ISOLATION AND CHARACTERISATION OF BIOACTIVE COMPOUNDS FROM LEAF EXTRACT OF *Combretum lamprocarpum(*DIELS)

BY

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BY

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DEPARTMENT OF CHEMISTRY,
FACULTY OF PHYSICAL SCIENCES,
AHMADU BELLO UNIVERSITY,
ZARIA, NIGERIA

MARCH, 2018

Declaration

I declare that the work in this dissertation entitled **'Isolation and Characterisation of Bioactive Compounds from Leaf Extract of *Combretum lamprocarpum*DIELS'** was carried out by me in the Department of Chemistry, Ahmadu Bello University, Zaria, Nigeria. 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.

Ibrahim Gadaka<u>KIZITO</u> _____ _____
Name of Student Signature Date

Certification

This dissertation entitled **'Isolation and Characterisation of Bioactive Compounds from Leaf Extract of *Combretum lamprocarpum*DIELS'** by **Ibrahim GadakaKIZITO**, meets the regulations governing the award of Master degree in Organic Chemistry of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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Dean School of Postgraduate Studies	Signature	Date

Dedication

This work is dedicated to the Glory of **God Almighty**, to my beloved wife Elizabeth, to my Children (Queen Esther and Kizito Jnr), and to the memory of Bulus Kizito.

Acknowledgement

First and foremost, I would like to give thanks and praise to God **Almighty** for his grace, protection, and blessings throughout the entire period of this research. My sincere gratitude is reserved to my supervisors, Dr. I. A. Bello and ProfessorR. G. Ayo for guidance during the laboratory work. I really appreciate my supervisors for their willingness to meet with me at short notice every time and going through several drafts of my dissertation. May God Almighty continue to direct you in all your endeavours and raise you to greater heights in life. Amen.

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Most importantly, I wish to thank my loving and supportive wife, Elizabeth for her understanding against all odds and for taking good care of the children. Her support and encouragement made this dissertation possible. To my children, Queen Esther and Kizito Jnr, a very big thank you for your endurance throughout the period of this study. I remain grateful forever to my parents, Mr andMrs. Kizito Abare Gadaka for their prayers, support and encouragement. My heartfelt appreciation goes to my beloved siblings, Aisha, Musa, Yohanna, Dauda and Hannatu. To all my laboratory mates, Ibrahim Isyaku Funtua, R.S. Ochu, and Hadiza Sada, I say thank you. Special thanks to Jonathan IlemonaAchikaand John Anyam Vershima for their assistance throughout my laboratory work.

Abstract

*Combretum lamprocarpum,*is a plant used in tropical and sub Saharan parts of Africa in the treatment of wounds, stomach ache, diarrhoea, and vomiting. The dried powdered plant material(leaves) was extracted using a microwave-assisted extraction technique.Phytochemical and antimicrobial screening of the extracts were carried out using standard methods.The compound was purified using conventional chromatographic techniques and characterised using spectroscopic techniques. The phytochemical screening of the extracts revealed the presence of steroids, terpenes, alkaloids, carbohydrates, saponins, tannins, cardiac glycosides and flavonoids. The antimicrobial screening of the crude extracts showed that all the extracts had significant activity against microbes with the exception ofhexane extract. The diameter of the zone of inhibition ranged between 12 and 29 mm, the minimum inhibitory concentration ranges between 3.13 and 50.00 mg/ml. The minimum bactericidal concentration/minimum fungicidal concentration was between 6.25 and 50.00 mg/ml. A bioactive triterpene coded K1, was isolated as a white crystalline compoundfrom the ethyl acetate extract and characterised using FT-IR, ^{1}H-NMR, ^{13}C-NMR analysis as lupeol. The isolated compound from the ethyl acetate extract was found to be active against*Staphylococcus aureus*(MBC=25.00µg/ml), *Salmonella typhi*(MBC=12.50µg/ml), *Escherichia coli*(MBC=6.25µg/ml),and *Pseudomonas aeruginosa* (MBC=12.50µg/ml). The structure of the compound was supported by comparing the experimental spectroscopic data with that of literature. In conclusion, thisstudy revealed that the leaves of*Combretum lamprocarpum*hasa potent medicinal value. The chloroform and methanol extracts showedbioactivity and therefore, should be studied for possible bioactive compounds.

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Abbreviations and Symbols

^{13}C NMR	Carbon 13 Nuclear Magnetic Resonance
1D	One Dimension
^{1}H NMR	Proton Nuclear Magnetic Resonance
CC	Column Chromatography
CFU	Colony Forming Units
FT-IR	Fourier-Transform Infrared
MAE	Microwave-Assisted Extraction
MBC	Minimum Bactericidal Concentration
MFC	Minimum Fungicidal Concentration
MHA	Mueller-Hinton Agar
MHB	Mueller-Hinton Broth
MHz	Megahertz
MIC	Minimum Inhibitory Concentration
NA	Nutrient Agar
CLSI	Clinical and Laboratory Standards Institute
ppm	Parts per million
R_f	Retardation factor
PDA	Potato Dextrose Agar
TLC	Thin Layer Chromatography
TMS	Tetramethylsilane
WHO	World Health Organisation
ZI	Zone of Inhibition
δ	Chemical shift in ppm

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

The use of medicinal plants in the treatment of diseases is as old as mankind. A medicinal plant is defined as a plant that is useful in therapeutics because it contains active constituents (Sher, 2004). These constituents are mostly secondary metabolites such as alkaloids, essential oils, flavonoids, glycosides, resins, saponins, tannins, and terpenoids (Cowan, 1999).Plants remain a rich source of secondary metabolites that have been widely used as bioactive constituents in therapeutically effective medicines for several ailments (Hoareau and Da Silva, 1999). Fossil records revealed that the use of plants as medicines by human may be traced back to about 60,000 years ago (Fabricant and Farnsworth, 2001). The search for plants that for remedy against diseases and ailments have been documented since ancient times in the form of traditional medicine. Historically,the beginning of the nineteenth century marked the era of "modern" drugs (Joo, 2014). In the year 1805, morphine, the first pharmacologically active compound was isolated from the opium plant by a German pharmacist, Friedrich Sertürner (Hamilton and Baskett, 2000).

Despite the breakthrough made by mankind in the synthesis of pharmaceutical drugs, the decreasing efficacy of synthetic drugs and the increasing contraindications of their usage, make the continueduse of plants as important sources of medicinal agents (Petrovska, 2012). According to the World Health Organization, traditional medicine is the sum total of the knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health, as well as in the prevention, diagnosis, improvement or treatment of physical and mental illnesses (WHO, 2005). Traditional medicines could not be risk-free, and due to

their rampant use, it is important that the population be informed of risks that may be linked to their use. One of such risks is associated with the contamination of these herbal remedies with potential toxins such as heavy metals and organic pollutants (Awodele *et al.*, 2013). Traditional medicinal practice has been documented for centuries in many parts of the world. Several plants and herbs are used worldwide by traditional medical practitioners. Extracts from the various plant parts (leaves, stem bark and roots) of various higher plants are used in herbal medicine prescription (Sofowora, 1993). Crude extracts from plants are usually administered as concoctions for the treatment of various diseases. However, about 75% of the world population depends on these various forms of concoctions and herbal decoctions for the treatment of infectious diseases (Robinson and Zhang, 2011).

Natural products, which constitute the secondary metabolites, have been used since ancient times in traditional medicine for the treatment of many diseases and illnesses (Dias*et al.*, 2012). In Nigeria, the use of potent medicinal plants in management of different diseases has been reported (Ogunshe *et al*., 2008; Weintritt, 2007; Aiyeloja and Bello, 2006; Blench and Dendo, 2003).

Phytochemicals are the sources of basic raw-materialfor the establishment of pharmaceutical industries (Mothana and Lindequist,2005). Phytochemical screening plays a vital role in identifying new sources of pharmacologicallyactive compounds such as alkaloids, anthraquinones, flavonoids, phenolic compounds, saponins, steroids, tannins and terpenoids (Akindele and Adeyemi, 2007). Most of these phytochemical constituents are potent bioactive compounds found in parts of the medicinal plant which serve as lead for modern drugs (Sofowora, 1993).

Phytochemistry has been developed in recent years as a separate discipline. It is concerned with the enormous variety of organic substances that are biosynthesized and

stored by the plants.They have been known to reduce the risk of many human diseases including cardiovascular disease, hepatorenal diseases, diabetes, cancers and neurodegenerative disorders (Modak *et al.,* 2007; Shakya and Shukla, 2011).

Phytochemicals can be classified on the basis of their chemical composition or functional group(s).These include alkaloids, glycosides, flavonoids, tannins, saponins, terpenes, steroids, anthraquinones (Shakya, 2016).

Alkaloids are one of the major classes of natural products that exhibit antimalarial activity.

The first antimalarial drug -quinine, isolated from the bark of cinchona specie (Rubiaceae) belongs to this class. However, over 100 alkaloids from higher plants were reported to exhibit significant antimalarial activity (Saxena *et al.,* 2003). Taxol a plant alkaloid (generic name paclitaxel) is a microtubule-stabilising drug that is approved by the Food and Drug Administration for the treatment of patientswith ovarian, breast, and lung cancer as well as Kaposi's sarcoma (Shoeb, 2006). Taxol® is considered the most efficacious developed anti-cancer drug in the past five decades and represents taxane family of drugs with worldwide sales of $1.5 billion in the year 1999 (Isah, 2015). Alkaloids have wide range of pharmacological activities including antispasmodic, antimalarial, analgesic, diuretic activities (Kittakoop*et al.,* 2014).

Terpenes are a large and diverse class of organic compounds.They can occur as monoterpenes, diterpenes, sesquiterpenes, triterpenes, and tetraterpenes (C10, C15, C20, C30 and C40 respectively). A number of terpenes or terpenoids are reported to be active against fungi (Arif *et al.,* 2009). The oxygen containing derivatives i.e.terpenoids are known for their antiviral, anthelmintic, antibacterial, anti-cancer, antimalarial, and anti-inflammatory properties (Chen *et al.,* 2012).

Flavonoids are phenolic compounds that continue to draw the interests of the scientific community because of its wide range of bioactivity. Flavonoids have an antioxidant, anti-allergic, antibacterial properties among others (Xia *et al.,* 2014; Nobakht *et al.,* 2017) and saponins are reported to have anti-inflammatory and antiviral activities (Chopra and Doiphode 2002; Maurya *et al.,* 2008).

Anthraquinones(9, 10-dioxoanthracenes) constitute a vital class of natural products with numerous applications. Apart from their use as colourants, anthraquinone derivatives have been used over the centuries for medicinal purposes such as laxatives, antimicrobial, and anti-inflammatory agents (Malik and Muller, 2016). The example of antifungal anthraquinone from medicinal species includes a new 1,3-dihydroxy-2-methyl-5,6-dimethoxy-anthraquinone (Fatope *et al.,* 2003).

