PHYTOCHEMICAL AND ANTI SNAKE VENOM STUDIES ON THE METHANOL AERIAL PART EXTRACT OF *TACAZZEA APICULATA* OLIV (PERIPLOCACEAE)

BY

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ZARIA, NIGERIA

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FACULTY OF PHARMACEUTICAL SCIENCES,

AHMADU BELLO UNIVERSITY,

ZARIA, NIGERIA

JANUARY, 2022

DECLARATION

I declare that the work in this theses entitled **"PHYTOCHEMICAL AND ANTI SNAKE VENOM STUDIES ON THE METHANOL AERIAL PART EXTRACT OF *TACAZZEA APICULATA* OLIV (PERIPLOCACEAE)"** has been performed by me in the Department of Pharmaceutical Chemistry. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this theses was previously presented for another degree or diploma at this or any other institution.

Muhammad Abdulkadir	Signature	Date

CERTIFICATION

This thesis entitled **"PHYTOCHEMICAL AND ANTI SNAKE VENOM STUDIES ON THE METHANOL AERIAL PART EXTRACT OF *TACAZZEA APICULATA* OLIV (PERIPLOCACEAE)"** by MUHAMMAD ABDULKADIR meets the regulations governing the award of the degree of Master of Science in Pharmaceutical Chemistry of the Ahmadu Bello University, and is approved for its' contribution to knowledge and literary presentation.

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ACKNOWLEDGEMENT

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ABSTRACT

Tacazzea apiculata (Olive) is a woody climber indigenous to Tropical Africa often used in traditional medicine for the treatment of pain, inflammation, skin infections, diabetes, epilepsy and snakebite. This study aimed to isolate and characterize some of the phytochemical constituent(s) present in aerial part extract of *T. apiculata* and to scientifically validate the ethno medicinal claim of the use of the plant in the management of snakebite envenomation. The powdered aerial part of *T. apiculata* was extracted with methanol using maceration method which afforded methanol aerial part extract (MAE). Portion of this was treated with *n*-hexane, chloroform and ethyl acetate to obtain *n*-hexane (HEF), chloroform (CHF) and ethyl acetate fractions (EAF). Preliminary phytochemical screening of the methanol extract revealed the presence of secondary metabolites including alkaloids, flavonoids, saponins, tannins, and steroids/triterpenes. Silica gel chromatographic separation of chloroform fraction (CHF) afforded total of eleven major fractions C1 – C11, fractions C2 and C5 were subjected to further silica gel chromatographic separation which resulted to C2A and C5C sub fractions respectively. Repeated purification of C2A using silica gel packed column (micro pipette) eluted with *n*-hexane 100 % isocratic led to the isolation of a brown oily compound Tam 4. Similarly, repeated purification of C5C using silica gel packed column (micro pipette) eluted with *n*-hexane: ethyl acetate (8:2) isocratic resulted in the isolation of a white powdered compound Tam 2. The structures of the compounds were elucidated using physico-chemical tests and spectroscopic techniques (IR, 1D and 2D NMR) and by comparison with reference spectral data. The oral and intraperitoneal route LD_{50} of the methanol aerial part extract (MAE) in mice were estimated to be 5000 and 894 mg/kg, respectively. The LD_{99} of the *Naja nigricollis* venom was estimated to be 4.6 mg/kg. Antivenin studies suggest that methanol extract possess significant antivenin activity against

Naja nigricollis venom both *ex-vivo* and *in-vivo* with maximum protections level of 100 % survival at 80 mg/kg dose and 16.67 % survival at 2000 mg/kg dose respectively. Spectral analysis of both Tam 4 and Tam 2 by way of NMR showed Tam 4 to be Squalene and Tam 2 β-sitosterol. The structures were confirmed with the literature data and methanol extract demonstrated significant antivenin activity and lends credence to traditional use of the plant in the management of snakebite.

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LIST OF ABREVIATIONS

^{13}C-NMR	^{13} Carbon nuclear magnetic resonance spectroscopy
1D	One dimentional
^{1}H-NMR	Proton nuclear magnetic resonance spectroscopy
2D	Two dimentional
PPM	Part per million
DCM	Di chloro methane
CH	Chloroform
CHF	Chloroform fraction
EA	Ethyl acetate
EAF	Ethyl acetate fraction
H_2SO_4	Tetraoxosulphate (vi) acid
HSQC	Hetero nuclear spin quantum correlation
I.P.	Intraperetoneal
IR	Infra-red
LD_{50}	Median lethal dose
$CDCl_3$	Deuterated chloroform
MAE	Methanol aerial part extract

NaOH	Sodium hydroxide
HEF	*n*-Hexane fraction
NMR	Nuclear magnetic resonance spectroscopy
δH	Proton chemical shift
R_f	Retention factor
T.apiculata	*Tacazzea apiculata*
SEM	Standard error of mean
CTM	Chinese traditional medicine
TLC	Thin layer chromatography
WHO	World health organization
ANOVA	Analysis of variance
Tam 2	Isolated compound 1
Tam 4	Isolated compound 2
SBE	Snake bite envenoming
MLD	Minimum lethal dose
MSD	Maximum survival dose
HOD	Head of department pharmaceutical chemistry

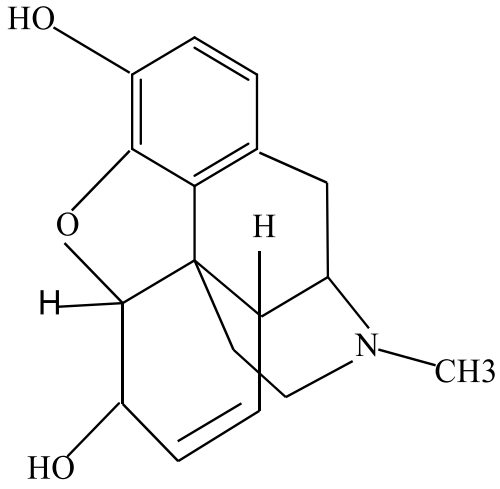
CHAPTER ONE

1.0 INTRODUCTION

1.1 Natural Product

Majority of African populations and other part of the developing countries depend on folkloric medicines for prevention and control of disease state or maintenance of well-being. World health organization (WHO) estimated that; about 80 % of the world population relies on natural product mostly of plant origin and other important sources, containing bioactive secondary metabolites such as alkaloids, tannins, terpenoids, steroids, flavonoids etc. known to produce definite physiological actions in living system (Yusuf *et al.,* 2015).

Nature has been a source of medicinal agents for millenniums in prevention and control of a disease state, and an impressive number of drugs used in present times have been derived from natural sources, many base on their use in folk medicine. Over the last century, a number of top selling drugs have been developed from natural products e.g morphine (i) from *Papaver somniferum*, and a potent anti – cancer drug taxol (ii) from *T. brevifolia*, etc (Sasidhran *et al*., 2011).



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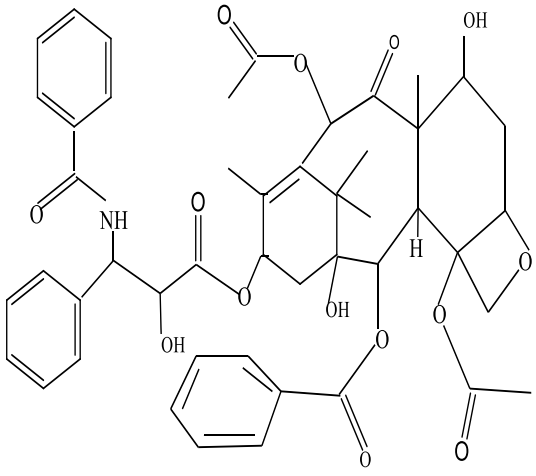

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Natural products are also used directly "natural" in the pharmaceutical industry, which is growing rapidly in developed countries (Europe and North America) as well as in traditional medicine programs being incorporated into the primary health care systems of Mexico, the people's Republic of China, Nigeria, and other developing countries (Newman and Cragg, 2007), the use of herbal drugs is once again becoming more popular in the form of food supplements, nutraceuticals, complementary and alternative medicine.

1.1.2 Contributions of natural product to search of new drugs

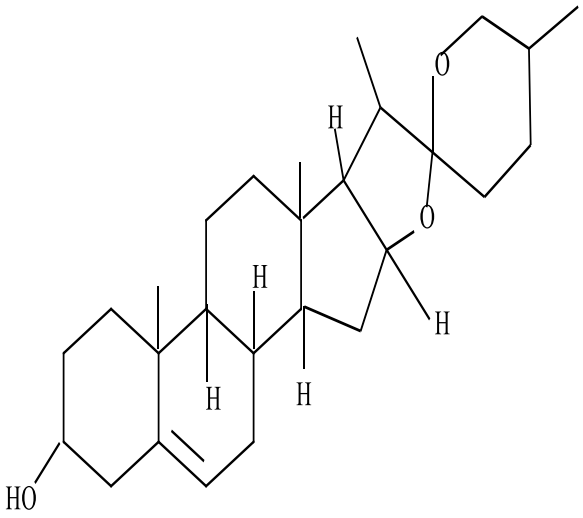
Natural product contributes to the search for new drugs in three different ways (Newman *et al.*, 2000):

1. By acting as new drugs that can be used in an unmodified state (e.g taxol (ii) from *T. brevifolia*).

2. By providing chemical (active principles) used to synthesize more complex molecule (e.g., diosgenin (iii) from *Dioscorea floribunda*) for synthesis of oral contraceptives.

3. By indicating new modes of pharmacological action that allow complete synthesis of novel analogs (e.g., synthetic analogs of Penicillin (iv)) (Newman *et al*., 2000).

Advent, introduction and development of several new and highly specific *in-vitro* bioassay techniques, chromatographic methods, and spectroscopic techniques, especially nuclear magnetic resonance (NMR), have made it much easier to screen, isolate, and identify potential drug lead compounds quickly and precisely. Automation of these methods now makes natural products viable for high-throughput screening (HTS). (Cragg *et al.,* 1997).



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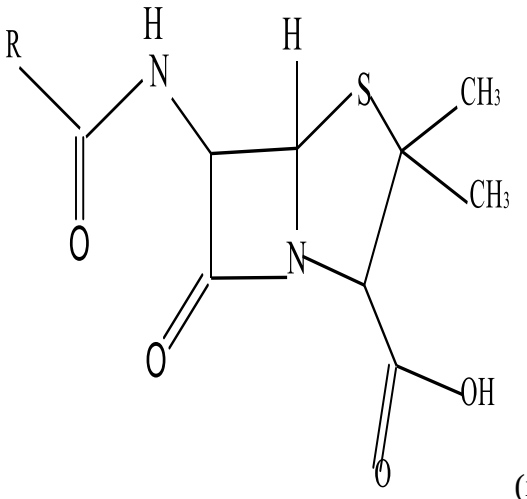


(iv)

1.1.3 Natural product as one of the major sources of new drugs

Natural products will certainly continue to be considered as one of the major sources of new drugs in the years to come because:

1. They offer incomparable structural diversity.

2. Many of them are relatively small (< 2000 Da).

3. They have drug-like properties (i.e., they can be absorbed and metabolized).

Only a small fraction of the world's biodiversity has been explored for bioactivity to date. For example, there are at least 250,000 species of higher plants that exist on this planet, but merely 5 – 10 % of these have been investigated so far (Newman *et al.,* 2003). In addition, research on previously studied plants has continued to produce new bioactive compounds that have drug potential (Newman *et al.,* 2003).

1.1.3.1 Plant as a source for new drugs

Medicinal plants contain chemical substance that can serve as precursors for the synthesis of useful drugs, for therapeutic purposes. The wide spread benefit of plants has been traced to the occurrence of natural products (active principles) with drug-like properties, which may act either individual, synergistically, or additively to improve health condition. A single plant may for example be used as anti-oxidant, anti-bacteria, anti-fungal and anti-venin etc. (Cragg *et al*., 1997).

1.2 Snake and Snake Venom

A snake is an elongated reptile of suborder serpentes comprising of venomous and non-venomous species inhabiting tropical and temperate regions of the world. It is a cold blooded, limbless, scaly animal (Russell, 1980). Snake bite is an injury inflicted by snake, often resulting in puncture wounds caused by the fangs of animal and sometimes result to important clinical pathological condition (Paul *et al*., 2011).

There are 3000 species of snakes known to science; in this, 30% of snakes are venomous and considered dangerous to human. Elapidae and viperidae are two families of species which causes human mortality worldwide (Sakthivel *et al*., 2013).

1.2.1 Classification of snakes

Snakes could be classified according to either their family or fangs orientation (Russell, 1980). There are four major groups of venomous snake base on the family:

1. Elapidae (e.g Cobras and Mambras)

2. Viperidae (e.g *Echis* and *Bitis*)

3. Hydrophiids (sea snakes)

4. Crotalidae (pit Viper)

Two species; black necked spitting cobra (*Naja nigricollis*), carpet viper (*Echis oscellatus*) belonging to the first two families; elapidae and viperidae respectively, are the most important snakes associated with envenoming in Nigeria (Ameen *et al*., 2015).

The fang is a long, sharp, hallows or grooved teeth of a venomous snake by which poison is injected. Three types of venomous snakes have been described on the basis of fang orientation (Russel, 1980) viz:

1. Opisthoglyphs (e.g boom-slang snakes)

2. Proteroglyphs (e.g Cobras, sea snakes and Mambras)

3. Solenoglyphs (e.g Carpet viper and puff adder)

1.2.2 Distribution

Venomous terrestrial and marine snakes are distributed throughout sub-tropical and tropical regions of the globe. Snakes are ectothermic poikilotherms that rely on external sources of heat, and as a result they are far more abundant in warmer climates. This restricts the hyper endemic regions for snake bite to tropical countries of Africa, South Asia, New Guinea and Latin America (Mohapatra *et al.,* 2011).

Species; carpet viper (*Echis ocellatus*), black – necked spitting cobra (*Naja nigricollis*) and puff adder (*Bitis arietans*), belonging to Viperidae and Elapidae families, are the most important snakes associated with envenoming in Nigeria, commonly found in middle belt particularly Plateau, Gombe, Bauchi, Kwara, Kogi, Kaduna and Taraba states as well as some parts of

Northern Enugu. Gombe has the highest number followed by Taraba and Plateau respectively (Ameen *et al.,* 2015). Viperidae represents the largest family of venomous snakes found in Africa, Europe, Asia and the America.

1.2.3 Snake venom

Snake venom (yellow, green or even color less) is an egg like viscous liquid mainly consisting of toxic protein, produced in modified parotid glands normally responsible for secreting saliva they are stored in structures called alveoli, behind the animal eyes and ejected through its hollow tubular fangs (Paul *et al.,* 2011). Fresh snake venom is neutral or weak acidic, and it is alkaline when it is placed for a long time and upon exposure to air, fresh venom produces foam and will be non-venomous and putrid when kept at room temperature for 24 hours (toxicity disappear following UV irradiation), treatment with heat plus formaldehyde action in activate the venom and makes it non – toxic, but antigenic property is retained (Paul *et al*, 2011).

However, snake venoms are mainly used by the snake to immobilized and digest the prey. It is also used as a defense mechanism against their natural predators (Sakthivel *et al.,* 2013). The most important venom components that lead to significant clinical effects after a bite are enzymes and polypeptide toxins as follows (Moses, 2007):

1. Pre-coagulant enzymes:

These are mainly found in vipers, they activate different steps of the blood-clotting cascade, this leads to the formation of fibrin in the blood. Most of the fibrin is broken down by the body's fibrinolytic system. This process depletes the body's own levels of clotting factors, and eventually the blood does not clot. This is also called consumption coagulopathy.

2. Cytolytic or necrotic toxins:

are digestive hydrolases (proteolytic enzymes and phospholipases) that destroy cell membranes and tissues and therefore increase the permeability of the vascular endothelium. This leads to local swelling, blistering and oedema.

3. **Hemolytic and myolytic phospholipases A2:**

this is a clinically important enzyme that damage cell membranes, endothelium, skeletal muscle, nerves and red blood cells.

4. Pre-synaptic neurotoxins:

are mainly found in elapid venoms, but they are also found in some vipers. They are phospholipases A2 that damage nerve endings, initially releasing acetylcholine followed by interfering with its release.

5. Post-synaptic neurotoxins:

are mainly found in elapid venoms, they are polypeptides that compete with acetylcholine for neuromuscular junction receptors and lead to curare-like paralysis.

6. Haemorrhagins:

are zinc metalloproteinases, that damage the endothelial lining of blood vessel walls causing spontaneous local and systemic haemorrhage.

