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A bioactivity guided study on the anti-gonococcal and anti-inflammatory activity of *Ficus abutilifolia* stem bark extracts

By

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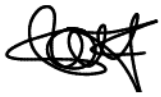
DECLARATION

I, **Leshaye Cynthia Theresia Domingo**, declare that the dissertation, which I hereby submit for the degree, Magister Scientiae in Medicinal Plant Sciences at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.



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DEDICATION

This dissertation is dedicated to my parents, Basil Christopher Domingo and Iris Carmelita Domingo, and my beloved grandmother, Elizabeth Chitrin. Thank you for believing in me and for encouraging me every step of the way.

"I can do all things through Christ who strengthens me"

Philippians 4:13

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ABSTRACT

Background: With high annual infection rates and rapidly increasing antimicrobial resistance (AMR), *Neisseria gonorrhoea* has become a major global public health concern, especially because delayed treatment often results in severe reproductive complications, and increases the risk of contracting the human immunodeficiency virus (HIV).

Aims of the Study: In South Africa, traditional healers use the stem bark of *Ficus abutilifolia* (Miq.) Miq. to manage gonorrhoea and to treat associated inflammatory conditions. Therefore, this study aimed to evaluate the anti-gonococcal and anti-inflammatory potential of extracts obtained from the stem bark of *F. abutilifolia*.

Materials and Methods:

The chemical constituents of the stem bark were extracted in dichloromethane, absolute methanol, 50% methanol (methanol: water, 1:1, v/v) and water, to yield four crude extracts. The anti-gonococcal potential of the crude extracts was determined using the *in vitro* broth microdilution assay, whilst the anti-inflammatory potential was determined by screening for inhibitory activity against 15-lipoxygenase (15-LOX). Active extracts were subjected to bioassay-guided fractionation and phytochemical analysis using proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy and gas chromatography time-of-flight mass spectrometry (GC-ToF-MS), to determine the compounds or classes of compounds responsible for the observed bioactivity.

Results: The polar crude extracts (absolute methanol, 50% methanol, water) all displayed good anti-gonococcal activity, with a minimum inhibitory concentration (MIC) value of 0.20 mg/mL. Low IC_{50} values comparable to that of quercetin (15.13 ± 0.19 µg/mL) were also reported for these extracts (absolute

methanol: 18.33 ± 0.70 µg/mL; 50% methanol: 23.82 ± 0.90 µg/mL; decoction: 25.58 ± 0.41 µg/mL), thus indicating significant anti-inflammatory potential. ^{1}H-NMR-based metabolomics revealed distinct chemical differences between the active and non-active fractions on the PCA, OPLS-DA and contribution plots, with bioactivity attributable to prominent peaks in the alcohol, vinylic, aromatic, phenolic and amide regions. GC-ToF-MS analysis was also used to tentatively identify compounds present in the active fractions, however, due to the low mass spectral similarity caused by the polar nature of these fractions, the identity of these compounds could not be established with certainty.

Conclusion: The results obtained in this study support the traditional use of *F. abutilifolia.* As this is the first report of anti-gonococcal activity in *F. abutilifolia*, the results may also help to discover new classes of compounds for the treatment of gonorrhoea and/or associated inflammatory conditions.

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
BHT	Butylhydroxytoluen
CC	Column chromatography
CFU	Colony forming units
CFR	Cape Floristic Region
COX	Cyclooxygenase
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
EtOAc	Ethyl acetate
FRAP	Ferric reducing antioxidant power
GC-ToF-MS	Gas chromatography to time-of-flight mass spectrometry
HBV	Hepatitis B virus
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
HPV	Human papillomavirus
HSV	Herpes simplex virus
LA	Linoleic acid
LOX	Lipoxygenase
LC-MS	Liquid chromatography-mass spectrometry
MeOH	Methanol
MVA	Multivariate analysis
iNOS	Inducible nitric oxide synthase

NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drug
NF-κβ	Nuclear factor Kappa-B
OPLS-DA	Orthogonal projections to latent structures discriminant analysis
PCA	Principle component analysis
PID	Pelvic inflammatory disease
PUFA	Polyunsaturated fatty acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
STD	Sexually transmitted disease
STI	Sexually transmitted infection
TLC	Thin layer chromatography
UPLC-Q-ToF-MS	Ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry
WHO	World Health Organization
XO	Xanthine oxidase
^{13}C-NMR	Carbon nuclear magnetic resonance
^{1}H-NMR	Proton nuclear magnetic resonance

CHAPTER 1

Introduction

1.1 Problem Statement

With an estimated daily average of one million new cases, sexually transmitted infections (STIs) have become a major global public health concern (Nthulane et al., 2020; Seleteng-Kose et al., 2019). Not only do they pose devastating effects on the reproductive health and well-being of individuals, but STIs also increase the risk of coinfection with the human immunodeficiency virus (HIV), thus placing a massive burden on healthcare systems, worldwide (Naidoo et al., 2013; Shannon and Klausner, 2018).

According to the World Health Organisation (WHO), up to 376 million cases of chlamydia, gonorrhoea, syphilis and trichomoniasis are reported annually, with higher incidences occurring in sub-Saharan Africa (Wand et al., 2020; World Health Organization, 2016). It is, however, speculated that this number may be a gross underestimate due to misdiagnosis, inadequate treatment or the fact that some STIs often present as asymptomatic (Farley et al., 2003). Furthermore, some individuals may not have access to adequate healthcare facilities, whilst others simply do not seek treatment due to the various social and moral stigmas associated with venereal diseases (Wand et al., 2020; Workowski, 2015).

Beyond the initial impact of infection, delayed diagnosis or treatment often results in severe complications or mortality (Shannon and Klausner, 2018). Furthermore, the risk of developing complications is often higher for women, than it is for men (Lawal et al., 2019; van der Eem et al., 2016), and very often, reproductive health is adversely affected, resulting in ectopic or preterm pregnancy, miscarriage or stillbirth (Lawal et al., 2019; Shannon and Klausner, 2018; Wynn et al., 2020). In severe cases, infection with chlamydia or gonorrhoea may even result in pelvic inflammatory disease (PID), which if left untreated,

may eventually cause infertility or facilitate the progression to ovarian cancer (Shen et al., 2016; Tien et al., 2020).

'Curable' venereal diseases such as chlamydia, gonorrhoea, syphilis and trichomoniasis are typically treated using antibiotic therapies. However, due to the misuse of these drugs, there has been a marked increase in antimicrobial resistance throughout the globe (Krupp and Madhivanan, 2015; Nazer et al., 2019). In particular, several cases of multi- and extensively drug-resistant gonococcal strains have been reported, thus limiting the number of effective treatment options available for this disease (Tien et al., 2020; Whelan et al., 2021). To combat the global STI pandemic and the challenges posed by antimicrobial resistance, scientists have begun to explore alternative treatment options, including ethnobotanical resources (Heinrich, 2000).

Ethnobotany can be described as the study or documentation of the different uses of medicinal plants within societies around the world (Schultes, 2008). Not only does this research field aim to preserve indigenous knowledge systems, but it also plays a profound role in the discovery and development of modern-day drugs, as it provides insight into indigenous plants with therapeutic potential (Heinrich, 2000). These remedies are often effective, as plants have an abundance of biologically active secondary metabolites, with unique and diverse chemical structures that can be used as precursor molecules or as lead compounds for drug development (Das et al., 2013; Mahomoodally, 2013; Pendota et al., 2017). However, before these remedies can be commercialised, there is a need for rigorous scientific evaluation, to validate traditional uses, and to ensure that these remedies are safe for human consumption (Seleteng-Kose et al., 2019). Therefore, the current study aims to evaluate the anti-gonococcal and anti-inflammatory efficacy of *Ficus abutilifolia* (Miq.) Miq.

1.2 Hypothesis

Ficus abutilifolia, a medicinal plant currently used by traditional healers to treat gonorrhoea and associated inflammatory sequelae, may exhibit *in vitro* anti-gonococcal and anti-inflammatory activity.

