

**PHYTOCHEMICAL STUDIES AND EFFECT OF METHANOL LEAF EXTRACT OF  
*LEPTADENIA HASTATA* (PERS.) DECNE (ASCLEPIADACEAE) ON ACETIC ACID  
INDUCED WRITHES IN MICE AND VENOM OF *ECHIS OCELLATUS***

**By**

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**AHMADU BELLO UNIVERSITY, ZARIA**

**NIGERIA.**

**OCTOBER 2014**

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**DEPARTMENT OF PHARMACEUTICAL AND MEDICINAL CHEMISTRY,  
FACULTY OF PHARMACEUTICAL SCIENCES  
AHMADU BELLO UNIVERSITY, ZARIA**

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**OCTOBER, 2014.**

## DECLARATION

I declare that the work in this Thesis entitled **PHYTOCHEMICAL STUDIES AND EFFECT OF METHANOL LEAF EXTRACT OF *LEPTADENIA HASTATA*(PERS.) DECNE (ASCLEPIADACEAE) ON ACETIC ACID INDUCED WRITHES IN MICE AND VENOM OF *ECHIS OCELLATUS*** has been carried out by me in the Department of Pharmaceutical and Medicinal Chemistry. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other Institution.

Maria M. Mailafiya

Name of Student

\_\_\_\_\_  
Signature

\_\_\_\_\_  
Date

## CERTIFICATION

This thesis entitled **PHYTOCHEMICAL STUDIES AND EFFECT OF METHANOL LEAF EXTRACT OF *LEPTADENIA HASTATA*(PERS.) DECNE (ASCLEPIADACEAE) ON ACETIC ACID INDUCED WRITHES IN MICE AND VENOM OF *ECHIS OCELLATUS***

by MARIA MANAGER MAILAFIYA meets the regulations governing the award of the degree of Master of Science in Pharmaceutical and Medicinal Chemistry of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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Dean, School of Postgraduate  
\_\_\_\_\_

## **DEDICATION**

This research work is dedicated to God Almighty.

## ACKNOWLEDGEMENT

I am grateful to God Almighty, who has been my strength, my provider and my all. To him who gives wisdom, understanding and for his blessings in successful completion of this work to him be all the glory forever more.

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## ABSTRACT

*Leptadenia hastata* (Pers.) Decne (Asclepiadaceae) is widely African plant species used in traditional medicine as an anti-venom, antihypertensive, anti-diabetic, analgesic and also used for catarrh and skin disease. The powdered leaves of *L. hastata* was extracted with methanol using maceration method and the resulting crude methanol extract (ME) was suspended in water and fractionated into hexane (HF), chloroform (CF), ethylacetate (EF) and n-Butanol (BF). Preliminary phytochemical screening of ME revealed the presence of carbohydrates, alkaloids, flavonoids, anthraquinones, saponins, tannins, glycosides, steroids and triterpenes while (HF) revealed the presence of steroids and triterpenes nucleus. HF was step-wisely eluted in a silica gel packed column to afford ten fractions H<sub>1</sub>-H<sub>10</sub> and fraction H<sub>4</sub> was selected for further purification using Preparative Thin Layer Chromatography, with the solvent system of hexane: ethyl acetate (8.5 : 2.5). This resulted in the isolation of a whitish crystalline compound M<sub>1</sub> ( $\beta$ -Sitosterol). The structure of the compound was elucidated using chemical test and spectroscopic techniques (IR, 1D & 2D-NMR) and by comparison with reference spectral data. The LD<sub>50</sub> of the methanol extract (ME) and the LD<sub>99</sub> of the *Echisocellatus* venom were estimated to be greater than 5000 mg/kg and 2.2 mg/kg respectively. Anti-venom studies suggest that ME possess significant anti-venom activity against *Echisocellatus* venom both *In vitro* and *In vivo*. Analgesic effect was evaluated using acetic acid induced writhing in mice. The results showed that ME (150 mg/kg) decreased writhing response with 84.6 % inhibition. However, the extract when compared to control showed statistically significant difference at (p  $\leq$  0.05)

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## ABBREVIATION

ME	Methanol Extract
HF	Hexane Fraction
EF	Ethylacetate Fraction
CF	Chloroform Fraction
BF	n-Butanol Fraction
TLC	Thin Layer Chromatography
PTLC	Preparative Thin Layer Chromatography
<i>L. Hastata</i>	<i>Leptadeniahastata</i>
<i>E. ocellatus</i>	<i>Echisocellatus</i>
<sup>1</sup> H-NMR	Proton Nuclear Magnetic Resonance
DEPT	Distortionless Enhancement by Polarization Transfer
COSY	Correlation Spectroscopy
HMBC	Hetero Nuclear Multiple Bond Correlation
HSQC	Homo Nuclear Single Quantum Correlation
<sup>13</sup> C-NMR	Carbon Nuclear Magnetic Resonance
NEOSY	Nuclear Overhauser Effect Spectroscopy
WHO	World Health Organisation
H <sub>2</sub> SO <sub>4</sub>	Sulphuric Acid
HCl	Hydrochloric Acid
NaOH	Sodium Hydroxide

## **CHAPTER ONE**

### **1.0 INTRODUCTION**

A natural product is a chemical compound or substance produced by a living organism – found in nature that usually has a pharmacological or biological activity for use in pharmaceutical drug discovery and drug design. A natural product can be considered as defined above even if it can be prepared by total synthesis (Newman and Cragg, 2007).

Traditional medicine is the sum total of the knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness.

Traditional use of herbal medicines refers to the long historical use of these medicines. Their use is well established and widely acknowledged to be safe and effective, and may be accepted by national authorities (WHO, 2013).

In recent years, the treatments and remedies used in traditional African medicine have gained more appreciation from researchers in Western science. Developing countries have begun to realize the high costs of modern health care systems and the technologies that are required, thus proving Africa's dependence to it. Due to this, interest has recently been expressed in integrating traditional African medicine into the continent's national health care system. In Africa, the importance of traditional healers and remedies made from indigenous plants play a crucial role in the health of millions.

According to the International Development Research Centre (IDRC), one estimate puts the number of Africans who routinely use these services for primary health care as high as 85% in Sub-Saharan Africa (Bob, 2006). The relative ratios of traditional practitioners and university trained doctors in relation to the whole population in African countries showcase this importance. For example, in Ghana, in Kwahu district, for every traditional practitioner there are 224 people, against one university trained doctor for nearly 21,000 (Converse Africa, 2002).

Natural substances are innately superior to man-made or synthetic substances with respect to their side effects and efficacy on human health. About 80 percent of people in developing countries still rely on traditional medicine—based largely on species of plants and animals—for their primary health care. In the United States, some 25 percent of prescriptions are filled with drugs whose active ingredients are extracted or derived from plants. Sales of these plant-based drugs in the U.S. amounted to some \$4.5 billion in 1980 and an estimated \$15.5 billion in 1990. Other drugs are derived from animals and microorganisms (Farnsworth *et al.*, 1991).

All 119 plant-derived drugs used worldwide in 1991 came from fewer than 90 of the 250,000 plant species that have been identified. Each such plant is a unique chemical factory, says Norman R. Farnsworth of the University of Illinois at Chicago in 1989, “capable of synthesizing unlimited numbers of highly complex and unusual chemical substances whose structures could [otherwise] escape the imagination forever.” In other words, scientists may be able to synthesize these plant compounds in the laboratory, but dreaming them up, rather than plucking them from the forest and then replicating them, is quite another matter.

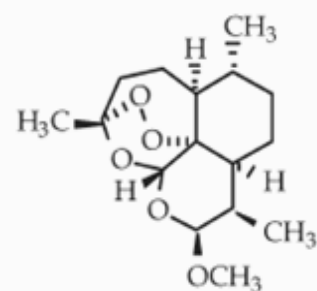
Since the mid-1960s, one-fourth of all prescription drugs dispensed from American pharmacies contained active ingredients derived from flowering plants. Commercially, these plant-derived medicines are worth about \$14 billion a year in the United States and \$40 billion worldwide. In 1985, Lilly Research Laboratories sold roughly \$100 million worth of vincristine and vinblastine – the periwinkle derivatives used to treat childhood leukemia and Hodgkin’s disease and turned a stunning 8 percent profit (Robin, 1991).

## **1.1 BRIEF HISTORY OF DRUG DEVELOPMENT**

Therapeutic drugs have played a major role in increasing average life expectancy in the world in the last century. However, while many of the drugs in use in the last fifty years or more have been of synthetic or semi-synthetic origin, the pharmacopoeias prior to that period were of natural origin. The medicinal value of plants has been recognized by almost every society on this planet. In the nineteenth and earlier centuries, natural product extracts, particularly those derived from botanical species, provided the main source of folk medicines (Dias *et al.*, 2012).

The discovery of new important anti-infective is not limited to searching the microbial kingdom, but includes plant and animal sources as well. Artemisinin, a sesquiterpene with an unusual endoperoxide moiety, was isolated from the Chinese medicinal plant commonly known as Qinghaosu (*Artemisia annua*), a herbal remedy that had been used in China for centuries for the treatment of malaria. Isolated in 1972 as the active constituent, artemisinin was discovered to be particularly effective for the deadly cerebral malaria. Subsequent efforts to develop artemisinin derivatives with more desirable pharmaceutical properties included many synthetic and semisynthetic studies, microbial bio-transformations, biological evaluations, mechanism of

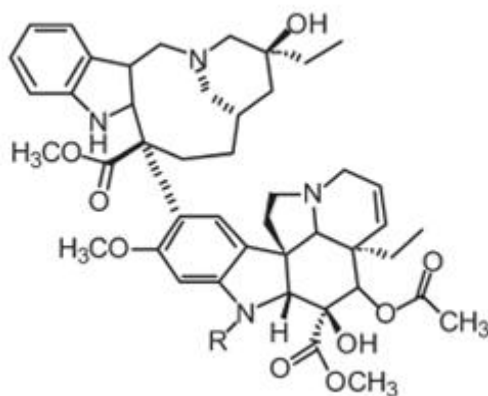
action studies, and pharmacological studies of artemisinin and a number of related analogs (Wu and Li, 1995; Lee and Hufford, 1990). From these efforts, the semisynthetic derivative artemether (i) was developed and is now approved for the treatment of malaria in many countries of the world. Thus, a natural substance was transformed into a related semisynthetic substance with superior therapeutic properties.



(i)

Natural products have provided the most important successes in the chemotherapy of cancer. Most of the major anticancer drugs are unmodified natural products obtained from plants or microorganisms (Loo and Freireich, 1995), and include such important anticancer drugs as bleomycin, doxorubicin, daunorubicin, vincristine (ii), vinblastine (iii), mitomycin, streptozocin, and paclitaxel (Taxol™). Irinotecan (a camptothecin derivative), and etoposide and teniposide (podophyllotoxin derivatives) are examples of semisynthetic derivatives of natural products that are important anticancer drugs. Vincristine (ii) and vinblastine (iii) are complex, dimeric indole-indolines obtained from the rosey periwinkle (*Catharanthus roseus*), and are among the most important therapies for the treatment of childhood leukemia, Hodgkin's disease, and metastatic testicular tumors. These unmodified natural products continue to be produced today by mass

cultivation and processing of the plant material (Tyler *et al.*, 1988). Vinorelbine is a semisynthetic analog and has been reported to have decreased hematological toxicity.

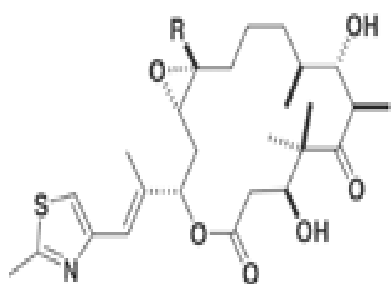


(ii)R=CHO

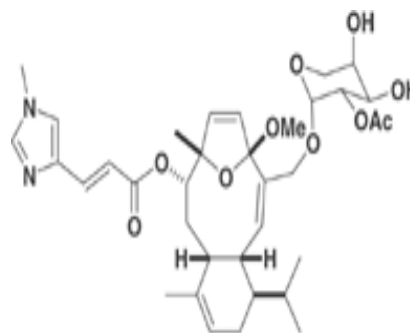
(iii)R= CH<sub>3</sub>

As part of a program undertaken over 40 years ago by the National Cancer Institute, which was aimed at examining higher plants as a source of anticancer agents, one of the most important discovery of this century occurred. Wall and Wani, (1995) reported that a complex di terpene, which they isolated from the bark of the Pacific yew tree and named taxol, was reported to possess significant cytotoxicity for cancer cells. Although it took some 15 years for the potential of paclitaxel to be realized, it is now recognized as a breakthrough in the treatment of ovarian breast cancers and one of the most exciting new drugs in recent history. Currently, both a semisynthetic derivative with improved water solubility, docetaxel (Taxotere®), and paclitaxel (Taxol®) are approved and used clinically. Paclitaxel also serves as a vivid example of the utility of bioactive natural products as biochemical probes. Studies on the paclitaxel's mechanism of anticancer action revealed a previously unknown mechanism (stabilization of

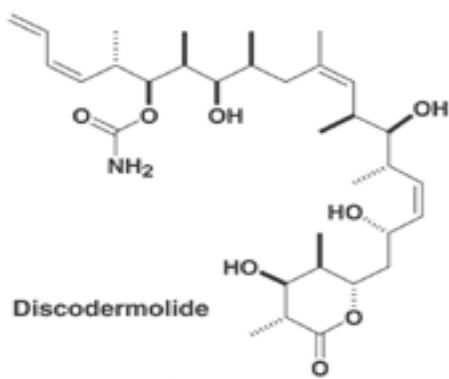
microtubules)(Schiff *et al.*, 1979). Using bioassays to detect this type of activity, more recently, other mechanistically similar but structurally unrelated natural products, including the epothilones (iv), eleutherobin (v) and discodermolide (vi), have been developed, and are in various stages of preclinical/clinical development.(Bollaget *et al.*, 1995), and intense efforts are underway to identify drug candidates from these classes.



(iv) R = H



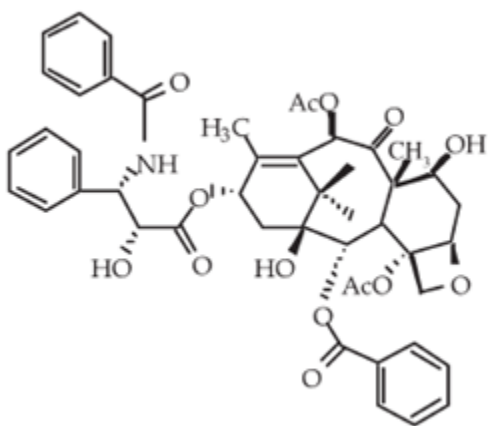
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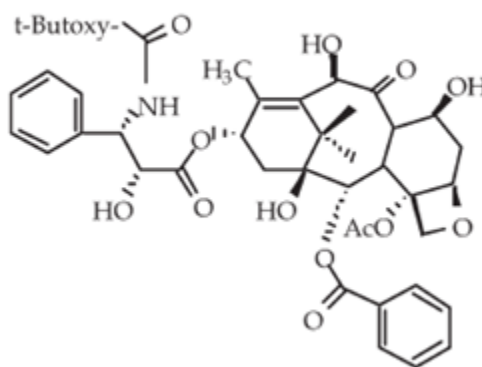
Discodermolide

(vi)

Since paclitaxel (vii) was the only known compound to exhibit this activity, without its discovery and development this new target for anticancer drug discovery and development would likely not have been identified

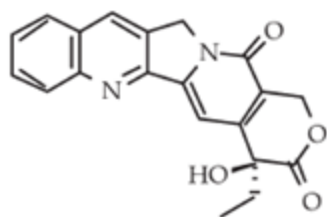


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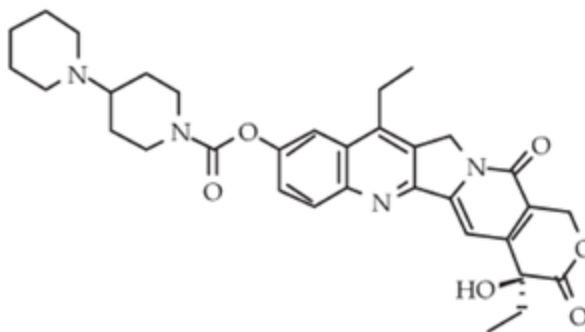


(viii)

Camptothecin (ix), an alkaloid from the Chinese tree, *Camptotheca acuminata* Descne (Wall *et al.*, 1966), was also discovered by Wall and Wani. Although showing promising anti-tumor activity, clinical studies were discontinued due to unpredictable side effects. Subsequent semisynthetic modifications led to the development of Irinotecan (x) (Topotecin<sup>TM</sup>, Campto<sup>TM</sup>), a derivative of camptothecin (ix) that is now clinically available. The camptothecins (ix) act by inhibition of the topoisomerase I.