1.2.4 General symptoms of snake bite

Snake bites cause considerable morbidity and mortality worldwide with the highest burden found in south Asia and sub-saharan Africa (Ameen *et al.,* 2015). The outcome of snake bites depends

on numerous factors, including the species and size of snake, the area of the body bitten, the amount of venom injected, the age and health conditions of the victim. Children are more likely to show severe symptoms because they receive a large concentration of venom due to their smaller body size (Paul *et al.,* 2011).

Feelings of terror and panic are common after snake bite and may produce a characteristic set of symptoms mediated by the autonomic nervous system. Most snake bites whether by a venomous snake or not, will produce some type of local effect. There is minor pain and redness in over 90 % of cases, although this varies depending on the site. Bites by vipers and some cobras may be extremely painful, with the local tissue sometimes becoming tender and severely swollen within 5 min., this area may also bleed and blister and can eventually leads to tissue necrosis (Paul *et al.,* 2011). However, clinical manifestations of snake bite occur in a wide spectrum with some bites resulting in minimal or no symptoms at all, while others are severely enough to result in systemic manifestations and even death (Kumar, 2009).

Other important clinical features of the bites of venomous snakes reflect the effect of constituent of the venom. These includes abnormal blood clothing and bleeding, hypotension and shock, neurotoxicity which sometimes leads to paralysis of respiratory muscles requiring assisted ventilation, and renal toxicity (Ameen *et al*., 2015).

1.2.5 Anti-snake venom

For more than a century, the cornerstone of snakebite treatment has been the administration of animal derived immunoglobulins (antivenins); which can either be poly-specific (polyvalent) antivenins or mono-specific (monovalent) antivenins, produced from the hyper immune plasma of horses or sheep. Consequently, the quality and safety of some antivenins remain poor,

production inefficiencies, storage limitations, and distribution problems combined with inadequate funding for procurement. As a result; antivenins become limited and highly costly (David *et al*., 2019).

However, production and supply of effective antivenins is still a big challenge in health facility of most African countries (Moses, 2007) and are associated with the following problems:

1. Most antivenins carry a risk of potentially dangerous early anaphylactic reactions. Antivenom is not always necessary; some patients are bitten by non-venomous snakes, and 10 - 50 % of those bitten may not be envenomed.

2. They have a defined range of specific and para - specific neutralizing activity, but are useless for venoms outside that range. Currently, specific antivenin is not available for the treatment of envenoming by some species.

3. They are very expensive and usually in short supply, particularly in Africa.

1.2.6 Plant constituents active against snake venom

The use of plants against the effect of snake bite has long been recognized, and has merited more scientific attentions, from the past three decades. Quite a number of reports, from different geographical areas, mention plants reputed to neutralize the action of snake venom; such activities are attributed to certain chemical compounds identified in them, and even less are concerned with the mechanism of action (Walter *et al*., 2000).

Furanoid diterpene lactone isolated from the rhizome of *Aristolochia albida* (family: Aristolochiaceae) was reported to be active against the venoms of *Naja nigricollis* (LD_{99}; 8.75 mg/kg) and *Bitis arientans* (LD_{99}; 4.20 mg/kg), administered intra-peritoneal (*i.p*.) to swiss

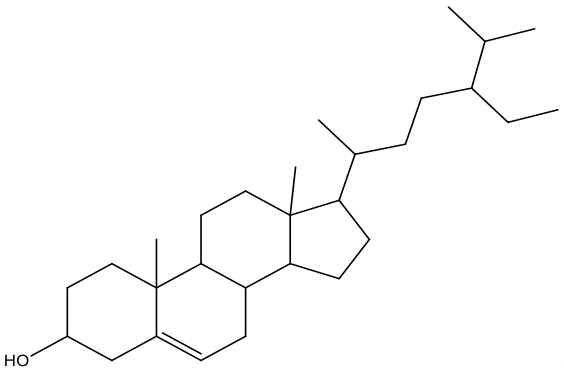
albino mice, the compound isolated was found significantly to reduce the toxic symptoms and protect the mice against the lethal doses (Haruna *et al*., 1995). Di-n-octyl phthalate (v) isolated from *Ceiba pentandra* leaves extract, was tested for its anti *Echis ocellatus* venom properties. It was biologically active in inhibiting phospholiphase A2 activity in a dose dependent manner (Ameen *et al*., 2015).

Several plant secondary metabolite including flavonoids, polyphenols and terpenoids are reported to possess protein binding and enzyme inhibiting properties. Tannins are also known to unspecifically inactivate proteins.

Triterpenoids may be involved in venom inactivation processes, penta cyclic triterpenes (free or as glycosides) are found widely in several anti-snake venom plants and provide nearly 20 % protection against snake venom (Yusuf *et al*., 2015).

Flavan-3-ol glycoside [(+)-Catechin-3'-O-rhamnopyranoside] (vi), isolated from the stem bark extract of *N. macrophylla* was evaluated for its antivenom activities. The compound (at 1, 5 and 10 mg/kg) was able to protect mice against lethality induced by *Naja nigricollis* venom with 100, 40, and 20 % survival rate, respectively; it was also found to be 33.7 % effective in combating the hydrolytic actions of *N. nigricollis* phospholiphase A2 enzyme (Yusuf *et al*., 2019). However, (Walter *et al*., 2000) reported that β-sitosterol (vii) and stigmasterol (viii) may play an important role in neutralizing snake venom-induced actions.

(V)



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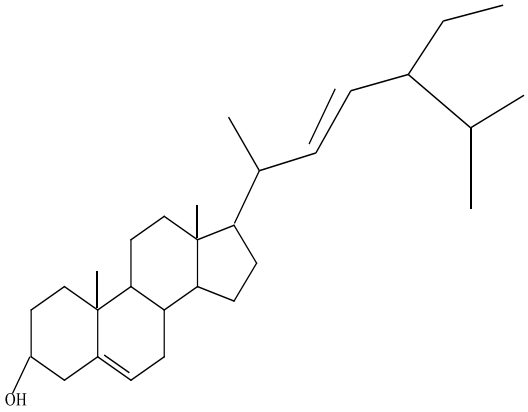

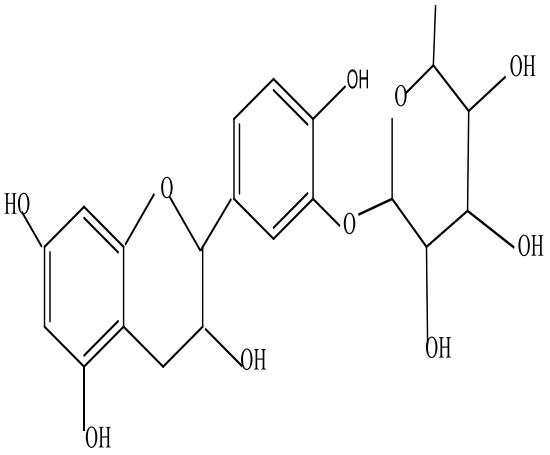

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(viii)

1.3 Statement of Research Problem

Envenomation resulting from snakebite is a public health problem that inflicts clinical pathological condition characterized by inflammation and hemorrhages, in many developing countries such as Nigeria (Yusuf *et al.*, 2015).

It is considered as one of the most neglected tropical disease, with vast and often devastated consequences (Isah *et al*., 2015), it affects as many as 2.7 million people every year, with annual mortality of 81,000 - 138,000 and 400,000 surviving victims suffering permanent physical and psychological disability (David *et al*., 2019).

The current available therapy for snakebite victims is the immediate administration of antivenin but, the supplies of the drug is limited and largely unavailable due to access, cost and lack of storage facility (Yusuf *et al.*, 2015)

1.4 Justification of the Study

Snake bite envenoming (SBE) is a medical emergency and, the sooner a victim receives effective treatment, the greater the likelihood of a full recovery and an early return to normal life (David *et al.*, 2019). Safe, effective, available and accessible antivenin in local communities is largely not guaranteed. This necessitates the search for effective alternative remedy that are less expensive, easily accessible and readily available antivenin, by paying more scientific attention to plant-derived antivenin.

Interestingly, *Tacazzea apiculata* has been reportedly used in ethno-medicine for the treatment of snake bites. Extensive literature search revealed that no work has been done on the anti-snake venom property of the arial part of *Tacazzea apiculata* nor phytochemical screening and isolation of any compound from this plant. This research is therefore the first extensive phytochemical studies on the aerial part of *Tacazzea apiculata.*

1.5 Aim of the Study

The aim of the research is to isolate and characterize some of the phytochemical constituent(s) present in *T. apiculata* and to scientifically validate, the ethno-medicinal claim of the use of the plant in the management of snakebite envenoming using laboratory models.

1.6 Specific Objectives of the Study

The specific objectives of the study are;

1. To carry out preliminary photochemical screening of crude aerial part extract of *T. apiculata*, using standard procedure.

2. To isolate some phytochemical compound(s) present in aerial part of *T. apiculata* using chromatography techniques.

3. To characterized the isolated compound(s) using spectroscopy techniques and physicochemical test.

4. To evaluate the antisnake venom activity of the crude aerial part extract of *T. apiculata* using laboratory models.

1.7 Research Hypothesis

The aerial part of *T. apiculata* contains phytochemical substances with antivenin activity.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Periplocaceae Family

Periplocaceae family is composed of approximately 50 genera and about 190 species in Africa, Madagascar, Asia and Australia. The taxa are predominantly climbers, some of which are huge lianas and shrubs found in Tropical and Sub-Tropical swamp, rain or mesophytic forests (Venta., 1997). In another related report by (Verhoeven, 1994) most of the genera are lianous and a small number are shrubs and as well as herbaceous.

2.1.1 Morphology of periplocaceae

The family periplocaceae is characterized with following: they have linear to spathulate leaves born in clusters, milky latex, flowers with coronas, fruits composed of paired follicles and seeds with comas of hairs, single-grained pollen. The xylem in the form of a continuous cylinder is traversed by uniseriate rays. The leaves are dorsiventral and hypostomatic with paracytic stomata (Venter *et al*., 1990).

2.2 Scientific Classification of *T. apiculata*

Kingdom:- Plantae

Phylum:- Tracheophyta

Class:- Magnoliopsida

Order:- Gentianales

Family:- Periplocaceae

Genus:- Tacazzea

Species:- *Tacazzea apiculate* (Venta *et al*., 1990).



Plate I: *Tacazzea apiculata* in its natural habitat.

2.2.1 Morphology of *Tacazzea apiculata* (Oliv)

Tacazzea apiculata (Oliv) is a woody climber indigenous to Tropical Africa, belonging to periplocaceae family. It is a liana (a woody vine). The stem has unicellular and multicellular non-glandular trichomes similar to those on the leaves, the xylem in the form of a continuous cylinder is traversed by uniseriate rays The leaves are dorsiventral and hypostomatic with paracytic stomata having petiole adaxially grooved. On the adaxial leaf surface; the density of the trichomes varies from scattered to almost glabrous. It is widely distributed throughout the tropics and sub-tropics of Africa (Venter *et al*., 1990).

2.2.2 Local names of *Tacazzea apiculata*

English – Craw-craw vine

Hausa – *Yaadiyar kada*

2.2.3 Ethno-medicinal uses of *T. apiculata*

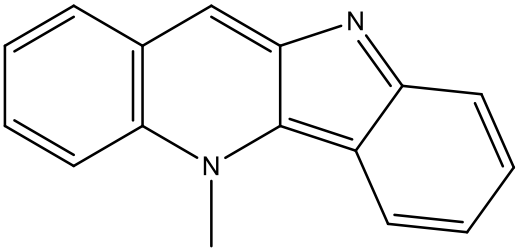
T. apiculata has been used extensible by traditional medical practitioners for treatment of wide range of diseases. In Nigeria the powdered root, mixed with milk or honey is taken orally as remedy to relief pains associated with pile, inflammatory conditions and cancers (Ahmad *et al.*, 2019). The leaf is used for skin diseases, flowers are considered edible, powdered *leaves* are used in snake bite and stings by venomous animals, decoction of the root is used for convulsion and epilepsy by the hausa people of north-western Nigeria, twig is powdered and taken in milk or water as "tonic" to improve general health condition of the body. The plant is also reported to be used in hausa traditional medicine for the treatment of diabetes (Gwarzo *et al*., 2014).

Other species of the Periplocaceae have been historically used as folk medicines in China, especially *Periploca sepium* and *Periploca forrestii*. The root bark of *P. sepium*, known as

"*Xiangjiapi*" is utilized as a traditional Chinese medicine (TCM) for the treatment of rheumatoid arthritis, bone and muscle pain, which is recorded in the Chinese pharmacopoeia (2015 version). The stems or the whole plant of *P. forrestii*, which is a well-known herb known as "Heiguteng," are widely used by the Miao nationality in China to treat many diseases, including rheumatic arthritis, traumatic injury, stomach ache. The usage of *P. forrestii* is usually decocted in water or soaked in wine for internal use, or it is smashed for external application (Mingjin *et al*., 2019).

2.2.4 Biological activities of *T. apiculata*

There are many studies that address the biological activities of species of periplocaceae. Gwarzo *et al.*, (2014) reported the level of serum glucose and malondialdehyde in Alloxan induced diabetic rats supplemented with methanol leaf extract of *T. apiculata*, Analgesic effects of ethanol and ethyl acetate fractions of crude ethanol extract of the root bark of *T. apiculata* has been reported (Ahmed *et al.*,2007), Antimicrobial activities of Crude methanol leave extracts of *Cryptolepis sanguinolenta* was carried out against six bacteria ie *Pseudomonas aeruginosa, Escherichia coli, Salmonella typhii, Staphylococcus aureus, Klebsiella pneumoniae, Bacillus subtilis* and two fungi which includes *Aspergillus niger* and *Candida albicans* using agar dilution method (Agboke *et at*., 2011). Mingjin *et al*., (2019) reported the cardiac glycosides isolated from stem back of *P. forrestii* showed hepatotoxicity and cardiotoxicity at certain doses, anti-malarial activity of two alkaloids; cryptolepine (viv) and isocryptolepine (x) isolated from the ethanol roots extract of *Cryptolepis sanguinolenta* has been reported (Philippe *et al*., 1996).



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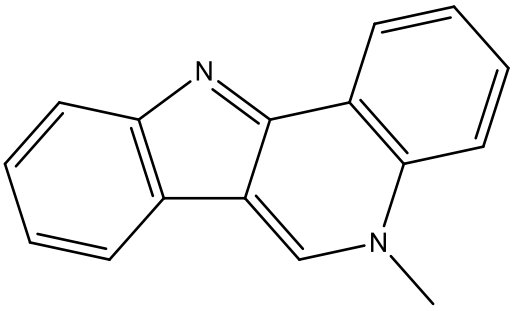

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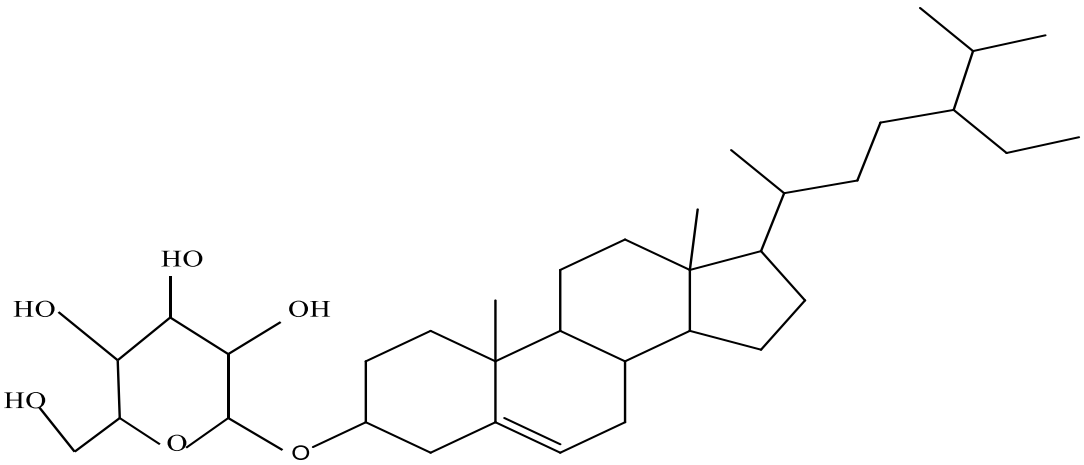
2.2.5 Chemical constituents of *T. apiculata* and other species

Phytochemical investigation of *T. apiculata* revealed predominant metabolites; such as steroids, carbohydrates, glycosides, tannins, alkaloids, terpenoids, flavonoids, quinones, cardiac glycoside, saponins (Philippe *et al*.,1996). To date, one hundred and forty-two steroids were isolated from *Periploca* species, including forty-six cardenolides, ninety-two C21 pregnane-type steroids, and some phytosterols [β-sitosterol (vii), β-daucosterol (xi), and stigmasterol (viii) from *P. laevigata* roots, *P. sepium* root back, *P. aphylla* and *P. calophylla* root back respectively. Approximately 80 % of the C21 pregnane-type steroids come from *P. sepium*, while *P. forrestii* is a rich source of cardenolides (Mingjin., 2019). Two alkaloids, cryptolepine (viv) and isocryptolepine (x), have been isolated from the ethanol roots extract of *Cryptolepis sunguinolenta* (Philippe *et al.*, 1996).



