ISOLATION AND PHYTOCHEMICAL SCREENING OF POTENTIAL ANTI-HIV AND ANTIMICROBIAL COMPOUNDS FROM THE LEAVES OF *MAERUA SCHINZII* AND *CATOPHRACTES ALEXANDRI*

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ABSTRACT

The validation of traditional medicines involves the isolation and characterization of bioactive compounds and the subsequent evaluation of their safety and efficacy. Traditionally used medicinal plants shortlisted for this study include *Maerua schinzii* Pax (Capparaceae) and *Catophractes alexandri* (Bignoniaceae) which are indigenous to Namibia. An extract of the leaves of *M. schinzii* is ethnomedicinally used to treat cough, headache, earache, acne and skin disorders, whereas the leaves extract of *C. alexandri* is used to treat worms, sore throat, stomach ache, diarrhoea, and inflammatory conditions. A literature review revealed that these plants have not been subjected to phytochemical analyses. This study therefore attempted the isolation and structure elucidation of the major metabolites from the leaves of the two plants and the subsequent evaluation of their antimicrobial activity as well as inhibitory activity against HIV protease and reverse transcriptase.

Air-dried leaves of each plant were separately pulverized and extracted with an equivolume mixture of dichloromethane and methanol at room temperature for 24 hours on an orbital shaker. The crude extracts so obtained were fractionated using Vacuum Liquid Chromatography (VLC) over silica gel with hexane, dichloromethane, ethyl acetate, acetone, methanol and distilled water - in order of increased solvent polarity. VLC fractions were pooled on the basis of their Thin Layer Chromatography (TLC) profiles and subjected to a combination of Preparative Thin Layer Chromatography (PTLC) and column chromatography techniques.

A yield of 10.84 mg/g of dry leaves was obtained for the crude extract of *M. schinzii*, whereas 16.52 mg/g of dry leaves was recorded for the crude leaf extract of *C. alexandri*. Purification of the VLC methanol fraction of the leaf extract of *C. alexandri* afforded four subfractions, namely DB1-4. The partially purified compounds CLDB11a and CLDB222 were obtained from DB1 and DB2 respectively, but the compounds could not be characterised due to the small amount isolated. The VLC MeOH fraction of *M. schinzii* was subjected to PTLC and yielded a partially pure compound, 0.041g MLCB51. Qualitative and phytochemical screen test revealed that the plants under study could serve as a potential source of flavonoids, saponins, bitter principles, alkaloids and coumarins. Semi-purified compounds isolated including crude leaf extracts displayed poor or no inhibitory activity against HIV protease and HIV-1 reverse transcriptase, respectively. Moderate antimicrobial activities were recorded for the semi purified compounds of both plants against four microorganisms: - gram-negative bacteria *Klebsiella pneumoniae, Escherichia coli,* gram-positive *Staphylococcus aureus* and fungal strain *Candida albicans*. However, no compound show activity with *E. coli* and best activity were recorded with *C. albicans*. Opportunistic fungal infection considered in the study were *Candida albicans* for the two plants but *C. alexandri* stand out for anti HIV and antimicrobial which merit for further study.

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LIST OF ABREVIATIONS

^{13}C – NMR	Carbon-13 Nuclear Magnetic Resonance spectroscopy
^{1}H-NMR	Proton Nuclear Magnetic Resonance spectroscopy
AIDS	Acquire immunodeficiency syndrome
AR	Analytical reagent grade
ART	Anti- retroviral therapy
CC	Column chromatography
$CHCl_3$	Chloroform
COSY	Correlation Spectroscopy
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
EA	Ethyl acetate
EC_{50}	50% effective concentration
GC	Gas Chromatography
GC-MS	Gas Chromatography – Mass Spectrometry
H_2O	Water
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
Hex	Hexane
HIV	Human Immunodeficiency Virus
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Correlation
HPLC	High Performance Liquid Chromatography
LC-MS	Liquid Chromatography – Mass Spectrometry

IR	Infrared
IC_{50}	50% Inhibitory concentration
MeOH	Methanol
MS	Mass Spectroscopy
NBRI	National Botanical Research Institute
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NOESY	Nuclear Overhauser Enhancement Spectroscopy
PrEP	Pre-exposure Antiretroviral
R_f	Retardation factor
RUF	Relative fluorescence unit
TLC	Thin-Layer Chromatography
TM	Traditional medicine
TOL	Toluene
UV	Ultraviolet
VLC	Vacuum liquid chromatography
WHO	World Health Organisation

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DEDICATION

I cried when you passed away. I still cry today. Although I love you dearly, I could not make you sit. A golden heart stopped beating, hardworking hands at rest. God broke my heart to prove to me he only takes the best. Deeply missed.

DECLARATIONS

I Lidia L.O Hamalwa, hereby declare that this study is a true reflection of my own research and that this work, or part thereof has not been submitted for a degree in any institute of high education.

..... Date.....

Lidia L.O Hamalwa

CHAPTER ONE: INTRODUCTION

1.1 Orientation of the study

Secondary metabolites, also known as natural products, offer a competitive advantage to organisms producing them and improve their survival by functioning as: (i) attractants of pollinators and seed dispersing animals, (ii) deterrent of herbivores and insects, and (iii) offering protection against microbial attack [1]. For humans, these structural diverse agents (natural products) have through the application and advances in spectroscopic techniques, played a pivotal role in drug discovery and development [1].

According to the global statistics of 2016, 36.7 million people were living with HIV/AIDS by the end of 2015. Since the start of the epidemic until the end of 2015, 35 million people died of AIDS related illnesses [2]. In 2015, approximately 210 000 people were infected with HIV/AIDS and 3100 died of related illnesses in Namibia [2]. The highest infection rates were recorded in Katima Mulilo in the Zambezi region [3]. The scourge of the HIV/AIDS pandemic and the negative effect it has on the economic growth of developing countries require expeditious introduction of alternative treatment and management methods.

Opportunistic infections which results from the weakened immune system of people living with HIV/AIDS, present a serious health threat and are a common cause of death [4]. Some of these opportunistic infectious diseases such as, malaria, tuberculosis, measles and cholera are preventable and curable [5]. There are studies with extracts of traditional medicines that display antiHIV activities, the treatment and prevention of opportunistic infections. For example, a phytochemical study by

Magadula & Suleimani (2010) on a medicinal plant from Tanzania, *Garcinia livingstonei,* revealed a potential lead compound that displayed antiHIV activity with an EC_{50} of 2.25µg/ml. The phytochemical screen indicated the dominance of phenolic compounds; isolation of the active principle from the active fraction is currently underway [6]. Some of the compounds that have been reported to inhibit HIV replication have not been allocated to a class of HIV inhibitors because their mechanism of action has not been elucidated [7]. Extraction of the active principles from medicinal plants and the subsequent evaluation of their mode of action, safety and efficacy therefore need to be undertaken [8]. People infected with HIV/AIDS in developing countries where TB is prevalent typically present with tuberculosis (TB) as the first manifestation of the disease [5].

In most developing countries, a large portion of the population consult traditional healers and/or use medicinal plants to meet their primary health care needs [9]. Although modern medicine may be available in these countries, herbal medicines have often maintained popularity because they are accessible, cheap and safe [10]. Historically most of drug discoveries are driven by the traditional use of plants species. The use of secondary metabolites such as alkaloids, coumarins, phenolics, flavonoids, tannins and terpenoids have been reported in most medical journals [11]. Thus, the shortlisting of plants for phytochemical preliminary studies for antiHIV activities and the lack of toxicity, thereof can be done on the base of evidence that such plants are used traditionally. This evidence would be confirmed by further purification and the isolation of potential drug leads through an HIV drug discovery program [12].

Ethnobotanical surveys conducted by Hedimbi & Chinsembu in 2010 and 2012 in the Ohangwena and Caprivi (now Zambezi) Regions respectively, led to the discovery of a hundred and fifty four (154) plant species that are used in the treatment of common diseases associated with HIV/AIDS [13,14]. Most of these medicinal plants have not been subjected to phytochemical analysis. It is believed that a detailed phytochemical analysis will aid in confirming the efficacy and safety of traditional formulations, through the application of scientific methodologies. More importantly the isolation and identification of bioactive compounds can contribute and be used as templates in antiHIV drug discovery development [13].

1.2 Introduction to plants under study

For this study, traditionally used medicinal plants that are abundant, not endangered and indigenous to Namibia and that have not been subjected to phytochemical studies, were selected. These plants are *Maerua schinzii* Pax and *Catophractes alexandri* [15].

1.2.1 Maerua schinzii

Maerua schinzii (Capparaceae) is also known as Riverbush-cherry (English), Lammerdrol (Afrikans), Knotenfruchtbaum (German), Goradab (Khoekhoegowab), and Omutengu (Otjiherero) [16,17]. It is often mistaken with *Maerua angolensis, Maerua kaokoensi* and sometimes with *Boscia albitrunca*, especially in North-West Africa. The similarities of these trees are in the leaves and fruit morphology. The difference is in the habitat and flowers. Usually *M. schinzii* have large and yellowish green leaves as shown in figure 1 [18].

A B

Figure 1: A photo of *Maerua schinzii* showing the (A) flowers, (B) leaves and fruits [17]

The tree is eye catching when in full bloom with the conspicuous flower having a cluster of light yellow stamens that gives a pleasant, sweet smell which attract bees and insects. Seeds are contained in a long beanlike pod [18]. The leaves are eaten by ostrich, giraffe, rhino and kudu, which might be an indication of their lack of toxicity. In Namibia, it is widely distributed as shown in figure 2. The plant is widely used throughout Africa to treat cough, headache, earache, acne, and skin disorders. A literature review revealed limited reports on phytochemical analysis conducted on *M. schinzii* [16,17].

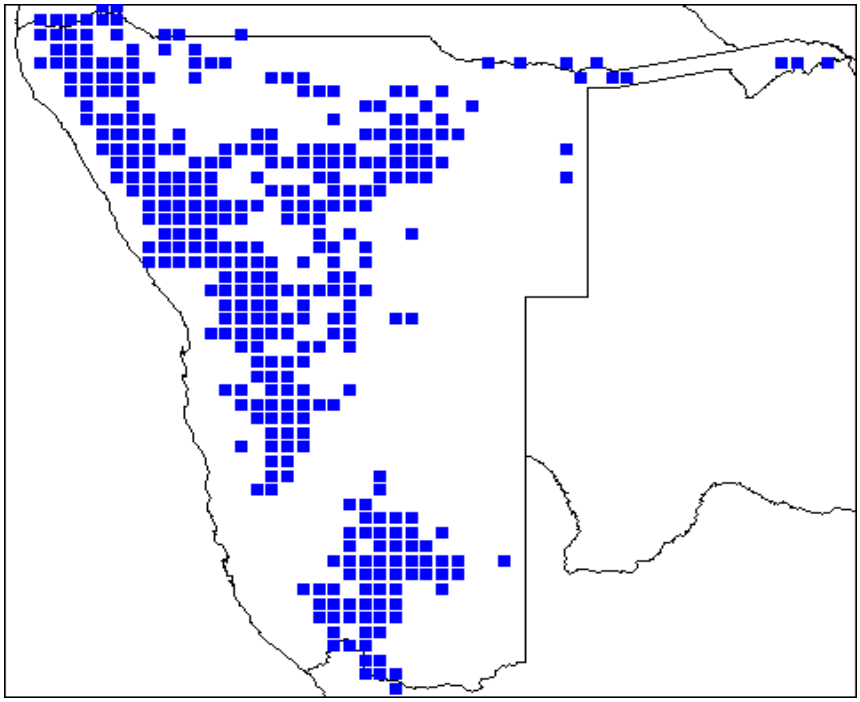


Figure 2: Geographical distribution of *Maerua schinzii* in Namibia [17].

1.2.2 Catophractes alexandri

Catophractes alexandri (Bignoniaceae), is also known as Gobbabos and Skaapbos (Afrikans), Trumpet thorn and Rattle tree (English), Schwarzdorn-Silberbusch (Germany), Omukaravize (Herero), Mutwatwa (Silozi), Okalyanzi in (Oshindonda) and Okalyadi (Oshikwanyama) [17]. It is a branched shrub that grows up to 2 meters high with greyish green foliage (figure 3), which zebra and springbok feed on.

A **B**

Figure 3: A photo of *Catophractes alexandri* showing (A) plant with flowers (B) leaves [17]

In Namibia, this plant is widely distributed as illustrated below (figure 4). It is used to treat worms, sore throat, stomach ache, diarrhoea and inflammatory conditions. A literature review did not reveal any phytochemical analysis conducted on the plant and it therefore merits further study [14].

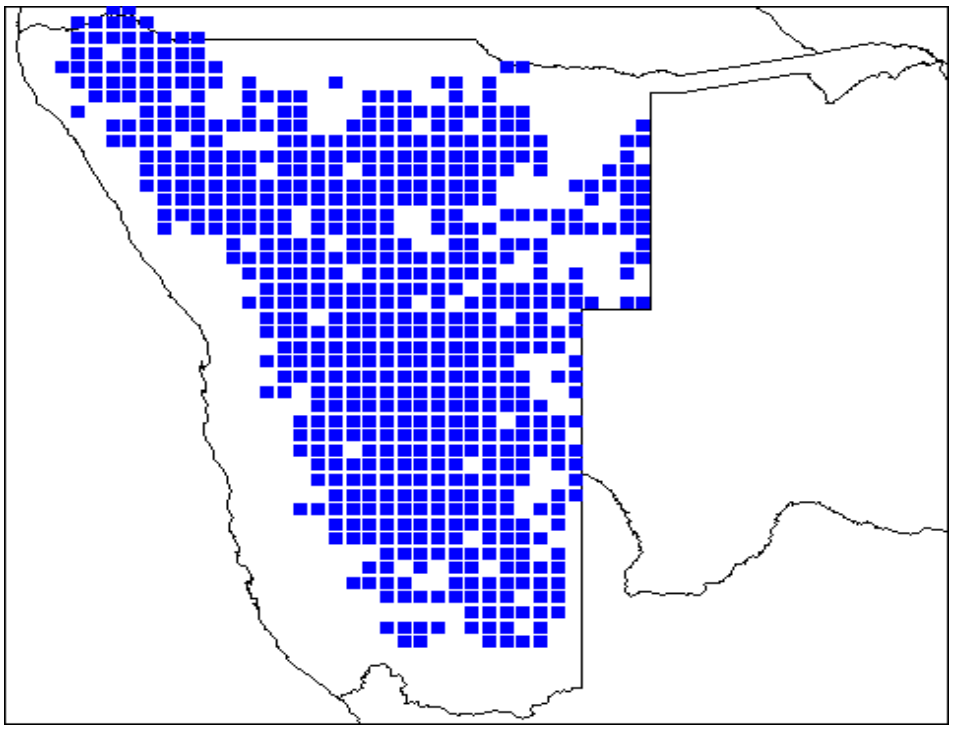


Figure 4: Geographical distribution of *Catophractes alexandri* in Namibia [17]

1.3 Statement of the problem

The chemotherapeutic treatment of HIV/AIDS is impeded by the ability of the virus to develop resistance against clinically used drugs. In addition, these drugs, do not cure but rather slow the progression of the disease, and are often of limited availability, more expensive or have adverse effects [19]. Traditional medicines remain a readily available and safe alternative in resource-poor countries and they are used extensively in the treatment and management of opportunistic diseases related to HIV/AIDS [19,20]. Some of the manifestations and symptoms of opportunistic infections associated with HIV/AIDS include bacterial and fungal infections, cold/cough, diarrhea, and skin infection. Some of these symptoms relates to the medicinal uses of *Maerua schinzii* and *Catophractes alexandri*, respectively [15]. Although used for the treatment of opportunistic infections, the major metabolites of the two plants species have not been identified nor have the leaf extracts or isolated compound been tested for antiHIV activities [20].

It is worth mentioning that biologically active plant extracts comprise a complex mixture of secondary metabolites. The different components in the extracts may work synergistically or antagonistically to elicit a biological effect [21]. For example, there have been reports that some metabolites isolated from the bioactive plants extracts are devoid of biological activity even though the parent/original fractions are biological active [22]. Despite this, the standardization of herbal formulation, which entails the quality control of these bio-resources, requires knowledge on the identity and composition of the major or bioactive secondary metabolites.

This study therefore intends to isolate the metabolites from the leaves of the two plants species used medicinally in the treatment of symptoms of opportunistic

infection, namely *Maerua schinzii* (Capparaceae) and *Catophractes alexandri* (Bignoniaceae), and to explore the potential of the isolated metabolites as antiHIV and antimicrobial agents [18].

1.4 Objectives of the study

The objectives of the study are:

1. To extract and purify the major metabolites from the leaves of *Maerua schinzii* and *Catophractes alexandri*.
2. To characterize the isolated major metabolites.
3. To evaluate the antimicrobial activities as well as the inhibitory activities of the isolated metabolites against HIV protease, and reverse transcriptase.

1.5 Significance of the study

It is envisaged that this study will contribute to the chemical knowledge of Namibian medicinal plants. It could further contribute towards the discovery of new antiHIV agents by exploring the bioactive potential of the major metabolites isolated from the plants under study against two HIV enzymes, protease and reverse transcriptase. Community awareness and conservation of the selected medicinal plants can be promoted if the study yields positive results. By subjecting these plants to phytochemical analysis the metabolites obtained can be used as templates in the synthesis of drugs with better active profiles, fewer side effects and better pharmacokinetics [23]. The results may inspire and motivate researchers to continue the research for lead compounds from these plants resources [24]. This study further aims to make a contribution to (i) the discovery of antiHIV drugs and (ii) the

preparation of standardized herbal formulation, that is should the leaves of the plants under study be considered as components in new herbal formulations.

CHAPTER TWO: LITERATURE REVIEW

2.1 Previous phytochemical studies

2.1.1 Bignoniaceae family

Bignoniaceae is a family of flowering plants in the order of *Lamiales* that comprises trees, shrubs, woody plants and herbs. It consists of 120 genera and 800 species [15,25]. Members of this family are mostly found in tropical and neotropical regions of America, Asia, and Africa, and some are cultivated in other regions as ornamental plants [25]. Of interest to this study is the genus *Catophracte.*

2.1.2 Previous phytochemical studies on the genus *Maerua*

The genus *Maerua* comprises of about 50 species and most are found in dry areas of tropical Africa, but some extending to the Middle East and tropical East Asia [25]. Preliminary phytochemical screening of the stem bark of *M. angolensia* revealed the presence of glycosides, tannins, saponins, terpenes, flavonoids, carbohydrates, proteins and alkaloids [24]. Extracts from *M. angolensia* has been evaluated and demonstrate to display anti-diabetic effects, antimicrobial and antimycobacterial activity [21]. *M. schinzii* Pax, the plant understudy is a species with coarse hairs that has not been subjected to any phytochemical screening before [18].