1.2 Statement of the Research Problem

About fifty percent of the number of death recorded in the tropical countries are largely due to infectious diseases (Iwu *et al.,* 1999). This can be linked to the increasing bacterial resistance to antibacterial drugs (Fair and Tor, 2014).The increasing occurrenceof resistance to antimicrobial drugs is attributed to the indiscriminate and abuse of the modern antimicrobial drugs (Usha *et al*., 2010). However, the ethno medicinal uses of the leaves of *C. lamprocarpum* in Nigeria for the treatment of ailments such diarrhoea, stomach-ache, healing of woundshave not been scientifically verified. It is against this backgroundthat there is need to develop a more convenient and very active therapeutic antimicrobial agent.

1.3 Justification for the Research

This research was conducted in order to search for newer and more effective antimicrobial agents because of the alarming increase in multidrug resistance. The use of

antimicrobial drugs from medicinal plants, have many advantages such as less side effects, cheaper, acceptance due to long history of use, and being renewable in nature (Gur *et al*., 2006). To the best of our knowledge, there is no report on the pharmacological activity or isolation and characterization of bioactive compounds fromthe plant. Therefore, there is need to validate the claims of the ethnomedicinal practices and to identify the active components of the leaf of *Combretum lamprocarpum*which are responsible for the treatment of numerous ailments such as diarrhoea, stomach-ache, healing of wounds etc.

1.4 Aim and Objectives of the Research

1.4.1 Aimof the research

The aim of this research was to validate the ethnomedicinal claim on *Combretum lamprocarpum*leaf by isolation and characterisation of bioactive constituents responsible for the ethnomedicinalclaims on the plant.

1.4.2 Objectives of the research

The aim of the research stated above will be achieved through the following objectives:

i. Preliminary phytochemical screening of the crude extracts.
ii. Antimicrobial screening of the crude extracts.
iii. Purification of the extracts and isolation of bioactive compound(s) using chromatographic techniques
iv. Structure elucidation of the isolated compound(s) using spectroscopic techniques (^{1}H,^{13}C NMR and IR).
v. Determination of the antimicrobial activity of the isolated compound(s).

1.5 Scope and Limitation of the Research

This research work is expected to cover extraction of the leaf of *C. lamprocarpum,* phytochemical screening of the plant extracts, antimicrobial screening of the crude extracts and any isolated compound(s), purification of the extracts, isolation of bioactive compound(s), and characterisation of the isolated compound(s).

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The family *Combretaceae*

The *Combretaceae* family consists of about 600 species of trees, shrubs and lianas in about 18-20 genus. Plants belonging to this family are found in tropical and sub-tropical regions, mostly in Africa and India. The largest genus is *Combretum* and *Terminalia* with about 370 and 200 species respectively (Roy*et al.,* 2014a). Several *Combretaceae* species are widely distributed in Africa and are used in folk medicine for treating numerous conditions. The leaves of *Combretum*species are widely used for treating disorders like pneumonia, syphilis, leprosy, abdominal pains, conjunctivitis, diarrhoea, scorpion bites and mumps (Hutchings *et al*., 1996).

2.1.1 The genus *Combretum*

*Combretum*is a genus of the plant whichbelongs to the family of*Combretaceae* of the order Myrtales. *Combretum* comprises of about 250 species. Some of the species are used extensively in folklore medicine against inflammation, infectious diseases, diabetes, malaria, bleeding, diarrhoea and digestive disorders among others (Gouveia *et al.,* 2011). The list of some *Combretum* species in Table 2.1 (theplantlist.org, 2016).

Table 2.1: List of some *Combretum* species

S/No	Species	S/No	Species
1	*Combretum apiculatum*	13	*Combretum micranthum*
2	*Combretum barbatum*	14	*Combretum oyemense*
3	*Combretum caffrum*	15	*Combretum platypterum*
4	*Combretum decandrum*	16	*Combretum racemosum*
5	*Combretum edwardsii*	17	*Combretum schumannii*
6	*Combretum falcatum*	18	*Combretum sundaicum*
7	*Combretum glutinosum*	19	*Combretum tarquense*
8	*Combretumhypopilinum*	20	*Combretumvendae*
9	*Combretum imberbe*	21	*Combretum villosum*
10	*Combretum kraussii*	22	*Combretum woodii*
11	*Combretum lamprocarpum*	23	*Combretum xanthothyrsum*
12	*Combretum loefl*	24	*Combretumzenkeri*

Adapted from The plant list (2016).

2.2 Taxonomic Classification of *Combretum lamprocarpum*

Kingdom: *Plantae*

Phylum:*Tracheophyta*

Class: *Magnoliopsida*

Order: *Myrtales*

Family: *Combretaceae*

Genus: *Combretum*

*S*pecies: *Combretum lamprocarpum* DIELS

Common names: English; *variable bush-willow* Hausa; *Bauli,* Fulfulde; *danehee*, Yoruba; *ajantiro*

2.3 Morphological Description of the Genus *Combretum lamprocarpum***DIELS**

Combretum lamprocarpum(*Combretaceae*) is a savannah tree of height up to 7m. The under surface of tender leaves is densely covered with white scales, densely covered with minute hairs on midrib, petiole and nerves, but only sparsely covered on the lamina. The matured leave has scattered white scales beneath, and is smooth except for a few hairs on the midrib. The lamina appeared to be ovate-elliptic to oblong-lanceolate, 8–14 cm long, and 4–6 cm broad respectively. The bark appeared grey to dark grey in colour, fissured, and peeling off in ragged strips leaving a red-brown powder under the rhytidome (Keay and Hepper, 2013). The flowers develop in spikes at the leaf-axils, and are greenish white to cream. The fruits have four-wings. The wings are greenish at first then turning to brownish green tinged red. The plant produce flowers from January to February and fruits from January to March (Gibreel, 2008).

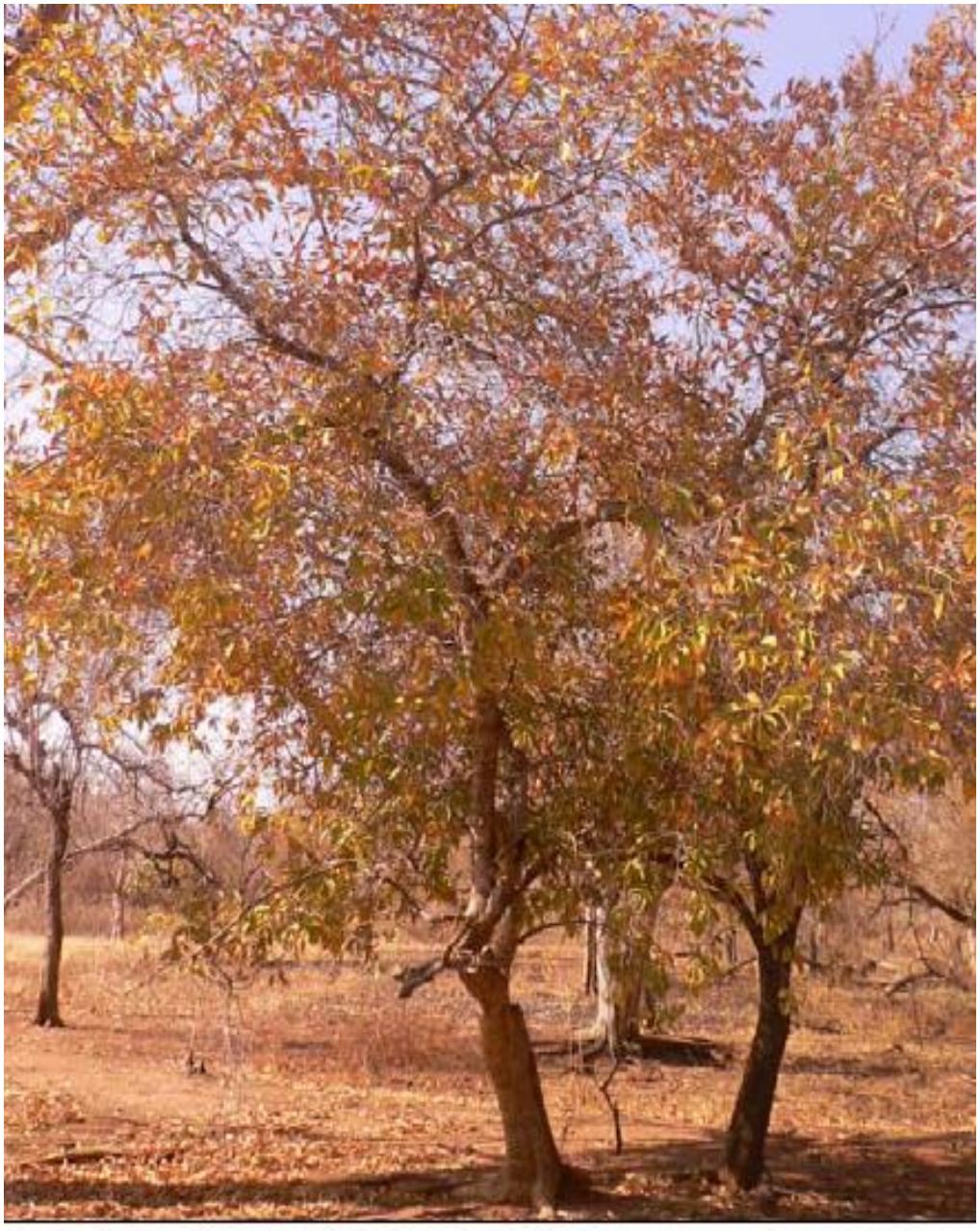


Plate I: The plant *Combretum lamprocarpum* (Gibreel, 2008).



Plate II: The leaf of *Combretum lamprocarpum* (Gibreel, 2008).

2.4 Ethno-medicinal uses of *Combretum lamprocarpum* and other species

The plant *Combretum lamprocarpum* based on reports from the ethnobotanical survey have been used in the treatment of wounds, diarrhoea, stomach ache, and vomiting (Offiah*et al.*, 2012; Gibreel *et al.*, 2013; Adjakpa *et al.*, 2016).

Many species of *Combretum* have been used in African traditional medicine for the treatment of various ailments and diseases. The fruits and seeds are considered poisonous by traditional healers in various African countries and have been reported to have toxic effects on humans (Rogers and Verotta, 1996). The root decoction of *Combretum apiculatum* is used for the treatment of mental illness and a necklace of the roots is used for the same purpose (Chhabra *et al.*, 1989).