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CHAPTER THREE

3.0 METHODOLOGY

3.1 Materials, Chemicals and Reagents

The chemicals and reagents used were of analytical grade (Sigma Chemical Co. St. Louis, USA). Silica gel of mesh size 60-120µm (Qualikems). Thin layer chromatography (TLC) precoated plates (Silica gel 60 F_{254}).

3.1.1 Equipment

Electro thermal melting point apparatus was used for melting point determination. ABB - MB3000 spectrophotometer was used for infrared spectroscopy available at the Multi-user laboratory, Ahmadu Bello University, Zaria, Nigeria. Bruker AVANCE III NMR (400 Hz for ^{1}H and 100 MHz for ^{13}C) available at University of Surrey, United Kingdom was used for both 1D- and 2D-NMR spectroscopy.

3.1.2 Experimental animals

Swiss Albino mice of either sexes weighing (15-33 g) were obtained from animal house facility of the department of pharmacology and therapeutics, Ahmadu Bello University, Zaria, Nigeria. They were fed with laboratory diet and water *ad libitum* and maintained under ambient environmental conditions in a propylene cage at room temperature.

3.1.3 Venom preparation

The venom was collected by the milking method of Markfarlane (1967) from locally caught *Naja nigricollis* by the skilled snake handler. The venom was pooled, lyophilized and stored at about 2 – 4 °C prior to the experiments.

3.1.4 Collection, Identification and Preparation of Plant material

The plant material (*T. apiculata*) was collected in March, 2019 at Zaria, Zaria local Government Area of Kaduna State. The plant material was authenticated by Malam Namadi Sanusi of the herbarium section, department of botany, faculty of life science, Ahmadu Bello University, Zaria by comparing with voucher specimen number (No. 066633). The aerial parts were removed, shade dried, pulverized and stored at room temperature for further use.

3.2 Ethical Clearance

The ethical clearance for the conduct of this work was taken from the ABU Committee on Animal Use and Care (ABUCAUC), with the approval No. ABUCAUC/2021/055

3.3 Extraction Procedures

Pulverized plant material (2700 g) of *T. apiculata* was macerated with 5 litres of methanol for 7 days with occasional shaking/swirling. The extract was decanted, filtered and concentrated using rotary evaporator at 40 ^{0}C to afford a reddish-brown methanol aerial-part extract (MAE). 200 g of MAE was successively extracted with *n*-hexane (1000 mL), chloroform (1500 mL) and ethyl acetate (1300 mL) in sequential order of polarity afforded *n*-hexane (HEF), chloroform (CHF) and ethyl acetate fractions respectively Figure 3.1.

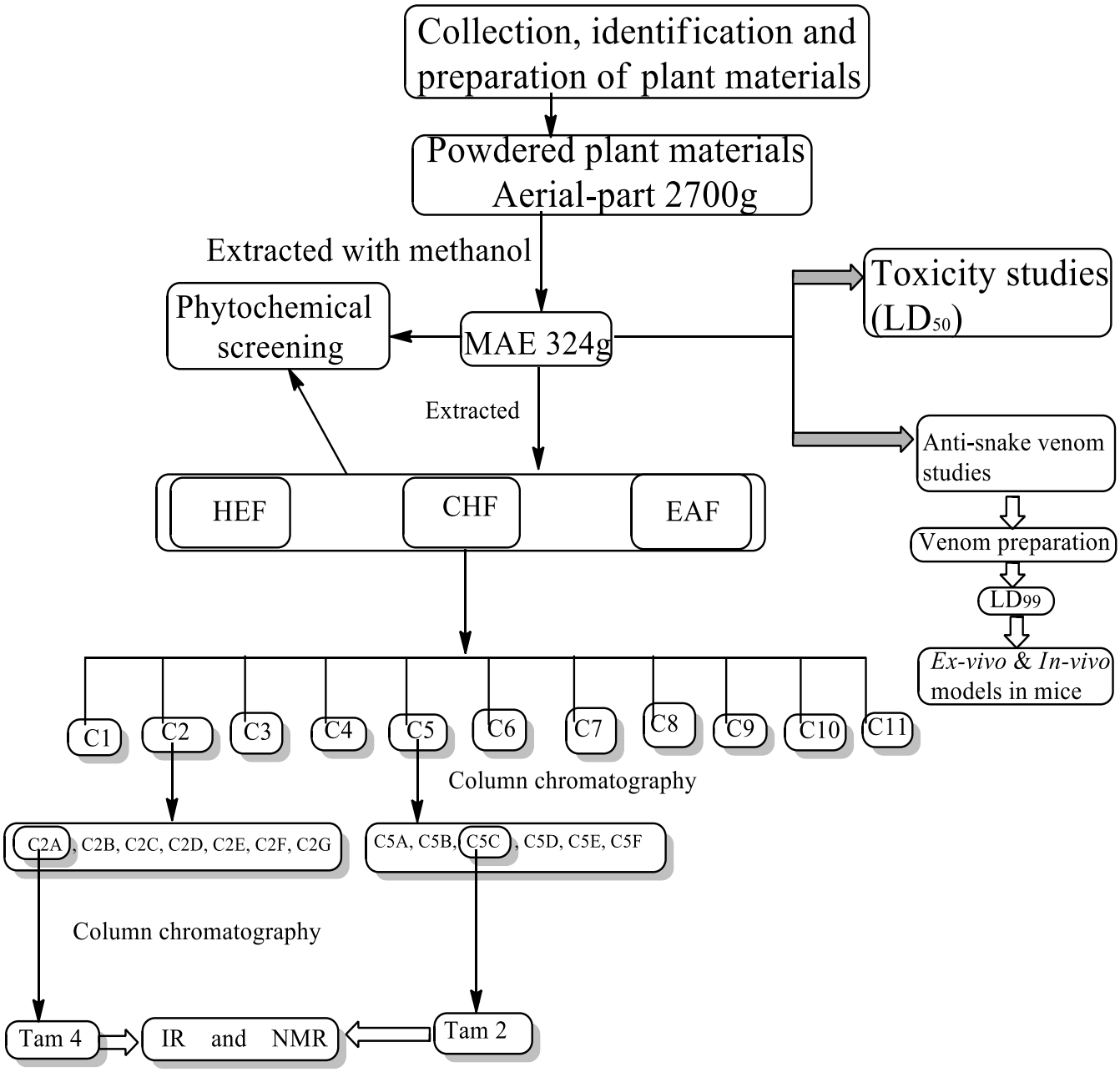

Figure 3.1: Schematic representation of work conducted on the aerial-part of *T. apiculata*

3.4 Preliminary Phytochemical Screening

Different chemical tests were carried out on the methanolic extract (MAE), *n*-hexane HEF, chloroform CHF and ethylacetate fractions EAF to identify the presence of various chemical constituents.

3.4.1 Test for alkaloids

0.5 g of the extract was stirred with 5 ml of 1 % aqueous hydrochloric acid on a water bath and filtered. 3 ml of the filtrate was divided into two. To the first 1 ml few drops of freshly prepared Dragendoff's reagent ware added, a rose red precipitate indicates the presence of alkaloids. To the second, few drops of Meyer's reagent ware added, a cream precipitate indicates the presence of alkaloids (Trease and Evans, 1996).

3.4.2 Test for flavonoids

Ferric chloride test

To the methanol extract (50 mg), 3 drops of ferric chloride solution ware added, green precipitate indicates the presence of flavonoids (Trease and Evans, 1996).

Sodium hydroxide test

Two drops of 10 % sodium hydroxide ware added to 50 mg solution of the methanol extract, yellow coloration indicates the presence of flavonoids (Silva *et al*., 1998).

3.4.3 Test for saponins (frothing test)

To a 50 mg portion of the methanol extract in test tube, 10 ml of distilled water was added and then shaken vigorously for 30 seconds. The solution was allowed to stand for 5 minutes; the formation of a persistent froth indicates the presence of saponins (Trease and Evans,1996).

3.4.4 Test for steroids and triterpenes

Liebermann-buchard test

To a 50 mg portion of the methanol extract equal volume of acetic anhydride was added and mixed gently. 1 ml concentrated sulphuric acid was added down the test tube. It was observed for instant colour change over a period of one hour. Blue to blue-green colour in the upper layer and a redish, pink or purple colour at the junction of the two layers indicates the presence of steroids and triterpene (Silva *et al.,* 1998).

Salkowski's test

A small quantity of the extract (50 mg) was dissolved in 2 ml chloroform and to it 2 ml of concentrated sulphuric acid was added down the test tube and observed. A red brown coloration at the interface indicates the presence of steroid and triterpene (Sofowora, 1993).

3.4.5 Test for tannins

Lead sub-acetate test

To a small portion of the methanol extract (50 mg), 3 – 5 drops of lead-acetate solution was added, cream coloured precipitate observed indicates presence of tannins (Trease and Evans, 1996).

3.4.6 Test for cardiac glycosides (Keller-kiliani test)

A small portion of the extract (50 mg) was dissolved in 1 ml glacial acetic acid containing traces of ferric chloride solution. The solution was then transferred into a dry test tube to which an equal volume of sulphuric acid was added, a brown ring obtained at the interface will indicates the presence of a deoxy suger (Trease and Evans, 1996).

3.5 Chromatographic Procedure

3.5.1 Thin-layer Chromatography (TLC)

Thin-layer chromatography was carried out using TLC pre coated plates (Silica gel 60 F_{254}) by using ascending technique. Spotting was carried out manually using capillary tubes and developed in an air-tight chromatographic tank, at room temperature.

Preliminary TLC of all the fractions were conducted.

Solvent systems: different solvent systems were used to develop the plates, including;

1. Hexane: Ethylacetate (9:1, 8:2, 7:3, 1:1, 1:2)

2. Dichloromethane: Ethylacetate (9:1, 25:1)

3. Dichloromethane: Hexane (8:2, 9:1, 1:1)

4. Ethylacetate: Methanol (25:1, 19:1, 9:1)

Developed chromatograms were air dried and visualized;

1. Under normal day light

2. Under ultra violet light (254 and 366 nm)

3. By spraying with 10 % sulphuric acid followed by heating at 105 ^{o}C for 5 – 10 minutes in an oven.

The chloroform fraction CHF was mainly used for this work because of presence of prominent spots on the preliminary TLC profile (Plate IV & V).

3.5.2 Column chromatography of CHF

The chloroform fraction CHF (11 g) was eluted in a silica gel packed column (flash column) starting with *n*-haxane (100 %), *n*-haxane:ethyl acetate 95:5, 90:10, 85:15, 80:20, 70:30, 50:50 to ethyl acetate (100%), followed by ethyl acetate:methanol 90:10, 70:30, 50:50 to methanol (100 %). A total of 100 fractions were collected and combined based on their TLC profiles to give eleven major fractions coded C_1 - C_{11}. Fractions C_2 and C_5 were subjected to further silica gel packed column chromatography separation, based on their prominent TLC profile.

3.5.3 Column chromatography of C_2

Fraction C_2 was eluted in a silica gel packed column (5 × 50 cm) starting with *n*-haxane (100 %), *n*-haxane:dichloromethane 95:5, 90:10, 85:15. Total of 50 collections were made and pooled in to 7 fractions coded C_2A - C_2G. Fraction C_2A was further purified using silica gel packed column (micro pipette) eluted with *n*-haxane 100 % isocratic, a brown oily compound was obtained and was coded Tam 4.

3.5.4 Characterization of Tam 4

Solubility test

Tam 4 was freely soluble in n-hexane and chloroform

Chemical test

Compound Tam 4 was subjected to Salkowski's test.

Spectral analysis of Tam4

The isolated compound Tam4 was subjected to IR and NMR analysis.

3.5.5 Column chromatography of C_5

Fraction C_5 was eluted in a silica gel packed column (5 x 50 cm) starting with *n*-haxane (100 %), *n*-hexane:ethyl acetate 9:1, 8:2. Total of 35 collections were made and pooled into 5 fractions coded C_5A - C_5F. Fraction C_5C was further purified repeatedly using silica gel packed column (micro pipette) eluted with *n*-haxane:ethyl acetate 8:2 isocratic, a white powdered compound was afforded coded Tam 2.

3.5.6 Characterisation of Tam 2

Solubility test

Tam 2 was soluble in ethyl-acetate, dichloromethane, chloroform and methanol.

Chemical test

Compound Tam 2 was subjected to Liebamann Buchard's test.

Melting Point determination

The melting point of compound Tam 2 was determined using melting point apparatus.

Spectral analysis of Tam 2

The isolated compound Tam 2 was subjected to IR and NMR analysis.

3.6 Biological Studies

3.6.1 Acute toxicity studies

The method described by Lorke (1983) was employed. Route of administrations were oral and *intra-peritoneal*.

For oral

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, 100 and 1000 mg/kg respectively. In the second phase, three animals were used based on the outcome of the first phase. Each of the three animal received different doses of the extract which are: 160, 290 and 5000 mg/kg. The median lethal dose was calculated using the following formula;

$$LD_{50} = \sqrt{Minimum\ Lethal\ Dose\ (MLD) \text{X}\ Maximum\ Survival\ Dose(MSD)}$$

For *intra-peritoneal*

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, 100 and 1000 mg/kg respectively. In the second phase, four animals were used based on the outcome of the first phase. Each of the four animals received different doses of the extract which are: 200, 400, 800 and 1600 mg/kg. The median lethal dose was calculated using the following formula;

$$LD_{50} = \sqrt{MLD \text{ X } MSD}$$

3.6.2 Lethality assay of the venom

The venom was reconstituted with normal saline and concentrations ranging between 1 to 7 mg /ml were obtained. The method of Theakston and Reid (1983) was adopted where five groups of mice containing 5 mice each were injected *intra-peritoneally* (*i.p.*) with 0.2 ml of different concentration of the reconstituted venom. The control group (5 mice) received only normal saline (0.2 ml each, *i.p.*). The time of death was recorded over a period of 24 h after the administration of the venom. The LD_{99} value (minimum lethal dose, MLD) was determined by probit analysis.

3.6.3 Venom detoxification effect by the methanol crude extract

Ex-vivo model

Method described by Abubakar *et al* (2000) was adopted, briefly; four groups of mice containing 6 mice each were used. The first group (control) received 0.2 ml of LD_{99} (4.6 mg/kg) of venom only. Groups 2, 3, and 4 (serving as treatment groups) were given an equivalent of LD_{99} (4.6 mg/kg) of the venom containing 80, 170, and 260 mg/kg extract, respectively. The venom and the extract ware incubated at 37 °C for 10 min and 0.2 ml of the incubated mixture was then injected (*i.p.*) into each animal in the treatment group. The number of deaths and survival time ware recorded within 24 h.

In-vivo model

The method described by Theakston and Reid (1983) was employed. Thirty mice were divided into five groups containing 6 mice each. Group 1 (control group) received 0.2 ml normal saline, group 2, 3, 4 and 5 (treatment groups) received 500, 1000, 1500 and 2000 mg/kg of the extract respectively (oral).

All the animals in the various groups ware then injected (*i.p.*) with LD_{99} (4.6 mg/kg) of the venom 1 h after injecting the extract. All animals were observed for mortality and survival within 24 h (Theakston and Reid.,1983).

3.7 Statistical Studies

The survival of experimentally protected laboratory animals (within 24 h after being challenged with lethal venom doses) were presented as mean $\pm$ standard error of mean (SEM). Statistical analysis for differences between the means was carried out by one-way analysis of variance (ANOVA) followed by multiple comparism of the control group with the values of the treated group using post hoc Dunnett's test. The results were considered significant at $p \leq 0.05$. All the analysis was done using Statistical Package for the Social Sciences (SPSS) version 20.0.

CHAPTER FOUR

4.0 RESULTS

4.1 Extraction and Fractionation Yield

The methanol aerial part extract obtained from 2700 g of pulverized aerial part of *T. apiculata* was 324 g representing (12 % w\w). When 200 g of MAE was partitioned with different solvents; the percentage yield obtained was given in Table 4.1 below.