1.3 Aims and Objectives of the Study

Aim

- To investigate the anti-gonococcal and anti-inflammatory potential of crude extracts from the stem bark of *F. abutilifolia*.

Objectives

- To determine the anti-gonococcal potential of extracts from the stem bark of *F. abutilifolia*.
- To determine the anti-inflammatory potential of these extracts, by evaluating them for inhibitory activity against 15-lipoxygenase.
- To conduct a bioassay-guided fractionation of the extract(s) with the most potent bioactivity.
- To conduct a ^{1}H-NMR-based metabolomics analysis on the crude extracts, to determine the major classes of compounds responsible for the observed bioactivity.
- To tentatively identify the compounds in the active fractions using GC-ToF-MS.

1.4 Structure of the Thesis

All the chapters in this thesis have been written according to the structure outlined by the South African Journal of Botany.

Chapter 1

This chapter provides a brief overview of the study, including a problem statement that highlights the burdens associated with venereal diseases and antimicrobial resistance, as well as the role of ethnobotanical research in modern drug development. The hypothesis, aim and objectives of the study are also presented in this chapter.

Chapter 2

This chapter contains a literature review on STIs, with a focus on gonorrhoea and associated inflammatory sequela. It also contains a section on the significance of ethnobotanical research in South Africa, and an extensive literature review on the ethnobotanical uses, phytochemistry and pharmacological activities of *F. abutilifolia*.

Chapter 3

This chapter focuses on the *in vitro* methods used to screen extracts from the stem bark of *F. abutilifolia* for activity against gonorrhoea and 15-lipoxygenase. A detailed discussion of the results obtained is also included in the chapter.

Chapter 4

This chapter focuses on the bioassay-guided fractionation of the anti-gonococcal and anti-inflammatory constituents of *F. abutilifolia,* followed by proton nuclear magnetic resonance (^{1}H-NMR) and gas chromatography to time-of-flight mass spectrometric (GC-ToF-MS) analysis, to identify compounds or classes of compounds attributable to bioactivity.

Chapter 5

This chapter contains a general discussion and conclusion of the findings of this study. Future prospects and recommendations are also included.

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CHAPTER 2

Literature Review

2.1 Background Information

Sexually transmitted infections (STIs) are caused by a wide variety of pathogens, including bacteria, viruses, and protozoa. STIs affect individuals of all ages and can be spread through unprotected sex, intravenous needles, blood transfusions, or from mother to child during birth (Kharsany et al., 2020). STIs can either be classified as curable or incurable, depending on the infectious agent. Curable infections are caused by bacteria or protozoa and include chlamydia, syphilis, gonorrhoea, and trichomoniasis, whilst incurable infections are usually caused by viruses and include the herpes simplex virus (HSV), hepatitis B virus (HBV), human papillomavirus (HPV), and human immunodeficiency virus (HIV) (Rowley et al., 2019; World Health Organization, 2018). According to the World Health Organisation (WHO), approximately 376 million cases of curable infection occur every year, thus making STIs a huge cause for concern (Rowley et al., 2019; World Health Organization, 2018).

STIs are a significant threat to human health, as the majority of these infections often present with mild to no symptoms (Francis et al., 2018). Not only do these infections affect the quality of life, but if left untreated, they can damage the reproductive organs, resulting in long-term complications such as infertility (Francis et al., 2018). Other complications that may arise include inflammatory diseases, miscarriage, premature or stillbirth, or even death (Wand et al., 2020). STIs have also been associated with an increased risk of either contracting or transmitting HIV (Kharsany et al., 2020; Wand et al., 2020).

The STI focused on in this study is gonorrhoea, which is the world's second most common STI (Unemo and Shafer, 2011; World Health Organization, 2016). Gonorrhoea is caused by the gram-negative diplococcus, *Neisseria gonorrhoea*,

which is spread through unprotected sexual intercourse (Erasmus et al., 2012). If left untreated, infections may result in severe complications, which can lead to death (Wand et al., 2020). Gonorrhoea is primarily treated with antibiotics, however, in recent years, there has been a surge in antibiotic-resistant strains. Globally, it has been estimated that up to 87 million people are infected with gonorrhoea, annually, with Africa bearing the highest burden of infection (Maduna et al., 2020; Rowley et al., 2019). This burden is particularly notable in South Africa, where an estimated average of 2 million cases are reported every year (Kularatne et al., 2018). The high number of STI cases in South Africa can be attributed to factors such as poverty, inadequate prevention, screening, and treatment strategies (Wand et al., 2020).

2.1.1 *Neisseria gonorrhoea*

Neisseria gonorrhoea is an obligate aerobe, that thrives in the warm mucosal membranes of the human urogenital tract, rectum, conjunctiva, pharynx, and synovium (Mabey and Mayaud, 2013). The disease affects both male and female populations, however, women often present as asymptomatic, thus posing an extreme risk to their sexual health, and increasing the chances of unknown transmission (Erasmus et al., 2012; Miller, 2006). The pathogen is typically transmitted through unprotected vaginal, anal, and oral sex, but can also be passed on from mother to child during pregnancy and birth (Rowley et al., 2019). Delayed diagnosis or treatment of the disease will often result in the development of complications. Pelvic inflammatory disease (PID), ectopic pregnancy, miscarriage, cervicitis, and infertility have been reported in female populations, whilst urethritis, prostatitis, and epididymitis have been observed in males (Miller, 2006; Tshikalange et al., 2016).

2.1.2 Antimicrobial Resistance and the Treatment of Gonorrhoea

Bacterial infections, including those caused by gonorrhoea, are typically treated through the use of antibiotics, which were first discovered by Alexander Fleming in 1928 (Tan and Tatsumura, 2015). This discovery revolutionized modern medicine however, over time, several microorganisms have developed resistance to a wide range of antibiotics (Unemo et al., 2019; Ventola, 2015). The ability of a microorganism to evolve and develop resistance to an effective drug is termed antimicrobial resistance (AMR) (Dadgostar, 2019). Resistance often arises when chromosomal mutations occur within microbial populations as a result of excessive or incorrect usage, as well as limited control of antimicrobial drugs (Dadgostar, 2019). This phenomenon is a major global concern as it prevents effective treatment, thus placing a severe burden on healthcare systems worldwide (Dadgostar, 2019; Prestinaci et al., 2015).

Sulphonamides were first prescribed as an effective treatment against *N. gonorrhoea* in the 1930s, however, by the 1940s, resistance to these drugs had become widespread (Hook and Kirkcaldy, 2018). In 1943, penicillin was identified as a highly effective antimicrobial agent with a low effective dosage, however, susceptibility towards this ‘miracle’ drug unfortunately gradually began to decrease over the years, and by the late 1970s, penicillin was no longer considered to be effective against this superbug (Unemo and Shafer, 2014, 2011). Since then, several alternative antibiotics have been discovered and recommended for the treatment of gonorrhoea. These antibiotics include tetracycline, spectinomycin, quinolones, macrolides, and early-generation cephalosporins (Unemo and Shafer, 2014). Although initially effective, throughout the years there have unfortunately been several reports of gonococcal strains that have also developed resistance to these drugs (Unemo et al., 2014). Furthermore, the WHO recently released reports on the prevalence of untreatable gonococcal strains (Wi et al., 2017).

To date, the WHO recommends a dual therapy consisting of azithromycin and ceftriaxone for the treatment of gonorrhoea (World Health Organization, 2016b). However, in addition to resistance to ciprofloxacin, gonococcal strains unique to South Africa are now beginning to show resistance to azithromycin (Maduna et al., 2020; Rambaran et al., 2019). Therefore, ceftriaxone remains the current antibiotic of choice for treatment in South Africa, as no resistance to this drug has been detected yet (Maduna et al., 2020).