2.1.3 Capparaceae family

Members of the *Capparaceae* or *caper* family are trees, shrubs or herbs that are usually found in tropical regions. They are the major group of angiosperms (flowering plants). It is a family of plants that contain 33 genera and 700 species. The large genera are *capparis* (with about 250 species), *maerua* (with about 37 species)

cadada (30 species) and *boscia* (37 species) [26]. *Capparaceae* has been closely related to *brassicaceae,* the mustard family, because both groups produce glucosinolate (mustard oil) compounds [25].

2.1.4 Previous phytochemical studies on the genus *Catophractes*

These species have not been extensively studied. Some metabolites extracted from *Cinchona* identified as quinines displayed potential as anti-insect agent [25]. Phytochemical screening of *catophractes* ethanolic extracts showed the presence of alkaloids, flavonoids, carbohydrates, terpenoids, coumarins and phenolics [26].

2.2 HIV/AIDS

2.2.1 Background

New HIV infections are still reported in all regions of the world with about 70% occurring in sub-Saharan Africa and it affects about 38% of people under the age of 25 [2]. It is reported that HIV/AIDS does not only affect the health of individuals but also affect the household, the development and economic growth of nations [2]. Despite these challenges, new global efforts have been launched to address the epidemic. In the last decade there have been signs that the epidemic may be changing course [28]. The number of people newly affected with HIV and the number of AIDS - related deaths has declined and it is mostly likely due to the understanding and stabilization of the epidemic [28]. Preventative measures exist to combat HIV and new tools such as vaccines are currently being researched [28]. Effective prevention strategies that are being used and/or researched include behaviour change programs, HIV testing, condoms use, male circumcision, blood supply safety and harm reduction efforts for injection drug user, are being used or researched. A recent study

showed that providing treatment to people with HIV significantly reduces the risk of transition to their HIV negative partners [2].

2.2.2 HIV life cycle

HIV infection is caused by the human immunodeficiency virus. It can be contracted through contact with infected blood, semen, vaginal fluid or by sharing needles with someone who is infected with the virus [29]. HIV gradually destroys the immune system by attacking and killing CD4 cells, a type of white blood cell which plays a major role in protecting the body from infection. It uses the CD4 cells to multiply itself following a series of seven stages. These stages are binding, fusion, reverse transcription, integration, replication, assembly and budding as shown in figure 5 below [29].

HIV binds to the receptors on the surface of the CD4 cell using its HIV glycoproteins. Upon entry the HIV envelope and the CD4 cell membrane fuse together allowing the HIV to enter the CD4 cell. The HIV releases and uses its enzyme, reverse transcriptase to convert its genetic material HIV-RNA into HIV-DNA which allows it to enter the CD4 cell nucleus and combine with the cell's genetic material, cell-DNA. Inside the CD4 cell nucleus, the HIV releases an enzyme integrase that facilitates the integration of its viral DNA and the CD4 cell-DNA, and uses the machinery of the CD4 cell to make long chains of HIV proteins which are responsible for the building blocks of the HIV [30]. The new HIV proteins assemble with HIV-RNA at the surface of the CD4 cell and form an immature non-infectious HIV which pushes itself out of its host. The new HIV releases an HIV enzyme protease which breaks up the long protein chains that form the immature virus. The

smaller HIV proteins then combine to form a mature infectious HIV, depleted in figure 5 below [29,30].

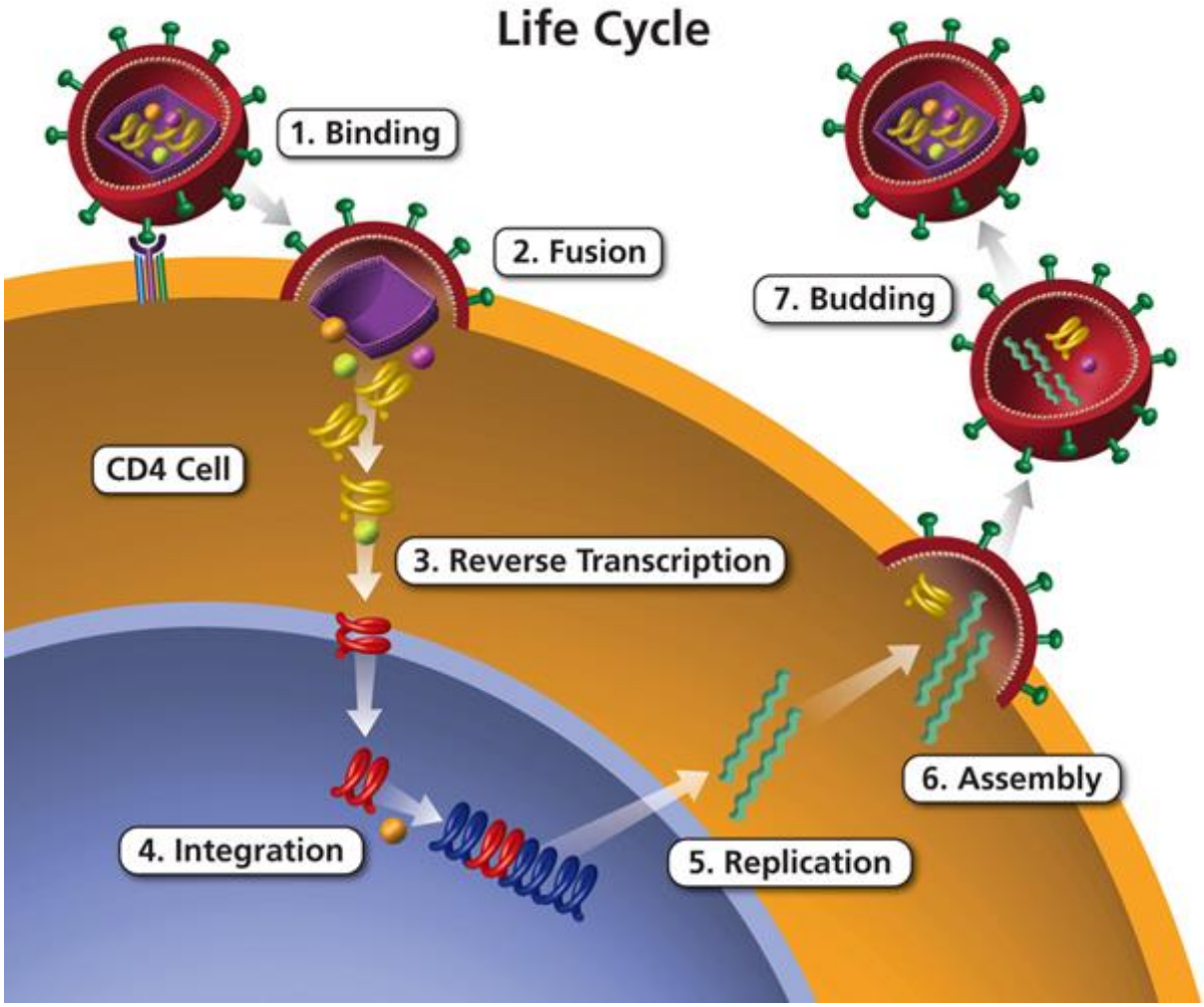


Figure 5: HIV life cycle showing the site of action of different classes of Anti-retroviral drugs [29,30].

2.2.3 Antiretroviral drugs

Antiretroviral therapy (ART) comprises a combination of HIV protease, integrase and reverse transcriptase inhibitors that are used to improve the immune system by blocking the growth and development of the HIV virus at different stages of its life cycle [31,32]. There are various combination inhibitors on the market which are

known as highly active antiretroviral therapies (HAART). They are usually comprised of three or more antiretroviral drugs (nucleoside analog reverse transcriptase (RT) inhibitors, non-nucleoside analog RT inhibitors, and protease inhibitors) which are very effective in suppressing viral replication and have led to significant reduction in the mortality rate of the disease. These combination inhibitors increase the life span of HIV/AIDS patients as well improve their quality of life [33].

The use of a combination of ARTs as standard antiretroviral therapy was first introduced in 1996, and has led to a dramatic reduction in morbidity and mortality. Access to ARTs has increased in recent years and has risen to 15.8 million people as of June 2015, achieving a goal set by world leaders in 2011 to have 15 million people on treatment by 2015. Globally, 40% of people living with HIV are receiving antiretroviral treatment, which include 41% adults and 32% children [28]. About 76% of all people receiving antiretroviral therapy in sub-Saharan Africa are viral suppressed, which means they are healthier and less likely to transmit the virus [31]. The percentage of pregnant woman receiving ART for the prevention of mother to child transmission of HIV increased to 73% in 2014 from 36% in 2009. Access to ART among children has also risen significantly, although they have less access to treatment than adults [30].

There are six classes of antiretroviral drugs that are used to treat HIV/AIDS. These groups are classified as follows:

- **Non-nucleoside reverse transcriptase inhibitor (NNRTIs**): these act by binding non-competitively to the reverse transcriptase enzyme causing a conformational change to the three-dimensional structure of the HIV enzyme and create the NNRTI

binding pocket (NNIBP) [34]. This affects the catalytic activity of the enzyme and blocks the HIV replication by inhibiting the polymerase active site of the reverse transcriptase. The conformational change additionally destabilizes the nucleic acid template of the enzyme and reduces its ability to bind nucleotides which results in a reduced rate of the transcription of viral-RNA to DNA [35].

- **Nucleoside reverse transcriptase inhibitor (NRTIs):** competitively inhibit the nucleoside by binding to the reverse transcriptase and terminating the DNA chain. These are analogues of the naturally occurring deoxynucleotides needed to synthesise the viral-DNA and they compete with natural deoxynucleotides for incorporation into the growing viral-DNA chain, but they lack the 3'-hydroxyl group on the deoxyribose moiety [36,37]. Their incorporation means that the incoming deoxynucleotide cannot form the next 5'-3' phosphodiester bond needed to extend the chain, leading to chain termination. The NRTIs also compete as substrates for both the viral and host cell DNA [36].
- **Protease inhibitor (PIs):** target protease, an enzyme that helps in making the proteins available that are required for building new HIV copies, thus lowering proliferation.
- **Entry or Fusion inhibitor:** block HIV from initially infecting CD4+ helper T cell through various mechanisms of actions.
- **Integrase strand transfer inhibitor (INSTIs):** disable integrase, which is the enzyme responsible for integration of copied HIV DNA into the host T-cell DNA [38,39]. This is achieved by forming chelates with Mg^{2+} and Zn^{2+}cations that are part of the structure of the integrase. The chelates subsequently disable the integrase active sites for integration [40].

2.2.4 Opportunistic infections

People living with HIV/AIDS are susceptible to fungal and bacterial opportunistic infections that result from immune-suppression that compromised their immune system. As mentioned in Section 1.1, these infections have been reported from the early days of the HIV/AIDS pandemic and are one of the leading causes of death. Treatment of these infections is therefore one of the most important reason for managing HIV/AIDS cases [5]. Opportunistic infections include: tuberculosis (TB), Herpes zoster (shingles), Herpes simplex (Genital herpes), oral candidiasis and Cryptococcal meningitis [5]. Oral candidiasis reportedly occurs in most HIV positive patient but is prevalent among patients with a low CD4 count. Other symptomatic conditions that, are undefined as opportunistic infections that trouble HIV/AIDS patients which are skin rashes and chronic diarrhoea [5]. Most AIDS patients on ART are using herbal/traditional medicine to relieve the symptoms they experience as well as to treat the side effect of ARTs [5].

2.3 Traditional medicine used in the management of HIV/AIDS

Studies have been conducted to evaluate the use of traditional medicine in the treatment of opportunistic infections associated with HIV/AIDS. Some of these studies have proven traditional medicine to be safe and effective compared to conventional medicine [5]. Pharmacological interaction between antiretroviral drugs and traditional herbal medicines need to be further examined as the herbal drug side effects and interactions are not yet known. It is of great concern among researchers, as traditional medicine (TM) may decrease the effectiveness of the ART [41]. Further investigations are needed to identify the potential risk, benefit and interaction

or non-interaction associated with ARV drugs and TM, and also to explore the effect of TM use among AIDS patients receiving ART in terms of mobility and mortality pattern [41].

A study done in Uganda by the WHO, recommended that health-care workers be more vigilant when AIDS patients that are combining ART with herbal medicines, because it may reduce the ART treatment effectiveness [10]. It is therefore imperative to find a considerable potential for medicinal plant to have an adjuvant effect on ARV drug efficacy, as has been done with chloroquine: the bioactive constituent *malagashanine* derived from the plant species *strychnos* in Madagascar that is used in a treatment of chloroquine resistant malaria [41]. A survey by Maroyi reported medicinal plants indigenous to sub-Saharan Africa that are recommended by herbalists and traditional healers as alternative sources of medicine for the management of HIV/AIDS. This study reported 74 plants which were distributed among 34 families and 65 genera. The majority of the plants belonged to the Fabaceae, Asteraceae, Euphorbiaceae, Combretaceae Lamiaceae, Myrtaceae, Cucurbitaceae, Phyllanthaceae, Apiaceae, Celastraceae and Xanthorrhoeaceae families [19]. Some of the plants listed are shown in Table 1.

Table 1: A summary of sub-Saharan medicinal plants recommended by traditional healers for treatment of HIV/AIDS related illnesses [19,20].

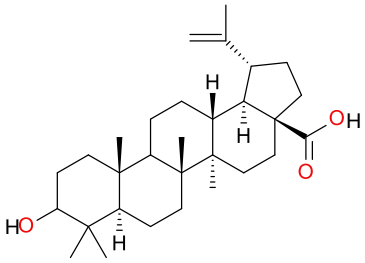
Scientific name and family:	**Plant parts used and condition treated:**	**Biological Activity:**
1) Kigelia africana (Bignonaceae)	Bark and fruits Herpes simplex, diarrhoea (Namibia, Tanzania), skin rashes (Uganda) [19,20].	Inhibit HIV-1 reverse transcriptase [19,42].
2) *Garcinia buchananii* Bak. And *Garcinia livingstonei (*Clusiaceae)	Bark and fruits Cryptococcal meningitis, diarrhoea, herpes simplex, herpes zoster, skin rash, tuberculosis (Namibia, Tanzania) [19,20].	HIV-1 protease inhibitory activity [6].
3) *Terminalia sericea* Burch. ex DC (Combretaceae)	Bark Cryptococcal meningitis (Namibia) [20].	Inhibit HIV-1 reverse transcriptase [19,43].
4) *Dichrostachys cinerea* (L) Wight & Arn (Fabaceae)	Leaves Oral candidiasis (Namibia, Tanzania) [19,20].	Inhibit HIV-1 reverse transcriptase [19,44].
5) *Senna occidentalis* (L.) (Fabaceae)	Leaves and roots Cough (Namibia), diarrhoea (Tanzania), jaundice, malaria (Sudan) [19,20].	Inhibit HIV-1 reverse transcriptase [19,45].
6) M*oringa oleifera (*Moringaceae)	Leaves and seeds Diarrhoea, skin infection, vomiting (Namibia, Cameroon), boost appetite and immunity (Uganda) [19,20].	Anti-HIV-1 activity [19,46].
7) *Syzygium guineense* (Myrtaceae)	Bark Diarrhoea (Namibia, Tanzania) [19,20].	Inhibit HIV-1 reverse transcriptase [19,47].
8) *Ximenia americana* L. (Olcaceae)	Bark and roots Oral candidiasis (Namibia), skin rash (Tanzania), contagious diseases, stomach ache, worms (Ethiopia)	Inhibit HIV-1 replication [19,48].

A study on *Plectranthus barbatus* (Lamiaceae), one of the plant species used in the management of HIV/AIDS and associated opportunistic infections, revealed that the ethanolic extract displayed inhibitory activity against HIV-1 protease with a 50% inhibitory concentration IC_{50} of 62.0µg/mL. This study moreover confirmed the ethnobotanical claim of the plant [49]. Another study by Kapewangolo *et al,* (2016) focussed on *in vitro* antiHIV and antioxidant activity of *Hoodia gordonii* (Apocynaceae), a plant that is used ethnomedicinally as an appetite suppressant [50]. This study found that the ethanol and ethyl acetate extracts of the leaves demonstrated good inhibitory activity against HIV with an IC_{50} of 73.55 and 69.81µg/mL, respectively as well as antioxidant activity with a phenolic content of 420 and 319 mg GAE/g. These results suggested new potential uses for *H. gordonii* [50].

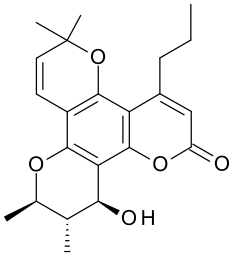
2.4 AntiHIV compounds sourced from plants

A literature study revealed several phytochemicals that show promising antiHIV activities and that could assist in the prevention and control of the opportunistic infectio. These promising natural products such as terpenoids, coumarins, alkaloids and polyphenols, tannins and flavonoids have been shown to possess antiHIV activities [51]. A review by J. Hupfeld and T. Efferth focused on betulinic acid (**1**, figure 6), a triterpene isolated from different plants, which showed inhibitory activity against HIV replicate *in vitro* with an EC_{50} of 1.4 µM. Structural modification of betulinic acid yielded more potent derivatives [52]. In addition coumarins such as calanolide A and B (**2**, figure 6) isolated from *Callophylum cordsto-oblongum* displayed anti-HIV-1 activity. It (calanolide) is a non-nucleoside reverse transcriptase inhibitor (NNRTI) for which an

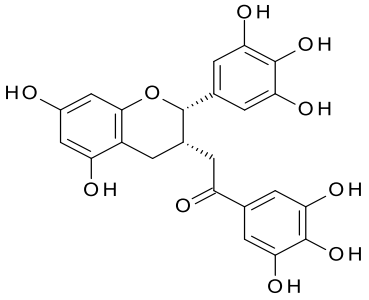
EC_{50} value below 0.02-0.5 µM was recorded [52]. It is reported that this compound is active against a broad range of HIV-1 strains, including other drug resistant variants. (-) Epigallocatechin gallate (**3**, figure 6), a flavanoid isolated from green tea showed inhibition of several steps in the HIV life cycle such as post-adsorption entry and protease kinetics. It also displayed antitumor promoting, anti-inflammatory and anti-oxidative activities [53]. Palicourein (**4**, figure 6), a cyclic polypeptide isolated from organic extracts of the tropical tree *Palicourea condensata* (Rubiaceae), inhibited the *in vitro* cytopathic effects of HIV-1 reverse transcriptase of the cell. Niruriside (**5**, figure 6), a polysaccharide fraction isolated from the methanolic extract of the leaves of *Phyllanthus niruri* L (Cuppressaceae), inhibited HIV-1 reverse transcriptase activity [54].