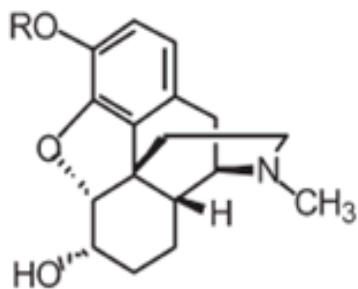


(ix)



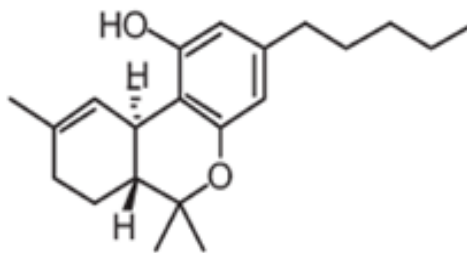
(x)

One of the oldest CNS drugs in use is d-tubocurarine, a neuromuscular blocker derived from a plant (curare) used as an arrow poison by South American Indians. Also, the opium alkaloids codeine (xi) and morphine (xii) are important analgesic drugs, and continue to be manufactured by processing opium exudate and extract (Tyler *et al.*, 1988). Delta-9-tetrahydrocannabinol (THC) (xiii), the component of *Cannabis sativa* responsible for its CNS effects, is an important drug (Marinol®) used to reduce nausea associated with cancer chemotherapy. Although prepared commercially using synthetic methodology, Marinol® is identical to natural THC.



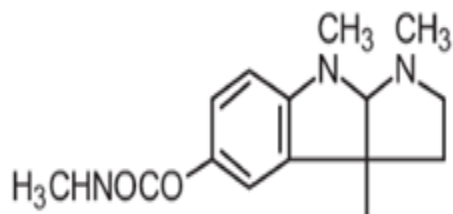
(xi) R=CH<sub>3</sub>

(xii) R = H

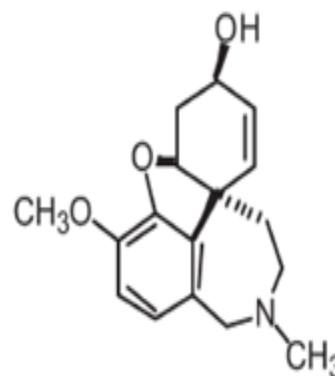


(xiii)

Physostigmine (xiv), a naturally occurring alkaloid, and its carbamate ester, neostigmine, are acetyl cholinesterase inhibitors used for the treatment of myasthenia gravis and as antagonists to neuromuscular blockage by non-depolarizing blocking agents. Galanthamine (xv), an alkaloid that occurs in the bulbs of daffodils, is also an acetyl cholinesterase inhibitor and is currently in clinical trials as a possible therapy for cognitive impairment in Alzheimer's disease (Tillotson, 1996).



(xiv)



(xv)

## 1.2

## SNAKE VENOM

Snake venom is highly modified saliva (Reptile Venom Research 2010) containing zootoxins used by snakes to immobilize and digest prey or to serve as a defense mechanism against a potential predator or other threat. The venom produced by the snake's venom gland apparatus is delivered by an injection system of modified fangs that enable the venom to penetrate into the target (Roland, 1994).

The glands that secrete the zootoxins are a modification of the parotid salivary gland found in other vertebrates and are usually situated on each side of the head, below and behind the eye and encapsulated in a muscular sheath. The glands have large alveoli in which the synthesized venom is stored before being conveyed by a duct to the base of channeled or tubular fangs through which it is ejected (Halliday and Tim., 2002, Biswas *et al.*, 2011)

Venoms contain more than 20 different compounds, mostly proteins and polypeptides (Halliday and Tim., 2002, Bottrallet *et al.*, 2010). A complex mixture of proteins, enzymes, and various other substances with toxic and lethal properties (Roland, 1994) serves to immobilize the prey animal (Mattison, 2007), enzymes play an important role in the digestion of prey (Biswas *et al.*, 2011), and various other substances are responsible for important but non-lethal biological effects (Roland, 1994). Some of the proteins in snake venom have very specific effects on various biological functions including blood coagulation, blood pressure regulation, and transmission of the nervous or muscular impulse and have been developed for use as pharmacological or diagnostic tools or even useful drugs.

Snake toxins vary greatly in their functions. Two major classifications of toxins found in snake venoms include neurotoxins (mostly found in elapids) and hemotoxins (mostly found in viperids). However, there are exceptions - a black-necked spitting cobra's (*Naja nigricollis*) venom consists mainly of hemotoxins, while the Mojave rattlesnake's (*Crotalus scutulatus*) venom is primarily neurotoxic. However, there are numerous other different types of toxins which both elapids and viperids may carry (Roland, 1994).

### **1.2.1 Effect of Snake Venom on Human Health**

Snake venom is a complex mixture of many different compounds. The composition and effects of venom varies considerably between species to species, but can broadly be divided into categories which include;

- i. cytotoxins causing local swelling and tissue damage
- ii. haemorrhagins which disturb the integrity of blood vessels
- iii. Compounds which inhibit blood coagulation
- iv. Neurotoxins causing neurotoxicity
- v. Myotoxins which cause muscle breakdown. (Seifert and vogel, 2012)

The clinical features of the bites of venomous snakes reflect the effects of these venom components. These include, local tissue damage ranging from swelling of the bitten limb to skin and muscle necrosis, abnormal blood clotting and bleeding, hypotension and shock, neurotoxicity sometimes leading to paralysis of respiratory muscles requiring assisted ventilation, and renal toxicity. Although the most obvious explanation for a confirmed snake-bite with no clinical manifestations is a bite by a non-venomous species, bites by venomous species

do not always cause symptoms, and only 50-70% of bites by a venomous species will actually cause envenoming. (Seifert and Vogel, 2012)

Viper venom (Russell's viper, saw-scaled vipers, bushmasters, rattlesnakes) acts more on the vascular system, bringing about coagulation of the blood and clotting of the pulmonary arteries; its action on the nervous system is not great, no individual group of nerve-cells appears to be picked out, and the effect upon respiration is not so direct; the influence upon the circulation explains the great depression which is a symptom of viperine envenomation says Martin Charles James and Lamb George in 1907, "the pain of the wound is severe, and is speedily followed by swelling and discoloration."

## **1.3**

## **PAIN**

Pain is an unpleasant feeling that is conveyed to the brain by sensory neurons. The discomfort signal is an actual or potential injury to the body. However, pain is more than a sensation, or the physical awareness of pain; it also includes perception, the subjective interpretation of the discomfort. Perception gives information on the pain location, intensity and something about its nature. The various conscious and unconscious responses to both sensation and perception, including the emotional response, add further definition to the overall concept of pain (Nijset al, 2014).

### **1.3.1 Description of pain**

Pain arises from any number of situations. Injury is a major cause, but pain may also arise from illness. It may accompany a psychological condition such as depression, or may occur in the absence of a recognizable trigger.

### 1.3.1.1 *Acute Pain*

Acute pain often results from tissue damage, such as a skin burn or broken bone. It can also be associated with headache or muscle cramps. This type of pain usually goes away as the injury heals or the cause of the pain (stimulus) is removed (Schonbeck and Uretsky, 2004).

### 1.3.1.2 *Chronic pain*

Chronic pain refers to pain that persists after an injury, cancer pain, pain related to a persistent or degenerative disease, and long term from an unidentifiable cause.

American chronic pain association defined chronic pain as the pain that may be caused by the body's response to acute pain. It is sometimes difficult to locate and control.

## **1.3.2 Causes and Symptoms of Pain**

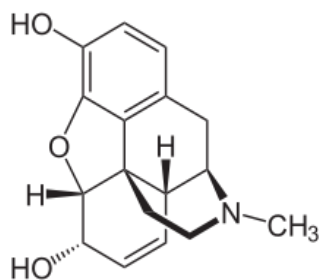
Pain is the most common symptom of injury and disease, and descriptions can range in intensity from a mere ache to unbearable agony. Nociceptors have the ability to convey information to the brain that indicates the location, nature, and intensity of the pain. Pain perception also varies depending on the location of the pain (Schonbeck and Uretsky, 2004).

## 1.4

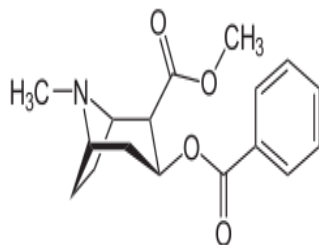
## ANALGESICS

An analgesic, or painkiller, is any member of the group of drugs used to achieve analgesia that is, relief from pain (Harper, 2001).

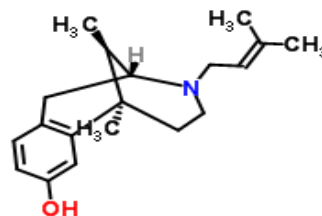
They are classified into narcotic and non- narcotic analgesics. Narcotic analgesics, otherwise called opioid analgesics are natural or synthetic compounds that produce morphine-like effects. They act by binding to specific opioid receptors in the central nervous system (CNS) to produce effect which mimics the action of endogenous peptide neurotransmitters, the opiopentins (e.g. the endorphins, enkephalins and dynorphins). Examples of Narcotic (opioid) analgesics include: Morphine (xvi), Cocaine (xvii) and Pentazocine (xviii). Their major drawbacks are tolerance, dependence and respiratory depression (Romulo *et al.*, 2013).



(xvi)



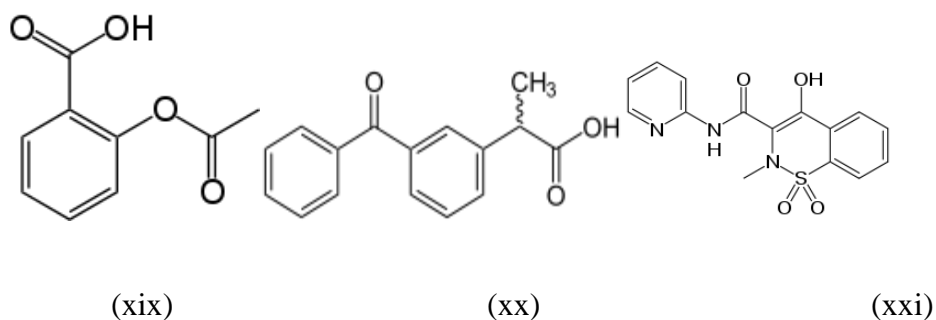
(xvii)



(xviii)

Non-narcotic analgesics commonly referred to as non-steroidal anti-inflammatory drugs (NSAIDs) are among the most widely used therapeutic agents (Insel, 1996). They are mild analgesics which are particularly effective when inflammation has caused sensitization of pain receptors. The expression of pain and inflammation is associated with prostaglandin synthase

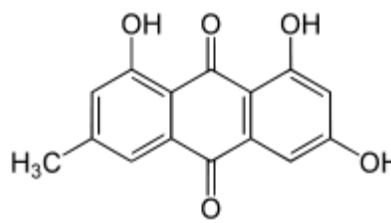
enzyme known as Cyclooxygenase (COX) which is classified into two types, COX-1 and COX-2. The inhibition of cyclooxygenase enzyme is the mechanism of action of most currently available NSAIDs. The NSAIDs inhibit both COX-1 and COX-2. Most NSAIDs are mainly COX-1 selective e.g., aspirin (xix), ketoprofen (xx) and piroxicam (xxi). Their major drawback is the increased gastric irritation leading to hyperacidity and aggravation of ulcer (Insel, 1996).



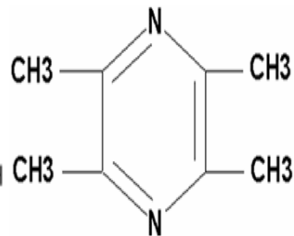
#### 1.4.1 Herbal constituents active against pain

Many African medicinal plants and have been reported to have analgesic activity in animal models; *Ptericarpuserinaceus* (Aliyuet *al.*, 2005), *Calliandrapororicensis* (Agunuet *al.*, 2005). Gossypin, a biflavonoid from yellow petals of *Hibiscus vitofolius* has been reported to possess antinociceptive activity similar to morphine with no tolerance and dependency effect of the opioids (Dahanukaret *al.*, 2000).

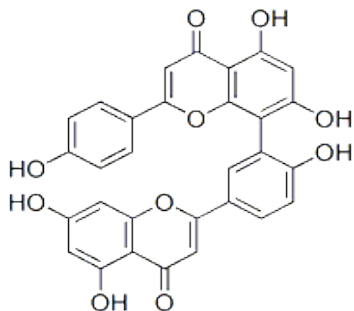
Some of the secondary metabolites isolated from plants that have been shown to have analgesic activity include: Emodin (xxii), Ligunstrazine (xxiii), Amentoflavone (xxiv) and Puerarin (xxv) (Rômuloet *al.*, 2013).



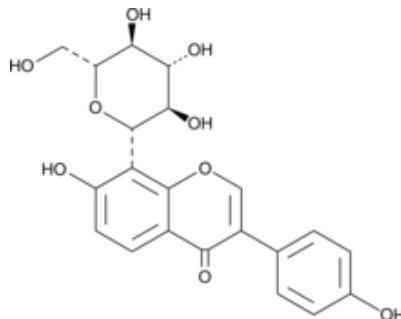
(xxii)



(xxiii)



(xxiv)



(xxv)

## 1.5 STATEMENT OF RESEARCH PROBLEM

Envenoming resulting from snake bites is a particularly important public health problem in rural areas of tropical and subtropical countries situated in Africa, Asia, Oceania and Latin America. A recent study estimates that at least 421,000 envenomings and 20,000 deaths occur worldwide from snakebite each year, but warns that these figures may be as high as 1,841,000 envenomings and 94,000 deaths. The highest burden of snakebites is in South Asia, Southeast Asia, and sub-Saharan Africa (Kasturiratneet *al.*, 2008).

Snake bite is primarily a problem of the poorer rural populations in these regions and affects mainly those involved in subsistence farming activities. Poor access to health services in these

settings and, in some instances, a scarcity of anti-venom, often leads to poor outcomes and considerable morbidity and mortality. Many victims fail to reach hospital in time or seek medical care after a considerable delay because they first seek treatment from traditional healers. Some even die before reaching hospital. Hospital statistics on snakebites therefore underestimate the true burden. In addition to mortality, some snakebite victims survive with permanent physical sequelae due to local tissue necrosis and, sometimes psychological sequelae. Because most victims are young, the economic impact of snakebite can be considerable (Kasturiratneet *al.*, 2008).

## **1.6 JUSTIFICATION OF THE STUDY**

Envenoming following snakebite is largely a neglected threat to public health. It affects mainly the poor in deprived rural areas where health facilities are limited and anti-venoms may be unavailable, difficult and expensive to obtain. Training of health staff in the management of envenoming is often neglected, despite good evidence that it improves outcome. Concerted action is needed to ensure supplies of effective anti-venoms and to develop systems that deliver good quality health care to snake bite victims so that we can deal effectively with this problem, which causes severe disability, brings misery to families and which kills thousands of people (WHO, 2013). Therefore this research will establish scientifically presence or absence of anti-snake venom activity and when present postulate mechanism for the activity and isolate some bioactive compounds.

## **1.7 JUSTIFICATION FOR PLANT SELECTION**

*Leptadeniahastata* has been used for wound healing (Nikiemaet *al.*, 1997) and considered safe to use due to its high LD quotient value of 0.78 (Tambouraet *al.*, 2007). Literature in scientific data based showed no phytochemical and pharmacological investigation aimed at isolating bioactive compounds of the methanol leaves extract was ever undertaken.

## **1.8 AIM OF THE STUDY**

The aim of this study is to carry out phytochemical studies on the leaf extract of *Leptadeniahastata* and evaluate its anti-snake and analgesic potential using laboratory animals.

## **1.9 OBJECTIVES OF THE STUDY**

The specific objectives of the study are;

1. To carry out preliminary phytochemical studies on *L. hastata* using standard procedures.
2. To undertake chromatographic studies of the leaves extract with a view to isolating and characterizing the compounds isolated, through spectroscopic analysis.
3. To determine the LD<sub>50</sub> of the methanol leaves extract of *L. hastata* using Lorke's method.
4. To determine the LD<sub>99</sub> of the venom of *E. ocellatus*
5. To determine the *in vitro* and *in vivo* detoxifying effect in mice, of the leaves extract of *L. hastata* against *E. ocellatus* venom.
6. To determine the analgesic activity of the extract on laboratory animals

## **1.10**

### **STATEMENT OF RESEARCH HYPOTHESIS**

The leaves of *Leptadenia hastata* contain secondary metabolite with anti-snake venom and analgesic activity.

## CHAPTER TWO

### 2.0 LITERATURE REVIEW

#### 2.1 LITERATURE REVIEW OF *LEPTADENIA HASTATA*

*Leptadeniahastata* (Pers.) Decne is a member of the Asclepiadaceae family. The Asclepiadaceae are mostly herbs and shrubs with white sap comprising about 250 genera and 2,000 species, many of which are lianous and some of which are cactus like succulents with reduced leaves (Thomas, 2013).