Considering the widespread increase in AMR, a large number of annual estimated cases, and the limited treatment options available, *N. gonorrhoea* has become a major public health concern, not only in South Africa but on a global scale (Mulaudzi et al., 2015). Consequently, the WHO has prioritized the search for new antibiotics to treat this superbug (World Health Organization, 2018). As a result, scientists are now leaning towards plants as a potential source of novel therapeutics, which may complement or enhance the effects of existing antimicrobial drugs in the fight against gonorrhoea (Taiwo et al., 2016; Unemo et al., 2014).

2.1.3 Inflammation

Pelvic Inflammatory Disease

Untreated gonococcal infections have been associated with a wide variety of inflammatory sequelae, including pelvic inflammatory disease (PID) (Lawal et al., 2019). PID occurs as a result of chronic inflammation caused by untreated infection, and only affects women due to the unique anatomical structure of the female reproductive system (Curry et al., 2019). The disease often arises when bacteria spread from the vagina and cervix, into the upper genital tract, causing infection and chronic inflammation of these organs (Lawal et al., 2019; Miller, 2006). PID results in severe discomfort and is associated with decreased fertility,

and an increased risk of miscarriage and ectopic or preterm pregnancy (Curry et al., 2019).

In addition to the risk of chronic inflammation, untreated infections also predispose patients to a higher risk of HIV (Lawal et al., 2019; Mulaudzi et al., 2015; Rowley et al., 2019). This is because infection triggers the secretion of pro-inflammatory cytokines, which attract leukocytes to the site of infection (Pahwa and Jialal, 2014). Leukocytes play an essential role in the removal of infectious agents, however, they are also the target site for HIV infection (Lawal et al., 2019). Additionally, cytokines also increase the chances of HIV infection by reducing plasma membrane integrity and promoting viral replication through the activation of transcription factors such as nuclear factor Kappa-B (NF-κβ) (Iwalewa et al., 2010; Masson et al., 2016; Smith and Murphy, 2016).

Mechanism of Inflammatory Responses

Inflammation is a natural defence mechanism that is elicited by the body, in response to either injury or exposure to an irritant, toxin, or infectious agent (Pahwa et al., 2020). Inflammation usually promotes wound healing and restoration, however, prolonged exposure to an infectious agent and uncontrolled or unresolved inflammatory responses often result in chronic inflammation, which may eventually lead to disease (Iwalewa et al., 2010; Pahwa et al., 2020).

The inflammatory cascade consists of a series of biochemical reactions, that are highly regulated by a variety of complex mediators (Lawal et al., 2019). These mediator molecules play an important role in either establishing or resolving inflammation (Arita, 2016). Eicosanoids are a group of lipid molecules that serve as precursors for the biosynthesis of inflammatory mediators (Arita, 2016; Gomes et al., 2018). Biosynthesis begins with the secretion of pro-inflammatory cytokines, which trigger the cleavage of arachidonic acid (AA) from the cell

plasma membrane, by the enzyme phospholipase A2 (Dobrian et al., 2011; Pahwa and Jialal, 2014). AA is a polyunsaturated fatty acid (PUFA), that serves as a substrate for the synthesis of eicosanoids through either the cyclooxygenase (COX) or lipoxygenase (LOX) pathways (**Figure 2.1**) (Arita, 2016; Dobrian et al., 2011; Rao et al., 2012). Both pathways contain several oxidative isoenzymes, which are responsible for the production of a wide variety of mediator molecules; COX isoenzymes are responsible for the synthesis of pro-inflammatory mediators, which include prostaglandins and thromboxanes, whilst the LOX isoenzymes are involved in the synthesis of leukotrienes and anti-inflammatory lipoxins (Ricciotti and Fitzgerald, 2011; Wisastra and Dekker, 2014).

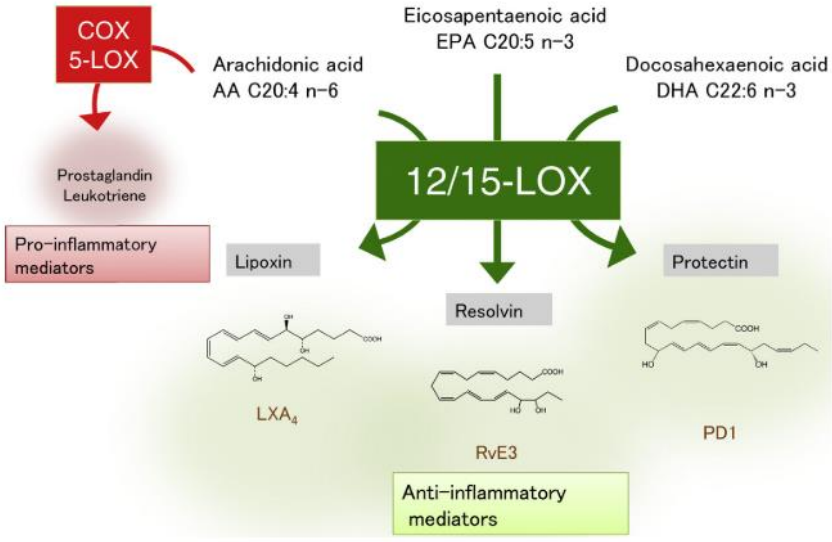

Figure 2.1. Lipid mediators involved in the initiation and resolution of inflammation (Arita, 2016).

Other oxidative enzymes that play an important role in the inflammatory cascade include xanthine oxidase (XO) and inducible nitric oxide synthase (iNOS) (Lawal et al., 2019). These enzymes, together with the COX and LOX isoenzymes, have been implicated in the development and progression of cancer and other life-threatening inflammatory diseases, such as PID (Gomes et al., 2018; Rao et al., 2012). This is because these enzymes all produce reactive nitrogen or oxygen

species (RNS or ROS) as a by-product of their metabolic reactions (Cho et al., 2011; Lawal et al., 2019). Reactive species are produced under aerobic conditions, and generally play an important role in the coordination and regulation of inflammation, however, overexpression or uncontrolled activity often leads to an accumulation of these molecules, resulting in oxidative stress (Cho et al., 2011; Djankou et al., 2020). Oxidative stress is harmful to the body, as it prevents the resolution of the inflammatory cascade, and may eventually cause chronic inflammation and disease (Djankou et al., 2020; Iwalewa et al., 2010).

Treatment of Inflammatory Diseases

Non-steroidal anti-inflammatory drugs (NSAIDs) are typically prescribed for the treatment of both pain and inflammation (dos Reis Nunes et al., 2020). These drugs reduce inflammation and tissue damage by inhibiting the production of inflammatory mediators produced by COX, LOX, iNOS, and XO (Pahwa et al., 2020). Although NSAIDs alleviate the discomfort of inflammation and are highly effective, they are also associated with several adverse side effects including nausea and gastrointestinal bleeding (Adebayo et al., 2015). The adverse side effects associated with these drugs have therefore prompted the search for alternative and effective therapeutic agents with fewer side effects.

Medicinal plants are an attractive alternative to NSAIDS, as they are naturally occurring agents, with a wide variety of biologically active compounds that can combat disease, often without any adverse side effects (Mahomoodally, 2013; Rao et al., 2012). In South Africa, a wide variety of plants have been implicated for use in the management and treatment of pain-related inflammatory diseases (Iwalewa et al., 2010). Several pharmacological studies have validated the traditional use of these plants and some have even identified the phytochemicals

responsible for the observed activity (Iwalewa et al., 2010). For example, flavonoids have been identified as inhibitors of the inflammatory cascade, with a similar mechanism of action to that of NSAIDs (Choy et al., 2019; Iwalewa et al., 2010; Serafini et al., 2010). Other metabolites that have been identified as potential inhibitors include alkaloids, phenolic compounds, phytoestrogens, terpenoids, tannins, coumarins, carotenoids, and curcumin (Adebayo et al., 2015; Iwalewa et al., 2010; Rao et al., 2012).