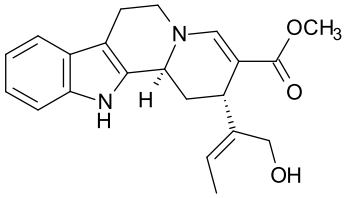
1. Betulinic acid



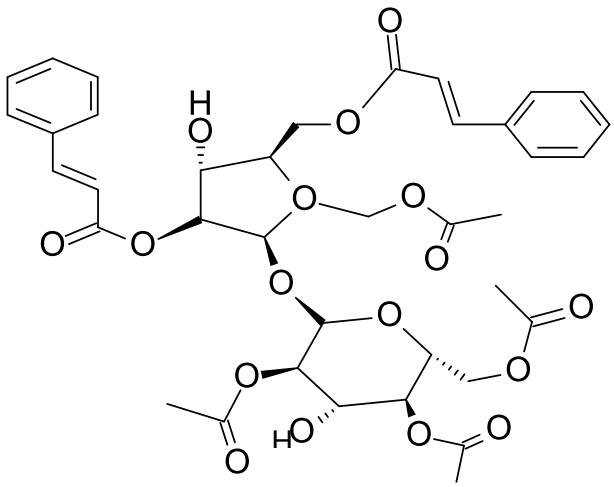
2. Calanolide A



3. Epigallocatechingallate



4. Palicourein



5. Niruriside

Figure 6: Chemical structures of natural products that show antiHIV activity.

2.5 Analytical methods for the isolation and characterization of bioactive compounds from plants.

2.5.1 Chromatographic methods

Chromatographic separation techniques are multistage separation methods in which the components of a sample are distributed between two phases, the stationary and the mobile phase [55]. The stationary phase can be a solid or a liquid supported on a solid or a gel and may be packed in a column, spread as a layer, distributed as a film, or by other techniques. The mobile phase may be gaseous or liquid or supercritical fluid [56].

The separation may be based on adsorption, mass distribution or partition ion exchange. It may also be based on differences among the physicochemical properties of the molecules such as size, mass and volume. The types of chromatography which are useful in qualitative and quantitative analysis are thin-layer, vacuum column, gas and high pressure liquid chromatography [55,56].

2.5.1.1 Thin layer chromatography (TLC)

TLC is a liquid chromatography technique similar to paper chromatography [56]. Separation is achieved by the difference in solubility, pK_a value and capacity of hydrogen bonding of analyte and mobile phase. The stationary phase is coated on a flat glass, metal sheet, plastic or aluminium plate, either a porous solid that is used alone or coated with a stationary liquid [55]. For TLC, the stationary phase can be a silica gel or aluminium oxide with an average particle size of 10- 50µm, fairly wide size distribution. Visualisation of separated compounds on a developed TLC plate can be done with the naked eye. Colourless spots can be visualised using a UV lamp, by exposing the TLC

plate to iodine vapour or by using spray reagent such as vanillin and p-anisaldehyde. A high-performance TLC (HPTLC) plate has an average particles size of 5µm i.e. a narrower particle size distribution. The HPTLC plates are far superior to the normal TLC plates making it faster, reproducible, sensitive and accurate for quantitative work [57]. TLC can be used: - (1) to test the purity of sample, where direct comparison is done between the sample and the standard or authentic sample; (2) to identify compounds, where purification, isolation, and identification of natural products are achieved on basis of retention factor (R_f) and colour of spots whereby visualisation of separated compounds can be done with naked eye and colourless spots can be visualised by the UV lamp or exposing it to iodine vapour or by using spray reagent such as vanillin and p-anisaldehyde; (3) to monitor the progress of reaction. Moreover the advantages of TLC are: (a) very little time for separation is required; (b) less equipment is required; (c) inexpensive; (d) have lower detection limit of analytical samples; and (e) very small quantity of sample can be used [58].

2.5.1.2 Vacuum Liquid Chromatography (VLC)

VLC is a technique where the sample and mobile phase are forced through the column by the application of a vacuum [55,57]. The elution pattern is started with a lipophilic solvent e.g. hexane and the polarity is gradually increased until a more polar solvent like water is used. VLC provides an ideal pre-treatment of large amount of samples of up to 50g extracts for separation and enables fractionation [57]. This technique is simple and quick. Another advantage is that it uses a variety of chromatographic supports such as silica gel, aluminium oxide, cyanide, diol and polyamide. [59].

2.5.1.3 Column Chromatography (CC)

Colum chromatography makes use of columns that vary in length and diameter. It is used for separation or isolation of pure compounds from a mixture of compounds, and involves elution of samples through the column to allow components to separates. Usually, the column is packed with silica or alumina stationary phase that can be chemically bonded. A non-polar solvent is used for a polar surface that uses partition mechanism and the solvents are changed stepwise. The mobile phase solvent can be forced through the column under pressure (using pump or pressure gas) or allowed to pass via gravity. The fractions are collected separately with the elution being monitored by TLC to confirm if the separation is successful [55].

2.5.1.4 Gas chromatography (GC)

GC is a technique used to separate volatile compounds on a stationary phase that is solid or liquid and the mobile phase is a gas [59]. The separation is achieved by varying the polarity of the stationary phase thus enhancing analyte interaction with the inert carrier gas. The chromatography can be gas-solid, which is mainly an adsorptive process and/ or gas-liquid which is partition based process [56]. The liquid stationary phase can be a packed column or a capillary column. In packed column GC, the stationary phase is deposited on a finely divided inert solid support such as diatomaceous earth, porous polymer or graphitized carbon which is packed into a column 1-3 meters (m) in length and 2-4 millimetres (mm) in internal diameter. Capillary columns contain no packed

support; the liquid stationary phase is deposited on the inner surface of the column and may be chemically bonded to it [55,60].

2.5.1.5 High Performance Liquid Chromatography (HPLC)

HPLC is a technique that separate mixture of compounds and is used in phytochemical and analytical chemistry to identify, quantify and purify individual components of mixtures [60]. HPLC columns are stainless steel or polymeric columns packed with a stationary phase comprising small size particles (3, 5, 10 nm) with a narrow size distribution [55]. The flow rate and column dimension can be adjusted to reduce band broadening. The required pressure is applied by the pump to force the mobile phase through the system at a high pressure. The system uses a normal phase column where a nonpolar solvent and polar surface such as silica is used, but it can also use a reverse phase (RP) column. A reverse phase column is usually used for large non-volatile molecules where polar solvent and nonpolar surface are used. The commonly used detector is a UV detector although others like refractive index detectors and evaporative light-scattering detectors are also used [56]

2.5.2 Selected spectroscopic techniques

2.5.2.1 Infrared (IR) spectroscopy

This is the study of the reflection, absorption or transmittance of radiant energy in the IR region of the electromagnetic spectrum at a wavelength of 0.8 to 500 μm. When a molecule is subjected to infrared, transitions take place between rotational and vibrational energy levels in the ground electronic state and the transitions give rise to an

absorption spectrum characteristic of the compound [49]. A more common unit of measurement is the frequency (waves per unit length) and is the wavenumber ($\tilde{\nu}$) in cm^{-1}, where;

$$\tilde{\nu} = \frac{104}{\lambda}$$

The infra-red spectrum is usually divided into three regions namely, Near-IR, Mid-IR and Far-IR. The associated of these region are 12500 to 4000 cm^{-1} (0.8 to 2.5 µm) which can excite overtone or harmonic vibration; 4000 to 400 cm^{-1} (2.5 to 25 µm) used to study the fundamental vibration and associated rotational-vibration structure; and 400 to 20 cm^{-1} (25 to 500µm) lying adjacent to the microwave region with low energy and may be used for rotational spectroscopy [48,49].

2.5.2.2 Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR spectroscopy is a non-destructive technique that allows the number, type and relative positions of certain atoms in a molecule to be determined. This type of spectroscopy applies to those atoms that have nuclear magnetic moments because of their nuclear spin properties [60]. Due to the associated charge and spin, a nucleus can behave like a magnet. NMR applies an external magnetic field to nuclei and then measure the amount of energy required to bring various nuclei to resonance. The amount of energy required for resonance differs due to electronic environments, which can be shielded or deshielded around the atom by a high electron density or lower electron density, respectively. An NMR spectrum is a plot of the strength of a magnetic field versus the intensity of the absorptions, hence the spectrum provides a peak representing

the energy necessary for nuclei resonance [56]. When hydrogen nuclei are studied then the number of each distinct type of hydrogen nuclei and information on the nature of the immediate environment of each type can be obtained. Similar information is determined for carbon nuclei. The combination of IR and NMR data is often used to determine the structure of unknown molecules [60]. The impact of NMR spectroscopy has been significant, because of the range of information it gives. It can work with a diversity of samples solutions and solids. NMR spectra are unique, well-resolved, tractable and often highly predictable for small molecules. In organic chemistry, it is used to confirm the identity of substances although large amounts (2-50 mg), is required, depending on the frequency of machine used [60].

One Dimensional NMR

One-dimensional nuclear magnetic resonance spectroscopy covers the variability in chemical shift of proton attached to carbon and electronegative element such as oxygen and nitrogen. It also deals with the special characteristic of proton attached to nitrogen, the effect of solvent on chemical shift, lanthanide shift reagents, and spin decoupling experiments [55,61]. In one-dimensional experiments, the signal is presented as a function of a single parameter usually a chemical shift [60]. Chemical shift is a resonant frequency of nucleus expressed with reference to standard in a magnetic field, it is often used as a diagnostic of structure of a molecule. In carbon spectra the numbers of non-equivalent carbons such as methyl, methylene, aromatic and carbonyl which may be present in a compound is determined and identified. It provides information about the carbon skeleton of a molecule. Structural determination of Carbon -13 NMR is much

easier than that of proton NMR, but both techniques are used together to determine the structure of an unknown compound [60].

Two dimensional NMR

In two-dimensional experiments there are two coordinate axes that also represent ranges of chemical shift. The data are plotted as a grid, one axis represent one chemical shift range, the second axis represent the second chemical shift range and the third dimension constitute the magnitude (intensity) of the observed signal [61]. Types of 2D NMR methods include: (a) homonuclear through bond Correlation Spectroscopy (COSY) Nuclear Overhauser Effect Spectroscopy (NOESY), and (b) Heteronuclear correlation, one-bond correlation Heteronuclear Single-Quantum Correlation Spectroscopy (HSQC), and long range correlation, Heteronuclear Multiple-bond Correlation Spectroscopy (HMBC) [61]. In experiments that use complex pulse sequences, such as distortion less enhancement by polarization transfer (DEPT), a preparative phase is included before data acquisition that determine the multiplicity of carbon atoms substituted with hydrogen [61]. In COSY NMR spectroscopy technique, the method is applied to a structure problem where the chemical shift range of the proton spectrum is plotted on both axes [56]. The splitting pattern of a certain proton can be obtained and interpret in terms of number of protons located on the adjacent carbon [53]. Selective decoupling is used to collapse or sharpen portions of the spectrum in order to obtain more direct information about the nature of coupling pattern. These methods can be tiresome and very difficult with complex spectra [60].

HMBC (Heteronuclear multiple bond correlation)

HMBC spectroscopy is a modified version of HMQC (heteronuclear multiple quantum correlation) and is suitable for determining long range ^{1}H- ^{13}C connectivity. Since it is a long range chemical shift correlation experiment, HMBC provides information about the chemical shift of carbon atoms that are about 2-3 bonds away from the proton to which they couple. HMBC allows coupling to exist across heteronuclear atoms such as oxygen and nitrogen, making it a powerful technique for structure characterisation. HMBC also allows for the detection of quaternary carbons atoms that are coupled to protons through multiple bonds [61].

A HSQC (Heteronuclear Single Quantum Correlation) experimental reveals a 2D heteronuclear chemical shift that correlates the proton that is directly bonded to carbon or a heteronuclei (commonly ^{13}C and ^{15}N). It is widely used because it is based on proton-detection, offering high sensitivity when compared with the conventional carbon-13 detected by 2D HETCOR (Heteronuclear correlation). At the bottom of the spectra is the proton and on the vertical axes is the carbon spectrum. Cross peaks gives the shift of the corresponding proton and carbon. This experiment utilizes the one-bond coupling between carbon and proton (J = 120-215Hz), where J is the coupling constant [61].

CHAPTER THREE: RESEARCH METHODS

3.1 Chemicals and Materials

All solvents, with the exception of analytical grade solvents for example hexane, purchased from Biodynamic, were distilled before use.

3.2 Collection and pre-treatment of plant material

The leaves of *Maerua schinzii* and *Catophractes alexandri* (Figure 7) were collected in January 2015 at plot 46 on the farm Dobra in the Khomas Region (22°20'55.1"S 17°10'06.7"E) about 25km from the capital Windhoek. The plants collected were taxonomically identified at the National Botanical Research Institute (NBRI) by Mr. D. Aiyambo. After collection, the leaves were air-dried at room temperature for a period of two weeks in the shade. Adulterated and diseased leaves were removed before the dry leaves were grounded to a uniform powder using a blender. The powders were stored in amber bottles in a fridge at 4^{o}C until they were needed for further analysis.

(a) (b)

Figure 7: Leaves of (a) *M. schinzii* and (b) *C. alexandri*.

3.3 Phytochemical screening

Two methods – TLC screen and qualitative test were used in testing for the presence of six classes of compounds such as alkaloids, flavonoids, bitter principles, coumarins, saponin and terpenes. The TLC screen methods entailed the separation of plant extracts on a TLC plate with the best solvent system, followed by the spraying of the plate with a recommended detection reagent. The qualitative test involves the use of a test tube according to reported procedure [62].

3.3.1 TLC screening method

Extracts of the leaves for each plant were prepared by suspending about1g of powdered leaves in different solvent extraction system depending on the metabolites being screened. The leaf extracts were spotted manually using a capillary tube on analytical TLC plate (pre-coated silica gel 60 F_{254}). The plates were developed in different solvent systems in order to select a suitable mobile phase that give a good separate of the tested compounds outlined in Section 3.4 [57].

Table 2: Solvent systems used for TLC

No	Solvent	Ratio
1	ethyl acetate: hexane	9:1
2	ethyl acetate: methanol	5:1
3	ethyl acetate: methanol: water	8:1:1
4	ethyl acetate: methanol: water	4:1:1

5	DCM: methanol	5: 2.5
6	DCM: methanol: water	7:1:1
7	chloroform: methanol: acetic acid	7:1:2
8	DCM	100%
9	methanol	100%

The developed plates were sprayed using different spray reagents as indicated in Table 2 for the identification of the different classes of compounds. The colours of the spots were noted and R_f values calculated using the formula below [62].

$$\text{Rf} = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by solvent}}$$

Table 3: List of spray reagents used for phytochemical screen test

Class of secondary metabolites	Spray reagent	Expected colour
Alkaloid	Dragendoff reagent (commercially prepared)	Orange spot
Saponin	*p*-anisaldehyde-sulphuric acid spray reagent: prepared by mixing 0.5mL of *p*-anisaldehyde, 50 mL of acetic acid, and 1mL of concentrated sulphuric acid [60]	Red colour
Bitter principle	*p*-anisaldehyde-sulphuric acid spray reagent: preparation method as outlined above	Brown colour
Coumarins	10% ethanolic (in H_2O)	Yellow colour
Terpenes	*p*-anisaldehyde-sulphuric acid spray reagent: preparation method as outlined above	Red violet

3.3.1.1 Test for the presence of saponins

Powdered leaves (1g) were dissolved in 10mL MeOH and heated on a water bath for 5 minutes at about 60^{o}C and then filtered. The filtrate was evaporated to about 5mL and then spotted on silica gel 60 F_{254} pre-coated TLC plates. The plates were developed in chloroform/glacialacetic acid/methanol/water (64:32:12:8), a blue violet colour at 365 nm and an intense yellow orange fluorescent colour with visible light, confirmed the presence of saponins [57].

3.3.1.2 Test for the presence of bitter principles

Well-grounded leaves (0.9757g *C. alexandri* and 0.9723g *M. schinzii*) were suspended in 10mL methanol and heated on a water bath for 15 minutes at about 60^{o}C and then filtered. The filtrate was evaporated to about 5mL and then spotted on a TLC plate. The plates were developed in EA/methanol/water (77:15:8). For detection, a spray reagent of *p*-anisaldehyde-sulphuric acid was used [58].

3.3.1.3 Test for the presence of alkaloids

Well-grounded leaves (0.9454g *C. alexandri* and 0.9718g *M. schinzii*) were mixed with 1mL of a 10% ammonia solution, exactly 5 ml of methanol was added and the mixture was heated under refluxed for 15 minutes. The extract was cooled to room temperature and filtered. The filtrate was evaporated to about 5mL and then spotted on a TLC plate. The plates were run in EA/methanol/water (100:135:10). For detection the developed plates were sprayed with Dragendorff reagent [57,62].

3.3.1.4 Test for the presence of coumarins

Well-grounded leaves (1.0046g *C. alexandri* and 1.0027g *M. schinzii*) were extracted by heating for 15 minutes under reflux with 10 mL DCM. The filtrate obtained was evaporated to dryness and the residue dissolved in 0.5mL toluene and then spotted on silica gel 60 F_{254} pre-coated TLC plates. The plates were developed in toluene/EA (93:7). For detection, a spray reagent of 10% ethanolic KOH was used [63].

3.3.1.5 Test for the presence of terpenes

Well-grounded leaves (1.0017g *C. alexandri* and 1.0015g *M. schinzii*) were extracted by heating for 15 minutes under reflux with 10mL DCM. The filtrate obtained was evaporated to dryness and the residue dissolved in 0.5 mL of toluene. Approximately 20 – 40µl was used for TLC. The plates were developed in toluene/EA (93:7). For detection, the *p*-anisaldehyde-sulphuric acid spray reagent was used and a blue-violet and red-violet spot showed the presence of terpenes [62].