**Plate I: Picture of *Leptadeniahastata***

Fresh leaves of *Leptadeniahastata* contains per 100 g: water 815, energy 226 kJ (54kcal), protein 4.9 g, fat 0.2 g, carbohydrate 11.3 g, fiber 4.7 g, calcium 417 mg, phosphorus 94 mg, iron 5.4 mg, Vitamin A 4915 mg, thiamin 0.25 mg, riboflavin 0.35 mg, niacin 1.9 mg, ascorbic acid 78

mg (Leung *et al.*, 1968). The latex contains triterpenelupeol and derivative of it which possess anti-inflammatory activity.

*Leptadeniahastata*(Pers.) Decne. (Asclepiadaceae) is a widely distributed tropical African herb used as vegetable. *Leptadeniahastata* is edible non-domesticated vegetable and it is collected in wild throughout Africa. It is typically grown in tropical dry lands in sandy soil. Wild foods like *L. hastata* provide food security during seasonal changes and are used medicinally in many areas (Thomas, 2013).

### **2.1.1 Botanical Features of *Leptadeniahastata***

*L. hastata* is a climbing latex containing herb, becoming woody at base, with strongly branched, finely pubescent stems becoming corky with age. Leaves opposite, simple; petiole 0.5cm-1.5cm long; blade variable, usually ovate, 2.5cm-12.5cm x 0.5-4cm, base rounded to cordate, apex acuminate, margin entire, both side puberulous, often glabrescent. Inflorescence umbellate, minutely tomentose, many-flowered; peduncle up to 1.5cm long. Flower bisexual, regular, 5-merous, yellowish, scented; pedicel up to 0.5cm long; calyx lobed nearly to the base, lobes 2-4mm long, inserted at the sinuses of the corolla, apex hairy. The fruit is a pair of follicles each one conical, up to 10cm long, greenish, glabrous, many seeded. The seeds are with a tuft of hairs at apex.

### **2.1.2 Local names of *Leptadeniahastata***

Vernacular names for *L. hastata* include: *hagalhadjar* (Arabic) in Chad, *yadiya* (Hausa) in Nigeria and Niger, *hayla* (Kusume) Ethiopia, *ekamongo* (Turkana) in Kenya, *lolongo* (Moore) in Burkina Faso, *tarhatordarhat* (Wolof), *busumbaamata* (Jola) in Senegal, and *nzongnè* (Bambara) in Mali.

### **2.1.3 Ethno-medical uses of *Leptadenia hastata***

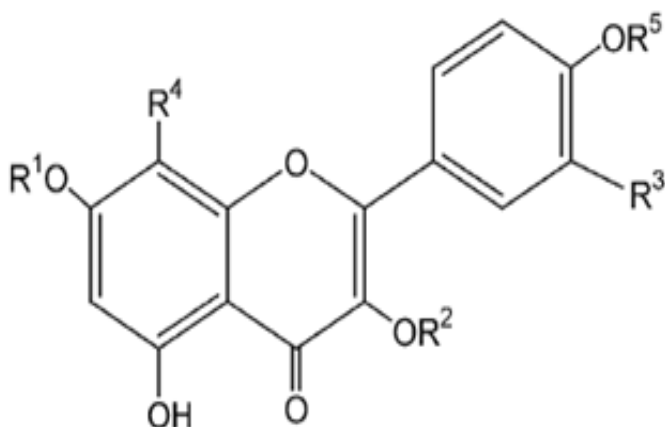
Decoction of the leaves of *L. hastata* with the bark of *Erythrina senegalensis* either taken orally or is used as a medicinal bath to treat onchocercosis in Mali (Togola *et al.*, 2008). In Chad, the roots are used to treat scabies (Bettiet *et al.*, 2011). This plant is commonly used in Hausa-speaking communities in Nigeria as a spice and used in sauces (Ibrahim *et al.*, 2012). Also in Nigeria, local healers use the plant for hypertension, catarrh and skin diseases (Dambatta and Aliyu, 2011). In Burkina Faso, locally it is used for sexual potency (chewing leaves), trypanosomosis (decoction of leaves), skin diseases and wound-healing (application of latex) (Tamboura *et al.*, 2007). In Senegal, the leaves have been reportedly used for lactation and as a purgative by Kerharo and Adam 1974, Arbonnier, 2000. Senegalese healers also use the *L. hastata* for prostate and rheumatism complaints (Mathieu and Meissa, 2007).

### **2.1.4 Phytochemistry of *Leptadenia hastata* and other species in the family Asclepiadaceae**

To the best of our research, there is no report on isolation of any compound from the whole leaf of *Leptadenia hastata* but there was on the latex of the leaves.

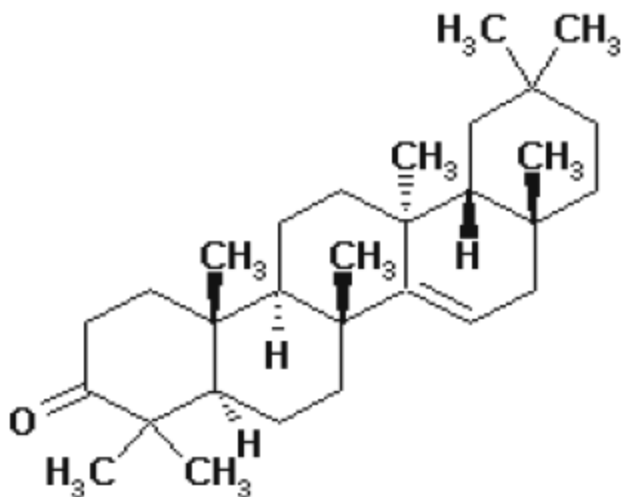
Seven flavonoids were isolated from the butanol fraction of the methanol extract of the aerial parts of *Cynanchum acutum* L. (Asclepiadaceae). All of which have been isolated for the first time from the genus *Cynanchum*. Their structures were established as quercetin 3-*O*-galacturonopyranoside (xxvi), quercetin 7-*O*- $\alpha$ -glucopyranoside (xxvii), tamarixtin 3-*O*-

galacturonopyranoside (xxviii), kaempferol 3-*O*-galacturonopyranoside (xxix), 8-hydroxyquercetin 3-*O* - galactopyranoside (xxx), tamarixtin 3-*O*- $\alpha$ -rhamnopyranoside (xxxi), and tamarixtin 7-*O*- $\alpha$ -arabinopyranoside (xxxii) on the basis of their chromatographic properties, chemical and spectroscopic data. (Ghadaet *al.*, 2008)



Compound	R <sup>1</sup>	R <sup>2</sup>	R <sup>3</sup>	R <sup>4</sup>	R <sup>5</sup>
xxvi	H	Galacturonide	OH	H	H
xxvii	Glucoside	H	OH	H	H
xxviii	H	Galacturonide	OH	H	CH <sub>3</sub>
xxix	H	Galacturonide	H	H	H
xxx	H	Galactoside	OH	OH	H
xxxi	H	Rhamnoside	OH	H	CH <sub>3</sub>
xxxii	Arabinopyranoside	H	OH	H	CH <sub>3</sub>

A pentacyclitriterpenoid compound has been reported to be Isolated from the fruits of *Dregeavolubilis* Benth *Asclepiadaceae* (Bikashand Haldar, 2009).



(xxxiii)

### 2.1.5 Pharmacological actions of *L. hastata* and other species in the family of *Asclepiadaceae*

The water and methanol extracts of *L. hastata* in normal and alloxan-induced diabetic rats model was reported to have potent hypoglycaemic and hypolipidaemic effects (Bello *et al.*, 2011)

Three quercetin glycosides (**xxvi**, **xxvii**, **xxx**), three tamarixtin glycosides (**xxviii**, **xxxi**, **xxxii**) and one kaempferolgalacturonoside (**xxix**) were isolated from the aerial parts of *C. acutum*. All isolated compounds are reported for the first time to have pharmacological activity.

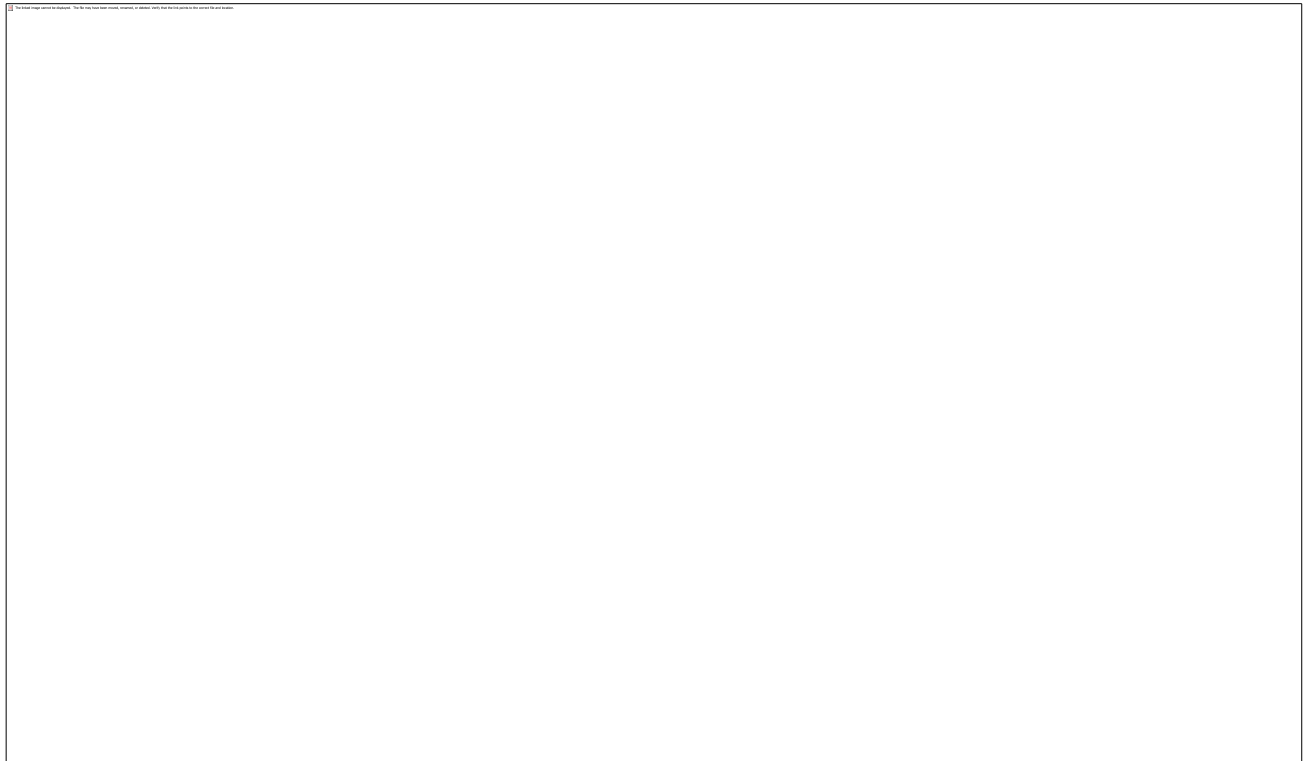
The major isolates **xxvi-xxviii** showed potent anti-diabetic activity in comparison with metformin through an increase of insulin and a decrease of glucose levels. By the same way, all tested isolates were very potent as antioxidant (Ghadaet *al.* 2008).

Taraxerone (Dfriedoolean- 14- en, 3 one) a pentacyclictriterpenoid Compound with Anti-leishmanial and Anti-tumor activities has been reported to be isolated from the Fruits of *Dregeavolubilis*Benth*Asclepiadaceaa*(Bikashand Haldar2009).

The triterpenes isolated from the latex of *Leptadenia hastata* latex showed anti-inflammatory activity Keratinocyte Proliferation (Nikeimaet *al.*, 2001).

## **2.2 LITERATURE REVIEW OF *ECHIS OCELLATUS***

*Echisocellatus* is a venomous viper species, named after the distinctive series of “eye-spots” (ocelli) which runs the length of its body. No sub species are currently recognized. The maximum total length (body + tail) is 65 cm, possibly more, while the average total length is 30–50 cm. The venom of *E. ocellatus* is hemotoxic (Spawls and Branch, 1995). Sexually mature females lay between 6 and 20 eggs, usually at the end of the dry season in February to March. Hatchlings are 10–12 cm (3.9–4.7 in) in total length.



**Plate II : Picture of *Echisocellatus***

It is found mainly in West Africa from Mauritania, Senegal and Guinea, through, Mali, Ivory Coast, Burkina Faso, Ghana, Togo, Benin, southern Niger, and Nigeria. It is also found in northern Cameroon and southwestern Chad (Mallow *et al.*, 2003). There are also reports of single specimens found in the Bangui in the Central African Republic, and in central Sudan. It is rarely found north of the 15<sup>th</sup> parallel, after which *E. leucogaster* becomes more common. The geographic range of *E. ocellatus* extends to the coast via the Dahomey Gap (Spawls and Branch, 1995).

### 2.2.1 Scientific classification of *Echisocellatus*

Kingdom:	Animalia
Phylum:	Chordata
Sub-phylum:	Vertebrata
Class:	Reptilia
Order:	Squamata
Sub-order:	Serpentes
Family:	Viperidae
Subfamily:	Viperinae
Genus:	<i>Echis</i>
Specie:	<i>E. ocellatus</i>

### 2.2.2 Binomial name

*Echisocellatus*

### 2.2.3 Common names

African saw-scaled Viper, West African carpet viper, *ocellated carpet viper*.

### 2.2.4 Synonyms

- *Echiscarinatusocellatus*
- *Echisocellatus*
- *Echis (Toxicoa) ocellatus*

## CHAPTER THREE

### 3.0 MATERIALS AND METHODS

#### 3.1 MATERIALS

##### 3.1.1 Chemicals and Reagents

The chemicals and reagents used were of analytical grade. Thin layer chromatography (TLC) pre-coated plate was 60 F<sub>254</sub> (20×20cm) and silica gel for column chromatography was of mesh size 60-120 μm (Qualikems).

##### 3.1.2 Equipment

Electro thermal melting point apparatus at the Department of Pharmaceutical and Medicinal Chemistry, Ahmadu Bello University Zaria was used.

Bruker AVANCE III NMR spectrometer 600 MHz at the School of Chemistry and Physics, University of Kwa-Zulu Natal-Durban, South Africa was used for 1D- and 2D-NMR spectroscopy.

Fourier Transform infrared spectrophotometer FTIR-8400S at National Research Institute for Chemical Technology Zaria, was used.

##### 3.1.3 Experimental animals

Adult Swiss albino mice of either sex weighing (15-38 g) were obtained from National Veterinary Research Institute, Vom and were housed at the animal house facility of the Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria, Nigeria. The

mice were fed with laboratory diet and water *ad libitum* and maintained under standard conditions (12 hrs light and 12 hrs dark cycle) in a propylene cages at room temperature.

#### **3.1.4 Snake venom**

The venom of *Echisocellatus* was obtained by milking the snakes (*Echisocellatus*) which were gotten from Billiri Local Government Area of Gombe State, Nigeria. The venom was milked and freeze-dried in the Department of Pharmacology and toxicology, Faculty of Veterinary Medicine, Ahmadu Bello University Zaria, Kaduna state of Nigeria.