2.2 The Use of Plants in Traditional Medicine

For centuries, medicinal plants have been used as a primary source of healthcare (Danmalam et al., 2017). According to the WHO, 80% of the population in the developing world is still largely dependent on traditional medicine as their primary source of healthcare (Nthulane et al., 2020; World Health Organization, 2019). Traditional medicine, as defined by the WHO, is ‘the sum total of knowledge, skill, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, that are used to maintain health, as well as to prevent, diagnose, improve or treat physical and mental illnesses’ (World Health Organization, 2019). This knowledge is often based on a combination of past experiences and observations, passed down through many generations to provide effective treatment for common ailments within these communities (Ozioma and Chinwe, 2019).

Rural communities in the developing world are still largely dependent on traditional medicine for several reasons. Very often, hospitals and clinics are absent within these areas, therefore patients do not always have reliable access to modern healthcare. Even when these services are available, patients would rather consult with traditional healers instead, as their services are often more affordable than western medicines (Lampiao et al., 2019). Furthermore, given the intimate nature of some medical conditions, patients often prefer to confide in traditional

healers instead of physicians, due to shared religious and cultural beliefs, and also because traditional healers often aim to establish close relationships, by offering counselling services (Ozioma and Chinwe, 2019; Semenya, 2013).

Traditional Medicine and Modern Drug Development

Home to the Cape Floristic Region (CFR), South Africa is a botanically diverse country that harbours up to 10% of the world's floral species, the majority of which are endemic species (Erasmus et al., 2012; Sobiecki, 2014). Given this immense diversity, the widespread use of traditional medicine throughout South Africa comes as no surprise, with an estimated average of 27 million consumers from both rural and urban communities alike (Mander et al., 2007; Mulaudzi et al., 2015).

This immense botanical diversity, along with the widespread use of traditional medicine, has in recent years resulted in an increase in ethnobotanical research throughout South Africa (Sobiecki, 2014). Ethnobotanical surveys play a very important role in the identification of plants that can be used to treat or manage disease (Ozioma and Chinwe, 2019). Once a potential medicinal plant has been identified, several scientific investigations are then carried out, to characterise biological activity, establish safety, and validate or document the traditional uses of the plant (Ozioma and Chinwe, 2019).

Medicinal plants are rich in phytochemicals that have protective properties, and that are effective against pathogens that cause disease in humans (Aliba et al., 2018). These phytochemicals disrupt microbial growth through several mechanisms including enzyme inhibition, plasma membrane disruption, metal ion complexation and substrate deprivation (Taiwo et al., 2016). Therefore, analytical techniques can be applied to isolate and purify these phytochemicals,

to develop new drugs to combat infection and improve human health (Mahomoodally, 2013; Taiwo et al., 2016).

2.3 Plant Selection: *Ficus abutilifolia* (Miq.) Miq.

2.3.1 Taxonomy

Kingdom : Plantae

Division : Magnoliophyta

Class : Magnoliopsida

Subclass : Rosidae

Order : Rosales

Family : Moraceae

Genus : *Ficus*

Subgenus : *Urostigma*

Species : *abutilifolia*

2.3.2 Distribution and Botanical Description

Ficus abutilifolia (Miq.) Miq., more commonly known as the large-leaved rock fig, is a plant belonging to the Moraceae family (Danmalam et al., 2012). Moraceae is a large family of angiosperm, that contains about 53 genera, including *Ficus*. This large genus is comprised of up to 800 woody species, shrubs, and hemi-epiphytes (Mawa et al., 2013; Somashekhar et al., 2013).

Ficus abutilifolia is a small to medium-sized deciduous tree, that is widely distributed throughout the hot, dry regions of southern and western Africa, where it can be found growing in rocky terrain (**Figure 2.2 A and B**) (Burring, 2006; Danmalam et al., 2012; Taiwo et al., 2016). In South Africa, the tree can be found growing throughout the provincial areas of Limpopo, Mpumalanga, Gauteng, KwaZulu-Natal, and the North West (Burring, 2006; Burrows and Burrows, 2003).

The leaves of *F. abutilifolia* have been described as broad and ovate, with a smooth surface and a cordate base (**Figure 2.2 C**) (Danmalam et al., 2012). Figs produced from the plant are often 15-25 mm in diameter, and are often sour but edible (**Figure 2.2 D**) (Danmalam et al., 2012). The trunk of the plant is unusually contorted and has a yellowish-white bark with a smooth, powdery, or slightly flaky texture (Aliba et al., 2018). Lastly, the plant has large conspicuous roots which can penetrate rocks and are often found growing as far as 60 m below the ground (Burring, 2006; Fern, 2014).

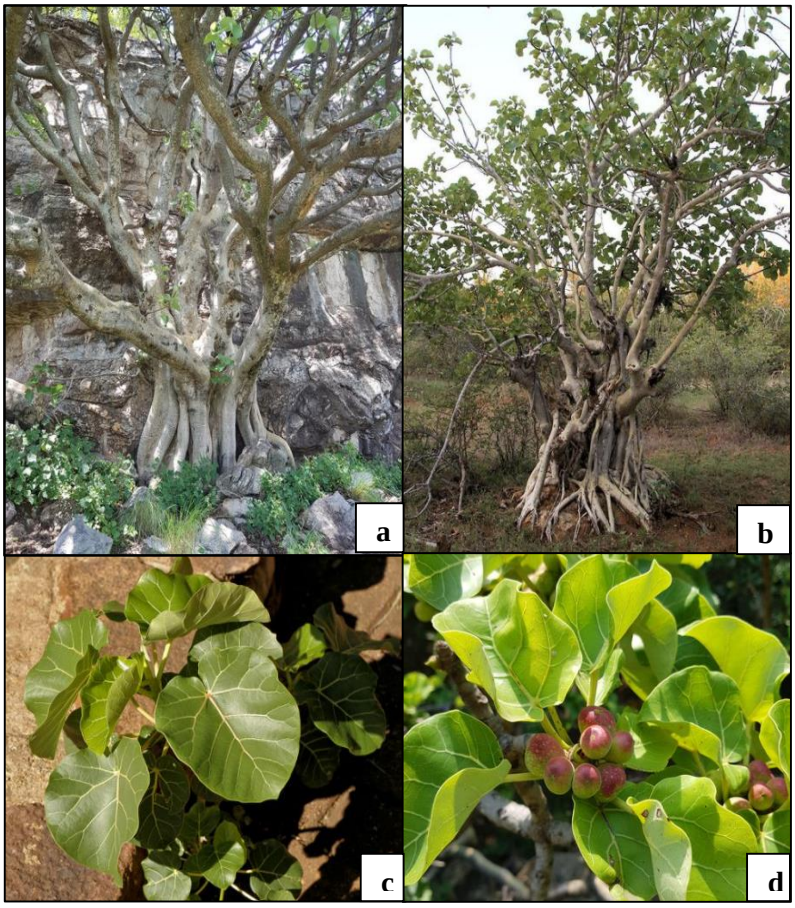

Figure 2.2. A pictorial view showing (a & b) the contorted trunk and roots; (c) foliage, and (d) a close-up of the figs produced by *Ficus abutilifolia.*

Image Reference: https://www.gbif.org/occurrence/gallery?taxon_key=5570182

2.3.3 Traditional Uses

Traditionally, the leaves, stem, and root bark of *F. abutilifolia* are prepared as decoctions, which are used to treat a wide variety of ailments throughout southern and western Africa (Chauke et al., 2015; Djankou et al., 2020). In South Africa, these decoctions are used to treat infertility and STIs (Chauke et al., 2015), whilst in Nigeria and parts of Cameroon, they are used to treat typhoid, dysentery, malaria, infertility, and a variety of STIs (Djankou et al., 2020; Taiwo et al., 2016). The traditional uses of *F. abutilifolia* are summarized in **Table 2.1** below:

Table 2.1. The traditional uses of *F. abutilifolia.*

Plant Part	Traditional Use	Country	References
Leaves	Typhoid; malaria; fever; chronic dysentery; infertility; STIs; jaundice; coughs, hypertension; rheumatism	Nigeria; Cameroon	(Taiwo et al., 2016); (Djankou et al., 2020)
Stem bark	STIs; strengthening tonic for men	Nigeria; South Africa	(Aliba et al., 2018) (Chauke et al., 2015); (Ukwubile, 2014a)
Roots	Epilepsy	Nigeria	(Danmalam et al., 2012); (Danmalam et al., 2017)
Milky latex	Removal of skin warts	Nigeria	(Aliba et al., 2018); (Danmalam et al., 2012)

2.3.4 Phytochemistry

A wide range of biologically active phytochemicals are present in the leaves, stem, and root bark of *F. abutilifolia* (**Table 2.2**). For example, the total phenolic content of the stem bark was investigated in two separate studies, and results from both of these studies indicated a significantly high level of phenolic compounds, including anthraquinones, flavonoids, and tannins (Djankou et al., 2020; Lawal et al., 2019). These compounds, in particular, are associated with radical scavenging activity and are also capable of inhibiting the activity of both cyclooxygenases and lipoxygenases (Djankou et al., 2020; Hussain et al., 2016). Other classes of phytochemicals that have been found in the leaves and stem bark of *F. abutilifolia* include alkaloids, carbohydrates, cardiac glycosides, cardenolides, phlobatannins, reducing sugars, saponins, steroids, and terpenoids (Djankou et al., 2020; Mikail et al., 2019; Taiwo et al., 2016).

Extensive ^{1}H-NMR and ^{13}C-NMR spectroscopic analysis of the stem bark has led to the isolation of β-sitosterol, a compound with antibacterial, antifungal, anticancer, antimutagenic, anti-inflammatory, and antioxidant properties (Aliba et al., 2018). Proanthocyanidins and terpenoids have also been detected in the stem bark of *F. abutilifolia.* These compounds have radical scavenging properties and are effective inhibitors of the lipoxygenase isoenzymes (Govindaraj et al., 2020; Lawal et al., 2019). Other phytochemicals that have been isolated from the stem bark through spectroscopic analysis include octatriacontane, lupeol acetate, α-amyrin acetate, β-amyrin acetate, daucosterol, and a new fatty acid known as pentacosyl-henicosanoate (**Figure 2.3**)(Djankou et al., 2020).

Volatile compounds that have also been isolated from the stem bark through gas chromatography-mass spectrometry (GC-MS) analysis include phenol 3-pentadecyl, methyl commate D, polygalitol, benzoic acid, and octadecanoic acid-dihydroxy propyl ester (Lawal et al., 2019).

Table 2.2. Phytochemical classes present in *F. abutilifolia*.

Plant Part	Phytochemicals Present	Biological Activity	Reference
Leaves	Alkaloids, anthraquinones, carbohydrates, cardiac glycosides, flavonoids, phlobatannins, saponins, tannins	Antibacterial	(Ukwubile, 2014a); (Taiwo et al., 2016)
Stem bark	Alkaloids, carbohydrates, cardiac glycosides, cardenolides, flavonoids, saponins, steroids, tannins, triterpenes **Isolated compounds**: Phenol 3-pentadecyl, methyl commate D, polygalitol, benzoic acid, octadecanoic acid-dihydroxy propyl ester, pentacosyl henicosanoate, octatriacontane, β-sitosterol, lupeol acetate, α-amyrin acetate, β-amyrin acetate, daucosterol,	Anti-inflammatory; Antioxidant; Sedative-hypnotic	(Ukwubile, 2014a); (Aliba et al., 2018); (Lawal et al., 2019); (Mikail et al., 2019); (Djankou et al., 2020)
Root bark	Flavonoids, saponins, tannins, terpenoids, steroids	Anticonvulsant	(Danmalam et al., 2012); (Ukwubile, 2014a)

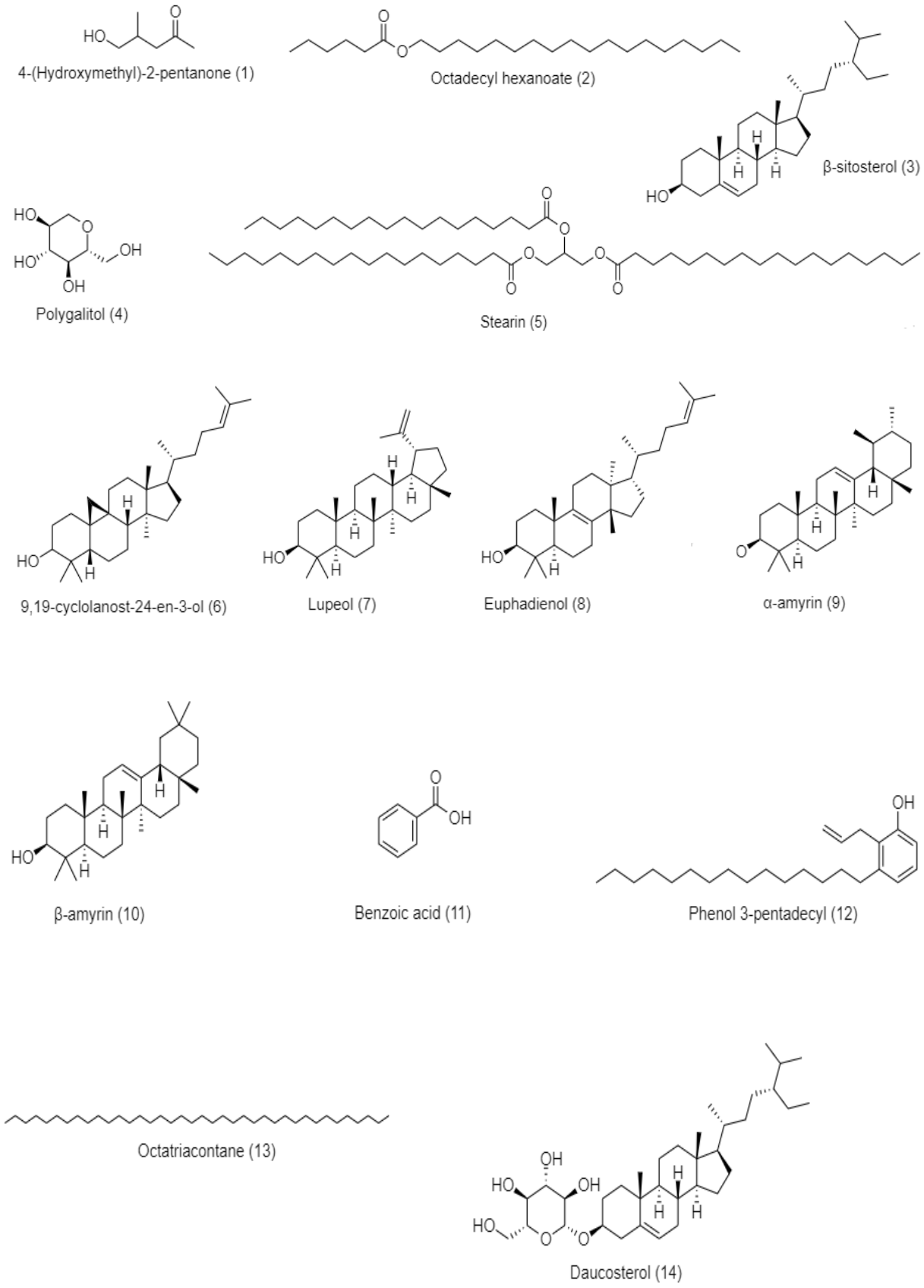

Figure 2.3. Chemical structures of selected compounds isolated from the stem bark of *F. abutilifolia*.

2.3.5 Biological Activity

Anticonvulsant activity

Ethanol extracts prepared from the root bark of *F. abutilifolia* have displayed anti-epileptic activity in mice, thus indicating a potential for use in the management of grand mal epilepsy (Danmalam et al., 2012).