3.3.2 Chemical test method

3.3.2.1 Test for the presence of saponins

Well-grounded leaves (0.9730g *C. alexandri* and 0.9745g *M. schinzii*) were suspended in 15mL of water in a test tube and the resulting mixture was heated on a water bath for 5 minutes at 60^{o}C. The extracts were cooled to room temperature and then filtered into a test tube (16 x 160 mm). The filtrate was shaken for 30 seconds and the height of the honeycomb froth which persists for 3 minutes was measured. Froth higher than 1cm confirmed the presence of saponins [62]

3.3.2.2 Test for the presence of flavonoids

Well-grounded leaves (0.9454g *C. alexandri* and 0.9718g *M. schinzii*) were extracted with 10 mL water and 5 mL methanol for 10 minutes and then filtered. To the filtrate, magnesium turnings were added and conc. HCl was added drop wise (cyaniding reaction). The subsequent development of the colours: orange for flavones, red for flavonols and pick for flavanones indicated the presence of flavonoids [63,64].

3.3.2.3 Test for the presence of coumarins

About 1g of powdered leaves (1g *C. alexandri* and 1g *M. schinzii*) was moistened with 5mL and the extracts of 1.05g were taken in a test tube. The mouth of the test tube was covered with filter paper and treated with 1N NaOH solution. The test tube was placed for a few minutes in boiling water upon which the filter paper was removed and examined under UV light; yellow fluorescence indicated the presence of coumarins [63].

3.4 Extraction and purification of metabolites

3.4.1 Chromatographic methods

Analytical thin layer chromatographies (TLCs) on alumina backed silica gel 60 F_{254} coated plates (Merck) were used. Visualization of spots on the plates was done using an ultraviolet (UV) lamp (254 nm/366 mm, Camag) before spraying the plates. Spray reagents used to detect and visualize the spots included (i) *p*-anisaldehyde – sulphuric acid [60]; and (ii) vanillin–sulphuric acid, prepared by dissolving 0.5g vanillin in 100mL sulphuric acid/ethanol (40:10). After application of the spray reagent, the plates were

heated at 110^{o}C for approximately three minutes [63]. R_f values were calculated as discussed in Section 3.3.1.

Vacuum Liquid Chromatography was done using sintered glass filter funnel (diameter of 75 mm, length of 50 mm) which was dry-packed with high purity grade silica gel (pore size 60Å, 230-400 mesh particles, Sigma Aldrich) as shown in figure 8. The packing was consolidated by the application of a vacuum and the column was washed with hexane before the crude extract was introduced as dried slurry. For the preparation of the dry slurry, the crude extract was dissolved in approximately 10mL of the extraction solvent system (DCM/MeOH-1:1). The mixture was then added drop wise to 10-20 g of coarse silica gel (60-200 mesh). Constant stirring of the silica gel allowed for a uniform adsorption of the extract onto the silica gel. The dried slurry was then introduced onto the VLC column and covered with filter paper. Solvents were eluted with the application of vacuum in the order of increasing polarity, starting with hexane (2 x 300 mL), DCM (2 x 300 mL), ethyl acetate (2 x 300 mL), acetone (2 x 300 mL), MeOH (2 x 300 mL) and finally H_2O (5 x 200mL).

Figure 8: Experimental setup for vacuum liquid chromatography (VLC) [65].

Column Chromatography (CC) columns (gravity) were prepared by using the slurry packing method with silica gel (70-230 mesh particles, Sigma-Aldrich). Hundred gram of silica gel was used to purify 1g of the crude sample. A glass column with a sintered glass filter was used. The introduction of bubbles in the column was avoided by stirring the slurry continuously while introducing it into the column. The adsorbent was allowed to settle before the sample was introduced as pre-adsorbed dried slurry. Fractions were eluted with solvents in order of increasing polarity starting with hexane, DCM, ethyl

acetate, acetone, MeOH and finaly 20% MeOH/H_2O. The fractions were subsequently collected in labelled test tubes.

Preparative TLC plates were prepared using high-purity grade (Merk grade 7749) silica gel with gypsum binder and fluorescent indicator. A slurry was prepared by mixing 30 g silica gel and 60mL distilled water in a stoppered Erlenmeyer flask. The flask was shaken vigorously before 90 mL (to obtain a 1 mm thick plates) of the slurry was uniformly distributed on a clean, dry 20 x 20 cm glass plate [66]. The plates were then allowed to dry in a cupboard for four days. Before use, the plates were activated in an oven at 110^{o}C for an hour and allowed to cool to room temperature. Approximately 100 – 150 mg of the combined VLC fraction was applied. Purification of compounds was also done using commercial preparative TLC plates (PLC Silica gel 60 F_{254}, 1 mm thick, Merck). Activation of the commercial PLC plates was done as described above.

3.4.2 Extraction

The powdered leaves of *C. alexandri*, (201.7g) was subjected to an extraction by suspending it in 1.5 L of a 1:1 mixture of dichloromethane/methanol (DCM/MeOH) solution on an orbital shaker. The extraction was done at room temperature over a period of 48 hours, after which the extract was filtered and concentrated using a rotary evaporator. The leave residue was further extracted with MeOH (400mL) for another 24 hours on an orbital shaker [63].

After filtration, the MeOH extract was concentrated. The TLC profile of the DCM/MeOH (1:1) and MeOH extracts, obtained using EA/MeOH/H_2O (4:2:1) as

solvent system, were found to be similar and thus the extracts were combined. The combined extracts were dried and the yields determined [63].

The combined extract for *M. schinzii* was prepared as outlined above with the exception that 300.2 g of powdered leaves was used for the extraction.

VLC fractionation was done for both *C. alexandri* (33.3234 g) and *M. schinzii* (32.5616 g) leave extracts as explained in the above sub-section. The glass funnel was charged with 100 g of silica gel for every 1 g of crude extract to be purified. For each solvent, 3 - 5 fractions were obtained yielding a total of 23 fractions for *C. alexandri* and 18 fractions for *M. schinzii*, respectevely. The fractions were profiled using TLC with EA/MeOH/H_2O (4:1:1) as mobile phase and then combined based on their TLC profile. In this manner five combined fractions labeled A- E were obtained for each plant. The combined VLC fractions (A-E) were then further purified using a combination of column chromatography, preparative TLC as well as recrystalizaion to afford semi-purified metabolites.

3.5 Structure elucidation of isolated metabolites

Structure elucidation was done using physical data such as melting point as well as spectroscopic data.

NMR analysis of samples was done by Dr Jacobus Brand at Stellenbosch University in South Africa. The ^{1}H-NMR data were recorded on Varian Spectrometers with operating frequency of 400 MHz and 600 MHz respectively. ^{13}C-NMR was done using the same

instruments at 75 and 150 MHz, respectively. For ^{13}C NMR, chemical shift values were reported without being assigned to specific carbon atoms. Chemical shift (δ) were recorded in part per million (ppm) in relative to the internal standard, tetramethylsilane (TMS). Deuterated solvents used include CD_3OD (δ_H 73.31, 4.78; δ_C 49.2) and DMSO (δ_H 5.32; δ_C 54.0). The multiplicity of the signal is indicated as: *s*- singlet, *d*– doublet, *dd*- double of doublets, *q*- quartet, and *m*- multiplet. Coupling constant (*J*), rounded off to one decimal, were recorded in Hertz (Hz). The infrared (IR) spectra were recorded at the Namibian University of Science and Technology on a Perkin Elmer Fourier-Transform Infrared Spectrophotometer and the relative intensities of the bands indicated as broad (br), strong (s) or weak (w). The melting points of the isolated compounds were measured on a Stuart melting point apparatus without correction.

3.6 Bioassay screening

In accordance with the objectives of the study, the semi pure compounds isolated from the two plants were screened for antiHIV activity (protease and reverse transcriptase) and antimicrobial activities.

3.6.1 AntiHIV assay

The HIV testing was done at the Chemistry and Biochemistry department at UNAM. Partially purified compounds isolated from *M. schinzii* and *C. alexandri* were tested for inhibitory activity against HIV protease and reverse transcriptase. Due to limited amounts of the compounds isolated and the limited amount of test kits available, the tests conducted could not be done in duplicate.

For the HIV-protease (PR) inhibitory activity evaluation, a fluorogenic HIV protease substrate 1 (Arg-Glu(EDANS)-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-Lys(DABCYL)-Arg) was dissolved in DMSO to make a 1 mM stock. The stock fluorogenic substrate was diluted to 10 μM using buffer (0.1 M sodium acetate, 1 M NaCl, 1mM EDTA and 1mM DTT, pH 4.5). An aliquot of the substrate (10 μM, 49 μM) and 1μl of HIV-1 PR solution (1 μg/ml; Bachem, Switzland) were added to the reaction mixture in an assay buffer in the presence of the extract to yield a final reaction volume of 100 μl. The mixture was incubated at 37 ^{o}C for 1 hour in black 96 well assay plates. The fluorescence intensity was measured at an excitation wavelength of 355 nm and an emission wavelength of 460 nm using a synergy microplate spectro-fluorometer (BioTek, Analytical & Diagnostic products, South Africa). Ritonavir (Rit) was used as a positive control for HIV-1 PR inhibition. The blank treatment, consisting of assay buffer with only the substrate, an untreated control of enzyme and substrate were also included. The percentage inhibition was calculated based on the formula:

$$100 - [(test\ reagent\ RFU - background)/(untreated\ control\ RFU - blank)X\ 100]$$

Where RFU = relative fluorescence units [49,67].

For inhibitory effect against HIV-1 reverse transcriptase (RT), extracts were tested using a reverse transcriptase colorimetric assay kit from Roche Diagnostics (Mannheim, German) and purified recombinant HIV-1 RT (Merck, Darmstadt, Germany). The extracts and the enzymes (0.2 U) were incubated for 1hour at 37 ^{o}C. Consequently 1 hour incubation involved addition of an antibody conjugated to peroxidate that bind to the digoxigenin-labeled DNA. At the final step, the peroxidase substrate solution [2,2'-

axino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)] is cleaved by the peroxidase enzyme, producing a coloured reaction product. The absorbance of the samples was read at 405 nm with a reference wavelength of 492 nm using a microtiter plate reader (Multskan Ascent; Thermo Lab systems; USA) and was directly correlated to the level of RT activity in the sample. A known RT inhibitor, doxorubicin, was used as a control [49,50].

3.6.2 Antimicrobial activity testing

In this study, two methods were used to evaluate the antimicrobial activities of the plant extracts:

i. The agar disc diffusion and;

ii. Minimum inhibitory concentration (MIC)

Reference isolates of gram-negative *Escherichia coli*, gram-positive *Staphylococcus aureus, Klebsiella pnemoniae* and fungal *Candida albicans* were used [67].

3.6.2.1 Agar Disc diffusion method

A concentration of 10 mg/ml of each semi-purified compound was prepared by dissolving 0.1g in 10 ml of DMSO. For positive controls 1mg/ml each of ampicillin, penicillin, and vancomycin were used and DMSO solvent as the negative control. This experiment was done in duplicate. Schaelau Nutrient agars were prepared by dissolving 28 g of agar powder in 1000 mL of distilled H_2O and by bringing the mixture to a boil to aid dissolution. The agar was sterilized by autoclaving at 121 ^{o}C for 15 min. After 15 min, the agar was allowed to cool for a while and 15ml of the agar was dispensed into

the plates. The agar plates were allowed to solidify and 100 µl of the microorganisms were inoculated onto the plates. A paper disc that had been soaked in the plant extract was placed at the centre of the plate. The plates were incubated for 24 hours at 37 ^{o}C for the plant extracts to find the true zone of inhibition [68].

3.6.2.2 Minimum inhibitory concentration (MIC)

A total of 17 agar plates were used for the three semi-pure compounds. Distilled water, nutrient broth and pipette tips were autoclaved and cultures of bacteria were prepared and left for 24 hours to grow. A concentration of 5 mg/ml of bacteria strain was prepared and 100µl of bacteria culture was then added to the entire plate and allowed to set. A stock solution of each semi-purified compounds were prepared by dissolving 0.1g of the compounds in 10ml of the DMSO solvent, then 800µl of the mixture was removed from stock solution A and transferred to B solution (serial dilutions) and 500µl from B downwards up to E solution. Ampicillin (25 µg), penicillin (10 µg), and vancomycin (30 µg) were used as a positive controls and DMSO as the negative control. The plates were covered and incubated overnight at 37^{o}C. The plates were visualised for growth activities the following day. A clear area around the disc indicates the presence of the activities [68].

CHAPTER FOUR: RESULTS AND DISCUSSION

4.1 Phytochemical screen test results

The isolation of bioactive compounds is a long and labour-intensive process. Phytochemical screen test however, allows for the detection of class of metabolites in crude plant extracts. The tests are simple to perform, but have a low sensitivity and selectivity of detection, which makes it difficult to detect components present in trace amounts within the sample [68]. Like most qualitative tests there are limitations, which include false-negative and false-positive results that may be recorded, e.g. amino acids may give a positive result for alkaloids. [65]. It is for this reason that two different methods, the TLC and chemical (Harborne) method, were used to detect the presence/absence of saponins, bitter principles, alkaloids, flavonoids, coumarins and terpenes in the leaves of the two plants under study.

4.1.1. *M. schinzii*

With the TLC method, a yellow to orange colour was observed for spots on TLC which indicated the presence of saponins for both plants under study, as presented in Table 4. The saponins R_f values with $CHCl_3$:CH_3COOH:MeOH:H_2O (64:32:12:8) were found to be the same as reported by Wagner which were in the range of 0.7 – 0.9 [65]. For the chemical test the height of the honeycomb forth for *M. schinzii* was higher than that of *C. alexandri* which further provides evidence of the presence of saponins as shown in Table 5. Saponins were detected in the leaves of *M. schinzii* as indicated in table 4.

Table 4: Results from TLC screening done on the two plants

Phytochemical class	Plant species	Colour observed	R_f value (solvent system)
Saponins	*C. alexandri*	Yellow-orange colour	0.81 ($CHCl_3$:CH_3COOH:MeOH:H_2O - 64:32:12:8)
	M. schinzii	yellow-orange colour	0.71 ($CHCl_3$:CH_3COOH:MeOH:H_2O - 64:32:12:8)
Bitter principles	*C. alexandri*	Brown-red colour	0.53 (EA:MeOH:H_2O - 77:15:8)
	M. schinzii	Brown-red colour	0.24 (EA:MeOH:H_2O - 77:15:8)
Alkaloids	*C. alexandri*	Orange-brown colour	0.5 (EA:MeOH:H_2O - 60:30:10)
	M. schinzii	Orange-brown colour	0.9 (EA:MeOH:H_2O - 60:30:10)
Flavonoids	*C. alexandri*	Orange colour	0.32 (EA:MeOH:H_2O - 60:30:10)
	M. schinzii	Pick colour	0.24 (EA:MeOH:H_2O - 60:30:10)
Coumarin	*C. alexandri*	Yellow colour	0.05 (TOL:EA - 93:7)
	M. schinzii	Yellow colour	0.35 (TOL:EA - 93:7)
Terpenes	*C. alexandri*	Blue violet	0.1 (TOL:EA - 93:7),
	M. schinzii	Red violet	0.6 (TOL:EA - 93:7)

Bitter principles are a class of substances that have a bitter taste but with no sharply defined chemical characteristics. They are heterogeneous compounds that do not belong to the class of alkaloids. Upon conducting the TLC method, a brown-red colour in the visible region was observed using *p*-anisaldehyde spray reagent and this signified the

presence of bitter principles, Table 4 [57]. The R_f values with EA:MeOH:H_2O (77:15:8) solvent system was 0.24 for *M. schinzii*, which is in line with the values stated by Wagner of R_f range 0.2- 0.5 [57].

The presence of alkaloids was determined using the TLC method and confirmed by the presence of yellow and orange-brown spots. The R_f values of the orange-brown spots are at 0.5 for *M. schinzii*. The brown colour was reported by Wagner, who reported that alkaloids form a brown precipitate with Dragendroff's reagent [57]. The blue spots observed on a sprayed TLC had R_f values with EA:MeOH:H_2O (60:30:10) of about 0.9 and this was also reported by Wagner [55]. Thus, the experiments concluded the presence of alkaloids in *M. schinzii* and this is supported by litreture as mentioned in Section 2.1.

The test for flavonoids has been described in literature by many researchers (e.g. Wagner and Tiwari) to give different colours depending on their classes [57]. In this research, the *M. schinzii* gave a pink colour. Flavones give an orange colour, flavonols give a red colour and flavanones give a pink colour [57]. Thus flavonoids were found to be present in *M. schinzii* and specifically, they contain flavones and flavanones, respectively. Previous studies on members in the genus M*aerua* also reported the presence of flavonoids [59].

The test for coumarins was done on two plants leaves where they were treated according to the chemical method (A) described in Section 3.3.2.5. Yellow fluorescence was observed on the filter paper under UV-254 nm. With the TLC method (B), the TLC spots showed yellow fluorescence spot with an R_f value ranging between 0.05 to 0.35.

The spot observed for *M. schinzii* were more intense than that of *C. alexandri*. Wagner also reported similar R_f values ranging from 0 to 0.38 and their research used a furanocoumarin standard, whereby the solvent system used was toluene/ethyl acetate/acetic acid (90:10:1). Coumarins are reported to have a distinct fluorescence quenching at UV-254 nm. At UV-365 nm, an intense blue or blue-green fluorescence was observed for simple coumarins whereas a yellow, brown, blue or blue-green fluorescence is observed for furano- and pyranocoumarins. The un-substituted coumarins also fluoresce with yellow-green in UV-365 nm, but only after treatment with the KOH reagent or ammonia vapour [57]. The observed yellow fluorescence marked the presence of coumarins in *M. schinzii*.

For the test of terpenes, red-violet spots were observed on the TLC for *M. schinzii*. The spots for *M. schinzii* were more intense than those for *C. alexandri* and the R_f values with TOL:EA (93:7) was 0.6. Wagner also observed a blue-violet and red to red-violet colour on TLC after spraying with *p*-anisaldehyde-sulphuric acid reagent with a triterpene standard [57]. The test for the presence of terpenes was positive for *M. schinzii*. Previous studies on the genera of *Maerua* also confirm the presence of terpenes [24].