Table 4.1 Percentage yield of fractions from MLE of *T. apiculata*

Fraction	Colour	Weight (g)	Yield (%) w/w
Ethyl acetate fraction	Light brown	13.0	6.50
Hexane fraction	Green	8.3	4.15
Chloroform fraction	Light green	13.8	6.90

4.2 Result of Phytochemical Studies

4.2.1 Result of preliminary phytochemical screening

Results of preliminary phytochemical screening of crude methanol extract (MAE), *n*-hexane fraction (HEF), chloroform fraction (CHF) and ethylacetate fraction (EAF) were shown in (Table 4.1).

Table 4.2: Result of Phytochemical Constituents of MAE, HEF, CHF and EAF

Constittuents	Test	Observation	Inference			
			MAE	HEF	CHF	EAF
Alkaloids	Mayer	Cream precipitate	+	+	+	-
	Dragendorff	Rose red precipitate	+	+	+	-
Flavonoids	Ferric chloride	Green precipitate	+	-	-	+
	Sodium hydroxide	Yellow color	+	-	-	+
Steroids and terpenes	Lieberman-Buchard	Purple colour at interface	+	+	+	-
	Salkoswski	Red brown color	+	+	+	-
Tannins	Lead Sub-acetate	Cream colored ppt	+	-	-	+
Saponins	Frothing	Froth persists for 15mins	+	-	-	+
Cardiac glycoside	Keller-kiliani	Brown ring at interface	+	-	-	+

+ means detected, - means not detected

4.3 Thin-layer Chromatography

4.3.1 Thin layer chromatography of various fractions

Thin layer chromatographic (TLC) analysis of ethyl acetate, chloroform, *n*-hexane fractions and crude methanol aerial part extract using *n*-hexane: ethyl acetate (9:1) as solvent system gave some spots shown in plates II, III, IV & V.

4.3.2 Thin-layer chromatography of chloroform fraction

Thin layer chromatographic (TLC) analysis using *n*-hexane: ethylacetate (9:1) and (8:2) as solvent systems revealed the presence of 7 major spots when sprayed with 10 % sulphuric acid shown in Plates VI & VII.

4.3.3 Column chromatographic separation of chloroform fraction (CHF)

Having conducted the TLC of all the collections and merged, the TLC of the various fractions of CHF made using hexane: ethylacetate (9:1) as solvent system revealed various spots as shown in Plate VIII.

4.3.4 TLC profile of column (micro pipette) chromatographic separation of C_2A

Thin layer chromatographic analysis of some of the column chromatography collections made using hexane: ethylacetate (9:1) as solvent system (Plate IX).

4.3.5 TLC profile of column (micro pipette) chromatographic separation C_5C

Thin-layer chromatographic analysis of some of the column chromatography collections made using *n*-hexane: ethylacetate (8:2) as solvent system (Plate X).

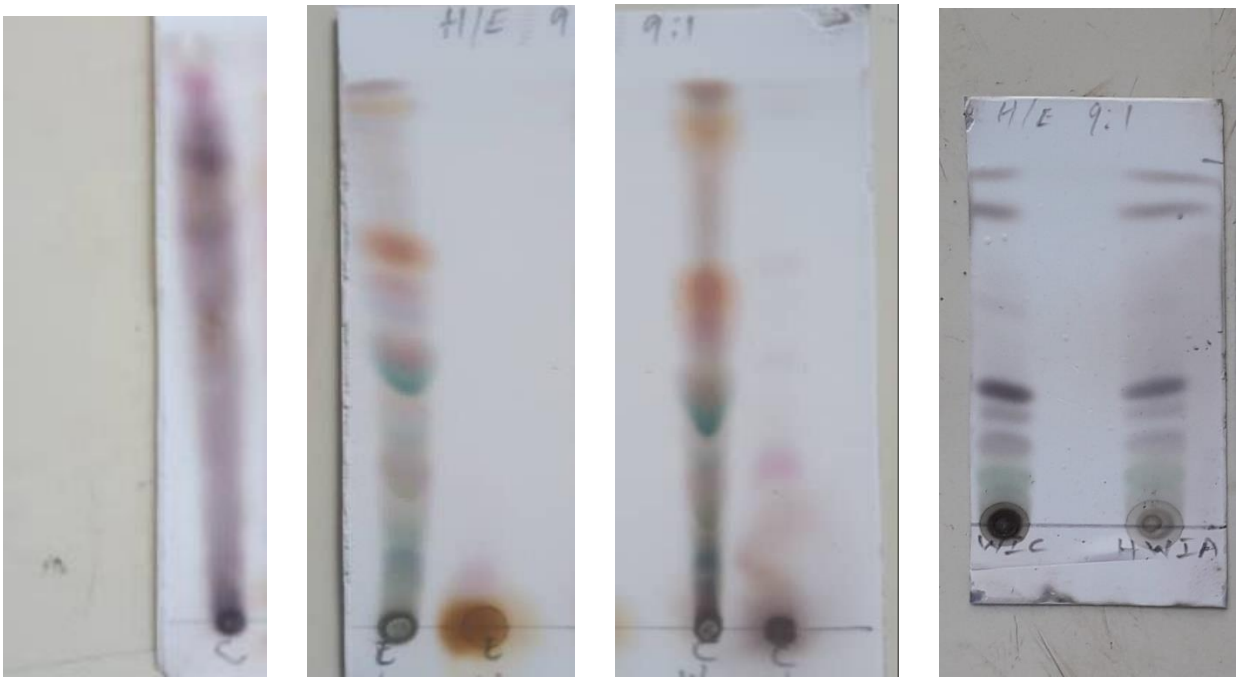

Plate II: H:E (9:1) **Plate III:** H:E (9:1) **Plate IV:** H:E (9:1) **Plate V:** H:E (9:1)

Plate II, III, IV and V: TLC profile of various fractions from the crude MAE





Plate VI: H:E (8:2)

Plate VII: H:E (9:1)

Plate VI& VII: TLC profile of chloroform fraction (CHF)

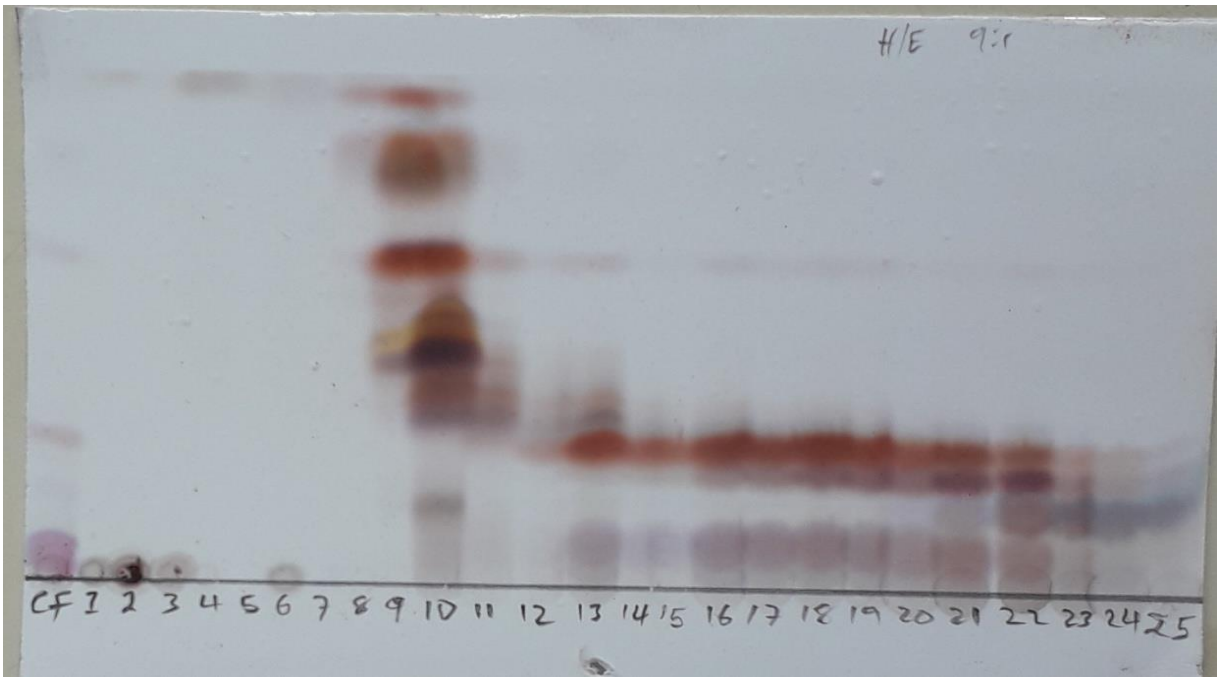

Plate VIII: TLC profile of various fractions of CHF

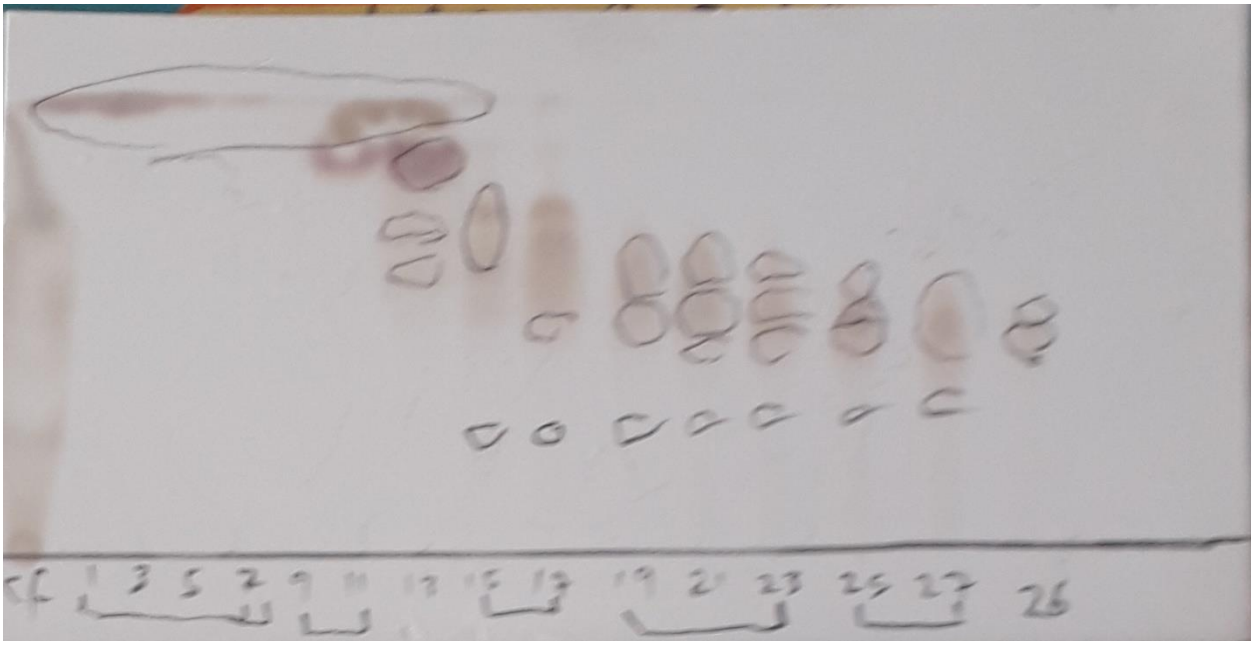

Plate IX: TLC profile of silica gel column chromatography of C_2A

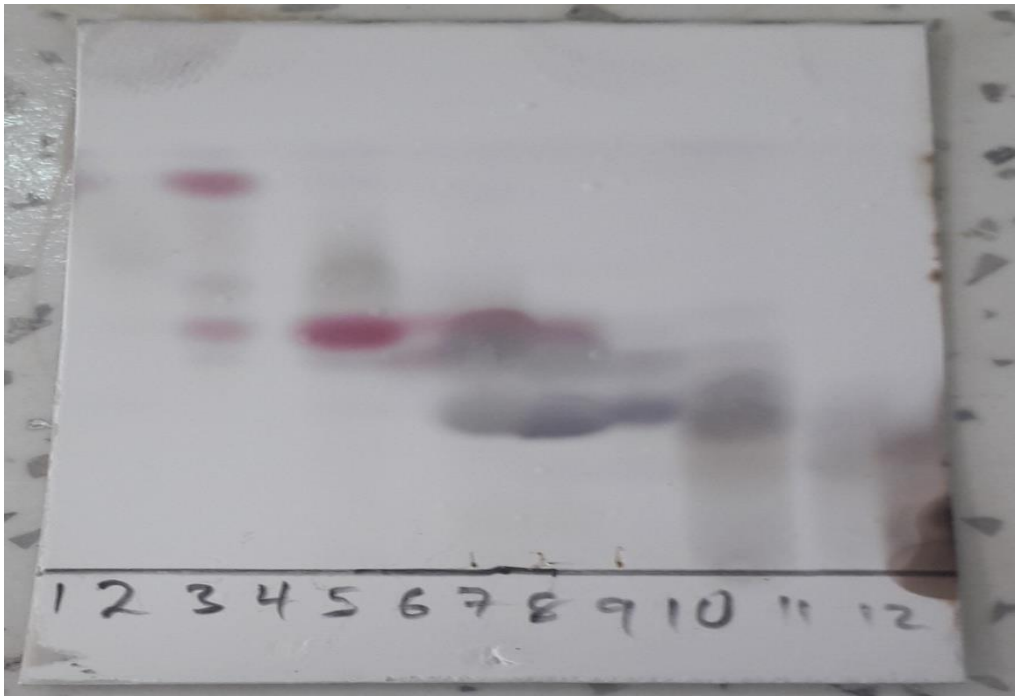

Plate X: TLC profile of silica gel column chromatography of C_5C

4.4 Isolation of Tam 2

4.4.1 Result of column chromatography of Tam 2

Fraction C_5C consisting of one major spot with little impurities was further purified repeatedly in silica gel packed column (micro pipette) eluted with *n*-hexane:ethyl acetate isocratic and gave single homogenous spot coded Tam 2 (Table 4.3).

Table 4.3: Pooled fractions from column chromatography of fraction C_5 and C_5C

No of eluates	Solvent ratio	Code
5 – 10	Hex 100 %	C_5A
11 – 17	Hex/Eth 9:1	C_5B
18 – 21	**Hex/Eth 8:2**	**C_5C**
22 – 29	Hex/Eth 8:2	C_5D
30 – 33	Hex/Eth 8:2	C_5E
34 – 35	Hex/Eth 8:2	C_5F
C_5C fraction		
3 – 7	**Hex/Eth 8:2**	**Tam2**

4.4.2 Thin-layer chromatographic profile of Tam 2

TLC analysis of the isolated compound Tam 2 using *n*-haxane:ethylacetate (8:2), dichloromethane:ethyl acetate (9:1) as solvent systems viewed under normal day light and sprayed with 10 % sulphuric acid revealed single homogenous spot with R_f value 0.50 (Plate VI and VII) respectively, (Table 4.2).

Table 4.4: R_f value of Tam 2

The R_f values of Tam2 was calculated in different solvent systems.

Solvent systems	R_f values	Color of spot after heating
Hex: EA (8:2)	0.50	Purple
DCM: EA (9:1)	0.52	Purple

4.4.3 Melting of Tam 2

The melting point of the isolated compound Tam 2 was found to be between 135 – 136 ^{O}C.

4.4.4 Solubility profile of Tam 2

Tam 2 was found to be soluble in ethyl acetate and chloroform.

4.4.5 Chemical test on Tam 2

Tam 2 (white compound) was positive to Liebermann Buchard test for steroidal nucleus (Table XI and XII)



Plate XI: H:E (8:2)

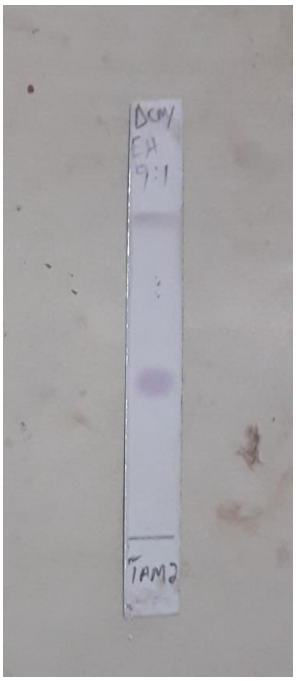

Plate XII: DCM:E (9:1)

Plate XI and XII: TLC Profile of Tam 2

4.5 Spectral Analysis of Tam 2

4.5.1 Infrared (IR) spectroscopy of Tam 2

The IR spectrum of Tam 2 revealed major peaks at: 3421 cm^{-1} (O-H stretching); 2933 – 2866 cm^{-1} (C-H aliphatic stretching); 1666 cm^{-1} (C=C olefinic stretching). Others include, 1461 cm^{-1} (CH_2 bending), 1379 cm^{-1}, 1058 cm^{-1} (cycloalkane), 801 cm^{-1} and 738 cm^{-1} (Figure 4.1).

