PHYTOCHEMICAL, ANALGESIC AND ANTI-INFLAMMATORY STUDIES ON THE METHANOL ROOT EXTRACT OF *COMBRETUM HYPOPILINUM* (DIELS) OKAFOR (COMBRETACEAE).

BY

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ZARIA, NIGERIA

JANUARY, 2022

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DEPARTMENT OF PHARMACEUTICAL AND MEDICINAL CHEMISTRY,

FACULTY OF PHARMACEUTICAL SCIENCES,

AHMADU BELLO UNIVERSITY,

ZARIA, NIGERIA

JANUARY, 2022

DECLARATION

I declare that the work in this dissertation entitled "**Phytochemical, Analgesic and Anti-inflammatory Studies on the Methanol Root Extract of *Combretum Hypopilinum* (Diels) Okafor *(Combretaceae)*** has been carried out by me in the Department of Pharmaceutical and Medicinal Chemistry. The information derived from literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

Amina Abubakar MUHAMMAD	_____	_____
Name of Student	Signature	Date

CERTIFICATION

This Dissertation "**Phytochemical, Analgesic and Anti-inflammatory Studies on the Methanol Root Extract of *Combretum Hypopilinum* (Diels) Okafor *(Combretaceae)***" by Amina Abubakar Muhammad meets the regulations governing the award of the degree of Master of Science in Pharmaceutical Chemistry of the Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

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Chairman, Supervisory Committee	Signature	Date

Dr. Y.M Sani

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Member, Supervisory Committee	Signature	Date

Prof. A. Musa

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Head of Department	Signature	Date

Prof. S.A. Abdullahi

_____	_____	_____
Dean School of Postgraduate Studies	Signature	Date

DEDICATION

This dissertation is dedicated to my dad Dr. Bala Muhammad and my aunt Amina Muhammed for their immense support and inspiration.

ACKNOWLEDGEMENTS

My profound gratitude goes to Almighty Allah for seeing me through this research work successfully. May His blessing be upon the noble prophet Muhammad (SAW) and the entire ummah till the Day of Judgement.

My sincere gratitude goes to my supervisors, Prof. H.S. Hassan and Dr. Y.M Sani for their patience, guidance, instructions, advice and encouragement during the course of this research work. May Almighty Allah bless them and their families.

I wish to express my sincere gratitude to all the staff, comprising the academic, non-academic, technical staff and postgraduate students of the department of Pharmaceutical and Medicinal Chemistry, Ahmadu Bello University, Zaria. I really appreciate all the help you all rendered to me one way or the other. My utmost gratitude is also expressed to Malam Balarabe and Mallama Maryam Salahudeen of Pharmacology department for their kind assistance in my biological activity experiments and interpretation. and Malam Kabiru of Pharmacognosy for his assistance with some reagents.

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ABSTRACT

Combretum hypopilinum (Diels) Okafor belongs to the family *Combretaceae,* and composed of 20 genera with about 500 species. It is a small to medium sized, deciduous shrub or tree growing from 4 - 18 metres in height, with a rounded or flat heavy crown. The plant is used in ethnomedicine for the treatment of pain, snake bites, jaundice, gonorrhoea, gastrointestinal disorder, infertility, tuberculosis and as blood tonic. The aim of the study is to isolate and characterize some of the phytochemical constituent present in the methanol root extract of *combretum hypopilinum* and to screen the extract for analgesic ant ant-inflammatory activity in mice and rats. The methanol root extract of the plant was subjected to phytochemical screening as well as analgesic and anti-inflammatory studies. The preliminary phytochemical screening of the methanol root extract of *Combretum hypopilinum* revealed the presence of steroids/triterpenes, flavonoids, tannins, cardiac glycosides, alkaloids, carbohydrates and saponins. The methanol root extract was partitioned successively with chloroform, ethyl acetate and acetone in order of increasing polarity to yield different fractions. Column chromatography separation of ethyl acetate fraction led to the isolation of stigmasterol and Lupeol which are white powdered and white crystalline material with melting points range of 169-171^{0}C and 212-214^{0}C respectively. The structures of these isolated compounds were elucidated using physical test, chemical test, IR and NMR analysis. The intraperitoneal LD_{50} of the extract was found to be greater than 5000 mg/kg body weight which implies that the extract is relatively safe. The analgesic activity was determined using acetic acid induced writhing, hot plate tests in mice while the anti-inflammatory activity was investigated using carrageenan-induced paw edema test in rats. The extract significantly and dose dependently inhibited the acetic acid-induced writhing in mice. However, the highest protection against writhing was produced by the MRE at a dose of 1000 mg/kg (66.58 %) when compared to the standard drug 69.57 %. The extracts at dose of 500 mg/kg at 90 min showed a significant (P<0.05) increase in the mean reaction time using hot plate method. The carrageenan induced paw oedema was employed in investigating the anti-inflammatory activity in rats. The extract showed significant decreases in paw

edema at 4 -5 h of carrageenan injection at all doses tested when compared to the control group at (P<0.05). These results thus, justify the use of the plant in ethnomedicine for the treatment of pain and inflammation. The biological activity might be as a result of the presence of bioactive phytoconstituents present in the methanol root extract.

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LIST OF ABBREVIATIONS

NMR	nuclear magnetic resonance
1D-NMR	1-dimensional nuclear magnetic resonance spectroscopy
^{1}H NMR	Proton nuclear magnetic resonance spectroscopy
^{13}C NMR	Carbon nuclear magnetic resonance spectroscopy
IR	Infrared spectroscopy
LD_{50}	Median lethal dose
TLC	Thin layer chromatography
δ	Proton chemical shift
WHO	World health organization
g	Gramm
Kg	Kilogram
mg	Milligram
nm	Nanometer
ppm	Parts per million

CHAPTER ONE

1.0 INTRODUCTION

1.1 Natural Products

Natural product is an organic chemical substance that is produced by living organisms found in nature by the pathways of secondary and primary metabolism (Woldeyes *et al.*, 2012). Natural products have pharmacological properties that can be useful in treating various kinds of diseases, and may act as active ingredients for both traditional medicine and modern medicines (Raafat, 2013). Natural products are the basis for synthetic and conservative herbal medicine. From time immemorial, natural products from medicinal plants and other sources have served as limitless source of pharmaceuticals (Parikh *et al.*, 2015). The phytochemicals in plants have been a pivotal pipeline for pharmaceutical discovery over the past century (Samuel *et al*., 2016).Throughout human history, plants have the ability to synthesize a wide variety of chemical compounds that are used to perform important biological functions, and to defend the plant against attack from predators such as insects, fungi and herbivorous mammals. It has been estimated that over 300,000 secondary metabolites exist (McMurry, 2010), and it's thought that their primary function is to increase the likelihood of an organism's survival by repelling or attracting other organisms. Many secondary metabolites are cytotoxic and have been selected and optimized through evolution for use as chemical warfare agents against prey, predators, and competing organisms (Hunter, 2008). Drugs from natural sources may fall into one of three categories of compounds. Plants are a major source of complex chemical constituents with pharmacological activities. Major classes of phytochemicals include phenols, polyphenols, tannins, terpenes, and alkaloids (Bhat *et al.*, 2005). Although the number of plants that have been extensively studied is relatively small, many pharmacologically active natural products have already been identified. Plant derived

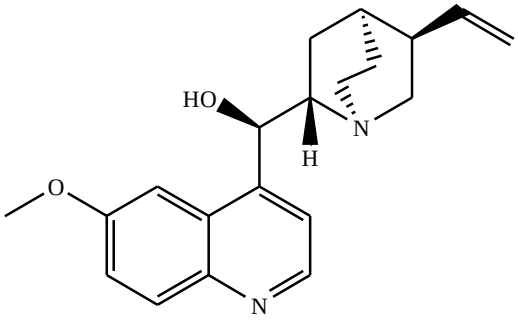
natural products such as flavonoids, terpenes and alkaloids have received considerable attention due to their diverse pharmacological properties including anti-inflammatory, antipyretic and analgesic activities (Shukla *et al*., 2010). the human use of plants as medicine may be traced back to at least 60,000 years according to fossil record, (Mugdha and Sapartashi, 2017). Animals can sometimes be sources of new pharmacologically active compounds (whether as simple compounds, venoms or toxins) or leads in the development of newer drugs (Dossey, 2010). In recent years there has been great interest in finding pharmacologically active natural products from marine sources e.g. coral sponges, fish, and marine microorganisms have biologically potent chemicals with interesting anti-inflammatory, anti- viral, anti-cancer and anti-tumor activity (Dossey, 2010). Micro-organisms such as bacteria and fungi have been invaluable for discovering pharmacologically active natural products; they provide large variety of ant-microbial agents which has evolved to give their host an advantage over their competitors in micro biological world (Chin *et al.,* 2006). Therefore, natural products continue to provide unique structural diversity in comparison to standard combinatorial chemistry, and presents opportunities for discovering mainly novel, low molecular weight lead compounds. Since less than 10% of the world's biodiversity has been evaluated for potential biological activity, many more useful natural leads compounds await discovery with the challenge being how to access this natural chemical diversity (Cragg and Newman, 2005). Thus, natural products are the active constituents not only of most traditional medicines but also many modern medicines.

1.2 Traditional Medicine

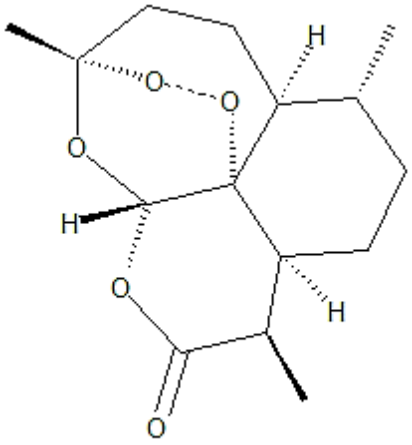
Traditional medicine has a rapidly growing economic importance and is used in all parts of the World, mainly through the use of medicinal plants, especially in developing countries (Agra *et al.,* 2007). Traditional medicine, often called indigenous or folk medicine has been described as the knowledge, skills and practices based on the theories, beliefs and

experiences indigenous to different cultures, used in the maintenance of health and in the prevention, diagnosis, improvement or treatment of physical and mental illness (WHO, 2013). Traditional medicine or ethnic medicine still plays a key role in combatting illness in many countries today (Haidan *et al.*, 2016). Traditional medicine remains the main use of a large majority of the population to solve their health problems (Kone, 2009). It is noteworthy that the primary health care for about 75% of the world's populace depend on traditional medicine (Gani *et al*., 2016). Traditional medicine has continuously been use of recent as complementary medical therapies for different types of diseases such as cancer, diabetes, hypertension (Goli,2018). A medicinal plant is any plant that has some of its parts containing substances that can either be used for therapeutic purposes or which are precursors for the synthesis of useful drugs (Maridass and John, 2008). Medicinal plants are a significant source of synthetic and herbal drugs and have been identified and used throughout human history. Plants have the ability to synthesize a wide variety of chemical compounds that are used to perform important logical functions, and to defend the plant against attack from predators such as insects, fungi and herbivorous mammals. Medicinal plants have provided a good source of compounds, such as phenolic compounds, nitrogen compounds, vitamins, terpenoids and some other secondary metabolites which are rich in valuable bioactivities such as antioxidant, anti-inflammatory, anti-tumor, anti-mutagenic, antibacterial and antiviral activities (Maridass and John, 2008). The plants used in traditional medicine have resisted the test of time and has immensely contributed to the development of novel compounds that are useful in curative and preventive medicine of modern science (Umadevi *et al.,* 2013). Plants may become the basis for developing medications, a natural blue print for the development of new drugs or a phytomedicine to be used for the treatment of diseases (Sharma *et al.*, 2012). The increased awareness of the significance of herbal medicine, particularly the devise to integrate it with orthodox medicine still have much to be achieved (Sofowora, 2008). The

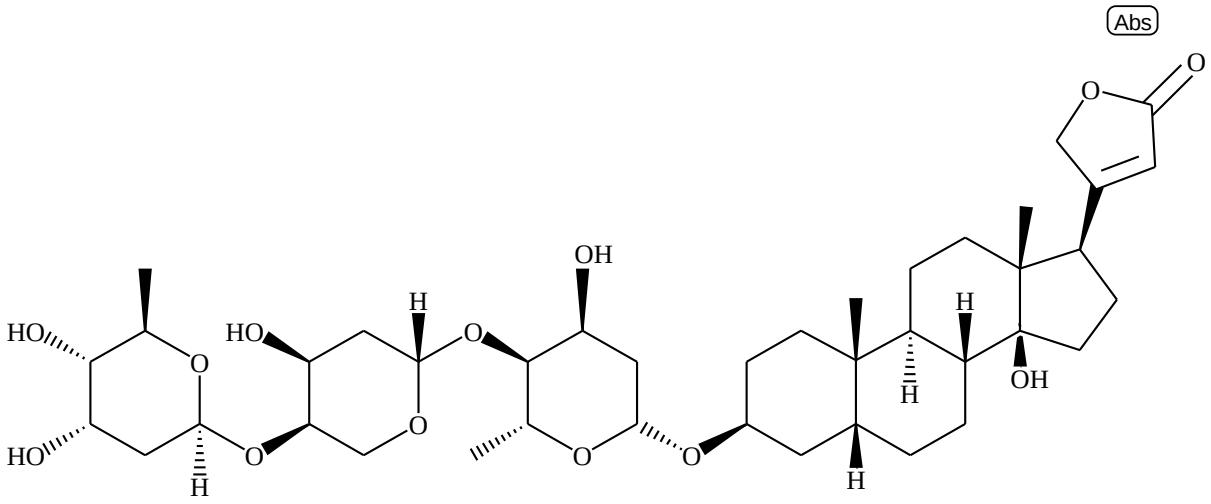
knowledge associated with traditional medicine (complementary or alternative herbal products) has presented further investigations of medicinal plants as potential medicines and has led to the isolation of many natural products that have become well known in modern medicine. Many drugs originated from plant sources and many modern pharmaceuticals are derived from chemical found in plants. Examples are quinine (I), an antimalarial agent isolated from the bark of *Cinchona succirubra* tree, artemisinin (II), an antimalarial agent isolated from the leaves of *artemisia annua,* digitoxin (III), a heart medication derived from foxglove (*Digitalis purpurea*).



I



II



III

1.3 Pain and Inflammation

Pain is an unpleasant sensory and emotional experience associated with, or resembling that with, actual or potential tissue damage (Alcock, 2017). Pain can also be defined as an unpleasant sensation that can be either acute or chronic and that is a consequence of complex neurochemical processes in the peripheral and central nervous system (Howland and Mycek, 2006). It is also described as a complex experience consisting of physiological response to noxious stimulus, which in some cases is followed by emotional response. Pain is a warning signal and primarily protective in nature, but often causes discomfort. Excessive pain may be unbearable and causes effects such as sweating, nausea, apprehension, tachypnea (Adib-Hajbaghery and Zohrehea, 2013) and can also be a symptom of injury, inflammation, heat or pressure, malaria and other diseases. Tissue injury is the immediate cause of pain as it releases different chemical mediators like prostaglandins, bradykinins and substance P which act on the nociceptors causing the sensation (Basbaum *et al.*, 2009).