Table 5: Chemical test results of the leaves of *M. schinzii* and *C. alexandri*

Phytochemical constituents	***C. alexandri* leaves**	***M. schinzii* leaves**
Saponins	++	+++

Bitter principles	+	++
Alkaloids	+++	++
Flavonoids	+++	+++
Coumarins	++	++
Terpenes	++	+++

+ faint (very weak) ++ clear (slightly visible) +++ - intense ++++ highly intense

4.1.2 *C. alexandri*

For the TLC method, a yellow to orange colour was observed for spots on TLC which indicated the presence of saponins for *C. alexandri*, as indicated in Table 4. The saponins R_f values with $CHCl_3$:CH_3COOH:MeOH:H_2O (64:32:12:8) were found to be in line with the work done by Wagner (range of 0.7 – 0.9) [55]. For the chemical test the height of the honeycomb forth was lower than of *M. schinzii*, as summarised in Table 5.

Regarding the bitter principles, the TLC method showed a brown-red colour in the visible region using *p*-anisaldehyde spray reagent and this signified presence thereof [63]. The R_f values with EA:MeOH:H_2O (77:15:8) was 0.53 for *C. alexandri* which is in line with the values stated by Wagner (R_f range of 0.2- 0.5) [63].

The presence of alkaloids was determined by TLC method and identified by the presence of yellow and orange-brown spots. The R_f values of the orange-brown spots was determined to be 0.9 for *C. alexandri*. The brown colour was reported by Wagner as well, who reported that alkaloids form a brown precipitate with Dragendroff's reagent [65]. Thus, from the experiments, alkaloids were also present in *C. alexandri* as

discussed in Section 2.1.5, which stated that the genus *Catophractes* has alkaloids present.

As stated before, flavonoids has been described in literature by many researchers (e.g. Wagner and Tiwari) to give different colours depending on their classes [65]. Many different colours have been reported according to the test scheme of the researcher. In this research, the *C. alexandri* extract produced an orange colour. Flavones give an orange colour, flavonols give a red colour and flavanones give a pink colour [65]. This means that flavonoids are present in *C. alexandri* and specifically they contain flavones respectively [67].

The test for coumarins was done on *C. alexandri* leaves whereby they were treated according to a chemical method (A), as discussed in Section 3.3.2.5. A yellow fluorescence was observed on the filter paper under UV-254 nm. With the TLC method (B), the TLC spots showed yellow fluorescence spots with an R_f value of 0.05. Wagner also reported similar R_f values ranging from 0 to 0.38 and their research used a furanocoumarin standard, whereby the solvent system used was toluene/ethyl acetate/acetic acid (90:10:1) [65].

Coumarins are reported to have a distinct fluorescence quenching at UV-254 nm. At UV-365 nm, an intense blue or blue-green fluorescence was observed for simple coumarins while yellow, brown, blue or blue-green fluorescence is observed for furano- and pyranocoumarins. The un-substituted coumarins also fluoresce with yellow-green at UV-365 nm, but only after treatment with the KOH reagent or ammonia vapour [65]. The observed yellow fluorescence marked the presence of coumarins in *C. alexandri*.

For the test of terpenes, blue-violet spots were observed on TLC for *C. alexandri*. The spots for *C. alexandri* had R_f values with TOL:EA (93:7) of 0.1. Wagner also observed a blue-violet and red to red-violet colour on TLC after spraying with *p*-anisaldehyde-sulphuric acid reagent and their research was using a triterpene standard [65]. The test for the presence of terpenes was positive for *C. alexandri*. Previous studies on the genus of *Catophrates* also confirm the presence of terpenes [26].

Previous phytochemical analysis on members of genus *Catophractes* and *Maerua* reveal the presence of the following classes of phytochemicals: Saponins, bitter principles, alkaloids, flavonoids, coumarins and terpenes as discused in Section 2.1. Alkaloids are mostly used as analgesic (morphine), anaesthetic (cocaine), stimulant (caffeine), antitumor (vinblastine) and antibacterial (berberine) agents [69]. Flavonoids are known to possess anti-inflammatory, anti-allergic, antiviral, antispasmodic and diuretic property (Bruneton, 1995; van Wyk et al 1997). Saponins are known to act as immune boosters, but also display cholesterol lowering and anticancer property. [69].

4.2 Extraction of the powdered leaves

The crude dried, extracts were prepared according to the specified method outlined in Section 3.5. The method used afforded an exhaustive extraction of plant material as evidence by the relative high crude yields reported in Table 6 below. The high chlorophyll content of the leaves presented a challenge for the purification and isolation of compounds.

Table 6: The amounts and percentage yield of crude extracts obtained for the leaves of *M. schinzii* and *C. alexandri*.

Plant type	Plant powder (g)	Amount (g)	Percentage yield
C. alexandri	201.70	33.3234g	16.52%
M. schinzii	300.20	32.5616g	10.85%

Table 6 shows the amount of powder extracted and percentage yield obtained with reference to the grounded plant material. *C. alexandri* had the highest percentage yield from the 201.70g compared to *M. schinzii*.

4.3 Fractionation of crude leaf extract of *M. schinzii*

The crude extract of *M .schinzii* (32.5616 g) was subjected to VLC as described in Section 3.4. A total of 18 fractions were obtained and Figure 9 (a) below shows the VLC fractions as different coloured solutions using a gradient elution as explained in Section 3.4. The fractions were spotted on TLC in EA/MeOH/H_2O (4:1:1) as observed in figure 9 (b) and fractions with similar spots in terms of R_f values were combined and yielded 5 pooled fractions (1-3, 4-6, 7-9, 10-15 and 16-18) that were labeled A-E respectively as presented in figure 9 (c).

From the TLCs in figures 9(b) and 9 (c), it was clear that fraction A was devoid of any compound and therefore no further work was done on this fraction. Fraction B in this figure 9 (b) and 9 (c) comprised mostly of chlorophyll, and it was also not subjected to further purification.

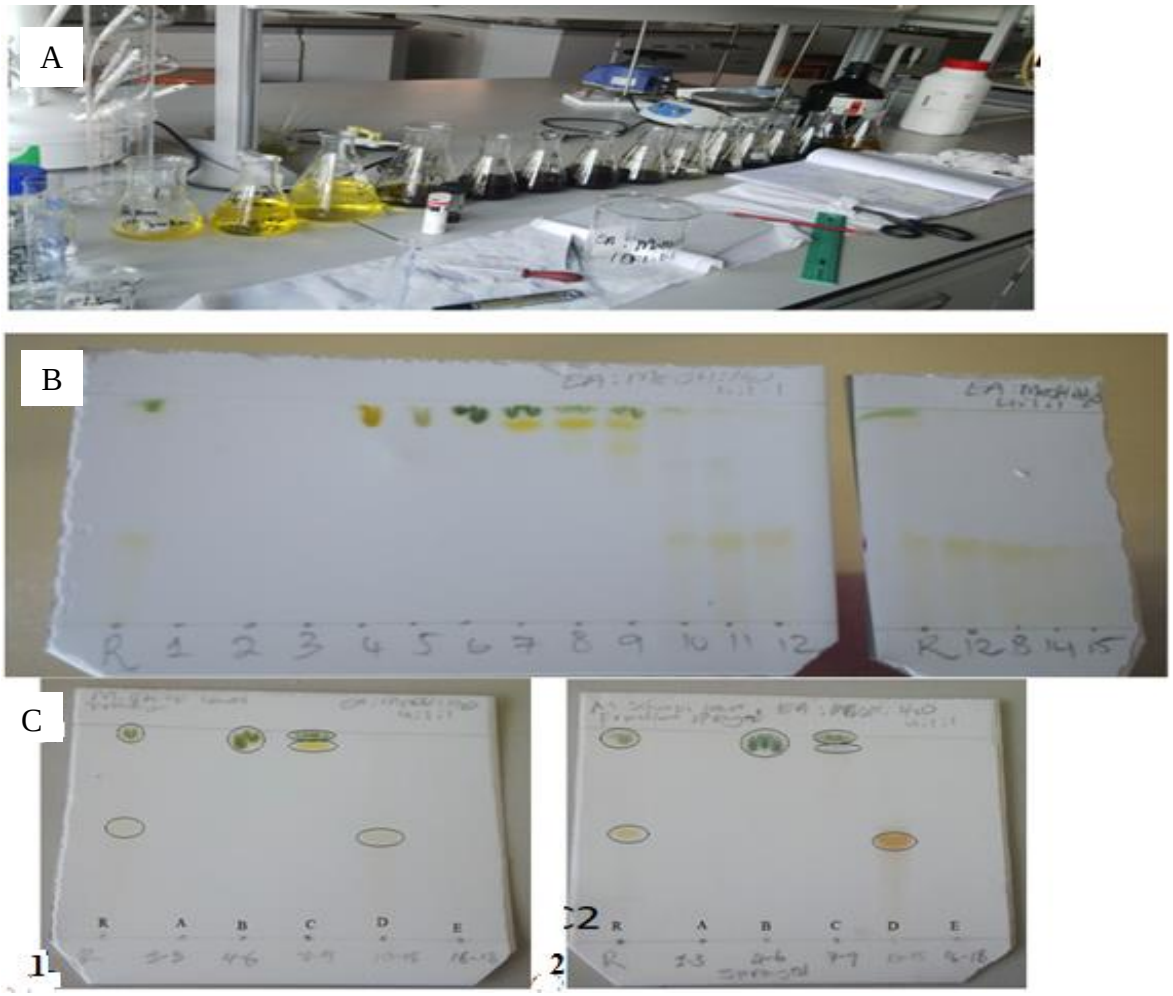


Figure 9: (A) VLC fractions of the crude leave extract of M. schinzii obtained with solvents of increasing polarity, (B) TLC profile of VLC fractions developed in EA/MeOH/H_2O (4:1:1), before the fractions were combined. (C) TLC plates of the combined VLC fractions of *M. schinzii*, showing the UV active and UV inactive compounds. The letter R (=reference) represent the crude, leave extract.

Fraction C (0.2672g) was loaded onto a commercial preparative TLC plate and developed in EA/MeOH/H_2O (4:1:1) to give 5 bands: (CB1- DC5). A whitish solid formed in fraction CB5 (0.0705g) and was therefore subjected to recrystallization using methanol to yield partial purified compound MLCB51 (0.0341g), as shown in figure 10.

Bands CLDB11a, CLDB221, CLDB222, CLDB331, MLCB21 and MLCB31 as well as semi-purified compound MLCB51were submitted for antiHIV analysis. Fraction E (16-18) was obtained by eluting the VLC column with distilled water. It was suspected to consist of polysaccharides and glycoside which are not of interest to the project; thus no work was further conducted on this fraction.

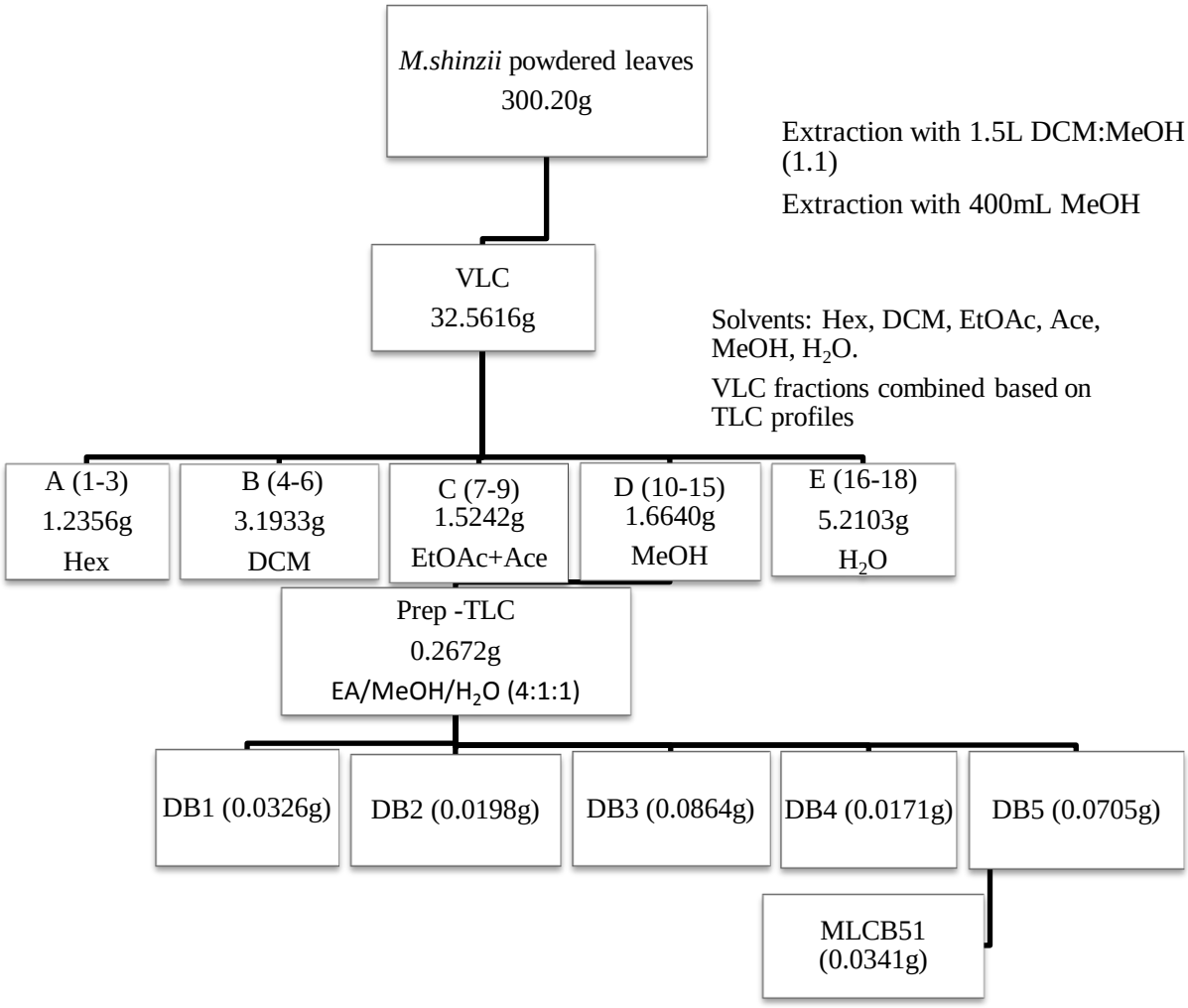


Figure 10: Schematic diagram of the isolated compounds from the leaves of *M. schinzii*.

From figure 10, only one compound MLCB51was isolated from the leaves extract of *M. schinzii* which could not be further purified nor could additional spectral data be obtained, to aid in determining the structure. This was due to the small amount of compound isolated.

4.4 Fractionation of crude leaf extract of *C. alexandri*

The crude leave extract of *C. alexandri* (33.3234 g) was subjected to VLC as described in Section 3.4. A total of 23 VLC fractions were obtained and subsequently spotted on TLC and developed with EA/MeOH/H_2O (4:1:1) solvent system. Fractions with similar TLC profiles were combined to yield 5 pooled fractions (1-5, 6-9, 10-13, 14-19, and 20-23) that were labeled A-E respectively as summarized in figure 11.

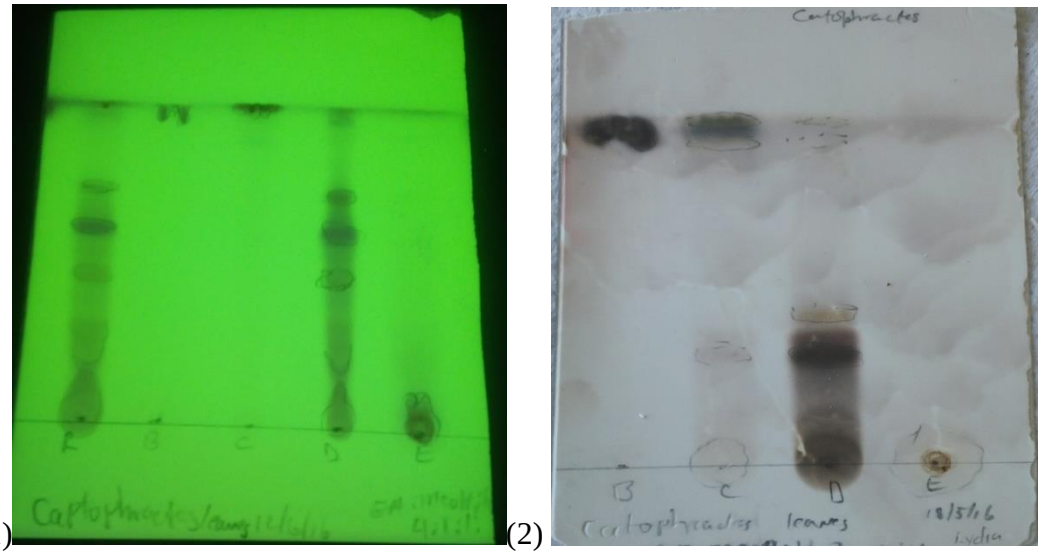


Figure 11: A TLC plate developed in EA/MeOH/H2O (4:1:1) of the combined VLC fractions for C. alexandri, showing (1) the UV active compounds and (2) the compounds visible after using anisaldehyde spray reagent. The letter R (= reference) represent the crude, unfractionated leaf extract.

Fraction A, is mostly likely to contained non-polar, lipophilic metabolites including waxes and fatty acids. Additionally, the TLC profile of fraction A confirmed the absence of potentially valuable compounds, thus it was not further investigated. Fraction C comprised mostly of chlorophyll as shown in figure 12 and it was also not further purified. Fraction E (20-23) was the distilled water extract and was not further purified.

