ISOLATION AND CHARACTERISATION OF ANTIBACTERIAL SECONDARY METABOLITES FROM *ODONTONEMA STRICTUM* (ACANTHACEAE)

By

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DECLARATION

This dissertation represents my own work, and it has not previously been submitted for a degree, diploma or other qualification at this or any other university.

Four articles have been published from this present work:

-Luhata, L.P. and N.M. Munkombwe, 2015. Isolation and Characterisation of Stigmasterol and *β*-Sitosterol from *Odontonema strictum (Acanthaceae).* Journal of Innovations in Pharmaceutical and Biological Sciences, 2, 88-96.

-Luhata, L.P., N.M. Munkombwe, P.M. Cheuka, and H. Sikanyika, 2015. Phytochemical screening and *in vitro* antibacterial activity of *Odontonema strictum (Acanthaceae)* against selected bacteria. International Journal of Development Research, 5, 4655-4659.

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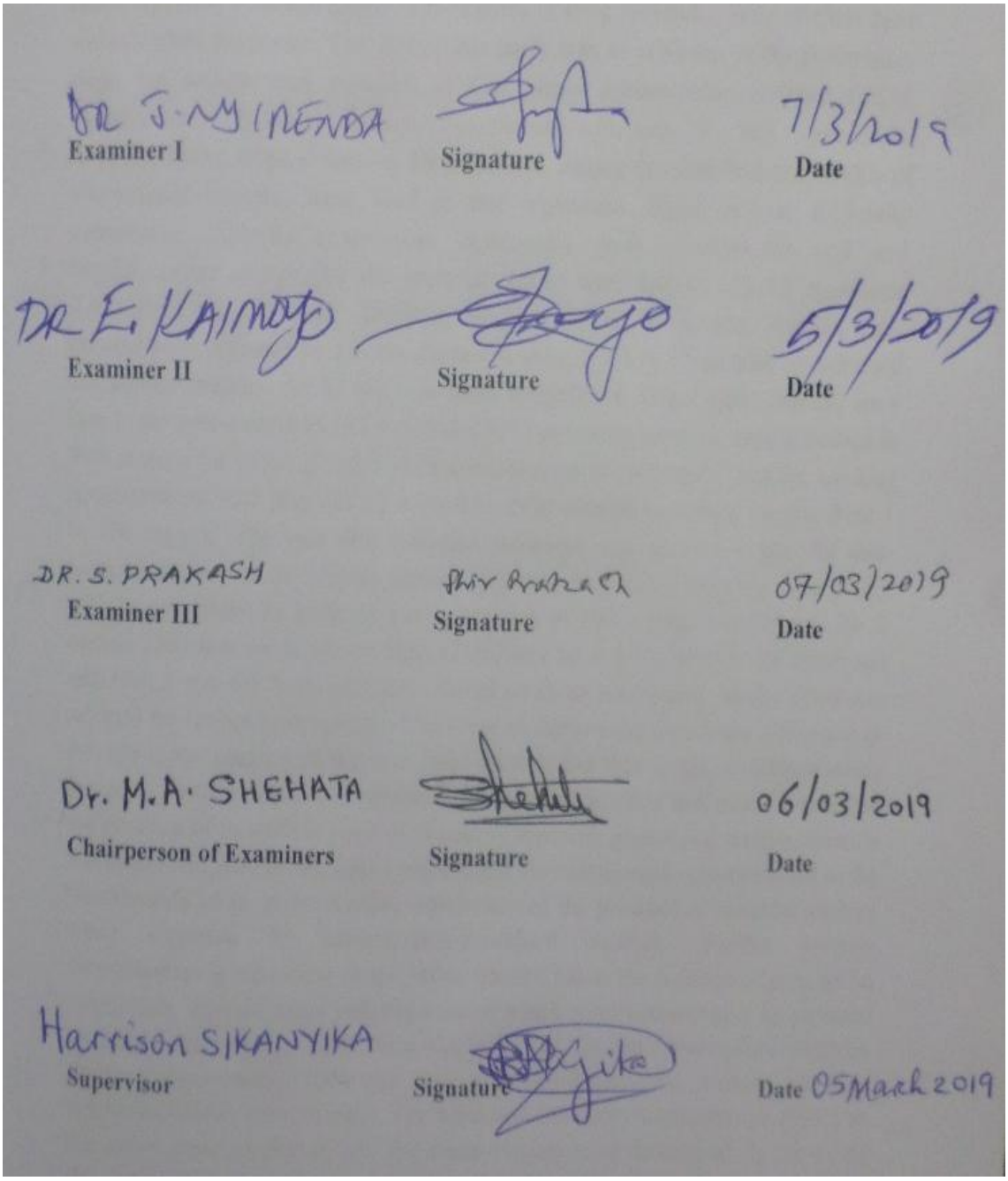
APPROVAL

This dissertation of **LUHATA LOKADI PIERRE** is approved as partial fulfilment of the requirements for the award of the degree of Master of Science in Chemistry by the University of Zambia.



ABSTRACT

It has been noted that an increasing number of bacteria are now developing resistance to commercial antibiotics. This problem necessitates the discovery of new classes of anti-microbial drug leads. Plants have always played an important role in the discovery of new medicines. Secondary metabolites which are present in almost all plants represent a special group of molecules in drug research. Some of them have anti-microbial properties. The aim of this study was to evaluate, in the preliminary stage, the anti-bacterial potential of four plants *(Odontonema strictum* (*OSM*) (Acanthaceae), *Solanum torvum*, *Symphytum officinale L,* and *Aphelandra squarrosa*) from tropical regions. Five bacteria, among the nine bacterial strains of international concern, were used as test organisms. These include *Klebsiella pneumoniae, Shigella dysenteriae, Salmonella typhi, Escherichia coli* and *Staphylococcus aureus*. All the bacterial strains were locally isolated organisms (LIO) from the University Teaching Hospital (UTH) in Lusaka, Zambia. Leaf extracts were prepared by solvent extraction using a 1:1 (v/v) mixture of methanol and dichloromethane for 24 hours at room temperature. Dried crude extracts were kept in air tight containers and stored at 4 °C. For testing, extracts were dissolved in 80% acetone (in water) giving a stock concentration of 100 mg/mL and the working concentrations were prepared by a ten-fold serial dilution technique ranging from 1 to 100 mg/mL. The agar disk diffusion technique was used to screen for anti-bacterial activity of the solvent extracts. Extracts from *OSM* were the most potent with largest inhibition zones at a concentration of 100 mg/mL (37±1.5 mm for *S. aureus*, 25±1 mm for *K. pneumoniae*, 12±0.7 mm for *S. typhi*, 20±0.9 mm for *E. coli* and 18±1.3 mm for *S. dysenteriae*). Based on these preliminary results, *OSM* was selected for further investigation. There was no statistically significant difference ($P > 0.05$) in the potency of the four plant extracts and that of the antibiotics which served as positive controls. Phytochemical screening of *OSM* leaf extracts indicated the presence of flavonoids (type of flavones), saponins, glycosides, tannins, steroids and terpenoids. Bio-guided fractionation using chromatographic separation led to the identification of an active fraction which showed the presence of steroidal nucleus when subjected to Liebermannn-Bourchard reaction. Further column chromatographic separation on the active fraction led to the isolation of pure active compounds, *beta*-sitosterol and stigmasterol which were characterized by physical (melting point and thin layer chromatography (TLC)) and spectroscopic methods (infrared spectroscopy (IR) and one- and two-dimensional nuclear magnetic resonance (NMR) spectroscopy). The Minimum Inhibitory Concentration (MIC) of the active pure compounds and the crude extracts were determined by the broth dilution method using *S. aureus* as a test organism. The active phytosterols (*beta*-sitosterol and stigmasterol) were found to have MIC values in the range 1.84 to 3.68 mg/mL. Compared to the total activity (TA) (MIC = 3.83 to 7.66 mg/mL) of the crude extract, these MIC values mean that 1 g of the plant material can be diluted 10.52 to 21.4 times and it will still inhibit the growth of *S. aureus*, implying that they are not the only active compounds or possibly there is a synergistic effect.

DEDICATION

To my beloved late dad, LUHATA DIOWO ESONGHO Jean-Denis, you have always been an inspiration and my strength; your memories are still fresh in my mind. May this thesis be an accomplishment of your will.

To mum M'BI ONAMEMBA Louise, your love to me is forever. Receive this thesis as a gift from the Lord our God who we worship every day.

To Doctor Namboole Moses MUNKOMBWE, you are gone before enjoying the fruits of your hard work. May you rest in peace and I will make sure that your work continues.

To my brothers and sisters and their children.
To all the youth of the Democratic Republic of Congo.

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ABBREVIATIONS

COSY:	Correlation Spectroscopy
HSQC:	Heteronuclear Single Quantum Coherence or Heteronuclear Single Quantum Correlation
AcO:	Acetone
ADCs:	Antibody Drug Conjugates
AIDS:	Acquired Immune Deficiency Syndrome
AI:	Activity index
AMR:	Antimicrobial Resistance
AMU:	Atomic Mass Unity
BPLC:	Balloon Pressure Liquid Chromatography
CFU:	Colony Forming Unit
CNS:	Central Nervous System
DCM:	Dichloromethane
ESBLs:	Extended Spectrum *beta*-lactamases
EtOAc:	Ethyl Acetate
***E. coli*:**	*Escherichia coli*
IR:	Infrared
***K. pneumoniae*:**	*Klebsiella pneumoniae*
Fr:	Fraction
H3D:	Drug Discovery and Development Centre
Hex:	Hexane
HIV:	Human Immunodeficiency Virus
HPLC:	High Performance Liquid Chromatography
MS:	Mass Spectrometry
MeOH:	Methanol
MRSA:	Methicillin Resistant Staphylococcus aureus
MIC:	Minimal Inhibitory Concentration
MLC:	Minimum Lethal Concentration
MDR:	Multidrug Resistant
M.P.:	Melting Point
N.B:	Nota Bene

NMR:	Nuclear Magnetic Resonance
NP:	Natural Products
OD:	Optical Density
OSM:	*Odontonema strictum*
ppm:	Parts per million
Rf:	Retention Factor
SAR:	Structure-Activity Relationship
***S. aureus*:**	*Staphylococcus aureus*
***S. dysenteriae* :**	*Shigella dysenteriae*
***S. enterica*:**	*Salmonella enterica*
TA:	Total Activity
TLC:	Thin Layer Chromatography
UV:	Ultra Violet
VLC:	Vacuum Liquid Chromatography
VRSA:	Vancomycin Resistant Staphylococcus aureus
WHO:	World Health Organization
ZI:	Zone of Inhibition

LIST OF SYMBOLS

Kgm^{-2}	Kilogram-force per square meter
°C	Degrees Celsius
MHz	Mega Hertz
δ	Chemical shift
°F	Degrees Fahrenheit
cm^{-1}	Inverse centimeter
nm	Nanometer
µL	Microliter
mL	Milliliter
g	gram
%	Percentage

APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Background

1.1.1 The Utility of Natural Products in Pharmaceutical Care

For thousands of years, folklore medicines and natural poisons have been used for human health. Clinical, pharmacological, and chemical studies of these traditional plant-based medicines have led to the discovery of conventional medicines. These include aspirin (used to treat pain, fever, and inflammation); digoxin, (used to treat various heart conditions); morphine (an analgesic for severe pain); quinine (an alkaloid used for the treatment of malaria and babesiosis); taxol otherwise known as paclitaxel (for cancer), vincristine and vinblastine (two Vinca alkaloids isolated from *Catharanthus roseus*, used in chemotherapy to treat different types of cancers) (Butler et al., 2014; Colegate and Molyneux, 2008; Beutler, 2009; Butler, 2004).

These compounds are called secondary metabolites and belong to many classes: aspirin (benzoic acids), morphine, quinine, vincristine (alkaloids), digoxin (steroids) and taxol (terpenoids). Others include artemisinin, limonene, β-carotene (terpenoids), prostaglandin (fatty acids), etc. Secondary metabolites are of great importance due to their preventive and curative properties in health care (Akinpelu et al., 2009). They are used either as pure compounds (like quinine) or as herbal products such as teas (e.g., the leaves of *Psidium guajava*). Secondary metabolism differs from primary metabolism in that the later involves the biosynthesis of carbohydrates, proteins, fats, and nucleic acids. Despite extremely varied characteristics, living organisms need carbohydrates, proteins, fats, and nucleic acids for their growth and reproduction. These compounds are found to be essentially the same in all organisms, apart from

minor variations (Cseke et al., 2006). In most cases, the term natural products refers to secondary metabolites, which are small molecules (molecular weight < 2000 amu) produced by an organism that are not strictly necessary for the survival of the organism. Natural products can be from any terrestrial or marine source: plants (e.g., paclitaxel [Taxol] from *Taxus brevifolia*), animals (e.g., vitamins A and D from cod liver oil), or microorganisms (e.g., doxorubicin from *Streptomyces peucetius*) (Sarker, 2006).

Secondary metabolism has evolved in nature in response to needs and challenges of the natural environment (Zhan et al., 2005). Over 40% of the medicinal chemicals used throughout the developed world today were originally isolated from natural sources. These sources include flowering plants, fungi, bacteria, and to a lesser extent, animals especially of marine origin (Rabia and Bano, 2012).

Plants use chemical building blocks, enzymes and energy to synthesize compounds. The metabolic sequences leading to various classes of secondary metabolites mentioned above are sub-divided into the acetate, shikimate, mevalonate, deoxyxylulose phosphate and alkaloidic pathways (Dewick, 2002).

1.2. Economic Impact of Natural Products and Herbal Remedies

Developing countries still depend, mainly, on medicinal herbs due to cheaper cost and effectiveness in the treatment of certain infectious diseases as well as lesser side effects (Shivani et al., 2012). Although the use of herbal products in the United States of America (USA) has never been as common as it was in continental Europe,

in recent years there has been an unprecedented increase in herbal sales, reaching a peak in 1998 and approaching $700 million (Trease and Evans, 2002). In 2014, sales of herbal dietary supplements in the United States increased by 6.8% reaching an estimated total of more than $6.4 billion. These statistics and other herbal sales figures are included in a new market report published in the current issue of Herbal Gram (2015), the peer-reviewed quarterly journal of the nonprofit American Botanical Council (ABC). The report indicates that the herbal/traditional products category in the USA continues to be dominated by dietary supplements, which accounted for 68% share of total sales in 2013. The cough, cold and allergic remedies represented 17% of herbal and traditional medicine product sales while digestive remedies accounted for 5% of sales.

The Natural products enterprise is expected to grow by 14% to reach $5.3 billion by 2018. Some consumers, afraid of complex chemical and drug interactions, will continue to look for more natural remedies and products and, therefore, the herbal product enterprise is expected to maintain a modest momentum with a Compound Annual Growth Rate (CAGR) of 3% in value sales at constant 2013 prices over the forecast period (http://www.euromonitor.com/herbal-traditional-products-in-the-us/report, accessed on 1st July 2014).

Many of today's small molecule therapeutics trace their origins to natural products, estimated variably as providing or inspiring the development of between 50–70% of all agents in clinical use today (Pye et al., 2017). Over 100 new products are in clinical development, particularly as anti-cancer and anti-infective agents (Patwardhan et al., 2015). According to Harvey (2008), almost half of the drugs

approved since 1994 are based on natural products. A total of 360 antiplasmodial natural products have been reported in the literature from January 2009 to November 2010 (Nogueira and Lopes, 2011).

Africa, with its rich fauna and flora, is a big resource of medicinal plants. In Africa, phytotherapy still plays an important role in the management of diseases, mainly among people of very low income (Geoffrey and Kirby, 1996; Kuete, 2017). In the streets of Lusaka are several stands where people deal in medicinal plants. Advertisements on the uses of natural products abound in the newspapers. Perhaps the most prominent in Zambia is the *Sondashi formula* (SF2000SD), a mixture of natural products that is commonly used in the management of HIV/AIDS. This formula has now completed the phase I clinical trials to determine the safety profile in healthy male volunteers (https://clinicaltrials.gov/ct2/show/NCT02941159, accessed on 4th December 2017).

Considering the exceptional floral biodiversity of the African continent and the high number of medicinal plants found in each African country, there is a need for African governments to adequately fund universities and research laboratories to investigate the efficacy and safety of medicinal plants used on the continent. This will encourage not only the use of these plants, but also the production of improved phytodrugs (Kuete, 2017).

1.3. Statement of the problem

Recently, the problem of multi-drug resistance of disease-causing microorganisms has reached an alarming level around the world and is posing a serious challenge to Public Health (Elbossaty, 2017). An increasing number of bacteria are now developing resistance to commercial antibiotics (Eloff et al., 2005; WHO, 2015). Antimicrobial resistance (AMR) threatens the effective prevention and treatment of an ever-increasing range of infections caused by bacteria, parasites, viruses and fungi. AMR results in reduced efficacy of antibacterial, antiparasitic, antiviral and antifungal drugs, making the treatment of patients difficult, costly, or even impossible. The impact on particularly vulnerable patients is most obvious, resulting in prolonged illness and increased mortality (WHO, 2014). For example, the most urgent threat level pathogen in the U.S. is the Gram-positive bacterium *Clostridium difficile*, causing 250, 000 infections with 14, 000 deaths per year (Martin Exner et al., 2017).

In 2014, the WHO released a report on the global status of Anti-Bacterial Resistance (ABR) and surveillance (**Table 1**). The information on resistance to antibacterial drugs commonly used to treat infections caused by nine bacteria of international concern was compiled:

- *Escherichia coli*: resistant to third-generation cephalosporins, including resistance conferred by extended spectrum beta-lactamases (ESBLs), and to fluoroquinolones;
- *Klebsiella pneumoniae*: resistant to third-generation cephalosporins, including resistance conferred by ESBLs, and to carbapenems;

- *Staphylococcus aureus*: resistant to *beta*-lactam antibacterial drugs (e.g., methicillin);
- *Streptococcus pneumoniae*: resistant or nonsusceptibility to penicillin (or both);
- *Nontyphoidal Salmonella* (NTS): resistant to fluoroquinolones;
- *Shigella species*: resistant to fluoroquinolones;
- *Neisseria gonorrhoeae*: decreased susceptibility to third-generation cephalosporins.

Table 1: Annual WHO report on the global status of Anti-Bacterial Resistance (ABR) and surveillance (2014)

Bacteria commonly causing infections in hospitals and in the community			
Name of *Bacterium*/resistance	Examples of typical diseases	N° out of 194 States providing Data	N° of WHO regions with national reports of 50% resistance or more
Escherichia coli/ - vs 3rd gen. Cephalosporins -vs. Fluoroquinolones	Urinary tract infections, blood stream infections	86 92	5/6 5/6
Klebsiella pneumonia/ - vs 3rd gen. Cephalosporins - vs. 3rd gen. Carbapenems	Pneumonia, blood stream infections, urinary tract infections	87 71	6/6 2/6
Staphylococcus aureus/ - vs Methicillin "MRSA"	Wound infections, blood stream infections	85	5/6
Bacteria mainly causing infections in the community			
Streptococcus pneumoniae/ -non-susceptible or resistant to penicillin	Pneumonia, meningitis, otitis	67	6/6
Nontyphoidal Salmonella/ -vs fluroquinolones	Foodborne diarrhea, Blood stream	68	3/6

	infections		
Shigella species/ - vs fluoroquinolones	Diarrhoea (bacillary dysenteria)	35	2/6
Neisseria gonorrhea/ -vs 3rd gen. cephalosporins	Gonorrhoea	42	3/6

- **Legend**: Gen = generation

At one time, methicillin, a beta-lactam antibiotic, was used to treat infections caused by a number of Gram-positive bacteria (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes,* and *Streptococcus pneumoniae)*. But today, infections with Methicillin-Resistant *Staphylococcus aureus* (MRSA) have become a major problem, particularly in hospitals (Rand and Dale, 2007). Resistance to vancomycin in the United States among patients infected with *Enterococcus faecium* amounts to approximately 20 to 30% (Lemke et al., 2008). According to Hsueh and Luh (2002), *Streptococcus pneumoniae* exhibits resistance to penicillins, cephalosporins, trimethoprim-sulfamethoxazole, macrolides and fluoroquinolones like ciprofloxacin. The magnitude of AMR worldwide and its impact on human health and wider society, as well as on costs for the healthcare sector are still largely unknown.

Scientists around the world have gained interest in the investigation of medicinal plants as sources of new medicines. Recent discovery shows that medicinal plants have fewer side effects than Allopathic medicines. Herbal medicine is gaining popularity all over the world (Rabia et al., 2011). In fact, NPs have been the single most productive source of leads for the development of drugs. There is a significant number of NP drugs in development (Butler et al., 2014). At the end of 2013, 100 NP

and NP-derived compounds and 33 antibody drug conjugates (ADCs) with a NP-derived cytotoxic component were evaluated in clinical trials or in registration. Thirty-eight of the NPs/NP-derived compounds and 33 ADCs were investigated as potential oncology treatments, 26 as anti-infectives, 19 for the treatment of cardiovascular and metabolic diseases, 11 for inflammatory and related diseases and 6 for neurology (**Figure 1.1**).

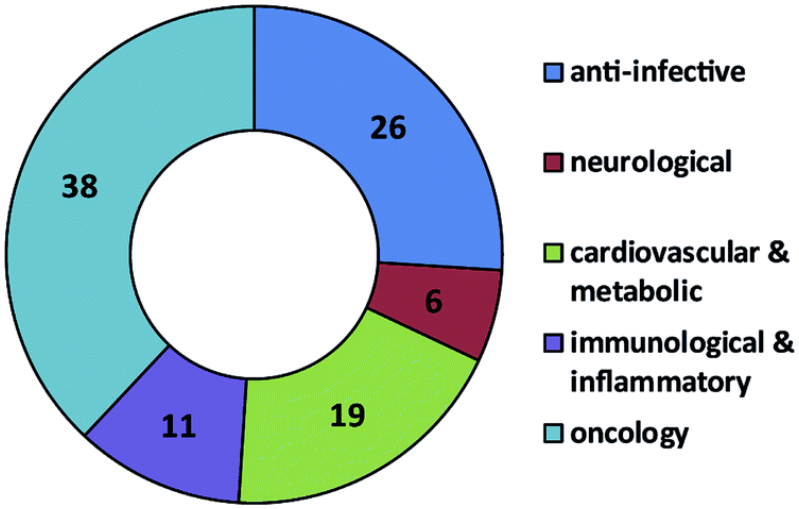


Figure 1.1: Natural product and natural product-derived drugs in clinical trials at the end of 2013 (Butler et al., 2014).

Africa has a plethora of medicinal plants used in folklore medicine. The use of medicinal plants as a fundamental component of the African traditional healthcare system is perhaps the oldest and the most assorted of all therapeutic systems. In many parts of rural Africa, traditional healers prescribing medicinal plants are the most easily accessible and affordable health resource available to the local community and at times the only source of therapy (Mahomoodally, 2013). Although these traditional medicinal preparations could be effective in treating a wide array of ailments, their safety remains unknown and they could potentially exert undesirable

side effects (Maroyi, 2017). Therefore, given the advent of widespread resistance to commonly used antibiotics and the threat of potential toxicities which could arise from the use of traditional medicinal preparations, it is important to intensify research efforts into the discovery of new therapies as well as scientifically validating traditional remedies.

In this study, leaf extracts from four plants (*Odontonema strictum*, *Aphelandra squarossa*, *Solanum torvum* and *Symphytum officinale L*) were investigated for their antibacterial potential. The most potent plant was then selected for further investigation.

1.4 Aim

The aim of this study was to evaluate the antibacterial activity of leaf extracts of four tropical plants (*Aphelandra squarrosa*, *Odontonema strictum*, *Solanum torvum* and *Symphytum officinale L*.) used in folklore medicine, select the most potent plant extracts, isolate and characterize its active secondary metabolites.

1.5 Objectives

The objectives of the study were to:

(1) Evaluate the antibacterial potential of 4 plant extracts (*Aphelandra squarrosa*, *Odontonema strictum*, *Solanum torvum* and *Symphytum officinale L*.) against selected bacterial species (*Klebsiella pneumoniae*, *Shigella dysenteriae*, *Salmonella typhi*, *Escherichia Coli* and *Staphylococcus aureus*);

(2) Isolate the active compounds from the most potent plant by chromatographic methods;

(3) Elucidate the structure of the active compounds using spectroscopic methods; and

(4) Evaluate the anti-bacterial potency of the active compounds.

1.6 Research questions

Three main questions guided this research and these were:

(1) Can the antibacterial potential of the selected plants be evaluated using Agar disk diffusion method?

(2) Can the active ingredients be isolated using chromatographic methods?

(3) Can the structure of the active compounds be elucidated?

(4) Can the minimum inhibitory concentration (MIC) suppressing the bacterial growth be determined visually?

1.7 Significance of the study

Different species of bacteria are now resistant to a wide range of synthetic medicines as mentioned in Section 1.4. Therefore, novel, affordable and structurally diverse chemotherapeutic interventions with new mechanisms of action are urgently needed to combat resistant strains of disease-causing organisms. This study has potential of finding both unknown and known antibacterial molecules. Such a discovery has the likelihood of providing drug candidates for further development into the clinic for the management of multi-drug resistant microorganisms. This study could also

potentially provide a scientific validation or invalidation of the traditional use of medicinal preparations from the studied plant species.

CHAPTER 2:

LITERATURE REVIEW

2.1 Medicinal Plants in Africa

The use of plants as medicines goes back to early human. Certainly, the great civilizations of the ancient Chinese, Indians, and North Africans provide written evidence of humanity's ingenuity in utilizing plants for treatment of a wide variety of diseases (Phillipson, 2001). Traditional African medicine is a holistic discipline involving indigenous herbalism and African spirituality. Diviners, midwives, and herbalists work together to resolve a variety of problems in the society. For example, while folkloric medicine takes a holistic approach where good health, sickness, success or misfortune are not seen as chance occurrences but are believed to arise from the actions of individuals and ancestral spirits according to the balance or imbalance between the individual and the social environment, conventional medicine, in contrast, is technically- and analytically-based where measurements are very important (Anyinam, 1987).