### **3.2**

## **METHODS**

#### **3.2.1 Collection, Identification and Drying of Plant material**

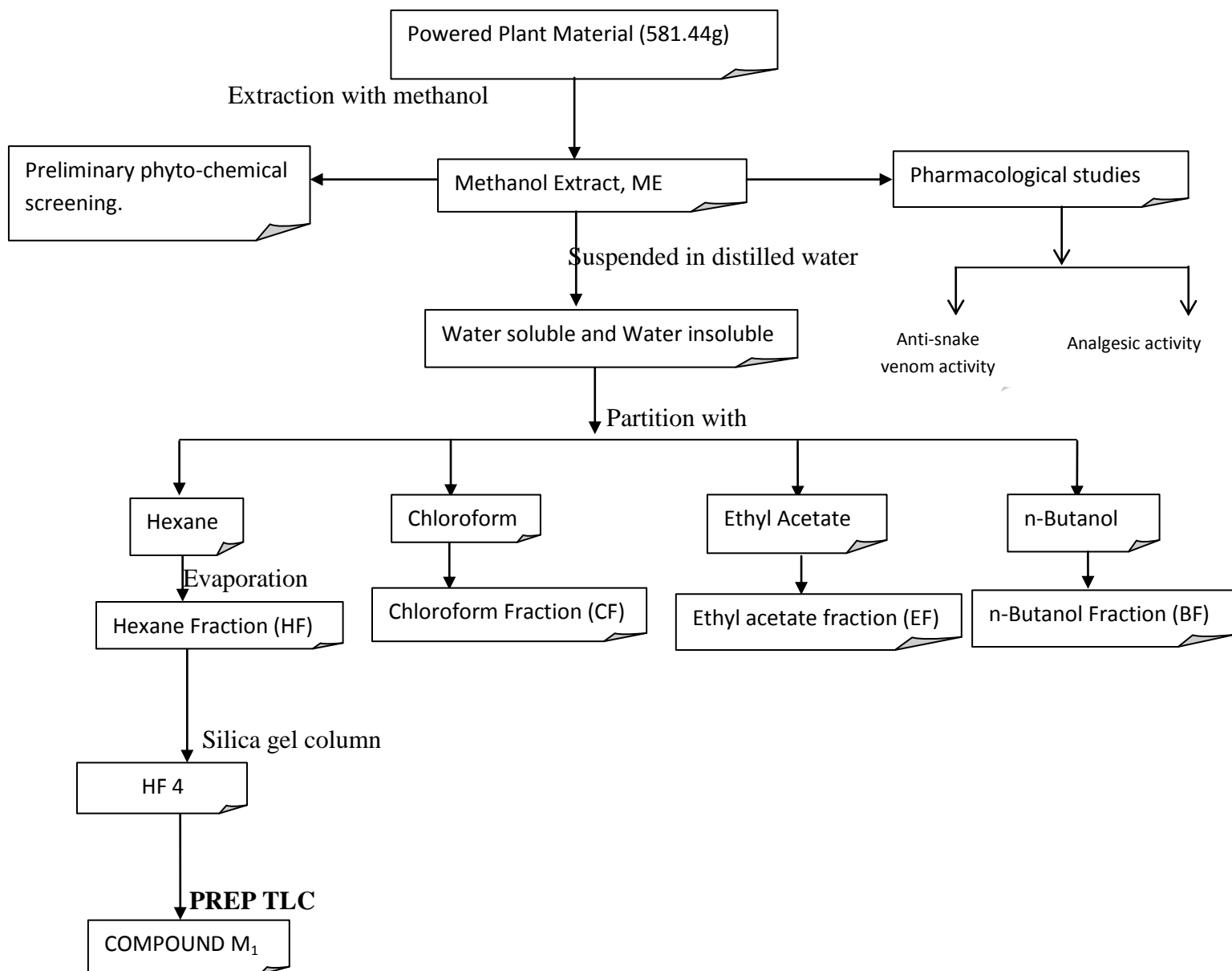
The plant sample of *Leptadenia hastata* was collected in May 2013, at Billiri Local Government area of Gombe State of Nigeria. It was identified at the Herbarium unit, Department of Biological Sciences, Ahmadu Bello University, Zaria Nigeria by comparing with herbarium reference voucher specimen (No. 900220). The leaves were shade dried, pounded to powder, labelled and stored for use.

#### **3.2.2 Extraction and Fractionation**

The powdered leaves (581.44 g) was extracted with methanol using maceration method. The extract was freed from solvent by evaporation *in-vacuo* using rotary evaporator to residue subsequently referred to as the methanol extract. The weight and color were recorded.

The methanol extract (186.44 g) was suspended in distilled water and partitioned successively with n-hexane, chloroform, ethylacetate, and n-butanol to obtain hexane fraction (HF), Chloroform fraction (CF), ethylacetate fraction (EF), n-butanol fraction (BF) and the residual aqueous fraction (AF) respectively. The water insoluble portion was washed with hexane to obtain HF.

Preliminary TLC analysis of crude extract and partitioned fractions was conducted. Of all the fractions, the TLC profile of HF (7.00 g) revealed the most prominent spots and was chosen for further study.



**Scheme I : The chart of extraction protocol**

### 3.2.3 Preliminary Phytochemical Screening

Different chemical tests were carried out on the methanol extract and the hexane fraction (water insoluble) to identify the presence of various chemical constituents like alkaloids, flavonoids, and tannins etc using standard procedures as summarized below:

#### 3.2.3.1 Test for Carbohydrates

Molisch test: To small portion of the extract, distilled water was added and mixed with a few drops of Molisch reagent. 1ml of concentrated H<sub>2</sub>SO<sub>4</sub> was carefully added down the side of the inclined tube so that the acid forms a layer beneath the aqueous solution without mixing with it. A reddish ring at the junction of the liquids was observed, which indicate the presence of carbohydrates (Silva *et al.*, 1998).

Fehling's Test: The extract (0.5g) was dissolved in distilled water and filtered. The filtrate was heated with equal volumes of Fehling's solution A and B. Formation of a red precipitate of cuprous oxide indicates the presence of reducing sugar(Sofowora, 1993).

#### 3.2.3.2 Test for Alkaloids

The extract (0.5g) was stirred with 5ml of 1% aqueous hydrochloric acid on a water bath and filtered. 3 ml of the filtrate was divided into three. To the first 1ml few drops of freshly prepared Dragendoff's reagent was added and observed for formation of orange to brownish precipitate. To the second, 1 drop of Meyer's reagent was added and observed for formation of white to yellowish or cream coloured precipitate. To the third, 1ml of Wagner's reagent was added to give a brown or reddish or reddish-brown precipitate when alkaloid(Evans, 2009).

### 3.2.3.3 *Test for Flavonoids*

#### Shinoda test

5mg of the compound was dissolved in ethanol. 3mg magnesium powder was then added followed by few drops of conc. HCl. An orange colouration indicates the presence flavonoid (Evans, 2009).

#### Ferric chloride test

A drop of ferric chloride was added to a solution of the extract. The solution was observed for the formation of a green precipitate (Evans, 2009)

#### NaOH test

2ml of the extract was dissolved in 10% aqueous NaOH solution. The solution was observed for the presence of a yellow colour, a change in colour from yellow to colorless on addition of dilute HCl indicates the presence of flavonoids (Silva *et al.*, 1998).

### 3.2.3.4 *Test for Anthraquinones*

The extract (0.5g) was shaken with 5ml carbon tetrachloride, filtered and 10% dilute ammonia solution was added. The mixture was shaken and appearance of pinkish color in the ammonical layer indicates the presence of free anthraquinones(Silva *et al.*, 1998).

### 3.2.3.5 *Test for Saponins*

0.5g of the extract was mixed with water in a test tube and shaken. A froth which persists for 15 minutes was taken as preliminary evidence for the presence of saponins (Silva *et al.*, 1998).

### 3.2.3.6 *Test for Steroids*

Liebermann-Buchard test: A small portion of the extract was dissolved in 2mls chloroform. Sulphuric acid was carefully added to form a lower layer. A reddish brown colour at the interface indicates the presence of a steroidal ring (Evans, 2009).

Salkowski test: A small quantity of the extract was dissolved in 1ml chloroform and to it 1ml of concentrated H<sub>2</sub>SO<sub>4</sub> was added down the test tube. Formation of red or yellow coloration was taken as an indication for the presence of steroidal ring nucleus (Sofowora, 1993).

### 3.2.3.7 *Test for Terpenes*

A little portion of the extract was dissolved in ethanol. To it 1ml of acetic acid was added followed by the addition of conc. H<sub>2</sub>SO<sub>4</sub>. Change of colour from pink to violet show the presence of terpenes (Sofowora, 1993).

### 3.2.3.8 *Test for Tannins*

#### Lead Sub-acetate Test

To a small portion of the fraction, 3-5 drops of lead acetate solution was added. Presence of precipitate was observed (Silva *et al.*, 1998).

### 3.2.3.9 *Test for cardiac glycosides*

Salkoski test was used to identify cardiac glycosides. 0.5g of the extract was dissolved in 2ml of chloroform and sulphuric acid carefully added to form a lower layer.

Formation of reddish-brown colour at the interface indicated the presence of steroidal ring (i.e. aglycone portion of the cardiac glycoside) (Sofowora 1993).

### **3.3 CHROMATOGRAPHIC STUDIES**

The TLC of all the fractions were ran. Hexane fraction happens to be the fraction with promising spots.

#### **3.3.1 Column Chromatography of Hexane Fraction**

7g of the hexane fraction was weighed and subjected to extensive column chromatography; some mls of hexane were added to dissolve the fraction. It was followed by addition of silica gel and dried before it was mounted on the column.

##### *3.3.1.1 Packing of the column*

The column was packed with n-Hexane and silica gel using wet slurry method. The sample was mounted on top of the silica gel, but prevented from having direct contact with the silica gel by the use of cotton. Wet method of packing was used in packing the column.

The column was eluted using hexane → hexane/ethyl acetate → ethyl acetate as gradient mixture solvent systems of increasing polarity. Several eluates collected were monitored using TLC, and those with similar Thin Layer Chromatographic profile were pooled together as a fraction, evaporated at reduced pressure.

### 3.3.2 Thin-layer Chromatography(TLC)

Thin-layer chromatography was carried out using TLC pre coated plates (Silica gel 60F<sub>254</sub>) by one way ascending technique. Spotting was carried out manually using capillary tubes and developed in an air tight chromatographic tank at room temperature.

#### 3.3.2.1 Solvent systems

Various solvent systems were used to develop the plates, including;

- |  |                           |
|--|---------------------------|
| a) Hexane: Ethyl acetate                     | (9:1, 8:2, 7:3,6:4, 1:1,) |
| b) n-hexane                                  | (100%)                    |
| c) Ethylacetate                              | (100%)                    |
| d) n-Butanol: Acetic acid: Water             | (4:1:5)                   |
| (Upper layer)                                |                           |
| e) Ethylacetate ; Hexane                     | (8:2, 7:3 )               |
| f) Chloroform: Ethylacetate: Methanol: Water | (15: 8: 4:1)              |

Developed chromatograms were air dried and visualized;

- Under normal day light
- Under ultra violet light (254nm & 366nm)
- By spraying with 10% sulphuric acid followed by heating at 105°C for 5-10mins in an oven

**Table 3.1: Column Chromatographic Studies of Hexane fraction**

S/No	Eluting solvent	Collections	TLC Status	Codes
1.	n-Hexane 100%	1-10	Similar profile	H <sub>1</sub>
2.	Hex : Ethyl acetate 97.5 : 2.5	11-17	No profile	Nil
3.	Hex : Ethyl acetate 92.5 : 7.5	18-32	18-26 similar profile 27-32 No profile	H <sub>2</sub> Nil
4.	Hex : Ethyl acetate 90 : 10	33-38	33-36 Similar profile 37-38 No profile	H <sub>3</sub> Nil
5.	Hex : Ethyl acetate 85 : 15	39-57	40-43 Similar profile 44-50 Similar profile 51-57 Similar profile	H <sub>4</sub> H <sub>5</sub> H <sub>6</sub>
6.	Hex : Ethyl acetate 80 : 20	58 – 69	Similar profile	H <sub>7</sub>
7.	Hex : Ethyl acetate 75 : 25	70 – 80	Similar profile with H7	H <sub>7</sub>
8.	Hex : Ethyl acetate 70 : 30	81 – 91	Similar profile	H <sub>8</sub>
9.	Hex : Ethyl acetate 65 : 35	92 – 97	Empty beakers (no profile)	Nil
10.	Hex : Ethyl acetate	98 – 102	Empty beakers	Nil

	60 : 40		(no profile)	
11.	Hex : Ethyl acetate 50 : 50	103 - 113	103-108 Similar profile 109-113 Similar profile	H <sub>9</sub> H <sub>10</sub>
12.	Hex : Ethyl acetate 50 : 40	114 – 119	Empty beakers (no profile)	Nil

Total of 119 fractions (10mls) were collected.

Fractions 40-47 gave major compound which was further purified using Preparative Thin Layer chromatography and solvent system of hexane – ethyl acetate (8.5 : 1.5) to afford compound coded M<sub>1</sub> a brownish red component which was worked-up to white homogenous crystalline .

### 3.3.3 Preparative Thin-layer Chromatography (PTLC) of Fraction H<sub>4</sub>

a) Silica Plates used: Preparative TLC was performed using pre-coated glass plate 20 x 20 cm and 0.25mm thickness.

b) Sample application: fraction H<sub>4</sub> was faintly dissolved in methanol. Capillary tubes were used to uniformly apply the dissolved sample on a thin line marked with pencil about 2.5cm from the bottom of the plate and allowed to dry.

c) Solvent system used: the dried plates were developed in a chromatographic tank using hexane: ethyl acetate (8.5:2.5) solvent system in sufficient quantity.

The region containing band of interest was marked and scrapped off. The scrapped sorbent was dissolved with methanol and filtered and repeatedly extracted with the mixture of hexane: ethyl acetate; the solution obtained was evaporated to afford a reddish brown compound M<sub>1</sub>.

### **3.3.4 Characterization of Compound M<sub>1</sub>**

#### a. Solubility Test:

The solubility of M<sub>1</sub> was observed in Dichloromethane, Chloroform, Acetone and Methanol.

#### b. Chemical Test

The isolated compound M<sub>1</sub> was subjected to Liebermann Burchard's test.

#### c. Melting Point determination

The melting point of the isolated compound M<sub>1</sub> was determined using melting point apparatus.

### **3.3.5 Spectral Analysis**

The isolated compound M<sub>1</sub> was subjected IR and NMR analysis.

## 3.4

## PHARMACOLOGICAL STUDIES

### 3.4.1 Acute toxicity studies

The method described by Lorke (1983) was employed. The route of administration was intraperitoneal. In the first phase, nine mice of either sex were divided into three groups containing three mice each. The first, second and third groups received 10 mg/kg, 100 mg/kg and 1000 mg/kg respectively. In the second phase, four animals were used. Each of the four animals received different doses of the extract which are: 1200 mg/kg, 1600 mg/kg, 2900 mg/kg and 5000 mg/kg. The median lethal dose was calculated as

$$LD_{50} = \sqrt{\text{Minimum animal dose died} \times \text{Maximum animal dose died}}$$

### 3.4.2 Venom sampling

The venom was collected by the usual milking method of Markfarlane (1967) from locally caught *Echisocellatus* (kept at the Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria). The venom was pooled separately, freeze-dried and stored prior to the experiments.



Plate III: Picture of *E. ocellatus*

Plate IV: Milking of *E. ocellatus*

### 3.4.3 Lethality assay of the venom

The venom was reconstituted with normal saline and concentration of  $10 \text{ mgml}^{-1}$  was obtained. The method of Tuner, 1965 was adopted where five groups of mice ( $n=10$ ) were injected intra-peritoneally with 10 ml of different doses of 0.00, 1.50, 1.80, 2.10, 2.40 and  $2.70 \text{ mgml}^{-1}$  respectively of the reconstituted venom. The control group which is group 1 (10 mice) received  $0.00 \text{ mgml}^{-1}$ . The time of death was recorded over a period of 24 h after the administration of the venom.

The assay was carried out for the venom of *E. ocellatus*. The  $\text{LD}_{99}$  value (minimum lethal dose, MLD) was determined by probit analysis.

#### **3.4.4 *In vivo* snake venom detoxifying effect of the methanol extract**

The method described by Theakston and Reid, (1983) was employed. Twenty four mice were used and they were divided into four groups (n=6). Group 1 (control) received MLD (LD<sub>99</sub>) of the venom. Groups 2, 3 and 4 (test groups) received three different doses (mg/kg) i.e LD<sub>50</sub> of the extract. The test groups were then injected with MLD of the venom 30 minutes after injecting the extract. The route of administration was intra-peritoneal. All animals were observed for mortality for 24hrs.

#### **3.4.5 *In vitro* snake venom detoxifying effect of the methanol extract**

The method described by (Abubakaret *al.*, 2000) was employed in this protocol. Twenty four mice were divided into four groups (n=6). Group 1 (control) received the MLD (LD<sub>99</sub>, 2.2 ((mg/kg) of the venom only. Groups 2, 3 and 4 (treatment groups) received MLD of the venom containing three different doses (mg/kg) i.e LD<sub>50</sub> of the extract. The venom and the extract were both incubated at 37<sup>0</sup>C for 10 minutes prior to injection. The route of administration was intra-peritoneal. The animals were observed for any sign of mortality for 24hrs

#### **3.4.6 Analgesic Studies**

##### **Acetic acid-induced abdominal constrictions in mice**

The method described by (Kosteret *al.*, 1959) was used. 30 albino mice were divided into 5 groups of 6 mice each. Group 1 was injected with 10 ml/kg *i.p* of normal saline (negative control). Groups 2, 3 and 4 were injected with 50 mg/kg, 100 mg/kg and 150 mg/kg *i.p* of the methanol extract (ME) respectively. While group 5 was injected with piroxicam 10 mg/kg

(positive control) Thirty minutes later, each mouse was injected with 10 ml/kg of aqueous solution of acetic acid (0.6%). The number of abdominal constrictions for each mouse was counted 5 minutes after injection of acetic acid for a period of 10 minutes.