Some of the secondary metabolites isolated from plants that have been shown to have analgesic activity include: Emodin, Ligunstrazin, Amentoflavone, and Puerarin (Rômulo, 2013). 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 (Dahanukar *et al*., 2000). Also, different classes of compounds from plants have been reported to possess anti-inflammatory activities, these include: triterpenoids, glycyrrhizin and glycyrrhizic acid from the roots of *Glycyrrhiza glabra* (liquorice) used as a non-steroidal anti-inflammatory drug NSAIDs (Wiart, 2006). Tryptanthrin an indoloquinazoline alkaloid, isolated from dichloromethane crude extract of *Isatis tinctori*a leaves exhibit its anti-inflammatory effect through COX-2 inhibition (Danz *et al.*, 2001).

1.3.1 Classification of pain

There are several variables for the classification of pain but the most commonly used system for pain classification is divided into two which includes:

1. Acute and Chronic pain classified based on duration
2. Nociceptive and Neurogenic pain classified based on physiology

1.3.1.1 Acute and chronic pain

Acute pain: acute pain often results from disease, inflammation or injury to tissues which is self-limiting confined to a given period of time and severity. It begins suddenly and sharp, it serves for warning of disease or threat to the body, it can be severe but last for a relatively short period of time until the source that triggered the pain is removed. Acute pain includes burn, post-operative, visceral, traumatic and acute headache. Acute and post-operative pains are most treated with the opioid analgesics (Kumar, 2007).

Chronic pain: chronic pain is pain that persist for a long period of time as a result of medical condition or damage to the body. It is pain lasting for long period of time. It persists 3 to 6 months or beyond the expected period of healing (Vadivelu *et al*., 2009; WHO, 2012). It may begin as acute pain and persist for long periods or may occur due to persistence of noxious

stimuli or repeated exacerbation of an injury. Chronic pain is sometimes difficult to locate or control.

1.3.1.2 Nociceptive and neurogenic pain

Nociceptive pain: nociceptive pain is pain elicited through the action of nociceptors by an intense stimulus that poses a threat to normal undamaged tissue. A nociceptor is a type of sensory neuron that is activated by a noxious stimulus. This type of pain is, therefore, physiologic, usually of rapid onset and limited duration (Roberts and Windsor, 2019). Nociceptive pain has also been termed inflammatory pain because peripheral inflammation and inflammatory mediators play major roles in its initiation and development. In general, the intensity of nociceptive pain is proportional to the magnitude of tissue damage and release of inflammatory mediators (Vadivelu *et al.*, 2009).

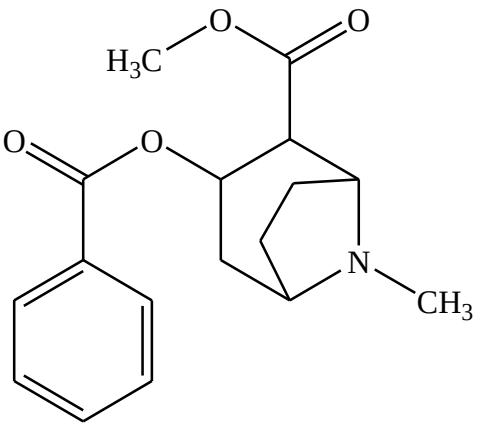
Neurogenic Pain: The term neurogenic pain refers to pain conditions associated with actual tissue damage (persistent, severe inflammatory pain) or trauma to the nervous system (neuropathic). It is characterized by a hyper- responsiveness of the patient to either a normally painful (noxious) stimulus (hyperalgesia), or to an innocuous stimulus (allodynia). Neurogenic pain can be categorized into acute and chronic depending on the underlying pathology (Guindon and Hohmann, 2008).

1.3.2 Analgesics and analgesia

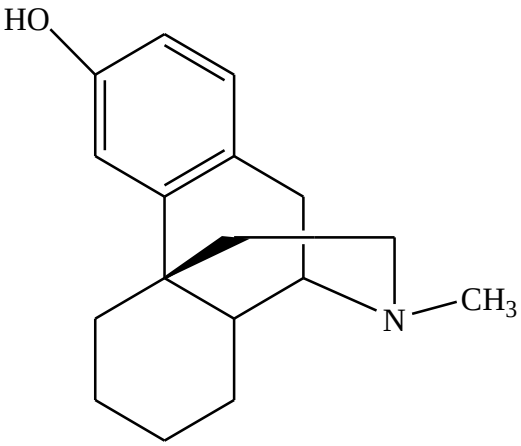
Analgesics are drugs that relieve pain without blocking the conduction of nerve impulses, markedly altering the sensory perception or affecting consciousness while analgesia is the loss or reduction of pain sensation (Tan *et al.,* 2015). The body modulates pains through complex processes including the endogenous opiate system, which consists of neurotransmitters. They are sometimes referred to as painkillers (Lilley *et al*., 2011; WHO, 2012). Analgesic drugs are broadly classified into narcotic (opioids) and non- narcotic analgesic.

1.3.2.1 Narcotic analgesics

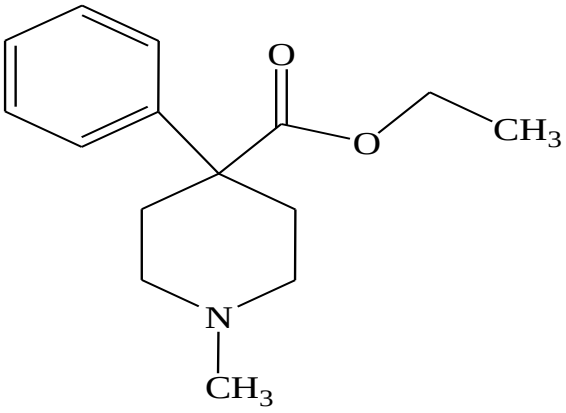
Narcotic analgesics is also known as opioid analgesics refer to any chemical, natural or synthetic compounds that produce morphine-like effects. They produce analgesia with depression of the central nervous system (Geofffery, 2005). They act by binding to specific opioid receptors in the central nervous system (CNS) to produce effect which mimics the action of endogenous peptide neurotransmitters. They may be classified as: Morphine and related substances e.g. cocaine (IV), Morphinans e.g. levorphanol (V), Phenylpiperidine e.g. pethidine (VI), Diphenylpropylamine e.g. methadone (VII).



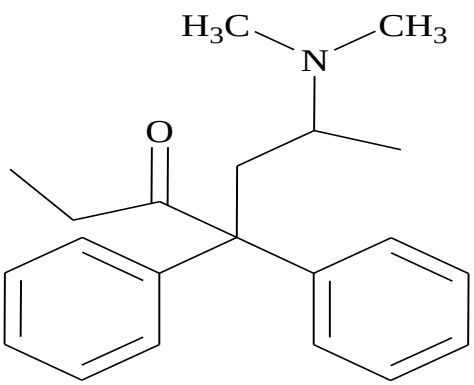
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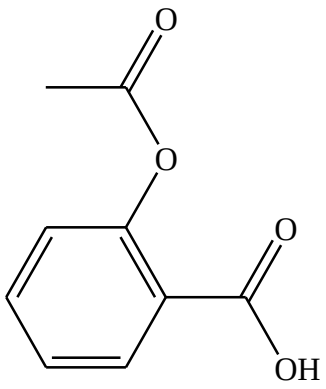
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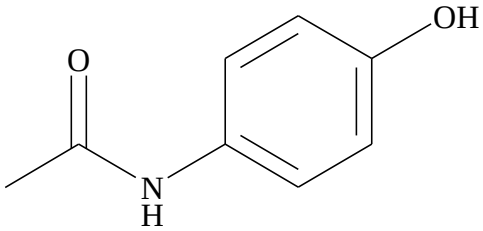
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1.3.2.2 Non-narcotic analgesics

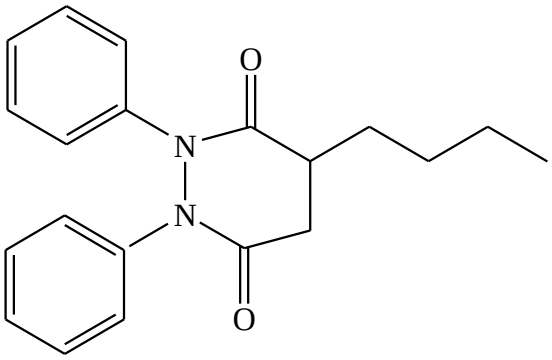
Non-narcotic analgesics commonly referred to as non- steroidal anti-inflammatory drugs (NSAIDs) are large chemically diverse group of drugs that are analgesics and also possesses anti-inflammatory and antipyretic activity but are not steroids (Lilley *et al.*, 2011). They are mild analgesics which are particularly effective when inflammation has caused sensitization of pain receptors. The analgesic effect of NSAIDs is not necessarily secondary to the anti-inflammatory response because they have clear analgesic effects in the absence of inflammation (Calixto *et al.*, 2000). They differ from narcotic analgesics in that they do not cause addiction, euphoria and respiratory depression (Hakkarainen *et al.*, 2015). Their major drawback is the increased gastric irritation leading to hyperacidity and aggravation of ulcer (Carmelo and Richard, 2010). They are classified into six classes namely: Derivatives of phenols e.g. aspirin (VIII), Derivatives of aniline e.g. paracetamol (IX), Derivatives of pyrazolone e.g. phenylbutazone (X), Arylacetic acid derivativese.g. indomethacin (XI), Anthranilic acid derivatives e.g. mefenamic acid (XII), Adrenocortical steroids e.g. cortisone (XIII).



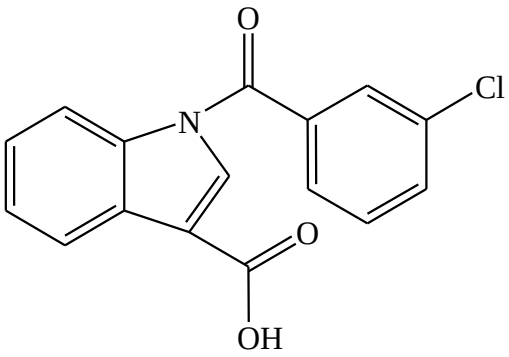
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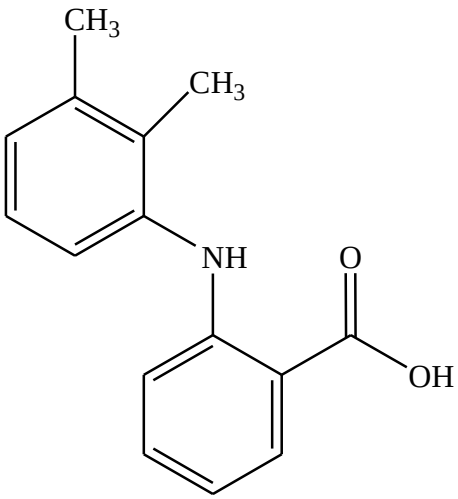
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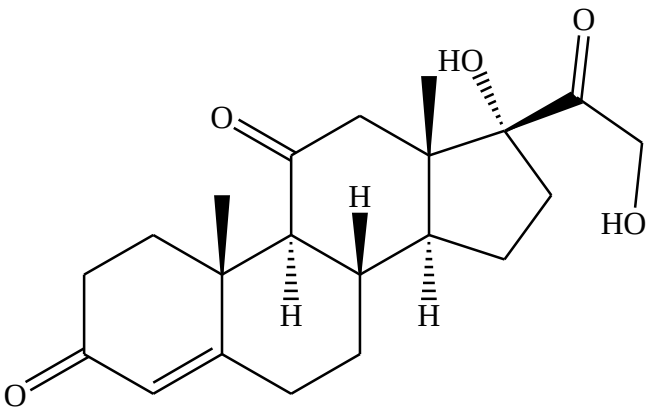
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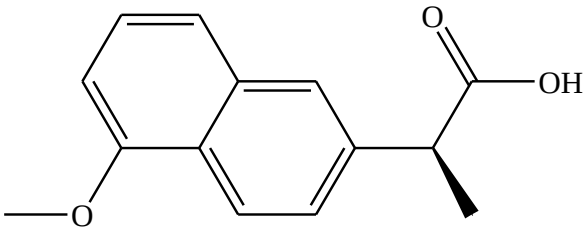
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1.3.3 Inflammation

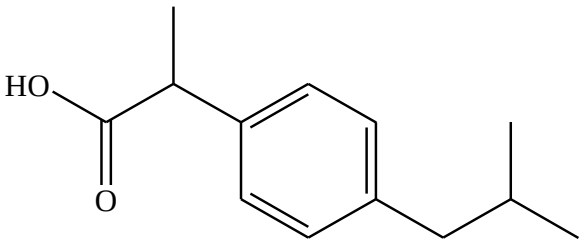
Inflammation is the immune system's response to harmful stimuli, such as pathogens, damaged cells, toxic compounds or irradiation (Medzhitov, 2010). It is a dynamic process that is elicited in response to mechanical injury, burns, microbial infections, and other noxious stimuli that may threaten the well-being of the host (Gautam and Jachak, 2009). It is the body's defense reaction in order to eliminate or limit the spread of injurious agents as

well as to remove or clear out necrotic cells and tissues (Anupama *et al*., 2012). It is a reaction of the body against an aggressive agent, characterized by vasodilatation, which allow access of fluid and cells to the target tissue (Schmid-Schonbein, 2006). One of the major signs of inflammation is the pain that can be triggered by direct stimulation of nociceptors or by action of inflammatory mediators (Isaac *et al.,* 2012). It is characterized by increased blood flow to tissue causing increased temperature, swelling, redness loss of function and pain. The function of inflammation is to eliminate the initial cause of the cell injury, and clear out tissues damaged from the original insult and the inflammatory process, and initiate tissue repair (Guindon and Hohmann, 2008). The usual treatment of inflammatory pain is done by non-steroidal anti-inflammatory drugs, but not without adverse side effects like gastrointestinal ulceration accompanied by anemia from the resultant blood loss, fluid retention, bronchospasm, and prolongation of bleeding time (Derle *et al;* 2006). Inflammation can be classified as acute and chronic inflammation:

I) Acute Inflammation: Acute inflammation is the body normal protective response to an injury, irritation or surgery. The damage may be purely physical, or it may involve the activation of an immune response. Acute inflammation is characterized by marked vascular changes including vasodilatation, increased permeability and increased blood flow, which are reduced by the action of various inflammatory mediators (Smith, 2005). Drugs used for the treatment of this inflammation are naproxen (XIV), ibuprofen (XV) etc.