Traditional African medicine is still very popular in both rural and urban areas for a good number of reasons that include accessibility and affordability. The medicines are accessible because they are found in the same environment as the user. Modern pharmaceuticals and medical procedures remain inaccessible to a large number of Africans due to their relatively high cost and low concentration of health care centers in both urban and rural centers. Furthermore, the inadequacy of basic medicines and medical personnel contributes to the unpopularity of Western medicine (Elujoba et al., 2000).

Africa is gifted with many plants that can be used for medicinal purposes to improve healthcare in the continent. In fact, out of the approximately 6000 plant species used in tropical Africa, more than 4000 are used as medicinal plants (Schmelzer et al., 2013). Pressure on medicinal plant resources has remained low in remote areas and in countries such as Mozambique and Zambia where the commercial trade in traditional medicines is still growing slowly due to the small size of major urban centers (Cunningham, 1993).

Traditional medical practitioners are attracted to urban centers where benefits can be good, as shown in studies undertaken in Nairobi (Kenya), Dar-Es-Salaam (Tanzania), Kampala (Uganda), Kinshasa (Democratic Republic of Congo) and Lusaka (Zambia) (Good and Kimani, 1980). Africa accounts for only 5% of global pharmaceutical trade. However, for crude drugs, it is next to Asia for the export of medicinal and aromatic plants. Medicinal plants are a common merchandise at urban markets. Most medicinal plant materials are exported to Europe, the USA and Asia. In 1996, about 26,500 tons of medicinal and aromatic plant materials were exported to Europe from West African countries such as Burkina Faso, Ivory Coast and Benin (Vasisht and Kumar, 2004). Many African plant species are a source of a number of active ingredients for the export market (Kuete, 2013). The fruit of *Srophanthus spp*., which contains the active ingredient ouabain, is exported to Europe by West African countries. Mozambique, DRC and Rwanda export the roots of the species *Rauvolfia vomitoria* which contains reserpine and yohimbine which are indole alkaloids used for the control of high blood pressure and for the relief of psychotic symptoms. The species *Catharanthus roseus* from Madagascar contains vincristine and vinblastine, two alkaloids used for treatment of a variety of cancers including acute leukemia, Hodgkin's and non-Hodgkin's lymphoma, neuroblastoma, rhabdomyosarcoma,

Ewing's sarcoma, Wilms' tumor, multiple myeloma, chronic leukemias, thyroid cancer and brain tumors. They are also used to treat some blood disorders (Vasisht and Kumar, 2004).

In Zambia, annual trade in traditional medicine is worth over US$ 43 million (Nswana, 1996). Trade, generally, takes place within and between neighboring countries. *Pterocarpus angolensisis* used by Traditional Medicine Practitioners (TMPs) is also exported. The plant is valued for several medicinal uses. It has been recorded to treat ringworm, eye problems, blackwater fever, stabbing pains, malaria, and to increase the supply of breast milk. Other locally used medicinal plants are *Eulophia petersiana* and *Selaginella imbricata* (Cunningham, 1993). The DRC exports *Cinchona spp.* and *Prunus africana* (Hook. f.) Kalkman, mainly to European countries (Vasisht and Kumar, 2004).

Traditional medicine practice in Zambia is used and accepted by about 70% of the population, regardless of ethnic, religious or social background. In addition, about 80% of all Zimbabweans have benefited from traditional medicinal plant practice voluntarily or involuntarily (Mwitwa, 2009). The needs of patients range from receiving simple herbal preparations to casting evil spirits (Duri, 2009). This includes remedies taken orally; through steaming; by anal insertion (for powders and liquids); by inhalation of smoke and fumes; by wearing and carrying on the body; by rubbing on the affected part; and by co-administration with a drink, porridge and solid food. In Zambia, traditional medicine is widely practiced in both urban and rural areas - the practice being recognized and supported by the Traditional Healers Practitioners Association of Zambia (THPAZ) which was founded in 1978 (Mudondo, 2000). The Association has now more than 35,000 registered members. It controls the profession

of traditional medicine but the practice is neither integrated with mainstream medicine nor with the national health-care system.

2.2. Protective uses of secondary metabolites

Phytochemistry studies describe the large number of secondary metabolic compounds found in plants. Many of these chemicals such as alkaloids, polyphenols, terpenes, flavonoids tannins, glycosides and quinones are known to provide protection against insect attacks and plant diseases. For example, tannins have a strong deleterious effect on phytophagous insects and affect insect growth and development by binding to proteins, which causes a reduction in nutrient absorption efficiency resulting in midgut lesions.

Both flavonoids and isoflavonoids protect the plant against insect pests by influencing the behavior, growth and development of insects. Condensed tannins are oligomeric or polymeric flavonoids, also known as proanthocyanidins. They have diverse structures and functions. They act as feeding deterrents against some insects such as *Lymantria dispar* (L.), *Euproctis chrysorrhoea* (L.) and *Operophtera brumata (*L*)*. In addition, plants indirectly defend themselves from herbivore feeding by emitting a blend of volatiles and non-volatile compounds (Abdul et al., 2012).

2.3 Medicinal plants as sources of drugs

Secondary metabolites also exhibit several curative and protective functions for human consumers. Phytochemistry is widely used in the field of herbal medicine. Phytochemical techniques mainly apply to quality control of traditional medicines or herbal medicines. Drug development from phytomedicines has focused, in the past, on the discovery and analysis of new lead compounds from natural products. The

search, aimed at the unravelling of the "active principle" in plants, is based on the assumption that a plant has one or more ingredients which determine its observed therapeutic effects (Ulrich-Merzenich et al., 2010). Although the use of bioactive natural products as herbal drug preparations dates back hundreds, even thousands of years ago, their application as isolated and characterized compounds to modern drug discovery and development only started in the 19th century, the dawn of the chemotherapy era (Liang and Fang, 2006). It was in the 19th century that humans began to isolate the active principles from medicinal plants and one particular landmark, in this regard, was the discovery of quinine from *Cinchona* bark by the French scientists Caventou and Pelletier in 1820. Such discoveries led to an interest in plants from the New World and expeditions scoured the almost impenetrable jungles and forests in the quest for new medicines (Phillipson, 2001).

During World War II, a number of natural products isolated from higher plants were used in clinical practice and many are still in use today. Quinine from *Cinchona bark*, morphine and codeine from the latex of the opium poppy, digoxin from *Digitalis* leaves, atropine (derived from (-) hyoscyamine) and (-) hyoscine) from the species of the *Solanaceae* family continue to be in clinical use. A whole series of natural products isolated from species of *Penicillium*, *Cephalosporium* and *Streptomyces* have antibacterial effects. In the post-war years, there were relatively few discoveries of new drugs from higher plants with the notable exception of reserpine from the *Rauwolfia* species heralding the age of the tranquillisers. Additionally, from *Catharanthus roseus* were obtained vinblastine and vincristine which are effective in cancer chemotherapy (Beutler, 2009). According to Phillipson (2001), the isolation and structure determination of Taxol from Western Pacific Yew,

Taxus brevifolia, followed on from experiments that showed that a bark crude extract was active against cancer cells in laboratory tests. Although this activity was discovered in the early 1960s, it was not until 1971 that complete structural elucidation of this complex diterpene was done. Artemisinin is another natural product which is an unusual sesquiterpene endoperoxide that has been identified as the active principle of the Chinese antimalarial herb *Artemisia annua* (Zongru, 2016). Clinical trials demonstrated that artemisinin is an effective antimalarial drug, and is currently used to treat infections of multi-drug resistant strains of *Plasmodium falciparum*, the cause of human malignant cerebral malaria. Artemisinin is very unstable and, therefore, is methylated for stability.

Despite competition from other drug discovery methods, NPs are still providing their fair share of new clinical candidates and drugs. NPs are still a significant source of new drugs, especially in the anticancer and antihypertensive therapeutic areas (Butler, 2004). There still is an urgent need to develop new clinical drugs for treatment of numerous diseases and it is possible that there are other drugs still to be found from nature (Phillipson, 1999).

Medicinal chemistry has evolved from the chemistry of bioactive compounds in early days to areas at the interface of chemistry and biology nowadays. Today, medicinal chemistry of bioactive natural products spans a wide range of fields such as isolation and characterization of bioactive compounds from natural sources, structural modification for optimization of their activity and other physical properties, and total and partial-synthesis for a thorough scrutiny of structure-activity relationship (SAR).

In addition, synthesis of natural products provides a powerful means in solving supply problems of a particular drug required in clinical trials and marketing.

2.4 Detection of biologically active metabolites

The starting point in the search for potentially useful compounds from plants is the detection of bioactive metabolites (Sarker et al., 2006). Folklore medicine has selected groups of plants that time and experience have shown to be beneficial. Scientists try to identify the active ingredients in plants using different techniques. They do screening of a large number of organisms for a particular effect. This includes searches for a particular type of compound, old metabolites being tested in new screens or, not the least important, from serendipitous discovery of new metabolites displaying pharmacological properties.

Testing large numbers of extracts or compounds to determine if they produce a biochemical or cellular effect is usually one of the first steps in the discovery of bioactive compounds. In principle, a bioassay is any *in vitro* or *in vivo* system used to detect the biological activity of an extract or a pure substance from a living organism. This may involve testing for antibiotic activity, *in vitro* inhibition tests, pharmacological, agricultural, or veterinary screens, which require diverse *in vitro* assays or *in vivo* animal models (Pye et al., 2017; Maroyi et al., 2017). The application of these assays to monitor the presence of a bioactive compound(s) during the isolation process is called bioassay-guided fractionation. Thus, all fractions generated from a crude extract are tested for biological activity, and those

giving a positive test are further processed until the bioactive agent is obtained in a pure form (Colegate and Molyneux, 2008).

2.5 Isolation and characterization of secondary metabolites

According to Colegate and Molyneux (2008), the isolation of natural products that have biological activity toward organisms other than the source has several advantages including the following:

- ✓ A pure bioactive compound can be administered in reproducible, accurate doses with obvious benefits from an experimental or therapeutic aspect.
- ✓ It can lead to the development of analytical assays for particular compounds or for classes of compounds.
- ✓ It permits the structural determination of bioactive compounds, which may enable the production of synthetic material, incorporation of structural modifications, and rationalization of mechanisms of action.

Chromatographic techniques are utilized in separation and purification of isolated bioactive constituents based on polarity or other gradient factors such as molecular size (**Figure 2.1)**.

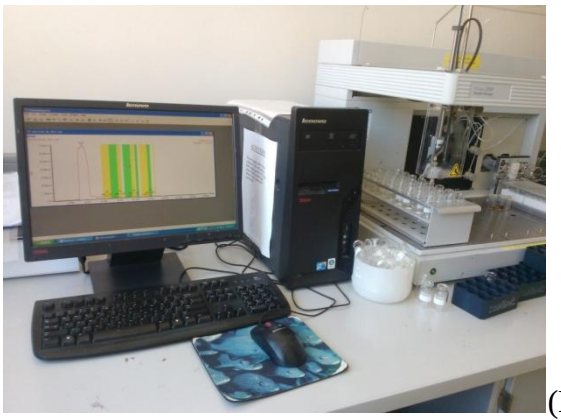

(A)

(B)

Figure 2.1: (A) A Biotage automated flash chromatography machine at Drug Discovery and Development Center (H3D), University of Cape Town (UCT); (B) A HPLC machine at H3D, UCT.

The isolated compound is characterized by spectroscopic methods. There are four basic types of spectroscopy utilized in the characterization of purified natural product compounds. These are ultraviolet to identify the presence of chromophores in a molecule, infrared (IR) spectroscopy to detect different functional groups in the molecule, chromatography to purify compounds (**Figure 2.2B**), HPLC-MS assembly (**Figure 2.2A**) to determine the molar mass of the molecule and nuclear magnetic resonance (^{1}H and ^{13}C NMR/ **Figure 2.2C**) techniques to elucidate the structure.


(A)


(B)

(C)

Figure 2.2: (A) A HPLC-MS assembly at H3D, UCT; (B) Prep-HPLC at H3D, UCT; (C) A 400 MHz NMR spectrometer at H3D, UCT.

Because each plant may contain hundreds to thousands of organic chemicals, one can assume that there are very large numbers of yet undiscovered medicinal chemicals existing in nature.

2.6 Presentation of the investigated plants

2.6.1 Botanical information of the family *Acanthaceae*

The *Acanthaceae* family is composed of about 250 genera with almost 2500 species, widely spread in tropical regions of the world but are poorly represented in temperate regions (Wasshausen & Wood, 2004). The four main distribution centers of this family are Indo-Malay (*Strobilanthes* and *Andrographis*), tropical Africa (*Barleria*), Brazil (*Ruellia*) and equatorial regions (*Aphellandra* and *Odontonema*). These plants show a wide variety in habitat and biological types. Most of them are shrubs or herbs and climbing plants. Xerophytes and marshy plants are also very common while trees are rare in the family (Berrondo *et al.*, 2003).

Justicia is the largest subfamily of *Acanthaceae*, with approximately fifteen genera comprising 600 species that are found in pantropical and tropical regions (Durkee, 1986).

According to Lucinda (1999), the genus *Odontonema* belongs to the *Justicia* lineage as indicated in **Figure 2.3**.

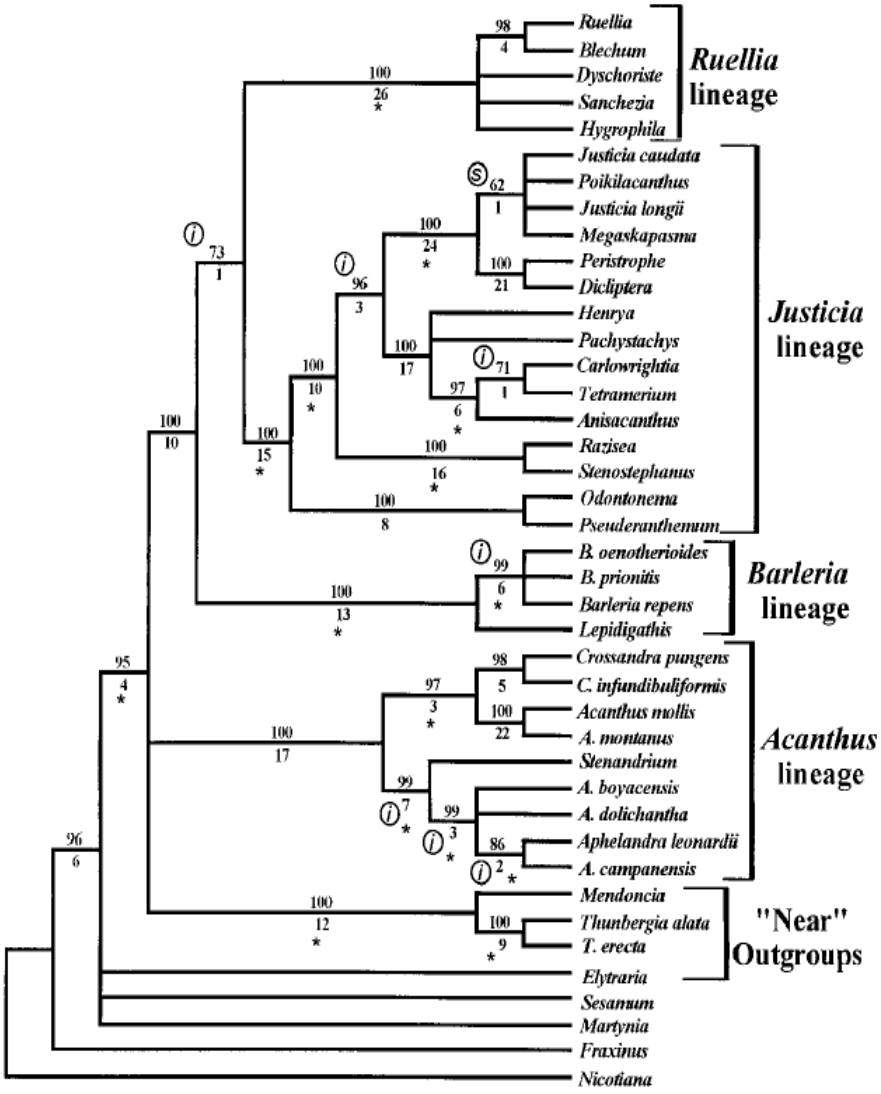


Figure 2.3: Phylogenetic relationships among the *Acanthaceae* family (Lucinda, 1999)

2.6.1.11 *Aphelandra squarossa*

Commonly called "zebra plant", *Aphelandra squarossa* (**Figure 2.4**) is a plant species in the family *Acanthaceae*, which is native to the Atlantic Forest vegetation

of Brazil. This plant is often used as a house plant. *Aphelandra* is a genus of about 170 species most of which are tropical flowering plants (www.wikipedia.com).

Figure 2.4: The species *Aphelandra squarossa*, one of the preferable plants for snails

The genus *Aphelandra* belongs to the subfamily *Acanthoideae*, tribe *Aphelandreae*. Its nearest relative is probably the Old-World genus *Crossandra*, consisting of about 50 species from Africa and Asia. In *Crossandra*, the upper lip is absent or the corolla is dorsally deeply clefted, whereas in *Aphelandra* the upper lip is present. In the New World, Aphelandra's nearest relative is the genus *Stenandrium*, distinguished from *Aphelandra* in that it is usually a subcaulescent or acaulescent herb, with short nonimbricate bracts, a narrowly cylindric, rarely enlarging corolla tube, and a slightly two-lipped corolla with subequal, spreading lobes (Wasshausen, 1975).

The Zebra plant is a fairly popular plant which is grown for its flower bracts and dark green leaves with prominent white colored veins. The plant flourishes when the temperature is in the range of 18-21 °C and will suffer if the temperature drops below 15 °C for a prolonged period (www.wikipedia.com, accessed on 16th April 2014).

2.6.1.1.1 Pharmacological activity

Traditionally, the most important plant part in the *Acanthaceae* family are the leaves which are used externally for wounds. Pharmacological properties of the genus *Aphelandra* include antibacterial, antifungal and immunomodulatory activities (Awan, 2014). The Terengganu people of Malaysia widely use Kuda Belang (*Aphelandra squarrosa*) as traditional herbal remedy. Khalili (2012) has also recently reported on the antibacterial potential of *Aphelandra squarrosa*.

2.6.1.1.2 Phytochemistry

Phytochemical reports on the genus *Aphelandra* have documented the presence of alkaloids, flavonoids, isoflavones, benzoxazinoids-cyclic hydroxamic acid and their corresponding glucosides. Benzoxazinoids in *Aphelandra squarrosa* are restricted to the root (Schullehner, 2008).

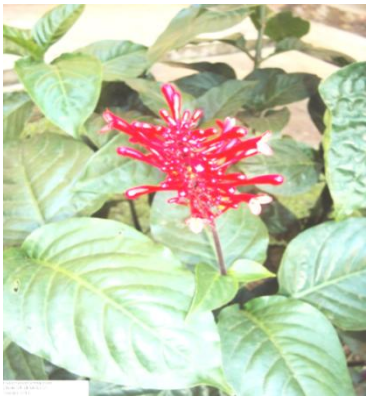
2.6.1.2 Odontonema strictum (OSM)

2.6.1.2.1 Botanical information of the genus *Odontonema*

The genus *Odontonema* includes flowering plants of the *Acanthaceae* family, native to Central America. *OSM*, also called *Fire spike* (tip fire) or *Cardinal Guard*

(custody of Cardinal) or *Scarlet Flame* (Flame Scarlet), is a plant belonging to the dicotyledonous angiosperm subclass of *Asteridae*, order of *Scrophulariales*, to the family *Acanthaceae*, sub family *of justiciaceae* and genus *Odontonema*.

There are many species in the genus *Odontonema*. The most known are: *O. callistachyum* (Schltdl. & Cham.) Kuntze (**Figure 2.5D**); *O. cuspidatum* (Nees.) Kuntze (**Figure 2.5C**); *O. tubiforme* (Bertol.) Kuntze(**Figure 2.5B**) and *O. strictum* (Bertol.) Kuntze (**Figure 2.5A**).



(A)

(B)

(C)

(D)

Figure 2.5: (A) *Odontonema strictum;* (B) *Odontonema tubiforme;* (C) *Odontonema cuspidatum;* (D) *Odontonema callistachyum*

OSM is a shrub, with an upright stem, and native to tropical regions, where it flourishes. The leaves are deciduous, green, shiny, opposite, simple, ovate, wavy

margins, pinnately veined and measure 10 to 15 centimeters long. Flowers are red, axillary or terminal, tubiform, hermaphrodite, in long panicles and erect (**Figure 2.6**). Fertilization is done by butterflies and the proboscis of hummingbirds. The propagation of the species is usually by cuttings.

In phylogenetic classification (as shown in **Figure 2.3**), *OSM* belongs to the order *Lamiales*. The plant is also sold under the name *Justicia coccinea* but is also synonymous with other plants as *Pachystachys coccinea, Jacobinia*. This is ambiguous at best and a potential source of error. There are many synonyms for *OSM*, including: *Justicia coccinea*, *Jacobinia*, *Odontonema tubaeforme* and *Odontonema* (Lucinda, 1999).

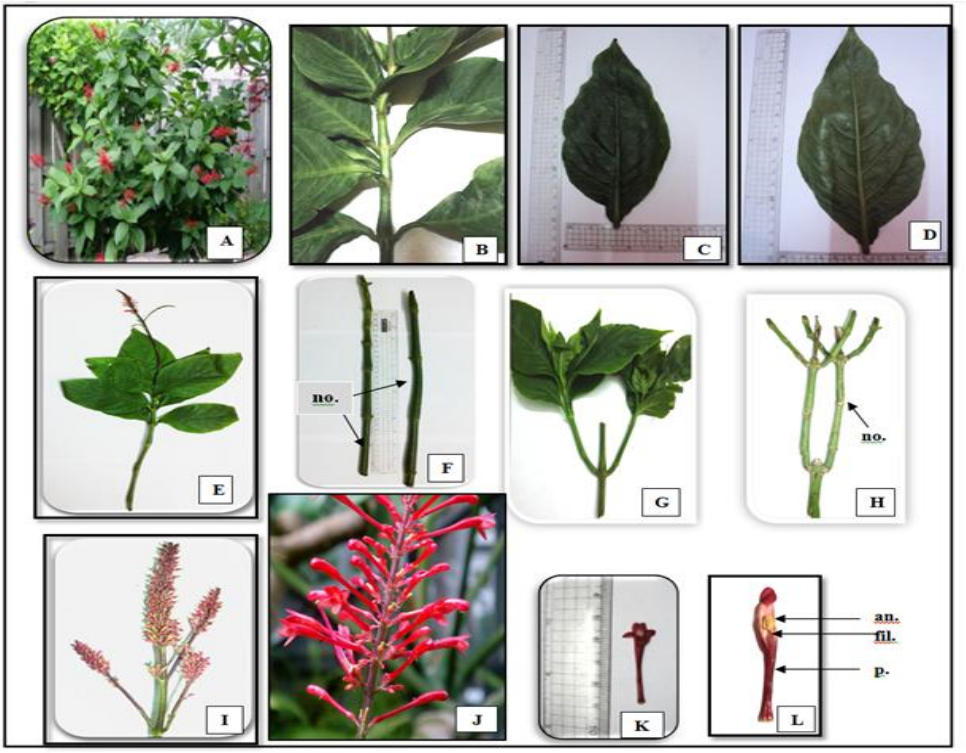


Figure 2.6: Photographs of *Odontonema cuspidatum* (Refaey et al., 2015).
(A) *O. cuspidatum* shrub (X = 0.035), (B) Leaves showing opposite decussate phyllotaxis (X = 0.31), (C) Upper surface of the leaf (X = 0.26), (D) Lower surface of the leaf (X = 0.26), (E) Part of *O. cuspidatum* showing leaves, stem and inflorescence together (X = 0.116), (F, G and H) Stem of *O. cuspidatum* showing branching and nodes (f, X = 0.128, g, X = 0.25 and H, X = 0.20), (I) Inflorescence of *O. cuspidatum* showing young dichasial raceme (X = 0.37), (J) Inflorescence of *O.*

cuspidatum showing mature flowers (X = 0.32), (K) Tubular petals of *O. cuspidatum* (X = 0.23), (L) Longitudinal cut in a petal (X = 0.47); an., anther; fil., filament; no., nodes; p., petal.

2.6.1.2.2 Ethnomedical importance of genus *Odontonema*

Three species of the genus *Odontonema* are used in folklore medicine (**Table 2.1**). However, the similarities observed in the genus are a source of confusion. For instance, *Odontonema tubiforme* (Bertol.) Kuntze, *Odontonema strictum* and *Odontonema cuspidatum* seem to be synonyms for naming one species. This could explain why the taxonomy of species in the genus *Odontonema* is flexible (Refaey et al., 2015).

In Sierra Mazateca (Mexico), the ground leaves and the stem of *Odontonema callistachyum* are applied on open wounds in order to speed up the healing process (Giovannini et al., 2003). The leaves of the species *Odontonema tubiforme (Bertol.)* are used as an anti-inflammatory drug and for inducing child birth (Caballero-George and Gupta, 2011). In addition, the aqueous extract of the leaves of OSM is used in Burkina Faso by traditional physicians for the treatment of arterial hypertension in humans (Ouedraogo et al., 2005; Kini et al., 2008). The genus *Odontonema* is, therefore, a likely source of new bioactive secondary metabolites.

Table 2.1 Scientific names, synonyms, and geographical distribution of the species of *Odontonema* with previous phytochemical and bioactivity information.

Species	Synonyms	Geographical distribution	Phytochemistry	Bioactivity	References
O. callistachyum	Purple Firespike, Naktam ay	Central America, Central Africa	No	Yes	Giovannini et al. 2003).
O. tubiforme	Bois indien ou bois	Central America,	No	Yes	Caballero-George and Gupta, 2011

Species	Synonyms	Geographical distribution	Phytochemistry	Bioactivity	References
	genou, Firespike, bwa kwapo, chapantye gwan bwa	Central Africa			
O. strictum	Firespike	Central America, Central Africa	Yes	Yes	Serme Ladiama, 2001; Ouedraogo et al., 2005; Felix Kini et al., 2008; Luhata et al., 2015 ; M. S. Refaey et al., 2015; Luhata et al., 2016
O. cuspidatum	The Cardinal's guard or the firespike	Central America, Central Africa	No	Yes	Refaey et al., 2015

2.6.1.2.3 Phytochemistry of *Odontonema strictum*

No compounds have been isolated so far from the genus *Odontonema*. However, many compounds have been isolated and characterized from the sub-family of *justiciaceae* including: flavonoids, triterpenoids, alkaloids, lignans (Geone and Alcântara, 2012).