Koenen (1996) reports that stomach problems are treated by a vapour bath of *Combretum apiculatum* and combining this treatment with drinking the leaf decoction of the same plant. The leaves are also used to disinfect the navel after birth (Van Wyk and Van Wyk, 1997). The roots of *Combretum zeyheri* are chewed for the treatment of schistosomiasis (Neuwinger, 2000). The leaves and roots of *Combretum imbibe* are used for the treatment of diarrhoea, coughs, and the ashes from the wood for toothpaste (Neuwinger, 2000). *Combretum erythrophyllum* is widely used for the treatment of abdominal pains and venereal diseases, and this indicates the presence of bioactive compounds in the leaves (Hutchings *et al.*, 1996). Moreover, *Combretummucronatum*Schumach. and Thonnhas been widely used in West African traditional medicine for treatment of wound and helminth infections (Spiegler *et al.*, 2015).

It has been reported by Nunes *et al*(2009) that *Combretum leprosum* is widely used as an expectorant, antimicrobial and antihemorrhagic agent. The different parts of *Combretum glutinosum* (roots, stem, bark and leaves) are used in the treatment of many ailmentssuch as scrotal elephantiasis, dysentery, ring worms, syphilis, typhoid fever, eye sore and ear ache (Nwaeze and Abarikwu, 2006). Moreover, Tony (2005) also reported that the stem bark and leaves of *Combretum glutinosum* are used as antipyretic and in the treatment of stomach ache, gonorrhoea and typhoid fever.

2.5 Phytochemical Studies on *Combretum* species

Phytochemical studies have been carried out on some species of the genus *Combretum* and have confirmed the existence of many classes of constituents, comprising of triterpenes, flavonoids, lignans and non-protein amino acids, among others (Pietrovski*et al.,* 2006). A research carried out on the preliminary phytochemical screening of the methanolic and aqueous extract of the stem bark and leaf of *Combretumglutinosum*revealed the presence of saponins, alkaloids, phenolics, tannins, glycosides, flavonoids and anthraquinones (Yahaya *et al.*, 2012).

Phytochemical screening carried out on the stem bark, leaves, fruits, and roots of *Combretumschumanii*confirmed the presence of natural products constituents – alkaloids, terpenoids, flavonoids, tannins and saponins (Mbwambo*et al.*, 2013).

2.6 Biological Activity of *Combretum* species

Combretum extracts or isolates have shown *in vitro* bioactivities such as antibacterial, antifungal, antihyperglycemic, cytotoxicity against various human tumour cell lines, anti-inflammatory, antisnake, antimalarial and antioxidant effects (Dawe *et al*., 2013).

2.6.1 Anti-inflammatory activity

Inflammation is part of the body's immune response, which can be indicated by pain, redness, heat, and swelling (Verma, 2016). The anti-inflammatory activity from the aqueous and acetone dried leaf extract of *Combretum edwardsii*, *Combretum kraussii*and *Combretum moggii* has shown potent activity *in vivo* (McGaw *et al.,* 2001). Also, anti-inflammatoryactivity was found in *Combretummicranthum*(Olajide *et al.,* 2003).

2.6.2 Anti-cancer activity

Cancer is one of the most obvious human diseases which has moved scientific attention to the discovery of novel anti-cancer agents from natural products. It was reported that more than 60% of cancer drug available in market or in clinical trials are established on natural products (Sen and Chakraborty, 2017). Isolation studies have shown that a bioactive compound, combretastatin A-4 (**I**) from the bark of *Combretum caffrum* has been found to be effective against lung and colon cancers (Prakash *et al.,* 2013).

Combretastatin A-4(**I**)

2.6.3 Antifungal activity

Fungi are common in the environment, and infection due to fungal pathogens has become more rampant (Dellavalle *et al.,* 2011). Plants are arich source of bioactive phytochemicals, reported to have *in-vivo* antifungal properties (Arif *et al.,* 2009). The methanol leaf extract of *Combretum caffrum*and *Combretumacutifolium* shows significant antifungal activity (Masoko *et al.,* 2007).

2.6.4 Antibacterial activity

Plant materials have been reported to be bioactive against microbes due to the presence of phytochemicals such as alkaloids, saponins, tannins, anthraquinones, steroids, flavonoids etc (Odugbemi, 2006). *Combretumschumannii*whole plant has been reported to have antibacterial activity (Mbwambo *et al.*,2013). *Combretum molle* has been widely used as herbal recipes for medicinal plant to treat various diseases such as parasitic, protozoan and other infectious diseases (McGaw*et al.*,2001). The extracts of *Combretumerythrophyllum*obtained with different solvents (acetone, hexane, chloroform, carbon tetrachloride and butanol) have shown antibacterial activity at different doses against *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Enterococcus faecal* (Martini and Eloff, 1998).

2.7 Compounds Isolated from Combretaceae Family

Several chemical constituents have been isolated from *Combretum* species and have been documented. Most of the isolated compounds belong to the class of terpenoids, steroids and flavonoids, and stilbenes (Eloff *et al.,* 2008). The isolation of Cardamonin (**II**) from *Combretum apiculatum* was reported byAderogba*et al*(2012). An important polyphenol,3-*O*-

methyl quercetin (**III**) was isolated from the methanol extract of the flowers of *Combretumlanceolatum* (Araujo *et al.,* 2013). A combretastatin (**IV**), which is a group of stilbenes, has been isolated from some species of *Combretum* (Fyhrquist *et al.*, 2006). A bioactive triterpene, betulinic acid (**V**) has been reported in *C. quadrangulare, C. laxum* and *C. yunnanense* (Roy *et al*., 2014b; Wang *et al.*, 2011; Bisoli *et al.*, 2008). Also, a flavan, 3′,4′,5,7-tetrahydroxy-flavan (**VI**) was isolated from *Combretum erythrophyllum* (Schwikkard *et al.,* 2000). A triterpene, arjunolic acid (**VII**) was isolated from the ethanolic root extract of *Combretum leprosum* in which the *in-vitro* activity showed activity against snake venom (Fernandes *et al.*, 2014).

4', 6'-dihydroxyl-2'-methoxychalcone(**II**)

3-*O*-methyl quercetin(**III**)

Combretastatin(**IV**)

Betulinic acid(**V**)

3',4',5,7-tetrahydroxyflavan(**VI**)

Arjunolic acid(**VII**)

2.8 Secondary Metabolites from Plants

Generally, some plant species have bioactive constituents which determine their potency. Research and studies show that plants contain some organic compounds that have different functions. These organic compounds are generally called secondary metabolites. These

constituents are capable of regulating biological systems because they are able to interact with various macromolecules in the body (Schmitt *et al.,* 2011). As a result, natural products have played a significant role in the development and discovery of novel drugs (Newman and Cragg, 2012). These active ingredients are alkaloids, saponins, tannins, cardiac glycosides, terpenes and terpenoids, steroids, flavonoids, and anthraquinones.

2.8.1 Alkaloids

Alkaloids are naturally occurring chemical compounds containing basic nitrogen atom. They are a large and structurally diverse group of compounds comprising about 5,500 known compounds that have served as scaffolds for important antibacterial drugs such as metronidazole and the quinolones. (Cushnie *et al*., 2014). Alkaloids constitute one of the widest class of natural products. Although, there is no satisfactory definition of the term alkaloid, but alkaloids generally include those basic substances which contain one or more nitrogen atoms, usually in combination as part of a cyclic system. Alkaloids are often toxic to man and many have dramatic physiological activities; hence they have wide application in medicine. They are usually colourless, usually optically active substances, most are crystalline while a feware liquids at room temperatures (Harbone, 1973).

Alkaloids have a lot of biological effects including antimalarial, quinine (**VIII**), anti-asthmaephedrine (**IX**), a vasodilator (vincamine), anti- hypertensive (reserpine), anti-tumour (vinblastine and vincristine) and anti-arrhythmic (quinidine) effects. Also alkaloids possess psychotropic and stimulant activities, cocaine **(X**), caffeine (**XI**) and nicotine (**XII**) and can exert an analgesic effect, such as in morphine (Ramona *et al.,* 2016).

Quinine (**VIII**)

Ephedrine(**XIV**)

Cocaine(**X**)

Caffeine(**XI**)

Nicotine(**XII**)

2.8.2 Saponins

Saponins aresecondary metabolites commonly found in different plant species. They are identified by their soap-like foaming when shaken in aqueous solutions. Saponins were classified into two groups based on the chemical structure of their aglycone skeleton (Vincken *et al.,* 2007). The first group consists of the steroidal saponinsfound mostly in monocotyledonous angiosperms and the second group consists of the triterpenoid saponins which occur mainly in the dicotyledonous angiosperms (Sparg *et al.,* 2004). Plants rich in saponins generally have anti-inflammatory properties (Yauan *et al*., 2006).A steroidal saponins, protodioscin (**XIII**) from *Tribulus terresteris* (Dinchev *et al.,* 2008) and a triterpenoid saponins (**XIV**)from *Trifolium argutum* Sol(Pérez *et al.,*2015) have been reported.

Steroidal saponin, Protodioscin(**XIII**)

Triterpenoid saponin(**XIV**)

2.8.3 Tannins

Tannins are known as tannic acids which are naturally occurring water soluble polyphenols that are present in many plants species. Tannins can be classified into two major groups based on their structural properties as hydrolysable and condensed tannins (Okwu, 2005).Hydrolysable tannins, upon hydrolysis, produce gallic acid and ellagic acid. The hydrolysable tannins are called gallotannins or ellagitannins. Tannins in some medicinal plants have been found to be responsible for the observed antiviral and antibacterial activities (Elegami *et al.,* 2002). They have been used as tanning agents and they possess astringent, anti-inflammatory, antidiarrheal, antioxidant and antimicrobial activities (Killedar and More, 2010).

Tannins are known to have anti-inflammatory and wound-healing properties (Piwowarski *et al*., 2011). Moreover, Tannins are found in many parts of plants such as bark, wood, leaves,

fruits and roots (Hoste *et al.*, 2006). Many tannin-rich plants are commonly used in traditional medicine as external anti-inflammatory, antioxidant and antimicrobial agents (Lipińska *et al*., 2014).Common example of pharmacologically active hydrolysable tannins (ellagitannin) includes Corilagin, (**XV**), a tannin isolated from *Phyllanthus niruri* (Jia *et al.*, 2013; Moreira*et al.*, 2013).Proanthocyanidin, condensed tannin (**XVI**)are complex flavonoids polymers found in a variety of plants (Santos-Buelga and Scalbert, 2000).