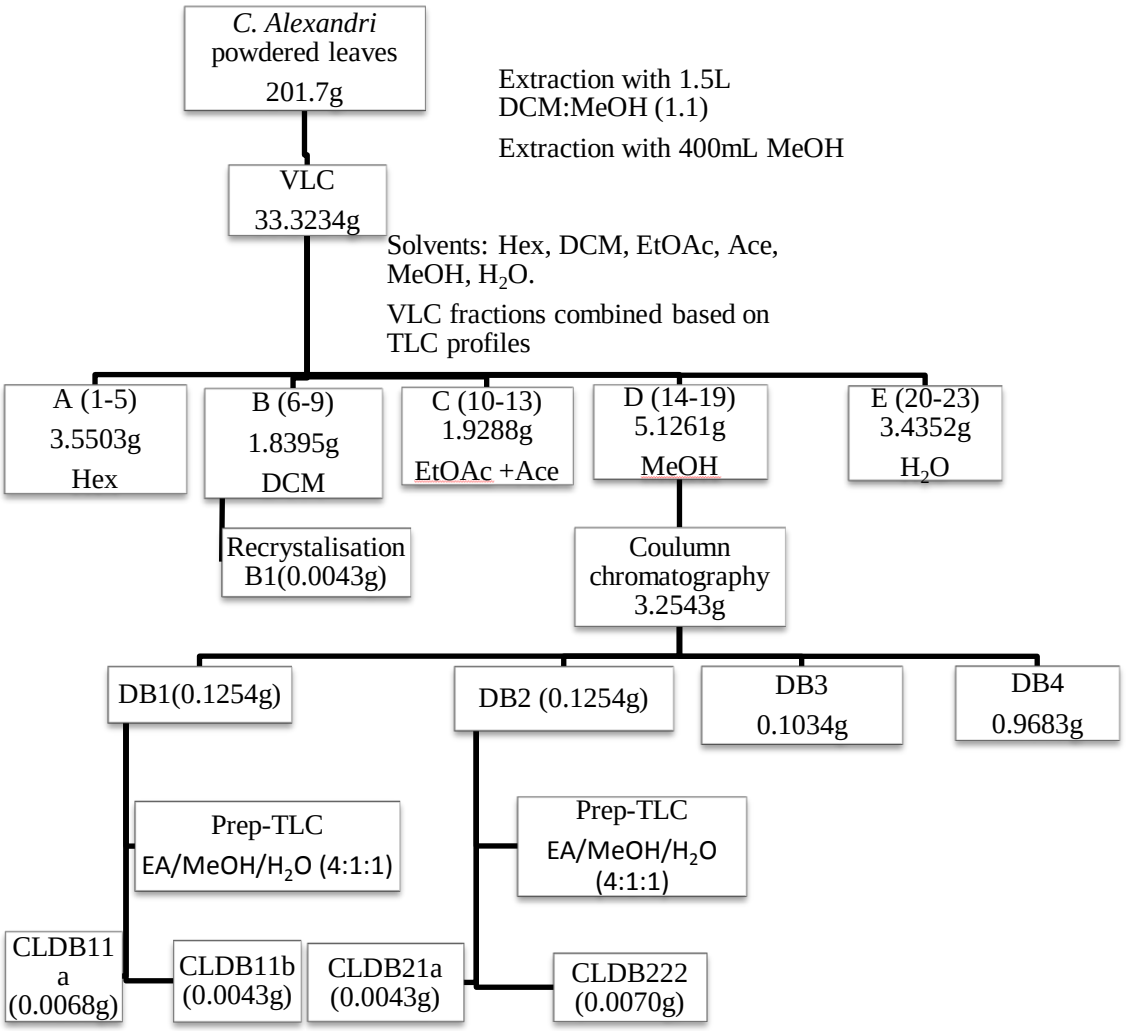


Figure 12: Schematic diagram of the isolated compounds from the leaves of *C. alexandri*.

Based on Figure 11, VLC fraction B (1.8395 g) which was eluted with DCM, consisted of a single brown spot with an R_f value of 0.9 (EA/MeOH/H_2O - 4:1:1). Minor purification was needed and therefore recrystallization using MeOH/ethyl acetate (2:1) was done which led to the isolation of 0.0043g of a white amorphous solid that was labeled B1 (figure12).

As shown in figure 12, fraction D required more extensive purification. Because of the amount of dried fraction and the fact that the compounds in the fraction were well resolved on TLC (3.2543g), fraction D was subjected to column chromatography using a gradient elution, starting with a EA/MeOH/H_2O (4:1:1) solvent system and end with. A total of 42 test tubes fractions were obtained, which were combined based on the TLC profile to yield four (4) major fractions DB1 –DB4. The two fractions DB1and DB2 were further purified on commercial preparative TLC plate with EA/MeOH/H_2O (4:1:1). DB1 (0.1254g) yielded sub-fraction CLDB11a (0.0068 g) and CLDB11b (0.0043g) as shown in figure 12. Compounds CLDB11a and CLDB11b had the same NMR profile but could not be subjected to further purification because they have peak overlapping.

The semi purified compound from DB2 (0.1254 g) was further purified on preparative TLC EA/MeOH/H_2O (4:1:1) and yielded CLDB21a (0.0043g) and CLDB222 (0.0070g) as shown in figure 12. TLC analysis shows the purity of the fractions and compounds which were submitted for antiHIV activity testing such as CLDB1a, CLDB11b, CLDB21a and CLDB222.

4.5 Structure elucidation of isolated metabolites

Compound MLCB51 (34.1 mg) was submitted for ^{1}H-NMR analysis and no other further analysis of ^{13}C-NMR and 2D-NMR was requested as the spectra showed the presence of impurities and peaks that were not well-resolved (figure 15 in appendix). It was further inferred that MLCB51 is lipophilic in nature. A yield of only 6.6 mg was obtained for CLDB11a and the ^{1}H-NMR (figure 16 in appendix) showed that the compound was partially purified as shown by TLC plates and ^{1}H NMR. Structural features of CLDB11a deduced from the ^{1}H-NMR spectra include: (i) the presence of one or more aromatic rings as evidence by signal in the region: δ6.30-8.06 ppm; (ii) the presence of a sugar unit indicated by signals in the region: δ3.42-4.20 ppm where some of the signals overlap with the solvent peak at δ4.87 ppm. The ^{13}C-NMR spectra (figure. 17 in appendix) indicated a total of 19 carbon signals with the remaining carbon assignable to the impurities present. The ^{13}C-DEPT (figure 18 in appendix) showed the absence of methyl and methylene carbons as well as the presence of 14 methane and five quaternary carbon atoms. Bands observed in the IR spectrum (figure 19 in appendix) include the broad OH (s) at 3308cm^{-1} and the C-O stretching frequency at 1022cm^{-1}. The yield obtained for the CLDB222 (7.0 mg) did not allow for it to be further purified or to be subjected to further spectral analysis. The ^{1}H-NMR (figure 20 in appendix) showed that CLDB222 comprised of a mixture of two compounds. Attempts to identify the spin systems using ^{1}H-^{1}H COSY for the compound CLDB222 (figure 22 in appendix) was unsuccessful as the amount of sample was too little and not all off-

diagonal peaks were clear. The FTIR (figure 23 in appendix) of compound CLDB222 indicated the presence of N-H band at 3706 cm^{-1}.

Physical and spectroscopic data of the isolated compounds with reasonable purities listed below:

Compound CLDB11a

Yellow brown amorphous solid, mp 135- 137 ^{o}C, yield 68.0 mg; R_f 0.9 (EA/MeOH/H_2O 4:1:1); (IRν_{max}/cm^{-1} 3308.25 (OH), 1022.04 (C-O)). δ_H (300MHz, MeOH-d_4), 8.04 (1H, d, *J* 7.45), 7.47-7.63 (3H, m), 6.77- 7.08 (6H, m), 6.37 (4H, m), 6.35 (4H, m), 5.07-5.18 (m, overlap with MeOH-d_4), 4.17 (3H, m), 3.43-3.96 (m,) 2.60 (4H, m), 1.89 (1H, s), 1.28 (s), 0.93 (2H, t, *J* 7.2).

^{13}C-NMR (75MHZ), 168.6, 149.4, 147.2, 146.4, 141.9, 133.9, 130.9, 129.2, 99.9, 78.1, 77.2, 74.4, 71.6, 66.4, 65.8, 62.5, 62.4, 43.3, 36.3.

Compound CLDB222

Yellow solid, mp 134- 137 ^{o}C, yield 7.0 mg; R_f 0.6 (EA/MeOH/H_2O 4:1:1), δ_H (600MHz, DMSO-d_6), 7.98 (2H, brd, *J* 07.6), 7.66 (1H, t, *J* 7.5), 7.54 (2H, t, *J* 7.7), 6.39 (1H, dd, *J* 1.9 & 5.9), 4.49-5014 (3H, m), 4.6 (1H, d, *J* 7.8), 4.28 (1H, d, *J* 12.6), δ 3.87 (1H, d, *J* 8.2), 3.69 (1H, d, *J* 11.2), 3.52 (1H, d, *J* 18.7), 3.13 (1H, m), 2.95 (1H, t, *J* 9.2), δ 2.86 (1H, t, *J* 8.2), 2.52 (1H, m), 2.15 (1H, m,), 2.0 (1H, M), 1.65 (1H, s), 0.80-1.4 (4H, m).

^{13}C-NMR (150MHz), 165.5, 140.4, 133.3, 129.8, 129.3, 128.8, 109.6, 103.1, 98.5, 93.6, 77.3, 77.1, 76.5, 73.7,70.1, 69.8, 63.1, 61.7, 61.5, 61.3, 60.8, 41.9, 37.3, 32.6, 31.3, 29.1, 29.0, 28.9, 28.7, 28.6, 25.5, 22.1, 14.0.

4.6 Anti-HIV activity

The inhibitory activities of the semi-purified compounds against HIV-1 protease (PR) and reverse transcriptase (RT) were investigated. The experiments was not carried out in duplicate due to the limited number of test kits available. The semi-purified compound CLDB11a demonstrated a remarkable inhibition of 100% with IC_{50} value of 50µg/mL for HIV-1 protease (figure 13), compared to the activity as performed *by* Ritonavir used as a positive control. Semi-purified compounds CLDB11a, CLDB222 and MLCB51 inhibited less than 50% at the highest concentration tested (100µg/mL) for HIV-1 reverse transcriptase, which is an indication of poor inhibitory activity (figure 14), in comparison of doxorubicin. Hence, the results showed that the semi-purified compound from *C. alexandri* has good *in vitro* inhibition against selected HIV-1 enzymes and could have potential anti-HIV agents. Thus further validation is required to confirm the observed activities. Isolated compounds from *M. schinzii* did not demonstrate any inhibitory activity with the two selected enzymes of HIV-1 protease (PR) and reverse transcriptase (RT).

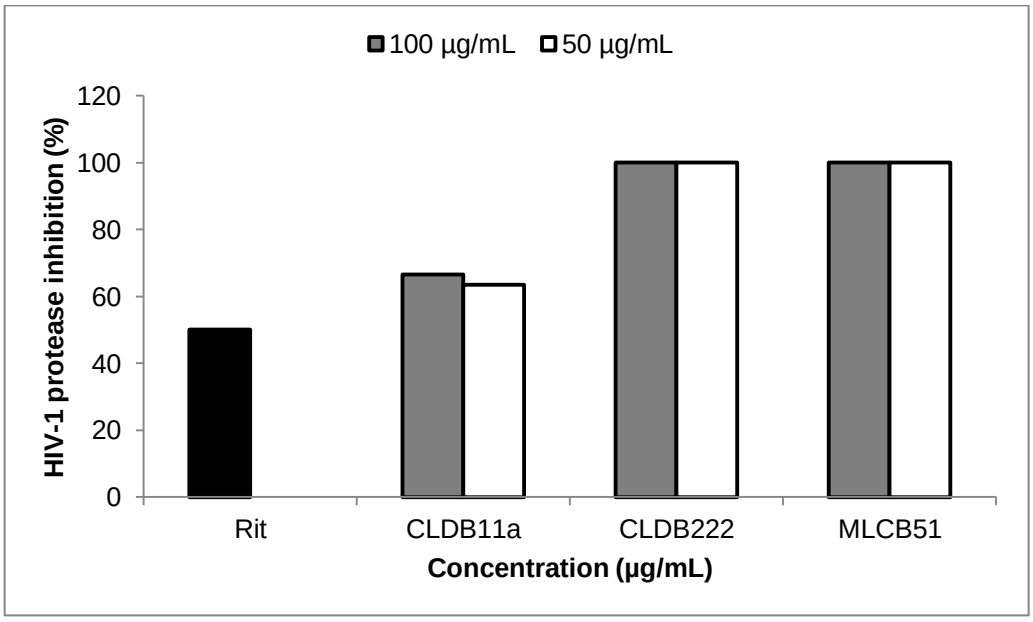


Figure 13: *In vitro* anti-HIV-1 protease inhibition activity of *M. schinzii* and *C. alexandri*. Rit= Ritonavir

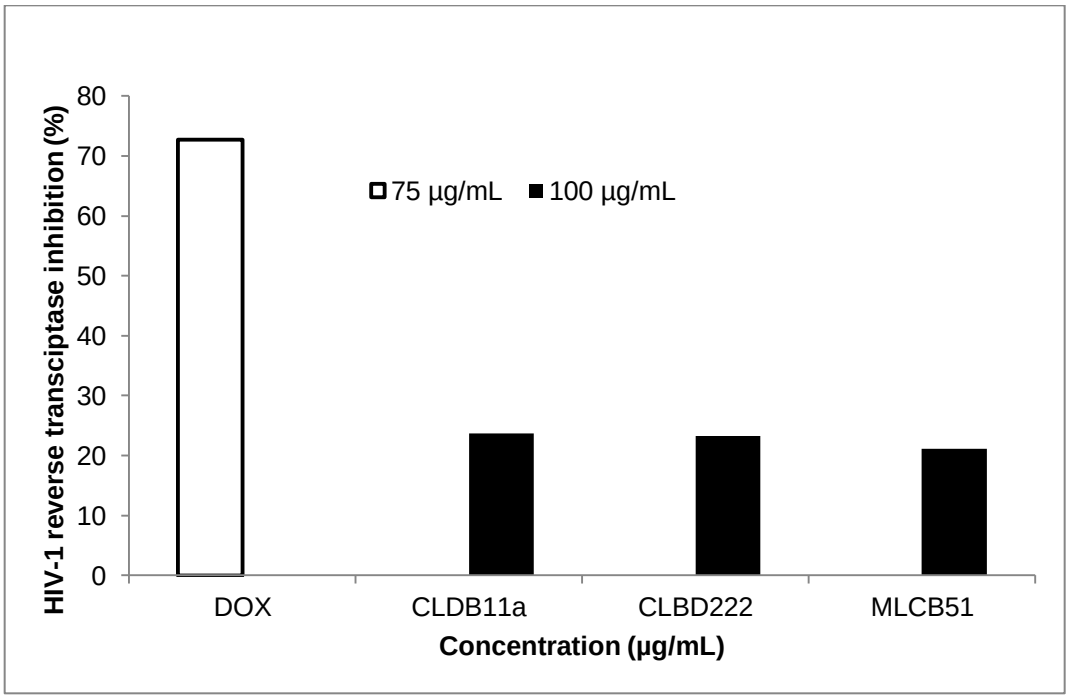


Figure 14: *In vitro* anti-HIV-1 reverse transcriptase inhibition activity of *M. schinzii* and *C. alexandri*, Dox = Doxorubicin

4.7 Antimicrobial activity

The negative impact of opportunistic infection on the management of HIV/AIDS has been discussed in chapter 2. The management of HIV/AIDS is further compounded by antimicrobial resistance which is a big concern worldwide.

The two plant extracts were tested for antimicrobial activity against four reference cultures of gram-positive (*Staphylococcus aureus)*, gram-negative (*Klebsiella pneumoniae*, *Escherichia coli*) and fungal strain (*Candida albicans*). The test was performed under aseptic conditions. Clear inhibition zone around the discs indicated the presence of antimicrobial activities. The zone of inhibition was measured and the strength was classified by diameter as ≥ 20 nm (very strong), 13-19 nm (strong), 6-12 nm (moderate), 11- 5 nm (weak), and ≤ 5 nm (negative) [63]. All the compounds tested were less active than the control ampicillin. The three semi-pure compounds tested only three microorganisms showed moderate active inhibition against three of the four microorganisms, with the inhibition ranging from 6 and 12 mm. It was of interest to note that none of the semi-purified compounds displayed growth inhibitory activity against *E. coli* as shown in Table 7.

Table 7: Antimicrobial activity of semi-purified compounds using the disc diffusion method

Microorganisms	**CLDB222 (inhibition zone, mm)**	**CLDB11a (inhibition zone, mm)**	**MLCB51 (inhibition zone, mm)**	**Ampicillin (inhibition zone, mm)**
Escherichia coli	-	-	-	19
Klebsiella pneumoniae	7.0	10.0	9.0	20

Staphylococcus aureus	-	6	-	18
Candida albicans	11.0	10.0	12.0	19

The minimum inhibition concentration (MIC) results (Table 8) showed that CLDB222 had the lowest value of 1.5mg/ml against *Klebsiella pneumoniae* and *Candida albicans.* This showed that, in addition to being the most active of all compounds, CLDB222 did not show selectivity, as it inhibited both the bacterial and fungal strains. The CLDB11a displayed equipotent activity against *Klebsiella pneumoniae* and *Candida albicans* whilst lower growth inhibitory activity of S*taphylococcus aureus* was observed. MLCB51 showed the highest growth inhibitory activity against *Candida albicans* with inhibition zone of 12mm as indicated in Table 7 below.

Table 8: Minimal inhibitory concentration (MIC) of semi-purified compounds with antibacterial activity

Microorganism	**CLDB222 (in mg/ml)**	**CLDB11a (in mg/ml)**	**MLCB51 (in mg/ml)**
Klebsiella pneumoniae	1.5	2.0	2.0
Staphylococcus aureus	-	10.0	-
Candida albicans	1.5	1.5	4.0

Candida is strain of fungus that causes an infection in skin, throat or mouth and is identified by white plaque growth on the oral mucous membrane [61]. *Klebsiella pneumoniae* is a bacterial infection that leads to pneumonia, bloodstream, wound, urinary tract infection and meningitis. It affects the skin, mouth and intestinal tract [61].

These are critical to this study as these diseases are among the opportunistic infection that is related to HIV positive individuals.

CHAPTER FIVE: CONCLUSION AND RECOMMENDATIONS

5.1 Conclusions

The objectives of the study have been partially met. The study aimed to extract metabolites from the two plants *C. alexandri* and *M. schinzii*. This was achieved although the yield obtained for the various semi-purified compounds were of very low quantities. This limitation prevented further purification of the compounds; hence semi-purified compounds were submitted for NMR analysis. As shown in figure 10 for the extraction of *M. schinzii*, significant a discrepancy in the amount of the crude extract (32g) and the total (recovered) mass of the fractions obtained (12g) after VLC was noted. This indicated a loss of 19g of the crude extracted which is most likely due to retention in the VLC column or some metabolites being volatile enough to have been lost during drying phase.