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Sample ID:TAM2 Method Name:Transmittance
Sample Scans:30 User:admin
Background Scans:16 Date/Time:2020-02-20T11:41:21.096+01:00
Resolution:8 Range:4000 - 650
System Status:Good Apodization:Happ-Genzel
File Location:C:\Program Files\Agilent\MicroLab PC\Results\TAM2_0000.a2r

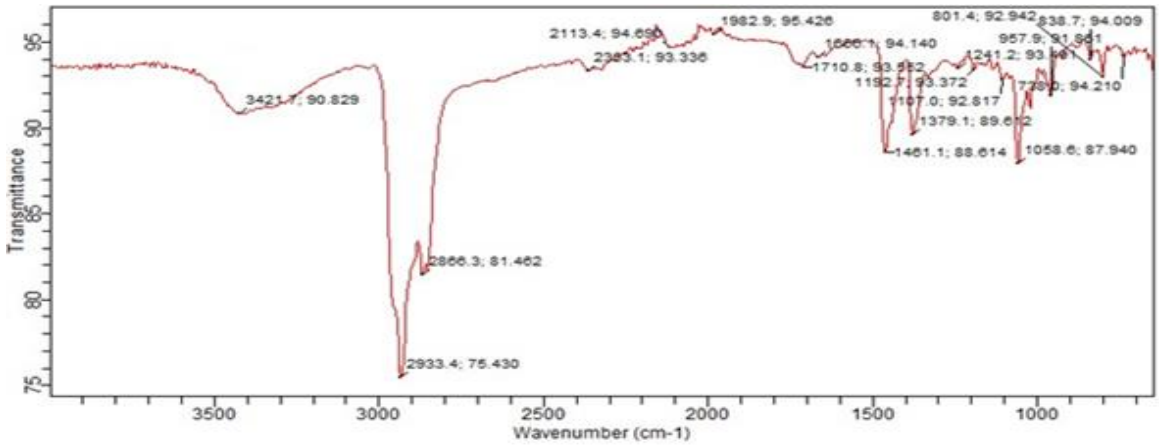

Figure 4.1: IR spectrum of Tam2

4.5.2 Proton nuclear magnetic resonance spectral analysis of Tam 2

The ^{1}H-NMR spectrum of compound Tam 2 in $CDCl_3$ (400 MHz) revealed signals at δ 5.29 (d, H-6), 3.46 (d, H-3), 2.22 (m, H-4), 1.81 (m, H-1), 1.58 (m, H-25), 1.50 (m, H-27) 1.22 (m, H-8), 1.18 (m, H-22), 1.17 (d, H-28), 1.09 (m, H-16), 0.95 (s, H-19), 0.89 (d, H-21), 0.81 (t, H-26), 0.78 (s, H-18), and 0.69 (d, H-29) (Figure 4.2) and (Table 4.5)

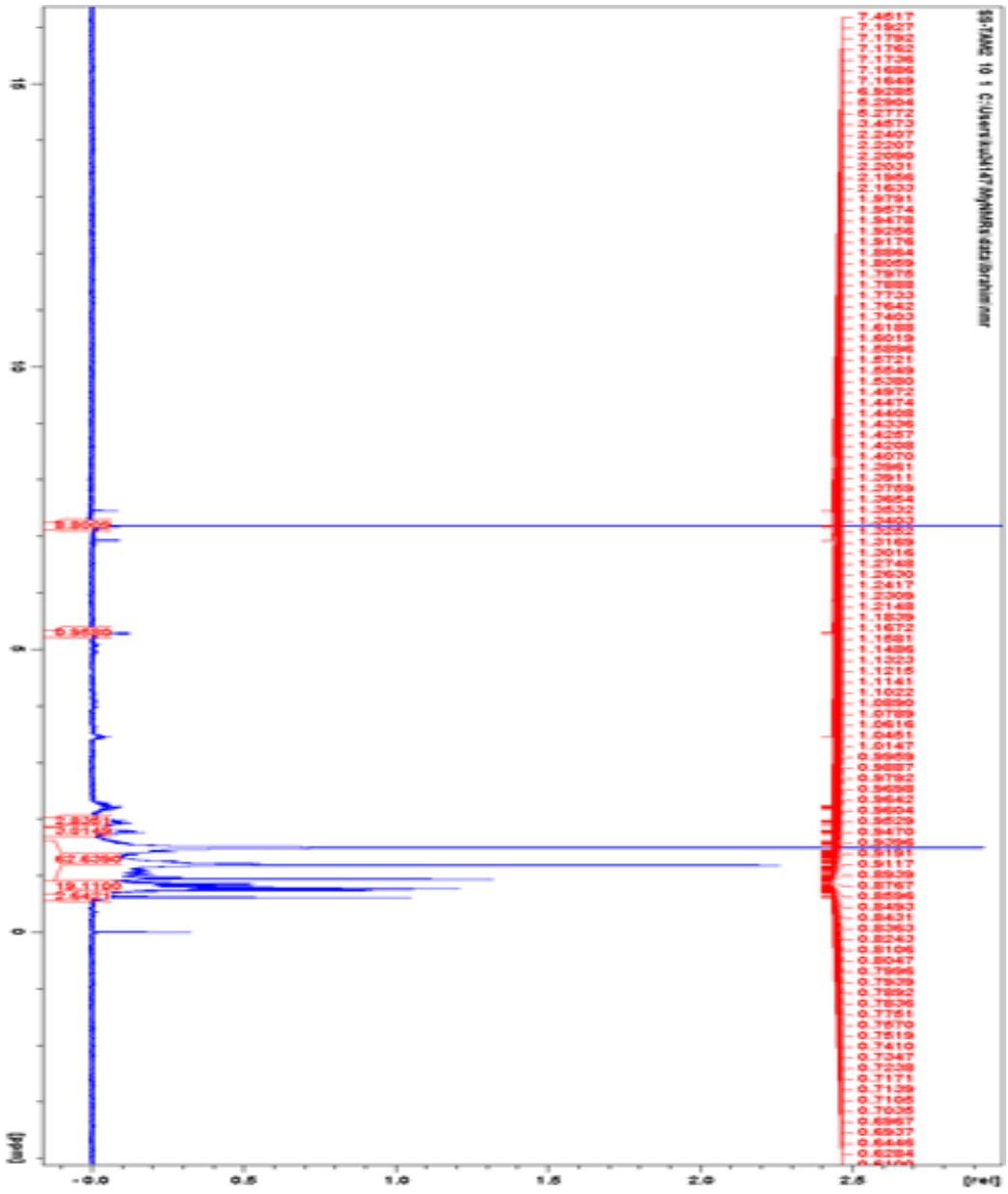

Figure 4.2: ^{1}H-NMR spectrum of Tam 2 in $CDCl_3$

4.5.3 Dept-Q 135 spectral analysis of Tam 2

The ^{13}C-NMR (δ ppm, 100 MHz, $CDCl_3$) indicated presence of 29 carbon atoms;140.80 (q), 121.75 (CH), 77.34-76.70 (solvent), 71.83 (HC-OH), 56.78 (CH), 56.17 (CH), 50.13 (CH), 45.88 (CH), 43.12 (CH_2), 42.31(q), 39.78 (CH_2), 37.25 (CH_2), 36.16 (q), 33.95 (CH_2), 31.93 (CH_2), 31.90 (CH_2), 31.67 (CH_2), 29.72 (CH), 28.26 (CH_2), 26.05 (CH_2), 24.36 (CH_2), 23.07 (CH_3), 21.31 (CH_2), 21.08 (CH), 19.50 (CH_3), 19.42 (CH_3), 18.79 (CH_3), 12.58 (CH), 12.14 (CH3), 11.87 (CH_3). Figure (4.3 – 4.4) and (Table 4.5)

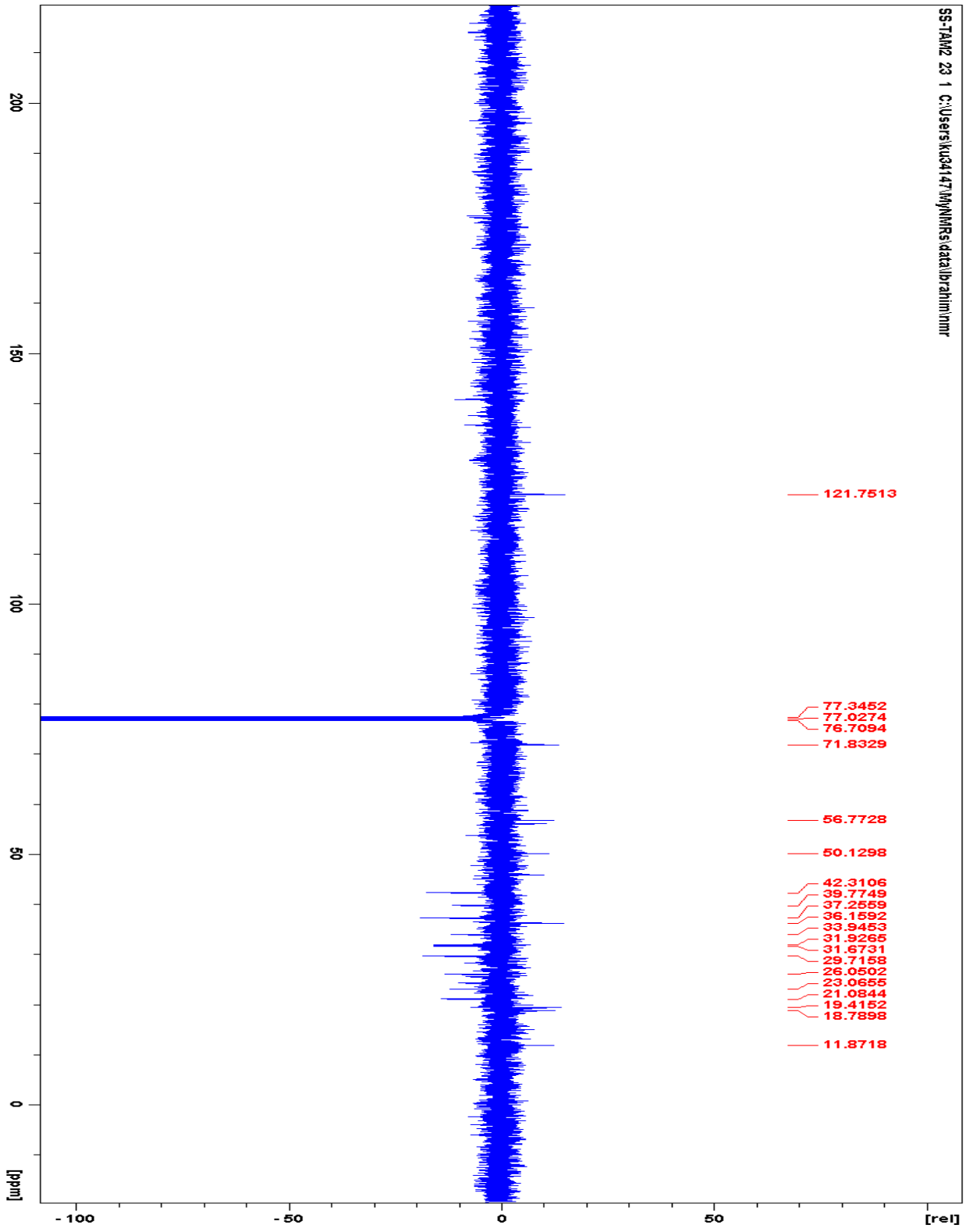

Figure 4.3: Dept-q 135 spectrum of Tam 2 in $CDCl_3$

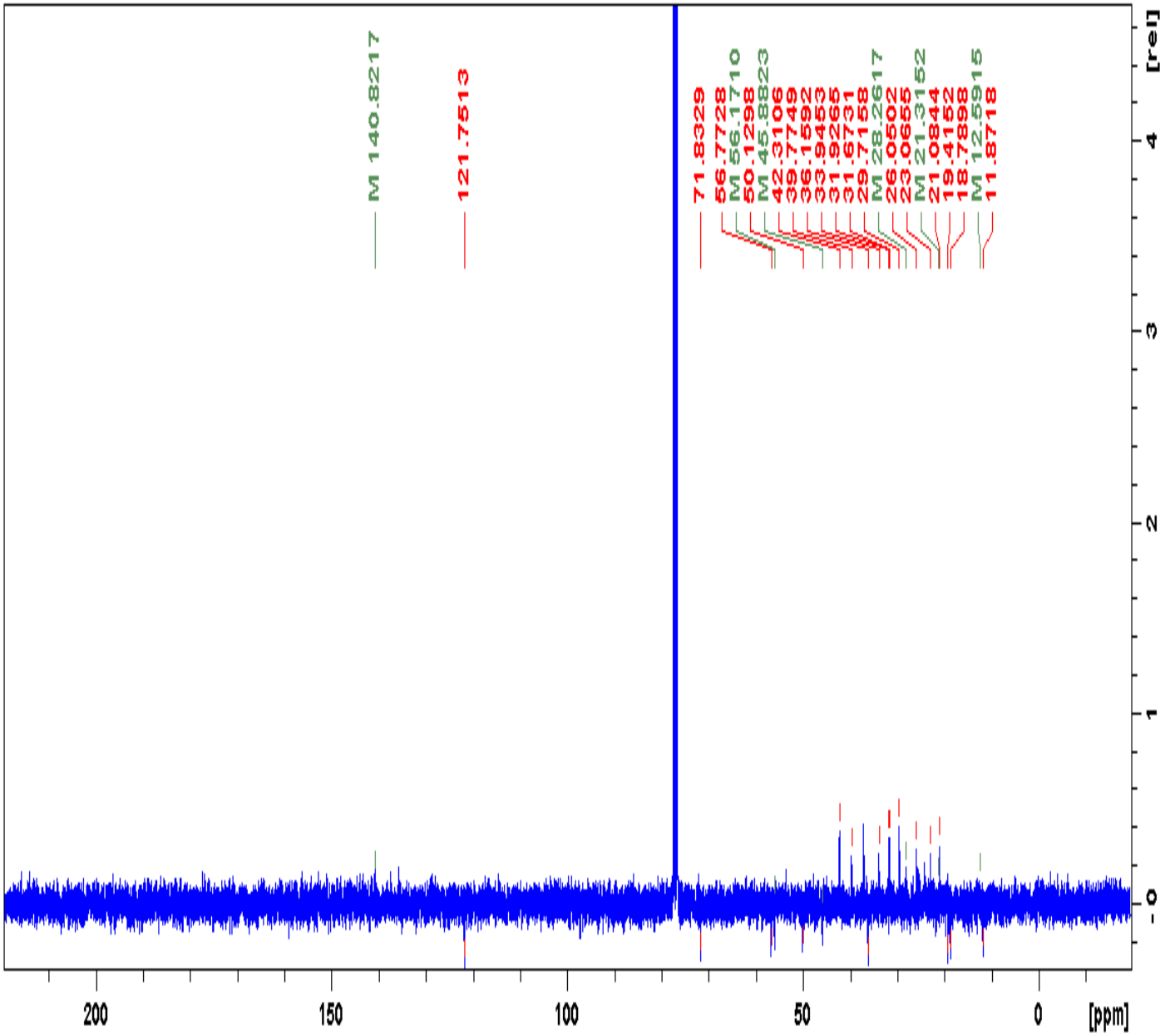

Figure 4.4: Dept-q spectrum of Tam 2 in $CDCl_3$ (Expanded)

4.5.5 ^{1}H-^{1}H COSY spectral analysis of Tam 2

The 2D^{1}H-^{1}H COSY correlation spectrum of Tam 2 indicates the protons that are situated at the adjacent carbon, major correlations include: H22 (1.17) # H23 (0.83), H25 (1.58) # H26 (0.81), H22 (1.17) # H21(0.89), H28(1,18) # H29 (0.62), H27 (1.45) # H29 (0.62), Figure 4.5