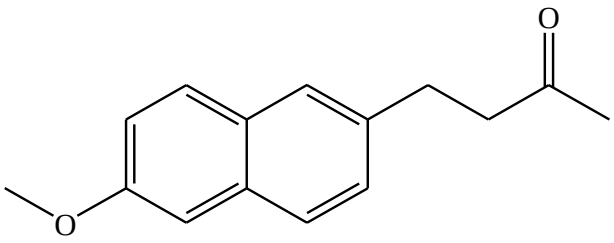


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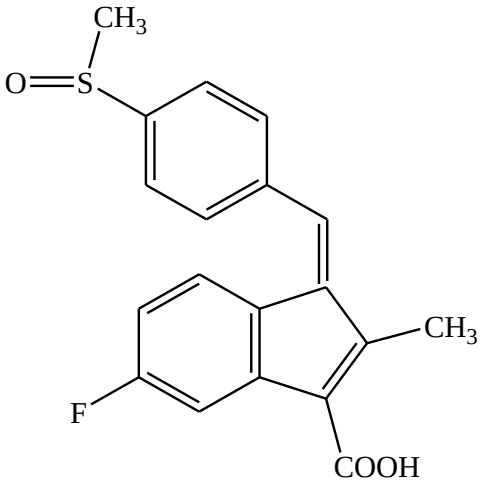


XV

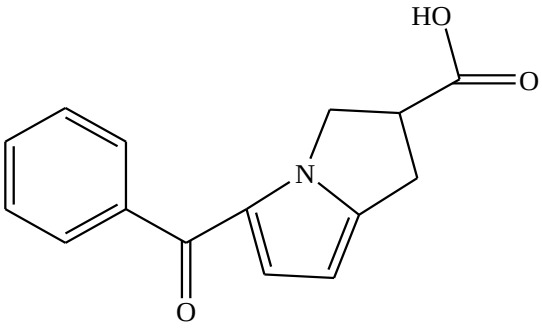
II) Chronic inflammation: inflammations are associated with disruption of anabolic signals initiating muscle growth. Chronic inflammation has been implicated as part of the cause of muscle loss that occurs with aging (Smith, 2005). The group of non-steroidal and ant-inflammatory drugs (NSAIDs) has received the most intensive attention for new clinical candidates. They show high analgesic potency in addition to their potent anti-inflammatory activity, needed for treating inflammatory diseases. Example of drugs in this class includes nabumethane (XVI), sulindac (XVII), ketorolac (XVIII), tolmetin (XIX) etc.



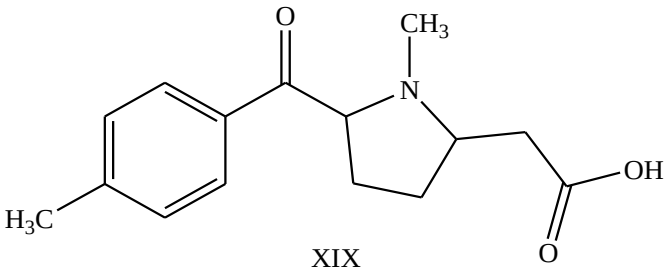
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XVII



XVIII



XIX

1.4. Statement of Research Problem

Pain is the most common pathological symptom associated with many diseases and injuries (Haddad, 2007). It is a global health problem and virtually all known disease conditions are accompanied by pain (Donkor *et al*., 2013). Globally, pain has accounted for 180 million disability adjusted life years as per systematic review of data from 2000–2014 (Henschke *et al.,* 2015). Pain of any type which can be mild, moderate or severe is one of the common complaints for patients seeking medical consultation in various health care deliveries worldwide. Example of diseases that cause such pain include burns, migraine, cancer, etc. which interfere with patient general functioning of the immune system (Mazur *et al.*, 2013). It is the hardest to manage despite availability of many analgesics and anti-inflammatory drugs and it is also one of the most common conditions constraining efficiency and diminishing quality of life (Caraceni *et al*., 2002; Mert *et al.,* 2013). Chronic pain and inflammatory condition remain one of the world's major health problems (Linton *et al*., 2003) and the use of commonly available analgesic and ant-inflammatory drugs are associated with serious limitation due to side effects such as dependency, tolerance, gastrointestinal irritation and liver disorder (Howland and Mycek, 2016). Therefore, these lead to the search for new analgesic and anti-inflammatory drugs which are relatively safe, cheap and considerable potent.

1.5 Justification of the Study

Medicinal plants have been found to be useful traditionally in the effective management of pain. The commonly used drugs for management of pain are the opioids and NSAIDs. Side effects such as nausea and vomiting, constipation, pruritus (itching), respiratory depression, tolerance, dependence and gastrointestinal disorder are associated with these drugs (Hanson *et al.,* 2009). *Combretum hypopilinum* has been employed traditionally as a therapeutic agent

in the effective management of pain and inflammation (Adamu *et al.*, 2005). Little or no work has been done on the analgesic and ant-inflammatory property of the root of *Combretum hypopilinum* and no detailed phytochemical screening and isolation of any compound from the root of the plant. Therefore, since little scientific data exist to validate the analgesic and anti-inflammatory properties of the root of *Combretum hypopilinum*, this research will therefore focus on the phytochemical studies and evaluation of analgesic and anti-inflammatory properties of root of *Combretum hypopilinum* in order to establish the efficacy and determine its potential as source to the discovery of new or active molecule to be used as analgesic and anti-inflammatory drugs.

1.6 Aim of the Study

The aim of the study is to isolate and characterize some of the phytochemical constituents present in the methanol root extract of *Combretum hypopilinum* and to screen the extract for analgesic and anti- inflammatory activity in mice and rats.

1.7 Specific Objectives of the Study

The specific objectives of the study are:

1. To carry out preliminary phytochemical screening on the methanol root extract of *Combretum hypopilinum* and various fractions using standard procedures.
2. To isolate some compound(s) present in the methanol root extract of *Combretum hypopilinum* using chromatography techniques.
3. To characterize the isolated compound(s) using spectroscopic techniques.
4. To determine the median lethal dose (LD_{50}) and evaluate the analgesic and anti-inflammatory activities of the methanol root extract using mice and rats.

1.8 Research Hypothesis

The methanol root extract of *Combretum hypopilinum* does not contain bioactive constituents with analgesic and anti-inflammatory activities.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Plant *Combretum hypopilinum*

Combretum hypopilinum belongs to the family *Combretaceae* which is composed of 20 genera and about 500 species. They are woody plants, shrubs, trees and lianas found in Tropical and Subtropical regions, mainly in African savannas and Asiatic monsoon forests (Christenhusz and Byng, 2016). Five genera are commonly found in Southern and Western Africa, of which four are tree species. The largest genera are *Combretum* and *Terminalia*, with about 370 and 200 species, respectively (Pietrovski *et al.,* 2006). *Combretum hypopilinum* is a small to medium sized, deciduous shrub or tree growing from 4 - 18 metres in height, with a rounded or flat heavy crown as shown in Plate I. The plant is harvested from the wild for local use of its gum and timber for medicinal purpose. The plant is highly dominant where it occurs and is widespread, extending from South Africa to West Africa, Central up to East Africa. Variably the *collinum* species has eleven sub-species recognised in Africa of which three are in West Africa. Sub-species *geitoxophyllum* is the most widely spread, occurring in the savannah from Senegal to Southern Nigeria but not beyond. Sub-species *binderiantum* occurs from Ghana to Nigeria and is widely spread in East Africa and sub-species *hypopilinum* is in Guinea to Southern Nigeria across to Sudan and Uganda. The living tree is tolerant of grass fires and so is suitable for planting as a savannah firebreak (Burkill, 1985).



Plate I: *Combretum hypopilinium* growing in its natural habitat.

2.2 Botanical Features

It is a synonym of *Combretum collinum* sub-species *hypopilinum.* The plant is commonly known as bush willow in English, *Jar taramniya* or *jar ganyee* in Hausa, *buski daneehi* in Fulfulde, *katankara* in Kanuri and *aro* in Yoruba language of Nigeria.

2.3 Taxonomy of the Plant

According to the international plant name index, the plant *"Combretum hypopilinum"* was classified under the following:

Kingdom: Plantae

Division: Streptophyta

Class: Magnoliopsida

Order: Myrtales

Family: Combretaceae

Genus: Combretum

Species: collinum

Sub-species: hypopilinum

2.4 Ethnomedicinal Uses of *Combretum hypopilinum.*

The plant has many uses in Africa, especially in traditional medicine. A twig bark and root decoction are given to relief pain and headache (Adamu *et al.,* 2005). Decoction of leaves is taken as a drink, and fresh roots are chewed to treat lung problems such as cough, bronchitis, and tuberculosis and also to treat jaundice. Also, a leaf-macerate is considered effective as depurative and chalagogue (Eloff *et al.,* 2008). Maceration or decoction of root is taken to treat gonorrhoea and infertility in women and men. The infusion of leaves and roots is taken as blood tonic. The leaves are used as a purgative and the roots are boiled and the decoction

drunk warm as a treatment for snakebites (Abreu *et al.,* 1999). Infusion of fresh and dried leaves and decoction of root bark are commonly taken as cholagenic, diuretic and purgative and also to treat gastrointestinal disorders, including diarrhea, dysentery, and stomach aches (Fyhrquist *et al.,* 2004). The gum exudes from injured branches are edible and used to cure toothache or plug a tooth with caries (Burkill, 1985). In Ivory Coast, the leaves are held to be strongly diuretic and of benefit in cases of general oedema (Recio *et al.,* 2004). A root decoction is taken for stomach ache in the Gambian and the roots are said to have unspecified medicinal use in the Southern-Guinean region of Africa (Idoh *et al.,* 2018).

2.5 Pharmacological action of *Combretum hypopilinum* and other species.

Only few studies were carried out on *Combretum hypopilnium* according to literature. It has been reported that the aqueous extract of stem bark and root of *C. hypopilinum* showed antibacterial activity against *Proteus mirabilis* while different leaf extracts showed weak antifungal activities *in vitro* (Fyhrquist *et al.,* 2004). Dried stem bark of the plant was reported to have anti-inflammatory activity (Recio *et al.,* 2004). Cardiovascular activity of methanol seed extract has been reported (Bamgbosa and Dramane, 2005). Gedson *et al*., (2012) also reported that methanol seed extract of *Combretum hypopilnium* has Central Nervous System depressant effect in mice. The protective effect of *combretum hypopilinum* root bark extract against tetrachloromethane (CCl_4) induced hepatotoxicity in wister rats has also been reported (Idoh *et al.,* 2018). Extract of *C. erythrophyllum* obtained with different solvents (acetone, hexane, chloroform, carbon tetrachloride and butanol) have shown antibacterial activity at different doses against *E. coli, P. aeruginosa, S. aureus* and *E. faecalis* (Eloff, 2000). Antipyretic activities of the leave and stem bark of *C. glutinosum* was also reported (Tony, 2005). Other *combretum* species, *C. molle* has also demonstrated

antibacterial, antifungal, anthelmintic, antiasthmatic and antitussive (Yeo *et al.,* 2008, Simon *et al.,* 2012). *C. micranthum* showed anti-fungal activity against *Candida albicans* (Chika and Bello, 2010).

2.6 Phytochemical Studies on *Combretum hypopilinum* and other species.

Although many species of Combretum have not been extensively investigated for their chemical constituents, nevertheless, various classes of secondary metabolites including terpenoids, flavonoids, stilbenoids and phenanthenes have been reported. Existing phytochemical investigation on *Combretum hypopilinum* indicates that combretastatin A and B which are stilbenoids and several phenanhrenes were isolated from the aerial part of the plant (Roger and Coobes, 1999). A series of amino acids has been reported to have been isolated from the gum exudates, the most important being aspatic acid, glycerine, glutamic acid and alanine (Idoh *et al.,* 2018)). Phytochemical studies carried out on other species of the genus combretum indicated the presence of Galic acid derivatives from *C. quadrangulare, and C. glutinosum* (Katerere *et al.,* 2004). Bisoli *et al;* (2008) isolated 11 triterpenes and their glycosides from *C. laxum,* among them, *oleanane-, ursane- and lupane-type.* flavonoids from *C. quadrangulare, C. micranthum, C. erythrophyllum, C. apiculatum* (Toume *et al.,* 2011; Runyoro *et al.,* 2013). triterpenoids from *C. molle* (Welch, 2010), *Combretum adenogonium* leaves powder is rich in natural chemical such as tannins, anthocyanins, flavonoids and triterpenoids (Marital *et al.,* 2017).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Solvents/reagents and chromatographic materials.

Solvents used were of analytical grade and they include: methanol, *n*- hexane, chloroform, ethyl acetate, and acetone. Reagents used were freshly prepared and include those for phytochemical screening such as; Molisch's, Meyer's, Salkowski's, Dragendorf's, Shinoda's, Wagner's and Borntrager's. Chromatographic materials used are aluminium pre-coated thin layer chromatographic plate ($F_{254,}$ 20 x 20 cm) obtained from Sigma Aldrich, Germany. Silica gel of mesh size 60-120 µm (Qualikems), Chromatographic tanks and column. Standard drugs used include piroxicam, pentazocine, ibuprofen, carrageenan and deionized water.

3.1.2 Equipment and apparatus.

Thermoelectro UV-Visible spectrophotometer machine at the Department of Pharmaceutical and Medicinal Chemistry, Ahmadu Bello University, was used for UV-spectroscopy. Shimadzu FTIR 8400S machine available at the Multi-user Laboratory, Ahmadu Bello University Zaria, was used for Infrared (IR) spectroscopy, Bruker AVANCE III Nuclear Magnetic Resonance (NMR) spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C) available at University of Surrey, United Kingdom was used for NMR with tetramethyl silane (TMS) as an internal standard. Apparatus used includes electro thermal melting point apparatus, Ohaus digital weighing balance, syringes and needles, mortar and pestle, sample bottles, beakers, separating funnel, conical flask, digital vernier caliper, and electric hot plate.

3.1.3 Experimental animals.

Swiss albino mice and adult Wistar rats of either sexes weighing (17-27 g) and (110 to 190 g) respectively were obtained from Animal House Facility of the Department of Pharmacology and Therapeutics, Ahmadu Bello University Zaria, Nigeria. The animals were kept in a well-ventilated room in propylene cages at room temperature and were fed with laboratory diet and water *ad libitum* and maintained under normal humidity, temperature and light for 7 days prior to the experiment.

3.2 Methods.

3.2.1 Collection, identification and preparation of plant material.

The plant sample; root of *Combretum hypopilinum* was collected from Dajin Biye, Sabon Gari Local Government area, Zaria, Kaduna State of Nigeria in December, 2018. It was identified by mallam Namadi Sanusi at the Herbarium unit, Department of Botany, Ahmadu Bello University, Zaria by comparing with herbarium reference voucher specimen number 900743. The root was air dried under shade and pulverized manually to a coarse powder using mortar and pestle, labeled and stored at room temperature for use.

3.2.2 Extraction and partitioning.

The plant material (1.2 kg) was extracted with 70% methanol using maceration method for 8 days with intermittent shaking. The extract was concentrated in a rotary evaporator to yield a brownish residue (110 g) subsequently referred to as the methanol root extract (MRE). Some part of the methanol root extract (100 g) was partitioned using chloroform, ethyl acetate and acetone in order of increasing polarity to obtain fractions coded CF (chloroform fraction), EAF (ethyl acetate fraction) and AF (acetone fraction) respectively, while 10 g of the methanol root extract was kept for the determination of the intraperitoneal $LD_{50,}$ analgesic and anti-inflammatory studies and to carry out preliminary phytochemical tests.