Antibacterial activity

Aqueous, ethyl acetate, n-butane, n-hexane, and dichloromethane fractions prepared from the leaves of *F. abutilifolia* were investigated for activity against Gram-positive (*Bacillus subtilis, Staphylococcus aureus, Enterococcus faecalis, Clostridium sporogenes*), and Gram-negative species (*Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli,* and *Salmonella typhi*) (Taiwo et al., 2016). All fractions, except for the aqueous, showed antibacterial activity (Taiwo et al., 2016). Ethyl acetate showed the highest activity against all isolates, especially against *S. aureus* and *P. aeruginosa*, with MICs ranging from 3.13 x 10^{-2} µg/ml to 6.25 x 10^{-2} µg/ml (Taiwo et al., 2016).

Anti-inflammatory activity

An acetone extract prepared from the stem bark was screened for anti-inflammatory activity against the oxidative enzymes, 15-LOX, and xanthine oxidase (XO). Moderate inhibition against 15-LOX was detected at a test concentration of 41.7 µg/ml, whilst noteworthy activity was also detected against XO (IC_{50}: 74 ± 6 µg/mL) (Lawal et al., 2019). The effect of the extract on inducible nitric oxide synthase (iNOS) was also determined. The extract showed higher activity (IC_{50}: 19.7 ± 0.2 µg/mL) as compared to the positive control, quercetin (IC_{50}: 30.0 ± 0.7 µg/mL), thus confirming the anti-inflammatory potential of the acetone extract (Lawal et al., 2019).

Cytotoxicity

An acetone extract prepared using the stem bark was screened for cytotoxicity using Vero cells (Lawal et al., 2019). The extract was found to be non-cytotoxic, with an IC_{50} value greater than 100 µg/mL (Lawal et al., 2019).

Sedative-hypnotic activity

A methanol extract prepared using the stem bark of *F. abutilifolia* was screened for sedative-hypnotic effects in mice. Mice that were pre-treated with a low dose (100 mg/kg) of the methanol extract, followed by ketamine administration exhibited longer sleeping cycles (Mikail et al., 2019). Furthermore, it was noted that the mice remained in a sedative state for some time after arousal from sleep, thus indicating the sedative-hypnotic potential of the plant extract (Mikail et al., 2019).

Radical Scavenging and Antioxidant Activity

The total phenolic content (TPC) of an acetone extract prepared from the stem bark of *F. abutilifolia* was evaluated to determine the radical scavenging and antioxidant potential of the plant (Lawal et al., 2019). The results indicated the presence of a high concentration of phenolic compounds (>500 mg/g GAE) (Lawal et al., 2019). The effect of the extract against 1,1-diphenyl-2-picrylhydrazyl (DPPH) was also investigated, and it was found that the extract had comparable activity (IC_{50}: 6.4 ± 0.4 µg/mL) to that of the positive control, quercetin (5.0 ± 0.3 µg/mL) (Lawal et al., 2019).

The effect of five stem bark extracts (hexane, EtOAc, acetone, CH_2Cl_2/MeOH (1:1), MeOH) against DPPH was also investigated in a separate study (Djankou et al., 2020). When compared to ascorbic acid (IC_{50}: 0.010 mg/mL), it was found that the acetone extract had the highest scavenging activity (0.038 mg/mL), followed by the CH_2Cl_2/MeOH (1:1) extract (0.053 mg/mL), the MeOH extract (0.060 mg/mL) and the EtOAc extract (4.532 mg/mL) (Djankou et al., 2020). The

hexane fraction showed no antioxidant activity. Further analysis also indicated that the extracts with the highest antioxidant activity also possessed a high TPC that corresponded with the observed activity (Djankou et al., 2020). The Ferric Reducing Antioxidant Power (FRAP) of the extracts was also determined against butylhydroxytoluene (BHT) and ascorbic acid, to further establish antioxidant potential. The results followed the same order of reactivity of the extracts as in the DPPH assay, and it was also noted that the observed activity of each extract increased proportionally with sample concentration, thus indicating potential antioxidant activity (Djankou et al., 2020).

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CHAPTER 3

The Anti-gonococcal and Anti-inflammatory Potential of Crude Extracts Prepared Using the Stem Bark of *Ficus abutilifolia* (Miq.) Miq.

3.1 Introduction

The use of medicinal plants in the treatment of disease is widely practised throughout South Africa, with approximately 80% of the population relying on the traditional healthcare system (Ozioma and Chinwe, 2019). This widespread use of traditional medicine is often attributed to personal preference, as well as cultural and religious practices (Mahomoodally, 2013; Mander et al., 2007). Studies have indicated that the use of traditional medicine in South Africa is not just confined to poor or rural communities, but also extends into urban areas, where it is often used as a supplement to modern medicine (Abdullahi, 2011; Mander et al., 2007).

A variety of medicinal plants are used by traditional healers to treat sexually transmitted infections (STIs) and pain-related inflammatory diseases in South Africa (Chauke et al., 2015; Iwalewa et al., 2010). For example, up to 115 different plant species have been implicated in the treatment and management of both pain and inflammation, whilst several plant species from various families are used throughout South Africa to treat STIs (Iwalewa et al., 2010; Semenya, 2013). Plants may be used individually to prepare these herbal remedies, or multiple plants may be combined, to produce an effective poly-herbal remedy (Dawurung et al., 2021; Naidoo et al., 2013).

The biological effects exerted by medicinal plants can be attributed to the presence of a large number of secondary metabolites (Dawurung et al., 2021; Ukwubile, 2014b). These metabolites are chemically diverse in structure, and are often effective against a wide range of infectious agents or can regulate various

metabolic pathways such as the inflammatory cascade (Iwalewa et al., 2010; Mahomoodally, 2013). Phenolic compounds, for example, play an active role in disease prevention, as they have both anti-inflammatory and antioxidant properties, and are also effective against several disease-causing microorganisms (Bouarab-Chibane et al., 2019).

Worldwide, traditional remedies are generally perceived as a safer, more affordable, and holistic alternative to modern medicine (Mahomoodally, 2013). These remedies are also thought to be associated with fewer adverse side effects and as a result, in recent years, their use has been widely embraced throughout the world (Ekor, 2014). However, despite this, there is still very little information available on the safety and efficacy of these remedies (Ekor, 2014; Naidoo et al., 2013). The risks associated with using these herbal remedies, therefore, remain high, unless both safety and efficacy are established through scientific evaluation (Ozioma and Chinwe, 2019).

In vitro and *in vivo* bioassays can be used to validate the traditional use of medicinal plants. These studies often result in the identification, characterisation, and isolation of biologically active phytochemicals, which can also be used as a lead for the development of new pharmaceutical drugs (Adebayo et al., 2015; Sasidharan et al., 2011; Shokeen et al., 2009). Therefore, this chapter focuses on the *in vitro* bioassays used to determine the anti-gonococcal and anti-inflammatory potential of crude extracts prepared from the stem bark of *Ficus abutilifolia* (Miq.) Miq. (**Figure 3.1**).