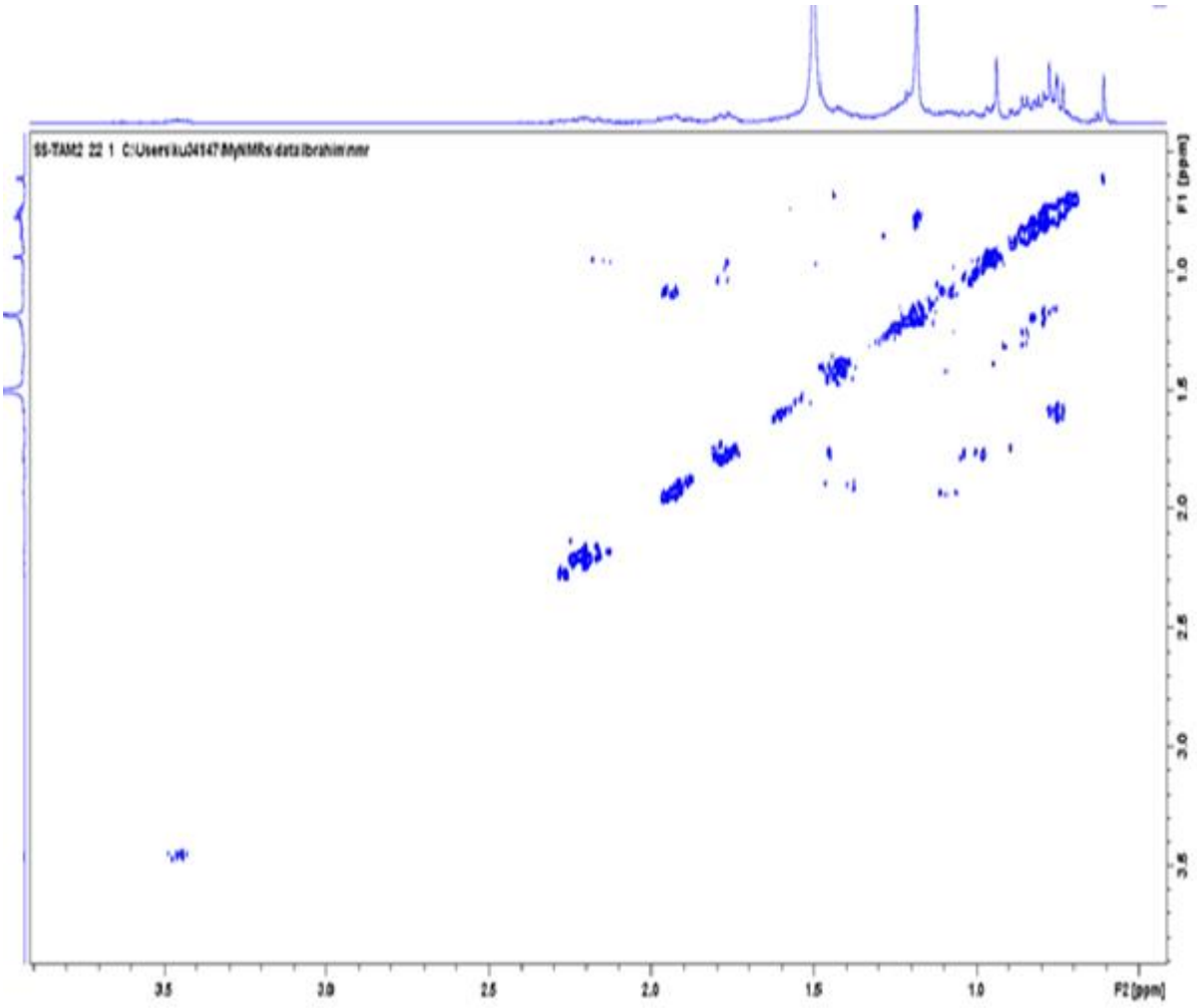

Figure 4.5: ^{1}H-^{1}H-Cosy of Tam 2 in $CDCl_3$

4.5.4 HSQC analysis of Tam 2

The HSQC spectrum (in $CDCl_3$) of Tam 2 showed the attachment of protons to their respective carbon atoms (Figure 4.6)

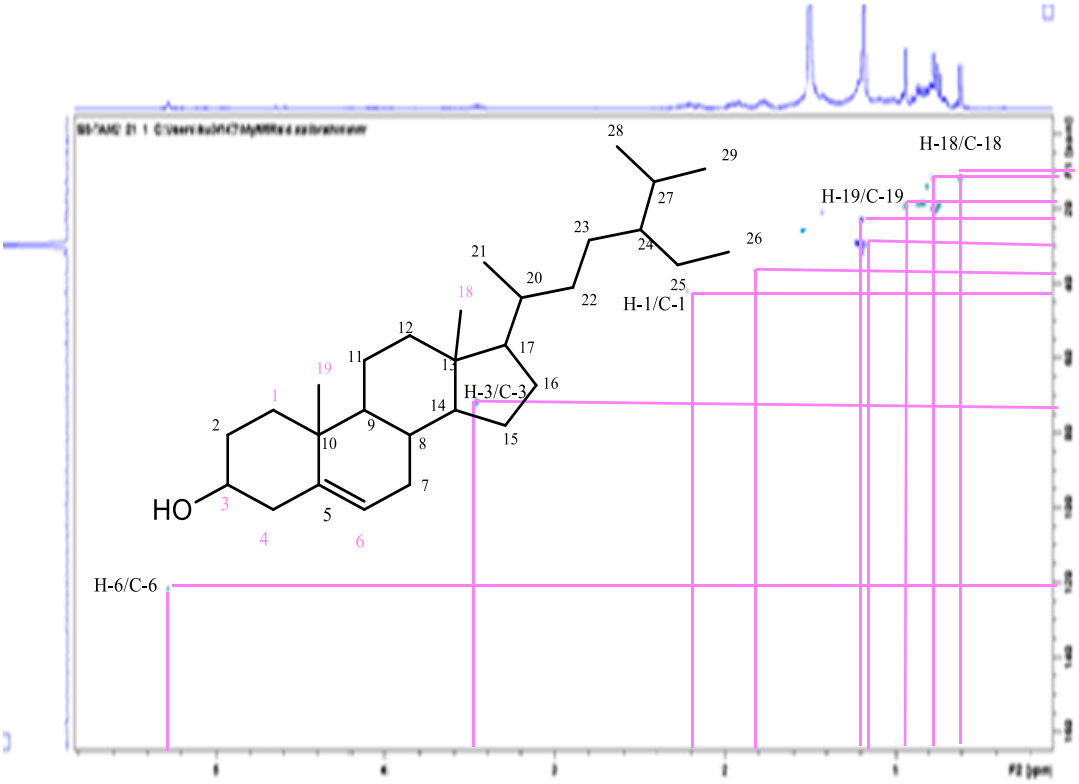

Figure 4.6: The HSQC spectrum of Tam 2 in CDCL3

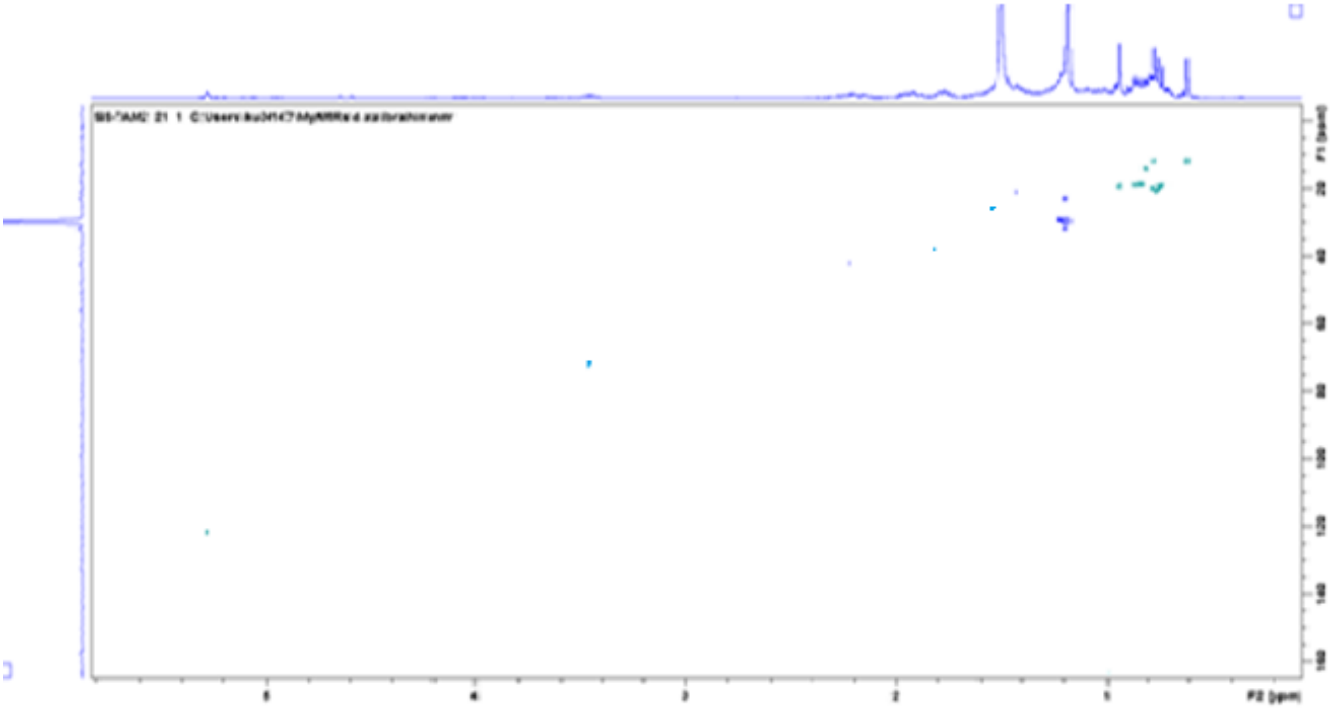

Figure 4.7: The HSQC spectrum of Tam 2 in $CDCL_3$ (expanded)

Table 4.5: ^{13}C-NMR and ^{1}H data of Tam 2 compared with those reported in literature

Positions	^{13}C-NMR Experimental	^{1}H-NMR Experimental	^{13}C-NM (Aliba *et al*., 2018)	^{1}H-NMR (Aliba *et al*., 2018)
1	37.25	1.81 (m, 2H)	37.39	
2	31.67		31.76	
3	71.83	3.46 (m, 1H)	71.98	3.53(tdd, 1H)
4	43.12	2.22 (m, 2H)	42.39	
5	140.80		140.88	
6	121.75	5.29 (d, 1H)	121.80	5.37 (d, 1H)
7	31.93		32.06	
8	29.72	1.22 (m, 1H)	29.30	
9	53.13		50.28	
10	36.16		36.65	
11	21.26		21.22	
12	39.78		39.92	
13	42.31		42.39	
14	56.78		56.91	
15	28.92		23.21	
16	31.90	1.09 (m, 2H)	28.39	
17	56.11		56.21	
18	12.14	0.78 (s, 3H)	12.12	0.70 (s, 3H)
19	19.42	0.95 (s, 3H)	19.18	1.03 (s, 3H)
20	40.41		36.29	
21	18.79	0.89 (d, 3H)	18.92	0.95 (d, 3H)
22	33.95	1.18 (m, 2H)	34.09	
23	24.36	1.2 (m, 2H)	24.44	
24	50.13		45.98	
25	26.05	1.58 (m, 2H)	26.23	
26	19.42	0.81 (t, 3H)	19.96	0.87 (t, 3H)
27	21.08	1.50 (m, 1H)	19.18	0.85 (d, 1H)
28	23.07	1.18 (d, 3H)	22.83	0.84 (d, 3H)
29	11.87	0.69 (d, 3H)	9.69	

4.6 Isolation of Tam 4

4.6.1 Result of column chromatography of Tam 4

Fraction C_2A consisting of one major spot with little impurities was further purified using silica gel packed column (micro pipette) eluted with n-hexane 100 % isocratic and a brown oily compound was afforded coded Tam 4 (4 mg). It was subjected to physicochemical tests and spectroscopic analysis to elucidate its chemical structure.

4.6.2 Thin-layer chromatographic analysis of Tam 4

TLC analysis of the isolated compound Tam 4 using *n*-hexane 100 % and *n*-hexane: dichloromethane (98:2) as solvent system viewed under normal day light and sprayed with 10 % sulphuric acid revealed single homogenous spot with R_f value 0.70 and 0.65 respectively (Plate XIII and XIV), (Table 4.7).

Table 4.6: R_f value of Tam 4

The R_f values of Tam4 was calculated in different solvent systems.

Solvent systems	R_f values	Color of spot after heating
Hex (100 %)	0.70	Brown
Hex: DCM (98:2)	0.65	Brown

4.6.3 Solubility profile of Tam4

Tam4 was found to be soluble in hexane and chloroform

4.6.5 Chemical test on Tam 4

Tam 4 (brown-oily) was positive to Salkowski's test for triterpenes.



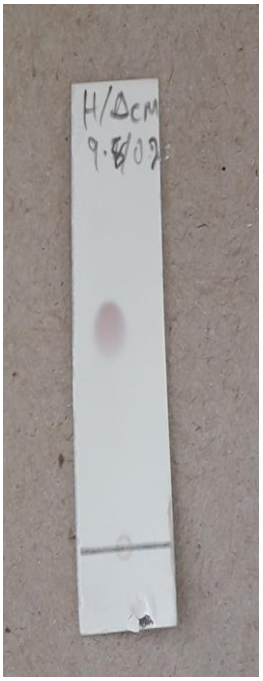

Plate XIII: Hex (100 %)

Plate XIV: H: DCM (98:2)

Plate XIII & XIV: TLC profile of Tam 4

4.7 Spectral analysis of Tam 4

4.7.1 Infrared (IR) spectroscopy of Tam 4

The IR spectrum of Tam 4 revealed major peaks at: 2968, 2922 and 2855 cm^{-1} (C-H stretching); four vibrational modes at 1461 cm-^{1}, 1375 cm-^{1}, 1151 cm-^{1}, and 879 cm-^{1}; 1636 cm^{-1} due to (C=C olefinic stretching). Figure 4.5

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Sample ID:TAM 4
Sample Scans:30
Background Scans:16
Resolution:8
System Status:Good
File Location:C:\Program Files\Agilent\MicroLab PC\Results\TAM 4_0000.a2r

Method Name:Transmittance
User:admin
Date/Time:2020-03-20T11:26:18.912+01:00
Range:4000 - 650
Apodization:Happ-Genzel

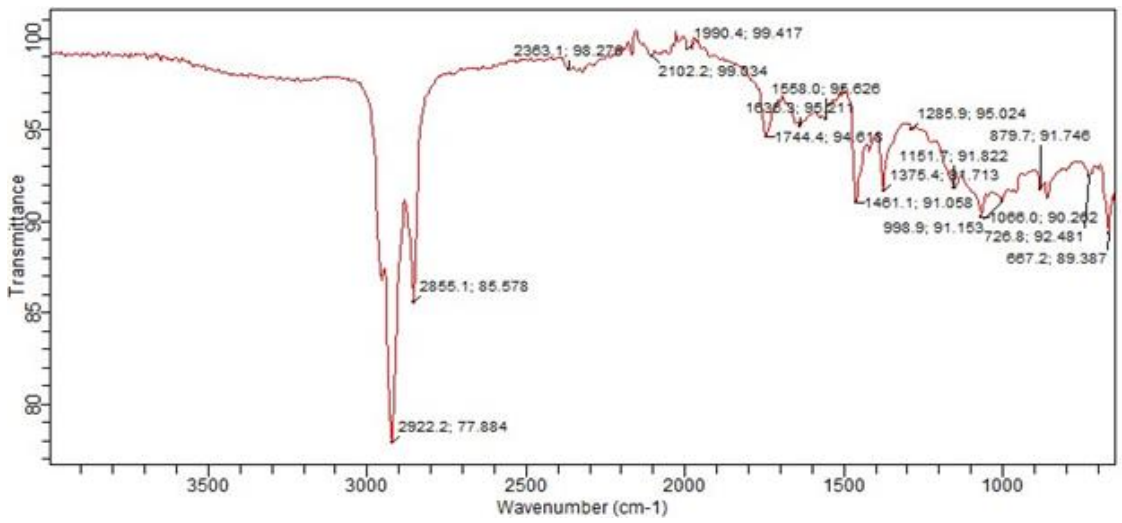


Figure 4.8: IR spectrum of Tam 4

4.7.2 Proton nuclear magnetic resonance spectral analysis of Tam 4

The ^{1}H-NMR spectrum of Tam 4 in $CDCl_3$ (400 MHz) revealed signals at δ 5.15 - 5.08 (6H, m, H-3, H-7, H-11, H-14, H-18 and H-22), 2.07 (4H, m, H-4 and H-21), 2.01 (4H, dd, H-12 and H-13), 1.68 (12H, s, H-5, H-8, H-9, H-16, H-17 and H-20), 1.60 (18H, s, H-25, H-26, H-27, H-28, H-29 and H-30), and 1.54 (6H, s, H-1 and H-24). Figure 4.10 and Table 4.8