The percentage yield of the extract was calculated using the formula:

$$\text{Percentage yield} = \frac{weight\ of\ extract}{weight\ of\ powdered\ material}\ x\ 100$$

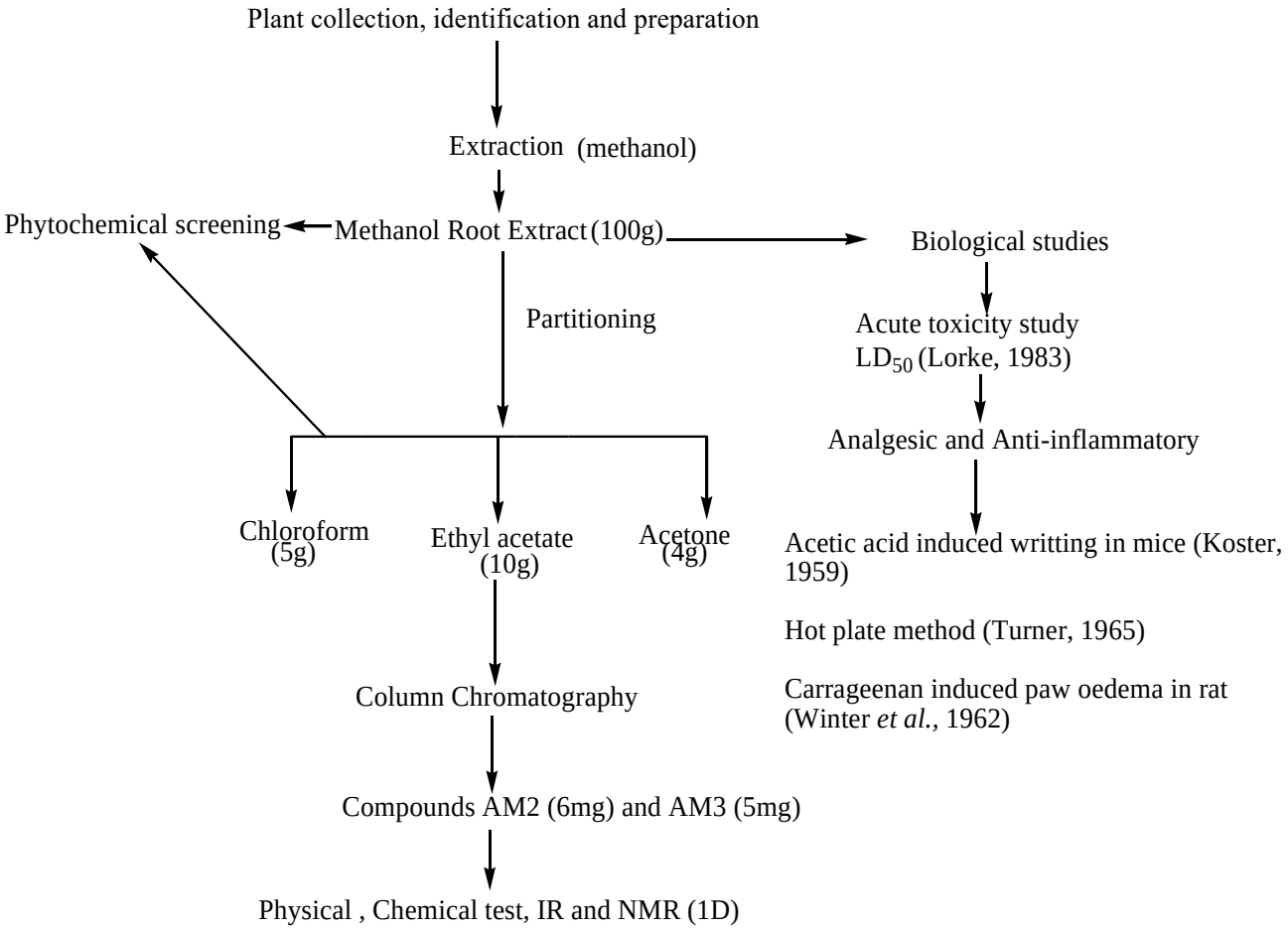


Figure 3.1: Schematic representation of the summary of the research methodology

3.2.3 Preliminary phytochemical screening.

Standard procedures were used to conduct chemical tests on the methanol root extract and partition fractions for the presence of various phytochemical constituents.

3.2.3.1 Test for carbohydrates

Molisch Test

2ml of the methanol root extract and the partitioned fractions were put in a test tube, and 3 drops of Molisch's reagent was added followed by concentrated sulphuric acid. The

formation of a reddish coloured ring at the interface indicated the presence of carbohydrates (Trease and Evans, 1996).

3.2.3.2 Test for saponins

Frothing Test

1 ml of the methanol root extract and partitioned fractions were put in a test tube and 5ml of distilled water was added to each and shaken vigorously for 30 seconds. The solution in the tubes was allowed to stand for 15 minutes, the formation of a persist froth indicated the presence of saponins (Trease and Evans, 1996).

3.2.3.3 Test for flavonoids

Shinoda test

0.5 g of the methanol root extract and partitioned fractions were dissolved in 2ml of methanol and few pieces of magnesium chips were added followed by few drops of concentrated hydrochloric acid, the formation of pink, orange, or red to purple coloration indicated the presence of flavonoid (Silva *et al.*, 1998).

Sodium hydroxide test

1 ml of the extract and partitioned fractions were put in a test tube and few drops of 10% sodium hydroxide solution was added to each. Formation of yellow coloration indicated the presence of flavonoids (Silva *et al.*, 1998).

Ferric chloride test

1 ml of the extract and partitioned fractions were put in a test tube and 3 drops of ferric chloride was added to each. Formation of green precipitate indicated the presence of flavonoids (Trease and Evans, 1996).

3.2.3.4 Test for tannin

Lead sub- acetate test

1 ml of the methanol root extract and partitioned fractions were put in a test tube and 3 drops of lead sub-acetate solution was added to each. Formation of cream-colored precipitate indicated the presence of tannins (Trease and Evans, 1996).

3.2.3.5 Test for steroids/triterpenes

Liebermann-Burchard's Test

1 ml of the methanol root extract and partitioned fractions were put in a test tube and equal voloume of acetic anhydride was added to each and was mixed gently. 1 ml concentrated sulphuric acid was added down the test tubes. It was observed for instant colour change and over a period of one hour. Blue to blue-green color in the upper layer and a redish color at the junction of the two layers indicated the presence of triterpenes (Trease and Evans, 1996).

Salkowski test

0.5 g of the extract and partitioned fractions were dissolved in 2ml of chloroform and 3 drops of concentrated sulphuric acid (Salkowski's reagent) were added at the side of the test tube and was observed. A red brown coloration at the interface indicated the presence of steroids (Trease and Evans, 1996).

3.2.3.5 *Test for alkaloids*

Extraction of Alkaloids

1g of the methanol extract and partition fractions were separately stirred with 20ml of 5% aqueous hydrochloric acid on a water bath and filtered. To the filtrate in a separating funnel, 30ml of chloroform were added resulting in the formation of two layers. The lower (chloroform) layer was drained off while the upper (aqueous) layer was made alkaline with ammonium hydroxide solution until basic to pH of 10. The base was further extracted with chloroform and the chloroform layer was washed with distilled water. The purified chloroform portion was kept with anhydrous sodium sulphate for a day in order to remove

any trace of water. The extracts were filtered and evaporated to dryness on water bath. The concentrated extracts were used for preliminary test for alkaloids.

Dragendoff's test

1 ml of the alkaloidal extract and partitioned fractions were put in a in a test tube and drops of Dragendoff's reagent was added to each, formation of red precipitate indicated the presence of alkaloids (Trease and Evans, 1996).

Mayer's test

1 ml of the alkaliodal extract and partitioned fractions were put in a in a test tube and 3 drops of Mayer's reagent was added to each, formation of a cream precipitate indicated the presence of alkaloids (Trease and Evans,1996).

Wagner's test

1 ml of the alkaliodal extract and partitioned fractions were put in a in a test tube and few drops of Wagner's reagent were added to each, formation of a reddish-brown precipitate indicated the presence of alkaloids (Silva *et al.,* 1998).

3.2.3.7 Test for anthraquinones

Bontrager's Test

1 g of the extract and partitioned fractions were dissolved in 5 ml chloroform each and was shaken and filtered. To the filtrates, an equal volume of 10% ammonia solution was added with continuous shaking absence of a bright pink colour in the aqueous upper layer indicated the absence of anthraquinone (Trease and Evans, 1996).

3.2.3.8 Test for cardiac glycosides

Keller-Kiliani Test

0.5 g of the extract and partitioned fractions were dissolved in 1 ml glacial acetic acid containing traces of ferric chloride solution. The solutions were then transferred into dry test

tubes to which an equal volume of sulphuric acid was added to each, formation of a brown ring at the interface indicated the presence of a deoxy sugar (Trease and Evans, 1996).

3.2.4 Chromatographic procedures

3.2.4.1 Thin layer chromatographic analysis (TLC)

Pre-coated TLC plates were used to carry out thin layer chromatography by ascending technique. Capillary tubes were used to manually apply spots on the TLC plate and the chromatogram was developed in an air tight chromatographic tank at room temperature employing different solvent systems. Visualization was done under ultra violet light (254 nm & 366 nm) and by use of a spraying reagent (10% Sulphuric acid) followed by heating in an oven at 105^{o}C for 2-3 minutes. The various TLC solvents system used to develop the plates include:

Ethyl acetate	100%
Ethyl acetate: Hexane	(9: 1), (8: 2), (7: 3), (1:5), (1:2) and (1:1)
Ethyl acetate: Chloroform: Methanol: water	(15:4:4:1) and (15:8:4:1)

3.2.4.2 Column chromatography of ethyl acetate fraction

Ethyl acetate fraction (10 g) of the extract was subjected to column chromatography packed with silica gel (60-120 mesh). The column was packed using wet slurry method. Slurry of silica gel was poured into column and allowed to settle. The sample was previously adsorbed on silica gel by dissolving it in a minimum amount of methanol, followed by adding small quantity of silica gel to form a paste. The paste was dried, triturated and loaded on the previously packed column. The column was eluted using gradient elution technique starting with *n*- hexane 100% followed by 95:5 n- hexane: ethyl acetate and increasing polarity

gradually by 5 up to ethyl acetate 100%. A total of 115 fractions of 100 ml each were collected and pooled together based on their TLC profiles to give 19 major fractions coded A1-A19. Fraction A4 was repeatedly treated with ethyl acetate and left behind a white amorphous substance which was soluble in chloroform and gave a single homogenous spot on the TLC plate coded **AM2**. The column fraction A6 was subjected to further purification using column of smaller diameter size (1cm x 50 cm). it was eluted isocratically with hexane: ethyl acetate 85:15. A total of 45 collections of 1 ml each were collected and collection 31-39 were pooled together and gave a white crystalline substance which gave a single homogenous spot on TLC plate coded **AM3**. Both compounds AM2 and AM3 were subjected to physicochemical tests as well as spectral analysis.

3.2.5 Pharmacological studies of methanol root extract (MRE) of *combretum hypopilinum*

3.2.5.1 Acute toxicity studies

The method described by Lorke (1983) was used for the study. This method was carried out in two phases. In the first phase, 3 groups of 3 animals each (mice/rats) were administered the methanol root extract of *combretum hypopilinum* of varying doses (10, 100 and 1000 mg/kg body weight) intraperitoneally and observed for the first 4 hours and intermittently for 24 hours for any sign of toxicity and mortality. The absence of toxicity and mortality led to the second phase. In the second phase three groups with one mouse/rat each were treated with doses of 1,600, 2,900 and 5000 mg/kg of the extract and observed for another 24 hours for signs of toxicity and death. The median lethal dose was calculated as a geometric mean of the highest non- lethal dose (with no death) and the lowest lethal dose (where death occurred).

$$LD_{50} = \sqrt{minimum\ lethal\ dose \times maximum\ tolerated\ dose}$$

3.2.5.2 Evaluation of analgesic activities in mice

Acetic acid-induced writhing test in mice:

The method described by Koster *et al.* (1959) was used for this study. Twenty-five albino mice were divided into five groups of five mice each. The first group served as negative control and received 10 ml/kg of normal saline, the second, third and fourth groups were orally administered 250, 500 and 1000 mg/kg of the methanol root extract respectively. While the fifth group served as positive control and was administered 10 mg/kg of piroxicam. Sixty minutes after oral administration each mouse was injected intraperitoneally 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. A reduction in the number of writhes as compared to the negative control was considered as evidence for the presence of analgesia and expressed as percent inhibition of writhes.

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

$$\text{Inhibition (\%)} = \frac{\text{mean number of writhing (control)} - \text{mean number of writhing (test)}}{\text{mean number of writhing (control)}} X100$$

Thermally-Induced pain test in mice (Hot plate method):

The method described by Turner (1965) was employed for this study. Twenty-five albino mice were divided into five groups of five mice each. The first group served as negative control and received 10ml/kg of normal saline. the second, third and fourth groups were orally administered 250, 500 and 1000 mg/kg of the methanol root extract respectively, while the fifth group served as positive control and was orally administered 5 mg/kg of pentazocine. Thirty minutes after treatment, each mouse was gently placed on Eddy's hot plate maintained at 55 ± 1ºC and a cut off period of 15 second (Franzotti *et al.*, 2000) was observed to avoid damage to the paw. Reaction time was recorded when animals licked their

hind paw or jumped at 0, 30, 60, 90 and 120 minutes after i.p administration of the test drug and it is referred to as the latency to pain response.

3.2.5.3 Evaluation of anti-inflammatory activities

Carrageenan-induced paw oedema test in rats:

The acute anti –inflammatory study was carried out using the carrageenan induced paw oedema in rat method as previously described by (Winter *et al.,* 1962). Twenty-five rats were divided into five groups of five rats each. The first group served as negative control and received 10 ml/kg of normal saline. The second, third and fourth groups were orally administered 250, 500 and 1000 mg/kg of the methanol root extract respectively, while the fifth group served as positive control and was orally administered 10 mg/kg ibuprofen. Sixty minutes after oral administration each rat was induced by injecting 0.1 ml of 1% carrageenan into sub plantar surface of rat hind paw. The paw diameter was measured at 0, 1, 2, 3, 4 and 5 hours, using vennier caliper to determine the diameter of oedema. The increase in paw diameter (oedema index) for each rat was calculated as the difference in paw diameter before carrageenan injection and after carrageenan injection at each time interval, while the percent inhibition of oedema was calculated for each group with respect to negative control group using the following relationship:

$$\frac{mean\ increase\ in\ paw\ volume\ of\ control - mean\ increase\ in\ paw\ volume\ of\ treated}{mean\ increase\ in\ paw\ volume\ of\ control} \text{ X } 100$$

3.2.6 Statistical analysis

The results were expressed as Mean±SEM and the mean values of the control groups were compared with the mean values of the treated groups using one way and repeated measures analysis of variances (ANOVA) followed by Bonferroni post hoc test for multiple comparison. The results obtained were considered statistically significant at *($p < 0.05$).*

CHAPTER FOUR

4.0 RESULTS

4.1 Extraction and Fractionation Yield

The extraction of 1.2 kg of *Combretum hypopilinum* root afforded a yield of 9.17% from 110 g of methanol root extract. The percentage yield from the methanol root extract and the partitioned fractions yield the following results. (Table 4.1).