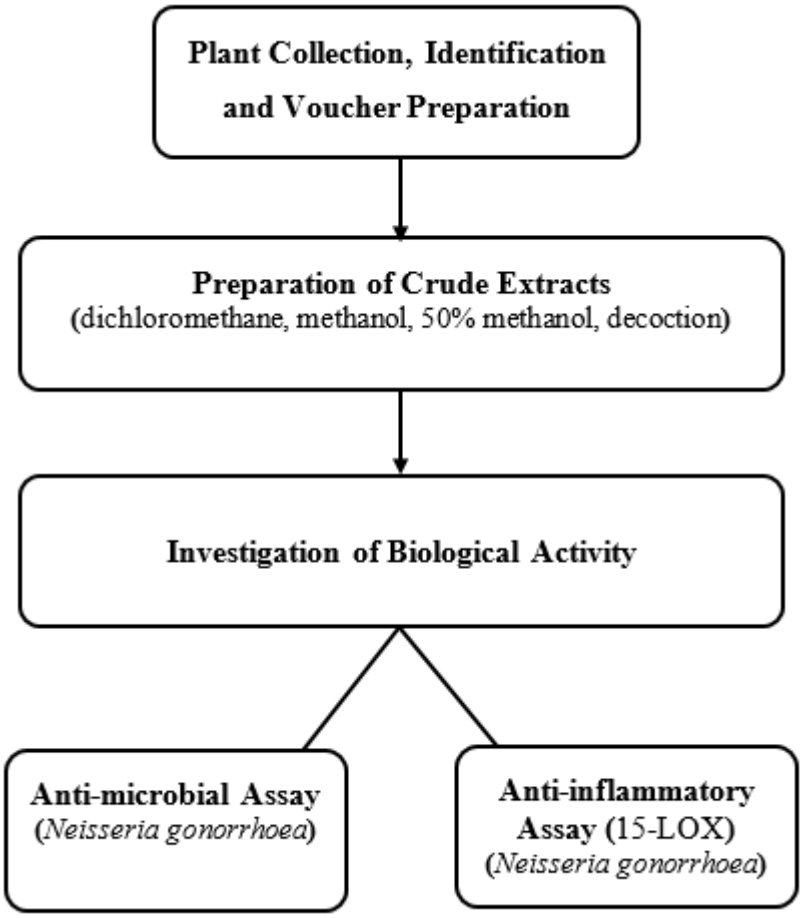

Figure 3.1. An overview of the methods used to determine the anti-gonococcal and anti-inflammatory potential of *F. abutilifolia*.

3.2 Materials and Methods

3.2.1 Plant Collection and Identification

The stem bark of *F. abutilifolia* (Moraceae) was collected in December 2019, from the region of Venda in Limpopo, South Africa. The plant material was identified, and a voucher specimen was deposited at the H. G. W. J. Schweickerdt Herbarium at the University of Pretoria, South Africa (voucher number: TE0211).

3.2.2 Plant Extraction

The fresh plant material was cleaned, cut into smaller pieces, and dried at room temperature, before being ground into a fine powder, using a Janke and Kunkel grinder (IKA Labortechnik, Germany). Three crude extracts were prepared from the dried plant material, through sequential extraction, starting with dichloromethane, absolute methanol, then 50% methanol (methanol: water, 1:1, v/v) (**Figure 3.2**). For the fourth extract, a decoction was prepared by boiling the plant material in distilled water.

3.2.2.1 *Sequential Extraction with Organic Solvents*

Dried plant material (700 g) was first extracted in 2 L of dichloromethane with continuous agitation (Labcon, South Africa) for 3 hours. The extract was filtered in a Buchner funnel (Buchi Vac V-500, South Africa), through Whatman No.3 filter paper. The retained filtrate was stored at 4°C, whilst the residue was extracted further using the same amount of fresh solvent, with continuous agitation for 3 hours, followed by a final repeat overnight. The filtrates from each extraction were combined (6 L), and the extraction procedure was repeated with absolute methanol and then finally 50% methanol (methanol: water, 1:1, v/v). The three filtrates were concentrated using a Heidolph Hei-Vap Rotary

Evaporator (Labotech, South Africa), and the dichloromethane and absolute methanol extracts were dried in a laminar flow (VividAir, South Africa). The remaining water in the 50% methanol extract was removed using a VirTis Manuel freeze dryer (United Scientific, South Africa).

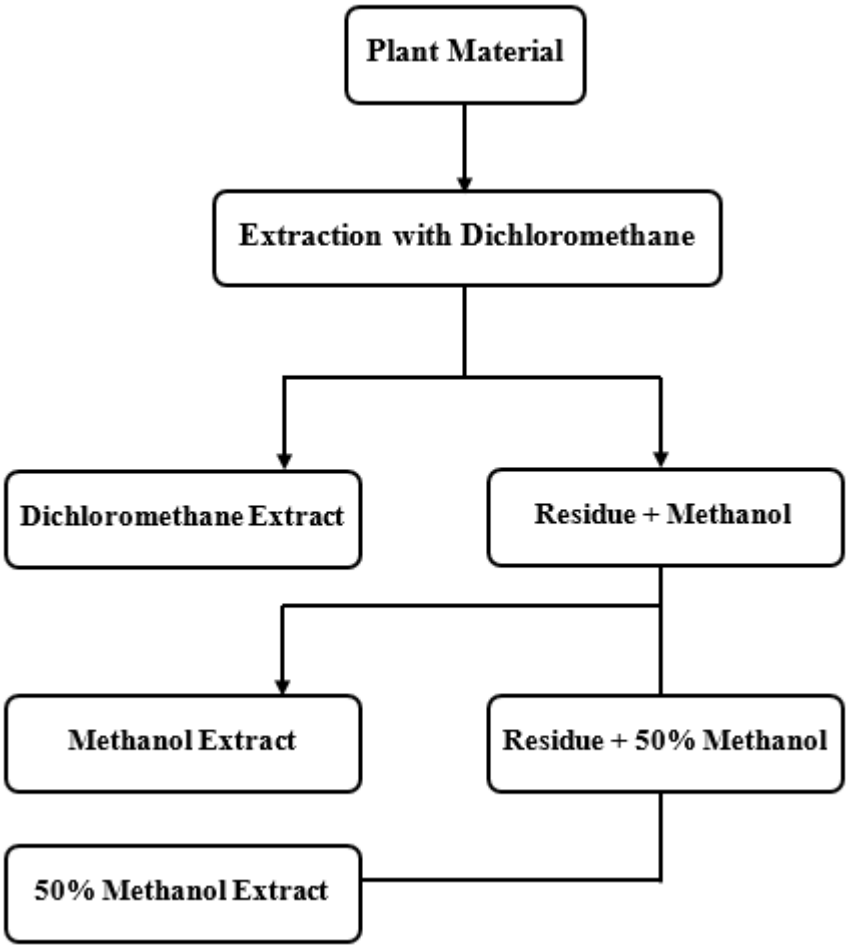

Figure 3.2. A flowchart of the sequential extraction.

3.2.2.2 *Water Extraction (Decoction)*

To imitate the preparation method followed by traditional healers, a decoction was prepared by boiling 300 g of the dry plant material in 3 L of distilled water for 30 minutes. The extract was cooled and filtered through a Buchner funnel (Buchi Vac V-500, South Africa) using Whatman No.3 filter paper, and the filtrate was frozen overnight at -80°C and dried in a VirTis Manuel freeze dryer (United Scientific, South Africa).

All four crude extracts were weighed and stored in airtight poly-top vials at 4°C.

3.2.3 Screening the Extracts for Anti-Gonococcal Activity

3.2.3.1 Culturing the bacteria

Agar plates containing chocolate blood agar were inoculated with *N. gonorrhoea* (ATCC 19424) and incubated under anaerobic conditions (37°C for 48 hours) with CO_2 supplementation (CO_2 Gen™ 2.5 L; Thermo Scientific, South Africa). Mature bacterial colonies were transferred into 100 mL of Mueller-Hinton (MH) broth and sub-cultured with mild agitation (37°C for 24 hours). The bacterial solution was prepared to an optical density (OD) of 1.5 x 10^8 CFU/mL (0.5 McFarland standard), using a DU® 720 General purpose UV/Vis Spectrophotometer (Labotec, South Africa), at a wavelength of 600 nm.

3.2.3.2 Microdilution Assay

The minimum inhibitory concentration (MIC) values for each extract were determined using the broth microdilution assay, as outlined by Eloff (1998), with slight modification. A 96-well microtiter plate was prepared and labelled as shown in **Figure 3.3**. Stock solutions (50 mg/mL) were first prepared by dissolving each extract in 10% dimethyl sulfoxide (DMSO). Next, sterile MH broth (100 µL) was pipetted into each of the wells in the microtiter plate. Extracts

(100 µL) were then loaded in triplicate, into the first row of the microtiter plate, creating a two-fold dilution.