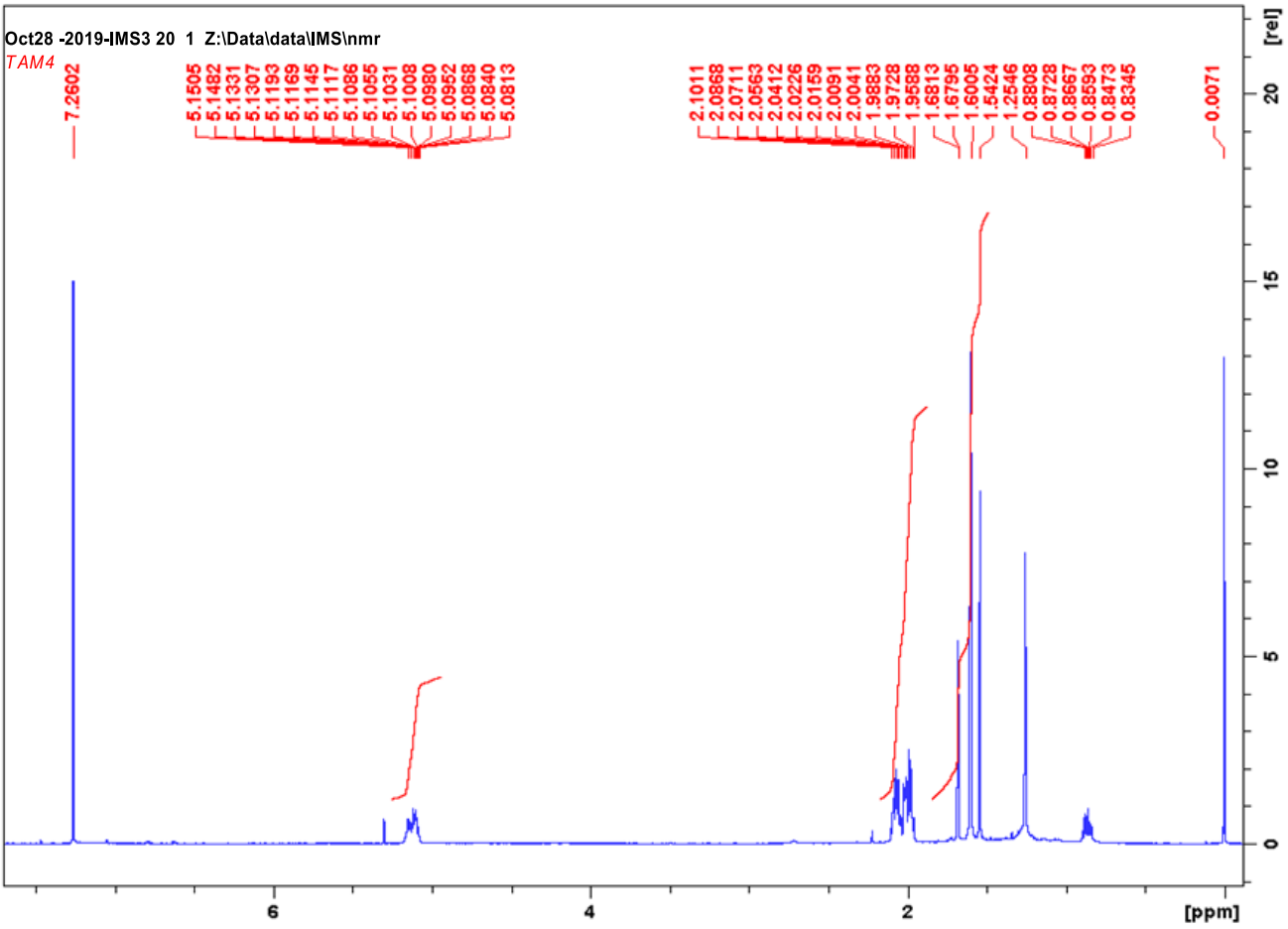


Figure 4.9: proton nuclear magnetic resonance spectrum of Tam 4

4.7.3 ^{13}C-NMR apectral analysis of Tam 4

The ^{13}C-NMR (δ ppm, 100 MHz, $CDCl_3$) indicated presence of 15 resonances; 135.32 (C-10 and C-15), 135.12 (C-6 and C-19), 131.47 (C-2 and C-23), 124.63 (C-11 and C-14), 124.52 (C-7 and C-18), 124.49 (C-3 and C-22), 39.97 (C-5, C-9, C-16 and C-20), 28.49 (C-12 and C-13), 26.99 (C-8 and C-17), 26.88 (C-4 and C-21), 25.91 (C-25 and C-30), 17.89 (C-1 and C-24), 16.21 (C-26, C-27, C-28 and C-29). The DEPT further revealed the following carbons: CH_3 (four), CH_2 (five), CH (two), Figure 4.11 - 4.12 and Table 4.7

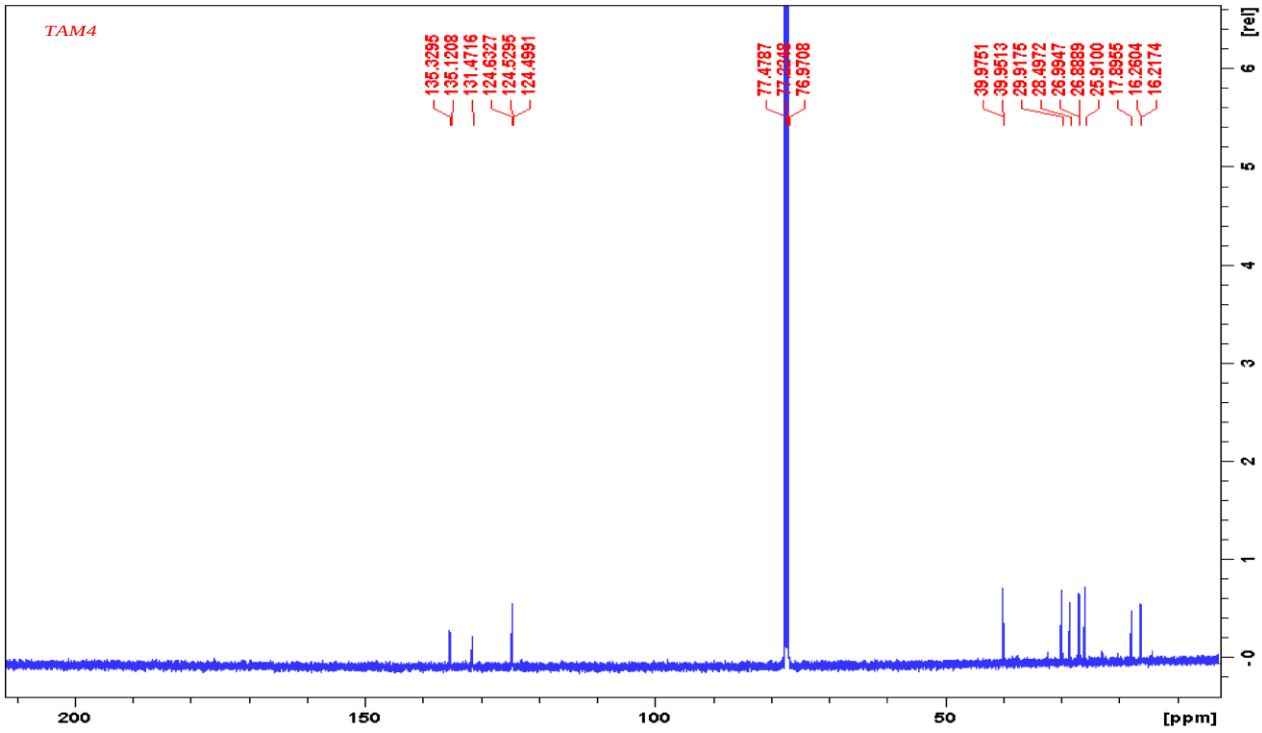


Figure 4.10: ^{13}C-NMR Spectrum of Tam **4**

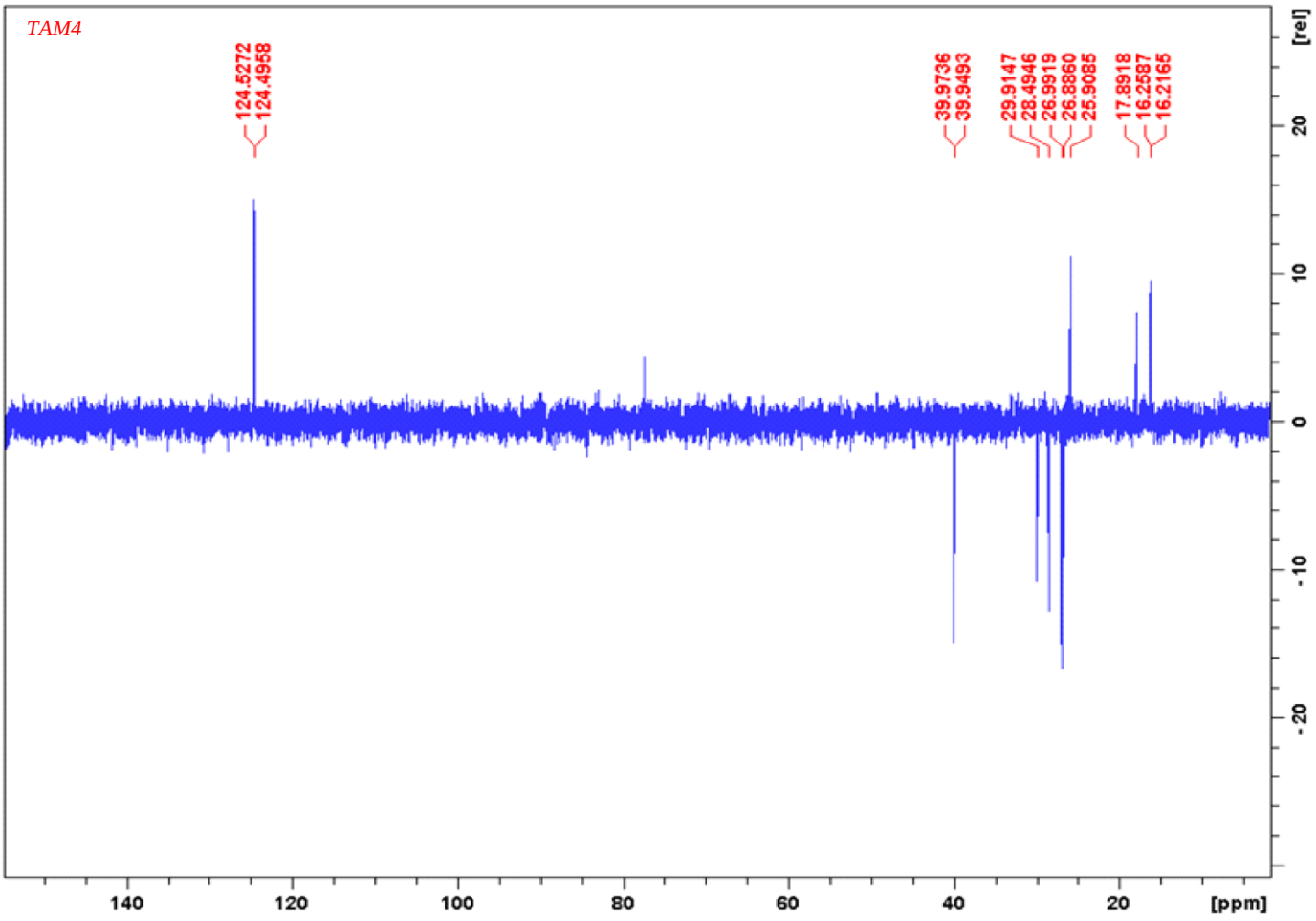


Figure 4.11: Dept spectrum of Tam 4

Table 4.7: ^{13}C-NMR and ^{1}H data of Tam 4 compared with those reported in literature

Position	^{13}C-NMR Experimental	^{1}H-NMR Experimental	^{13}C-NMR Literature (Mohd *et al*., 2019)	^{1}H-NMR Literature (Mohd *et al*., 2019)
1/24	17.89	1.54	17.8	1.68
2/23	131.47	-	131.4	-
3/22	124.49	5.08	124.3	5.15
4/21	26.88	2.07	26.7	2.07
5/20	39.97	1.68	39.8	1.68
6/19	135.12	-	135.0	-
7/18	124.52	5.10	124.4	5.14
8/17	26.99	1.68	26.8	1.99
9/16	39.97	1.68	39.8	1.99
10/15	135.32	-	135.2	-
11/14	124.63	5.12	124.5	5.13
12/13	28.49	2.01	28.3	2.01
25/30	25.91	1.60	25.8	1.60
26/29	16.21	1.60	16.1	1.60
27/28	16.21	1.60	16.1	1.60

4.8 Result of Acute Toxicity Studies

Table 4.8: Determination of median lethal dose LD_{50} (oral) of Methanolic Extract of *Tacazzea apicula*

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
160	1	0/1
290	1	0/1
5000	1	0/1

The LD_{50} (oral) of the methanolic extract was found to be above 5000 mg/kg, as there is no mortality in both the phases.

$$LD_{50} = \sqrt{5000 \times 5000}$$

$$= 5000 \text{ mg/kg oral}$$

Table 4.9: Determination of median lethal dose LD_{50} (*i.p*) of Methanolic Extract of *Tacazzea apiculata*

First Phase

Dose (mg/kg)	Number of mice used	Mortality
10	3	0/3
100	3	0/3
1000	3	2/3

Second Phase

Dose (mg/kg)	Number of mice used	Mortality
200	1	0/1
400	1	0/1
800	1	0/1
1600	1	1/1

The *intraperitoneal* LD_{50} of the methanolic extract was found to be 894 mg/kg, as calculated below:

$LD50 = \sqrt{800 \times 1000}$

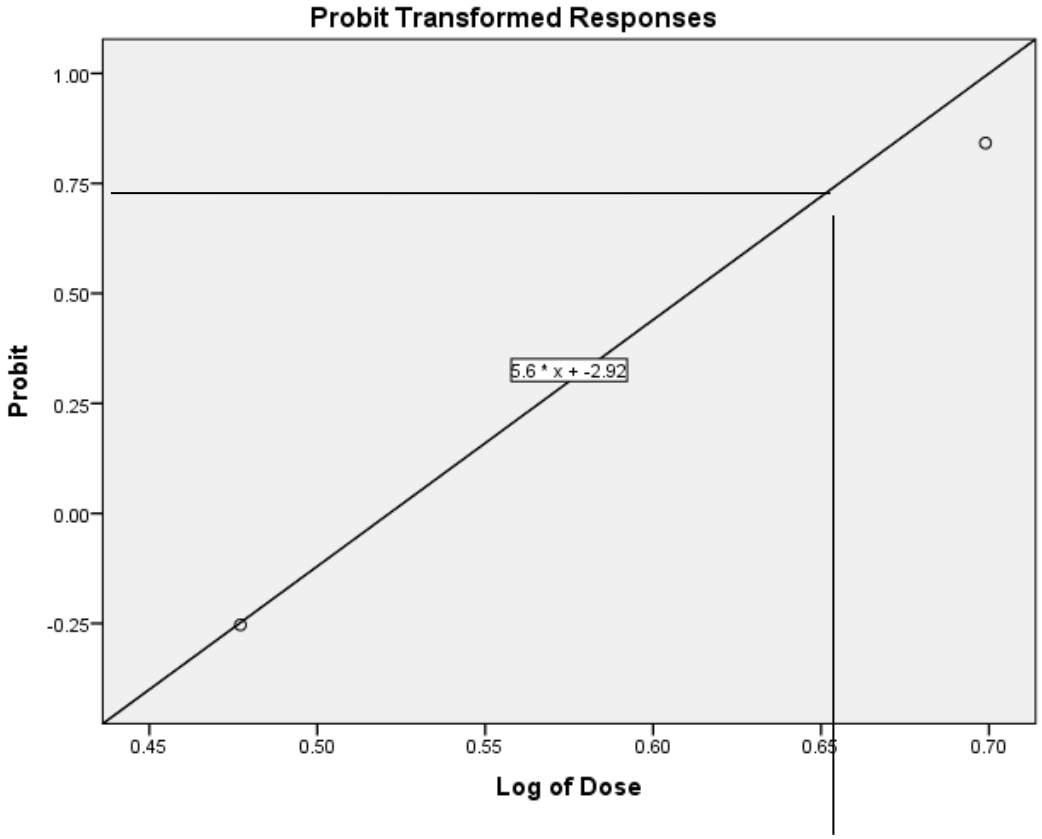
= 894 mg/kg *i.p*

4.9 Result of the Lethality Assay of the Venom

Table 4.10: Determination of minimal lethal dose (LD_{99}) of *Naga nigricollis* venom

Groups	Dose (mg/ml)	Dead/Total	Death %
1	0	0/5	0
2	1	0/5	0
3	3	2/5	40
4	5	4/5	80
5	7	5/5	100

LD_{99} was found to be 4.6 mg/kg, by probit analysis, as shown below;



0.66

LD_{99} = anti log of 0.66
= 4.6

Figure 4.12: Calculation LD_{99} of *Naja nigricollis* venom in mice receiving various doses of venom by Turner's method (n = 5)

Table 4.11: *Ex-vivo* detoxification effect of the Methanol extract of *T. apiculata* against *Naja nigricollis* venom.