4.2 Result of Preliminary Phytochemical Screening

Preliminary Phytochemical screening of the methanol root extract and partition fractions of the extract revealed the presence of carbohydrates, flavonoids, tannins, saponins, steroids/triterpenes, cardiac glycosides and alkaloids. (Table 4.2).

4.3 Thin Layer Chromatography of MRE, CF, EAF and AC

The chromatography profile of methanol root extract, chloroform, ethyl acetate, and acetone fractions are shown in Plates II-III and (Table 4.3).

4.4. TLC Profile of Column Chromatography of Ethyl acetate Fraction

Plate IV-XI shows the TLC Profile of column chromatography of ethyl acetate fraction

4.5 Summary of Pooled Column Chromatography of Ethyl acetate fraction

The summary of the TLC profiles of pooled column chromatography of ethyl acetate fraction (Table 4.4.)

Table 4.1: Weights and percentage yield of methanol root extract (MRE) and partition fractions of *Combretum hypopilinum.*

Solvents	Weight (g)	Yield (%)	Color
MRE	110	9.17	Dark brown
Chloroform	5.00	0.42	Greenish brown
Ethyl acetate	10.00	0.83	Light brown
Acetone	4.00	0.33	Light brown

Table 4.2: Phytochemical constituents of methanol root extract and partitioned fractions

Constituents	Test	MRE	CF	EAF	AF
Alkaloids	Meyer	+	+	+	+
	Wagner	+	+	-	-
	Dragendoff	+	+	-	-
Carbohydrates	Molisch	+	+	+	+
Flavonoids	Shinoda	+	-	+	+
	Sodium hydroxide	+	-	+	+
	ferric chloride	+	-	+	+
Saponins	Frothing	+	-	+	+
Triterpenes	Lieberman-Bucchard	+	+	+	-
Steroids	Salkowski	+	+	+	-
Tannins	Lead acetate	+	-	-	+
Cardiac glycosides	Keller-Kiliani	+	-	+	+

+= present, - = absent, MRE =methanol root extract,

CF= chloroform fraction, EAF= ethyl acetate fraction,

AF= acetone fraction.

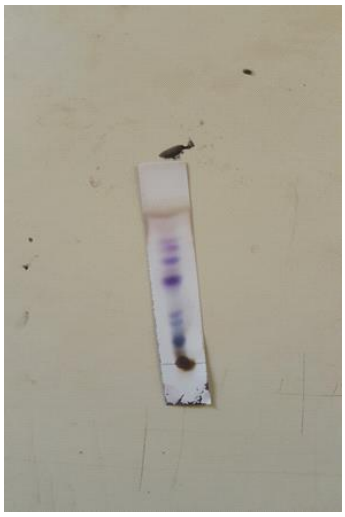


PLATE II: TLC profiles of crude Extract EA:C:M:W: 15:8:4:1

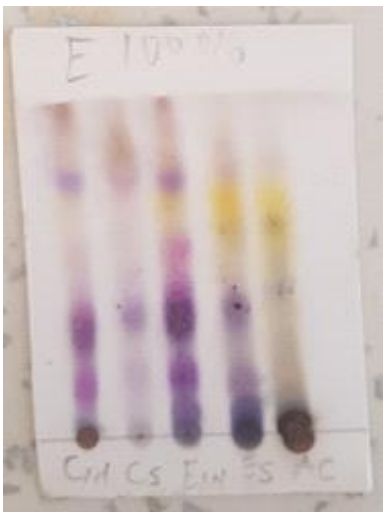


PLATE III: TLC profiles of EA, CF and AC fraction EA: 100%

Table 4.3: Summary of TLC profiles of methanol root extract and partition fractions

Extract	No. of spots	R_f values
Methanol root	8	0.07,0.09,0.08,0.21,0.23,0.24,0.25,0.26
Chloroform	5	0.06,0.08,0.09,0.11,0.13
Ethyl acetate	6	0.03,0.04,0.06,0.07,0.08,0.09
Acetone	4	0.03,0.06,0.07,0.09

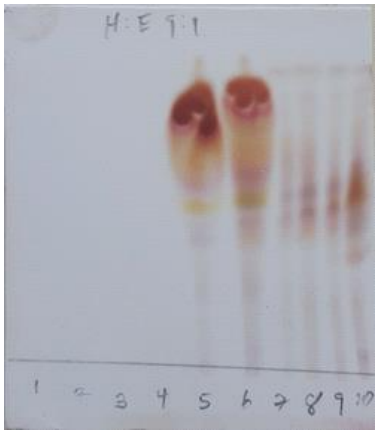


PLATE IV:TLC profile
of column collection 1-10
H:EA 9:1

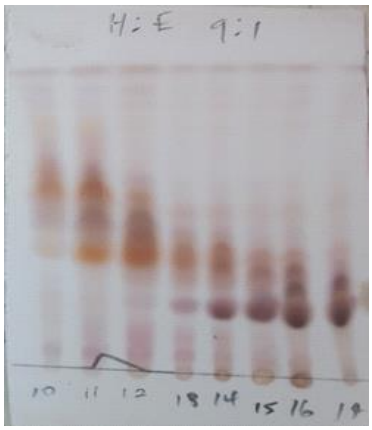


PLATE V: TLC profile
of column collection 10-17
H:EA 9:1

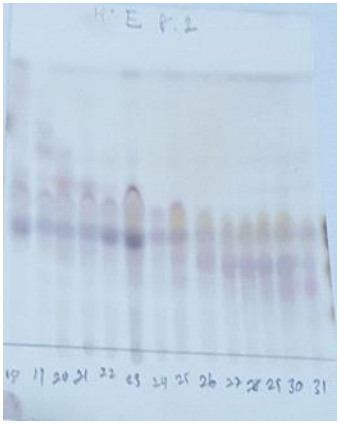


PLATE VI: TLC profiles
of column collections 18-31
H:EA 8:2

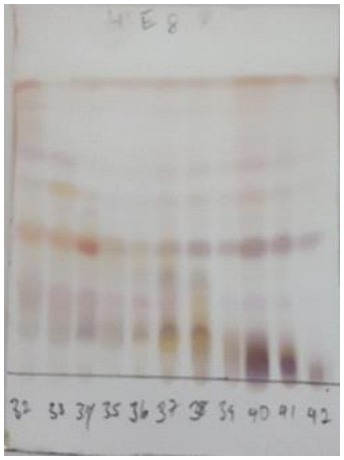


PLATE VII: TLC profiles
of column collections 32-42
H:EA 8:2

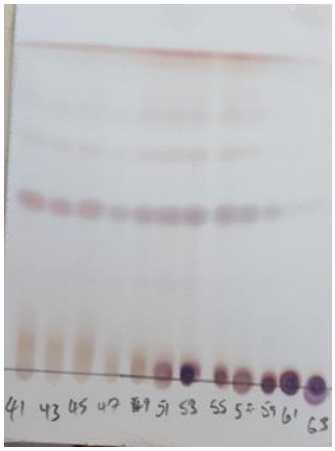


PLATE VIII: TLC profiles
of column collections 41-63
H:EA 8:2

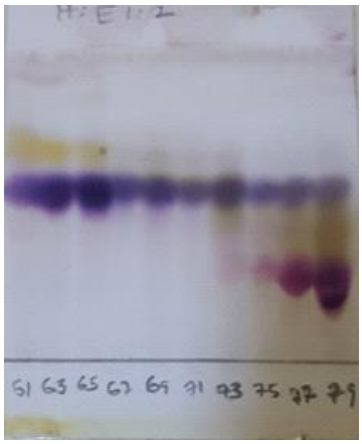


PLATE IX:TLC profiles
of column collections 61-79
H:EA 8:2

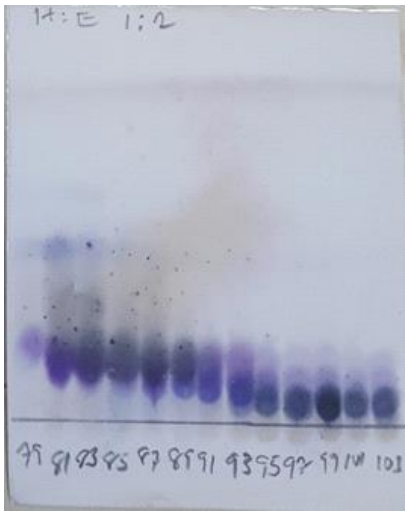


PLATE IX: TLC profiles of column
fractions 80-103
EA:C:M:W 15:8:4:1

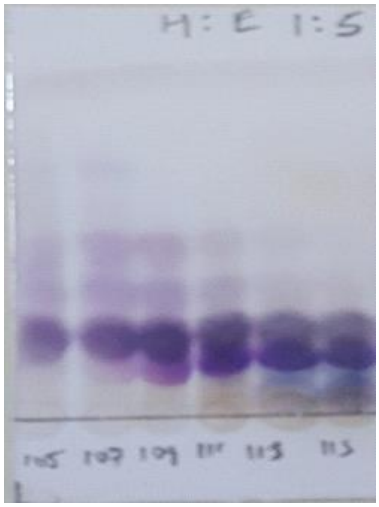


PLATE X: TLC profiles of column
fractions 105-115
H:EA 1:5

Table 4.4: Summary of pooled column chromatography of ethyl acetate fraction

Fraction	No. of spots	Solvent system Hexane: Ethyl acetate	Code
1-4	0	Hexane 100%	-
5-6	4	95:5	A1
7-12	4	90:10	A2
13-15	5	85:15	A3
16-17	**3**	**85:15**	**A4**
18-25	4	85:15	A5
26-31	**3**	**80:20**	**A6**
32-34	4	75:25	A7
35-38	4	70:30	A8
39-47	3	65:35	A9
48-55	3	60:40	A10
56-59	4	55:45	A11
60-66	4	50:50	A12
67-71	3	45:55	A13
72-79	4	40:60	A14
80-82	4	35:65	A15
83-93	3	30:70	A16
94-103	4	20:80	A17
104-109	4	10:90	A18
110-115	4	100% Ethyl acetate	A19

4.6 TLC Profile, Physical and Chemical Properties of Compound AM2

Compound AM2 was isolated as white powdered material, the melting point of AM2 was determined to be in the range of 169 – 171^{0}C with R_f values 0.73 and 0.39 using Hex: EA 8:2 (Plate XII) and 9:1 (Plate XIII) respectively as solvent systems using 10% H_2SO_4 as spray agent. AM2 is completely soluble in chloroform and Dichloromethane and tested positive to Salkowski's test.

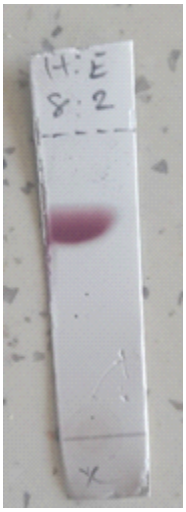


PLATE XI: H:EA:8:2



PLATE XII: H: EA: 9:1

4.7 Spectroscopic Data of Compound AM2

4.7.1 1R spectral data of compound AM2

The IR spectrum of compound AM2 showed stretching and bending vibration frequencies at 3399.3 cm^{-1}, 3291.2 cm^{-1}, 2933.4.2 cm^{-1}, 2862.6 cm^{-1}, 1640 cm^{-1},1461.1 cm^{-1} and 1051.1 cm^{-1} (Figure 4.1).

4.7.2 Proton nuclear magnetic resonance (^{1}H NMR) analysis of compound AM2

The ^{1}H NMR ($CDCl_3$, 400 MH_Z) spectrum of AM3 varied between δ_H 0.73 and 5.38 ppm. It revealed the presence of six methyl protons at δ 0.73, .0.87, 0.88, 0.89, 0.95 and 1.08, a carbinol proton at δ 3.50 and three olefinic protons at δ 5.38, 5.18 and 5.08 (Figure 4.2).

4.7.3 ^{13}C NMR of compound AM2

The ^{13}C NMR ($CDCl_3$, 100 MH_Z) spectrum of AM3 revealed the presence of twenty-nine (29) Carbon signals. It showed recognizable signals for four olefinic carbon at δ 140.91, 138.46, 129.44 and 121.87 which were assigned as C5, C22, C23 and C6 respectively, δ 71.97 for the carbinol carbon (C3), δ 12.02 and 19.98 for angular methyl carbon atoms for C18 and C19 respectively. Other signals include δ 37.42 (C1), 36.31 (C2), 42.48 (C4), 34.12 (C7), 36.31 (C8), 50.30 (C9), 39.85 (C10), 29.33 (C11), 40..64 (C12), 42.40 (C13), 56.90 (C14), 29.33 (C15), 34.12 (C16), 56.13 (C17), 12.02 (C18), 19.98 (C19), 39.85 (C20), 18.94 (C21), 46.01 (C24), 32.07 (C25), 21.24 (C26), 19.56 (C27), 23.24 (C28), 12.15 (C29) (Figure 4.3).

4.7.4 Comparison of spectral data of AM2 with literature

Comparison of 1D and 2D NMR data of compound AM2 with literature data* (Jephthah *et al*., 2014) revealed them to be fully in agreement. (Table 4.5).