According to Ouedraogo (2005), the water, ethyl acetate and methanol extracts of *OSM* were investigated for vascular activity in isolated rat tail and pig coronary arteries. The ethyl acetate fraction appeared to contain the active principle and this extract was thus recommended for further investigations.

Kini and his colleagues (2008) also worked on *OSM* and identified flavone glycosides (C-heterosides and *O*-heterosides present in 0.37 and 1.13 percent

respectively). No antimicrobial studies have been undertaken and no pure compound has been isolated so far.

The genus *Odontonema* possesses a variety of pharmacological activities. Unfortunately, the phytochemistry of the different species is not well documented.

2.6.1.2.4 Observations of a dog's behavior on the species *Odontonema strictum*

An observation made at a Jesuit Community in Lubumbashi Democratic Republic of Congo was that a dog was found regularly digging and eating the roots of the plant. This was very strange indeed since other plants were not attacked in the same way. It was further observed that the dog did not suffer any immediate observable consequences. Since the dog did not die, the plant root material may be assumed to be safe at the dosages the dog was consuming.

It is possible that the dog may have been using the roots for some kind of remedy or supplementation. Dogs usually dig for small animals that hide in the holes. So the question was "why should a dog eat those roots?

2.6.1.3 Solanum torvum

Solanum torvum (**Figure 2.7**) is also known by vernacular names such as mélongène-diable, bellangère bâtarde, aubergine pois (French), pea eggplant, cherry eggplant, devil's fig, flat brush, Turkey berry.

(A)

(B)

Figure 2.7: (A) Aerial parts of the species *Solanum torvum*; (B) *Solanum torvum's fruits*

2.6.1.3.1 Botanical presentation

The family *Solanaceae* comprises about 80 genera and 3000 species, from which 1500 belong to the genus *Solanum*. This genus is widespread all over the world although it is concentrated mainly in the tropics and subtropics. In Mexico, there are about 150 species.

According to Pérez-Amador (2007), *Solanum torvum* is native to Central and South America where it is found from Mexico to Brazil and Peru and has become widespread in the Caribbean. It has now become a pantropical weed. In West and Central Africa, it is grown locally in the kitchen gardens, and is probably found in

other parts of Africa as well. It is grown as a small-scale vegetable in southern and eastern Asia and is particularly popular in Thailand.

2.6.1.3.2 Ethnopharmacological reports

Solanum torvum is used in traditional medicine. Its fruits and leaves are used against a variety of microbial infections. Solasodine, a glycoalkaloid found in its leaves and fruits, is used in India in the manufacture of steroidal sex hormones for oral contraceptives. The plant has sedative, diuretic and haemostatic properties. The ripened fruits are used in the preparation of tonic and haemopoietic agents and also for the treatment of pain. It has antioxidant properties. It is intensively used worldwide in traditional medicine as a poison anti-dote and for the treatment of fever, wounds, tooth decay, reproductive problems and arterial hypertension (Ndebia, 2007).

The antimicrobial properties of leaves have been known for some time in Central America and India and also in Gabon where people apply the leaves on cuts and wounds. In Sierra Leone, a fruit decoction is administered to children as a cough medicine, while in Senegal, the plant is used to treat throat pain and stomach pain. In India, the leaves are dried and ground into a powder that is used as a medicine by diabetics. In Côte d'Ivoire, the plant is known to cause an instant mental alienation when consumed raw and it has been used to poison humans. In soils infested with Meloidogyne nematodes and bacterial wilt, *Solanum torvum* is sometimes used as a rootstock for eggplant and to a lesser extent for tomato (Ajaiyeoba, 1999; Chah et al., 2000).

2.6.1.3.3 Phytochemistry

Several *Solanum* species contain free and glycosilated alkaloids as well as other important substrates for the synthesis of steroidal hormones (Maití et al., 1979). Many valuable phytoconstituents of therapeutic importance such as steroidal alkaloids, chlorogenone, neochlorogenone, isoflavonoid sulfate, steroidal glycosides, 2, 2 *O*-spirostannol (torvonin-A), solasonine, sterolin (sitesterol-D-glucoside), protein, fat and minerals have been isolated (Watt, 1962; Wiart, 2004; Vijayakumari, 2012).

2.6.1.4 Symphytum officinale L.

This plant species (**Figure 2.8**) is also known by vernacular names which include grande consoude, oreille d'âne, langue de vache, confée (French); comfrey, knitback and knitbone.


(A)

(B)

Figure 2.8: (A) and (B) the species *Symphytum officinale*

Symphytum officinale is a perennial flowering plant of the genus *Symphytum* in the family Boraginaceae. Along with thirty four other species of *Symphytum*, it is known

as comfrey. To differentiate it from other members of the genus *Symphytum*, this species is known as common comfrey or true comfrey. Other English names include Quaker comfrey, cultivated comfrey, boneset, knitbone, consound, and slippery-root. It is native to Europe and it is known elsewhere, including North America, as an introduced species and sometimes a weed. The flowers are mostly visited by bumblebees.

2.6.1.4.1 Ethnopharmacological reports

Symphytum officinale roots have been used in the traditional Balkan medicine internally (as tea or tincture) or externally (as an ointment, compresses, or alcoholic digestion) for treatment of disorders of the locomotor system and gastrointestinal tract. The leaves and stems have also been used for the treatment of the same disorders, and, additionally, also for treatment of rheumatism and gout (Vogl et al., 2013). Comfrey has been used in folk medicine as a poultice for treating burns and wounds. However, internal consumption, such as in the form of herbal tea, is discouraged, due to concerns it might cause serious liver damage (Oberlies et al., 2007).

The antibacterial potential of *Symphytum officinale* was evaluated by Sumathi (2011). It has been concluded that the ethanol, ethyl acetate and methanol extracts of leaves of *Symphytum officinale* L possess reasonable antibacterial activities.

2.6.1.4.2 Phytochemistry

Various pyrrolizidine alkaloids, such as lasiocarpine, lycopsamine, intermedine, symlandine, riddelline and mainly symphytine have been identified in the plant

(Gomes et al., 2007).The plants' roots contain allantoin (0.6-2%), pyrrolizidine alkaloids (0.02–0.07%), polyphenolic acids, triterpenic saponosides, proteins, caffeic acid, chlorogenic acid, rosmarinic acid, tannins (2.4%), carotene (0.63%), choline, asparagine, coniferin, mucopolysaccharides, starch, gumiresins, phytosterols, carotenoids, vitamins (A, C, E, riboflavin and B_{12}). Moreover, they also contain an antigonadotropic principle - lithospermic acid and immuno-stimulant polyosides, as well as high amounts of mineral substances (Ca, K, P, Mg, Fe, Mn, Na, Zn) (Neagu et al., 2011).

CHAPTER 3: MATERIALS AND METHODS

3.1 Selection of plant species

This research was prompted by the initial observation of episodes in which a dog dug out and consumed the roots of *OSM* without suffering any observable side effects. We hypothesized the dog could have been using the plant for therapeutic effect or as a supplement. To carry out the research, three other plants were selected for preliminary antibacterial testing. Two of them are used in folklore medicine in Zambia (*Solanum torvum* and *Symphytum officinale* L.), and the other two are from the *Acanthaceae* family (*Aphelandra squarrosa* and *OSM*). The main reason for including the three other plants was to increase the probability of obtaining one plant which exhibits potent anti-bacterial activity.

3.2 Plant materials: collection, taxonomy, preparation and extraction

Plant specimens were collected in Lusaka (January-February 2014) and identified by Dr. Chuba and his team in the Department of Biological Sciences at University of Zambia (Appendix O). Voucher specimens were prepared and deposited in the Herbarium of the Department of Biological Sciences (**Table 3.1**).

Table 3.1 Name of specimens

	Plant species	Name of specimens
1	*Solanum torvum Swartz* (Solanaceae)	PLL1
2	*Symphytum officinale* L.(Boraginaceae)	PLL2
3	*Odontonema strictum* (Nees) Kuntze (Acanthaceae)	PLL3
4	*Aphelandra squarrosa* (Nees) (Acanthaceae)	PLL4

The plant samples (leaves) were shade-dried at room temperature and finely powdered using a blender. The powder (50 g) was soaked in a mixture of methanol and dichloromethane (v/v) 1:1 for 24 hours at room temperature, filtered and solid residue rinsed with the same mixture of solvents and the combined filtrate evaporated at room temperature. The dried crude extracts were kept in air tight containers and stored at 4 °C. Each extract was used further for antimicrobial activities and chromatographic separations (Naz et al., 2011).

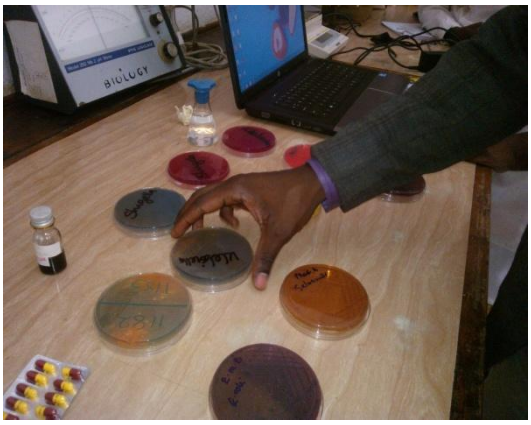
3.3 Samples for antibacterial testing

The dry extracts were dissolved in 80 % acetone (in distilled water) giving a stock concentration of 100 mg/mL and the working concentrations were prepared by ten-fold serial dilution technique ranging from 1 to 100 mg/mL. Acetone was selected based on its ability to extract compounds with a wide range of polarities and it is less toxic to bacteria (Eloff, 1999).

3.4 Test microorganisms

The bacteria were obtained from the University Teaching Hospital (UTH) of Lusaka in Zambia. The organisms available for microbial studies (one gram-negative and four gram-positives) were: *Klebsiella pneumoniae, Shigella dysenteriae, Salmonella typhi, Escherichia coli* and *Staphylococcus aureus*(**Figure 3.1**). All these bacteria were locally isolated organisms (LIO). The selection of bacteria was based on the WHO report (2014) on the global status of ABR and surveillance. In this regard, the five organisms are among the nine bacteria of international concern.

The experiment on antibacterial activity of plant extracts was directed by Dr Doctor Kaimoyo at the department of Microbiology, School of Sciences, University of Zambia, main Campus.



(A)

(B)

Figure 3.1: (A) and (B), test organisms, locally isolated from UTH/Lusaka

3.5 Agar disk diffusion method and turbidity measurement

To determine the turbidity of different concentrations of the selected microorganisms, a colorimeter, Auto Colorimeter ME 51 housed in the Department of Microbiology at University of Zambia (**Figure 3.2A**) was used. The optical density (OD) of the broth culture was read at 600 nm. Total population counts were determined by measuring the turbidity of the culture quantitatively against known turbidity standard (0.5 McFarland standards). A plot of the optical density on the Y-

axis against the concentration on the log X-axis was used and points along the straight portion of the line were used to calculate the viable cell count from the dilution factor using the formula as expressed in equation 1:

Equation 1:

$$\textbf{Absorbance (OD) = 2 - log \% Transmittance}$$

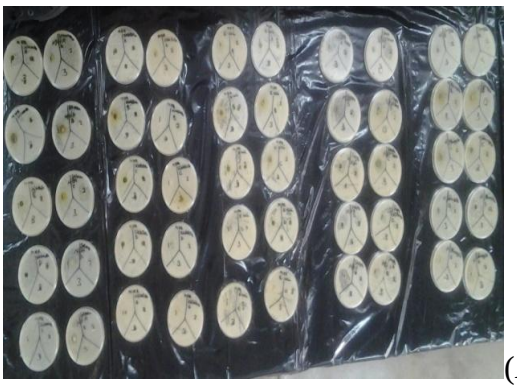
The absorbance (OD) obtained were in the range 0.08 to 0.1 which correspond to approximate cell density of 1.5 x 10^8 Colony Forming Unity (CFU)/mL (McFarland standards). The number of microorganisms was read directly from the standard curve. Inoculants were prepared from subcultures of bacteria as follows. Four to five colonies of the isolates (*K. pneumoniae, S. dysenteriae, S. typhi, E. coli* and *S. aureus*) were emulsified in sterile normal saline and turbidity adjusted to 1 × 10^8 CFU/ mL (corresponding to 0.5 McFarland standards). A sterile cotton swab was dipped into the standardized bacterial suspension and used to evenly inoculate the Mueller Hinton agar plates (Oxoid, Basingstoke, England). The plates were allowed to dry for 3 to 5 min. Wells about 6 mm in diameter were aseptically punched with a sterile cork borer (5 holes per plate) and filled with 50 µL extracts of different concentrations (100, 10 and 1 mg/mL). The plates (in duplicate) were left for 30 min in order for the extracts to diffuse into the agar before incubation (**Figure 3.2B**). Thereafter, they were incubated at 37 °C for 24 h and the zone of inhibition measured to the nearest millimeter using a ruler (Ndip et al., 2007).

(A)

(B)

Figure 3.2: (A) Auto Colorimeter ME 51 (UNZA, Department of Microbiology), used for the measurement of bacterial density. The traditional approach for examining growth dynamics of bacterial cells is by optical density (OD) measurements using a spectrophotometer. At a desired wavelength, visible light is passed through a cell sample. Light scatters as a result of the turbidity of a cell sample, and it provides an OD value. (B) Preparation of *inocula*.

The mean zone diameter of inhibition was calculated for each concentration of extract (1, 10 and 100 mg/mL) while 80 % acetone in distilled water was used as a negative control (Boyonova et al., 2005). Ampicillin, Tetracycline, Chloramphenicol and Amoxicillin (10 μg/disc) were used as positive reference standards to determine the sensitivity of each bacterial species tested. All tests were performed in ten replicates and repeated five times to minimize test error (**Figure 3.3**).


(A)

(B)

Figure 3.3: (A) and (B) reading of the results of the antibacterial experiment after 24 hours.

3.6 Activity index (AI)

The activity index gives an indication of how strong a medicine is compared to a standard. Comparison of antibacterial activity of medicinal herbs and standard antibiotics will be recorded via activity index. (Eloff, 2004; Sharma and Kumar, 2009; Perales and Leysa, 2012; Awan et al., 2013). It is calculated using **equation 2** shown below:

Equation 2:

$$\mathbf{AI=}\ \frac{\boldsymbol{Inhibition\ zone\ of\ the\ sample(mm)}}{\boldsymbol{Inhibition\ zone\ of\ the\ standard\ (mm)}}$$

3.7 Total Activity (TA)

Total activity is an important parameter which indicates the degree to which the active compound in one gram of plant material can be diluted and still inhibits the growth of the tested bacterial microorganisms (Eloff, 2004). TA can be calculated using the following formula:

Equation 3:

$$\mathbf{TA=}\ \frac{\mathbf{Quantity\ of\ material\ extracted\ from\ 1\ g\ of\ plant\ material\ in\ mg}}{\mathbf{MIC\ in\ mg/mL}}$$

3.8 Qualitative phytochemical analysis

The following lines give the general procedure for qualitative phytochemical analysis as described by Trease and Evans (2002). The dry powdered leaf material (50 g) of

OSM was extracted with 800 mL of a 1:1 (v/v) mixture of methanol (MeOH) and dichloromethane (DCM) for 24 hours. The supernatant layer was filtered through Whatman N°1 filter paper, and the residue was rinsed with MeOH. The filtrates were combined and left to dry at room temperature (**Figure 3.4**). The evaporation of the solvent afforded a crude extract (4.5 g) which was later subjected to qualitative phytochemical analysis.


(A)


(B)

Figure 3.4: (A) Maceration, filtration and (B) evaporation of solvents in open air. The leaves (powdered) of *OSM* were macerated in a container for 24 hours and the supernatant was filtered through Whatman N°1 filter paper using a funnel.

3.8.1. *Test for tannins*

About 0.5 g of the extract was boiled in 10 mL 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 coloration.

3.8.2 *Test for saponins*

To 0.5 g of the extract in a test tube, was added 5 mL of distilled water. The solution was shaken vigorously and observed for a stable persistent froth. Formation of a stable foam indicated the presence of saponins.

3.8.3 *Identification of sterols and terpenoids (Liebermannn-Bourchard reaction)*

To 0.5 g of the extract in a test tube, was added 2 mL of chloroform and 2 mL of acetic anhydride. Concentrated H_2SO_4 (3 mL) was carefully added to form a layer. The appearance of a red-brown or violet ring at the contact zone between the two liquids as well as a green-bluish or violet upper layer after 5 – 10 minutes shows presence of sterols or terpenoids. A reddish-brown coloration of the interface indicates the presence of terpenoids.

3.8.4 *Test for flavonoids (Shinoda reagent test)*

A 0.5 g of the extract was dissolved in 3 mL of methanol and mixed with 1-2 fragments of metallic Mg, then 2 mL of concentrated HCl were added; after 5 minutes a red color appeared– for the *flavonols*, orange – for the *flavons*, red-violaceous - characteristic to *flavanones* or green – in the case of *flavanols*.

3.8.5 *Test for alkaloids*

A 3 mL aqueous extract (0.1 g/mL) was stirred with 3 mL of 1% HCl in water on a steam bath. Mayer's reagent was then added to the mixture. Turbidity of the resulting precipitate was taken as evidence for the presence of alkaloids.

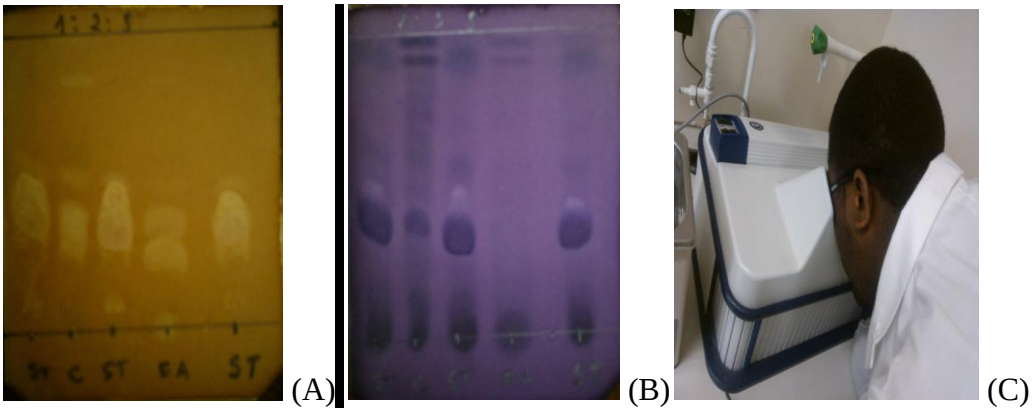
3.8.6 *Test for glycosides (Keller-Kiliani test)*

To a 0.5 g of extract diluted to 5 mL in water was added 2 mL of glacial acetic acid containing one drop of ferric chloride solution. This was underlaid with 1 mL of concentrated sulphuric acid. A brown ring at the interphase indicates the presence of a deoxy sugar, characteristic of cardenolides.

3.9 Visualisation of phytochemicals

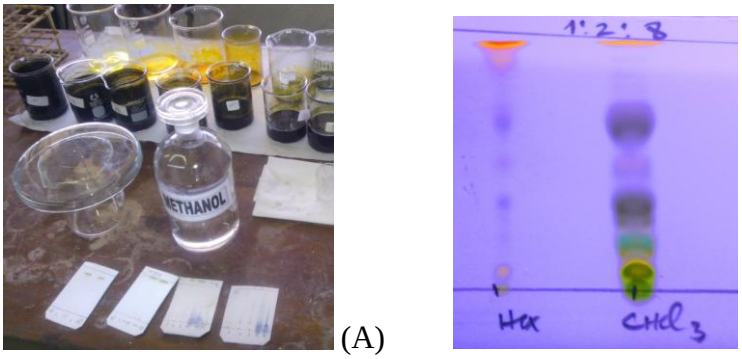
All the extracts were reconstituted in acetone (10 mg/mL) before being analyzed by aluminium-backed thin layer chromatography (TLC) (Merck, silica gel F254). The TLC plates were developed in saturated chambers (covered beakers) using eluent systems of different polarities.

The developed TLC plates were visualized under ultraviolet light and then sprayed with vanillin-sulphuric acid (0.1 g vanillin (sigma), 28 mL methanol and 1 mL sulphuric acid) (Stahl, 1969) and heated in an oven at around 110 °C to detect the phytochemicals (**Figures 3.5 and 3.6**).



(A) (B) (C)

Figure 3.5: Visualization of the chromatogram on TLC, (A) after spraying with vanillin-sulphuric acid, (B) after heating at around 110°C, (C) under the ultraviolet light (254 and 360 nm, Camac universal UV lamp TL-600, H3D, UCT).



(A) (B)

Figure 3.6: (A) Saturated chamber (covered beaker), (B) Chromatogram of hexane and chloroform extracts in the eluent system EtAc-CH_3Cl-Hex (1:2:8).

3.10 Chromatographic methods: general procedures

3.10.1 *Normal column chromatography*

There are two common methods of packing a gravity column: the slurry (wet) method and the dry pack method. In this project, the former method was used. In this method, the sample was dissolved in an appropriate solvent where after silica gel was added. The solvent was removed in vacuo to obtain the dried sample adsorbed on silica gel. The sample, adsorbed on silica, is then poured onto the wet-loaded column. The advantage of the slurry method is that it minimizes chances of formation of air bubbles in the column. The column is then securely fastened in a vertical position (**Figure 3.7**). The mobile phase is added to the column either isocratically or in a gradient fashion to elute compounds in different fractions or groups of fractions (Skoog et al., 2004 and Li et al., 2007).

Figure 3.7: A column chromatography run underway

3.10.2 *Size exclusion or gel filtration chromatography*

Column chromatography (CC) is a technique for separating large amounts of compounds. It relies on the relative polarities of the molecules involved (Li et al.,

2007). In contrary, gel filtration depends on differences in size of molecules for separation. In this method, molecules are eluted in the reverse order of their molecular weight. Big molecules are eluted first, followed by small molecules. In this regard, a biopolymer such as Sephadex LH-20 is used as the stationary phase. It was suspended in MeOH and Chloroform (1:2) and then loaded onto the column. The sample was redissolved in MeOH-Chloroform (1:2) before loading it into the column. The mobile phase was added to the column and fractions were collected using beakers or test tubes (Skoog et al., 2004; Colegate and Molyneux, 2008; Coskun, 2016).

3.11 Fractionation of crude plant material

The powdered leaf material (310 g) of OSM (Acanthaceae) was extracted with 1.6 L of a mixture of MeOH and DCM (v/v) 1:1 for 24 hours. The supernatant layer was filtered through a Whatman N°1 filter paper (**Figure 3.8A**). The extract was then left to dry at room temperature to obtain 25 g of the extract.

Vacuum liquid chromatography (VLC) using silica gel as a stationary phase was chosen to prepare crude extracts using different solvent systems (**Figures 3.8B** and **3.8C**). 70 g of TLC-grade silica gel (Merck) was mixed with hexane and stirred with a stirring rod to form a slurry which was poured onto a sintered funnel. The three quarter-filled sintered funnel was placed over an Erlenmeyer filtration flask connected to water. Vacuum was applied by opening the water tap to compact the silica and hexane was flashed through twice for further compaction. The column was left under vacuum to dry. The sample (25 g), adsorbed on 3 g of silica (slurry), was

introduced on top and a filter paper was used to cover the slurry. Assisted by vacuum, the packed column was flashed with 150 mL of n-Hexane (n-HeX) to remove fats, waxes and some chlorophyll. The column was then flashed with different portions of a mixture of ethyl acetate (EtOAc) and hexane in increasing polarity (0 -100 %). The column was further flashed in a similar fashion with a mixture of EtOAC and MeOH (0 - 50%) as the Flowchart of extraction and fractionations is represented in **Figure 3.9**. In total, 25 fractions were collected (50 mL each). Fractions were mixed according to the results obtained from TLC. The fractions were then left to dry at room temperature and subjected to bioactivity tests later.


(A)

(B)

(C)

Figure 3.8: (A) extraction of the powdered leaf material (310 g) of *OSM*; (B) Collection of fractions *via* VLC; (C) Collection of fractions by VLC.

The following quantities of dried material were obtained in different fractions numbered 1 - 25: 1 - 3 (180 mg), 4 - 6 (3300 mg), 7 and 8 (870 mg), 9 - 11 (650 mg), 12 - 14 (650 mg), 15 (270 mg), 16 and 17 (500 mg), 18 and 19 (425 mg), 20 (3050 mg), 21 and 22 (5100 mg), and 23 - 25 (8500 mg).

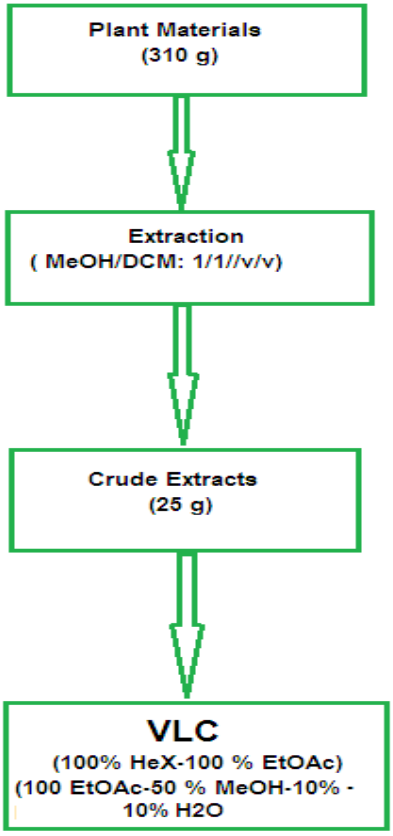


Figure 3.9: Flowchart of extraction and fractionations

A 1 mg sample of each group of fractions was dissolved in 2 mL of Acetone and used for antibacterial testing following the Agar well diffusion method as described in section 3.5.