Groups	Treatment (mg/kg)	Mean survival time (hr)	Total animal survival/total no. of animals in group	% survival (within24h)
1	LD_{99} + NS	2.833 ± 0.3333	0/6	0.00
2	LD_{99} + 80	24.000 ± 0.0000*	6/6	100
3	LD_{99} + 170	22.250 ± 1.7500*	5/6	83.33
4	LD_{99} + 260	20.083 ± 2.4780*	4/6	66.67

Result were expressed as Mean ± SEM, * represent $P \leq 0.05$ compared with normal saline (one way ANOVA and Dunett post hoc test, n = 6, NS = normal saline, LD_{99} = minimum lethal dose).

Table 4.12: *In vivo* detoxification effect of the Methanol extract of *T. apiculata* against *Naja nigricollis* venom.

Groups	Treatment (mg/kg)	Mean survival time (hr)	Total animal survival/total no. of animals in group	% survival (within 24h)
1	LD_{99} + NS	0.967 ± 0.6653	0/6	0.00
2	LD_{99} + 500	1.767 ± 0.5007	0/6	0.00
3	LD_{99} + 1000	2.200 ± 0.3162	0/6	0.00
4	LD_{99} + 1500	5.117 ± 1.7371	0/6	0.00
5	LD_{99} + 2000	8.433 ± 7.6935*	1/6	16.67

Result were expressed as Mean $\pm$ SEM, * represent $P < 0.05$ compared with normal saline (one way ANOVA and Dunett post hoc test, n = 6, NS = normal saline, LD_{99} = minimum lethal dose).

CHAPTER FIVE

5.0 DISCUSSION

5.1 Phytochemical Studies

Preliminary phytochemical screenining on crude methanol extract of *T. apiculata* revealed the presence of steroids and triterpenes, tannins, flavonoids, alkaloids, saponings, and cardiac glycosides (Table 4.1). The *n*-hexane fraction revealed the presence of steroids/triterpenes nucleus, and alkaloids, chloroform fraction revealed the presence of Alkaloids, steroids/triterpenes, and the ethylacetate fraction revealed the presence of flavonoids, cardiac glycoside, tannins and saponings.These constituents have been reported to be associated with different pharmacological activities in plants (Cowan, 1999).

Column chromatographic separation of chloroform fraction (CHF) followed by further repeated purification of column fraction C5C in micro pipette led to the isolation of a white compound (Tam 2). Result obtained from chemical test suggest the presence of steroidal nucleus (Silva *et al.,* 1998). The melting point was found to be 135 – 136 ^{0}C.

The IR spectrum of Tam 2 showed absorption peaks at 3421 cm^{-1} that is characteristic of O-H stretching; 2933 - 2866cm^{-1} is due to C-H aliphatic stretching (Arif *et al*., 2019). 1650 cm^{-1} is due to C=C olefinic stretching, other absorption frequency includes 1461 cm^{-1} for (CH_2 bend), 1379 cm^{-1}, 1058 cm^{-1} for cycloalkane and 738 cm^{-1} (Padmini *et al*., 2012). These absorption frequencies resemble those of β-sitosterol (Arif *et al*., 2019; Padmini *et al*., 2012).

The ^{1}H-NMR spectrum of Tam 2 exhibited one olefinic proton at δH 5.29, the H-3 proton which appeared as a multiplet at δ 3.46 and a cluster of resonances up field between δ 2.22 - δ 0.69 suggesting steroidal nucleus (Vitus *et al*., 2016). The six methyl proton signals were evident which include two methyl singlet at δ 0.78 and δ 0.95 which corresponds to the angular methyl singlet, three methyl doublets at δ 0.89, δ 1.18 and δ 0.69, and a methyl triplet at δ 0.81 corresponding to the methyl protons (Aliba *et al*., 2018).

The Dept-Q 135 (100 MHz) spectral analysis exhibited 29 carbon signals, comprising 6 methyls (CH_3), 11 methylene (CH_2), 9 methine (CH) and 3 quaternary (C) carbons; further indicating the steroidal nature of the compound (Vitus *et al*., 2016). The down field resonance at δC 140.80 and δ 121.75 were due to quartenary olefinic carbon C-5 and an olefinic carbon C-6 respectively. The signals at δC 19.42 and 12.14 correspond to angular methyl carbon atoms, the signal at δC 71.83 is assignable to the beta hydroxyl group attached to the carbon at position 3; suggest the compound as β – sitosterol (Arif *et al*., 2019).

The results of the 2D (homonuclear correlation ^{1}H-^{1}H COSY and the heteronuclear correlation HSQC spectroscopy) confirmed the relationship between the various protons and carbons in the molecule. The ^{1}H-^{1}H COSY experiment established the correlations between the protons at H22(1.17) # H23(0.83), H21(0.89) # H22(1.17), H25(1.58) # H26(0.81), H29(0.69) # H28(1.18), H26(0.81) # H27(1.45).

Based on these result and also by comparison with a reference NMR data (Aliba *et al.,* 2018), the spectral peaks are typical of β – sitosterol.

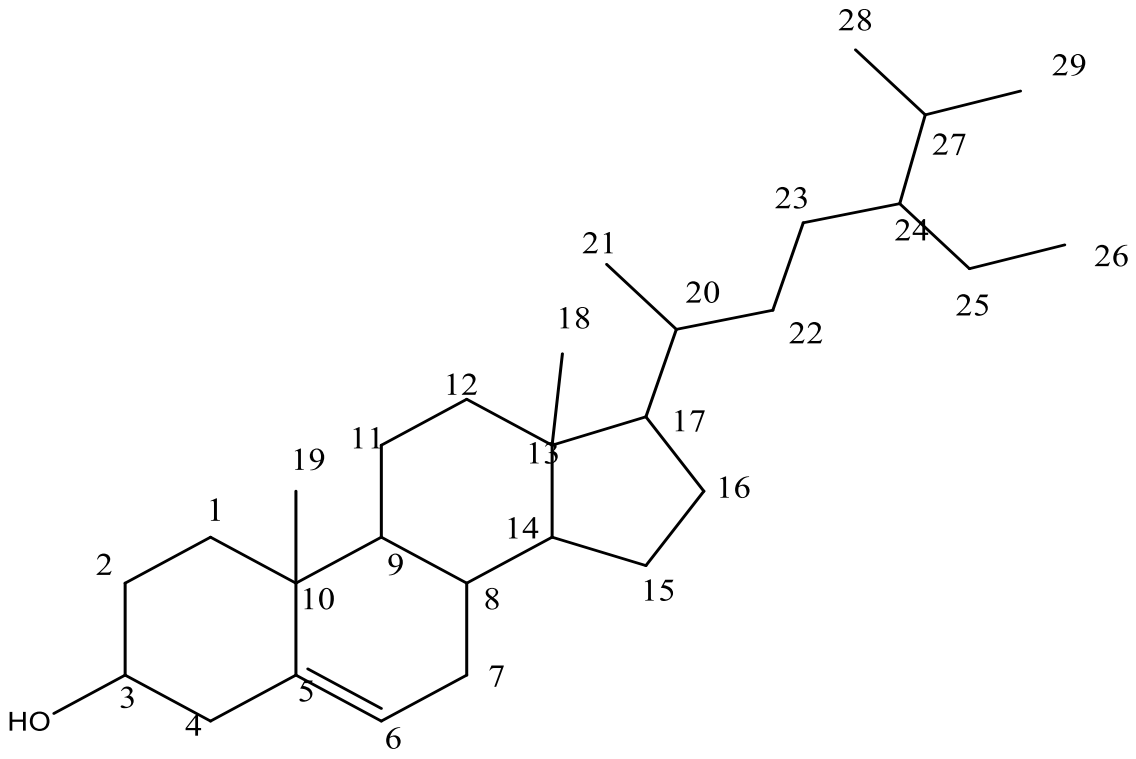


Figure 5.1: β-sitosterol (22,23-Dihydrostigmasterol, Stigmast-5-en-3-ol, β-Sitosterin) $C_{29}H_{50}O$

Further purification of C_2A fraction using silica gel packed column (micro pipette) collected from C_2 sub-fraction of silica gel column chromatoghraphy of the CHF fraction of the methanol extract led to the isolation of a brown oily compound coded Tam 4. It was positive to Salkowski's test suggesting the presence of triterpenes (Sofowora, 1993).

The IR spectrum of compound Tam 4 showed stretching peaks at 2968 cm^{-1} - 2855 cm^{-1} that is due to C-H aliphatic stretching; four vibrational modes at 1461, 137, 1151 and 879 cm^{-1} (David *et al*., 2015). The peak at 1636 cm^{-1} due to C=C olefinic stretching has also been observed (Mohd *et al*., 2019). These absorption frequencies resemble those of squalene (David *et al*., 2015; Mohd *et al*., 2019).

The ^{1}HNMR and ^{13}C NMR spectra of Tam 4 exhibited chemical shift values typical of triterpene with a centre of symmetry between position 12 – 13, each resonance integrating for two positions (Anne-mar *et al*., 2017). The resonance between δH 5.15 – 5.08 were due to vinylic signals assigned to H-3, H-7, H-11, H-14, H-18 and H-22 (Anne *et al*., 2017). The CH_2 region is characterized by three cluster of proton resonances centerd near δH 2.07 (4H, m, H-4 and H-21), δH 2.01 (4H, dd, H-12, and H-13) and δH 1.68 (12H, s, H-5, H-8, H-9, H-16, H-17 and H-20). Methyl groups proton appeared at δH 1.60 (18H, s, H-25, H-26, H-27, H-28, H-29 and H-30), and δH 1.54 (6H, s, H-1 and H-24) (Mohd *et al.*, 2019).

The ^{13}C-NMR and DEPT (figure 4.11 and 4.12) spectra showed the presence of 15 resonance. There were eight methyl carbons at δC 16.26 to 25.91, ten methylene carbons at δC 26.88 to 39.97 and six olefinic carbon at δC 124.49 to 124.63. Signals for quaternary carbon appered at δC 131.47 to 135.32, these amount to 30 carbons resonating at 15 positions because of the symmetrical nature of the compound.

The results of the 1D NMR expirements, as summarized on Table (4.6) confirmed the structure of Tam 4 as squalene, Figure (5.2). Comparison with reference NMR data (Mohd *et al*., 2019) showed a good match and further confirmed the proposed structure.

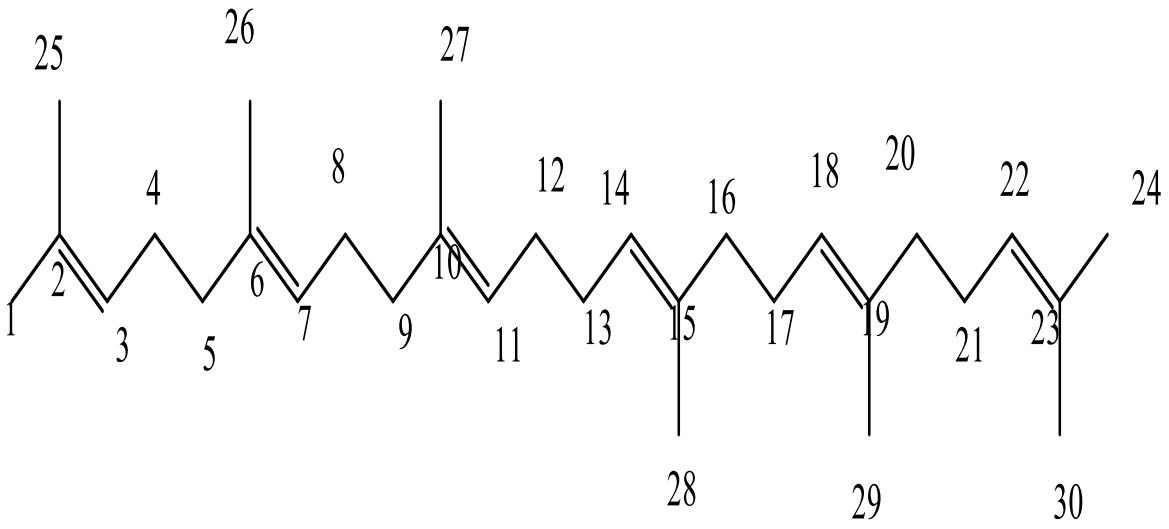


Figure 5.2: Squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene) $C_{30}H_{50}$

5.2 Biological Studies

The oral and intraperitoneal route LD_{50} of the methanol crude extract in mice were found to be 5000 mg/kg and 894 mg/kg respectively. These suggest that the former route is relatively non toxic and the later relatively toxic (Lorke, 1983).

The survival of experimentally protected laboratory animals (within 24 h after being challenged with lethal venom dose) was used to deduce the antivenin activity. The results of the present investigation suggest that methanol extract of aerial part of *T. apiculata* possesses significant antivenin activity against *Naja nigricollis* venom. It exhibited a significant activity against the *Naja nigricolis* venom in mice *ex-vivo*, maximum and minimum protection level against lethality were observed at 80 and 260 mg/kg (which corresponds to lowest and highest administered doses respectively) of the extract when compared with the normal saline treated group (Table 4.11). The result of the *ex vivo* studies suggests that the extract might act by neutralizing the enzymatic activity of the venom at the site of bite, thereby reducing the severity of the toxic effects (Yusuf *et al*., 2015).

The result of the *in-vivo* studies revealed a significant activity against *Naja nicricolis* venom in mice. There was significant protection level against lethality at the highest administered dose of 2000 mg/kg of the extract when compared with the norma saline treated group (Table 4.12). The activity observed by the mice administered with the venom/extract may be attributed to the presence of any of the constituents such as steroids, alkaloids, flavonoids, tannins, saponins etc. present in the plant extract. Flavonoids and terpenoids are reported to possess protein binding and enzyme inhibiting properties (Yusuf *et al*., 2015).

Tannins are known to unspecifically inactivate proteins (Abubakar *et al*., 2000). Triterpenoids may be involved in venom inactivation processes. The pentacyclic triterpenes (free or as glycosides) are found widely in several anti-snake venom plants and provide nearly 20 % protection against snake venom (Mors *et al*., 2000). Beta-sitosterol and stigmasterol have been reported to play an important role in neutrilizing snake venom-induced actions with nearly 70 % and 80 % protection, respectively (Yusuf *et al* 2015).

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

Preliminary phytochemical screening of aerial part of the methanol crude extract of *T. apiculata* revealed the presence steroids and triterpenes, tannins, flavonoids, alkaloids, saponins, and cardiac glycosides. The *n*-hexane fraction revealed the presence of steroids/triterpenes and alkaloids, chloroform fraction revealed the presence of steroids/triterpenes, alkaloids and the ethylacetate fraction revealed the presence of flavonoids, cardiac glycoside, tannins and saponins.

Chromatographic studies of the chloroform fraction resulted in the isolation of two compounds, Tam 2 which is a steroid (β-sitosterol) and Tam 4 a triterpene (squalene).

The methanol crude extract exhibited a significant *ex-vivo* antivenin activity against *Naja nigricollis* venom in mice with 100 % protection at 80 mg/kg. *In-vivo* protection against lethality was seen only at 2000 mg/kg of the extract with 16.67 % survival.

6.2 Conclusion

Base on the results presented, the plant demonstrated antivenin activity both *ex-vivo* and *in-vivo* at the doses of (80, 170, 260 mg/kg) and 2000 mg/kg, respectively, in mice rationalizing its ethno medicinal claim for the treatment of snakebite envenoming. The activity observed might be linked to the secondary metabolites present in the plant, steroids has been reported to possess antivenin activity in mice.

Hence, the research hypothesis is accepted.

6.3 Recommendations

1. There is a need for a bioassay guided isolation to be carried out to ascertain the exact secondary metabolites responsible for the observed antivenin activity.

2. Chronic toxicity studies should be done to establish its safety, in view of its widespread usage in traditional medicine.

3. *Ex-vio* venum detoxifying action of extract should be investigated at 10 % LD_{50} extract as the highest dose.

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