Corilagin (**XV**)

Proanthocyanidin gallate(**XVI**)

2.8.4 Cardiac glycosides

Cardiac glycosides are plant steroids that occur as glycosides. They possess the property of being able to stimulate heart muscles. A common therapy for heart ailments is cardiac glycosides for example, digitoxigenin (**XVII**) and have been part of folk medicines since time immemorial as heart tonics, diuretics and emetics (Kelly, 1990).

Digitoxigenin (**XVII**)

2.8.5 Steroids

A steroid is an organic compound with four rings arranged in a specific molecular configuration. The steroid core structure (**XVIII**) is composed of seventeen carbon atoms, bonded to four fused rings. Steroids possess a fully or partially reduced cyclopenta[α]phenanthrene structure i.e. ursane (**XIX**), sometimes bearing methyl groups at C-10 and C-13. However, the backbone of the side chain at C-17, its length, and the stereochemistry of some of its chiral centers lead to different steroid skeletons.

The three cyclohexane ring (designated as ring A, B, C as shown in the structure of cholestane) form the skeleton of phenanthrene; ring D has a cyclopentane structure. A number of steroids are found in plants. Steroids have been reported to have antibacterial properties (Epand *et al.*, 2007).

Steroid skeleton(**XVIII**)

Ursane(**XIX**)

2.8.6 Flavonoids

Flavonoids are a group of natural compounds with variable phenolic structures and are commonly found in plants. Chemically flavonoids are based upon a fifteen-carbon skeleton consisting of two benzene rings A and B linked via a heterocyclic pyran ring C (Vukics and Guttman, 2010). Flavonoids aresubdivided into subclasses of flavones, isoflavones, flavonols, flavanonols, flavanone, flavans, aurones, chalcones, catechins and anthocyaninsm

(Cushnie and Lamb, 2005). Apigenin (**XX**) was isolated from leaf of *N. laevis* has demonstrated antihyperglycemic and antidiabetic activity (Osigwe *et al.,* 2017). Several studies shown that quercetin (**XXI**), has a significant role against cancer(Baghel *et al.,* 2012). The catechin(**XXII**) in green tea hasrevealed a wide range of antiviral activity against a variety of viruses (Mahmood*et al.,* 2016).

Apigenin(**XX**)

Quercetin(**XXI**)

Catechin(**XXII**)

2.8.7 Anthraquinones

Anthraquinones are a class of aromatic compounds with a 9,10-dioxoanthracene core. Anthraquinonesoccur naturally and these include emodin (**XXIII**), rhein (**XXIV**), and physcion (**XXV**). The naturally occurring anthraquinones possess a broad spectrum of bioactivities, such as cathartic, anti-cancer, anti-inflammatory, antimicrobial, diuretic, vasorelaxant, and phytoestrogen activities, suggesting their possible clinical application in many diseases (Fouillaud*et al.*, 2016).

Emodin, which is commonly present in laxative herbs is therefore one of the most well-studied anthraquinones. Moreover, emodinis reported to be active against cancer, constipation, inflammation, microorganisms, and peptic ulcers (Srinivas*et al.*, 2007).

Emodin (1,3,8-trihydroxy-6-methylanthracene-9,10-dione)(**XXIII**)

Rhein (4,5-dihydroxy-9,10-dioxo-9,10-dihydroanthracene-2-carboxylic acid)(**XXIV**)

Physcion (1,8-dihydroxy-3-methoxy-6-methylanthracene-9,10-dione)(**XXV**)

2.8.8 Terpenes and Terpenoids

Terpenes are a diverse class of compounds that contain 10, 15, 20, 25, 30, or 40 carbons. They are usually found in oils extracted from aromatic plants. The oxygen containing terpenes are called Terpenoids. Terpenes and terpenoids have been used as spices, perfumes and medicines for thousands of years. They are classified according to the number of carbons atoms they contain (Bruice, 2016).

The pentacyclic lupane type of triterpenoids represents one of the important classes of natural products. The compounds of this class including betulin and betulinic acid showed significant anti-tumor activity in different types of cancers (Kommera*et al.,* 2011; Laszczyk, 2009). Several studies showed that lupeol, betulin, betulinic acid, oleanolic (**XXVI**) and ursolic acid (**XXVII**) are multi-target agents with differences in their efficacy in several assays (Mokoka *et al.,* 2013).

Oleanolicacid(**XXVI**)

Ursolic acid(**XXVII**)

2.9 Some Bacterial/Fungal Agents and their Effect on Human

The selected microorganisms in this research have been used in previous studies for the anti-microbial test of plant extracts. These pathogens cause different health problems. They include the following:*Staphylococcus aureus, Salmonella typhi, Escherichia coli, Pseudomonas aeruginosa, Candida albicans*and *Aspergillus niger.*

2.9.1 *Aspergillus niger*

Aspergillus niger is a fungus and one of the most common specie of the genus *Aspergillus.* Although there are several species in the *Aspergillus*genus, however, few species have considerable impacts on human health. Infections are typically caused by*Aspergillus flavus, Aspergillus fumigatus, Aspergillus nidulans, Aspergillus terreus,* and *Aspergillus niger* among other species (Baddley *et al*., 2001; Enoch *et al*., 2006). Infections due to *Aspergillus* species cause significant morbidity and mortality. It has been documented that

Aspergillus niger causes tracheobronchitis and invasive pulmonary aspergillosis in a lung transplant recipient (Xavier *et al*., 2008).

2.9.2 *Candida albicans*

Candida albicans is a type of yeast. It is a common member of human gut flora and does not multiply outside mammalian hosts. *Candida albicans* lives in the human body symbiotically, it usually affects the host with low immune system. *Candida albicans* is the leading cause of invasive fungal infections (Horn *et al*., 2009) and life threatening conditions including oral thrush and vaginitis (Mapfunde *et al*., 2016).It causes a serious public health challenge with increasing mortality rates and increased costs of care and duration of hospitalization (Lai *et al*., 2012).

2.9.3 *Escherichia coli*

Thebacteria *Escherichia coli* wasnamed after its discoverer Theodor Escherich, the genus Escherichia encompasses a group of Gram-negative, rod-shaped and non-spore-forming bacilli which belong to the family *Enterobacteriaceae*, order *Enterobacteriales,* class *Gammaproteobacteria*, phylum *Proteobacteria* (Lim *et al.,* 2010). *Escherichia coli* is classified into non-pathogenic and pathogenic groups. The non-pathogenic *E. coli* is a commensal present in the colon. The pathogenic *E. coli* is accountable for three main types of disease in humans; gastroenteritis, urinary tract infection, and neonatal meningitis (Clements *et al.*, 2012). Raju and Ballal (2009) reported that isolate of *E. coli* strains showed multidrug resistance to ampicillin, nalidixic acid, and trimethoprim-sulfamethoxazole on a disk diffusion-based test.

2.9.4 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa, a Gram-negative, rod-shaped bacterium has become an important cause of infection in plants and animals, including humans. *P. aeruginosa* is an important plant pathogen, usually affecting vegetables.

Pseudomonas aeruginosa is frequently present in small numbers in the normal intestinal flora and on skin of humans and it is a major pathogen of the group. *Pseudomonas aeruginosa* is a notorious opportunistic pathogen because of its innate resistance to many anti-microbial agent and disinfectants and therefore become dominant and important when more susceptible bacteria of the normal flora are suppressed (Percival*et al*., 2012).

Pseudomonas is increasingly recognized as an emerging opportunistic pathogen of clinical relevance. Infections caused by *Pseudomonas aeruginosa* include endocarditis, respiratory system infections, central nervous system infection, urinary tracts (Rossolini and Mantengoli, 2005). Pseudomonas infection can be treated with a combination of gentamycin and carbenicillin.

2.9.5*Staphylococcus aureus*

Staphylococcus species belong to the kingdom, Bacteria: Phylum, Firmicutes: Class, *Bacilli*: Order, *Bacillales*: Family, *Staphylococcaceae*: Genus, *Staphylococcus* (Todar, 2004). *S. aureus*a gram-positive bacterium, may occur as a commensal on human skin; it also occurs in the nose frequently in about a third of the population (Slayers and Whitt,2002) and less commonly in the throat. It can survive for some hours on dry environmental surfaces (Slayers and Whitt, 2002).

2.9.6 *Salmonella typhi*

The bacteria *Salmonella* are named after Dr. Daniel Salmon who discover it. *Salmonella typhi* is a Gram-negative flagellated bacterium that can cause food and water-borne gastroenteritis and typhoid fever in humans (Mathur *et al.,* 2013). In developing countries, typhoid fever is common and became difficult to treat due of the increasing resistance of *Salmonellatyphi* to antibiotics (Lanh *et al.*, 2003).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipment

The equipment used include the following:

Microwave oven (Kenwood K25MSS11)

Top loading balance (Denver Instrument, XP 600×0.02 g)

Rotary evaporator (BUCHI RE110)

UV lamp 254-326nm (Hitachi U-3200)

Oven (Gallenkamp OV-440)

Melting Point Apparatus (Stuart SMP40)

Agilent-NMR-vnmrs 400 MHz spectrometer

FTIR Agilent Technologies Cary 630 FTIR spectrometer

Astell Scientific Autoclave(ASB300BT+AVC001)

Incubator (Gallenkemp IH-150)

3.1.2 Reagents

10% Ammonium solution

10% Hydrochloric acid

20% sodium hydroxide solution

1% Ferric chloride solution

10% Sodium nitrate

10% H_2SO_4

Wagner's reagent

Dragendorff's reagent

Molisch reagent

Mayer's reagent

3.1.3 Solvents

The solvents used were: n-hexane, chloroform, ethyl acetate, and methanol. All solvents were distilled before use. Solvents used were of general-purpose grade.

3.1.4Culture media

Mueller Hinton Agar (MHA)

Mueller Hinton Broth (MHB)

Nutrient Agar (NA)

Potato Dextrose Agar (PDA)

3.1.5 Test microorganisms

The microorganisms used for the biological studies were acquired from the Department of Microbiology, Faculty of Life Sciences, Ahmadu Bello University, Zaria. The test microorganisms were;Gram-positive bacteria: *Staphylococcus aureus*, the Gram-negative

bacteria; *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi*, and the fungi *Candida albicans, Aspergillus niger*.

3.2 Methods

3.2.1 Collection of plant material

*Combretum lamprocarpum*leaf was collected fresh from Makurdi, Benue State, Nigeria. The plant was identified and authenticated by Mr. Namadi Sanusi at the Herbarium in Department of Biological Sciences, Ahmadu Bello University, Zaria and specimen voucher number 900743 was deposited for future reference. The plant material was air dried in a well-ventilated room for 3 weeks and then pulverized in a wooden mortar. The grounded plant material was put in air-tight containers until required.