Fractions 7 and 8 (870 mg) exhibited the highest anti-bacterial activity compared to others. Therefore, these were selected for further investigations and purifications.

3.12 Isolation of antibacterial secondary metabolites from active fractions

A Balloon Pressure Liquid Chromatography (BPLC) was used for purification (**Figure 3.10**). The solvent system used was n-HeX-EtOAc-$CHCl_3$ (5:1:2).

Thirty grams of fine silica gel (TLC-grade) were mixed with *n*-HeX to form a slurry which was poured onto a dried glass tube column. The balloon was inflated with air and then connected to the column to raise the pressure inside the Column. *n*-HeX was eluted to allow silica gel to settle. Fractions 7 and 8 (870 mg) were mixed with 1 g fine silica gel and n-HeX- EtOAc-$CHCl_3$ (5:1:2) to form a slurry and left to dry at room temperature. The slurry was later poured on top of the column and eluted isocratically with a mobile phase of HeX-EtOAc-Chloroform (5:1:2) using pressure from the balloon. Seventeen (17) fractions (30 mL each) were obtained and were mixed according to the chromatogram obtained from TLCs. Fractions 5 to 11 exhibited antibacterial activity therefore were mixed and left to dry at room temperature.

Figure 3.10: Dr Namboole Moses Munkombwe and the BPLC.

Dried extracts from fractions 7, 8 (VLC) and 5-11 (BPLC) were mixed with silica gel and HeX-EtOAc-$CHCl_3$ (5:1:2) to form a slurry which was left to dry at room temperature. Thirty (30) grams of silica gel was used to prepare a column as described previously. Upon loading the column with the sample adsorbed on silica, the column was eluted with HeX-EtOAc-$CHCl_3$ (5:1:2). In total, 62 fractions (20 mL each) were collected. Each eluate was monitored by TLC. Fractions 7-24 showed largely a single violet spot when sprayed with acidified vanillin followed by gentle heating. These fractions also showed antibacterial activity. They were combined and dried, upon which crystals were formed. The crystals were recrystallized from ethanol to give compound **1** (57 mg, white crystals/**Figure 3.11**): Melting Point (m.p.) 134-136 °C and a Retention Value (R_f) 0.55 (EtAc/Hex: 1/3).

Fractions 25 to 29, which also exhibited antibacterial activity, were recombined and left to dry to give compound **2** as white needle-like crystals (8 mg); m.p. 147-149 °C; R_f =*0.58* EtAc/Hex: 1/3).

Figure 3.11: Crystals of compound 1

3.13 Isolation of compounds from non-active fractions

3.13.1 *Compound isolated from fraction 20*

The dried residue obtained from fraction 20 (3050 mg), eluted from preliminary VLC, was subjected to a SEPHADEX-20 column for purification (EtOAc : MeOH, 2 : 1), from which eight fractions were collected (25 mL each). TLC was used to monitor the separation of compounds. Fractions 3 - 5 were mixed as dictated by TLC. The impure material obtained was further subjected to column chromatography over Sephadex (EtOAc-$CHCl_3$-MeOH (4:2:1). In total, 4 fractions (A, B, C and D) were collected (15 mL each) (**Figure 3.12**).

After evaporation of the solvent, yellow crystals formed in fractions C and D (m.p. 289 – 291 °C; R_f. 0.27 (EtOAc-MeOH(2:1)).

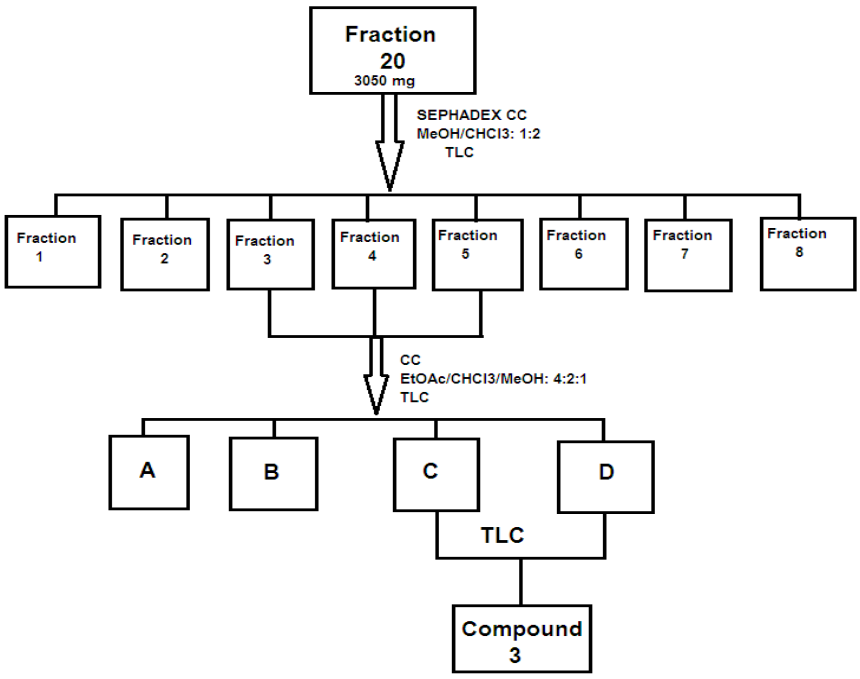


Figure 3.12: Schematic representation of the isolation of compound 3 using SEPHADEX-20 Column Chromatography (EtOAc-MeOH(2 : 1)).

3.13.2 *Compounds isolated from fractions 21 and 22*

Fractions 21 and 22 (5100mg) were redissolved in $CHCl_3$-MeOH (2:1), made into slurry with silica gel, dried and poured onto a SEPHADEX-20 packed column and run with the same solvent system.

Figure 3.13 shows a yellowish spot on the TLC of a pure compound after running the combined fractions (21 and 22) in solvent system: $CHCl_3$ / MeOH(4 : 1). This compound is still under investigations in order to elucidate its structure.

Twelve fractions were collected (25 mL each) and left to dry at room temperature (**Figure 3.14**). Crystals formed in the following fractions: 2 and 3 (yellow crystals, m.p. 289-291 °C), 7 and 8 (yellow crystals, m.p. 301-303 °C). The yellow crystals from fractions 7 and 8 were later purified by dissolving in MEOH and identified as a glucosidic flavonoid (a tiliroside) using physical and spectroscopic methods.

Figure 3.13: Chromatogram obtained from Fractions 21 and 22 (solvent system: $CHCl_3$: MeOH, 4 : 1)

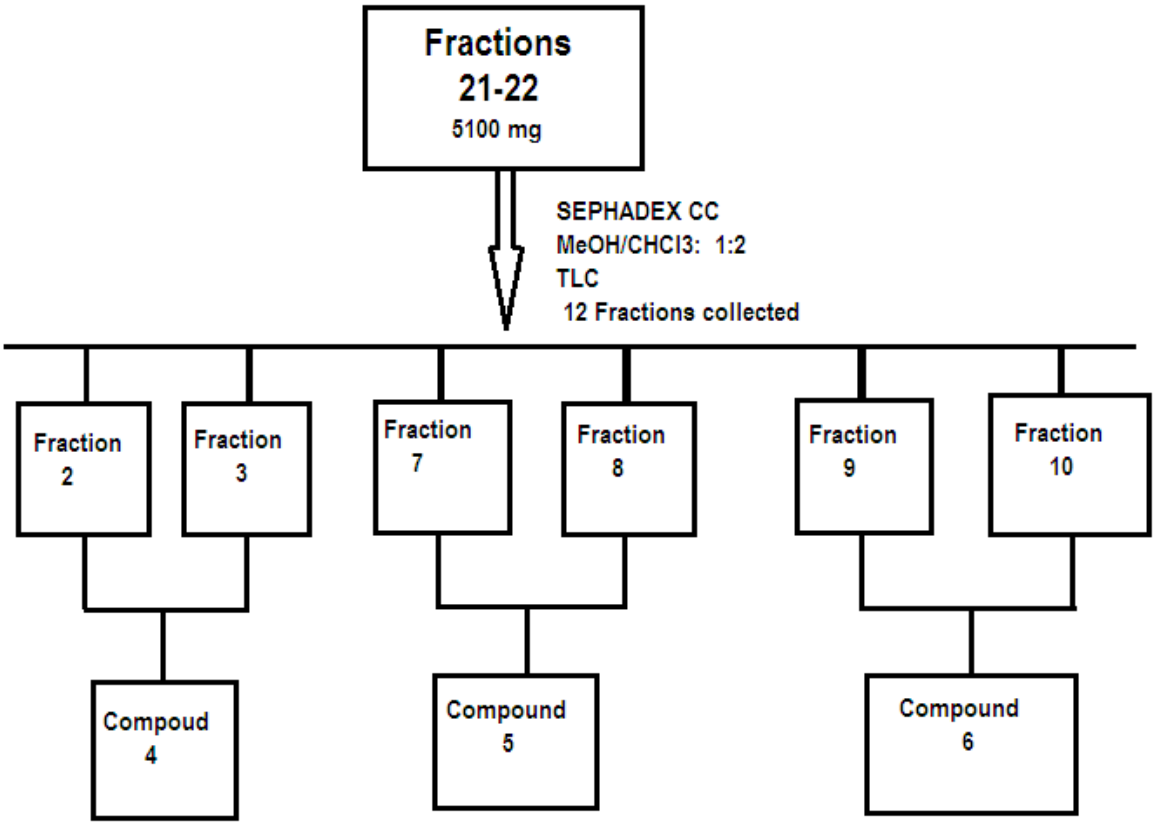


Figure 3.14: Flow chart for isolation of compounds from fractions 21 and 22

3.14 Structure elucidation of the active compounds

All the secondary metabolites isolated from *Odontonema strictum* were analyzed at the University of Cape Town Drug Discovery and Development Centre (H3D), under the supervision of Professor Kelly Chibale. Structures of all the compounds were elucidated under the supervision of Dr. Namboole Moses Munkombwe (UNZA).

3.14.1 Infra-Red analysis

A PerkinElmer, Spectrum 100, FT-IR Spectrometer (**Figure 3.16**) was used for the detection of functional groups with samples analyzed as potassium bromide (KBr)

pellet discs. In this method, 200 mg of KBr (kept in a desiccator) was mixed with 3 mg of compound. The mixture was grounded with an agate mortar and pestle and subjected to a pressure of about 1.575 x 10^5 kgm^{-2} in an evacuated die to produce a clear transparent disc. The prepared disc sample was put into the disc holder and analyzed.

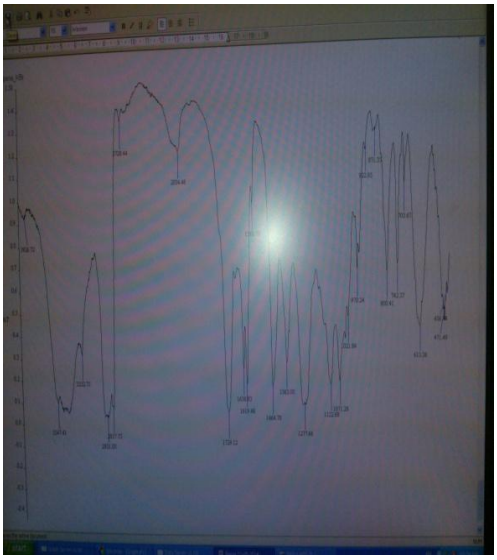

(A)

(B)

Figure 3.15: (A) A PerkinElmer, Spectrum 100, FT-IR Spectrometer (H3D, UCT); (B) IR spectrum of Compound 1.

3.14. 2 NMR analysis

All NMR spectra were acquired in deuterated chloroform ($CDCl_3$) on the 400 MHz spectrometer. Apart from ^{1}H-NMR and ^{13}C-NMR, correlation spectroscopy (COSY) and heteronuclear single quantum coherence (HSQC) spectra were also acquired to facilitate accurate structural elucidation. The interpretation of the spectra was done by Dr. Namboole Moses Munkombwe.

3.15. Minimum inhibitory concentration (MIC) of the active compounds

Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of antimicrobial that will inhibit the visible growth of a micro-organism after overnight incubation (Mazzola et al., 2009; Vipra et al., 2013; Owuama, 2017). The objective of this experiment was to determine the lowest concentration of the isolated active compound and the crude extract at which *S. aureus* cannot produce visible growth after overnight incubation.

Experiments for the determination of the MIC values were conducted at the School of Veterinary Medicine, UNZA, Main Campus under supervision of Professor Hangombe and Mr Mulenga.

3.15.1 *Test Microorganisms*

3.15.1.1 Broth preparation

Broth (4.0 g) was added to distilled water (300 mL) and stirred to a homogenous mixture. The mixture (1 mL each) was put in 16 autoclaving test tubes and sterilized using an autoclave for 15 minutes under pressure (121 °C) then cooled to 40 °C. The test tubes were divided into two batches A1-A8 and B1-B8.

Only one nosocomial gram positive pathogen, *Staphylococcus aureus* (Locally Isolated Organism, LIO), was selected for antibacterial evaluation due to insufficient quantities of the active compound.

The culture was maintained on Mueller-Hilton agar at 4 ºC. The cells were inoculated and incubated at 37 ºC in Mueller-Hilton broth for 12 hours prior to the screening procedure. The density of bacterial culture for use in the screening procedures was 2.6×10^{12} cfu/mL for the species *S. aureus* (Section 3.5).

3.15.1.2 Sample preparation

Sample solutions were prepared in two test tubes. To test tube 1 was added the pure compound (7.56 mg) in 80 % acetone in water (1 mL). To test tube 2 was added the extract (15.23 mg) in 80 % acetone in water (1 mL). Test tube 1 test solution was added to A1 to make a mixture (2 mL) which was recorded as concentration A1. 1 mL of A1 was added to A2 to give a mixture (2 mL) which was recorded as concentration A2. This was repeated all the way to A8.

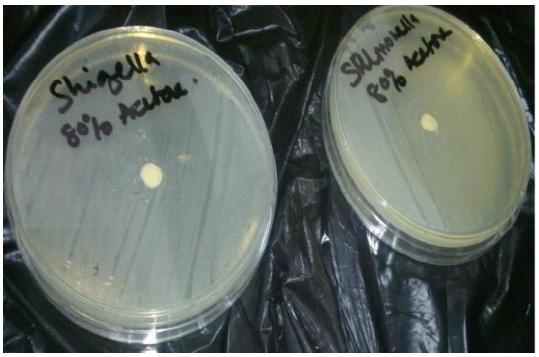
Parallel solutions of the extract were prepared using test tube 2 extract solution from B1 to B8.

S. aureus (LIO from UTH) was provided on a nutrient agar plate. Using a sterile inoculating needle, the bacteria were inoculated in each of the 16 test tubes, picking a different colony each time. The test tube contents were mixed using a vortex machine to ensure an even distribution of the bacteria within the broth. On the next day, the test tubes were observed visually by checking the turbidity. Turbidity increased with a decrease in concentration of the extracts and of the pure compound. The initial dilutions for both compound **1** and the crude extracts were clear. Nutrient agar was then prepared and each petri dish was divided into 4 sections. Each separated section was inoculated with the inoculant of *S. aureus* from each of the 16 test tubes. The plates were then incubated overnight at 37 °C. The following day, the MIC was taken as the lowest concentration that prevents the bacterial visible growth.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Antibacterial activities of selected plant extracts

The evaluation of the antibacterial activity using five bacteria (*Kblesiella pneumoniae*, *Shigela dysenteriae*, *Salmonella typhi*, *E. coli* and *S. aureus*) showed that the most active leaves' extract was that of *OSM* compare to that of *Solanum torvum*, *Symphytum officinale L.*and *Aphelandra squarrosa* comparing the inhibition diameters as reported in **Table 4.1**. No resistance was observed at the concentration of 100 g/mL for all the testing micro-organisms include *Kblesiella pneumoniae* for *OSM*. At the concentration of 1 mg/mL, all the plant extracts exhibited no or less activity. Furthermore, the *OSM* extracts showed relative activity at 10 mg/mL and was the only plant extracts active on *K. pneumoniae*(**Figure 4.2**). Hence the leaves of *OSM* were selected for further investigations. No activity was observed for the negative control (80% Acetone in distilled water) (**Figure 4.1A**).



(A)

(B)

Figure 4.1: (A) No Activity observed for negative control (Acetone 80% in distilled water), (B) Antibacterial activity of Ampicillin, Amoxicillin and Chloramphenicol (35±0.7 mm) against *E. coli*. The first two positive controls are no active.

Table 4.1 Susceptibility tests of different test organisms to different medicinal plant extracts (50 µL/disc) measured as zone of inhibition (ZI) in millimeters

	OSM (Leaves), ZI (mm)			*OSM* (flowers), ZI (mm)			*Aphelandra squarossa*, ZI (mm)			*Solanum torvum*, ZI (mm)			*Symphytum officinale* L, ZI (mm)		
	1 mg/mL	10 mg/mL	100 mg/mL	1 mg/mL	10 mg/mL	100 mg/mL	1 mg/mL	10 mg/mL	100 mg/mL	1 mg/mL	10 mg/mL	100 mg/mL	1 mg/mL	10 mg/mL	100 mg/mL
SA	na	11±1.2	37±1.5	na	13±1.2	26±1.4	Na	15±0.5	17±0.8	na	15±1.1	18±1.1	6±1.1	13±1.2	23±1.1
Ec	na	18±1.3	20±0.9	na	na	na	Na	na	na	na	na	na	na	na	na
SaL	na	Na	12±0.7	na	na	na	Na	15±1.6	18±1.3	na	na	na	10±1.3	17±1.3	20±0.9
ShI	9±0.8	15±1.1	18±1.3	na	17±1.4	37±0.8	11±1.8	14±1.8	17±1.3	na	19±1.3	20±1.6	na	18±1.1	25±1.5
KlB	11±0.8	12±1.3	25±1	na	na	na	Na	na	na	na	na	Na	na	na	na

Inhibition zone diameters are expressed as means ± standard deviation of five measurements to minimize test error; na = no activity; there was no inhibition found in negative control (Acetone). *SA*, *S. aureus*; *Ec*, *E. coli*; *SaL*, *Salmonella typhi*; *ShI*, *Shigella dysenteriae*; *KlB*, *Klebsiella pneumonia*, ZI= Inhibition zone.

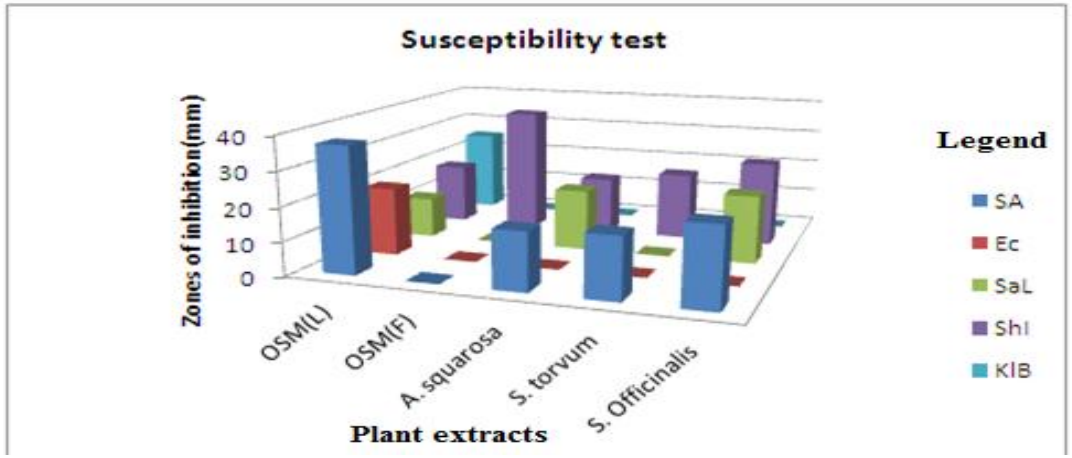


Figure 4.2: Histograms of zones of inhibition (mm) of plant extracts against *S. aureus*, *E. coli*, *S. typhi*, *S. dysenteriae* and *K. pneumoniae* at a concentration of 100 mg/mL. At this concentration, *OSM* is the only plant which exhibited antibacterial activity on all the selected microorganisms.

Positive controls, which are pure compounds, were more potent at the concentration of 0.025 mg/mL compare to the plants extracts. However, Ampicillin and Amoxicillin were not active against *E. coli, S. dysenteriae and K. pneumoniae* (**Figure 4.1B**). According to **Table 4.2**, Tetracycline and Chloramphenicol were active on all the testing bacteria. Chloramphenicol was more potent against four bacterial strains (*S. aureus*(37±1.4 mm), *E. coli*(35±0.7 mm), *S. typhi*(20±0.9 mm) and *S. dysenteriae*(35±1.3 mm)) compare to other positive controls. It should be strongly emphasized on the antibacterial sensitivity exhibited by *OSM leaf* extracts (100 mg/ml) on *S.aureus* (37±1.5 mm), *E.* coli (20±0.9 mm) and *K. pneumonia* (25±1 mm). At that concentration, *OSM* leaf extracts exhibited approximatively equal activity compare to positive controls.

Table 4.2 Zone of inhibition for the positive controls (mm)

Bacterial species	**Antibiotic (0.025 mg/mL)**			
	Chloramphenicol	Ampicillin	Amoxicillin	Tetracycline
S. aureus	37±1.4	35±1.9	21±1.8	40±1.6
E. coli	35±0.7	na	na	13±1.3
S. dysenteriae	35±1.3	na	na	09±1.3
Salmonella typhi	20±0.9	30±2.6	15±1.1	26±1.1
K. pneumoniae	06±0.9	na	na	08±1.1

N.B.: Inhibition zone diameters are expressed as means ± standard deviation of five measurements to minimize test error; na = no activity.

4.1.1 *Activity Index of OSM*

The AI values of *OSM* were determined using chloramphenicol as the standard antibiotic (Equation 2/ Section 3.6). The best results obtained from *OSM* against selected microorganisms were at the concentration of 100 mg/mL and the most potent positive control was Chloramphenicol(0.025 mg/mL). The comparison between the activity of a plant extracts and standards gives an indication how potent is a particular plant for further investigations (Awan et al., 2013**)**. In fact, crude extracts from *OMS* contain different types of secondary metabolites compare to the standard which is a pure compound. The activity exhibited by *OSM* could be the result of a single compound or the result of synergic effect.

The histograms (**Figure 4.3**) of AI reveals that *OMS* has quantitatively the same bactericidal properties as the standard against *S. aureus* and less potent for *Salmonella typhi*. Unexpectedly, *OSM* is for four more potent than the standard on *Klebsiella pneumoniae* as expressed clearly in **Figure 4.4**.

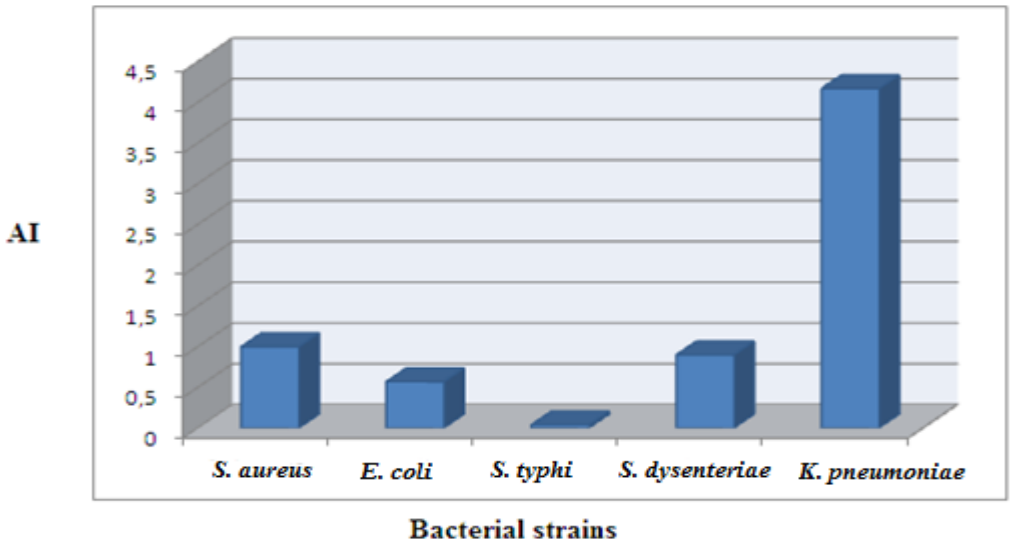


Figure 4.3: Histogram of Activity index (AI) for *OSM* leaf extracts with Chloramphenicol as a standard.

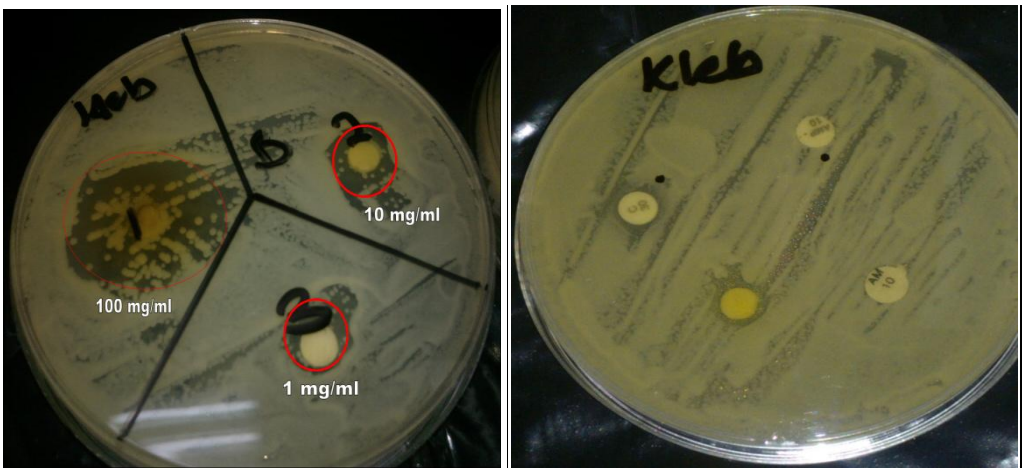


Figure 4.4: Antibacterial sensitivity of *OSM* and Chloramphenicol on *Klebsiella pneumoniae*. The standard was inactive compare to *OSM* which exhited antibacterial activity at the concentration of 1 mg/mL.