The percentage inhibition of abdominal constrictions was calculated using the following formula:

$$\text{Inhibition (\%)} = \frac{\text{Mean no. of writhes (control)} - \text{Mean no. of writhes (Test)}}{\text{Mean no. of writhes (control)}} \times 100$$

## **CHAPTER FOUR**

### **4.0 RESULTS AND DISCUSSIONS**

#### **4.1 YIELD**

The powdered leaves of *Leptadenia hastata* gave a greenish residue weighed 20.34 g. The hexane fraction HF gave a green residue (oily) weighed 7.20 g and the ethylacetate fraction (EF) yielded a reddish brown residue weighed 1.0 g. n-butanol fraction (BF) gave a reddish brown residue weighed 2.0 g and chloroform Fraction (CF) gave a brown residue weighed 1.0 g.

#### **4.2 RESULT OF PRELIMINARY PHYTOCHEMICAL SCREENING**

Preliminary phytochemical screening of the methanol extract revealed the presence of carbohydrates, alkaloids, flavonoids, anthraquinones, saponins, tannins, cardiac glycoside, steroids and terpenes as shown in table 4.1

**Table 4.1: Phytochemical Constituents of ME, HF, EF, CF and BF**

<b>TEST</b>	<b>Methanol extract (ME)</b>	<b>Hexane Fraction (HF)</b>	<b>Chloroform Fraction (CF)</b>	<b>Ethyl acetate Fraction (EF)</b>	<b>Butanol Fraction (BF)</b>
Carbohydrate	+	-	-	-	+
Alkaloids	+	-	-	-	-
Flavonoids	+	-	-	+	-
Anthraquinones	+	-	-	-	-
Saponins	+	-	-	+	+
Triterpenes	+	+	+	+	-
Steroids	+	+	+	+	-
Tannins	+	-	+	+	+
Cardiac glycosides	+	-	+	+	+

keys:           + = presence

                  □ = absence

### 4.3

### RESULT OF THINLAYERCHROMATOGRAPHY

#### 4.3.1 Result of Thin-Layer Chromatography of Crude Methanol fraction

Thin-layer chromatographic analysis of crude methanol fraction using chloroform: ethyl acetate: methanol : water (4: 8: 4: 1) as the solvent system, revealed the presence of some major spots.



**Plate V: TLC Profile of Methanol extract**

#### 4.3.2 Result of Thin-layer Chromatography of Hexane fraction HF

Thin-layer chromatographic analysis of hexane fraction and chloroform fraction using Pet ether: ethyl acetate (8:2) as solvent system revealed the presence of 7 and 3 major spots respectively when sprayed with 10% sulphuric acid (Plate VI)



**Plate VI: TLC profile of Hexane fraction (HF) and Chloroform fraction (CF)**

### 4.3.3 Result of Thin-layer Chromatography of Various fractions of HF obtained from column chromatography

Having conducted the TLC of all the collections and merged, the TLC of the various fractions of HF made using hexane: ethyl acetate (3:1) as solvent system revealed various spots as shown below (Plate VII) and (Plate VIII);

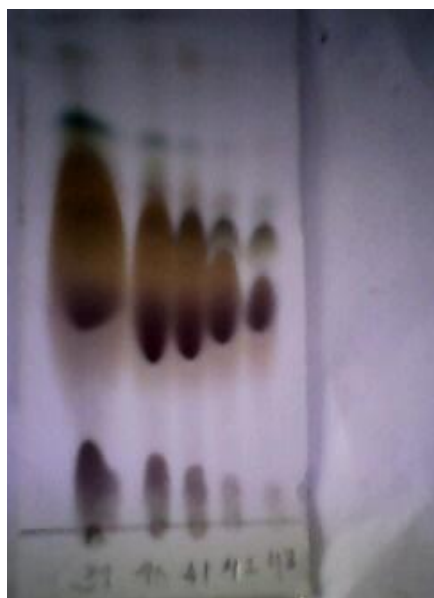


Plate VII

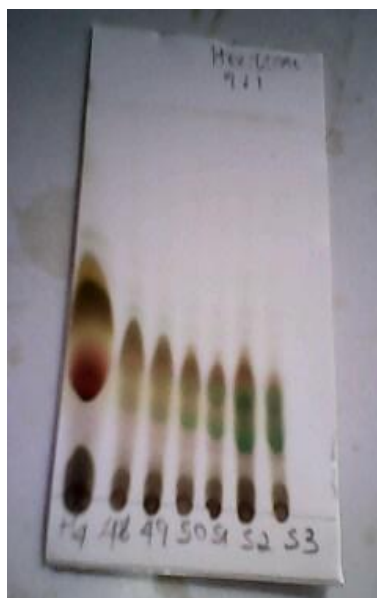


Plate VIII

**Plate VII and VIII: TLC of various fractions of HF**

**Table 4.2: Result of Column Chromatography of the Hexane fraction**

<b>Fraction</b>	<b>Eluting solvent</b>	<b>Collections</b>	<b>No of clear spots</b>
H <sub>1</sub>	Hexane 100%	1-10	2 spots
H <sub>2</sub>	Hexane : Ethyl acetate 92.5:7.5	18-32	3 spots
H <sub>3</sub>	Hexane : Ethyl acetate 90:10	33-36	2 spots
H <sub>4</sub>	Hexane : Ethyl acetate 85:15	39-43	3 spots
H <sub>5</sub>	Hexane : Ethyl acetate 85:15	44-50	5 spots
H <sub>6</sub>	Hexane : Ethyl acetate 85:15	51-57	No clear spots
H <sub>7</sub>	Hexane : Ethyl acetate 80:20, 75:25	58-80	No clear spot
H <sub>8</sub>	Hexane : Ethyl acetate 70 : 30	81-91	2 spots
H <sub>9</sub>	Hexane : Ethyl acetate 50 : 50	103-108	3 spots
H <sub>10</sub>	Hexane : Ethyl acetate 50 : 50	109-113	2 spots

## 4.4

## ISOLATION

### 4.4.1 Isolation of $M_1$

#### 4.4.1.1 Result of Preparative Thin Layer Chromatography of $M_1$

Fraction  $H_4$  containing one major spot with little impurity was purified using preparative Thin Layer Chromatography, hexane: ethyl acetate (8:2) and hexane: ethyl acetate (8.5 : 2.5) as solvent system for plate IX and Plate X respectively. 10% Sulphuric acid was used as the spraying reagent. It was subjected to chemical and spectroscopic analysis to elucidate its chemical structure.



Plate IX : Hexane: Ethylacetate (8:2)



Plate X : Hexane : Ethylacetate (8.5 : 2.5)

**Plate IX and X: TLC profile of Hexane fraction 4 ( $H_4$ )**

#### 4.4.1.2 Thin-layer Chromatographic Analysis of $M_1$

TLC analysis of the isolated compound  $M_1$  using hexane: ethylacetate (8:2) and (7:3) as solvent system viewed under normal day light and sprayed with 10% sulphuric acid respectively revealed single homogenous spot.

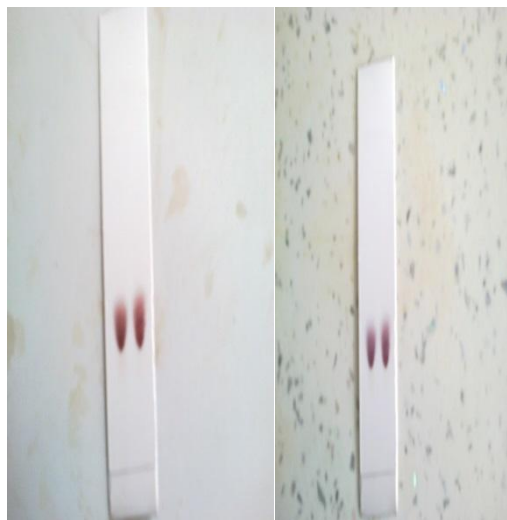


Plate XI: Hex:Ethylacetate (8:2)

Plate XII: Hex:Ethylacetate (7:3)

#### **Plate XI and Plate XII: TLC Profile of isolated compound $M_1$**

#### 4.4.2 Melting point determination

The melting point of the isolated compound  $M_1$  was found to be between  $120^{\circ}\text{C} - 121^{\circ}\text{C}$

#### 4.4.3 Chemical test on Compound $M_1$

$M_1$  (white crystalline substance) gave positive test to Liebermann Buchard reagent for steroidal nucleus.

## 4.5

## SPECTRAL ANALYSIS OF $M_1$

### 4.5.1 Infrared Spectral Analysis of $M_1$

Major bands:  $3427.62\text{cm}^{-1}$  (O-H stretching);  $2940\text{ cm}^{-1}$ (C-H aliphatic stretching);  $1457.27\text{cm}^{-1}$  (C=C olefinic stretching),  $1054\text{cm}^{-1}$  (C-O Stretching) as shown in figure 4.1.

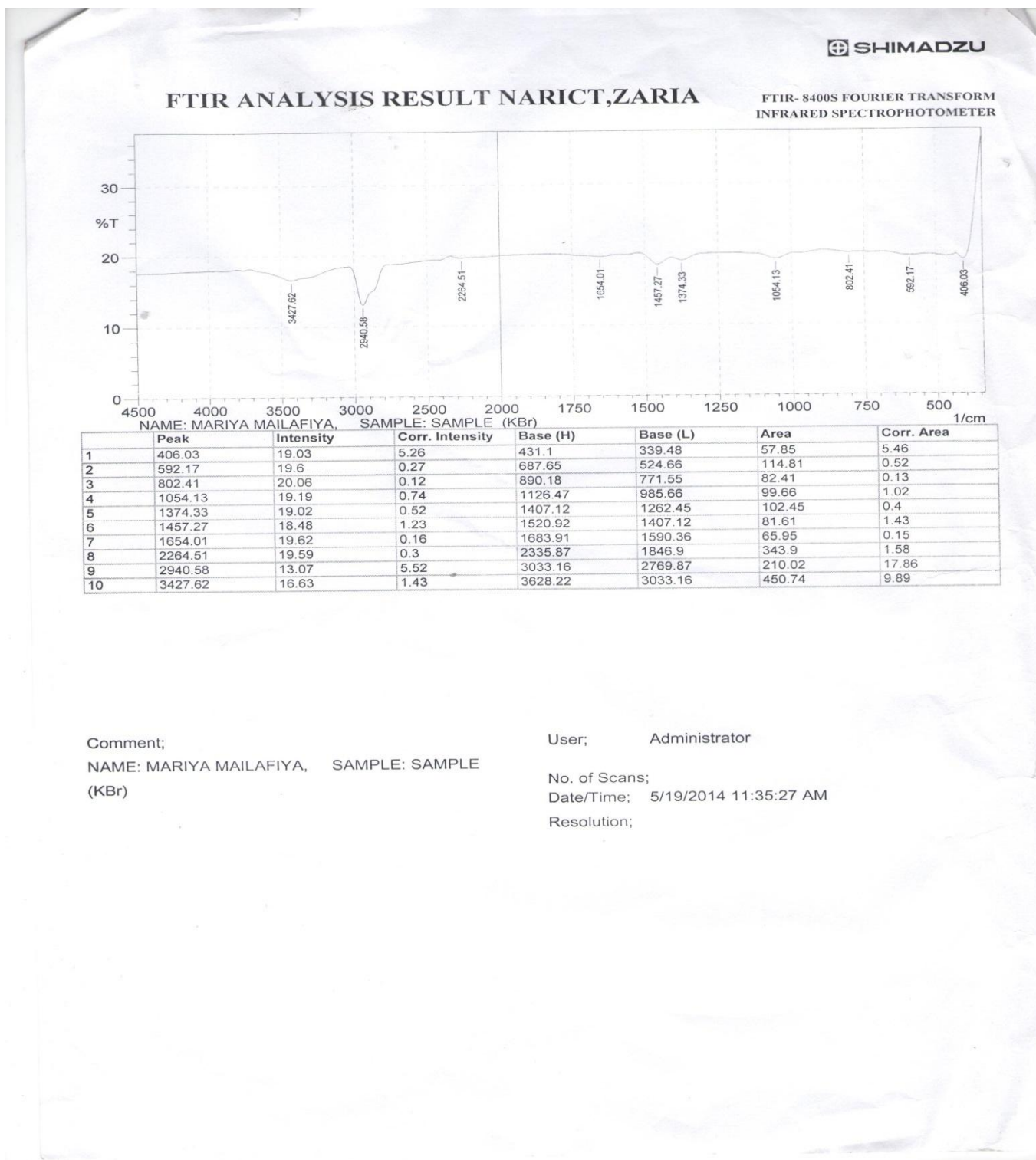


Figure 4.1: Infrared spectrum of M<sub>1</sub> in KBr

#### 4.5.2 Proton Nuclear Magnetic Resonance Spectral Analysis of M<sub>1</sub>

Overlapping protons between, six methyl protons at  $\delta_{\text{H}}$  0.74, 0.87, 0.88, 0.89, 0.93 and 1.06 (3H each),  $\delta_{\text{H}}$  2.30. (2H, m H-4),  $\delta_{\text{H}}$  3.50 (1H, m H-3) suggesting a carbinol proton in steroids or triterpenes nucleus and  $\delta_{\text{H}}$  5.39 (1H, d H-6) representing an olefinic protons as shown in figure 4.2 (Thomas *et al.*, 2006)

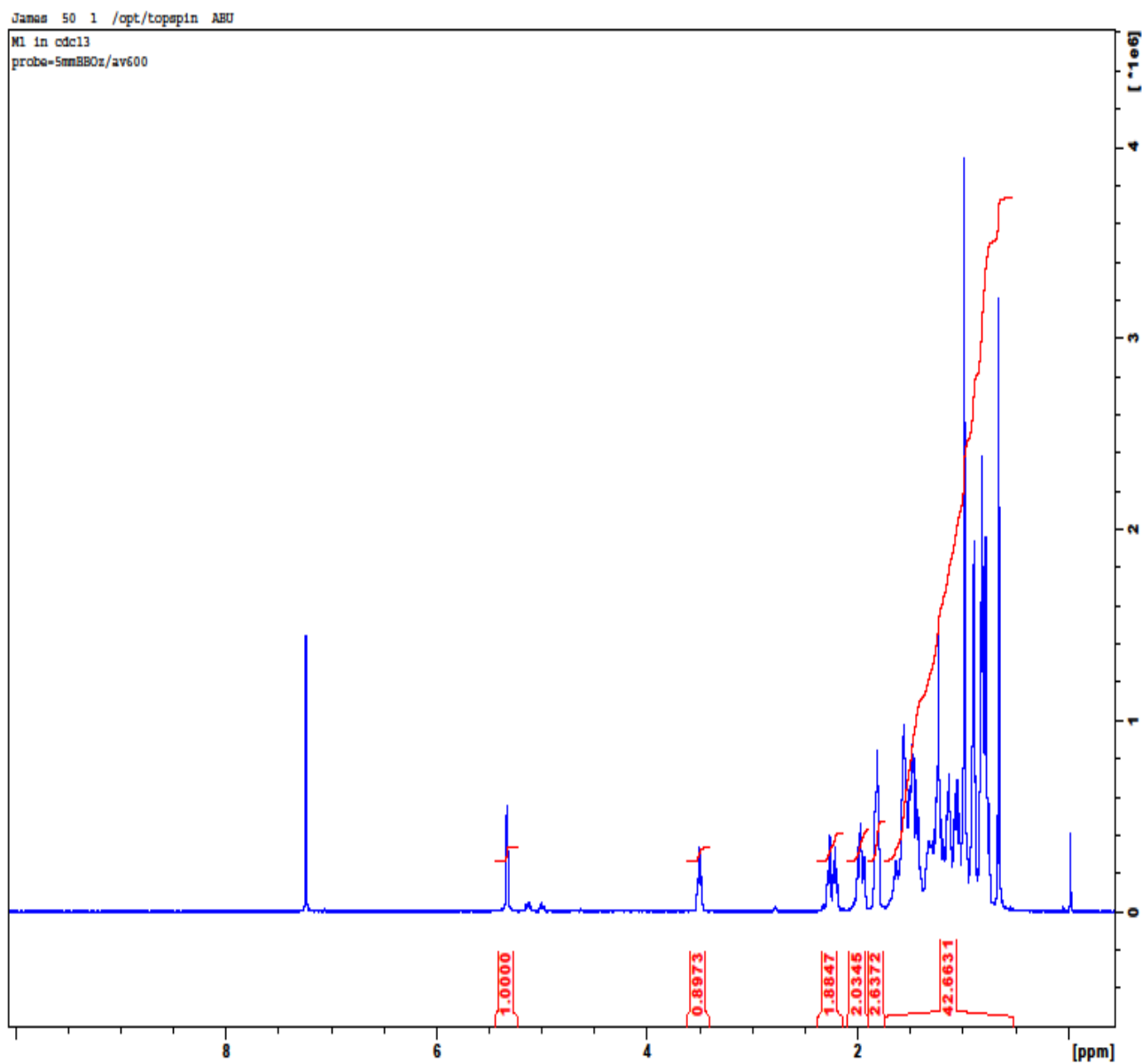


Figure 4.2:  $^1\text{H}$ -NMR spectrum of  $\text{M}_1$  in  $\text{CDCl}_3$

### 4.5.3 $^{13}\text{C}$ NMR and $^{13}\text{C}$ -DEPT NMR Spectral Analysis of $\text{M}_1$

$^{13}\text{C}$ -NMR ( $\delta$  ppm, 125 MHz,  $\text{CDCl}_3$ ) revealed the presence of 29 C-signals & DEPT- indicated the following carbons and their multiplicity: C=3, CH=11,  $\text{CH}_2$ =9,  $\text{CH}_3$ =6.