3.2.2 Extraction of the plant material

The powdered plant material was extracted using Microwave-Assisted Extraction (MAE)

The pulverized plant material (1 kg) was divided into three different portions and placed in mason jars. n-hexane was added to the three portions until it just covered the top. The bottles were covered tightly and then irradiated in the microwave oven under lowest power for 3 minutes. The bottles were removed from the oven and allowed to cool and vented. The process was repeated 5 times. The samples were washed 3 times with n-hexane and filtered through muslin cloth.The procedure was repeated using chloroform, ethyl acetate, and methanol respectively. Eachof the extractswere concentrated on a rotary evaporator at 40 °C. The procedure for the MAE technique is represented in the Figure 3.1

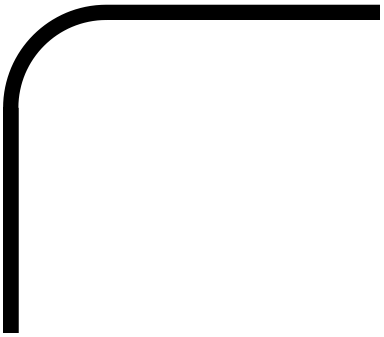


Figure 3.1: Line diagram for Microwave-Assisted extraction, adapted from Dubey and Goel (2013) with some modifications.

3.2.3 Preparation of stock solution for antimicrobial studies

Exactly 1.0 g each of the n-hexane, ethyl acetate, chloroform and methanol extract were weighed into Eppendorf tubes and dissolved in 10ml dextrose saline to produce extract solutions of 100 mg/ml stock solution. Other extract concentrations (50mg/ml, 25mg/ml, and 12.5mg/ml) were prepared from the stock extract solution by appropriate dilution with Dextrose saline. These concentrations were used for the *in vitro* studies. Extract solutions were prepared prior to use(Eze *et al.,* 2013).

3.3 Phytochemical Screening

The four extracts were analyzed for the presence of carbohydrates, alkaloids, flavonoids, saponins, tannins, anthraquinones, cardiac glycosidesand steroids/terpenes by chemical test using standard procedures as described by Edeoga *et al*(2005).

3.3.1 Test for carbohydrates

Each of the extracts (0.5g) was mixed with Molisch reagent and concentrated H_2SO_4 was added along the sides of the test tube to form layers. AReddishViolet ring was observed, the interference indicated the presence of carbohydrates (Ladan *et al.,* 2014).

3.3.2 Test for anthraquinones glycosides

a) Free anthraquinones

The extract (0.5 g) was shaken with 10 ml of benzene, the content was filtered, and 5ml of 10% ammonia solution was added to the filtrate, the mixture was shaken. Presence of a pink, red or violet colour in the ammoniacal layer (Lower phase) indicates the presence of free anthraquinone (Trease and Evans, 2002).

b) Combined anthraquinones

The extract was boiled with 10 ml of aqueous sulphuric acid and filtered hot. The filtrate was shaken with 5ml benzene, the benzene layer was separated and half its own volume, 10% NH_4OH was added. A pink, red or violet colouration in the ammonia phase (lower phase) indicates the presence of combined anthraquinone or anthraquinone derivative (Trease and Evans, 2002).

3.3.3 Test for saponins glycosides

Distilled water (5 ml) was added to each extract (0.5g) in a test tube and shaken vigorously for 30 seconds then allowed to stand for 45 minutes. A honey-comb froth that persisted for 30 minutes or more was observed indicative of the presence of saponins (Silva *et al.,* 1998)**.**

3.3.4 Test for steroids and terpenoids

The Salkowski's test for steroids: The extract (0.5g) was dissolved in chloroform and concentrated sulphuric acid (2ml) was carefully added down the side of the test tube to form a lower layer. A reddish-brown colour at the interface indicated the presence of a steroidal ring (Sofowora, 1993).

The Liebermann-Burchard's test: The extract (0.5g) was dissolved in chloroform (2ml) and filtered into a clean, dry test tube. Acetic anhydride (2ml) was added to the filtrate and shaken. Few drops of concentrated sulphuric acid were added carefully down the side of the tube to form a lower layer. A brownish-red or violet ring at the interface of the two liquids and the upper layer turning green denotes the presence of sterols and terpenes (Tajudeen*et al.*, 2015).

3.3.5 Test for tannins

The dried powdered sample (0.5g) was boiled in 20ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black colouration which shows the presence of tannins (Trease and Evans, 2009).

3.3.6 Test for cardiac glycosides(Keller-Killiani test)

Exactly 1 ml of concentrated H_2SO_4 was put in a test tube. The aqueous extract (5ml) from the plant sample was mixed with glacial acetic acid (2ml) containing 1 drop of $FeCl_3$. The abovemixture was carefully added to the 1 ml of concentrated H_2SO_4. The appearance of a brown ring indicated the presence ofcardiac glycosides (Ayoola *et al.*, 2008).

3.3.7 Test for flavonoids

Shinoda Test: A small quantity of magnesium powder and a few drop of conc. HCl was added to an alcoholic solution of each plant extract (2 ml). The appearance of an orange, or pink or red to purple colour indicated the presence of flavonoids (Sofowora, 1993).

Sodium Hydroxide Test: The extract (2 ml) was dissolved in 10 % aqueous sodium hydroxide solution and filtered to give yellow colour, a change in colour from yellow to colourless on the addition of dilute HCl indicates the presence of flavonoids (Cannell, 1998a).

3.3.8 Test for alkaloids

The extract (0.5g) was stirred with 5ml of 1% aqueous hydrochloric acid on a water bath and filtered. The filtrate (3ml) was divided into three portions. To the first portion, few drops of freshly prepared Dragendoff's reagent was added and observed for the formation of an orange to brownish precipitate. To the second portion, 1 drop of Mayer's reagent was added and observed for the formation of a white to yellowish or cream coloured precipitate. To the third portion, 1 drop of Wagner's reagent was added to give a brown or reddish or reddish-brown precipitate (Silva *et al.,* 1998).

3.4 Antimicrobial Screening

3.4.1 Cultivation and standardisation of test organisms

The McFarland's turbidity standard scale 1 was used to standardise the organisms. The scale was prepared by adding 9.9 ml of 1 % sulphuric acid to 0.1 ml of 1% barium chloride ($BaCl_2$). Suspension of the organisms were made in sterile distilled water and compared with

the McFarland's turbidity standard, until the opacity matched with the number 1, which corresponds to 1.5×10^6CFU/ml.

3.4.2 Preparation of culture media

The bacteriological and fungal culture mediaused were; Mueller Hinton agar (MHA), Mueller Hinton Broth (MHB), Nutrient Agar (NA), and Potatoes Dextrose Agar (SDA). All the media were prepared according to manufacturer's instructions. The media were dispensed into clean bottles and sterilised at 121 °C for 15 minutes in an Astell Scientific Autoclave.

3.4.3Screening for antibacterial assay using agar well diffusion method (susceptibility test)

The agar well diffusion method was used (Nostro *et al*., 2000; CLSI, 2014). The antimicrobial activities of the n-hexane, chloroform, ethyl acetate and methanol leaf extracts of the plant was determined using stock concentration of 100 mg/ml. Standardized inocula of the isolates were uniformly streaked unto freshly prepared Mueller Hinton Agar (MHA) plates with the aid of a sterile swab stick. Using a sterile cork borer (6 mm diameter) 5 appropriately labelled wells were punched into each agar plate. Thereafter, 0.2 ml of the appropriate extract concentration was placed in each well and then allowed to diffuse into the agar. An extra plate will also be streaked with the inocula and Ciprofloxacin standard (5 µg/disc) wasplaced on it. The plates were incubated at 37°C for 24 hours. The antibacterial activities were expressed as diameter zones of inhibition produced by the plant extracts and reported in millimetre (mm).

3.4.4Screening for antifungal assay using agar well diffusion method

Antifungal activity was screened by agar well diffusion method (Perez *et al*., 1990; Prince and Prabakaran, 2011). The n-hexane, chloroform, ethyl acetate and methanol extracts of the

aerial part of plant was tested against the fungi, *Candida albicans*, *Aspergillus niger*. The PDA medium was poured in to the sterile petri plates and allowed to solidify. The test fungal culture was evenly sprayed over the media by sterile cotton swabs. Then wells (6 mm) were made in the medium using sterile cork borer. 200µl of each extracts were transferred in to the separate wells. The plates were incubated at 27°C for 48-72 hours. After the incubation the plates was observed for formation of clear incubation zone around the well indicating the presence of antifungal activity. The zone of inhibition will then be calculated.

3.4.5 Determination of minimum inhibitory concentration (MIC**)**

The minimum inhibitory concentration (MIC) of the extracts was determined using the tube dilution method as outlined by Eloff (2004); CLSI (2014). Dilution of concentration of extract that exhibited sensitivity against the test organisms was prepared in the tube containing the Mueller Hinton Broth (MHB). The organisms were inoculated into each of the tubes containing the dilution extracts. The tubes were inoculated at 37 °C for 24 hours for bacteria. The lowest concentration in the series showing no visible growth of the test organism was considered the MIC.

3.4.6 Determination of minimum bactericidal/fungicidal concentration (MBC/MFC)

The minimum bactericidal concentration of the extract was determined as outlined by Eloff (2004); CLSI (2014).The MBC was determined by assaying the test tube content of the MIC determinations. A loop of the content of each tube was inoculated by streaking on a solidified nutrient agar plate and then inoculated at 37 °C for 24 hours and 25°C for 48 hours, for bacteria and fungi respectivelyafter which it was observed for microbial growth. The lowest concentration of the subculture with no growth was considered as the MBC.

3.5 Chromatographic Separation of Extracts

3.5.1 Thin layer chromatography (TLC)

Thin layer chromatography was carried out on all the crude extracts, using silica gel $60F_{254}$ pre-coated TLC plates of 0.2mm thickness.

Technique used:one-way ascending.

Spotting and development: capillary tubes were used to spot the TLC plates and they were developed at room temperature using a Shandon chromato-tank and hot air blower was used to dry the TLC plate.

Detection of Spots:Spots on TLC plates were visualised under UV light (254 and 366 nm) and spraying with 10% H_2SO_4 acid, followed by heating at 110 °C for 2-5 min

3.5.2 Column chromatography (CC)

The following column settings were employed in running the column chromatography.

(i) Technique - Gradient elution.

(ii) Column - A glass column of dimensions 75 by 3.5cm was used.

(iii) Stationary phase - Silica gel, 60 – 120 mesh size.

(iv) Column packing - Wet slurry method.

(v) Sample loading - the sample was dissolved in minimum amount of suitable organic solvent, mixed with a small quantity of silica gel, dried, triturated and then loaded on top of the previously packed column (Cannell, 1998b).

