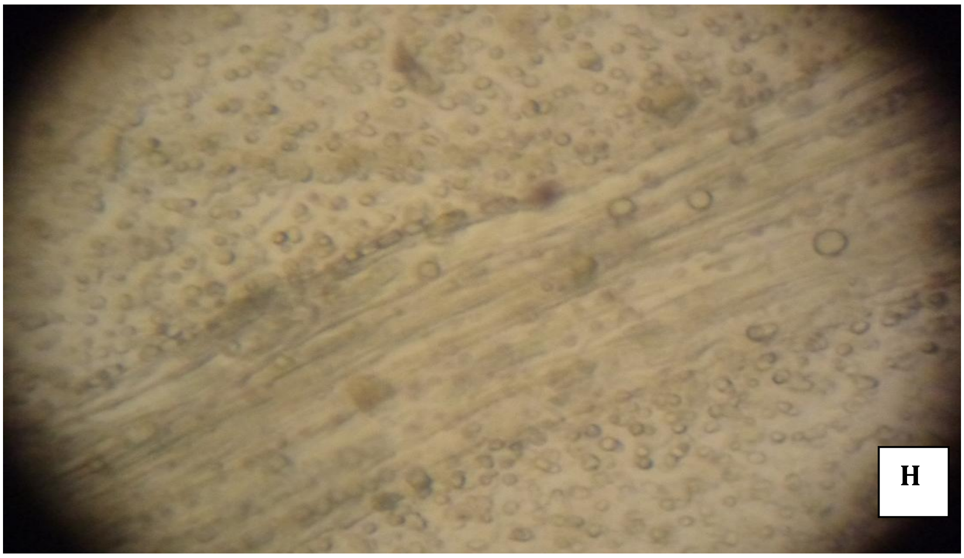
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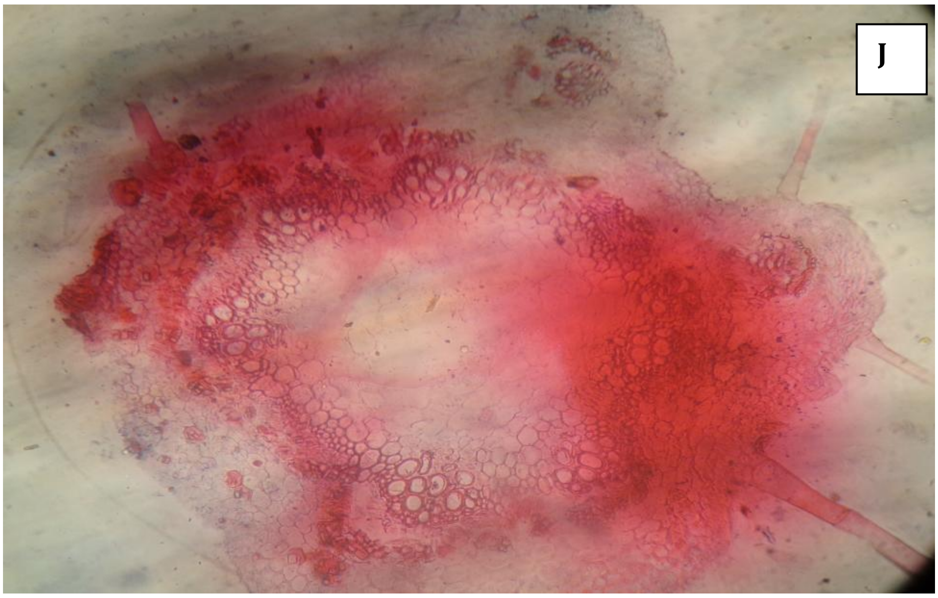

J

A. Slide of the Transverse Section through the Lamina of *C. capitatum* leaf. x100

B. Slide of the Transverse Section through the Midrib of *C. Capitatum* Leaf. x100

C. Slide showing a multicellular covering trichome on the upper epidermis. X1000

D. Slide showing a section of the lower epidermis X100

E. Slide showing a section of the upper epidermis x400

F. Slide showing secondary Cells x1000

G. Slide showing a larger view of the upper epidermis. X1000

H. Slide showing a larger view of the Conducting vessel. X1000

I. Slide showing the longitudinal section X400

J. Slide showing the transverse section through the midrib X400

APPENDIX II

Determination of some physical constant of the powdered leaf of *C. capitatum*

a) **Determination of Ash Value of powdered leaf of *C. capitatum***

Description	1	2	3
Constant weight of crucible (g)	24.08	24.57	24.25
Weight of crucible and content (g)	26.08	26.57	26.25
Weight of crucible and Ash (g)	24.32	24.99	24.51
Weight of Ash (g)	0.24	0.22	0.26
Ash Value (%)	12.00	11.00	13.00
Average mean (%)		12.00	