4.2 Phytochemical analysis

OSM leaves crude extract was subjected to qualitative phytochemical analysis in order to determine the groups of secondary metabolites which are present in the plant. According to **Table 4.3**, *OSM* contains tannins, saponins, flavonoids (strong presence), phytosterols and glycosides. Alkaloids are present in a very small quantity in the leaves but not in the flowers.

Table 4.3 Chemical constituents in leaf and flower extracts of *OSM*

Chemical Constituents	Tannins	Saponins	Sterols and Triterpenoids	Flavonoids	Alkaloids	Glycosides
Leaves	+++	+++	+	+++	±	+
Flowers	++	+++	+	+++	-	+

Legend: - = absent; ± = low present; + = present; ++ = abundant; +++ = very abundant

The anti-bacterial activity observed earlier may be the result of one compound or synergistic effect arising from a group of secondary metabolites.

4.3 Isolation of the bioactive compounds

The bioguided fractionation of *OSM* extracts revealed that the active molecules were not polar. In other words, the antibacterial secondary metabolites were not soluble in polar solvents. Subjected to the Liebermannn-Bourchard reaction, the active fraction gave a positive indication for steroidal nucleus. The purification of the active fraction (870 mg) led to two phytosterols, compound **1** (57 mg, white crystals; m.p. 134-136 °C; and R_f value 0.55, EtOAc-Hex(1:3) and compound **2** (8 mg, white needle crystals; m.p. 147-149 °C; and R_f value 0.58 (EtAc/Hex: 1/3). However, Compound **2** had exhibited no activity.

4.4 Structure elucidation of Compound 1

The structures of isolated compounds were elucidated using spectroscopic data from IR and NMR.

4.4.1 Infra-Red Spectrum

On subjection to IR spectroscopic analysis (**Figure 4.5**), compound **1** exhibited an absorption band at 3547.41 cm^{-1} that is characteristic of O-H stretching. Other absorption bands included 3232.75 cm^{-1} (cyclic olefinic –HC= CH- stretching), 3025 cm^{-1} (=CH stretching) and 2857.75 cm^{-1} (C-H str), 1638.83 cm^{-1} (C=C stretching, weak) (Pretsch et al., 2000). 1462 cm^{-1} (cyclic $(CH_2)_n$ bend) and 1382 cm^{-1} (–CH_2 (CH_3) stretching). The absorption frequency at 1071.28 cm^{-1} signifies a cycloalkane.

These absorption frequencies resemble the absorption frequencies observed for a phytosterol named Stigmasterol (Grasselli, 1973).

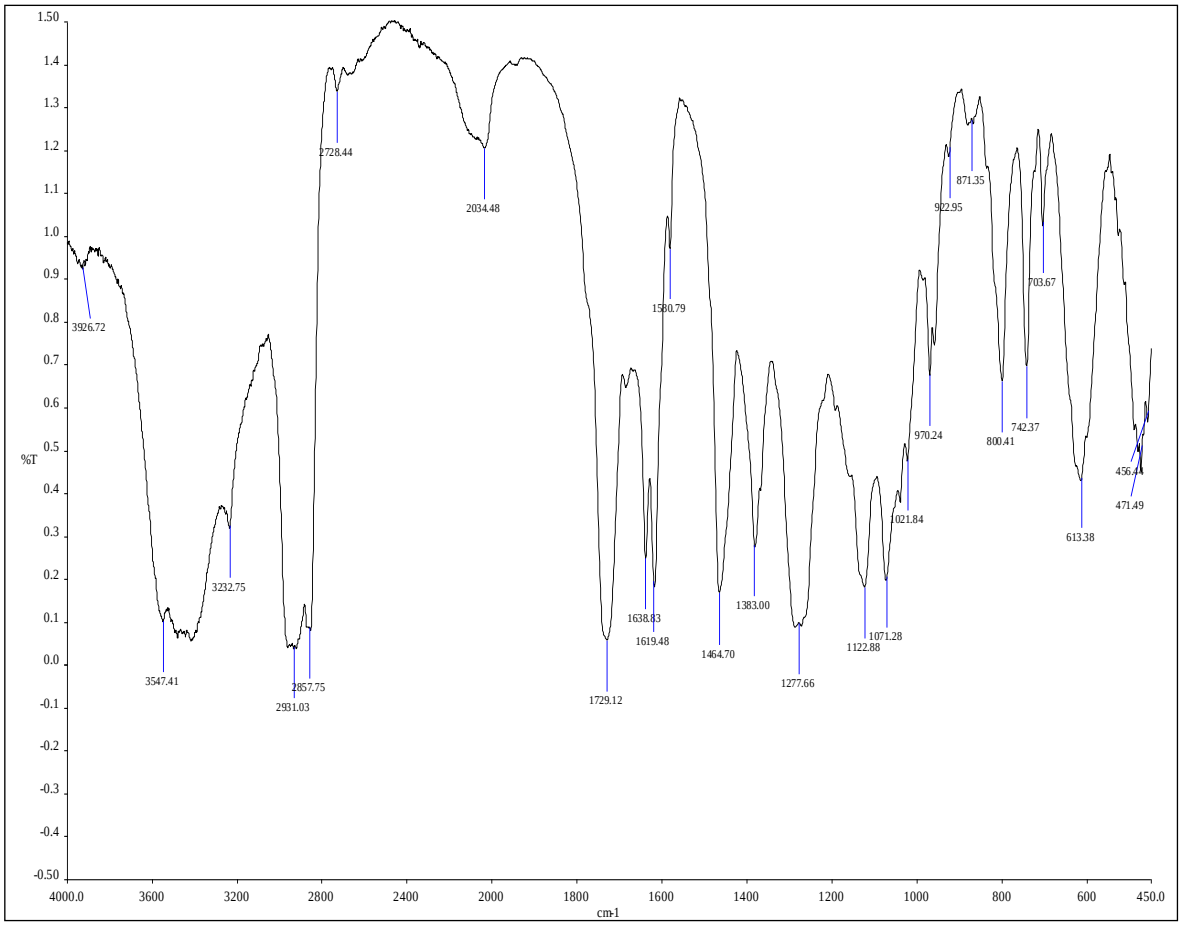


Figure 4.5: IR spectrum of compound **1**

4.*4.4.2. NMR spectrum*

4.4.2.1 ^{1}H NMR of Compound 1

In the ^{1}H NMR spectrum of compound 1 (**Figure 4.6**), peaks are ranging from 0.736 to 5.378 ppm (**Table 4.4**). This spectrum showed the presence of 6 high intensity peaks indicating the presence of six methyl groups at δ 0.736, 0.843, 0.967, 1.037, 1.200 and 1.534 ppm.

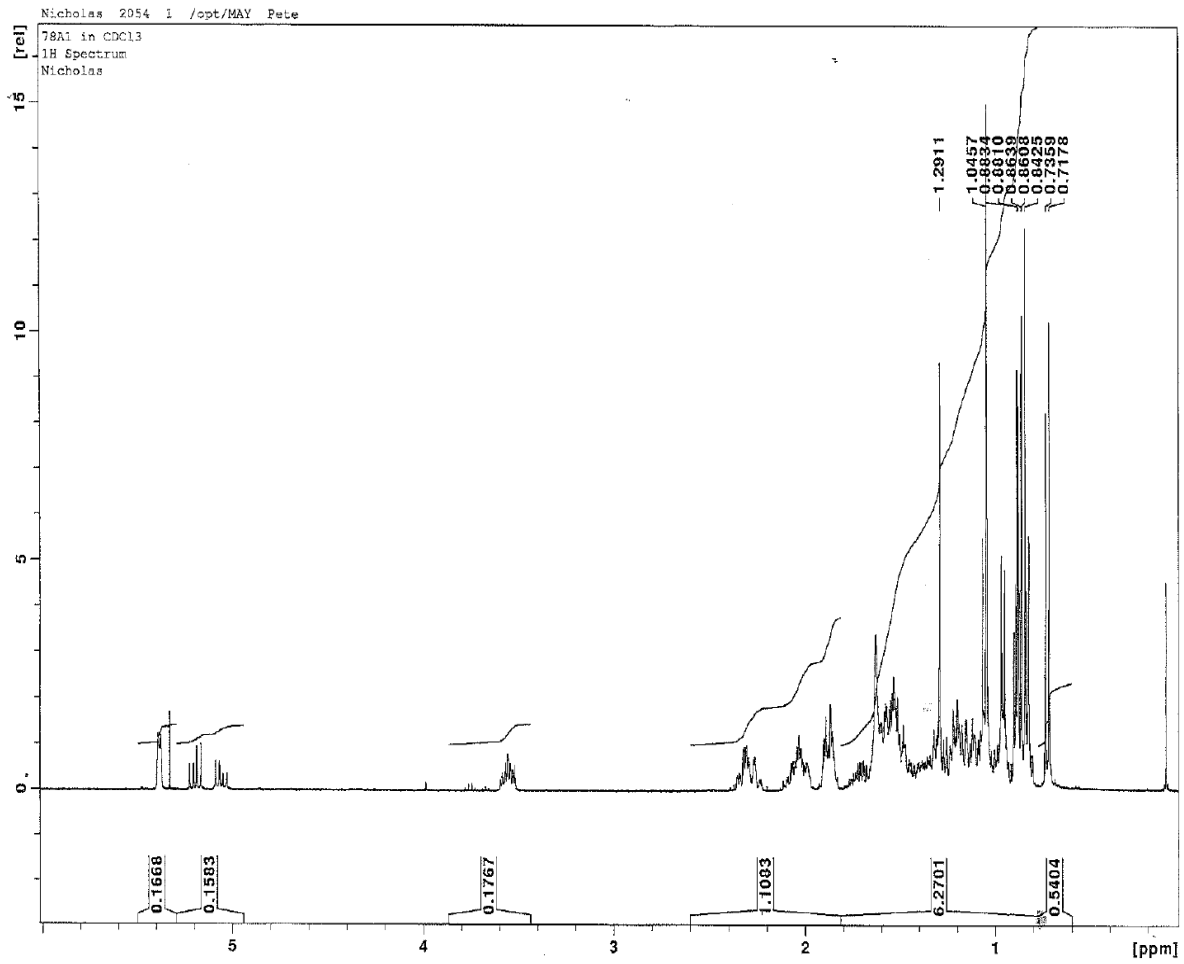


Figure 4.6: ^{1}H NMR spectrum of stigmasterol/beta-sitosterol isolated from *Odontonema strictum*

Proton H-3 of a sterol moiety appeared as a triplet of doublets at δ 3.529 ppm. Peaks at δ 5.197 ppm and δ 5.378 ppm are characteristic of three ethylene protons attached to trisubstituted and disubstituted olefin bonds (Kamboj and Saluja, 2001).

4.4.2.2 ^{13}C NMR of Compound 1

^{13}C NMR spectrum (**Figure 4.8**) had signals at 140.943 and 121.321 ppm for C5=C6 double bond respectively; 71.974 ppm for C-3 substituted with *β*-hydroxyl group;

19.064 and 12.060 for angular methyl carbon atoms for C-19 and C-18 respectively (**Table 4.4**). The signal at 138.404 ppm was attributed to C-22 while C-23 accounted for another signal at 129.341. C-5, C-6, C-22 and C-23 appeared to be alkene carbons. The three quaternary carbons appear at 140.943 and 39.849 ppm(**Figure 4.7**).

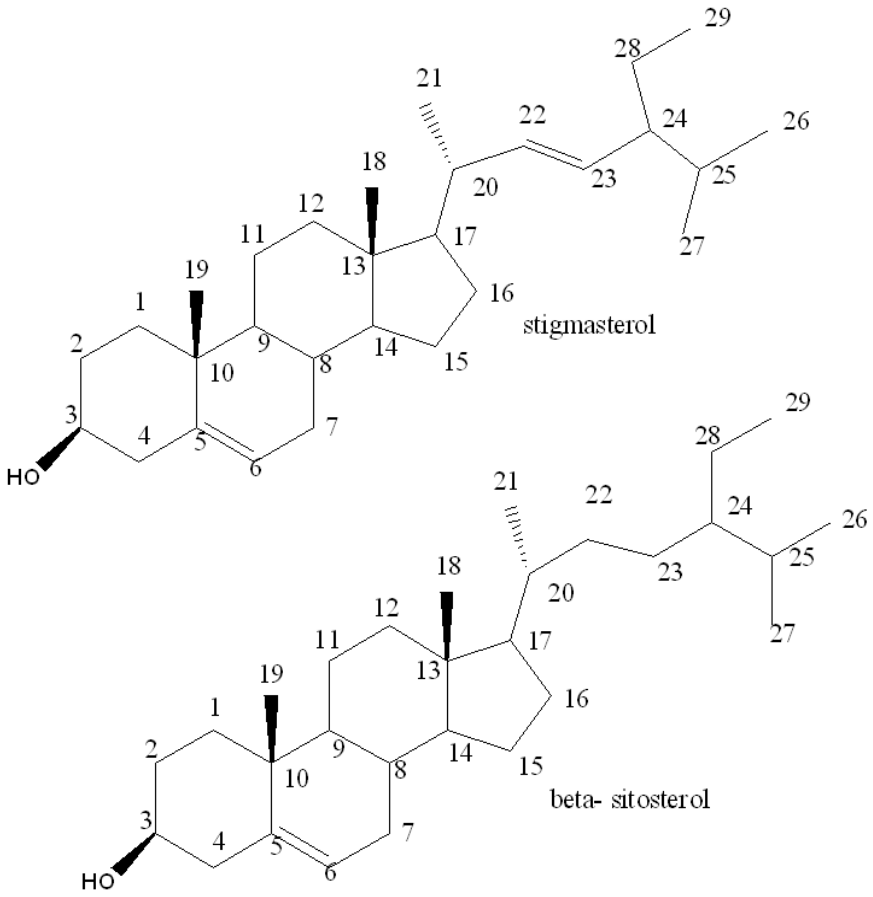


Figure 4.7: Numbered structure of *beta*-sitosterol and Stigmasterol from *OSM*

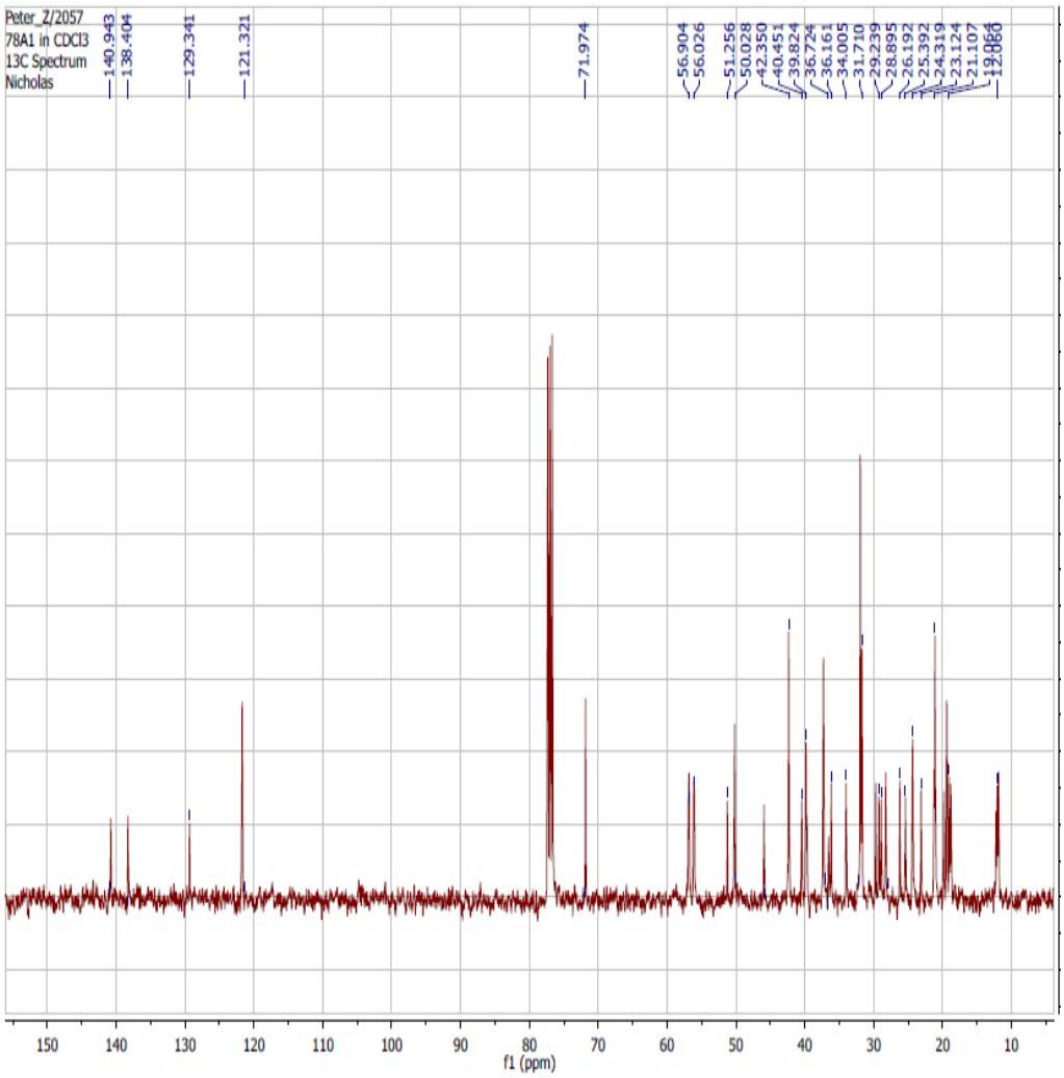


Figure 4.8: ^{13}C NMR spectrum of compound **1**

β-sitosterol and stigmasterol (**Figure 4.7**) are always reported as a mixture with stigmasterol being the major component (Trivedi and Choudhrey, 2011; Ahmed et al., 2013). The only difference between the two compounds is the presence of C22=C23 double bond in Stigmasterol and C22-C23 single bond in *β*-sitosterol, hence, the lack of practical difference in their R_f values.. Furthermore, literature sources have also shown that *β*-sitosterol is difficult to obtain in pure state (Pollock and Stevens, 1965; Kamboj and Saluja, 2011; Pateh et al., 2009).Therefore, compound 1 is not pure but is a mixture of two compounds (1A and 1B) (**Figure**

4.9). Compound 1A has the same R_f value as compound 1B after being run in different solvents systems such as EtOAc : $CHCl_3$: HeX (1:2:5), EtOAc : HeX (1:4), MeOH : $CHCl_3$: HeX (1:2:6). 1B is a colorless needle-like solid with a melting point of 147-149°C.

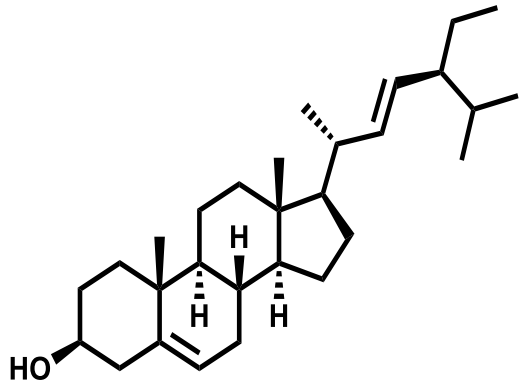
Table 4.4 ^{1}H and ^{13}C NMR chemical shift values for compound 1 recorded in $CDCl_3$ *(400 MHz)*[a].

Carbon atom	^{13}C NMR Experimental chemical shift (ppm)	^{13}C NMR Literature chemical shift (ppm)	^{1}H NMR Experimental chemical shift (ppm)	^{1}H NMR Literature chemical shift (ppm)	Nature of Carbon
C-1	36.72	37.15			CH_2
C-2	29.71	31.56			CH_2
C-3	71.97	71.71	3.53 (tdd, 1H)	3.51 (tdd, 1H)	CH
C-4	42.35	42.19			CH_2
C-5	140.94	140.81			C=C
C-6	121.32	121.62	5.38 (s, 1H)	5.31 (t, 1H)	C=CH
C-7	31.71	31.56			CH_2
C-8	29.24	31.79			CH
C-9	50.03	50.02			CH
C-10	36.16	36.16			C
C-11	24.32	21.12			CH_2
C-12	39.82	39.57			CH_2
C-13	40.45	42.10			C
C-14	56.90	56.76			CH
C-15	24.32	24.27			CH_2
C-16	28.90	28.83			CH_2
C-17	56.03	55.84			CH
C-18	12.06	12.15	1.29 (d, 3H)	1.03 (s, 3H)	CH_3
C-19	19.06	19.88	0.74 (d, 3H)	0.71 (s, 3H)	CH_3

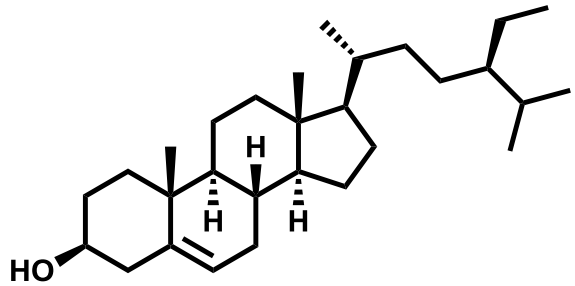
C-20	39.82	40.40-40.51			CH
C-21	23.12	20.99	1.20 (d, 3H)	0.91 (d, 3H)	CH_3
C-22	138.40	138.23	5.07 (m, 1H)	4.98 (m, 1H)	C=C
C-23	129.34	129.16-129.60	5.20 (m, 1H)	5.14 (m, 1H)	C=C
C-24	51.26	51.13-51.30			CH
C-25	34.01	31.94			CH
C-26	21.12	21.23	0.84 (d, 3H)	0.80 (d, 3H)	CH_3
C-27	22.82	19.01	0.97 (d, 3H)	0.82 (d, 3H)	CH_3
C-28	25.32	25.40-25.50			CH_2
C-29	12.06	12.25-25.30	1.04 (t, 3H)	0.83 (t, 3H)	CH_3

a- assignments made on the basis of COSY and HMQC correlations;

(See Appendixes A and D for COSY and HMQC).



Stigmasterol



beta-Sitosterol

Figure 4.9: Structure of *beta*-sitosterol and Stigmasterol from *OSM*

4.5 MIC value of compound 1

After 24 hours of incubation at 37 °C, the lowest concentration of compound 1 and the crude extracts of *OSM* at which no visible growth of *S. aureus* was determined (**Tables 4.5** and **4.6**). For compound 1, the lowest concentration to inhibit the visible growth of bacteria was observed between 3.68 and 1.84 mg/mL (**Figure 4.10**).

Table 4.5 MIC determination for compound 1

compound 1			
Test tube	Concentration (mg/mL)	Observation 1 Solution	Observation 2 Agar
A1	7.56	Clear	No growth
A2	3.68	Clear	No growth
A3	1.84	Cloudy	Growth
A4	0.92	Cloudy	Growth
A5	0.46	Cloudy	Growth
A6	0.23	Cloudy	Growth
A7	0.12	Cloudy	Growth
A8	0.06	Cloudy	Growth

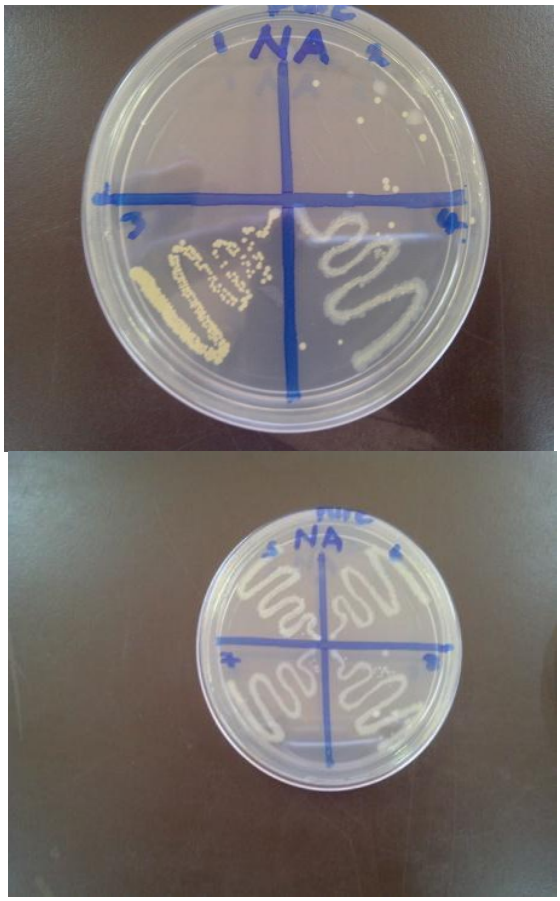


Figure 4.10: MIC determination of compound **1** *via* serial dilution method.

No growth was observed in A1 and A2. A little growth has started in A3. According to table 10, the MIC for the test compound **1** is between A2 and A3 (1.84 to 3.68 mg/mL).

The same procedure was applied for crude extracts and the MIC was found to be at the range of 3.83 and 7.66 mg/mL (**Figure 4.11**).