3.5.3 Solvent system

The solvent system hexane:ethyl acetate mixtures were used in eluting the column by gradient elution.

3.5.4 Analytical thin layer chromatography of the ethyl acetate extract

The ethyl acetate extract of *Combretum lamprocarpum* was subjected to thin layer chromatography using pre-coated aluminium TLC plate and Hexane:Ethyl acetate (9:1) as the solvent system was used to determine the profile of this extract. Two prominent spots were observed under ultraviolet (UV) light.

3.5.5 Column chromatography of ethyl acetate extract

The Ethyl acetate extract (10g) was chromatographed over silica gel-packed column of dimension 75 by 3.5cm (Appendix I). The column was eluted continuously using neat n-Hexane, followed by n-Hexane:Ethyl acetate mixtures as solvent systems (8:2, 7:3). Seventy-four (74) fractions, of 100ml each were collected. These fractions were merged based on their retardation factor (R_f) profile. Fivefractions (I_1, I_2, I_3, I_4, and I_5) were merged to give fraction I (Plate: III). Finally, the column was washed with methanol. Fraction I, showed one prominent spot and some minor impurities. It was purified further by preparative TLC. A pure white crystalline compound which showed a single spot on TLC, was isolated and coded as K1. The isolated compound was subjected to spectroscopic analysis.

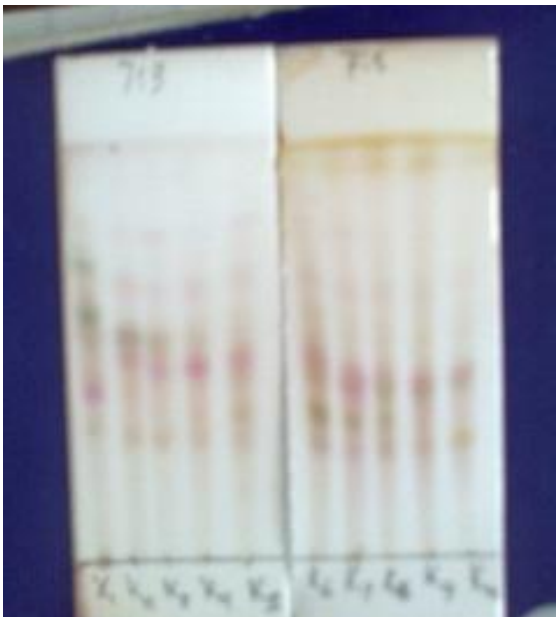


Plate III: Thin Layer Chromatography (TLC) profile of ethyl acetate collections

3.6 Melting Point Determination

The melting point of the isolated compound was determined on digital melting point apparatus (Stuart SMP40).

CHAPTER FOUR

4.0 RESULTS

4.1 Extraction of plant material

The powdered leaf (1kg) of *Combretum lamprocarpum*was extracted with n-Hexane, chloroform, ethyl acetate, and methanol. The percentage recovery of the extractswas calculated. The methanol extract had the highest recovery followed by the chloroform extract, hexane extract, and lastly the ethyl acetate extract (Table 4.1).

Table 4.1: Extracts obtained and their percentage recovery

Extract	**Mass of Extracts (g/kg)**	**Percentage recovery (%)**
Hexane	22.30	10.58
Chloroform	38.94	18.49
Ethyl acetate	17.33	8.23
Methanol	132.06	62.70

4.2 Result of preliminary phytochemical analysis

The preliminary phytochemical investigation of the extracts revealed the presence of alkaloids, carbohydrates, cardiac glycosides, saponins, steroids, tannins, terpenes and flavonoids. However, anthraquinones was found to be absent (Table 4.2).

Table 4.2: Phytochemical analysis of the crude extracts

Phytochemical	H.E	C.E	E.E	M.E
Alkaloids	-	+	+	+
Anthraquinones	-	-	-	-
Carbohydrates	-	+	+	+
Cardiac glycosides	-	+	+	+
Saponins	-	-	-	+
Steroids	+	+	+	+
Tannins	-	-	+	+
Terpenes	+	+	+	+
Flavonoids	-	+	+	+

Key: H.E= Hexane Extract, E.E= Ethyl acetate Extract, C.E= Chloroform Extract, M.E= Methanol Extract,
+ = Present, - = Absent

4.3 Result of Zone of Inhibition studies

The antimicrobial test results (Table 4.3**)**, shows the zones of inhibition (mm) of the crude extracts of the leave of *Combretum lamprocarpum*. Three of the extracts had significant activity against the test microorganisms.

Table 4.3: Determination of inhibitory activity (Sensitivity Test) of Chloroform, Ethyl acetate, and Methanol, extracts of *Combretum lamprocarpum*.

	Chloroform (mg/ml)				**Ethyl acetate (mg/ml)**				**Methanol (mg/ml)**				**Ciprofloxacin/Fluconazole 10 µg/disc**
Test organisms	**100**	**50**	**25**	**12.5**	**100**	**50**	**25**	**12.5**	**100**	**50**	**25**	**12.5**	
A. niger	-	-	-	-	-	-	-	-	-	-	-	-	35
C. albicans	-	-	-	-	-	-	-	-	-	-	-	-	35
E. coli	14	-	-	-	14	12	-	-	16	14	12	-	37
P. aeruginosa	18	14	-	-	17	14	-	-	29	26	14	20	36
S. aureus	17	15	13	-	21	18	15	13	26	23	20	18	35
S. typhi	16	12	-	-	14	13	-	-	19	17	14	12	38

Key: - = No inhibition zone

4.4 Result of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of the ethyl acetate, methanol, and chloroformextracts (Table 4.4) showed that the bacterial agents tested were responsive to the extracts at low concentration.

Table 4.4: Minimum inhibitory concentration of the crude extracts

Test organisms	**C.E (mg/ml)**	**E.E (mg/ml)**	**M.E (mg/ml)**
A. niger	ND	ND	ND
C. albicans	ND	ND	ND
E. coli	50.00	50.00	12.50
P. aeruginosa	25.00	25.00	3.13
S. aureus	25.00	12.50	3.13
S. typhi	25.00	25.00	6.25

Key: ND= Not determined, E.E= Ethyl acetate extract, M.E= Methanol extract, C.E= Chloroform extract, H.E= Hexane extract.

4.5 Result of minimum bactericidal/fungicidal concentration(MBC/MFC)

The minimum bactericidal/fungicidal concentration (MBC/FBC) of the extracts showed that *S. aureus, S. typhi, P. aeruginosa*followed by *E. coli* were reactive to the extracts at low concentrations.

Table 4.5: Determination of the Minimum Bactericidal/ Minimum Fungicidal Concentration of the extracts against the microbes.

Test organisms	**C.E (mg/ml)**	**E.E (mg/ml)**	**M.E (mg/ml)**
A. niger	ND	ND	ND
C. albicans	ND	ND	ND
E. coli	ND	ND	25.00
P. aeruginosa	50.00	50.00	6.25
S. aureus	50.00	25.00	6.25
S. typhi	50.00	50.00	12.50

Key: ND= Not determined, E.E= Ethyl acetate extract, M.E= Methanol extract, C.E= Chloroform extract, H.E= Hexane extract.

4.6Result of FT-IR spectrum of Compound K1

The IR spectrum of K1 (fig. 4.1) showed bands at 3403 cm^{-1}, 2926 cm^{-1}, 2855cm^{-1} and other bands in the finger print region.

Sample ID:k1
Sample Scans:16
Background Scans:16
Resolution:8
System Status:Good
Method Name:Transmittance Method
User:Admin
Date/Time:2017-03-16T13:47:44.891+01:00
Range:4000 - 650
Apodization:Happ-Genzel
File Location:C:\Program Files\Agilent\MicroLab PC\Results\k1_2017-03-16T13-47-44.a2r

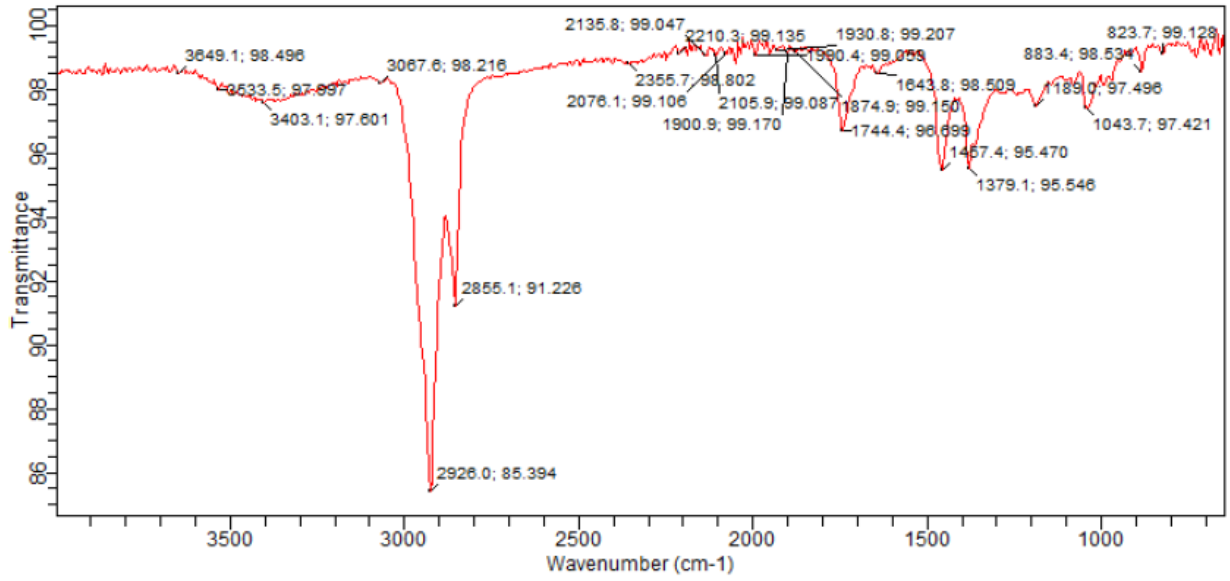


Figure 4.1: FTIR spectrum of K1

4.7 Result of 1D NMR Analysis of Compound K1

The proton NMR spectrum of compound K1(fig. 4.2) has revealed the presence of seven methyl at δ 0.77 (3H, d), 0.84(3H, s), 0.96(3H, d), 1.047(3H, s), 1.28 (3H, s), 1.40 (3H, d) and 1.66(3H, s) all in (ppm) and a pair of singlets at δ4.54 and 4.67 (1H each). The presence of secondary hydroxyl group at δ 3.18 (dd, J=11.2, 4.8 Hz).