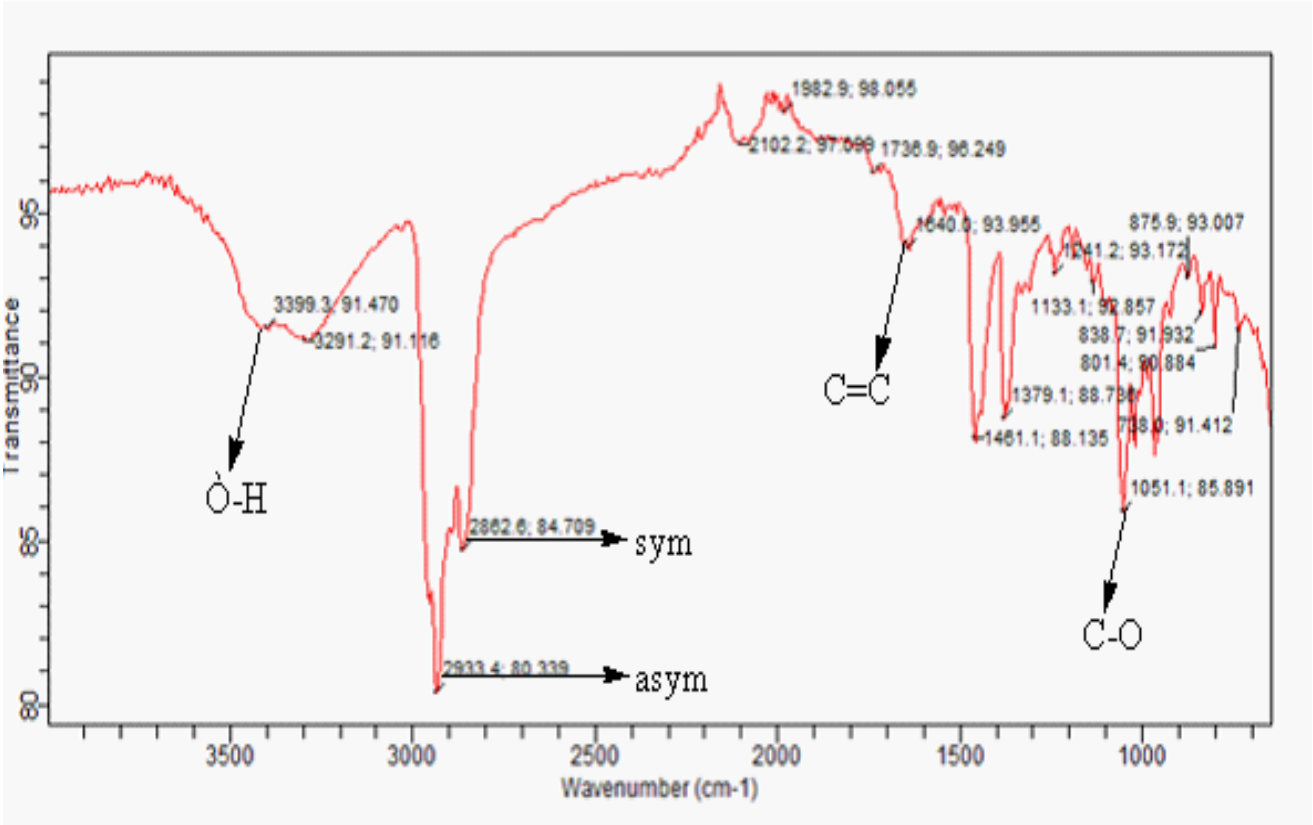


Figure 4.1: IR spectrum of compound AM2

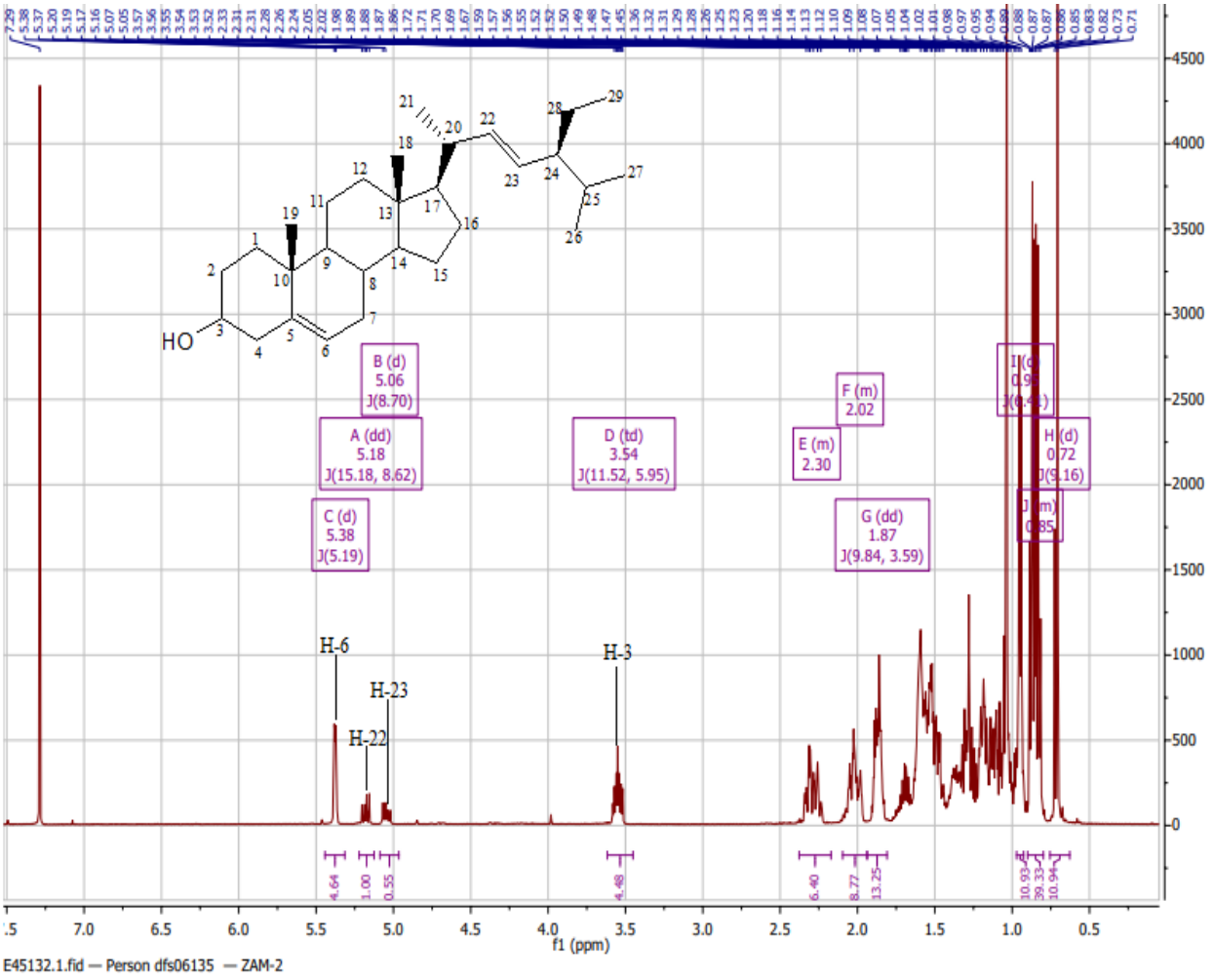


Figure 4.2: ^{1}H NMR spectrum of AM2

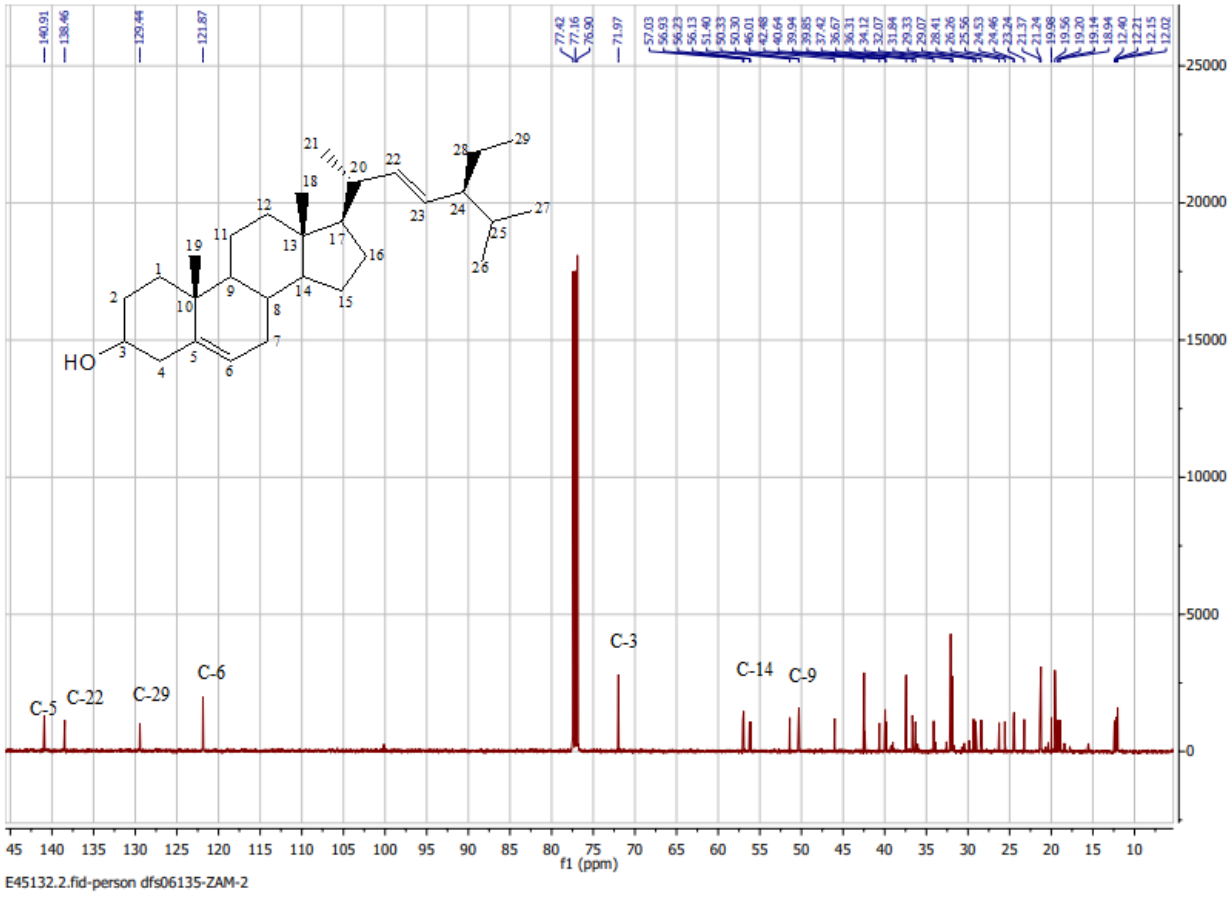


Figure 4.3: ^{13}C NMR Spectrum of AM2

Table 4.5: Comparison of spectral data of AM2 with literature data

Position	^{1}H NMR	^{1}H NMR*	^{13}C- NMR	^{13}C- NMR*
1	1.88	1.90	37.42	37.30
2	1.29	1.30	36.31	35.90
3	3.55	3.50	71.97	71.80
4	2.32	2.30	42.48	42.3
5	-	-	140.91	140.80
6	5.38	5.39	121.87	121.70
7	1.51	1.51	34.12	33.90
8	1.98	1.98	36.31	36.10
9	0.98	0.98	50.30	50.10
10	-	-	39.85	39.80
11	1.29	1.28	29.33	29.70
12	2.05	2.06	40.64	39.70
13	-	-	42.40	42.30
14	1.05	1.04	56.90	56.80
15	1.29	1.28	29.33	29.20
16	1.32	1.30	34.12	33.70
17	1.05	1.05	56.13	56.10
18	0.73	0.74	12.02	11.90
19	1.08	1.06	19.98	19.80
20	2.02	2.00	39.85	38.80
21	0.95	0.93	18.94	18.30
22	5.18	1.07	130.46	36.50
23	5.06	1.22	129.44	31.70
24	0.97	0.97	46.01	45.80
25	1.71	1.74	32.07	31.90
26	0.88	0.88	21.24	21.10
27	0.86	0.87	19.56	19.40
28	1.36	1.31	23.24	23.10
29	0.89	0.89	12.15	12.00

4.8 TLC Profile, Physical and Chemical Properties of Compound AM3

Compound AM3 gave single spot when developed with Hex: EA 9:1 (Plate XIV) and Hex: EA 7:3 (Plate XV) solvent system with R_f values of 0.32 and 0.78 respectively using 10% H_2SO_4 as spray reagent. The melting point was found to be in the range of 212-214^{0}C. AM3 is completely soluble in chloroform and tested positive to Leibarman Burchad's test for triterpenes.



PLATE XIV:H:EA 9:1



PLATE XV:H:EA 7:3

4.9 Spectroscopic Data of Compound AM3

4.9.1 IR spectral data of compound AM3

The IR spectrum of AM3 showed characteristic absorption frequencies at 3291.2 cm^{-1}, 3063.9 cm^{-1}, 2922.2 cm^{-1}, 2855.1 cm^{-1}, 1636.3 cm^{-1}, 1453.7 cm^{-1}, 1379 cm^{-1} and 1039.9 cm^{-1} (Figure 4.4).

4.9.2 Proton nuclear magnetic resonance (^{1}H NMR) data of compound AM3

The ^{1}HNMR spectrum of AM3 in $CDCl_3$ (400MHz) revealed signals at δ 4.65 (d, H-29a), 4.75 (m, H-29b), δ 3.20 (dd, H-3), 2.38 (m, H-19), 1.60 (3H, s, H-30), 0.77 (3H, s, 24), 1.01 (3H, s, 23), 1.02 (3H, s, 26), 0.86 (3H, s,27), 0.78 (3H, s, 25) (Figure 4.5).

4.9.3 ^{13}C NMR spectral data of compound AM3

The ^{13}C-NMR (δ ppm, 100 MHz, $CDCl_3$) spectrum revealed signals at δ 39.90 (C-1), δ 27.64 (C-2), δ 79.22 (C-3), δ 39.08 (C-4), δ 55.52 (C-5), δ 18.54 (C-6), δ 34.50 (C-7), δ 41.05 (C-8), δ 50.66 (C-9), δ 37.39 (C-10), δ 21.15 (C-11), δ 25.36 (C-12), δ 38.27 (C-13), δ 43.05 (C-14), δ 27.64 (C-15), δ 35.80 (C-16), δ 43.22 (C-17), δ 48.52 (C-18), δ 48.21 (C-19), δ 151.20 (C-20) , δ 30.07 (C-21), δ 40.22 (C-22), δ 28.20 (C-23), δ 15.59 (C-24), δ 16.34 (C-25), δ 16.90 (C-26), δ 14.77 (C-27), δ 18.22 (C-28), δ 109.54 (C-29), δ 19.52 (C-30) (Figure 4.6).

4.9.4 Comparison of spectral data of AM3 with literature

Comparison of 1D and 2D NMR data of compound AM3 with literature data* (Shehu *et al*., 2015) revealed them to be fully in agreement (Table 4.6).