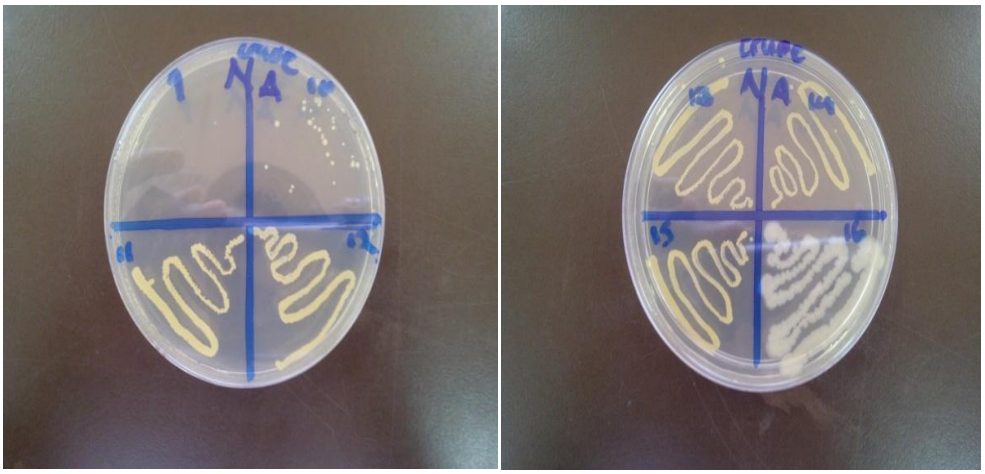


Figure 4.11: MIC determination of the Crude extract *via* serial dilution method

Table 4.6 MIC determination of the crude extract

Crude extract			
Test tube	Syrup (mg/L)	Observation 1 Solution	Observation 2 Agar
B1	15.23	clear	No growth
B2	7.66	cloudy	No growth
B3	3.83	cloudy	Growth
B4	1.92	cloudy	Growth
B5	0.96	cloudy	Growth
B6	0.48	cloudy	Growth
B7	0.24	cloudy	Growth
B8	0.12	cloudy	Growth

4.6. Total Activity (TA)

TA was calculated using the equation 3 (Section 3.7):

$$TA = \frac{\text{Quantity of material extracted from 1 g of plant material in mg}}{\text{MIC in mg/mL}}$$

310 g of plant material gave 25 g of crude extract which represents 8.06 %. Therefore, by extrapolation it was estimated that 0.0806 g of the extract could be obtained from 1g of plant material.

$$TA1 = \frac{80.60 \text{ mg}}{3.83 \text{ mg/mL}} = 21.04 \text{ mL}$$

$$TA2 = \frac{80.60 \text{ mg}}{7.66 \text{ mg/mL}} = 10.52 \text{ mL}$$

This result means 1 g of the plant material can be diluted 10.52 to 21.4 times and it will still inhibit the growth of *S. aureus*.

There was no growth on initial concentrations for both the pure and the crude extracts. Little growth was observed on the second dilutions and it increased on the subsequent sections (**Figure 4.11**). The most growth was observed on the 8th sections for both compound 1 and the crude extracts. From 25 g of dried extract, Fr 7 and Fr 8 (870 mg) gave 57 mg of compound 1 which represents 0.228 %. In 15.23 mg of crude extract used for the determination of MIC, compound **1** represents approximately 0.228% which means 0.0175 mg (17.5 μg). In contrast, compound **1**, in pure form, did not give a better MIC value than the crude extract. Compound **1** has a MIC value of 3.68 mg/mL compared to 0.0175 mg contained in 15.23 mg of the crude extract. In other words, in pure form, compound **1** is 210 less active than the

crude extract. This implies that compound **1** is not the only active compound or possibly there is a synergistic effect. The qualitative phytochemical screening of OSM revealed the presence of many groups of secondary metabolites (flavonoids, terpenoids, steroids, tannins, saponins, glycosides etc) which together may play an essential role in the observed antibacterial activity.

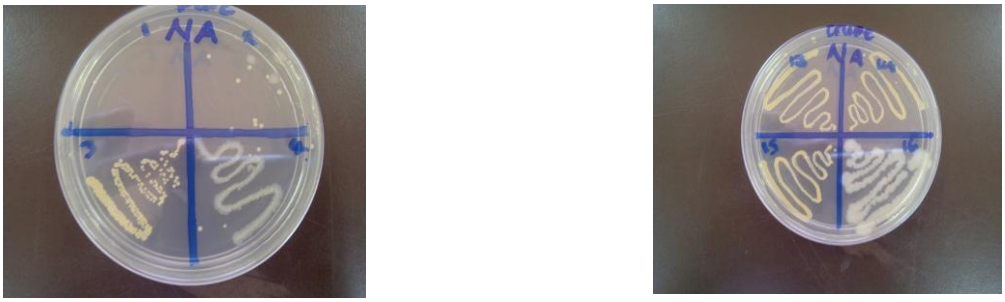


Figure 4.12: MIC determination of compound **1** and crude extract

CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

Three main objectives guided this research: the evaluation of the antibacterial activity of leaf extracts of *OSM*, the isolation, and the characterization of the antibacterial secondary metabolites. Preliminary work was done to compare the antibacterial effects of 4 plants and to identify the opportunity of doing further research. Five bacterial species were selected for testing, namely *Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Salmonella typhi* and *Shigella dysenteriae*. Four bacteria were gram-negative and one was gram-positive. These bacteria are the most known among the most harmful to humans (WHO, 2014). All selected microorganisms were locally isolated at the University Teaching Hospital in Lusaka, Zambia.

After preliminary testing, *OSM* was found to exhibit inhibition of all the selected bacteria at a concentration of 100 mg/mL. In fact, *OSM* exhibited good activity against *S. aureus* (ZI = 37±1.5 mm), *E.* coli (ZI = 20±0.9 mm) and *K. pneumoniae* (ZI = 25±1 mm). The results showed that *OSM* has exhibited equal antibacterial sensitivity as the standard chloramphenicol on *S. aureus*.

25 g of crude extract was obtained after maceration of 310 g of dry plant material of *OSM* (leaves) in MeOH and DCM (v/v), 1:1 for 24 hours. Chromatographic methods were used to separate compounds playing around solvent systems such as HeX, EtOAc, $CHCl_3$ and MeOH. The bioguided fractionation revealed that the

antibacterial secondary metabolites were in fractions 7 and 8 (870 mg). The two fractions were recombined and subjected to further purification. Two compounds with nearly the same R_f value were isolated: compound **1** (m.p. 134-136 °C) and compound **2** (m.p. 147-149 ^{o}C). The interpretation of the spectroscopic data confirmed that compound **1**, the active molecule, was a mixture of stigmasterol and *β*-sitosterol. Compound **2**, a phytosterol, was inactive.

Other compounds were also isolated from other fractions. Compound **3** (m.p. 289-290 °C) and compound **4** (m.p. 301-303 °C). The structure of compound **3** is yet to be elucidated. A tiliroside moiety has been identified in compound **4**. Further interpretations might lead to the complete structural elucidation of this compound.

The bioassays revealed that compound **1** has a MIC value range 1.84 - 3.68 mg/mL and the TA showed clearly that compound **1** contained in 1 g of plant extract can be diluted 10.52 to 21 times and will still be able to inhibit the growth of bacteria. The crude extract is, therefore, more bactericidal than compound **1**. The synergetic effect had been suggested to be responsible for the observed results.

5.2. Recommendations

This present research work was not easy to achieve. We encountered several difficulties. The biggest problem was the lack of some lab facilities. For example, accessibility to the unique rotary evaporator of the Chemistry Department was problematic. Thus, to circumvent the problem of solvent extraction, the protocol was modified by the supervisor, Dr. Munkombwe. The evaporation process was

conducted at room temperature. Consequently, more days were needed to move from one stage of fractionation to another.

Another difficulty encountered pertained to the spectroscopic analysis to characterize the active molecules. In this regard, our study was also inconvenienced by the need to travel to Cape Town to work in the H3D / UCT laboratory led by Prof. Kelly Chibale. Without the contribution of Professor Kelly Chibale and Peter Cheuka, spectroscopic analysis would not be possible.

The insufficient amounts of the pure active compounds did not allow us to perform some bioassays. Thus, we recommend additional experiments with standard pure stigmasterol and sitosterol to evaluate the differences if any between these known compounds and those we isolated from the species *OSM*.

Our project also suffered from lack of adequate funding. Indeed, research in phytochemistry requires a high consumption of solvents some of which are very expensive. Additionally, air travel and accommodation in South Africa required large sums of money. In future, we recommend that a phytochemical project must have substantial funding to achieve the objectives.

The unknown compounds that have been isolated from *OSM* could be of importance to human health. The work to follow is to characterize them and evaluate the antibacterial activity of others.

REFERENCES

Abdul, R.W., M.G. Paulraj, T. Ahmad, A.A. Buhroo, B. Hussain, S. Ignacimuthu, and H.C. Sharma, 2012. Mechanisms of plant defense against insect herbivores. Plant Signal Behav, 7, 1306–1320.

Ahmed, Y., S. Rahman, P. Akhtar, F. Islam, M. Rahman and Z. Yaakob, 2013. Isolation of steroids from n-hexane extract of the leaves of *Saurauia roxburghii*. International Food Research Journal, 20, 2939-2943.

Ajaiyeoba, E.O., 1999. Comparative phytochemical and antimicrobial studies of Solanum macrocarpum and Solanum torvum leaves, Fitoterapia, 70, 184 – 186.

Akinpelu, D.A., O.A. Aiyegoro and A.I. Okoh, 2009. The bioactive potentials of two medicinal plants commonly used as folklore remedies among some tribes in West Africa, African Journal of Biotechnology, 8, 1660-1664.

Anjoo, K. and A.K. Saluja, 2011. Isolation of stigmasterol and beta sitosterol from petroleum ether extract of aerial parts of *Ageratum conyzoides* (asteraceae), International Journal of Pharmacy and Pharmaceutical Sciences, 3, 94-96.

Awan, A.J., M.S. Aslam, C.B. Ahmed and M. Uzair, 2014. In vitro biological evaluation of crude extract of aerial parts of Aphelandra squarossa. Indian Research Journal of Pharmacy and Sciences, 1, 75-79.

Elujoba, A.A., O.M. Odeleye and C.M. Ogunyemi, 2005. Traditional medicine development for medical and dental primary health care delivery system in Africa, Afr. J. Trad. CAM., 2, 46- 61.

Anyinam, C., 1987. Availability, accessibility, acceptability and adaptability: four attributes of African ethnomedicine. Social Science and Medicine, 25, 803-811.

Alan, L.H., 2008. Natural products in drug discovery, Drug Discovery Today, 13, 894-900.

Banfi, E., G. Scialino and C. Monti-Bragadin, 2003. Development of a microdilution method to evaluate Mycobacterium tuberculosis drug susceptibility. J. Antimicrobial. Chemother, 52, 796-800.

Basile, A., S. Giordano, J.A. Lopez-saez and R.C. Cobianchi, 1999. Antibacterial activity of pure flavonoids isolated from mosses. Phytochem., 52, 1479-1482.

Berrondo, L.F., F.T. Gabriel, S.B. Fernandes and F. de S. Menezes, 2003. Dirhamnosyl flavonoid and other constituents from Brillantaisia palisatii. Quim. Nova, 26, 922-923.

Beutler, J.A., 2009. Natural Products as a Foundation for Drug Discovery, Curr Protoc Pharmacol., 46, 1–9.

Boyanova, L., G. Gergova, R. Nikolov, S. Derejian, E. Lazarova, N. Katsarov, I. Mitov and Z. Krastev, 2005. Activity of Bulgarian propolis against 94 Helicobacter pylori strains in vitro by agar-well diffusion, agar dilution and disc diffusion methods. J. Med. Microbiol., Rev., 54, 481-483.

Butler, M.S., 2004. The role of natural product chemistry in drug discovery, Journal of Natural Products. Rev., 67, 2141-2153.

Butler, M.S., A.A. Robertson and M.A. Cooper, 2014.Natural product and natural product derived drugs in clinical trials. Nat Prod Rep., 31, 1612-61

Caballero-George, C. and M.P. Gupta, 2011. A Quarter Century of Pharmacognostic Research on Panamanian Flora: A Review. Planta Med; Rev., 77, 1189–1202.

Chah, K.F., K.N. Muko and S.I. Oboegbulem, 2000. Antimicrobial activity of methanolic extract of Solanum torvum fruit, Fitoterapia, 71, 187 -189,

Chaturvedula, V.S.P., and I. Prakash, 2012. Isolation of Stigmasterol and β-Sitosterol from the dichloromethane extract of *Rubus suavissimus*, International Current Pharmaceutical Journal, 1, 239-242.

Ciccia, G., J. Coussio and E. Mongelli, 2000. Insecticidal activity against *Aedes aegypti larvae* of some medicinal South American plants. J. Ethnopharmacol., Rev., 72, 185-189.

Colegate, S.M. and R.J. Molyneux (ed), 2008. Bioactive Natural Products. Detection, Isolation, and structural determination. Second edition, CRC presse, USA.

Corfull, P. and C. Benned, 1981. Composition containing Stigmasterol for hair treatment. Fr. Demande Fr. Rev., 2, 831.

Coskun, O., 2016. Separation techniques: chromatography. North Clin Istanb., 3, 156-160.

Cseke, L.J., C.R. Lu, A. Kornfeld, P.B. Kaufman and A. Kirakosyan, 2006. How and Why These Compounds Are Synthesized by Plants, Natural Products from Plants, Second Edition, Taylor and Francis Group.

Cunningham, A.B., 1993. African medicinal plants: setting priorities at the interface between conservation and primary health care, People and Plants Working Paper1, UNESCO, Paris, France.

Dahiru, D., E.T. William and M.S. Nadroi, 2005. Protective effects of Ziziphus mauritiana leaf extract on carbon tetrachloride-induced liver injury. Afr. J. Biotech. Rev., 4, 1177-1179.

Dätwyler, P., H. Bosshardt, H.O. Bernhard, M. Hesse and S. Johne, 1978. D. Struktur des Spermin-Alkaloides Aphelandrin aus Aphelandra squarrosa NEES. 170. Mitteilung über organische Naturstoffe.Helvetica, 61, 2646–2671.

Department of Health and Human Services, 2010. Phytosterols and Risk of Coronary Heart Disease; Proposed Rule. Federal Register 75, 76526-71.

Dewick, P.M., 2002. Medicinal Natural Products, A Biosynthetic Approach, Second Edition, John Wiley & Sons.

Durkee, L.H., 1986. Flora Costaricensis: *Acanthaceae*. Fieldiana Botany, 18, 1-87.

Duri, J. Z., 2009. The status of traditional medicinal plant practice in the miombo eco-region of Southern Africa: Zimbabwe Case Study. Consultancy report prepared for WWF Southern Africa Regional Programme Office. Harare. Zimbabwe.

Eloff, J.N., 1999. A sensitive and quick method to determine the minimum inhibitory concentration of plant extracts for bacteria. Plant Medica, 60, 1-8.

Eloff, J.N, 1998. Which extractant should be used for the screening and isolation of antimicrobial components from plants? J. Ethnopharmacol., 1, 1-8.

Eloff, J.N., J.O. Famakin and D.R.P Katerere, 2005. Isolation of antibacterial stilbene from *Combretum woodii* (Combretaceae) leaves. Afr. J. Biotechnol. Rev., 4, 1167-1171.

Elujoba, A.A., O.M. Odeleye and C.M. Ogunyemi, 2005. Traditional medicine development for medical and dental primary health care delivery system in africa. African Journal. Traditional, Complementary and Alternative Medicines. Rev., 2, 46-61.

Exner, M., S. Bhattacharya, B. Christiansen, J. Gebel, P. Goroncy-Bermes, P. Hartemann, P. Heeg, C. Ilschner, A. Kramer, E. Larson, W. Merkens, M. Mielke, P. Oltmanns, B. Ross, M. Rotter, R.M. Schmithausen, H.G. Sonntag and M. Trautmann, 2017. Antibiotic resistance: What is so special about multidrug-resistant Gram-negative bacteria? GMS Hyg Infect Control., 12, Doc (5).

Farnsworth, N.R., 1984. Recovery and susceptibility testing of Mycobacterium tuberculosis from extra pulmonary specimens by the BACTEC radiometric method. J. Clin. Microbiol. Rev., 19, 720-721.

Fieser, L.F. and M. Fieser, 1962. Organic Chemistry 3rd Ed. Wiley New York.

Geoffrey, C. and M. Kirby, 1996. Medicinal plants and the control of protozoa disease with particular reference to malaria. Transaction of the Royal Society of Tropical Medicine and Hygiene. Rev., 90, 605-609.

Geone, M.C. and A.F. de C. Alcântara, 2012. Chemical constituents and biological activities of species of Justicia - a review, Brazilian Journal of Pharmacognosy. Rev., 22, 220-238.

Gibbons, S., 2004. Anti-staphylococcal plant natural products, Nat. Prod. Rep. Rev., 21, 263–277.

Giovannini, P.H., M.X.Yoma and X. Tienda, 2008. Interface between traditional and modern medicine among the Mazatecs of Oaxaca, Mexico, Journal of Ethnopharmacology. Rev., 10, 1012-1016.

Gomes, M.F.P.L., Cristina de Oliveira M., J. G. Xavier, L.V. Bonamin, 2007. Comfrey (*Symphytum Officinale L.*) and experimental hepatic carcinogenesis: A short-term carcinogenesis model study, e CAM, 1-6.

Good, C.M. and Kimani, V.N., 1980. Urban traditional medicine: A Nairobi case study. East African Medical Journal. Rev., 57, 301 - 316.

Gupta, S.S., 1994. Prospects and perspectives on natural plant products in medicine. Ind. J. Pharmacol.Rev., 26**,** 1-2.

Harvey, A.L. 2008. Natural products in drug discovery, Drug Discovery Today,13, 19-20

Habib, M.R., F. Nikkon, M.E. Rahma and M.R. Karim, 2007. Isolation of stigmasterol and beta sitosterol from methanolic extract of root of bark of Calotropis gigantean (Linn). Pak. J. Biol. Sci. Rev., 10, 4174-4176.

Hostetmann, K. and A. Martson, 2002. Twenty years of research into medicinal plants: results and perspectives. Phytochem. Rev., 1, 275- 285.

Hsueh, P.R. and K.T. Luh, 2002. Antimicrobial Resistance in Streptococcus pneumoniae, Taiwan. SYNOPSIS, Emerging Infectious Diseases, 8, 1487-1491.

IPGRI, 2002. Regional Report Sub-Saharan Africa 1999–2000. International Plant Genetic Resources Institute, Rome, Italy.

Karan, V. and V. Kumar, 2004. Compendium of Medicinal and Aromatic Plants AFRICA, volume 1. ICS-UNIDO.

Khalili, R.M.A., A.H. Norhayati, E.S. Shafekh, M.G. Fazari , M. Azlina, T. Zahili and A.H.A. Zubaidi, 2012. Screening of seven types terengganu herbs for their potential antibacterial activity against selected food microorganisms, Borneo science, 31, 9-23.

Kini, F., A.Saba, M. Titis, L. Angbnot and P.I. Guissou, 2008. Analyse par chromatographie et par spectrométrie électronique des extraits de feuilles de *Odontonema strictum* (Acanthaceae). Mise en évidence de flavonoïdes du type flavone. Journal de la Société Ouest-Africaine de Chimie. Rev., 25, 117-121.

Kuete, V. (ed), 2013. Medicinal Plant Research in Africa. Pharmacology and Chemistry. Academic Press, Elsevier.

Kuete, V. (ed), 2017. Medicinal spices and vegetables from Africa: Therapeutic Potential against Metabolic, inflammatory, Infectious and Systemic Diseases. Academic Press, Elsevier.

Lemke,T.L., D.A. Williams, V.F. Roche and S. W. Zito, 2008. Foye's principles of Medicinal Chemistry, 6e Edition.

Li, J. J., C. Limberakis and D.A. Pflum, 2007. Modern Organic Synthesis in the Laboratory, A Collection of Standard Experimental Procedures. Oxford University Press.

Lucinda, A.M. and M.L. Moody, 1999. Phylogenetic relationships among Acanthaceae: Evidence from Noncoding TRNL-TRNF chloroplast DNA sequences. American Journal of Botany. Rev., 86, 70–80.

Maiti, P.C., S. Mookherjea, R. Matew and S.S. Dan (1979). Studies on Indian Solanum I. Alkaloid Content and Detection of Solasodine. Economic Botany 33, 75-77.

Mahomoodally, M.F., 2013. Traditional Medicines in Africa: An Appraisal of Ten Potent African Medicinal Plants, Evidence-Based Complementary and Alternative Medicine,Volume 2013, Article ID 617459.

Maroyi, A., 2017. Elephantorrhiza elephantina: Traditional Uses, Phytochemistry, and Pharmacology of an Important medicinal Plant species in Southern Africa. Evidence-Based Complementary and Alternative medicine, volume 2017.

Mazzola, P.G., A.F. Jozala, L.C.de Lencastre Novaes, P. Moriel and T.C. Vessoni Penna, 2009. Minimal inhibitory concentration (MIC) determination of disinfectant and/or sterilizing agents. Brazilian Journal of Pharmaceutical Sciences, 45, 241-248.

Mwitwa, J., 2009. The status of traditional medicinal plant practice in the miombo eco-region of southern Africa: Zambia Case Study. Consultancy report prepared for WWF Southern Africa Regional Programme Office. Lusaka. Zambia.

Mohanasundari, C., D. Natarajan, K.Srinivasan, S.A. Umamaheswari and A. Ramachandran, 2007. Antibacterial properties of Passiflora foetida L. – a common exotic medicinal plant. Afr. J. Biotech**.** Rev., 6, 2650-2653.

Moronkola, D.O., 2012. Developments in Phytochemistry. In: Drug Discovery Research in Pharmacognosy, Prof. Omboon, V., and S. M. Olimat (Ed.), 11-21, Croatia, Intech.

Mudondo, C.M., 2000. The role of traditional medicine in the health system: Zambian situation, Paper presented at African forum on "The role of Traditional Medicine in Health Systems", 16-18 Feb., Harare, Zimbabwe.

Ndebia, E.J., R. Kamga and B. Nchunga-Anye Nkeh, 2007. Analgesic and anti-inflammatory properties of aqueous extract from leaves of Solanum torvum (Solanaceae), AJTCAM, 4, 240–244.

Ndip, R.N., A.E.M. Tarkang, S.M. Mbullah, H.N. Luma, A. Malongue, L.M. Ndip, K.W.C. Nyongbela and S.M.N. Efange, 2007. In vitro anti- Helicobacter pylori activity of extracts of selected medicinal plants from North West Cameroon. J. Ethinopharmacol. Rev., 114,452-457.

Neagu, E., G. Paun and L. G. Radu, 2011. Phytochemical study of some symphytum officinalis extracts concentrated by membranous procedures. U.P.B. Sci. Bull., Series B, 73, 65-74.

Nogueira, C.R. and L.M.X. Lopes, 2011. Antiplasmodial Natural Products, Molecules. Rev., 16, 2146-2190.

Nostro, A., M.P. Germanŏ, D'Angelo V, A arino and M.A. Cannattelli, 2000. Extraction methods and bioautography for evaluation of medicinal plants antimicrobial activity. Lett. Appl. Microbiol. Rev., 30, 379-384.

Nswana, A., 1996. Preliminary study on cosmetic and traditional medicine in Central, Copperbelt and Luapula provinces.

Nyenje, M. and R.N. Ndip, 2011. In-vitro antimicrobial activity of crude acetone extract of the stem bark of Combretum molle against selected bacterial pathogens, Journal of Medicinal Plants Research. Rev., 5, 5315-5320.

Oberlies, H. N., N. C Kim, D. R. Brine, B. J. Collins, R. W. Handy, C. M. Sparacino, C. Wani, Mansukh and Wall E. Monroe, 2007. "Analysis of herbal teas made from the leaves of comfrey (Symphytum officinale): Reduction of N-oxides results in order of magnitude increases in the measurable concentration of pyrrolizidine alkaloids". Public Health Nutrition., 7, 919–24.

Okpuzor, J., O. Adebesin, H. Ogbunugafor and I. Amadi, 2008. The potential of medicinal plants in sickle cell disease control: A review. International Journal of Biomedical and Health Sciences. Rev., 4, 122-123.

Ouedraogo, S., F. Kini, L. Serme, J. B. Nikiema, A. Traore, P. I. Guissou, M. Ndiaye, B. Bucher and R. Andriantsitohaina, 2005. Assessment of the hypotensiveand vasodilator effects of extract and fractions from *Odontoneme strictum* (Acanthaceae). Ethnopharmacologia. Rev., 36, 74-77.

Owuama, C.I., 2017. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using a novel dilution tube method.African Journal of Microbiology Research, 11, 977-980.

Panda, S., M. Jafri, A. Kar and B.K. Meheta, 2009. Thyroid inhibitory, antiperoxidative and hypoglycemic effects of stigmasterol isolated from *Butea monosperma*. Fitoterapia. Rev, 80, 123–126.

Pateh, U. U., A.K. Haruna, M. Garba, I. Iliya, I.M. Sule, M.S bubakar and A.A Ambi, 2009. Isolation of stigmasterol, b-sitosterol and 2-hydroxyhexadecanoic acid methyl ester from the rhizomes of stylochiton lancifolius pyer and kotchy (Araceae), Nig. Journ. Pharm. Sci., March. Rev., 8, 19 – 25.

Patwardhan, B., G. Mutalik and G. Tillu, 2015. Intergrative Approaches for Health. Biomedical Research, Ayurveda and Yoga. Elsevier.

Perales, Y.J. and M. Leysa, 2012. Phytochemical Screening and Antibacterial Acitivity of Bougainvillea Glabra Plant Extract as Potential Sources of Antibacterial

and Resistance-modifying Agents. International Conference on Life Science and Engineering, 45, 121-125.

Pérez-Amador, M.C., V.M. Ocotero, J.M. García Castañeda and A.R. González Esquinca, 2007.Alkaloids in *Solanum torvum Sw* (Solanaceae),International Journal of Experimental Botany, 76, 39-45.

Phillipson, J.D., 1999. New drugs from nature It could be yew. Phytother Res., 13, 2-8.

Phillipson, J.D., 2001. Phytochemistry and medicinal plants. Phytochemistry, 56, 237-43.

Pollock, J.R.A. and R.S. Stevem (ed.), 1965. Dictionary of organic compounds 4th ed., vol. 5, Eyre and spottiswoode (Publishers) Ltd.

Po-Ren Hsueh and Kwen-Tay Luh, 2002. Antimicrobial Resistance in *Streptococcus pneumoniae*, Taiwan, Emerging Infectious Diseases. Rev., 8, 56-61.

Pye, C.R., M.J. Bertin, R. S. Lokey, W.H. Gerwick and R.G. Linington, 2017. Retrospective analysis of natural products provides insights for future discovery trends, PNAS, 114, 5601-5606.

Rabia, N., A. Bano, Y. Humaira, Samiullah and Uzma Farooq, 2011. Antimicrobial potential of the selected plant species against some infectious microbes used. Journal of Medicinal Plants Research. Rev., 5, 5247-5253.

Rabia, N. and A. Bano, 2012.Antimicrobial potential of *Ricinus communis* leaf extracts in different solvents against pathogenic bacterial and fungal strains. Asian Pac J Trop Biomed, 2, 944-947.