The research has shown the presence of phytochemical compounds in the extracts of *M. schinzii*. These phytochemical compounds include saponins, bitter principles, alkaloids, flavonoids, coumarins and terpenes as discussed in Section 4.1. Semi-purified compound isolated from *C. alexandri* also show presence of phytochemical compounds which include saponins, bitter principles, alkaloids, flavonoids, coumarins and terpenes as discussed in Section 4.1. Two methods were used for phytochemical analysis, namely the TLC and Chemical method. The TLC method was found to be more efficient than the Chemical method and this supported by literature as this method requires less solvent and have variety selection of solvent system for separation.

Structure elucidation could not be made as the NMR spectral results showed presence of impurities and due to low quantity yield of the compounds submitted. Further purification could thus not be performed.

The anti-HIV activities displayed by fractions from the leaves of the two plants under studied, are reported here for the first time. This assay gave good result of 100 % HIV-1 protease for compound CLDB11a and lower than 50% at a high concentration tested 100μg/mL for HIV-1 reverse transcriptase for compounds CLDB11A, CLDB222 and MLCB51. Extraction of compounds could not be repeated to increase the yield, due to inadequate resources therefore; the yielded obtained were very little that made the analysis inconclusive. Traditionally, the plants are known to treat diseases that are commonly related to HIV opportunistic infections. Thus, antimicrobial activity tested were performed and found that the extracts showed antimicrobial activities. For the two plants extract that were tested *C. alexandri* and *M. schinzii* plant extracts possessed antimicrobial activities against *Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli* and *Candida albicans*.

5.2 Recommendations

It is recommended for preparation of fractions to be done at large scale so that sufficient yields will be obtained from VLC, to enable further analysis to be performed efficiently. Assay tests of the compounds that gave low results could be repeated to confirm the result for both HIV-1 protease and HIV-1 reverse transcriptase. There is a need to improve the isolation and extraction methods to achieve better yield and the need to perform the phytochemical test, anti-HIV assay and antimicrobial studies in duplicate.

Full characterisation of the isolated compounds should be done using both ^{1}H NMR and ^{13}C NMR for structure elucidation.

CHAPTER SIX: REFERENCES

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APPENDIX A

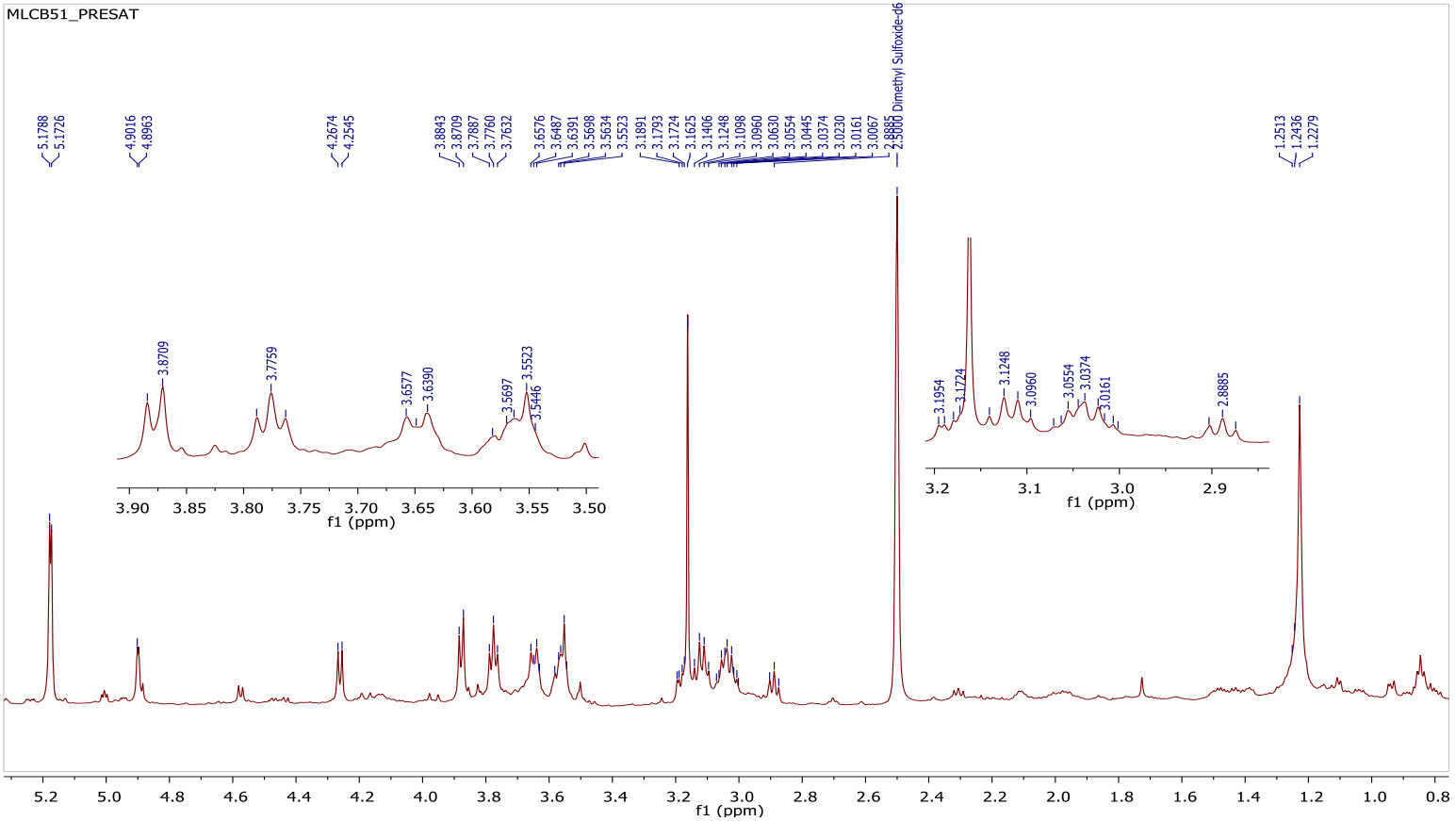

Figure 15: ^{1}H-NMR spectrum of MLCB51 in DMSO-d_6 at 600MHz.

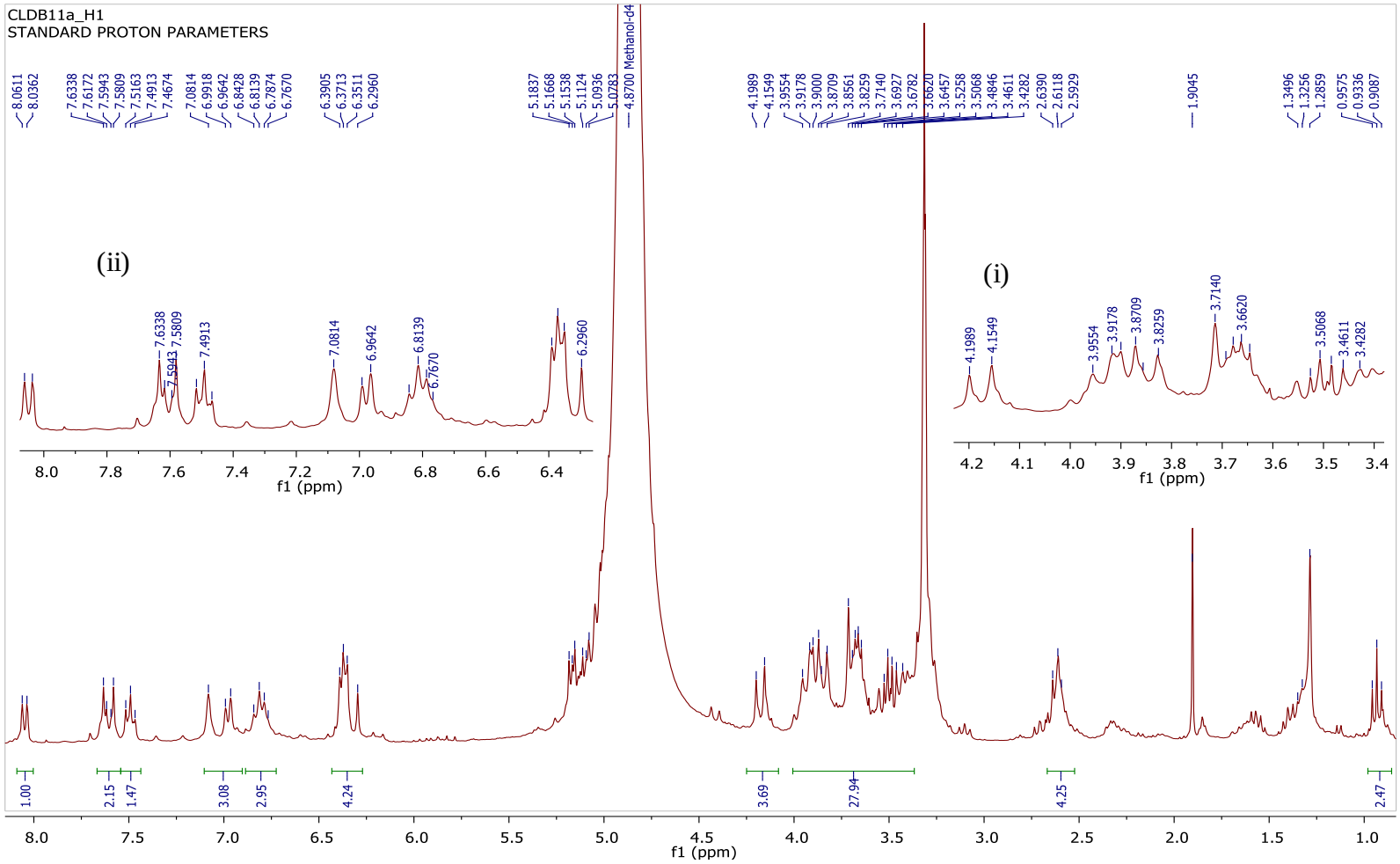

Figure 16: ^{1}H-NMR of CLDB11a in MeOH-d_4 at 300MHz with expanded regions: (i) 3.4-4.2ppm and (ii) 6.3-8.5ppm

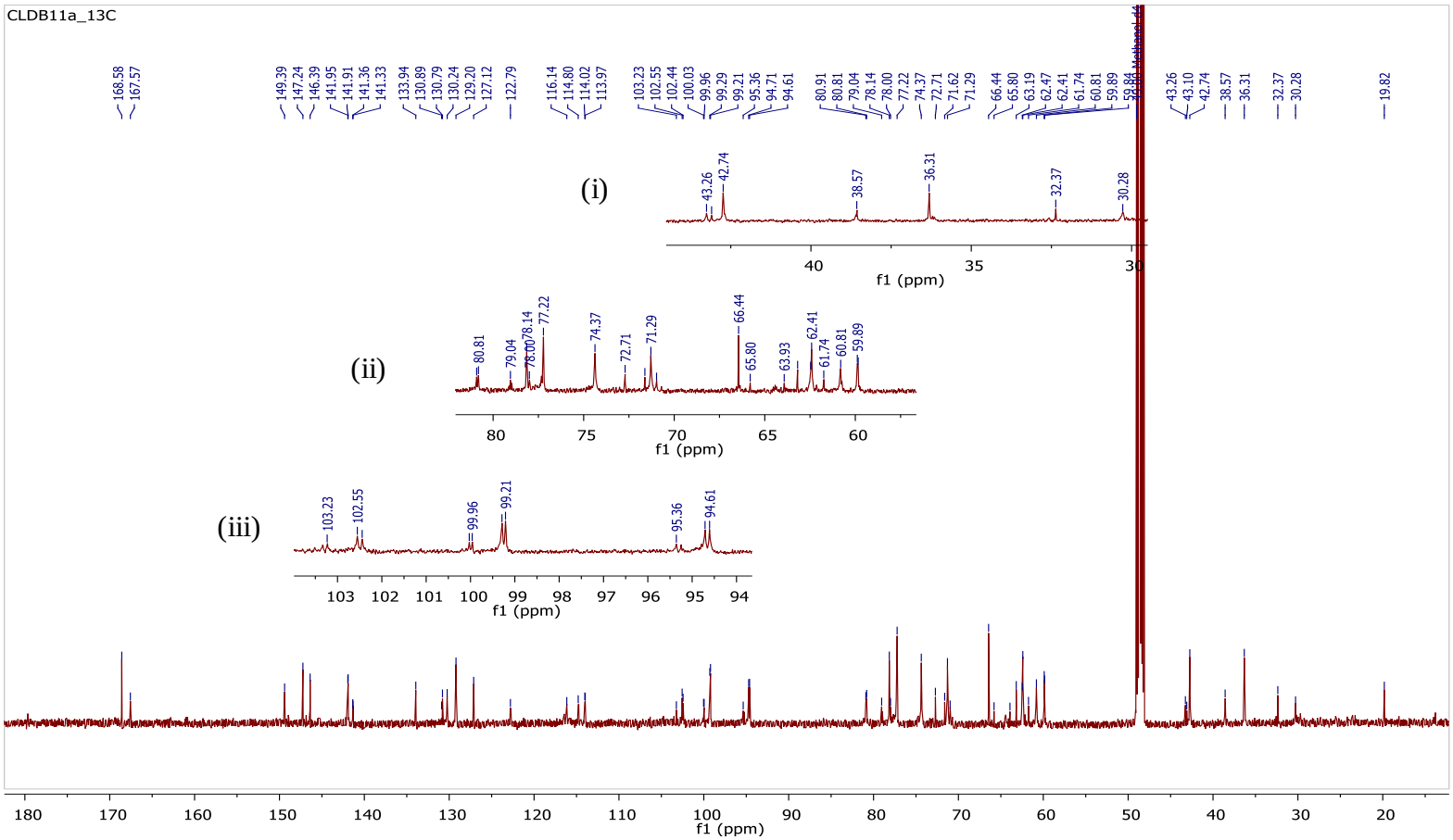

Figure 17: ^{13}C NMR spectrum of CLDB11a in MeOH-d_4 at 75MHz with expanded regions: (i) 30-45ppm, (ii) 55-80ppm, and (iii) 94-104ppm.

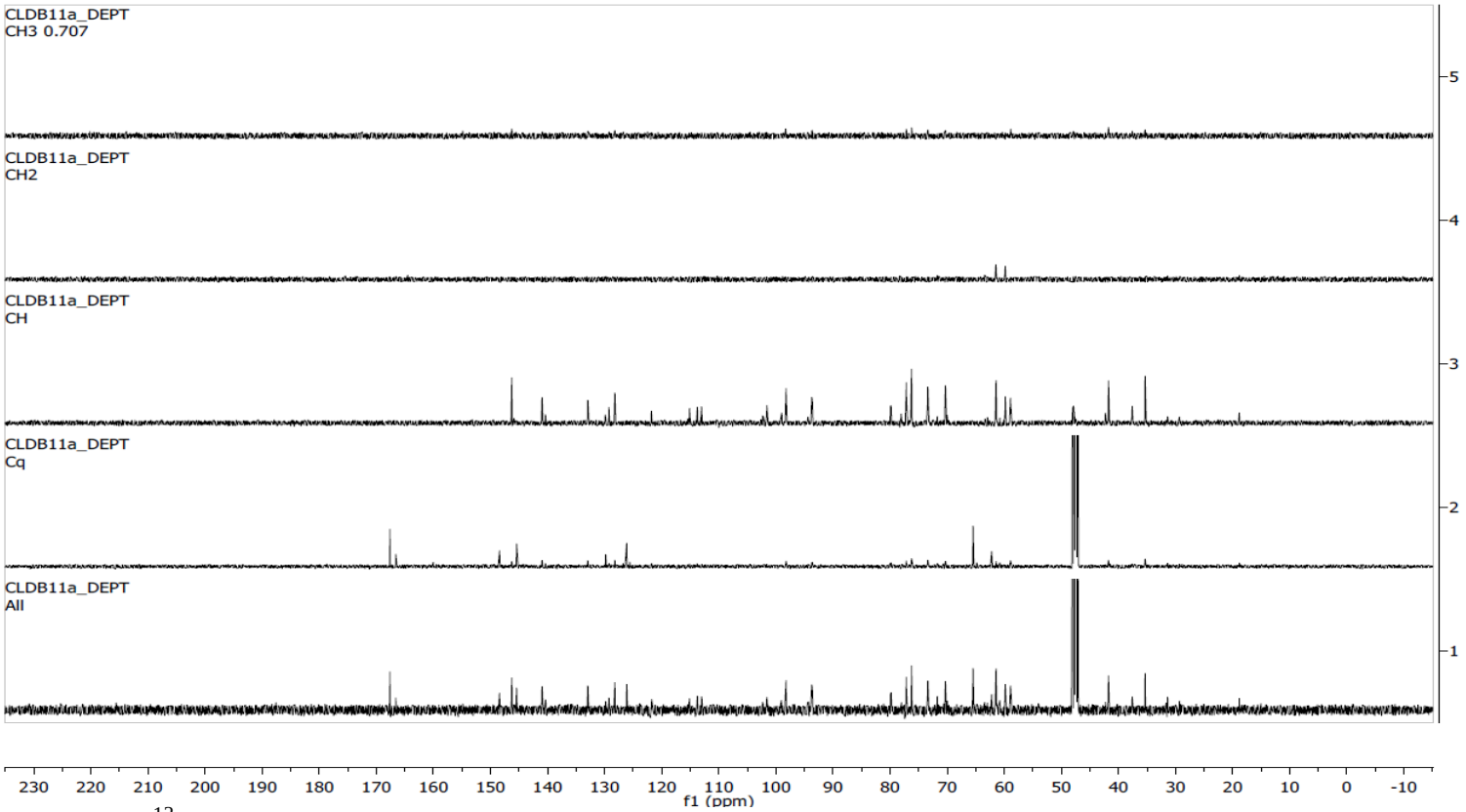

Figure 18: ^{13}C-DEPT spectrum of CLDB11a in MeOH-d_4.