Sample calculation

$$\text{Ash value} = \frac{\text{weight of Ash}}{\text{Initial weight of drug}} \times 100$$

$$\text{Ash Value} = \frac{0.24 \times 100}{2\ g} = 12.00\ \%w/w$$

APPENDIX III

Contd of (a)

b) Determination of Acid insoluble Ash of powdered leaf of *C. capitatum*

Description	1	2	3
Constant weight of crucible (g)	24.08	24.57	24.25
Weight of crucible and Acid insoluble ash (g)	24.16	24.67	24.36
Weight of Acid insoluble ash (g)	0.08	0.10	0.11
Acid Insoluble Ash Value (%)	4.00	5.00	5.50
Average mean (%)		4.83	

Sample calculation

$$\text{Acid Insoluble Ash(AIA) value} = \frac{\text{weight of AIA}}{\text{Initial weight of drug}} \times 100$$

$$\text{AIA Value} = \frac{0.08 \times 100}{2\ g} = 4.00\ \%w/w$$

APPENDIX IV

Contd of (b)

c) **Determination of water soluble Ash of powdered leaf of *C. capitatum***

Description	1	2	3
Constant weight of crucible (g)	24.08	24.57	24.25
Weight of crucible and Ash (g)	24.28	24.79	24.46
Weight of Ash (g)	0.20	0.22	0.21
Weight of water Insoluble ash (g)	0.16	0.15	0.10
Weight of water soluble ash (g)	0.04	0.07	0.04
Water soluble Ash Value (%)	2.00	3.50	2.00
Average mean (%)		2.50	

Sample calculation

$$\text{Water soluble Ash(WSA) value} = \frac{\text{Wt of Ash - Wt of Water Insoluble Ash}}{\text{Initial weight of drug}} \times 100$$

$$\text{AIA Value} = \frac{(0.20 - 0.16) \times 100}{2\ g} = 2.00\ \%\text{w/w}$$

APPENDIX V

Determination of Extractive value of powdered leaf of *C. capitatum*

a) **Alcohol – Soluble Extractive value**

5 g of the powdered leaves was used in 100 ml of 90% ethanol

Description	1	2	3
Constant weight of crucible (g)	24.08	24.57	24.25
Weight of crucible and content before heating (g)	25.35	25.67	25.35
Weight of crucible and content after heating (g)	25.10	24.42	25.10
Alcohol extractive content (g)	0.25	0.25	0.25
Alcohol extractive Value (%)	5.00	5.00	5.00
Average mean (%)		5.00	

$$\text{Alcohol extractive val} = \frac{\text{Wt of cruc. \& content after heat (g) - Constant wt. of cruc. (g) X 100}}{\text{Initial weight of Drug.}}$$

APPENDIX VI

b) **Water – Soluble Extractive value**

5 g of the powdered leaves was used in 100 ml of 0.25% chlolroform water.

Description	1	2	3
Constant weight of crucible (g)	24.08	24.57	24.25
Weight of crucible and content before heating (g)	25.10	25.57	25.45
Weight of crucible and content after heating (g)	24.32	24.77	25.18
Water extractive content (g)	0.28	0.27	0.27
water extractive Value (%)	28.00	27.00	27.00
Average mean (%)		27.40	

$$\text{Water extractive val} = \frac{\text{Wt of cruc.\& content after heat (g) - Const wt. of cruc. (g) X 5 X 100}}{\text{Initial weight of Drug.}}$$