140.8(q), 121.7(CH), 56.8(CH), 77.33-76.81(solvent), 71.80(HC-OH), 56.1(CH), 50.2(CH), 45.90(CH), 36.20(CH), 31.90(CH), 29.20(CH), 42.30(q), 39.68( $\text{CH}_2$ ), 37.30( $\text{CH}_2$ ), 36.50(q), 31.90( $\text{CH}_2$ ), 31.90(CH), 42.20( $\text{CH}_2$ ), 29.15(CH), 21.10( $\text{CH}_2$ ), 39.80( $\text{CH}_2$ ), 24.30( $\text{CH}_2$ ), 19.80( $\text{CH}_3$ ), 28.30( $\text{CH}_2$ ), 33.90( $\text{CH}_2$ ), 26.10 ( $\text{CH}_2$ ), 23.10( $\text{CH}_2$ ), 19.4 ( $\text{CH}_3$ ), 12.20( $\text{CH}_3$ ) (Table 4.3) (Figure 4.3 and 4.4)

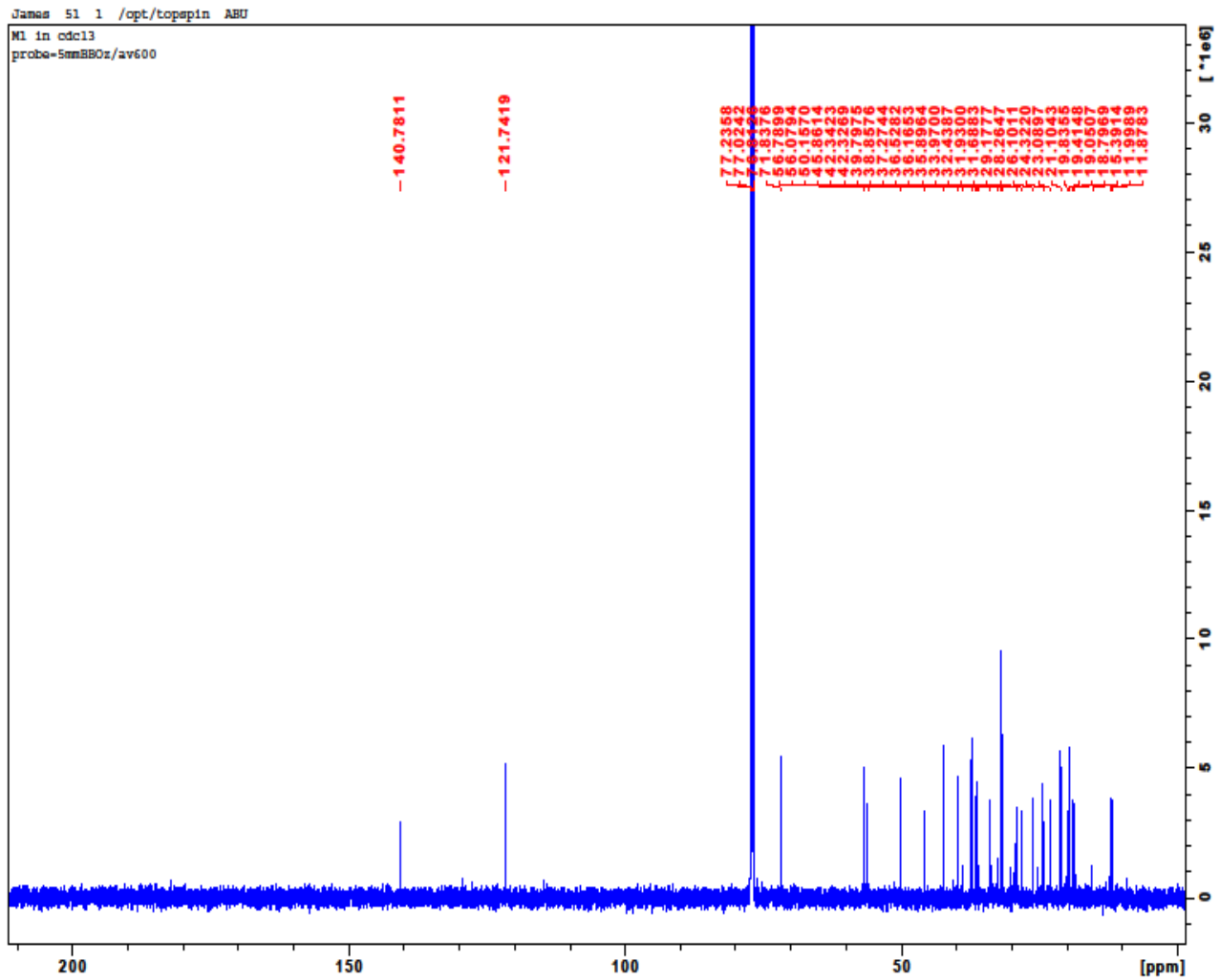


Figure 4.3:  $^{13}\text{C}$ -NMR spectrum of  $\text{M}_1$  in  $\text{CDCl}_3$

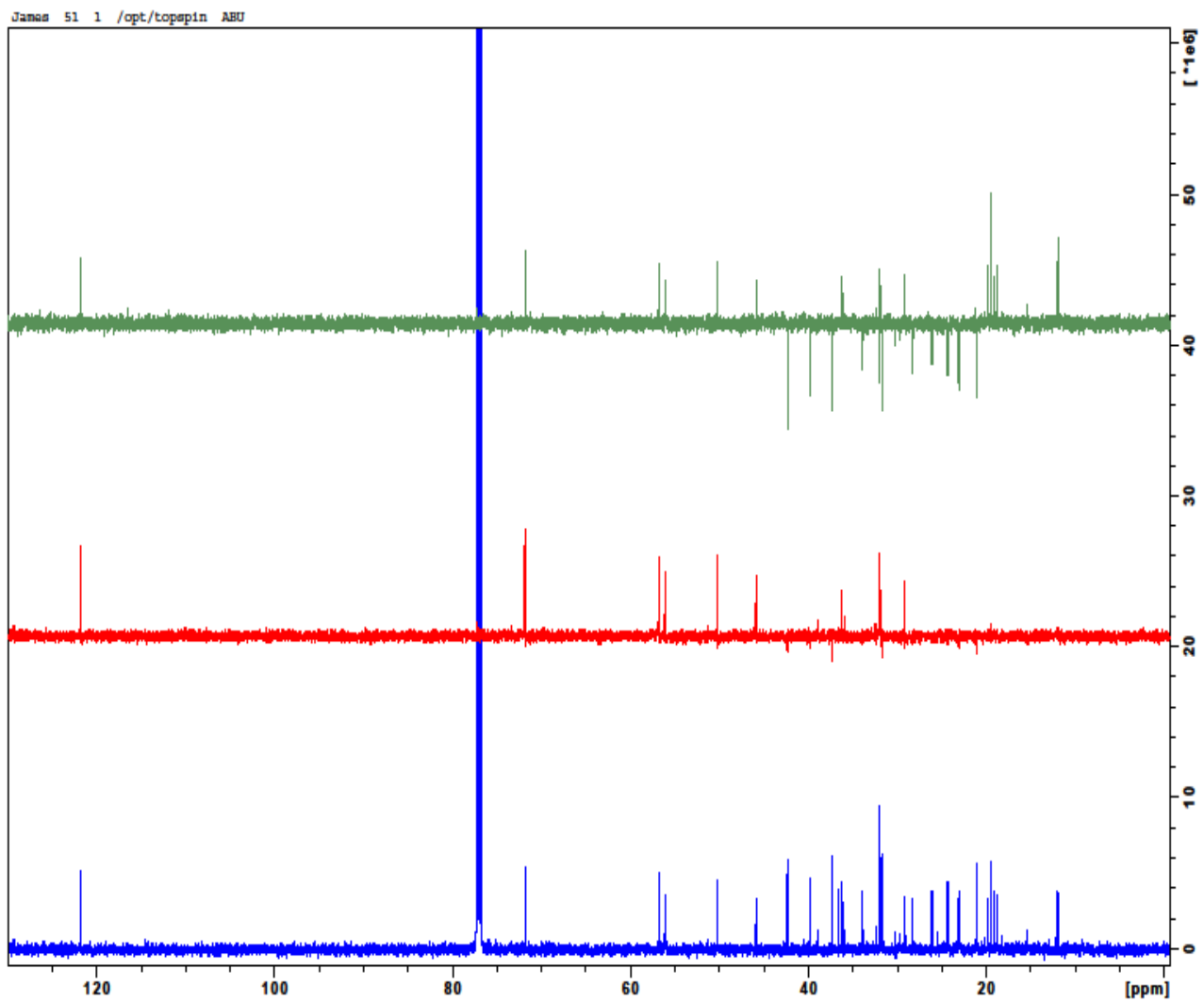


Figure 4.4:DEPT-NMR spectrum of M<sub>1</sub> in CDCl<sub>3</sub>

#### 4.5.4 HSQC Analysis of M<sub>1</sub>

The HSQC spectrum of M<sub>1</sub> figure (4.5) showed the attachment of protons to their respective carbon atom at (Table 4.3)

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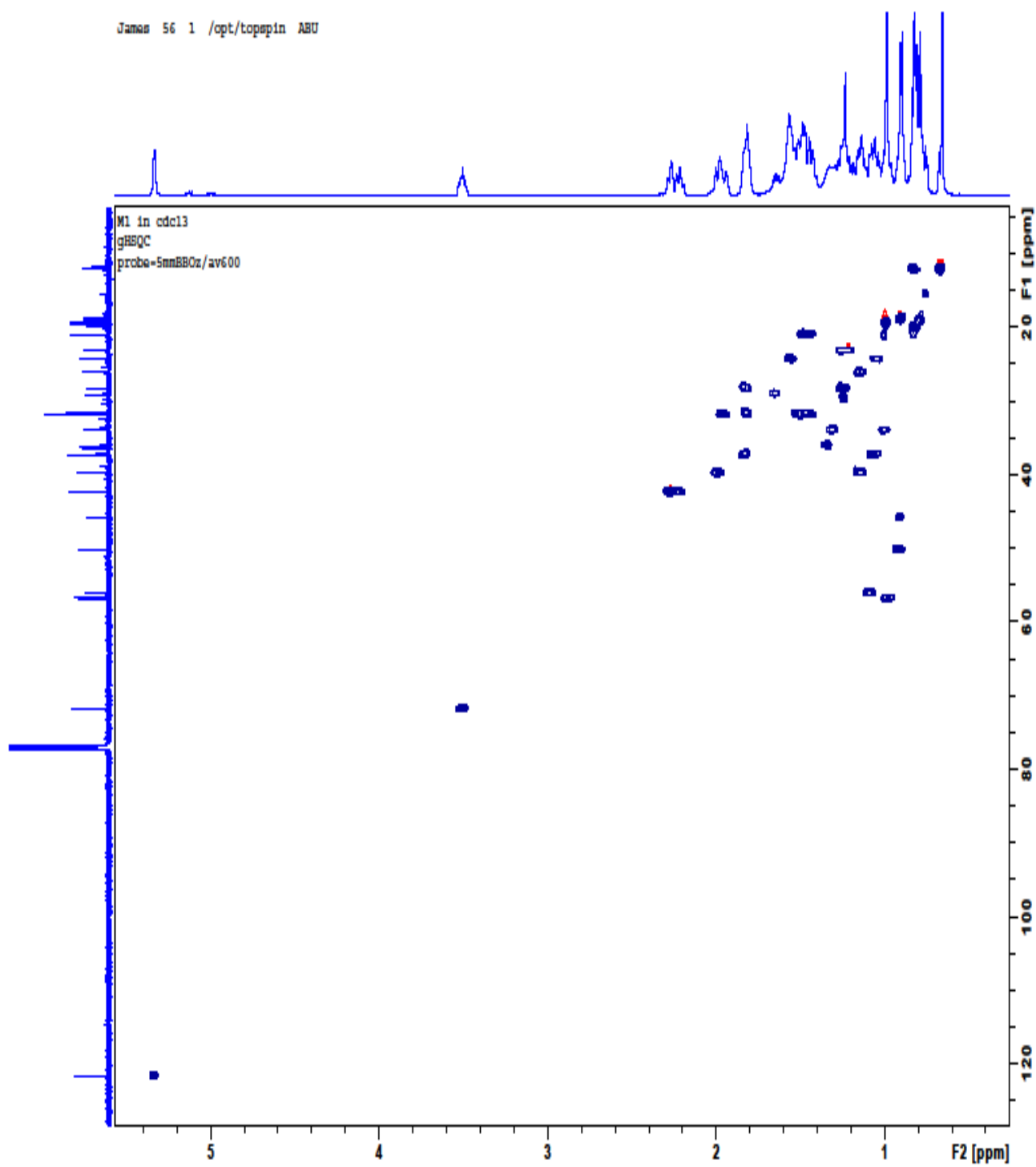


Figure 4.5: HSQC spectrum of M<sub>1</sub> in CDCl<sub>3</sub>

#### 4.5.5 $^1\text{H}$ - $^1\text{H}$ COSY Spectral Analysis of $\text{M}_1$

The 2D  $^1\text{H}$ - $^1\text{H}$  COSY correlation spectrum of  $\text{M}_1$  indicates the protons that are situated in the same environment, major correlations include: H6(5.35) / H7 (1.91), H6(5.35) / H8 (1.91), H3(3.52) / H4(2.27), H2(1.50) / H3(3.52), H13(1.75) / H16(1.22), H12(1.98) / H18 (0.76), H19(1.10) / H1(1.83), H1(1.83) / H2(1.50), H14(0.95) / H15(1.52), H17(1.04) / H20(1.28), H20(1.28) / H21(0.83), H9(0.91) / H11(1.43), H8(1.91) / H14(0.95), H25(1.22) / H26(0.81), H25(1.22) / H27(0.83), H24(0.50) / H25(1.22) e.t.c. (Figure 4.6)

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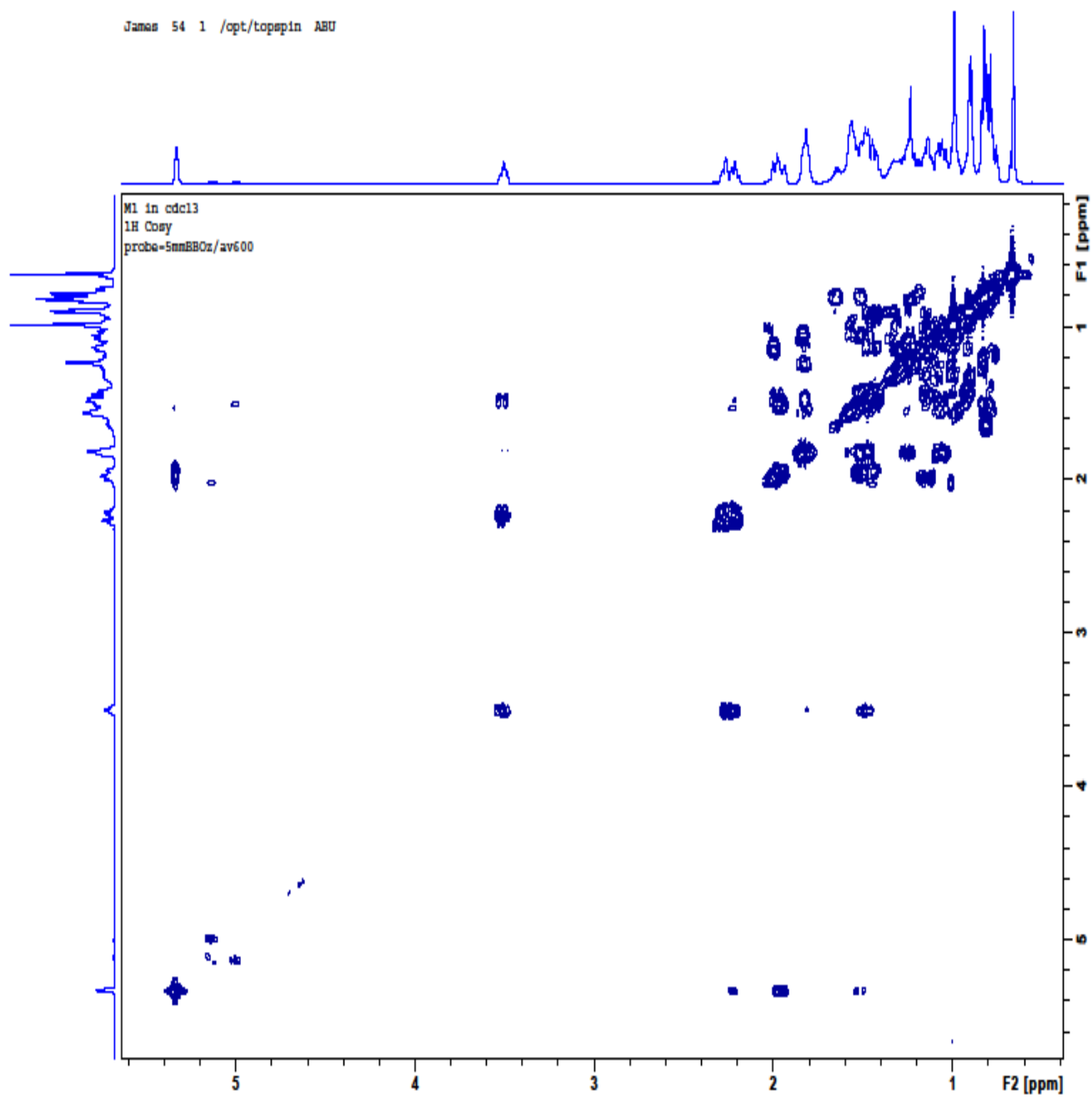