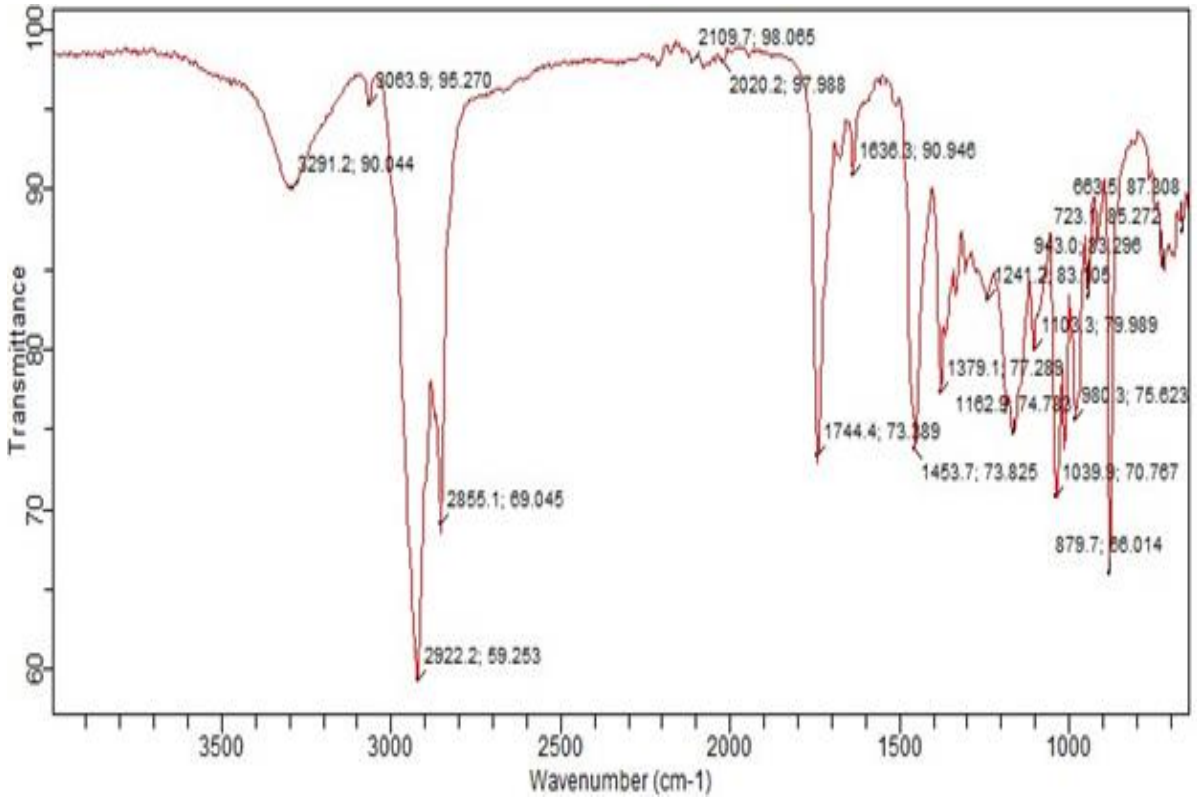


Figure 4.4: IR spectrum of AM3

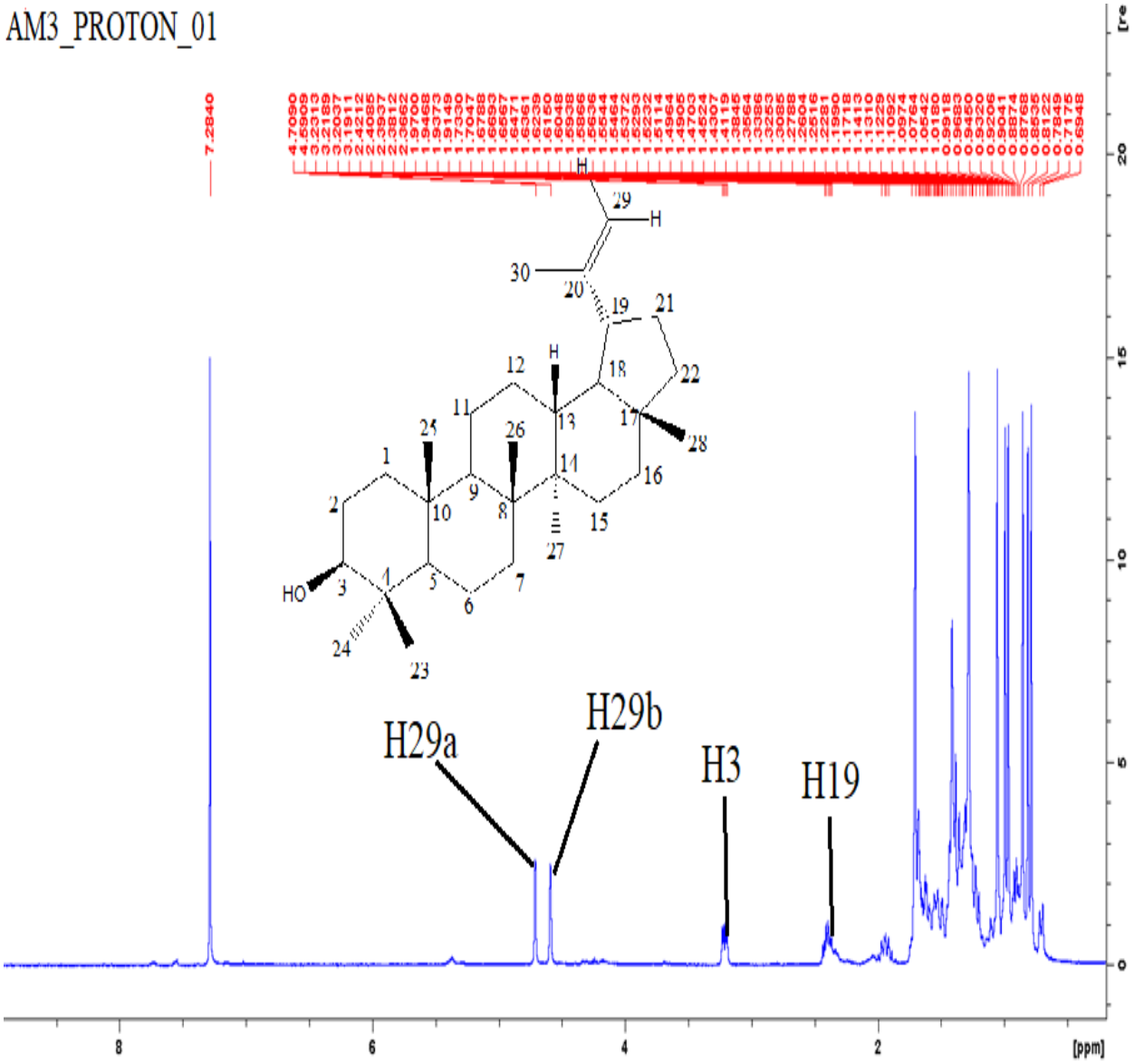


Figure 4.5: ^{1}H-NMR spectrum of AM3 in $CDCl_3$

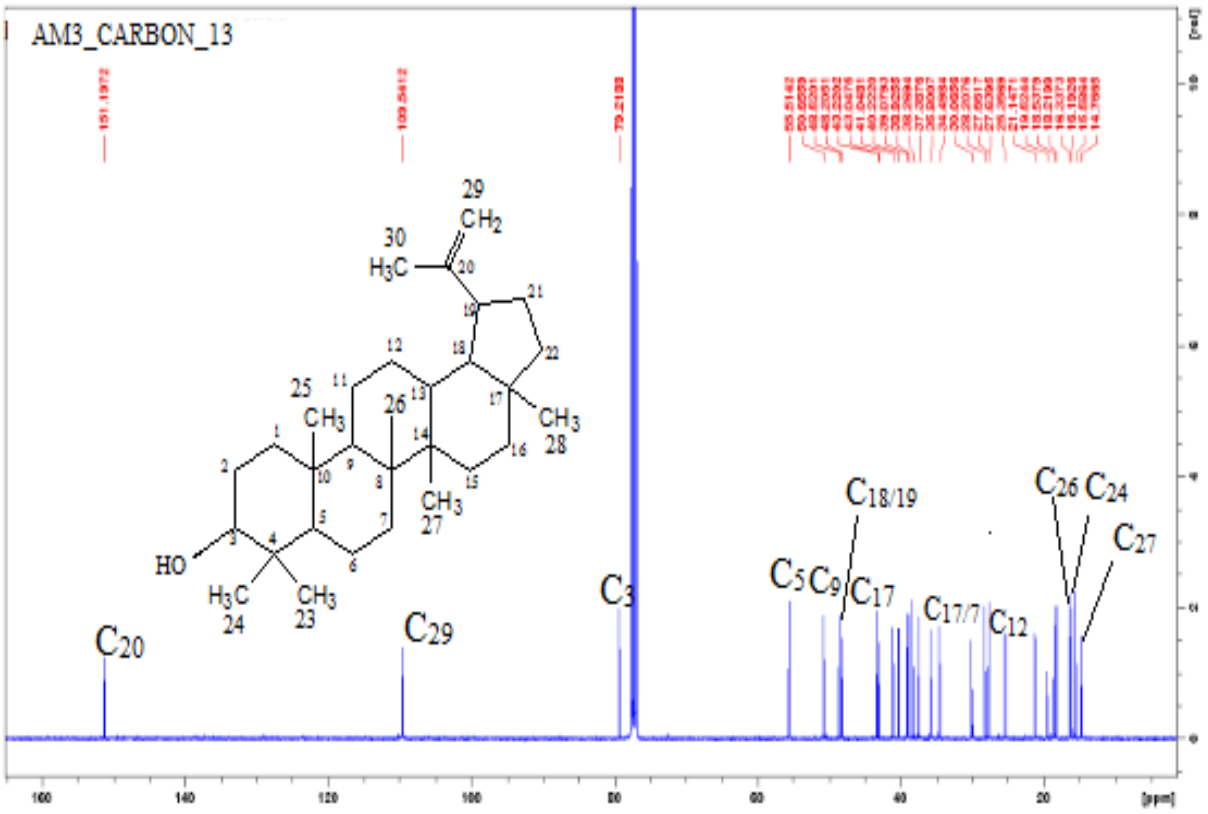


Figure 4.6: ^{13}C NMR Spectrum of AM3 in $CDCl_3$

Table 4.6: Comparison of spectral data of AM3 with literature

Position	^{1}H NMR	^{1}H NMR*	^{13}C-NMR	^{13}C-NMR*
1	0.94, m	0.92, m	38.9	38.80
2	1.62, m	1.60, m	27.64	27.20
3	3.20, dd	3.19, dd	79.22	79.30
4	-	-	39.08	39.10
5	0.67, m	0.68, m	55.52	55.30
6	1.50, m	1.40, m	18.54	18.30
	1.20, m	1.19, m		
7	1.37, m	1.38, m	34.50	34.20
8	-	-	41.05	41.10
9	1.28, m	1.28, m	50.66	50.60
10	-	-	37.39	37.20
11	1.43, m	1.40, m	21.15	21.10
12	1.44, m	1.45, m	25.36	25.50
	1.68, m	1.68, m		
13	1.62, t	1.63, t	38.27	38.30
14	-	-	43.05	43.20
15	0.97, m	0.97, m	27.64	28.00
	1.60, m	1.60, m		
16	1.46, m	1.45, m	35.80	35.20
17	-	-	43.22	43.30
18	1.36, m	1.38, m	48.52	48.30
19	2.38, m	2.36, m	48.21	48.10
20	-	-	151.20	150.10
21	1.25, m	1.28, m	30.07	30.00
22	1.17, m	1.17, m	40.22	40.50
23	0.98, s	0.99, s	28.21	28.00
24	0.77, s	0.75, s	15.59	15.60
25	0.79, s	0.80, s	16.34	16.20
26	1.01, s	1.02, s	16.19	16.00
27	0.86, s	0.91, s	14.77	14.80
28	0.76, s	0.75, s	18.22	18.20
29	4.75, s	4.75, s	109.54	109.50
	4.65, s	4.56, s		
30	1.68, s	1.68, s	19.52	19.40

4.10 Pharmacological Studies

4.10.1 Median lethal dose

The oral route median lethal dose (LD_{50}) of the methanol root extract of *combretum hypopilinum* was found to be above 5000 mg/kg in both the mice and rats suggesting the extract to be practically non-toxic.

4.10.2 Analgesic studies

4.10.2.1 Effect of the extract on acetic acid induced writhing test in mice

The methanol root extract significantly at *($p<0.05$)* decrease the number of writhing in the animals at the doses tested in dose dependent manner except at dose of 250 mg/kg when compared to the negative control as shown in Table 4.8. Percentage inhibition of pain was calculated to be 24.84 % at dose of 250 mg/kg, 40.49 % at dose of 500 mg/kg and 66.58 % at a dose of 1000 mg/kg. The percentage inhibition was found to be higher at dose of 1000 mg/kg compared to the standard drug used (piroxicam) which showed a percentage inhibition of 69.57% (Table 4.7).

Table 4.7: Effect of methanol root extract of *Combrertum hypopilinum* on acetic acid induced writhing in mice.

Treatment	Dose (mg/kg)	Mean number of writhing (± SEM)	Inhibition (%)
Distilled water	10 ml/kg	32.20 ± 2.22	-
MRE	250	24.20 ± 3.15	24.84
MRE	500	19.20 ± 2.08*	40.49
MRE	1000	10.80 ± 1.80*	66.58
Piroxicam	10	9.80 ± 2.11*	69.57

Data were analyzed using one-way anova followed by Dunnett post-hoc test. Values are expressed as Mean ± SEM, n=5, MRE = Methanol Root Extract of *Combretum hypopilinum*, SEM= Standard Error of Mean, * = $p<0.05$ statistically significant compared with distilled water.

4.10.2.2: Thermally – induced pain test in mice (Hot Plate)

The result of the hot plate test experiment shows statistically significant *($p<0.05$)* increase in mean reaction time of the extract only at a dose of 500 mg/kg at 90 mins when compared to distilled water. Only 500 mg/kg dose of the extract was found to have central activity compared to the standard drug (Table 4.8).

Table 4.8: Effect of methanol root extract of *Combretum hypopilimum* on hot plate test in mice.

		Mean reaction time (sec) ± SEM				
Treatment	**Dose (mg/kg)**	**0 min**	**30 min**	**60 min**	**90 min**	**120 min**
DW	10 ml/kg	5.00 ± 0.44	3.40 ± 0.68	5.40 ± 1.03	4.00 ± 0.63	4.60 ± 0.24
MRE	1000	4.800 ± 0.37	4.00 ±0.55	4.40 ± 0.81	5.20 ± 0.58	4.80 ± 0.58
MRE	500	4.80 ± 0.37	4.80 ± 0.66	5.60 ± 0.75	8.00 ± 0.89*	5.20 ± 0.73
MRE	250	5.60 ± 0.93	2.20 ± 0.37	5.80 ± 0.97	5.20 ± 0.37	4.80 ± 0.58
Pentazocine	5.0	6.20 ± 1.07	8.50 ± 0.58	9.80 ± 1.07	7.40 ± 0.81*	4.20 ± 0.37

Data were analyzed using repeated measure anova followed by Bonferoni post-hoc test. Values are expressed as Mean ± SEM, n=5, DW= Distilled water, MRE = Methanol Root Extract of *Combretum hypopilinum*, SEM= Standard Error of Mean, * = $p<0.05$ statistically significant compared with distilled water.

4.10.3 Anti-inflammatory studies

4.10.3.1 Carrageenan induced paw edema in rats

In the carrageenan induced paw edema test, the extract showed decrease in paw edema after 1 hour of carrageenan injection only at a dose of 250 mg/kg and another decrease in paw edema after 2 hours at dose of 250 and 500 mg/kg which shows a dose dependent decrease in paw edema when compared to the control. There was however a statistically significant *($p<0.05$)* decrease in paw edema at 4 -5 hour of carrageenan injection at all doses tested when compared to the control (Table 4.9).

Table 4.9: Effect of methanol root extract of *Combretum hypopilinum* on carrageenan induced paw edema in rats

		Mean paw diameter (cm) ± SEM					
Treatment	Dose (mg/kg)	0hour	1hour	2hour	3hour	4hour	5hour
DW	10 ml/kg	1.97 ± 0.08	3.47±0.11	4.09±0.03	3.65±0.08	3.60±0.09	2.77±0.24
MRE	1000	2.04±0.09	3.20±0.15	3.79±0.06	3.41±0.08	3.20±0.01*	2.40±0.04*
MRE	500	1.99±0.05	3.30±0.14	3.50±0.08*	3.59±0.08	2.94±0.24*	2.41±0.04*
MRE	250	1.79±0.03	2.80±0.06*	3.45±0.03*	3.46±0.06	2.40±0.06*	2.38±0.06*
IBU	10	1.89±0.01	2.64±0.10*	3.40±0.02*	2.32±0.04*	2.17±0.04*	2.13±0.03*

Data were analyzed using repeated measure anova followed by Bonferoni post-hoc test. Values are expressed as Mean ± SEM, n=5, DW= Distilled Water, MRE = Methanol Root Extract of *Combretum hypopilinum*, SEM= Standard Error of Mean, * = $p<0.05$ compared with distilled water.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Phytochemical Screening and Spectral Analysis

The preliminary phytochemical screening of the methanol root extract of *Combretum hypopilinum* revealed the presence of flavonoids, terpenoids/steroids, tannins, carbohydrate, saponin and cardiac glycosides (Tables 4.2). These phytochemical constituents have been reported to have different degrees of pharmacological activities (Sokenfu *et al*., 2017).