Refaey, M.S., A.M.M. Hassanein, M.A.H. Mostafa, A.M. Nafady and A.A. Ali, 2015. Botanical Studies of The Aerial Parts of Odontonema cuspidatum (Nees)

Kuntze, Family Acanthaceae, Cultivated in Egypt, Pharm. Sci. & Res. Vol. 7, 1076-1089.

Rahman, A.H.M.M., Biswas, M.C., Islam, A.K.M.R. and Zaman, A.T.M.N. (2013): Assessment of Traditional Medicinal Plants Used by Local People of Monirampur Thana under Jessore District of Bangladesh. Wudpecker Journal of Medicinal Plants. Nigeria, 2, 099-109.

Rambeloson, V.H.V., L.H. Rasoanaivo , A. Wadouachi and A. Raharisololalao, 2014. A new triterpene and stigmasterol from *Anthostema madagascariense* (Euphorbiaceae), International Journal of Chemical Studies, 1, 42-48.

Ragasa, C.Y., E.C.S. Javier and I.G.Tan, 2003. Antimutagenic Terpenes and Sterol from Vitex parviflora.Philippine Journal of Science. Rev., 132, 21-25.

Rang, H.P., M.M. Dale, J.M. Ritter and R.J. Flower, 2007. Rang and Dale's pharmacology. Churchill, Livingstone. Elsevier, 6th Edition.

Sarker, S.D., Z. Latif and A.I. Gray, 2006. Natural Products Isolation. An Overview .Second edition,Humana Press,Totowa, New Jersey.

Schilke, J.L., 2009. Artemisinin-based combination therapy (ACTs) drug resistance trends in Plasmodium falciparum isolates in Southeast Asia". Graduate School Theses and Dissertations, University of South Florida.

Schmelzer, G.H., A. Gurib-Fakim, R. Arroo, C.H., Bosch, A de Ruijter and M.S.J. Simmonds, 2008. Plant Resources of Tropical Africa 11(1) – Medicinal Plants 1. Backhuys Publishers, Wageningen, Netherlands.

Schulz, V., Hänsel, R. and V.E. Tyler, 2001. Rational Phytotherapy. A Physician's Guide to Herbal Medicine, 4th Ed., Berlin, Springer-Verlag.

Shivani, J., D. Mishra, K.S. Khetwal and G. Bisht, 2012. Antimicrobial evaluation of Buddleja asiatica Lour. Leaves and flowers extract, Research Journal of Phytochemistry. Rev., 6, 92-95.

Skoog, D.A., D.M. West, F.J. Holler, S.R. Crouch, 2004. Fundamentals of Analytical Chemistry,Eighth Edition.THOMSON Brooks/Cole, Belmont, U.S.A.

Soodabeh, S., A. Manayi, A.R. Gohari and M. Abdollahi, 2014. The Story of Beta-sitosterol- A Review. European Journal of Medicinal Plants. Rev., 4, 590-609.

Stahl, E., 1969. Thin Layer Chromatography, 2nd Edn. Springer-Verlag. New-York.

Sumathi, S., S. Senthil kumar, V. Bharathi and S. Sahaya Sathish, 2011. Antibacterial Activity of the plant extract of *Symphytum officinale L*. against selected Pathogenic Bacteria, International Journal of Research in Pharmaceutical Sciences, 2, 92-94.

Suparb, B. and P. Amorn, 1991. Chemical constituent of the roots of Birdelia Tomentosa BL., J. Sci. Soc. Thailand. Rev.,1, 61-69.

The EFSA Journal, 2008. Plant Sterols and Blood Cholesterol. Rev. 781, 1-12.

Lemke, T.L., D.A. Williams, V.F. Roche and S.W. Zito, 2008. Foye's principles of Medicinal Chemistry, 6e Edition.

Trease and Evans, 1996. Pharmacognosy. 14th Revised. W.B. Sounders Company limited, London, 545- 546.

Ulrich-Merzenich, G., D. Panek, H. Zeitler, H. Velter and H. Wagner, 2010. Drug development from natural products: exploiting synergestic effects. A review article, Indian Journal of experimental Biology, Rev., 48, 208-219.

Vasisht K., and V. Kumar, 2004. Compendium of Medidincal and aromatic plants. Volume 1: Africa. ICS-UNIDO, Trieste, 23–56.

Vijayakumari, P., K.Shanthi , Bharathi K, Kayalvizhi J, Muruganandham G, Thirumurugan V and Sethuraman M., 2012. Studies on the physico-phytochemical and Anti-diabetic properties of Cissus quadrangularis L and Solanum torvum swartz. International J of drug discovery and herbal research, 2, 323-328.

Vipra, A., S.N. Desai, R.P. Junjappa, P. Roy, N.Poonacha, P.Ravinder, B. Sriram and S. Padmanabhan, 2013.Determining the Minimum Inhibitory Concentration of Bacteriophages: Potential Advantages.Advances in Microbiology, 3, 181-190

Vogl, S., Picker, Paolo; Mihaly-Bison, Judit; Fakhrudin, Nanang; Atanasov, Atanas G.; Heiss, Elke H.; Wawrosch, Christoph; Reznicek, Gottfried; Dirsch, Verena M.; Saukel, Johannes; Kopp and Brigitte , 2013. "Ethnopharmacological in vitro studies on Austria's folk medicine — An unexplored lore in vitro anti-inflammatory activities of 71 Austrian traditional herbal drugs". Journal of Ethnopharmacology, 149, 750–71.

Wiart, C., S. Mogana, S. Khalitah, M. Mohan, S. Ismail and M. Buckle, 2004. Antimicrobial screening of plants used for traditional medicine in the state of Perak Peninsular Malayasia: Fitoterapia January, 75: 68-73.

Watt, J.M. and M.G. Breyer-Brandwijk, 1962. Medicinal and Poisonous plants of southern and eastern Africa: Edinburgh E and S Livingstone.

Wasshausen, D.C., 1978. The Genus *Aphelandra* (Acanthaceae), Smithsonian Institution Press, City of Washington.

World Health Organization, 1993. Regional Office for Western Pacific, Research Guidelines for Evaluating the Safety and Efficacy of Herbal Medicines. Manila.

World Health Organization, 2014. Antimicrobial Resistance, Global Report on surveillance, 1211 Geneva, Switzerland.

World Health Organization, 2015. Antibiotic Resistance: Multi-Country public awareness survey. 1211 Geneva, Switzerland.

Woldeyes, S., A. Legesse, T. Yinebeb, M. Diriba and B. Tadesse, 2012. Evaluation of Antibacterial Activities of Compounds Isolated From *Sida rhombifolia Linn*. (Malvaceae), Natural Products Chemistry & Research. Rev., 1, 114-116.

Liang, X.T and W.S. Fang(ed), 2006. Medicinal Chemsitry of Bioactive Natural Products, A John Wiley & Sons, Inc., publication.

Zhang, L. and A.L. Demain (ed), 2005. Natural Products, Drug Discovery and Therapeutic Medicine; Humana Press.

Zongru, G., 2016. Artemisinin anti-malarial drugs in China, Acta Pharm Sin B., 6, 115–124.

Web Sites

<http://www.euromonitor.com/herbal-traditional-products-in-the-us/report> [Accessed 01 July 2014].

<https://clinicaltrials.gov/ct2/show/NCT02941159> [Accessed 04 December 2017].

<https://www.scribd.com/doc/147098125/Medicinal-Plants-Rural-Resource-Pack-Netherlands> [Accessed 23 July 2015].

<http://www.anniesremedy.com/chart.php?use_ID=192 Medicinal Herbs From African Traditions> [Accessed 12 May 2014].

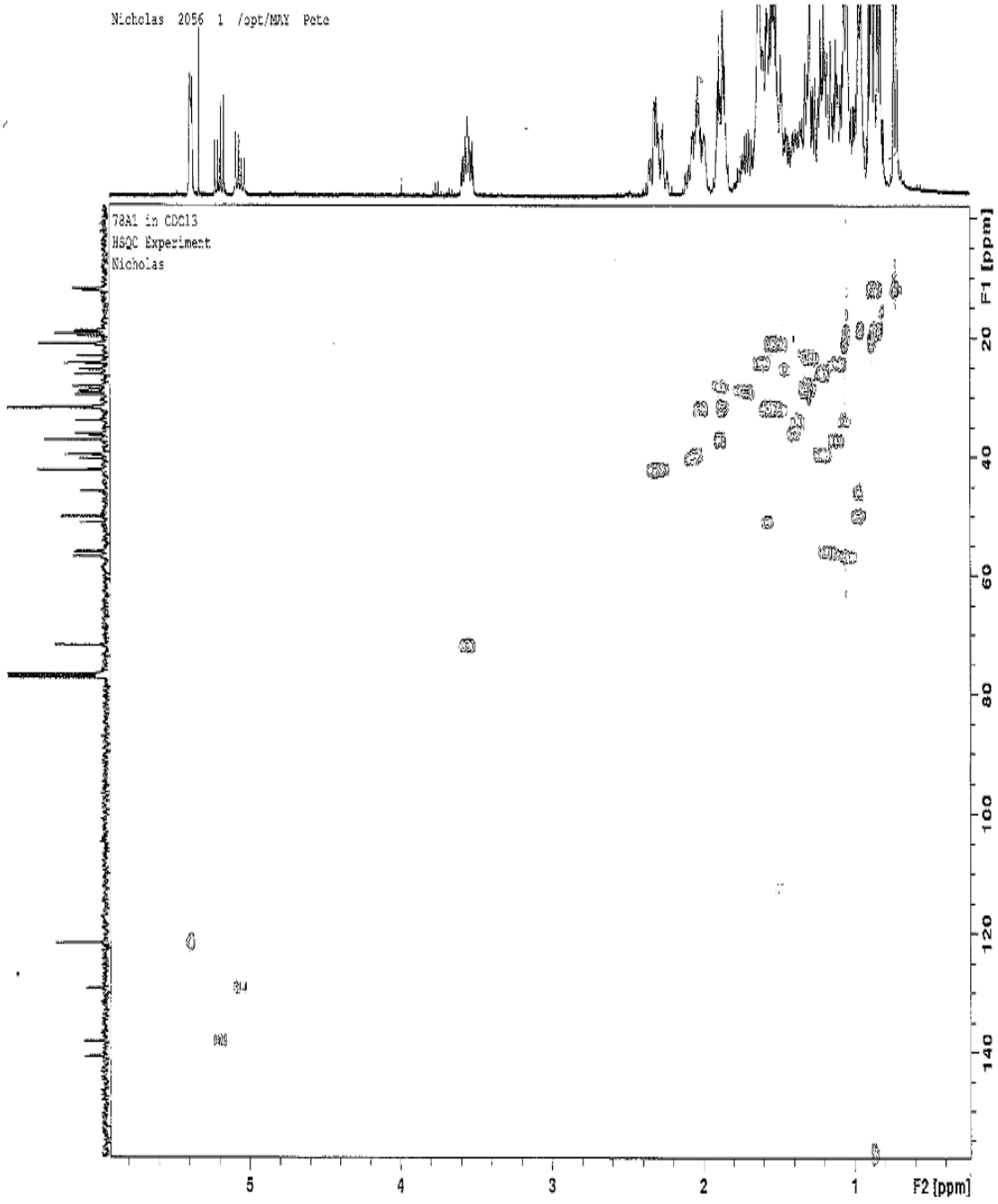
<www.Wikipedia.com> [Accessed 16 April 2014].

<http://cms.herbalgram.org/press/2015/HerbalDietarySupplementSalesinUSRisein2014.html> [Accessed 2 February 2016].

<www. Annies remedy.com> [Accessed 16 April 2014].