4.7.1 ^{1}H-NMR spectrum of compound K1 (δ ppm, 400 MHz, $CDCl_3$)

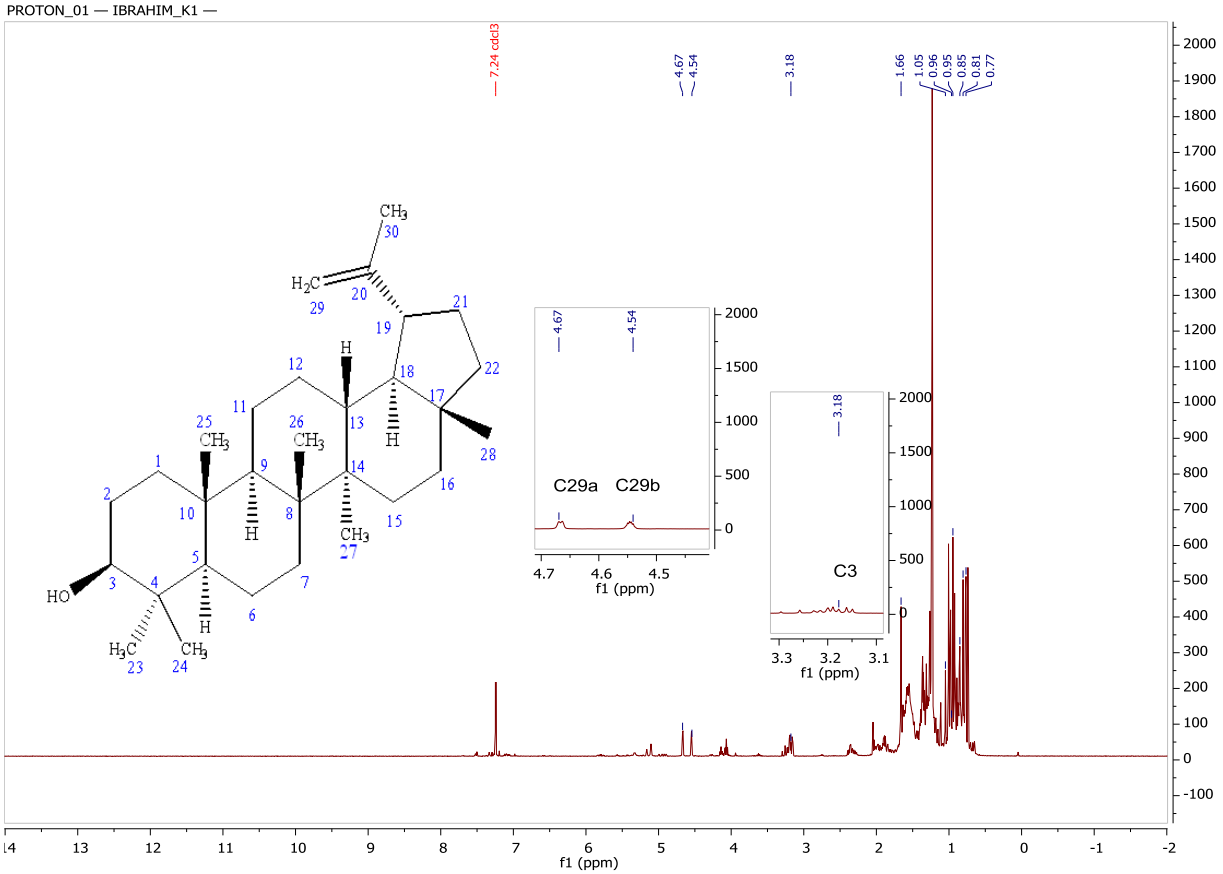


Figure 4.2: ^{1}H-NMR spectrum of K1

4.7.2 ^{13}C NMR spectrum of Compound K1

The ^{13}C NMR spectrum of the compound K1 showed 30 observed signals were; seven methyl groups [δ 28.07 (C-23), 17.98 (C-28), 16.11 (C- 25), 16.93 (C-26), 15.36 (C-24), 14.52 (C-27) 19.28 (C-30)]. Also, ten methylenes, five methines and five quaternary carbons. Also signals at δ150.98 and 109.32 ppm (C-20 and C-29) respectively and the presence of secondary hydroxyl group at 78.99 ppm (C-3).

The ^{13}C NMR spectrum of compound 1 revealed a total of 30 carbon signals.

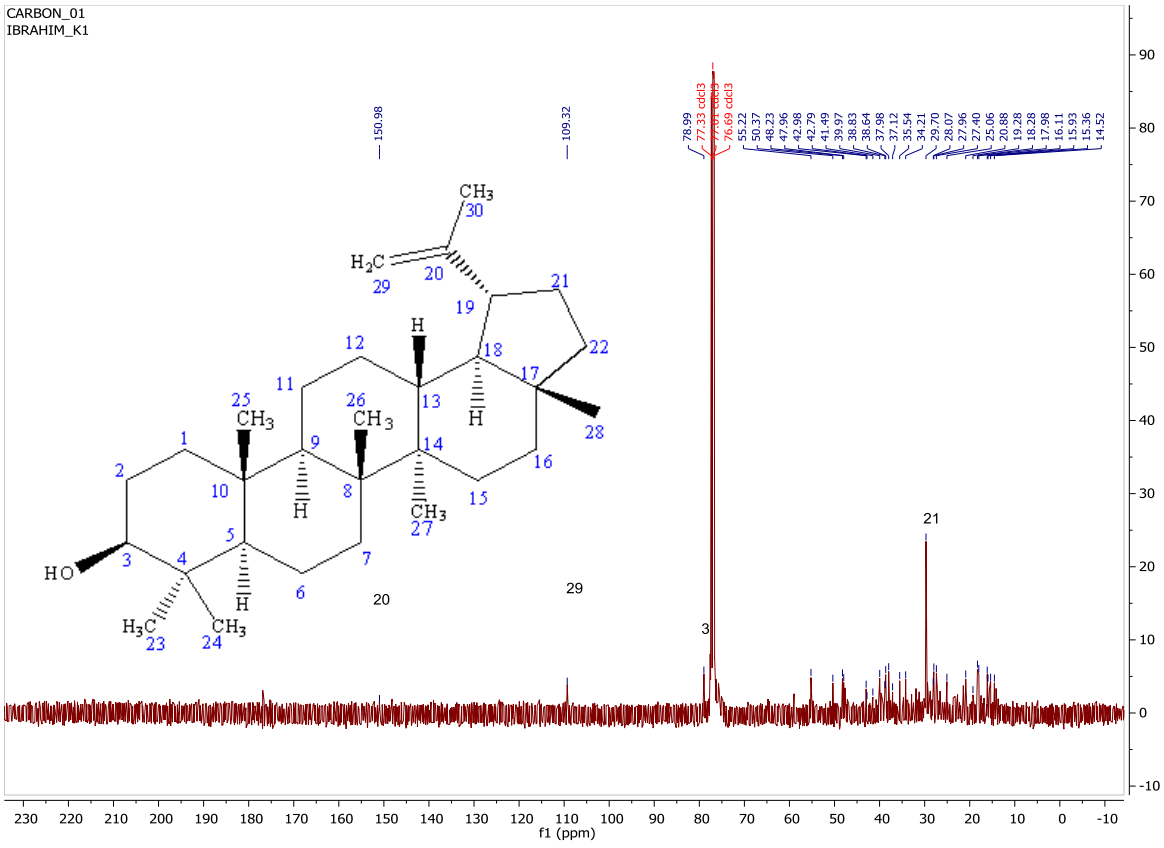


Figure 4.3: ^{13}C-NMR spectrum of K1

Table 4.6: Comparison of $^{13}C(CDCl_3)$ NMR data of K1 with literature data

Carbon Position	**^{13}C δ(ppm) (Isolated K1)**	**^{13}C δ(ppm) (Sánchez-burgos *et al.*, 2015).**	**^{13}C δ(ppm) (Roy *et al.*, 2016)**	**Carbon type**
C-1	38.83	38.76	38.2	CH_2
C-2	27.96	27.42	25.3	CH_2
C-3	78.89	78.98	79.2	CH
C-4	38.64	38.84	38.9	C
C-5	55.22	55.28	55.5	CH
C-6	18.28	18.30	18.5	CH_2
C-7	34.21	34.23	34.5	CH_2
C-8	41.49	40.80	41.0	C
C-9	50.37	50.40	50.6	CH
C-10	37.12	37.14	37.3	C
C-11	20.88	20.90	21.1	CH_2
C-12	25.06	25.10	27.5	CH_2
C-13	37.98	38.02	39.0	CH
C-14	42.98	42.80	43.0	C
C-15	27.40	27.39	27.6	CH_2
C-16	35.54	35.56	35.8	CH_2
C-17	42.79	42.98	43.2	C
C-18	48.23	48.27	48.5	CH
C-19	47.96	47.96	48.1	CH
C-20	150.98	150.96	151.1	C
C-21	29.70	29.82	30.0	CH_2
C-22	39.97	39.98	40.2	CH_2
C-23	28.07	28.07	28.2	CH_3
C-24	15.36	15.36	15.6	CH_3
C-25	16.11	16.10	16.3	CH_3
C-26	16.93	15.95	16.2	CH_3
C-27	14.52	14.53	14.7	CH_3
C-28	17.98	17.98	18.2	CH_3
C-29	109.32	109.32	109.5	CH_2
C-30	19.28	19.28	19.5	CH_3

The proposed structure of K1 (**XXVIII**)

Compound K1 ($C_{30}H_{50}O$)(**XXVIII**)

4.8 Result of Zone of Inhibition of the Isolated Compound K1

The antimicrobial test results (Table 4.7**)**, shows the zones of inhibition (mm) of the pure isolate from ethyl acetate. The pure isolate had significant activity against the test microorganisms except the fungal agents.

Table 4.7: Antimicrobial screening of K1 showing Zone of Inhibition (ZI)

Test organisms	**K1(mm)**	**Ciprofloxacin/fluconazole**
A. niger	0	30
C. albicans	0	30
E. coli	14	32
P. aeruginosa	18	25
S. aureus	23	32
S. typhi	18	25

4.9 Result of Minimum Inhibitory Concentration of the Isolated Compound K1

The minimum inhibitory concentration (MIC) of the pure isolate shows that the organisms tested were responsive to the extracts at low concentration.

Table 4.8: Result of minimum inhibitory concentration (MIC) of the compound K1

Test microorganism	**K1 (µg/ml)**
A. niger	0
C. albicans	0
E. coli	6.5
P. aeruginosa	12.5
S. aureus	12.5
S. typhi	6.50

4.10 Result of MBC/MFC of the Isolated Compound K1

The minimum bactericidal/fungicidal concentration (MBC/MFC) of the pure isolate showed that the organisms tested were responsive to the extracts at low concentration (Table 4.9).