Figure 4.6:  $^1\text{H}$ - $^1\text{H}$ -Cosy of  $\text{M}_1$  in  $\text{CDCl}_3$

#### 4.5.6 HMBC Spectrum Analysis of M<sub>1</sub>

The HMBC spectrum allowed establishing the connectivity between the various units of the molecule. Proton H1 showed correlation with C2, C3 and C4, Proton H4 showed correlation with C1, C2, C3, C5, and C6, Proton H6 correlated with C4, C5 and C7, Proton 29 showed correlation with C24, C25, C28, Proton H11 showed correlation with C1, C10, C12, C14 e.t.c. (Figure 4.7)

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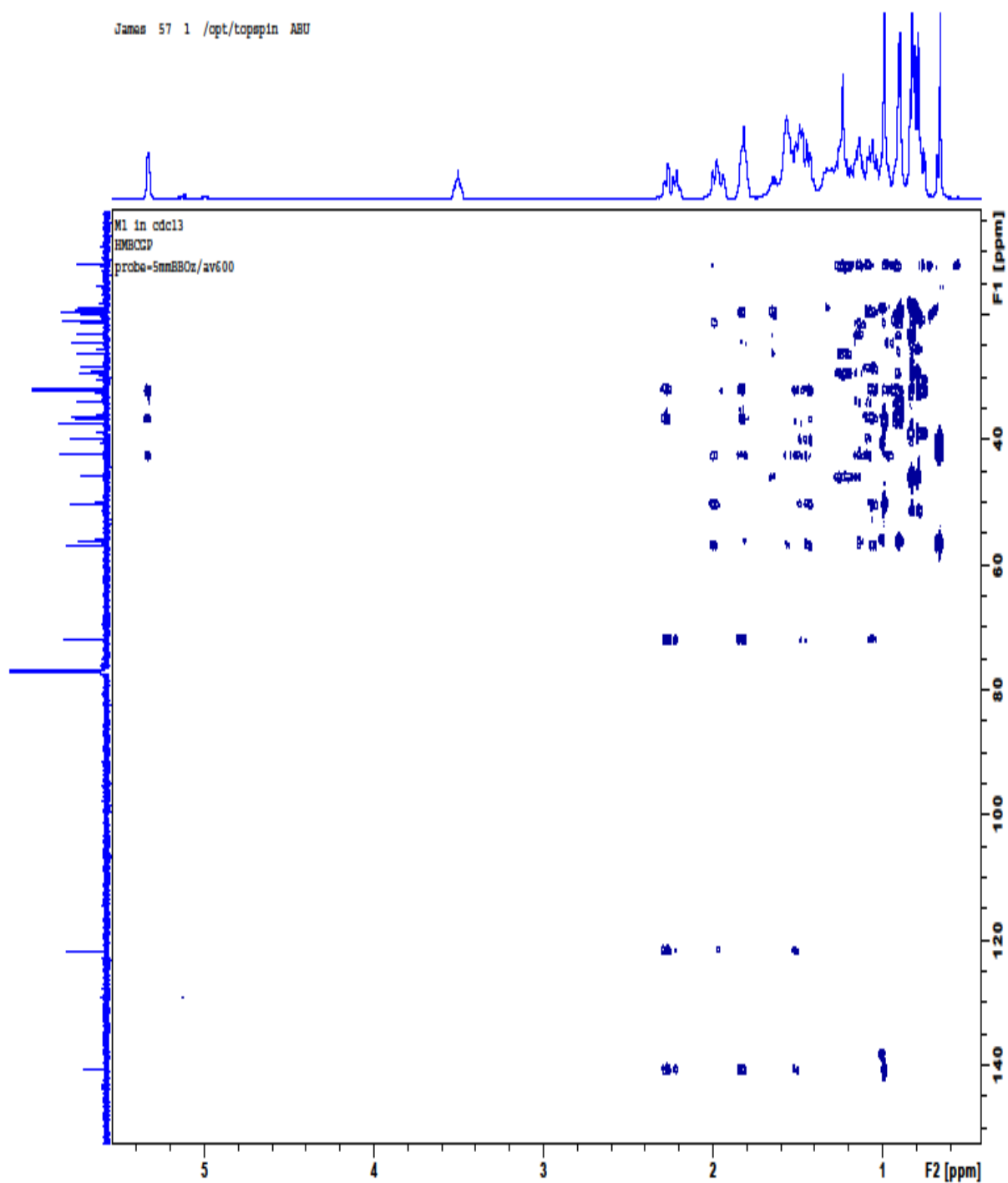


Figure 4.7: HMBC of M<sub>1</sub> in CDCl<sub>3</sub>

#### 4.5.7 NOESY Spectrum Analysis of M<sub>1</sub>

Protons coupling through space. H (5.32) / 2.27, 1.50 and 1.92, H (3.52) / 2.27, 1.75, 1.50 and 1.04 e.t.c. (Figure 4.8)

James 55 1 /opt/topspin ABU

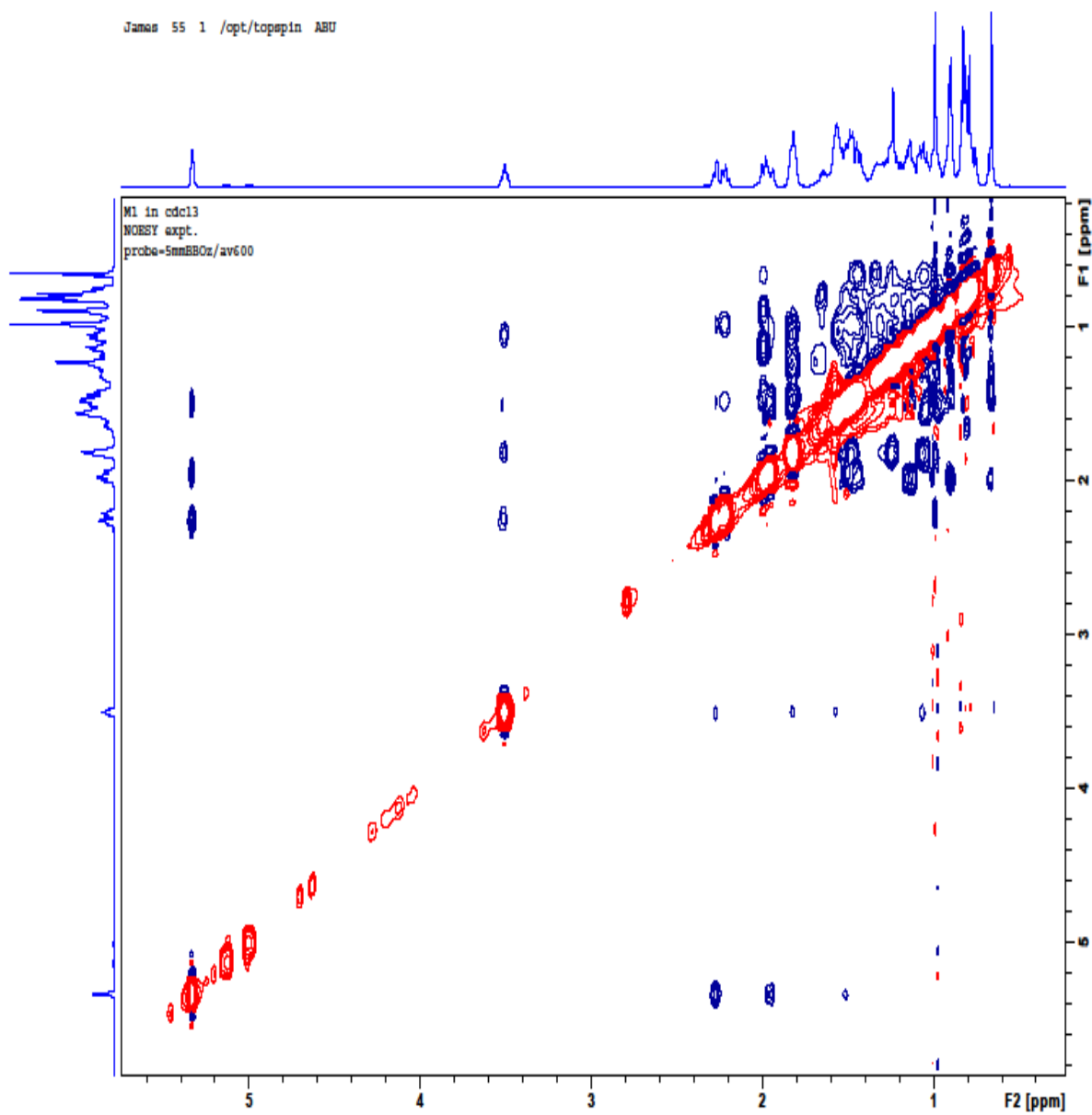


Figure 4.8: NOESY of M<sub>1</sub> in CDCl<sub>3</sub>

**Table 4.3:**  $^1\text{H}$  and  $^{13}\text{C}$ -NMR spectral summary data of compound  $\text{M}_1$  ( $\text{CDCl}_3$ )

Position	$\delta^{13}\text{C}$	$\delta^1\text{H}$ (J in Hz)
1	37.25	1.83
2	31.68	1.50
3	71.80	3.52
4	42.30	2.27
5	140.80	□
6	121.71	5.35
7	31.90	1.91
8	31.90	1.91
9	50.20	0.91
10	36.51	1.30
11	21.10	1.43
12	39.81	1.98
13	42.30	1.75
14	56.80	0.95
15	24.31	1.52
16	28.30	1.22
17	56.10	1.04
18	11.90	0.76
19	19.40	1.10s
20	36.20	1.28
21	18.80	0.83
22	34.00	1.30
23	26.10	1.10, 1.60
24	45.91	0.50
25	29.20	1.22
26	19.81	0.81
27	19.11	0.83
28	23.10	1.20
29	12.00	0.80

**Table 4.4:** 1D and 2D NMR spectral summary data for compound M<sub>1</sub> (CDCl<sub>3</sub>)

Position	$\delta$ <sup>1</sup> H (J in Hz)	$\delta$ <sup>13</sup> C	DEPT
1	37.25	1.83	CH <sub>2</sub>
2	31.68	1.50	CH <sub>2</sub>
3	71.80	5.52	CH
4	42.30	2.27	CH <sub>2</sub>
5	140.80	□	C
6	121.71	5.35	CH
7	31.90	1.91	CH <sub>2</sub>
8	31.90	1.91	CH
9	50.20	0.91	CH
10	36.51	1.30	C
11	21.10	1.43	CH <sub>2</sub>
12	39.81	1.98	CH <sub>2</sub>
13	42.30	1.75	C
14	56.80	0.95	CH
15	24.31	1.52	CH <sub>2</sub>
16	28.30	1.22	CH <sub>2</sub>
17	56.10	1.04	CH
18	11.90	0.76	CH <sub>3</sub>
19	19.40	1.10	CH <sub>3</sub>
20	36.20	1.28	CH
21	18.80	0.83	CH <sub>3</sub>
22	34.00	1.30	CH <sub>2</sub>
23	26.10	1.10, 1.58	CH <sub>2</sub>
24	45.91	0.50	CH
25	29.20	1.22	CH
26	19.81	0.81	CH <sub>3</sub>
27	19.11	0.83	CH <sub>3</sub>
28	23.10	1.20	CH <sub>2</sub>
29	12.00	0.84	CH <sub>3</sub>

**Table 4.5:**  $^1\text{H}$  &  $^{13}\text{C}$ -NMR data of  $\text{M}_1$  compared with those reported in literature

Position	$^{13}\text{C}$ -NMR $\text{M}_1$	$^1\text{H}$ -NMR $\text{M}_1$	$^{13}\text{C}$ -NMR (literature) (Patehet <i>et al.</i> , 2009)	$^1\text{H}$ -NMR
1	37.30	1.83	37.30	
2	31.68	1.50	31.60	
3	71.80	3.52m	71.80	3.52m
4	42.30	2.27	42.20	
5	140.80	□	140.80	
6	121.71	5.32s	121.70	5.358br,s
7	31.90	1.91	31.90	
8	31.90	1.91	31.90	
9	50.20	0.91	51.20	
10	36.51	1.30	36.50	
11	21.10	1.43	21.10	
12	39.81	1.98	39.80	
13	42.30	1.75	42.30	
14	56.80	0.95	56.80	
15	24.31	1.52	23.30	
16	28.30	1.22	28.30	
17	56.10	1.04	56.00	
18	11.90	0.68s	11.90	0.680s
19	19.40	1.10s	19.40	1.01s
20	36.20	1.28	36.20	
21	18.80	0.83	18.80	
22	34.00	1.30	33.90	
23	26.10	1.10, 1.58	26.10	
24	45.91	0.50	45.90	
25	29.20	1.22	29.20	
26	19.80	0.81	19.80	0.814(d, 6.5)
27	19.11	0.83	19.30	0.833(d, 6.5)
28	23.10	1.20	23.10	
29	12.00	0.84	12.00	0.845(t, 7.5)

## 4.6

## PHARMACOLOGICAL STUDIES

### 4.6.1 Result of acute toxicity studies

**Table 4.6: Determination of medianlethaldose (LD<sub>50</sub>) of Methanol extract of *Leptadeniahastata***

#### First Phase

Dose (mg/kg)	Number of mice used	Mortality
10	3	0/3
100	3	0/3
1000	3	0/3

#### Second Phase

Dose (mg/kg)	Number of mice used	Mortality
1200	1	0/1
1600	1	0/1
2900	1	0/1
5000	1	0/1

The leaves extract of *Leptadeniahastata* was found to be safe. The highest dose that did not kill any of the experimental animals (mice) was found to be a median lethal dose *i.p* (LD<sub>50</sub>). Hence, the LD<sub>50</sub> value was found to be greater than 5000 mg/kg.

$$\text{LD}_{50} > 5000 \text{ mg/kg i.p}$$

#### 4.6.2 Result of Median Lethal Dose (LD<sub>50</sub>) of the venom

**Table 4.7: Determination of lethal dose (LD<sub>50</sub>) of the venom of *Echisocellatus***

##### **First Phase**

Dose (mg/kg)	Number of mice used	Mortality
0.00	10	0
1.00	10	0
2.00	10	10
3.00	10	9
4.00	10	10
5.00	10	10

##### **Second**

Dose (mg/kg)	Number of mice used	Mortality
0.00	10	0
1.50	10	8
1.80	10	10
2.10	10	7
2.40	10	8
2.70	10	9

The intra-peritoneal LD<sub>50</sub> of the *Echisocellatus* venom was found to be 2.2 mg/kg using the probit analysis