Column chromatography separation of ethyl acetate fraction followed by column chromatography of fraction A4 led to the isolation of a white amorphous substance coded compound AM2 which gave a single homogenous spot on thin layer chromatography using two different solvent systems with R_f value of 0.39 and 0.73 using HEX: EA (9:1) and HEX: EA (8:2) respectively indicating the purity of the compound. AM2 was completely soluble in chloroform and dichloromethane, melted at 169-171^{0}C. The appearance of reddish-brown ring at the interphase to Salkowski's test suggests that compound AM2 might be a steroid (Rahman *et al*., 2017).

The IR spectrum of AM2 showed absorption band at 3399.3 cm-^{1} typical of OH stretching, 3291.2 cm-^{1} was due to SP^2 C-H stretching vibration of alkene, 2933.4 cm^1 and 2862.6 cm^1 was as a result of asymmetric and symmetric stretching respectively, 1640 cm-^{1} was due to aromatic C=C in plane stretching vibrations, a bending frequency at 1461.1 cm^1 was due to cyclic methylene. The band at 1051.1 cm^1 was due to cycloalkene (Figure 4.1) (Bulama *et al.*, 2015).

The ^{1}H NMR ($CDCL_3$) spectrum of AM2 (Figure 4.2) revealed the presence of six high intensity peaks of methyl protons at δ0.73 (H-18), 1.08 (H-19), 0.95 (H-21), 0.87 (H-26), 0.83 (H-27) and 0.89 (H-29), and eleven methylene protons. The carbinol proton (H-3)

resonance was at 3.50 ppm. It also revealed the presence of olefinic protons signal at δ 5.38, 5.18 and 5.08 ppm. The proton signals between 0.73- 1.08 are attributed to resonance of overlapping methyl and methylene protons which is a characteristic framework of steroids (Arjun *et al*., 2010; Jephthah *et al*., 2014).

The ^{13}C NMR ($CDCl_3$, 100 MH_Z) (Figure 4.3) further substantiated the structural assignment of compound AM2 revealing the presence of twenty-nine carbon atoms which comprises six methyl carbons, nine methylene carbons, eleven methine carbons and three quaternary carbons. It showed some recognizable signals at δ140.91 and 121.87 for C5 and C6 double bond respectively and δ138.46 and 129.44 for C22 and C23 double bond respectively as well as signals at δ12.02 and 19.98 for the angular methyl carbons (C18 and C19) respectively and the carbon attached to OH groups at 71.97 (C-3). Based on the result of IR, 1D NMR, and comparison with literature data shown in Table 4.5, compound AM2 was suggested to be stigmasterol (Figure 5.1).

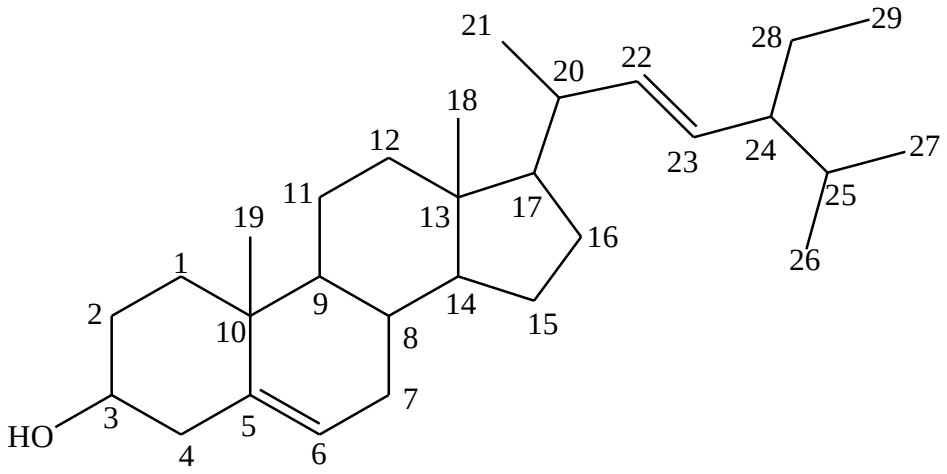


Figure 5.1: Structure of compound AM2 Stigmasterol

Phenolic compounds are reported to exert physiological effects such as to accelerate blood clothing, reduce blood pressure, decrease the serum lipid level, produce liver necrosis and

modulate immunoresponses (Ozcan *et al*., 2014). Steroids and triterpenes, among other phytochemicals have been reported to possess anti-inflammatory, analgesic and anticonvulsant activity (Musa *et al*., 2014). Stigmasterol has been reported to possess potent antioxidant, hypoglycemic and thyroid inhibiting properties (Panda *et al*., 2009). It has also been reported to be used in the prevention of certain cancers such as ovarian, prostate, breast and colon cancers (Gabay *et al*., 2010).

Column chromatographic separation of column fraction A9 led to the isolation of compound AM3 which gave a single homogeneous spot on TLC using two different solvent systems with R_f value of 0.32 using HEX: EA (9:1) and R_f value of 0.78 using HEX: EA (7:3) indicating the purity of the compound. AM3 was completely soluble in chloroform, melted at 212-214^{0}C and tested positive to Lieberman Burchard's test for triterpenes suggesting that compound AM3 might be a triterpene.

The IR spectrum of compound AM3 (Figure 4.4) showed characteristic absorption frequency at 3291.2 cm^{-1} typical of O-H stretching bands. Absorption frequency at 3063.9 cm^{-1} is found to be due to sp^2 C-H stretch. Asymmetric and symmetric stretching vibrations due to methyl groups were represented by the bands at 2922.2 and 2855.1 cm^{-1} respectively. C=C double bond stretching peaks was observed at 1636.3 cm^{-1}. Absorption bands at 1453 and 1379.1cm^{-1} are typical of methylene (CH_2) groups bending and CH_3 bending vibrations. C-O bond at C-3 can be observed at absorption frequency at 1039.9 cm^{-1} (Shehu *et al.,* 2015).

The ^{1}H NMR spectrum of AM3 revealed the presence of seven tertiary methyl protons at δ 0.72, 0.78, 0.81, 0.85, 0.99, 1.01and 1.70 (integrated for 3H-each, s, CH_3). A sextet of one proton at δ 2.38 ascribable to 19β- H is characteristics of lupeol. The H-3 proton showed a multiplet at δ 3.20 while a pair of broad singlets at δ 4.65 and δ 4.75 (1H, each) was indicative of two olefinic protons (exocyclic double bond) at H-29 a and b (Figure 4.5).

These assignments are in good agreement for the structure of lupeol (Shehu *et al.*, 2015*;* Abdullahi *et al.,* 2013).

The structural assignment of AM3 was further identified by the ^{13}C NMR experiments as shown in Figure 4.6 showed 30 signals for the pentacyclic triterpenoid of lupane skeleton which include seven (7) methyl groups at δc: 28.21 (C-23), 18.22(C-28), 16.34 (C-25), 16.90(C-26), 15.59 (C-24), 14.77(C-27), and 19.52 (C-30); eleven (11) methylene, six (6) quaternary carbon atoms. The signals at δc 151.20 ascribed to (C-20) and at δc 109.54 ascribed to (C-29) were deshielded due to olefinic bond between them. Also, signal at δc 79.22 assigned to (C-3) was deshielded due to the hydroxyl group attached to the (C-3). There was a marked difference observed between δc C-23 and C-24 despite being on the same carbon atom, which can be due to the close proximity of C-23 to the electronegative atom in space. Therefore, comparing its IR, ^{1}H and ^{13}C spectra with the reported literature values shown in Table 4.6, AM3 was confirmed to be a lupeol (Figure 5.2).

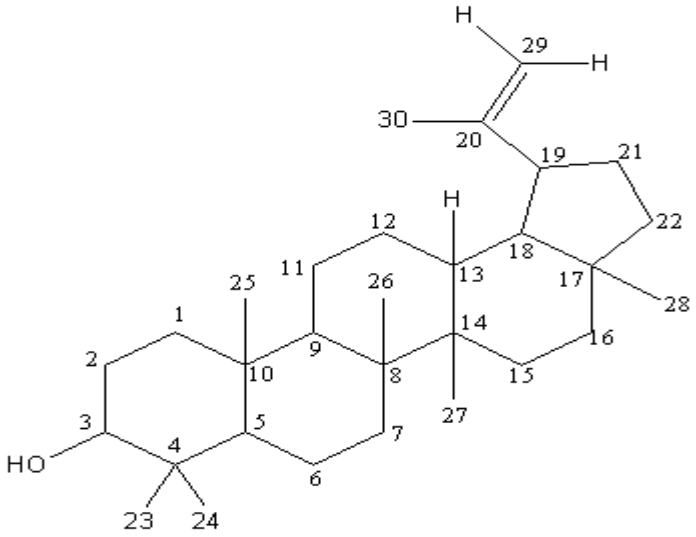


Figure 5.2: Structure of compound AM3 Lupeol

triterpenes with several medicinal potentials has been known to exhibit anti-inflammatory, analgesic and anti-arthritic activities (Agarwal *et al*., 2003). They are known to boost the inhibitory effects of anti-cancer drugs and also cure patients suffering from Alzhiemer's and Parkinsonism, (Sharma and Zafar, 2015). Lupeol is known to inhibits the growth of highly aggressive human metastatic melanoma cells (Saleem *et al*., 2008), lupeol and its related compounds have also been reported to possess some activity in the nervous system (Martini *et al.,* 2007).

5.2 Pharmacological Studies

5.2.1 Acute toxicity (LD_{50}) studies

The oral median lethal dose (LD_{50}) value of the methanol root extract of *combretum hypopilinum* was found to be greater than 5000 mg/kg body weight in swiss albino mice and rats which implies that the extract is relatively safe (Chakroborty *et al.,* 2004). The acute toxicity study is of utmost importance as it provides information for traditional medicine practitioners and researchers regarding the maximum tolerable dose and safety of the plant. All the doses used throughout the research procedure are less than or equal to 30 % of the (LD_{50}) which have been demonstrated to be relatively safe for ethnopharmacological research (Vongtau *et al.,* 2004).

5.2.2 Analgesic and anti-inflammatory studies

The acetic acid- induced writhing test also called abdominal constriction response is a sensitive procedure and is used for the evaluation of peripheral analgesic activity. It produces writhing reflex in animals by activating the chemo sensitive nociceptors, so the indication for level of analgesia in acetic acid-induced writhing models is reduction in the number of abdominal constrictions (Marchioro *et al.,* 2005). The methanol root extract significantly at ($P<0.05$) decrease the number of writhing in the animals at the doses tested in a dose

dependent manner except at dose 250 mg/kg. Percentage inhibition was highest (66.58 %) at the highest dose of 1000 mg/kg and relatively low (40.49 %) at the dose of 500 mg/kg experimental dose when compared with the distilled water (Table 4.7). The result showed that the extract possesses appreciable anti-nociceptive effect at the highest dose which was comparable to that of the standard drug (pentazocine 10 mg/kg) which showed 69.57 % nociceptive inhibition. The analgesic effect of this extract could be through suppression of prostaglandin pathway because it has been reported that any agent that decreases the number of writhing will demonstrate analgesia preferably by inhibition of prostaglandin synthesis, peripheral mechanisms (Ezeja *et al.,* 2011).

The hot plate test is found to be suitable by many investigators for the evaluation of centrally acting analgesics (Vogel, 2007). It evaluates the reaction time of the animal when exposed to a heated stimulus on the planter surface of their paws. The paws are sensitive to heat at temperatures which are not damaging to the skin and the responses commonly observed are jumping, withdrawal of the paws and licking of the paws. In this experiment, the extract shows significant effect only at a dose of 500 mg/kg at 90 min when compared to the control (Table 4.8). Pain induced by thermal stimulus of the hot plate is specific for centrally mediated nociception (Aiyelero *et al.,* 2009). The significant increase in pain threshold produced by the extract in this model suggests involvement of central pathways and the ability of the extract to significantly prolong the reaction latency to thermally –induced pain in mice by the hot plate at 90 min suggest that the extract have a weak central analgesic activity. Pain is centrally modulated via a number of complex processes including opiates, dopaminergic descending noradrenergic and serotonergic systems (Debasis *et al.,* 2011).

Carrageenan- induced paw edema is a commonly used test for the screening of anti-inflammatory activities and its reactions have been shown to be due to the release of inflammatory mediators (Ndebia *et al*., 2007). It has been reported that neutrophil infiltration

plays major role in the inflammation induced by carrageenan in hind paw (Mansouri *et al.,* 2015). The extract significantly inhibited the carrageenan- induced inflammation in the second hour and later at 4-5 hours (Table 4.9). The carrageenan- induced paw edema in rats is believed to be biphasic; the first phase is due to the release of histamine, serotonin and kinins while the second phase is attributed to the release of bradykinin, prostaglandins and lysosome emthymes (Achinta *et al.,* 2007). Therefore, it can be assumed that the inhibitory effect of the extract on carrageenan induced inflammation could be due to the inhibitory activity of cyclogenase-2 (COX-2) leading to the inhibition of synthesis of prostaglandins.

This result showed that the extract has significant analgesic and anti-inflammatory properties, and it justifies the traditional use of the plant in the treatment of pain and inflammation. The analgesic and anti-inflammatory effect of the extract may be due to the presence of bioactive constituents such as flavonoids, tannins, steroids/terpenoids and saponins. This is because these secondary metabolites have been reported to have those activities (Ahmadiani *et al.,* 2000).

CHAPTER SIX

6.0 SUMMARY, COCNCLUSION AND RECOMMENDATION

6.1 Summary

The preliminary phytochemical screening of the methanol root extract of *Combretum hypopilinum* revealed the presence of carbohydrates, flavonoids, saponins, triterpenes, steroids, tannins and cardiac glycoside. Column chromatography analysis of ethyl acetate fraction led to the isolation of two compounds characterized to be stigmasterol and lupeol. The methanol root extract of *Combretum hypopilinum* showed significance at *($p<0.05$)* analgesic and anti-inflammatory activities in experimental animal models.

6.2 Conclusion

Based on the results presented, the plant showed significant analgesic and anti-inflammatory activities which inferred that the methanol root extract of *Combretum hypopilinum* contains phytochemical constituent(s) with potential analgesic and anti-inflammatory properties, thus provides scientific justification for the use of the plant in ethnomedicine for the treatment of pain and inflammation. Hence, rejecting the research hypothesis.

6.3 Recommendation

1. Bioassay-guided isolation should be carried out with the view of isolating other bioactive constituents responsible for the observed analgesic and anti-inflammatory activity
2. Chronic toxicity test of the extract should be done to ascertain the reported claim of less toxicity as observed in the study.
3. Aqueous fraction of the extract should be worked on so as to isolate polar compounds as well as validating other ethnomedicinal claim.

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APPENDIX

Appendix 1: Determination of intraperitoneal median lethal dose (LD_{50}) of the methanil root extract of *Combretum hypopilinum*

First phase

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

Second phase

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