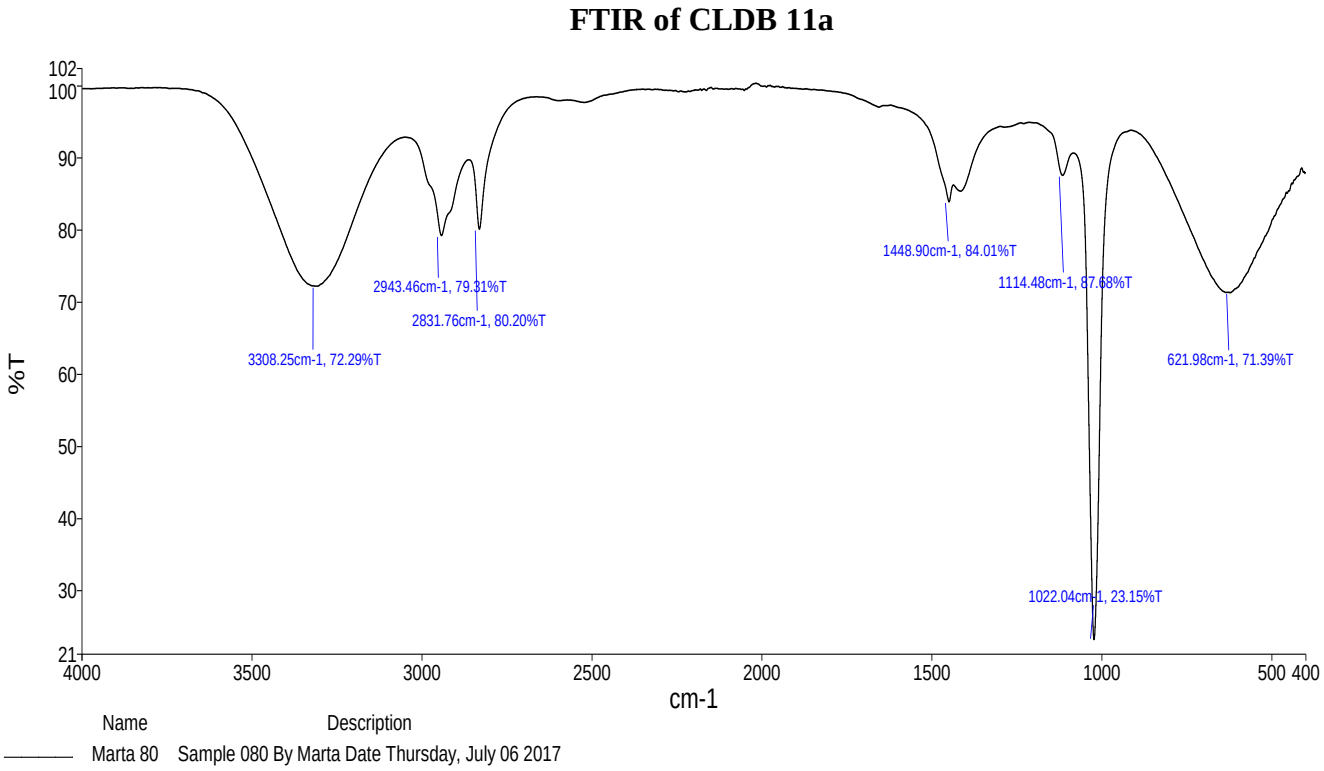

Figure 19: IR spectrum of CLDB11a in MeOH.

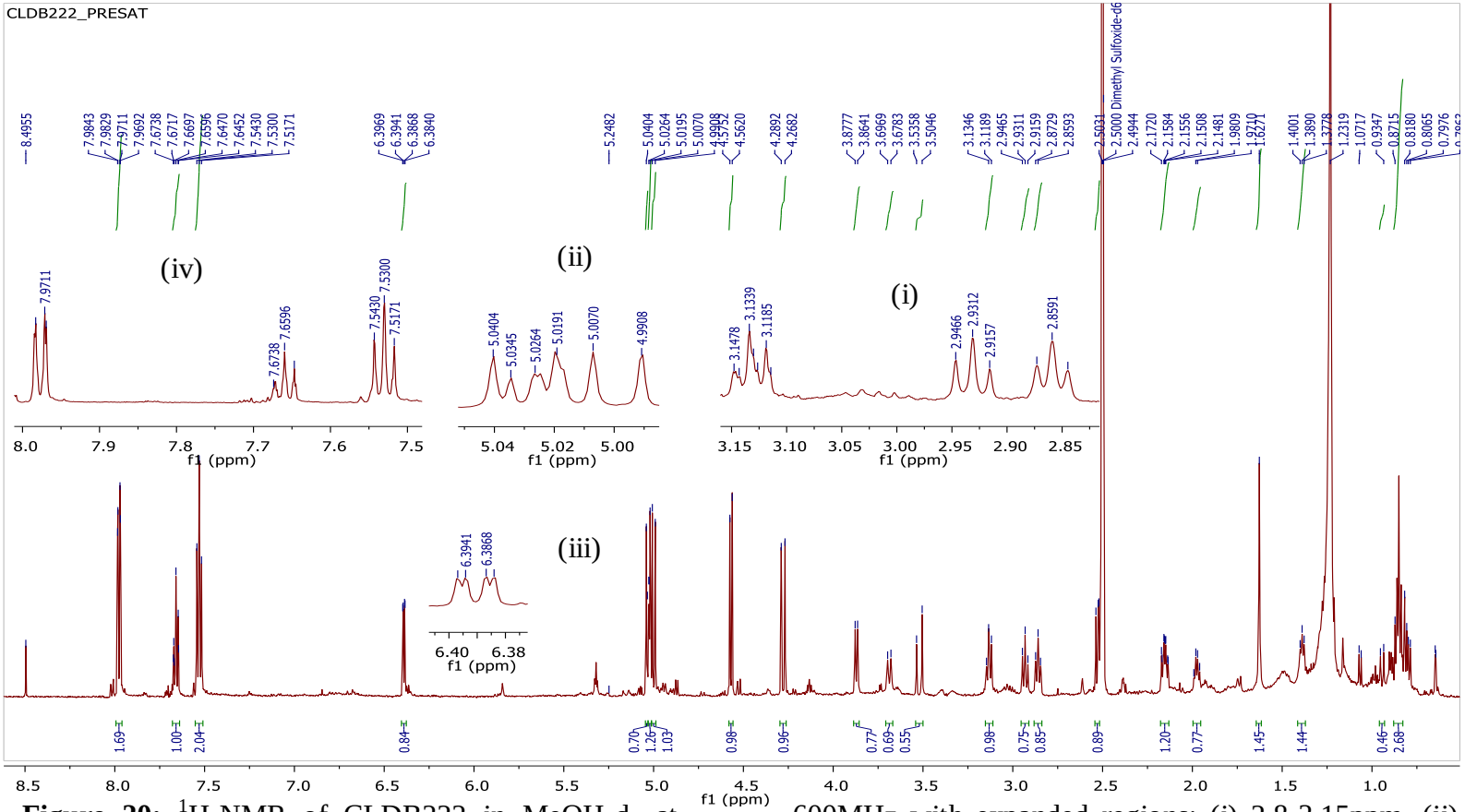

Figure 20: ^{1}H-NMR of CLDB222 in MeOH-d_4 at 600MHz with expanded regions: (i) 2.8-3.15ppm, (ii) 4.5-5.06ppm, (iii) 6.3 -6.48ppm and (iv) 7.5 – 8.0ppm.

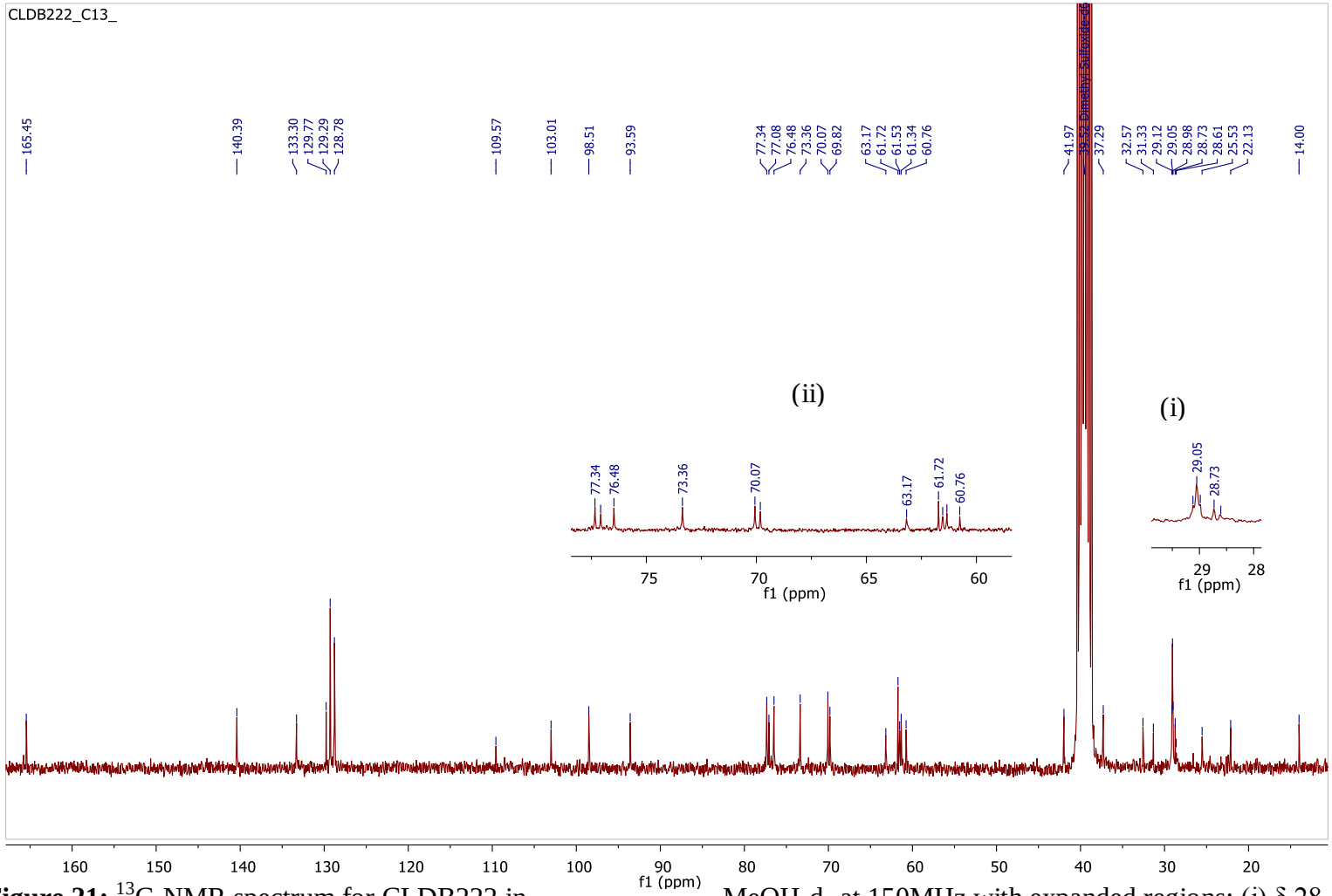

Figure 21: ^{13}C-NMR spectrum for CLDB222 in MeOH-d_4 at 150MHz with expanded regions: (i) δ 28-30, and (ii) δ 60-80ppm, with 19 total number of carbon

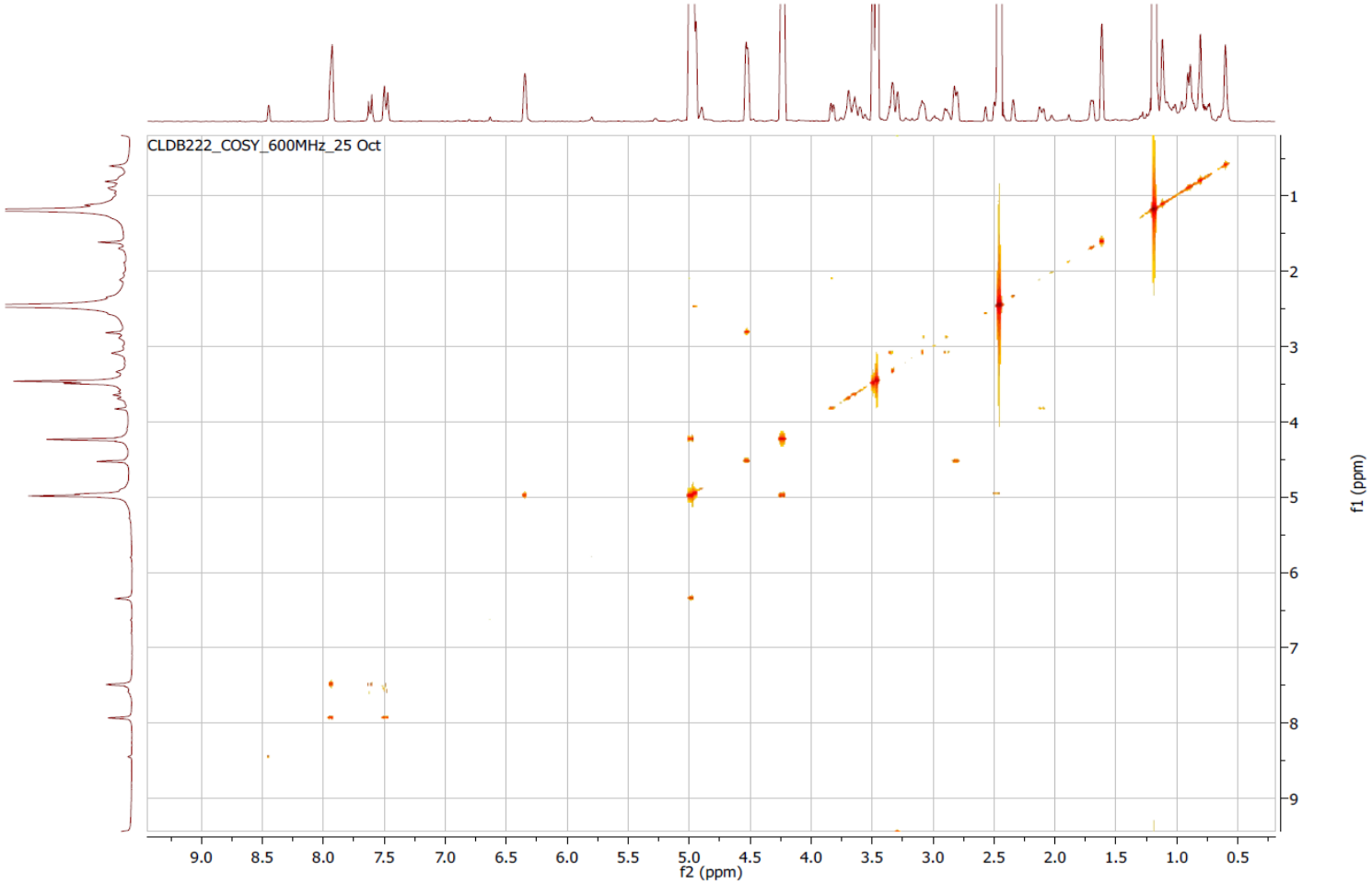

Figure 22: ^{1}H-^{1}H COSY spectrum for CLDB222 at 600MHz

FTIR of CLDB 222

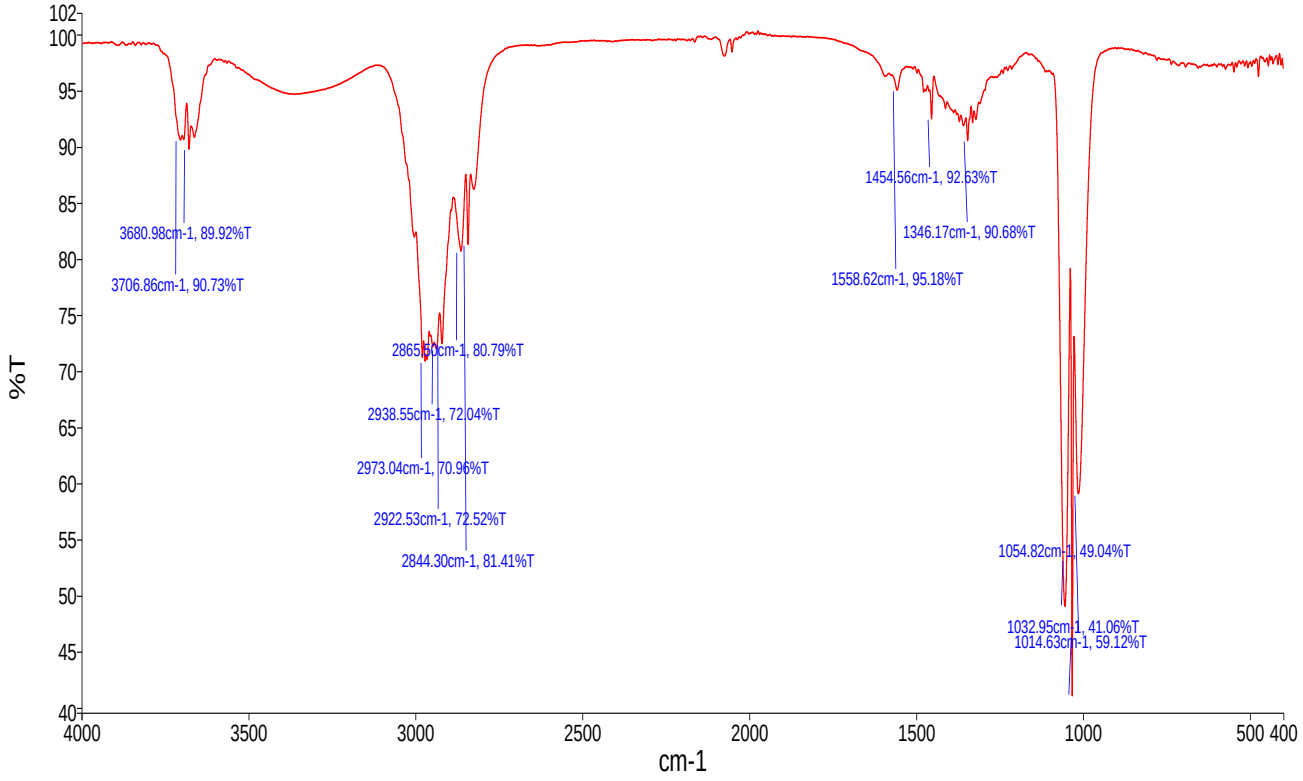

Figure 23: IR spectrum of the isolated compound CLDB222.

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