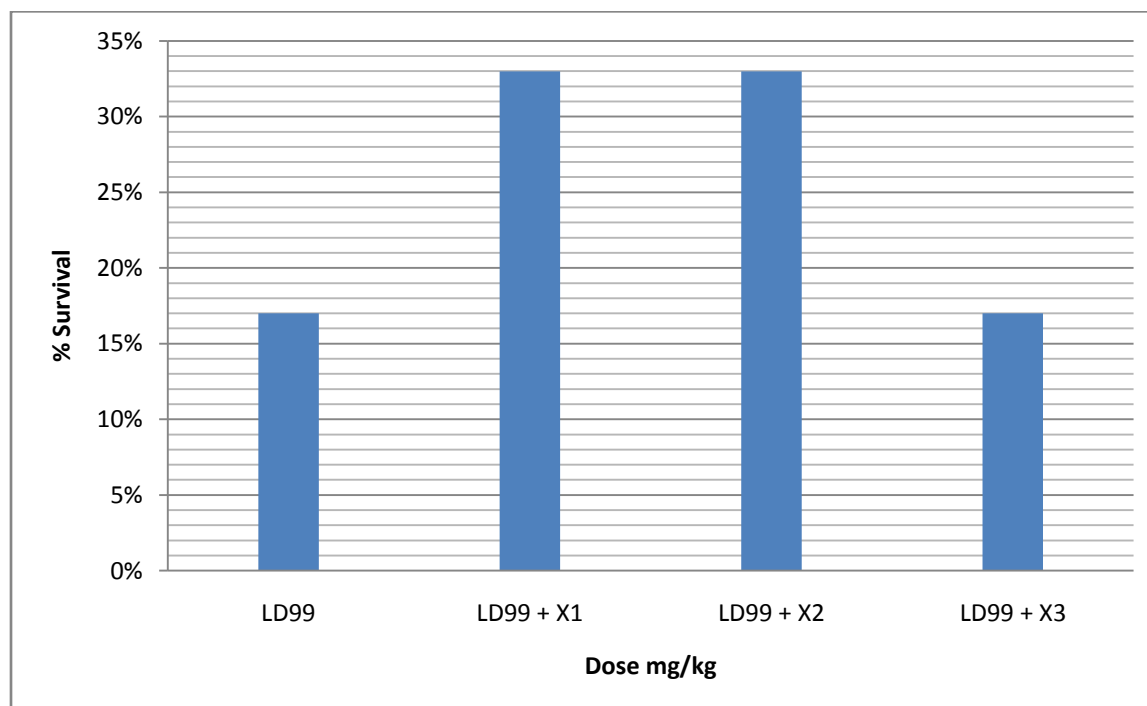
**4.6.3 Result of *In vitro* detoxifying effect of the methanol extract of *Leptadeniahastata* against *Echisocellatus* venom**

**Table 4.8: *In vitro* detoxifying effect of the methanol extract of *Leptadeniahastata* against *Echisocellatus* venom**

Group	Treatment	Number of Death	% Survival (within 24hrs)
n=6			
1	LD <sub>99</sub>	5	17
2	LD <sub>99</sub> + X <sub>1</sub>	4	33
3	LD <sub>99</sub> + X <sub>2</sub>	4	33
4	LD <sub>99</sub> + X <sub>3</sub>	5	17

Number of mice used (n) = 6

LD<sub>99</sub>= 2.2 mg/kg of the venom, X<sub>1</sub> = 150 mg/kg, X<sub>2</sub> = 100 mg/kg and X<sub>3</sub> = 50 mg/kg of the extract.



**Figure 4.9: Survival rate (%) for venom-injected mouse administered with different concentrations of *L. hastata* extract**

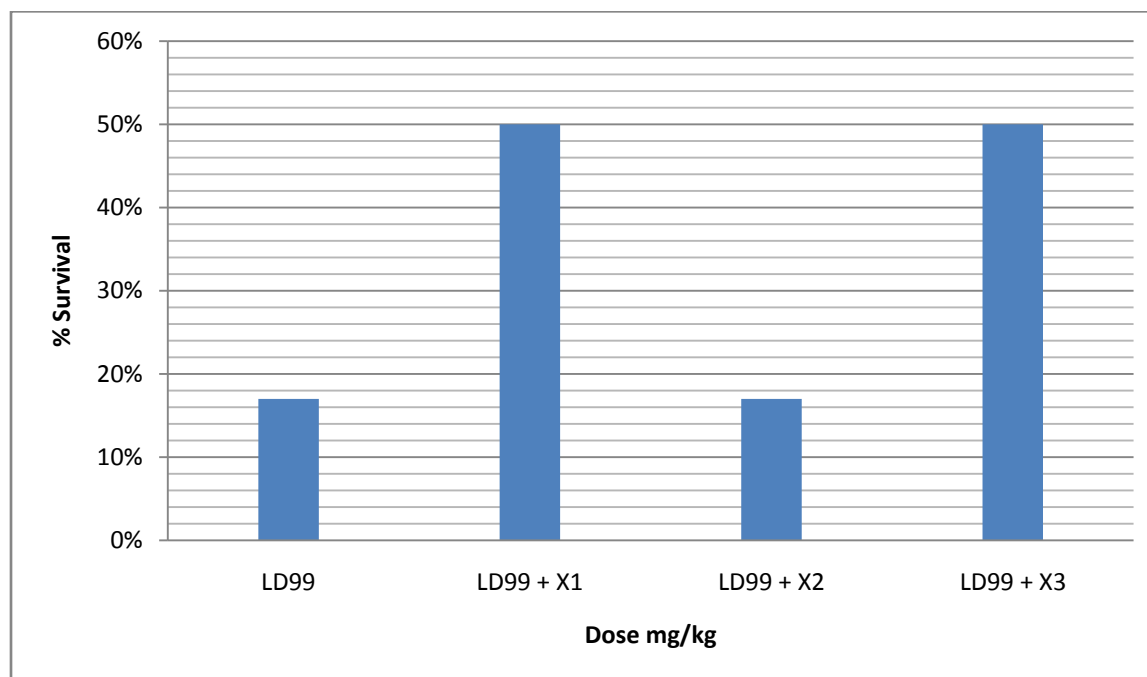
**4.6.4 Result of *In vivo* detoxifying effect of the methanol extract of *Leptadeniahastata* against *Echisocellatus* venom**

**Table 4.9: *In vivo* detoxifying effect of the methanol extract of *Leptadeniahastata* against *Echisocellatus* venom**

Group	Treatment	Number of Death	% Survival (within 24hrs)
<b>n=6</b>			
1	LD <sub>99</sub>	5	17
2	LD <sub>99</sub> + X <sub>1</sub>	3	50
3	LD <sub>99</sub> + X <sub>2</sub>	5	17
4	LD <sub>99</sub> + X <sub>3</sub>	3	50

Number of mice used (n) = 6

LD<sub>99</sub>= 2.2 mg/kg of the venom, X<sub>1</sub> = 150 mg/kg, X<sub>2</sub>= 100 mg/kg and X<sub>3</sub> = 50 mg/kg of the extract.



**Figure 4.10: Survival rate (%) for venom-injected mouse administered with different concentrations of *L. hastata* extract**

**4.6.5 Result of the effect of methanol extract of *Leptadenia hastata* against acetic acid induced writhing in mice**

**Table 4.10: Effect of methanol extract of *Leptadenia hastata* on acetic acid-induced writhing in mice**

Treatment (mg/kg)	Mean no. of writhes	% inhibition
Normal saline	31.2 ± 1.1	
ME 50 mg/kg	16.3 ± 1.2*	47.8
ME 100 mg/kg	8.00 ± 2.2*	74.4
ME 150 mg/kg	4.8 ± 0.7*	84.6
Piroxicam 10 mg/kg	10.1 ± 3.2	67.6

Each value represents mean ± SEM. \* $P < 0.05$  compared with control (student's t-test); n=6

## CHAPTER FIVE

### 5.0

### DISCUSSIONS

### 5.1

### PYTOCHEMICAL STUDIES

Preliminary phytochemical screening of methanol extract of the leaves of *Leptadeniahastata* indicates the presences of steroids, triterpenes, tannins, cardiac glycosides, flavonoids and saponins while the hexane fraction indicates the presence of triterpenes, steroids and cardiac glycosides.

Preparative Thin Layer chromatography carried out on the fraction H<sub>4</sub> collected from column chromatography of hexane fraction obtained from the methanol extract of *Leptadeniahastata* result in the isolation of a white crystalline compound of M<sub>1</sub> and revealed single homogenous spot using two different solvent.

The melting point of the compound was 120 °C – 121°C and it showed positive with Liebermann Buchard reagent for steroidal nucleus (Kandatiet *al.*, 2012; Rajuet *al.*, 2012 and Patehet *al.*, 2009)

The <sup>13</sup>C NMR revealed 29 C- signals. Six methyl groups at δc: 11.9 (C-18), 19.8 (C-19), 18.3 (C-21), 21.1 (C-26), 19.4 (C-27), and 11.9 (C-29), olefinic carbons appeared at δc: 140.8 (C-5) and 121.7 (C-6), and secondary hydroxyl bearing carbon at (δc: 71.8 (C-3) and Distortion less Enhancement by Polarization Transfer DEPT revealed the following carbons and their multiplicity; six methyl carbons at C-18, C-19, C-21, C-26, C-27, and C-29; eleven methylenecarbons at C-1, C-2, C-4, C-7, C-11, C-12, C-15, C-16, C-22, C-23, and C-28; nine

methinecarbons at C-3, C-6, C-8, C-9, C-14, C-17, C-20, C-24 and C-25; Three quaternarycarbons at C-5, C-10, and C-13 and The de-shielded signal at  $\delta_c$  71.8 was due to C-3 with a hydroxyl group attached to it.

The HNMR spectra of  $M_1$  showed the presence of six methyl signals that appear as two methyl singlet at  $\delta$  0.68 and 1.01; three methyl doublets that appear at  $\delta$  0.81, 0.83 and 0.93 and a methyl triplet at 0.84. The HNMR spectra of  $M_1$  also showed one olefinic proton at 5.36. The absence of protons corresponding to the double bond between C-20/C-21 in  $M_1$  suggested the presence of a di substituted double bond at C-5/C-6 in its structure. The HNMR spectra of  $M_1$  showed a proton corresponding in to the proton connected to the C-3 hydroxyl group which appeared as a triplet of doublets of doublets at  $\delta$  3.53.

The results of the 2D homo-nuclear correlation  $^1\text{H}$ - $^1\text{H}$  COSY and the hetero-nuclear correlation HSQC spectroscopy confirmed the relationship between the various protons and carbons in the molecule. The  $^1\text{H}$ - $^1\text{H}$  COSY experiment established the correlations between the protons at H(5.35) / H(1.91), H(3.51) / H(2.27), H(3.51) / H(1.50), H(1.91) / H( 1.50), H(1.91) / H(1.22), H(1.83) / H(1.04), H(1.60) / H(0.76) among others. The correct assignments of the protons, carbons and their linkages in the molecule were confirmed through the cross peaks detected on the Hetero nuclear Multiple Bond Correlation spectroscopy (HMBC). Some of the major correlations observed between protons and carbons include; Proton H1 (1.82) showed correlation with C26, C7, C8, C1, C4 and C3, Proton H4 (2.20) showed correlation with C3, C6, C20 and C22, Proton H6 (5.35) correlated with C12, C13 and C22, Proton H13 (1.75) showed correlation with C26, C24 e.t.c (Figure 4.7 and Table 4.7)

The  $^{13}\text{C}$  NMR together with DEPT, COSY, HMQC and HMBC showed twenty nine carbon signal including, six methyls, eleven methylenes, nine methine and three quaternary carbons suggesting the steroidal nature of the compound and the structure of  $\text{M}_1$  was assigned as  $\beta$  – sitosterol that was consistent to the reported literature values (Habibet *al.*, 2007; Jamal *et al.*, 2009 and Patehet *al.*, 2009) and was supported by the key COSY and HMQC correlations. Hence the proposed structure of  $\text{M}_1$  is shown in Figure 9

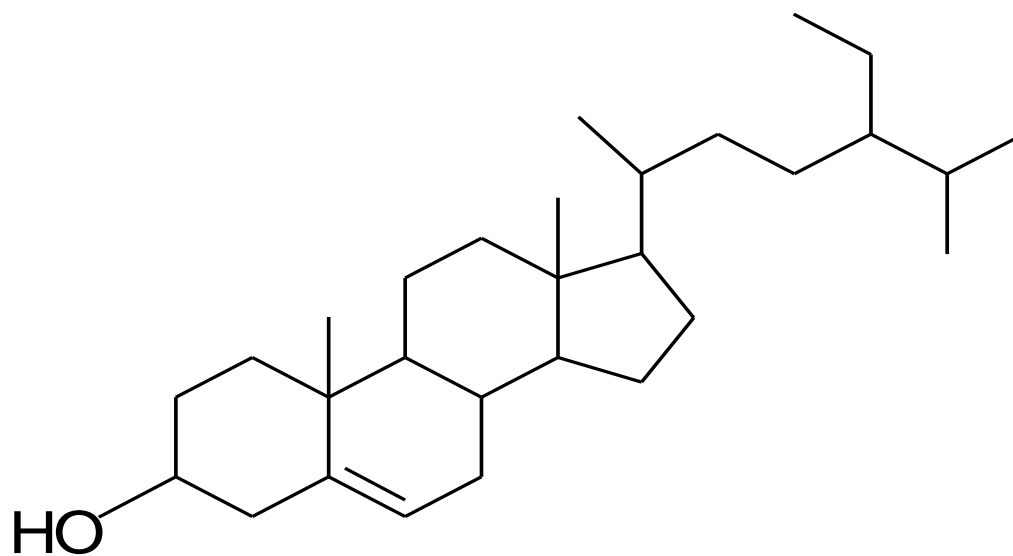


Figure 9: Stigmast-5-en-3 $\beta$ -ol ( $\beta$ -Sitosterol)

## 5.2

### PHARMACOLOGICAL STUDIES

The acute toxicity studies (LD50) of the methanol extract of *Leptadenia hastata* was found to be LD50 > 5000 mg/kg, this suggests that it is relatively safe, because there was no death up to 5000 mg/kg (Lorkes, 1983).

The result of this investigation suggests that the methanol extract of the leaves of *Leptadenia hastata* has a good anti-snake activity on *Echis ocellatus* venom. It showed a fair anti-snake venom *in vitro* activity against the *Echis ocellatus* venom in mice. 33% protection was observed against lethality at an administered dose 150 mg/kg of the extract, 33% at 100 mg/kg of the extract and 33% at 50 mg/kg of the extract. The result of the *in vitro* studies suggests that the extract might act by neutralising the activity of the venom at the site of bite, thereby reducing the harshness of the toxic effects.

The result of the *in vivo* studies revealed a significant activity against *Echis ocellatus* venom in mice. There was 50% protection against lethality at an administered dose of 150 mg/kg, 17% at 100 mg/kg and 17% at 50 mg/kg.

The extract exhibited analgesic potency. It significantly inhibited the abdominal constriction induced by acetic acid in mice. Acetic acid causes an increase in peritoneal fluids of PGF<sub>2</sub> and PGF<sub>2</sub>α involving in part, peritoneal receptors (Deraedt *et al.*, 1980; Bentley *et al.*, 1983) and is a very sensitive method of screening analgesic effect of compound (Collier *et al.*, 1968).

The observed effect of the extract in this study suggests that postaglandins may be involved in the action of the extract (Adzu *et al.*, 2003), the ability of the extract at 100 mg/kg and 150 mg/kg to produce comparable result to Piroxicam group in the study indicate high level of analgesic activity.

## CHAPTER SIX

### 6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

#### 6.1 SUMMARY

Preliminary phytochemical screening of methanol extract of the leaves of *Leptadenia hastata* indicates the presences of steroids, triterpenes, tannins, cardiac glycosides, flavonoids and saponins while the hexane fraction indicates the presence of triterpenes, steroids, cardiac glycosides, carbohydrate and saponins.

Extensive phytochemical studies of the hexane fraction resulted in the isolation of compound M<sub>1</sub> which is a steroid whose structure was elucidated using NMR spectroscopic data to be  $\beta$ -sitosterol.

The methanol extract exhibited a good *in vivo* activity against *Echis ocellatus* venom in mice with 50% protection. *In vitro* protection against lethality was seen at 150 mg/kg of the extract with 33% survival.

The methanol extract produced strong analgesic effect on the acetic acid-induced writhing, which was in the same order of magnitude as that observed after piroxicam administration.

## 6.2

## CONCLUSION

From the study carried out it can be concluded that the leaves *Leptedania hastata* contains flavonoid, steriod, triterpenes, cardiac glucoside, tannis and saponins. The n-hexane fraction afforded a steriod which was identified as  $\beta$ -Sitosterol.

On the other hand the pharmacological studies reveals a significant anti-snake venom and analgesic activity validating the ethnomedical use for the treatment of snake bite which resulted due to the secondary metabolite the plant appears to have.

To the best of our knowledge, this is the first report on isolation of this compound from the leaves of *Leptadeniahastata* and on its evaluation of anti- snake venom and analgesic activity.

### **6.3**

### **RECOMMENDATION**

1. A phytochemical screening should be carried on other parts of the plant.
2. Isolation of the active constituent responsible for both anti-snake venom and analgesic should be carried out.
3. An anti-venom studies should be carried out on the plants using different snake venoms.
4. Detailed activity guided isolation of the bioactive constituents responsible for antivenin activity should be undertaken.
5. Studies on possible mechanism(s) of action of the isolated drugs should be carried out.

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