APPENDIX VII

Determination of moisture content of powdered leaf of *C. capitatum*

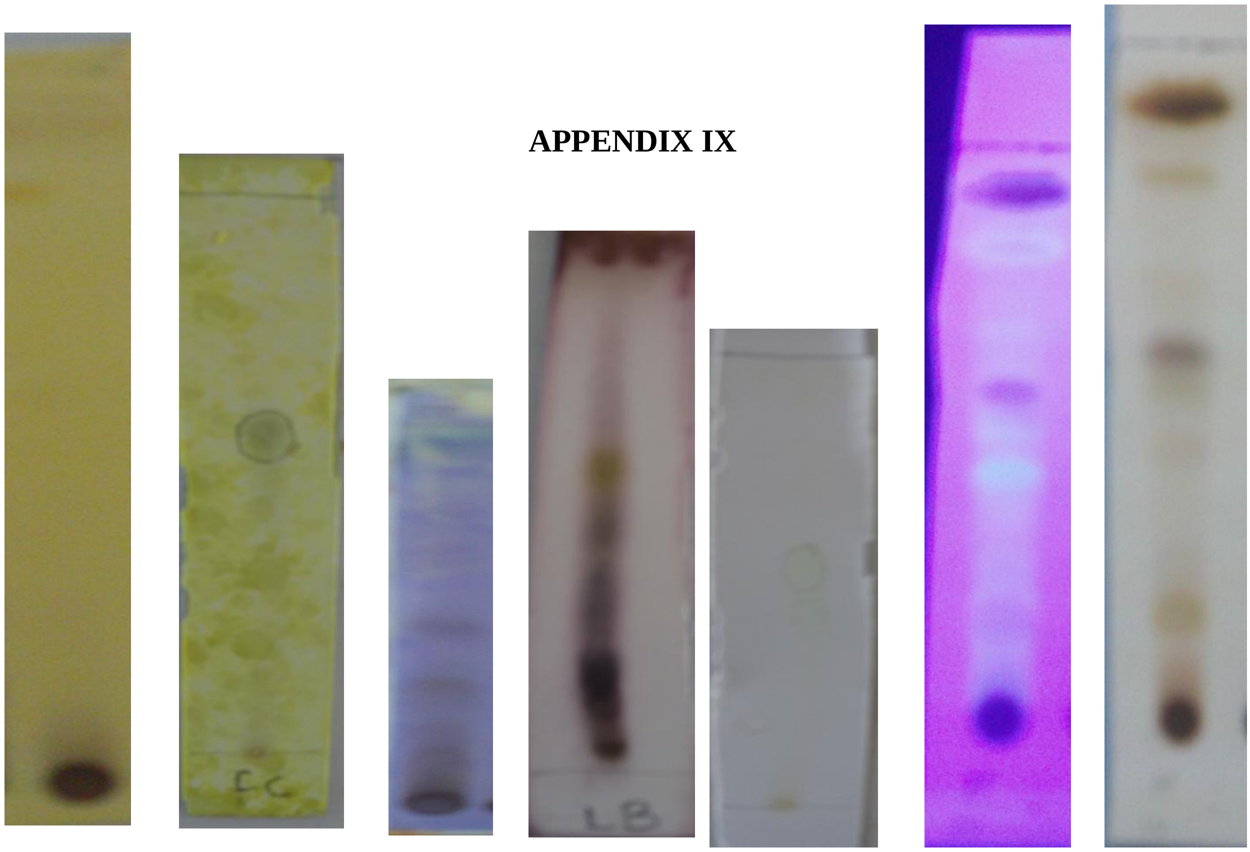
s/n Description	1	2	3
1. Constant weight of crucible (g)	24.08	24.57	24.25
2. Weight of crucible and powder before heating (g)	26.08	26.57	26.25
3. Weight of crucible and content after heating to constant weight (g)	25.80	26.33	25.99
4. Moisture content (g)	1.72	1.76	1.74
5. Loss on drying	0.28	0.24	0.26
6. (%) Loss on drying	14.00	12.00	13.00
Average mean (%)		13.00	

APPENDIX VIII

Results of Preliminary Phytochemical screening using test tubes

S/N	Phytoconstituents in aqueous Methanol Extract	Inference
1	**Carbohydrate**	**Present**
	Molish's test (General test):	**Present**
	Barfoed's test:	**Present**
2	**Tests for Proteins:**	
	Biuret test	**Present**
	Millon's test	**Present**
	Precipitation Test 5% $HgCl_2$ solution, 5% $CUSO_4$ solution, 5% lead acetate, 5% ammonium sulphate.	**Present**
3	**Amino acids**	
	Ninhydrin test (General test):	**Present**
4	**Tests for Alkaloids:**	
	Dragendorff's test:	**Present**
	Hager's test	**Present**
	Wagner's test	**Present**
5	**Flavonoids**	
	Shinoda test	**Present**
	NaOH test	**Present**
	Lead Acetate test	**Present**
	Ferric chloride test:	**Present**
6	**Tannins and phenol compounds**	
	5% $FeCl_3$ solution:	**Present**
	Lead acetate solution:	**Present**
	Bromine water	**Present**
	Acetic acid solution:	**Present**
	Dilute iodine solution:	**Present**