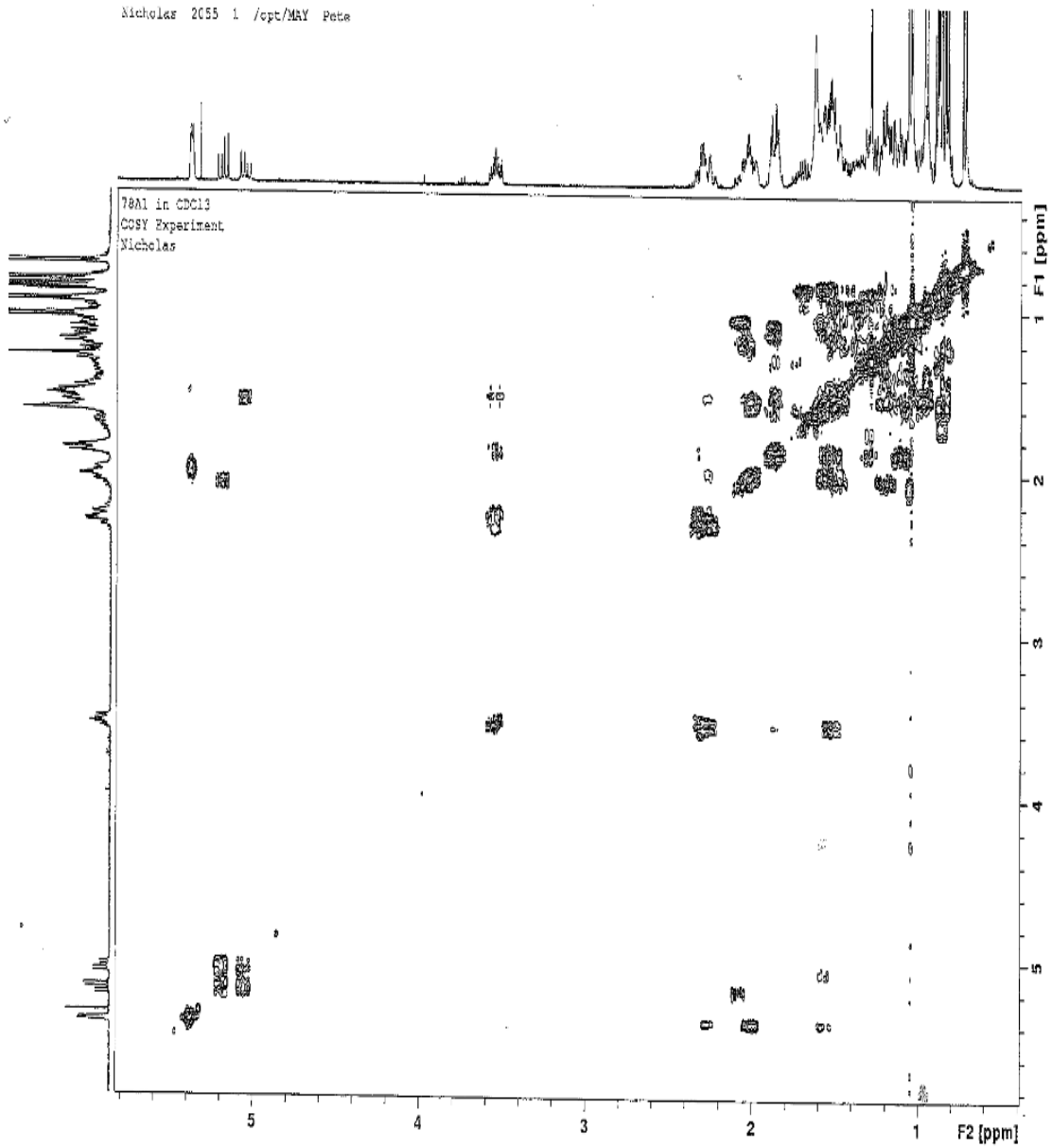
APPENDIX A

2D NMR: HSQC spectrometer of stigmasterol isolated from *OSM*



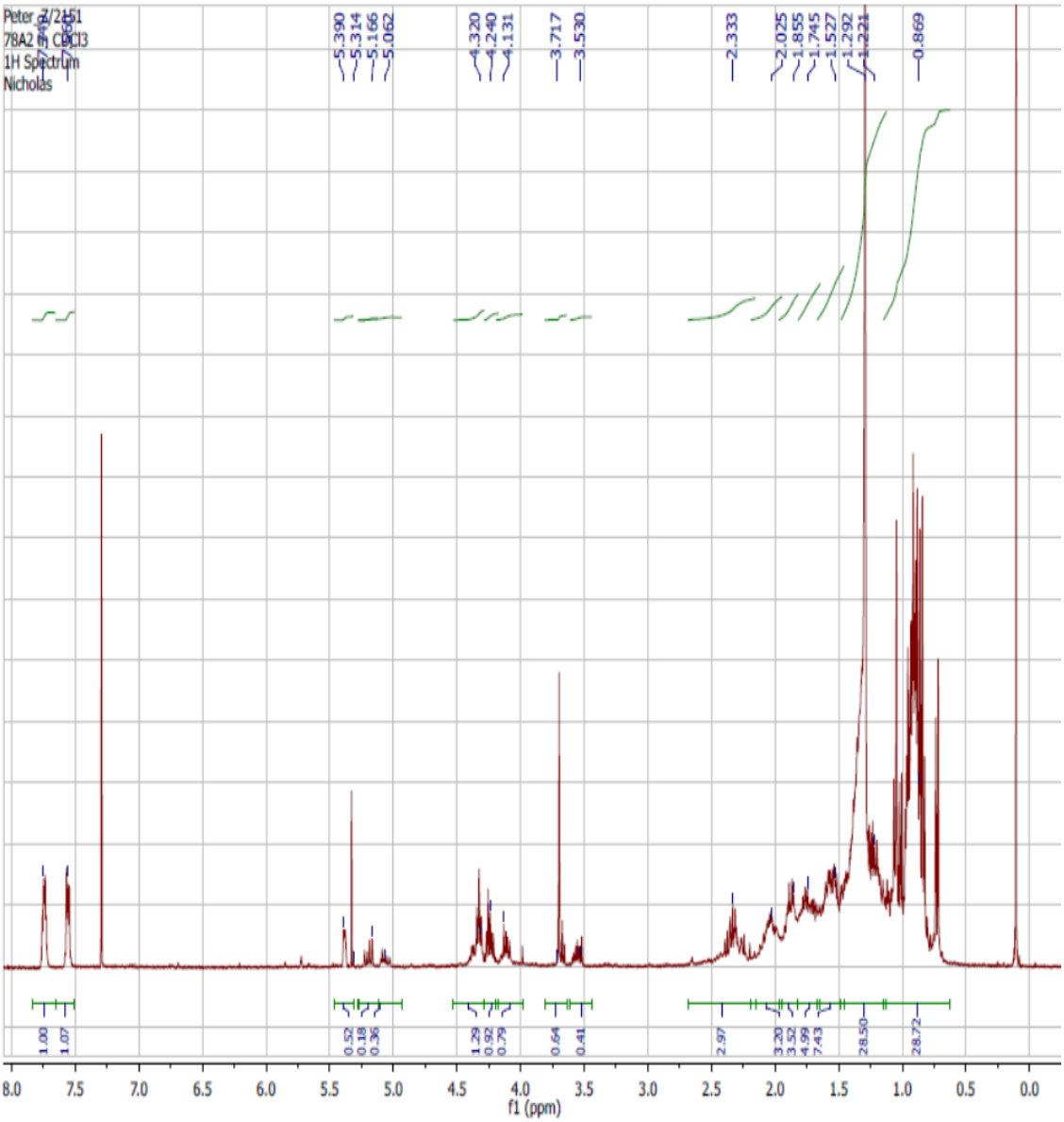
APPENDIX B

2D NMR: COSY spectrum of stigmasterol isolated from *OSM*



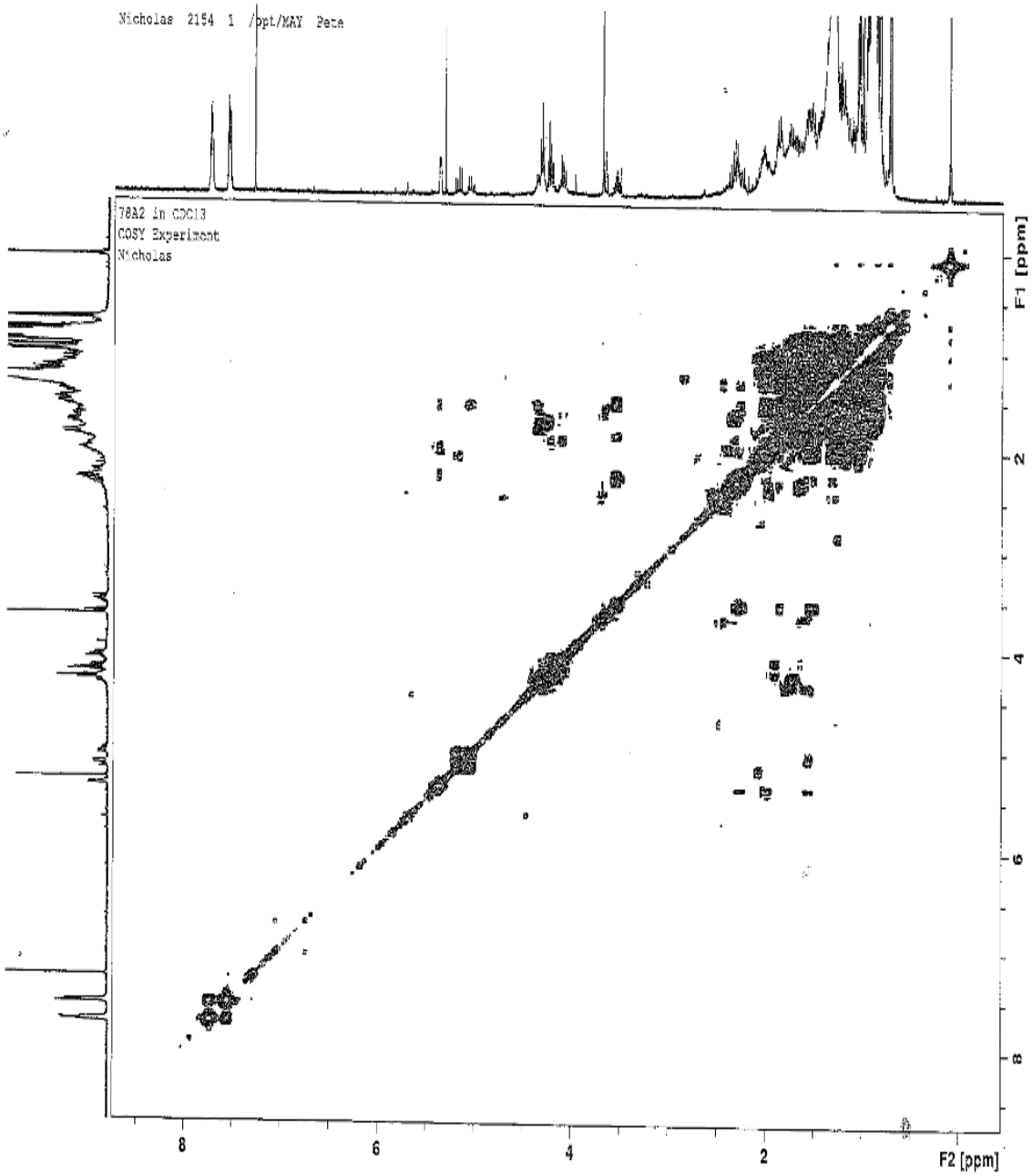
APPENDIX C

1H NMR spectrum of compound 2 isolated from *OSM*



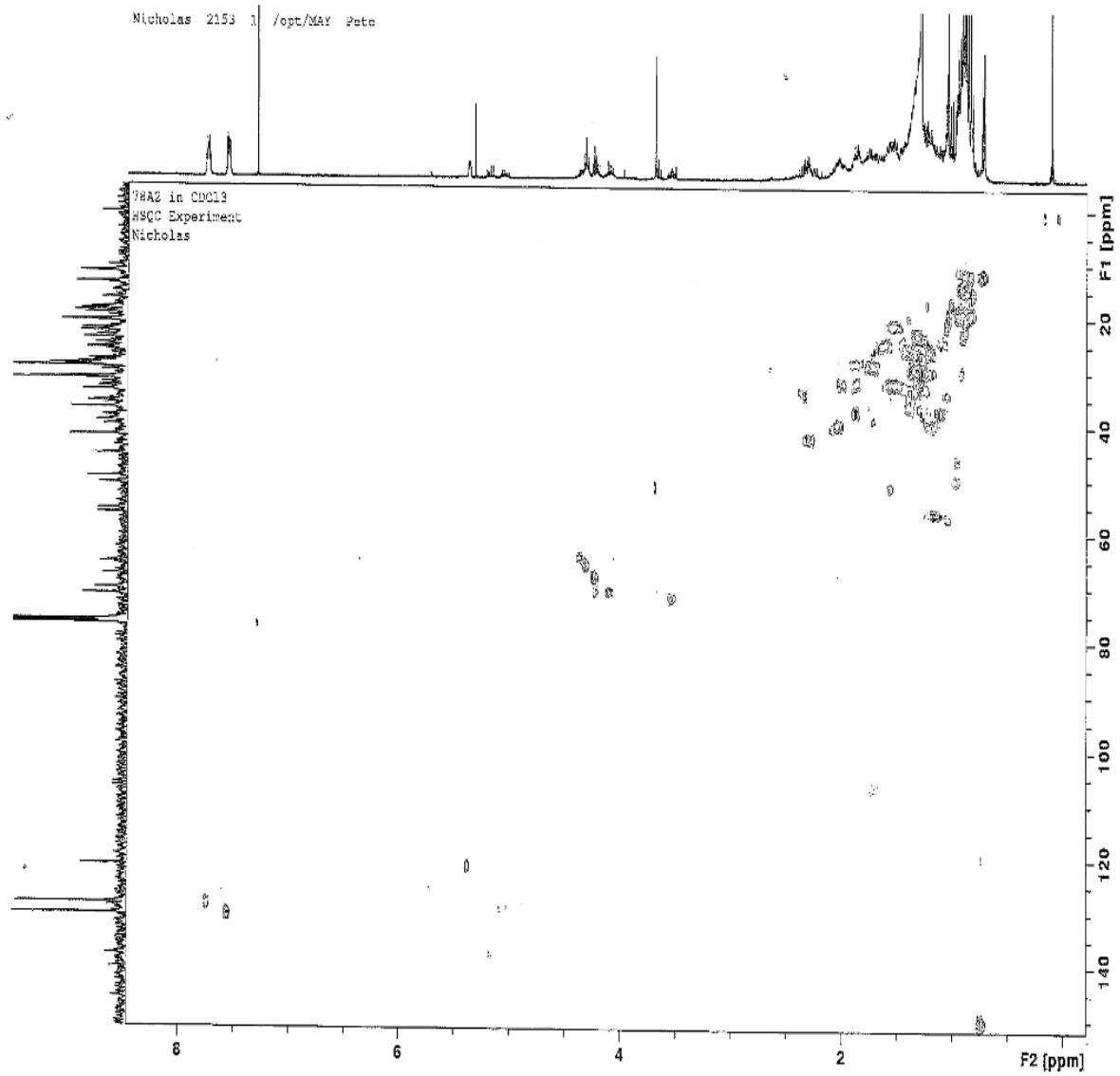
APPENDIX D

2D NMR: COSY spectrum of compound 2 isolated from *OSM*



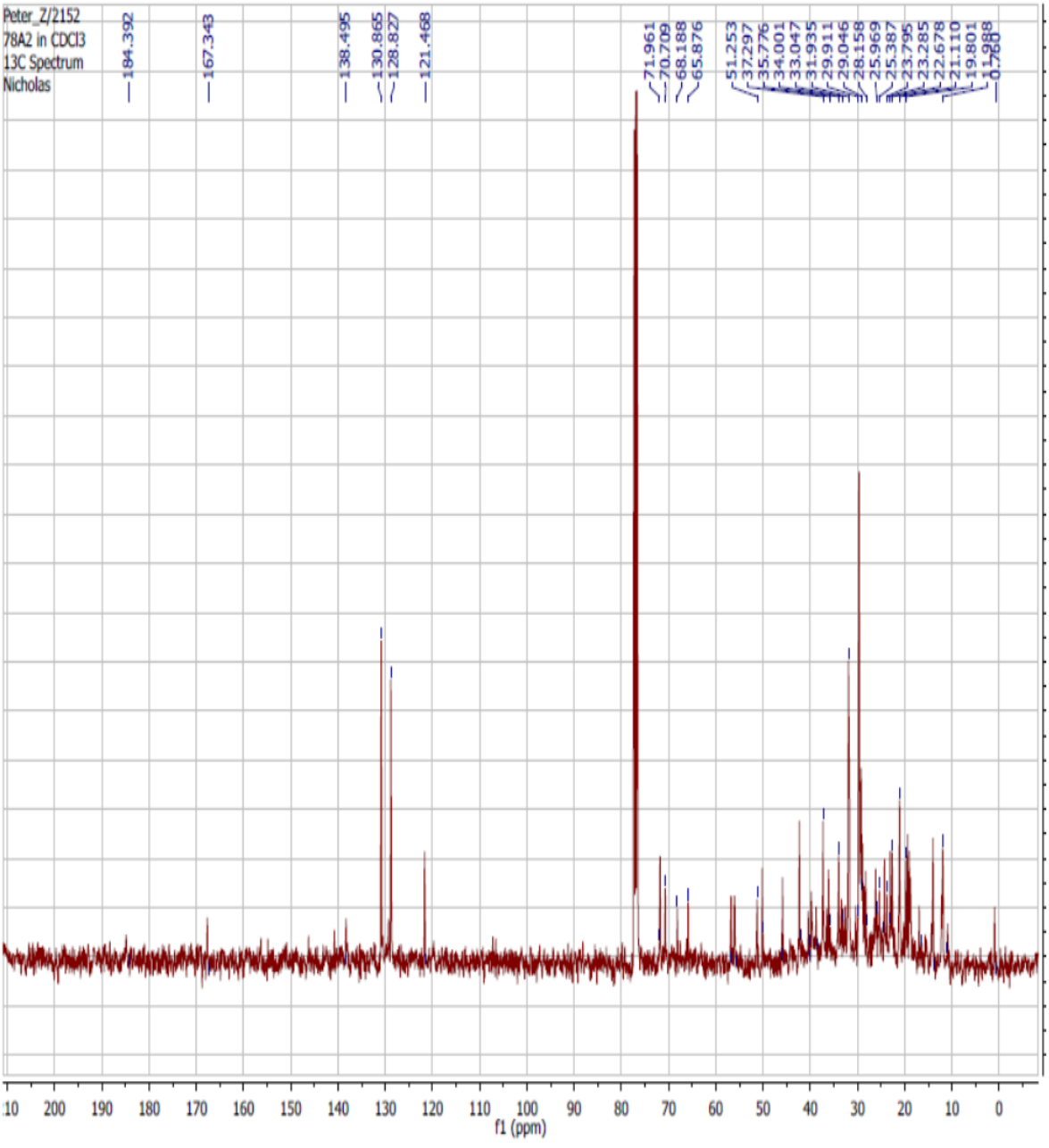
APPENDIX E

2D NMR: HMQC spectrum of compound 2 isolated from *OSM*



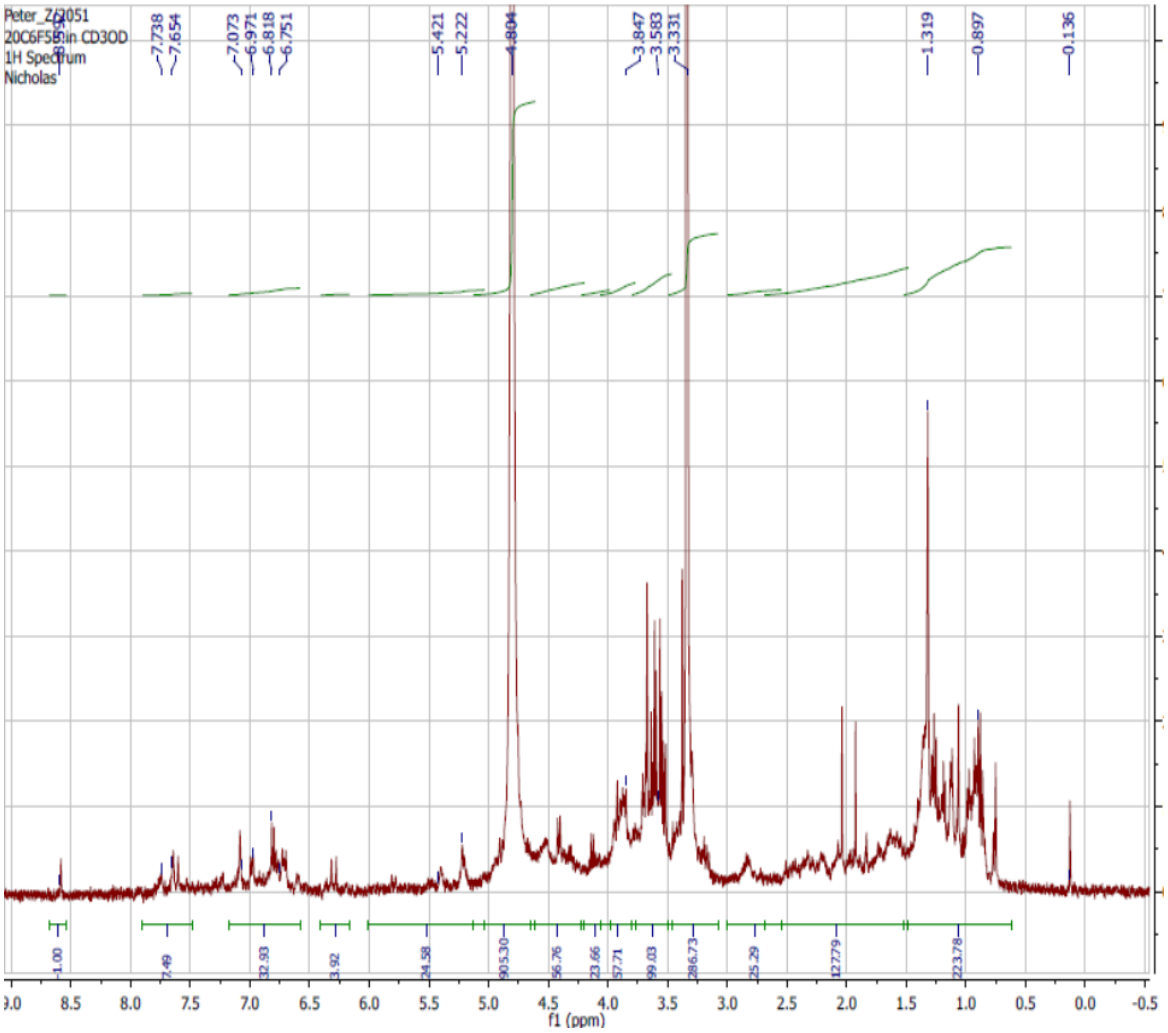
APPENDIX F

^{13} C NMR spectrum of compound 2 isolated from *OSM*



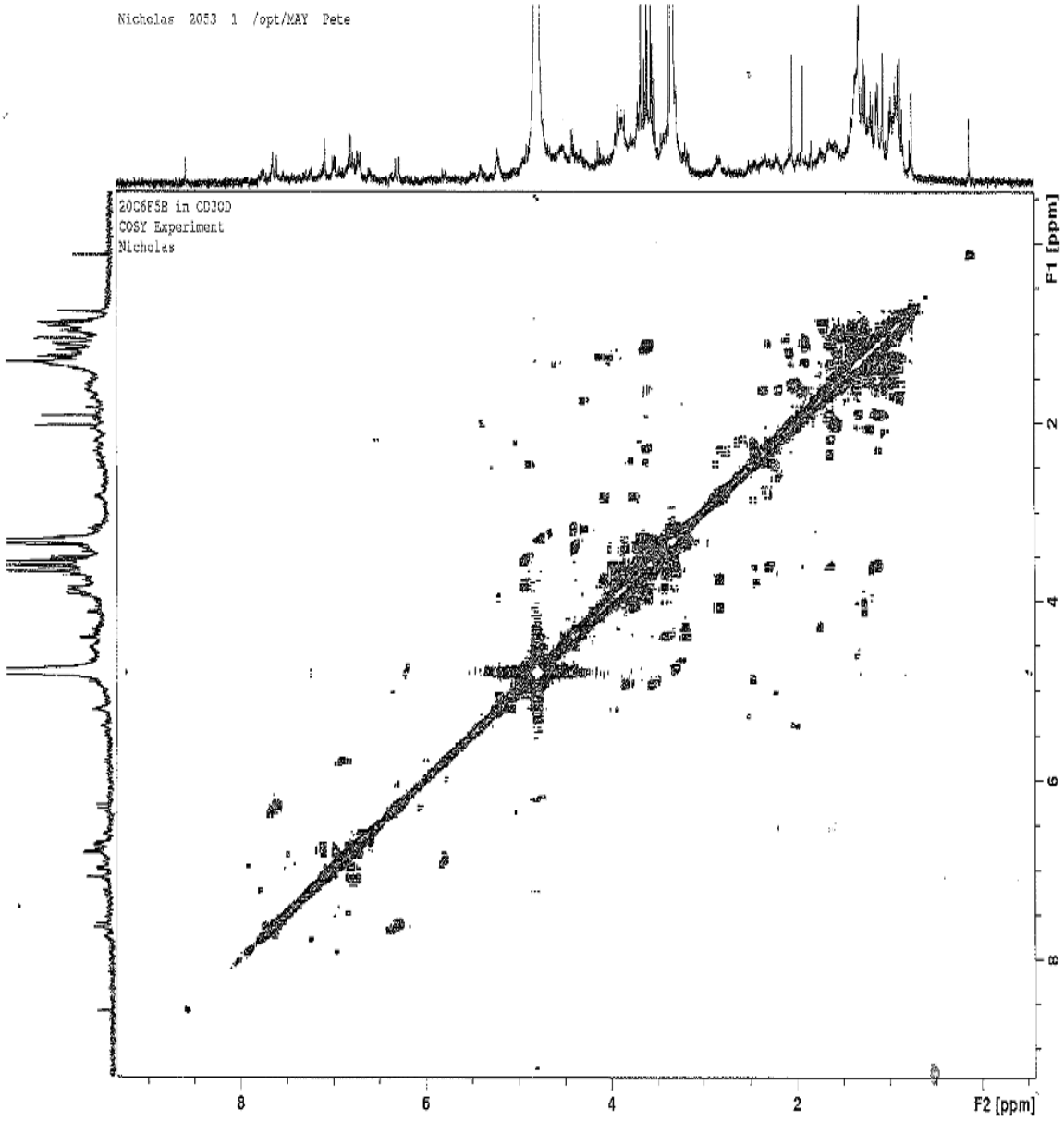
APPENDIX G

1H NMR spectrum of compound 3 isolated from *OSM*



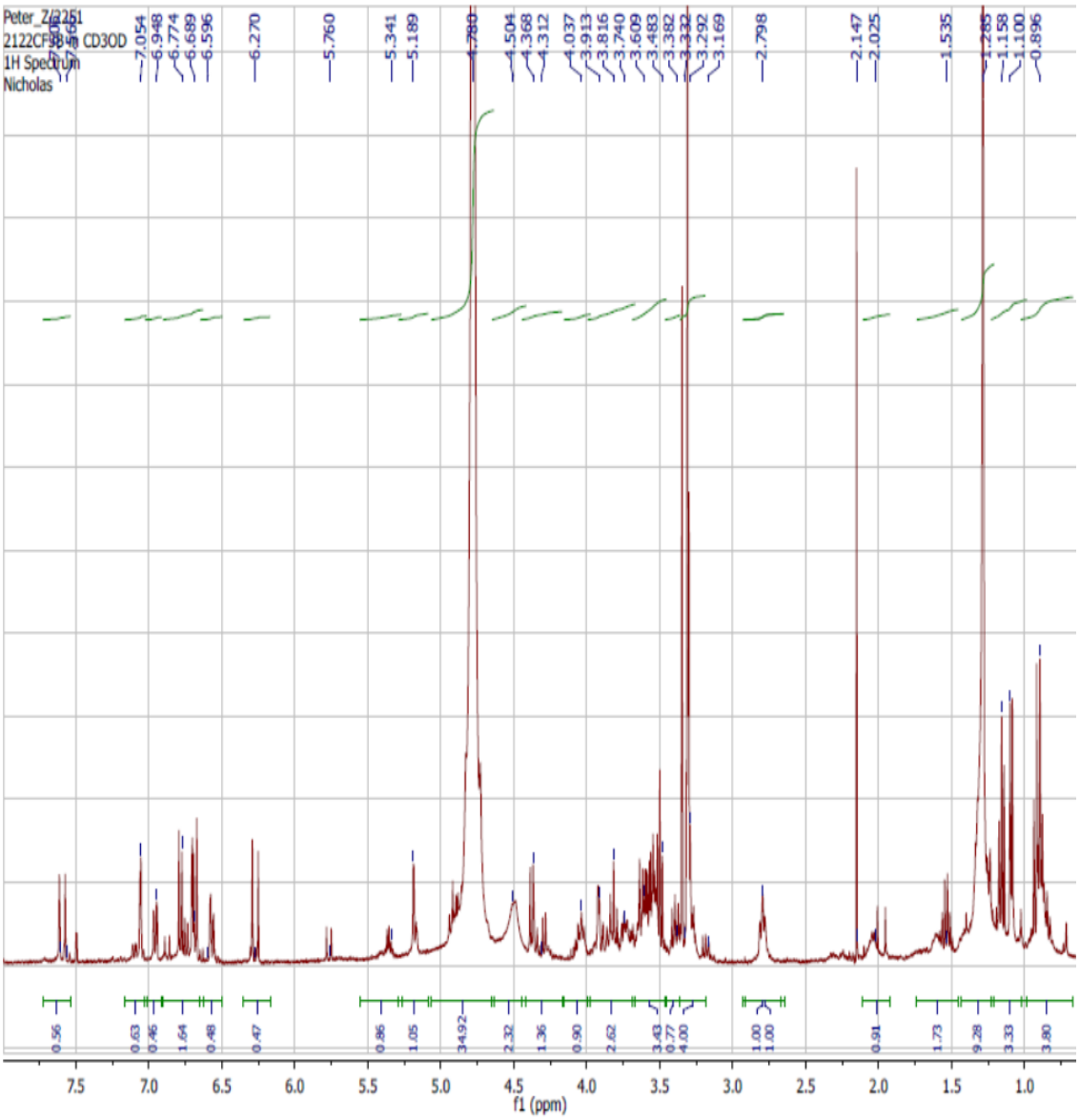
APPENDIX H

2D NMR: COSY spectrum of compound 3 isolated from *OSM*



APPENDIX I

^{1}H NMR spectrum of compound 4 isolated from *OSM*



APPENDIX J

NAME OF SPECIMENS

THE UNIVERSITY OF ZAMBIA

DEPARTMENT OF BIOLOGICAL SCIENCES

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P.O. Box 32379
Lusaka
Zambia

Your ref:
Our ref:

IDENTIFICATION RESULTS

Date: 31/01/2014.

Name of specimen (s):

1. *Solanum torvum* Swartz (Solanaceae); PLL1.
2. *Symphytum officinale* L. (Boraginaceae); PLL2
3. *Odontonema strictum* (Nees) Kuntze (Acanthaceae); PLL3
4. *Aphelandra squarrosa* Nees (Acanthaceae); PLL4

Number of specimens: **4**

Identified by: **Florence Nyirenda** (MSc.)

Designation: **Senior Technician/ Herbarium Assistant**

Signature: 

APPENDIX K

PUBLICATION

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Research Article

Isolation and Characterisation of Stigmasterol and B -Sitosterol from *Odontonema Strictum* (Acanthaceae)

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Abstract

Phytochemical screening of the extracts obtained from the leaves of *Odontonemastrictum*, a plant used in folklore medicine in Burkina-faso for its anty-hypertensive properties, indicated the presence of: flavonoids (type of flavones), saponins, glycosides, tannins, steroids and terpenoids. Column chromatography of the crude extracts lead to a number of fractions. TLC fingerprinting and the spraying reagent (Concentrated H_2SO_4 and vanillin in methanol) were used to identify the fraction containing phytosterols. The isolation and purification afforded white crystalline powder which was subjected to physical, chemical and spectral identification by IR, 1H-NMR, 2D-NMR and 13C-NMR. The compound was identified as a mixture of stigmasterol and β-sitosterol.

Key words: *Odontonema strictum, phytosterols, stigmasterol and β-sitosterol*

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1. Introduction

The genus *Odontonema* includes flowering plants of the *Acanthaceae* family and it is mostly found in tropical region and common garden inclusions. The plant belonging to the dicotyledonous angiosperm subclass of *Asteridae*, order of *Scrophulariales*, to the sub family of justiciaceae and gender *Odontonema*.

Three species are used in traditional medicine: the ground leaves and the stem of *Odontonema callistachyum* are applied on open wounds in order to heal them in Sierra Mazateca (Mexico) [1]. The leaves of the species *Odontonema tubiforme (Bertol.) kuntze* is used by Kuna, Ngöbe-Buglé, and Teribe Indians as an anti-inflammatory and for inducing child birth [2]. *Odontonema strictum* is used in Burkina Faso for the treatment of hypertension [3]. The genus *Odontonema* is the likely sources of bioactive secondary metabolites.

Stigmasterol and sitosterol are two phytosterols well spread in plants and animals as well as fungi, and have structural similarity to cholesterol. The most important benefit for these two secondary metabolites is their enrolment amongst the health promoting constituents of natural foods which

APPENDIX L

PUBLICATION

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Full Length Research Article

PHYTOCHEMICAL SCREENING AND *IN VITRO* ANTIBACTERIAL ACTIVITY OF *ODONTONEMA STRICTUM (ACANTHACEAE)* AGAINST SELECTED BACTERIA

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Odontonema strictum,
Phytochemicals,
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Klebsiella

ABSTRACT

The evaluation of the antibacterial activity using five bacterial strains (*Klebsiella, Shigela, Salmonella, Escherichia coli* and *Staphylococcus aureus*) showed that the most active leaf extract was that of *Odontonema strictum (Acanthaceae)* compared to that of *Solanum torvum, Symphytum officinale L.* and *Aphelandra squarrosa*. The inhibition diameters were measured and no resistance was observed at the concentration of 100 g/ml across all bacterial strains. The Activity Index (AI) values of *Odontonema strictum (OSM)* were determined using chloramphenicol as a positive standard antibiotic. The results showed that *OSM* is four fold more bactericidal than the standard on *Klebsiella*. Qualitative phytochemical screening of the extracts indicated the presence of flavonoids, carbohydrates, saponins, glycosides, tannins, steroids, and terpenoids.

INTRODUCTION

Plant natural products have always played an important role in discovery of new medicines. It has been estimated that between 25-50% of medicines have their origins in medicinal plants (David A. Akinpelu *et al.*, 2009). Secondary metabolites which are ubiquitous in plants represent a special group of molecules in drug discovery research some of which possess antimicrobial properties. The recent emergence of multi-drug resistant microorganisms poses a serious challenge in Public Health. An increasing number of bacteria are now developing resistance to commercial antibiotics (Eloff *et al.*, 2005). For instance, methicillin, a beta-lactam antibiotic, was used to treat infections caused by certain Gram-positive bacteria (*Staphylcoccus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, and *Streptococcus pneumonia)* but today, infections with Methicillin-Resistant *Staphylococcus Aureus* (MRSA) have become a major challenge , particularly in hospitals (Rand and Dale, 2007). Resistance to Vancomycin in the United States among patients infected with *Enterococcus faecium* amounts to approximately 20 to 30% (Thomas L. Lemke *et al.*, 2008). According to Po-Ren Hsueh and Kwen-Tay Luh (2002), *Streptococcus pneumoniae* exhibits resistance to penicillins, cephalosporins, trimethoprim-sulfamethoxazole, macrolides and fluoroquinolones (ciprofloxacin). Furthermore, vancomycin-resistant *Staphylococcus aureus* (VRSA) and multidrug-resistant (MDR) strains of this organism have been reported (Simon Gibbons, 2004). Therefore, the emergence of drug resistant bacterial strains across most commercially marketed antibiotics, necessitates intensified research efforts in the discovery and development of novel antibiotics. Africa, with its rich fauna and flora, is a big resource for medicinal plants. However, there is a dearth of phytochemical knowledge on many herbal medicines mainly, due to lack of laboratory facilities, qualified scientists, and financial support. This research originated from an observation made on a dog which was found regularly digging and eating the roots of *OSM*, a plant mainly found in tropical regions of the world. This was very strange indeed since the dog selectively dug and ate the roots from *OSM* over other plants. It was further observed that, after consuming the roots, the dog did not suffer any immediate observable consequences. Since consumption of these roots neither resulted in death nor any observable biological response, it may be assumed that the plant material

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APPENDIX M

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Isolation and ^{1}H-NMR identification of a tiliroside from *Odontonema strictum* (Acanthaceae)

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Abstract
The dried leaves (310g) of *Odontonema strictum* (OSM) were poured and extracted in MeOH/DCM (1/1//v/v) for 48 hours to yield 25g of dry residue which were purified using chromatographic methods (vacuum liquid chromatography column (VLC), Sephadex LH-20 column and Thin Layer Chromatography (TLC)). Fractions containing flavonoids were mixed for further purifications. Compound 1, yellow amorphous powder (8 mg) was re-dissolved in MeOH and developed in EtAOc/MeOH (2:1) to give an Rf value of 0.27 and mp 301-303°C. The compound reacted positively to HCl-Mg and Molish reactions. The 1 H-NMR (400 MHz, CDCl3) spectrum displayed the typical signals for tilirosides. The presence of glycosidic flavonoids may possibly contribute to the pharmacological properties of OSM.

Keywords: *Odontonema strictum*, identification, flavonoids, tilirosides, 1H NMR spectroscopy.

Introduction

OSM (Figure 1), a plant mostly found in tropical regions, is used as a folk medicine in Burkina-Faso to treat hypertension [1]. Felix Kini and his colleagues [2] have identified flavone glycosides (C-heterosides and O-heterosides in 0.37% and 1.13% respectively) as the active secondary metabolites. In our previous work, we have isolated stigmasterol and beta-sistosterol from OSM and confirmed the antibacterial properties of these phytosterols [3-4]. Recently, the hepatoprotective and antioxidant activity of OSM against CCl_4-Induced Hepatic Injury in Rats have been confirmed by M. S. Refaey and his colleagues [5]. In the continuing study on this plant, we isolated and identified a compound which belongs to the group of tilirosides (TLDs). We are still working to elucidate the complete structure of the isolated compound. TLDs are glycosidic flavonoids which exhibit several pharmacological properties such as anti-diabetic and anti-hyperlipedemic [6], antiviral and cytotoxic activity [7], anti–inflammatory, anti-rheumatism [8], anti-microbial and antioxidant [9-10]. Kaempferol-3-O-β-D (6-O-transp-cinnamoyl) glucopyranoside (trans-tiliroside,) revealed significant anti-hyperglycemic effects when compared with phenethyldiguanide in alloxan mice. As a part of trans-tiliroside, kaempferol-3-O-β-D-glucopyranose and related analogues revealed weak anti-diabetes activity [11]. Structurally, Tilirosides are composed of three distinct parts: a flavonoid, a phenyl propanoid and a sugar (Figure 2).

Fig 1: *Odontonema strictum*

APPENDIX N

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Phytochemical and Pharmacological Profiles of the Genus *Odontonema* (*Acanthaceae*)

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Authors' contributions

This work was carried out in collaboration between all authors. Author LPL designed the study, wrote the protocol and wrote the first draft of the manuscript. Author NMM managed the analyses of the study and performed the spectroscopy analysis. Author HS managed the experimental process and author PMC corrected the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Odontonema is a group of tropical plant species used in folklore medicine because of its wide range of pharmacological properties. These plants are known to be anti-bacterial, anti-inflammatory, anti-hypertensive, anti-viral, hepatoprotective, sedative and anti-oxidant. Furthermore, some species have been reported to induce child birth and trigger bronchodilatation. Since this group of plants is associated with a plethora of pharmacological properties, a review of reported medicinally-relevant investigations is warranted. Herein, we review the ethnopharmacology, bioactivity reports, and phytochemistry of the plant species belonging to the genus *Odontonema*. To compile this review, an extensive literature search was conducted using Google Scholar, SciFinder, ScienceDirect, Web of Science, and Scielo web sites, updated to May 2015. Although there are a number of pharmacological and ethnopharmacological reports on the

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