Table 4.9: Result of minimum bactericidal concentration/minimum fungicidal concentration (MBC/MFC) of the compound K1

Test organism	**K1 (10 μg/disc)**
A. niger	-
C. albicans	-
E. coli	6.25
P. aeruginosa	12.5
S. aureus	25.0
S. typhi	12.5

4.11 Result of Thin Layer Chromatography and Melting Point of Compound K1

The solvent system ethyl acetate:hexane (8:2) was used to develop the chromatogram of compound K1 and it showed a single homogeneous spot. After spraying with 10% H_2SO_4 in H_2O, a brown coloured spot was observed. The melting point was found to be 213-215 ºC.



Plate IV: TLC Profile of compound K1 using Hexane:Ethyl acetate (8:2)

CHAPTER FIVE

5.0 DISCUSSION

5.1 Extraction

The result of the percentage recovery of the extracts reported in (Table 4.1) showed that the methanol extract had the highest recovery followed by the chloroform extract, hexane extract, and ethyl acetate extract.

5.2 Phytochemical Screening

The result of the preliminary phytochemical screening showed that the crude extracts contained secondary metabolites which are known to possess biological activity (Fabricant and Farnsworth, 2001). Steroids and terpenes were observed in all the four extracts. The presence of alkaloid, carbohydrates, cardiac glycosides, and flavonoids was observed in ethyl acetate, chloroform and methanol extracts. Anthraquinones were absent in all the extracts. Saponins were present in methanol extract only. Tannins were present in ethyl acetate and methanol extracts (Table 4.2). Similar phytochemical investigation on *Combretum racemosum*revealed the presence of alkaloids, anthraquinones, tannin, steroids, cardiac glycosides, saponin, reducing sugars, flavonoids, terpenoids (Oghenejobo, 2014).

5.3 Antimicrobial Screening

The zones of inhibition (Table 4.3), the minimum inhibitory concentration (mic) (table 4.4) and minimum bactericidal/fungicidal concentration (MBC/MFC) (Table 4.5) of chloroform,ethyl acetate and methanol extracts of the leaf of *Combretum lamprocarpum*showed significant activities against four out of the six microorganisms tested. All the extracts had significant activity against the test microorganisms except on *Candida*

albicans and *Aspergillus niger*.The anti-bacterial and anti-fungal activities of the leaf extracts also reveal that Combretum racemosum has inhibitory effect on *Staphylococcusaureus, Pseudomonasaeruginosa,Escherichiacoli* and *Salmonellatyphi*and fungal activities was shown in *Aspergillusniger* (Oghenejobo, 2014).

5.4 Isolation and Characterisation of Compound K1

The compound was obtained as a white powder (1.38mg) with a melting point of 213-215 ºC. The IR spectrum of compound K1 showed absorption bands characteristic of the hydroxyl group at (3403cm^{-1}). The presence of terminal double bond (representing the exocyclic C=C double bond) was confirmed by bands at 1643 cm^{-1}. The stretching and bending vibrations of methyl part were noticed by the intense band 2926 cm^{-1}. The vibration of the methylene part was shown by the band at 2855 cm^{-1}. The moderate intense band at 823 cm^{-1} was attributed to the rocking movement of methylene part. The corresponding C-C vibration was shown as weak intense band at 1043 cm^{-1}.

The ^{1}H-NMR spectrum of compound K1 showed the presence of seven methyl at δ 0.77 (3H, d), 0.84(3H, s), 0.96(3H, d), 1.047(3H, s), 1.28 (3H, s), 1.40 (3H, d) and 1.66(3H, s) all in (ppm). Also, a pair of singlets at δ4.54 and 4.67 (1H each) was due to the vinyl protons at carbon 29. The presence of a doublet of a doublet with one proton intensity at δ 3.18 (dd, J=11.2, 4.8 Hz) was seen which was due to the proton attached to the carbon bearing the hydroxyl group at C-3. These characteristics indicated that compound K1 belongs to the lupane class of triterpenoids (Abdullahi *et al.*,2013).

The ^{13}C NMR of the compound K1 showed 30 signals. The observed signals were; seven methyl groups [δ 28.07 (C-23), 17.98 (C-28), 16.11 (C- 25), 16.93 (C-26), 15.36 (C-24),

14.52 (C-27) 19.28 (C-30)]. Also, ten methylenes, five methines and five quaternary carbons. The characteristic sp^2 carbon signals of lupeol are observed at δ 150.98 and 109.32 ppm (C-20 and C-29) respectively and a hydroxyl group bonded to (C-3) at 78.99 ppm. All together accounts for the 30 carbon signals. These spectroscopic data are in agreement with the literature values (Sánchez-Burgos *et al*., 2015).

5.5 Biological Activity of Compound K1

The effect of the isolated compound on *E. coli, P. aeruginosa, S. aureus S. typhi*showed significant activity as compared to that of Ciprofloxacin (10 μg/disc) drug used as the positive control. This shows that the compound may be used as drug lead. The isolated compound will have a remarkable effect in the treatment of diseases caused by *E. coli, P. aeruginosa, S. aureus S. typhi*. Lupeol, a pentacyclic lupane-type triterpenoid, isolated from the plant source is well-known for its beneficial activity against inflammation, and cancer (Fernández *et al.*, 2001).

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

The preliminary phytochemical investigation of the n-hexane, chloroform, ethyl acetate and methanol crude extracts of*Combretum lamprocarpum*, revealed the presence of steroids/terpenes, alkaloids, carbohydrates, saponins, tannins, cardiac glycosides, and flavonoids.Column chromatography of the ethyl acetate extract followed by thin layer chromatography led to the isolation of K1 and characterised by spectroscopic technique as lupeol.The antimicrobial screening of the crude extracts showed that the plant had activities against the test microorganisms.The isolated compound also exhibited antimicrobial activity at low concentrations.

6.2 Conclusion

The isolated compound, lupeol($C_{30}H_{50}O$) has been isolated from different plant species and has shown a variety of bioactivities such asanti-inflammatory, anti-tumour and antibacterial activity. Based on literature search and to the best of our knowledge, this is the first time lupeol was isolatedfrom the leaf of *Combretum lamprocarpum*. The result of antimicrobial activities of the isolated compound has justified the use of the plant in the treatment of various ailments as claimed by the traditional healers.

6.3 Recommendations

This research work was carried out on the leaf of *Combretum lamprocarpum*with a view to justify its ethno-medicinal uses for the treatment of ailments such diarrhoea, stomach-ache, healing of wounds. Thus, the following recommendations are made:

i. The need to exploit the whole plant, since only the leaf was used.

ii. Only the ethyl acetate extract was used, other extracts; methanol and chloroform shows activity and should be further studied.

iii. Structural modification of the isolated compound to improve its activity can be carried out as well.

iv. Acute toxicity test of the extracts should be studied.

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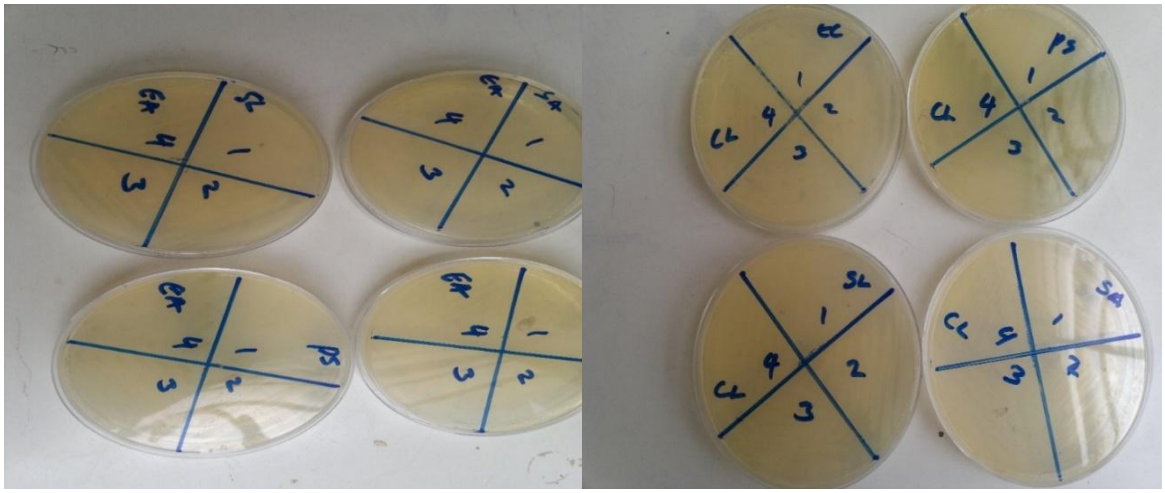
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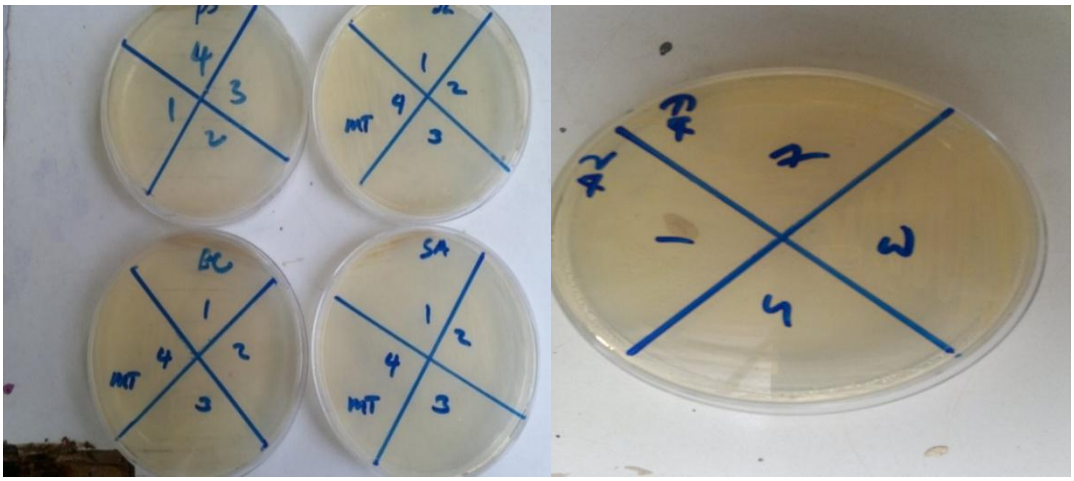
APPENDIX



Appendix I: Column chromatography of ethyl acetate extract



Appendix II: Petri dish for the antimicrobial studies on ethyl acetate and chloroform extracts



Appendix III: Petri dish for the antimicrobial studies on methanol extract