APPENDIX IX



IV **V** **VI** **VII** **VIII** **IX** **X**

Chromatographic analysis of aqeous methanolic leave extract of *C. capitatum* that was carried out on TLC plate (Silica gel) using appropriate solvent system and sprayed with the following spray reagents. The chromatograms are presented on plates IV - VIII

PlateIV: Chromatogram Sprayed with Dragendorff reagent shows absence of alkaloids and other nitrogen-containing compounds.

PlateV: Chromatogram Sprayed with Ferric chloride for presence of phenols and phenolic compounds

Plate VI: Chromatogram using Ninhydrin Spray for amino acid,

PlateVII: Showing spots using Acetic anhydride - sulfuric acid for Δ5-3-sterols (cholesterol and esters), steroids and triterpene glycosides (Liebermann-Burchard reagent)

PlateVIII: Spray using Aluminium chloride for flavonoids.

Plate IX: Chromatogram in UV light at 366.

Plate X: Chromatogram using 10% v/v Sulphuric acid as spray

APPENDIX X

Determination of Acute toxicity of extract of *C. capitatum* using Lorke's Method

Phase I

Group	Dose $mgKg^{-1}$	No. Of death/ No. Of Survival
1	10	0/3
2	100	0/3
3	1000	0/3

Phase II

Group	Dose $mgKg^{-1}$	No. Of death/ No. Of Survival
1	1200	0/1
2	1600	0/1
3	2900	0/1
4	5000	1/1

$LD_{50} = \sqrt{\quad}$

$= \sqrt{5000 \times 2900}$

$= \sqrt{14500000}$

$= 3,807.887 \text{ mg Kg}^{-1}$

APPENDIX XI

Determination of Acute toxicity of extract of *C. capitatum* using Karber's arithmetic method

LD_{50} of extract of *C.capitatum* calculated by arithmetic method of Karber

Group	Dose(mg)	Dose difference (Dd) mg	No. Of Dead (n)	Mean dead (Md)	Dd x Md
1	300		0,0,0	0	
2	600	300	0,0,0	0	0
3	1200	600	0,0,0	0	0
4	2400	1200	0,0,0	0	0
5	4800	2400	0,1,0	0.5	1200

$$LD_{50} = \frac{\text{Highest dose that killed the animals} - \text{Sum of (Dose difference} \times \text{Mean dose)}}{\text{No. Of group}}$$

$$LD_{50} = H.D - \frac{\sum(Dd \times Md)}{N}$$

LD_{50} = 4800 - 1200/5

LD_{50} = 4800 – 400

LD_{50} = 4400 mg/kg

APPENDIX XII

Determination of Inhibitory concentration IC_{50} of *N. nigricollis venom* against Acetyl choline induced contractions

Venom Concentration (mg/ml)	Lenght of inhibition (cm)	inhibition (cm)	% inhibition
0.002	2.8	0.2	6.67
0.004	2.3	0.7	23.33
0.008	1.8	1.2	40.00
0.01	1.0	2.0	66.67

Length of Optimum Contraction = 3 cm

From Linear regression plot, y = mx + b (m = gradient). Thus, at y = 50%, i.e 0.5,

IC_{50} = (0.5 – b)/ a where a and b = x axis and intercept respectively.

APPENDIX XIII

Determination of Phospholipase A2 Activity of *N. nigricollis venom* against *C. capitatum*

X-Values	Blank	Standard	T3 (1.25 mg/ml)	T2 (2.5 mg/ml)	T1 (5 mg/ml)
-0.6					
-0.5					
-0.4					
-0.3					
-0.2					
-0.1					
0					
0.1	0.54	0.61	0.83	0.88	1.22
0.2	0.61	0.73	1.1	1.3	1.6
0.3					
0.4	0.76	0.82	1.43	1.64	2.3