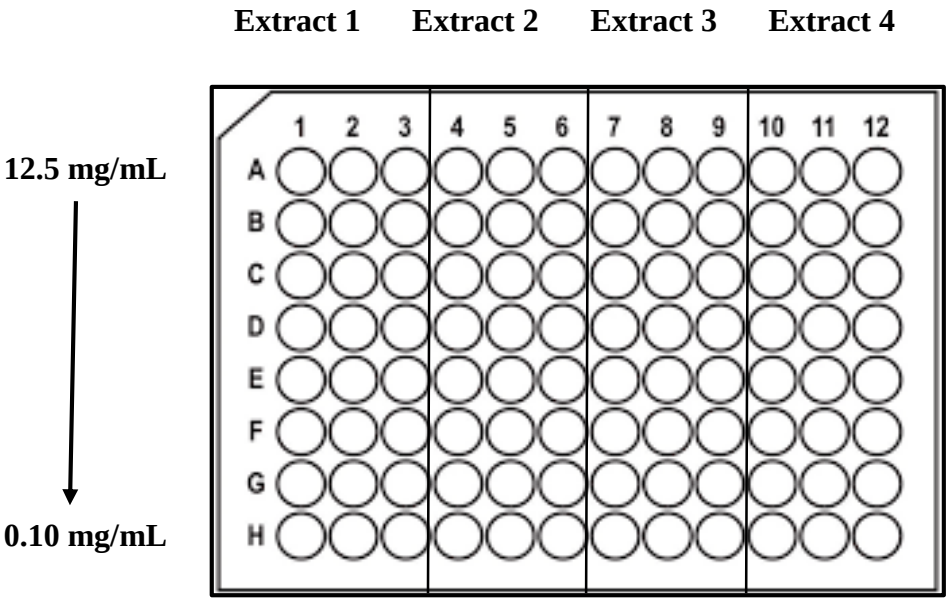

Figure 3.3. A microtitre plate depicting the experimental set-up for the *in vitro* microdilution assay.

The extracts were then serially diluted down the microtiter plate, starting with a concentration of 12.50 mg/mL, and ending with 0.10 mg/mL. Each well was then inoculated with the bacterial solution (100 µL), and incubated under anaerobic conditions (37°C for 24 hours) with CO_2 supplementation. The antibiotic, ciprofloxacin (0.2 mg/mL) was included as the positive control, whilst solutions of sterile MH broth and 10% DMSO were included as the negative controls in the experiment. Bacterial growth was determined by adding 10 µL of PrestoBlue® Cell Viability Reagent (Thermo Scientific, South Africa) to each well, before further incubation (37°C for 30 minutes). A pink colour change indicated microbial growth, and the MIC values were visually determined and recorded as the lowest concentration of plant extract with a blue well.

3.2.4 *In Vitro* Anti-Inflammatory Assay

The anti-inflammatory potential of the plant extracts was determined by screening for their ability to inhibit 15-lipoxygenase (15-LOX), using a modified version of the experimental technique outlined by Lyckander and Malterud (1996). 15-LOX is an oxidative enzyme that catalyses the breakdown of linoleic acid (LA) to hydroperoxy-octadecadienoic acid (HPODE) (Sadeghian and Jabbari, 2015). The *in vitro* anti-inflammatory bioassay, therefore, monitors the ability of plant extracts to inhibit the formation of this product at a wavelength of 234 nm.

The formation of HPODE from LA (final concentration, 134 µM) was monitored at 234 nm using a DU® 720 General purpose UV/Vis Spectrophotometer (Labotec, South Africa). Enzyme and substrate solutions were both prepared in cold borate buffer (0.2 M, pH 9.00), and to preserve activity, the enzyme solution was kept on ice for the duration of the bioassay. The plant extracts were dissolved in DMSO (10 mg/mL) and used to prepare six different concentrations, which were all tested in triplicate. The test samples were prepared in a quartz cuvette, by dissolving the plant extract (50 µL) in the substrate-buffer solution (2.9 mL). To begin the reaction, an enzyme solution (50 µL) was added to the cuvette, and the increase in absorbency was measured against a blank and recorded at 30-second intervals, for 5 minutes. The negative control contained DMSO, whilst the positive control contained quercetin. The experimental procedure was repeated 3 times for each concentration of extract, and inhibitory activity was determined by calculating enzyme inhibition (%) from the changes in absorbance values over 5 minutes.

$$\text{Percentage Inhibition} = [100 \text{ x } \frac{\Delta A0 - \Delta A1}{\Delta A0}]$$

Where ΔA_0 is the absorbance of negative control and ΔA_1 is the absorbance of the extract.

3.2.5 Statistical Analysis

Data obtained from the study were presented as the mean +/- standard error of the mean (SEM) of repeats from the experiment (n = 3). Statistical analysis was conducted using GraphPad Prism 4 software (GraphPad Software, Inc., La Jolla, CA, USA). These values were then compared using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test, where $p < 0.05$ indicated statistical significance.

3.3 Results and Discussion

3.3.1 Plant Extraction

The stem bark of *F. abutilifolia* was dried, ground, and extracted in four different solvents with increasing polarity (dichloromethane, absolute methanol, 50% methanol (methanol: water, 1:1, v/v), and distilled water). Extraction is the first step in the analysis of medicinal plants, which is used to extract biologically active phytochemicals from plant material, using specific organic solvents (Sasidharan et al., 2011; Truong et al., 2019). The choice of solvent and extraction technique influences the percentage yield, phytochemical composition and bioactivity of an extract. This is because phytochemicals have different functional groups that interact uniquely with each solvent, thus influencing their solubility properties (Dirar et al., 2019)

Sequential extraction was performed using dichloromethane, absolute methanol, and 50% methanol. This technique involves a series of successive extractions with multiple solvents of increasing polarity, to ensure the extraction of a broad range of phytochemicals (Nawaz et al., 2020). A decoction was also prepared by boiling the plant material in distilled water, to mimic the preparation method that traditional healers often use to prepare herbal remedies (Fongang et al., 2021; Sasidharan et al., 2011).

3.3.2 Anti-gonococcal Activity

The anti-gonococcal activity of the plant extracts was determined through the *in vitro* microdilution assay, as described above. This simple broth dilution technique is carried out in a 96-well microtiter plate and is often favoured over other techniques as it is rapid, reproducible, and generally less labour-intensive compared to other techniques (Eloff, 1998; Foerster et al., 2017). The technique

is also adaptable and can be used to determine the antimicrobial susceptibility of a wide range of microorganisms, including *N. gonorrhoea* (Chen et al., 2020).

Antimicrobial susceptibility is determined by calculating the minimum inhibitory concentration (MIC), which is the lowest concentration of any antimicrobial agent, that can prevent the visible growth of a microorganism following a 24 hour incubation period (Andrews, 2001; Gillespie, 1994). Microbial growth can then be visualized by adding an indicator solution such as PrestoBlue® Cell Viability Reagent, which contains a blue dye known as resazurin, that rapidly reduces into a fluorescent pink compound known as resorufin, in the presence of actively respiring bacterial cells (Kowalska-Krochmal and Dudek-Wicher, 2021). Therefore, in this experiment, the MIC of each plant extract was recorded as the lowest concentration of wells in the microtiter plate that remained blue.

The results obtained from the microdilution assay are presented in **Table 3.1**. The highest tested concentration for the crude extracts was 12.50 mg/mL whilst the lowest was 0.10 mg/mL. According to literature, MIC values that are lower than 0.10 mg/mL are considered to be noteworthy, whilst MIC values ranging from 0.10 to 0.80 mg/mL are considered to be moderate (Eloff, 2004; Kuete, 2010; Pendota et al., 2017). Thus, the dichloromethane extract displayed moderate to low anti-gonococcal activity with a MIC value of 6.25 mg/mL whilst the polar crude extracts displayed moderate to high activity against *N. gonorrhoea*, with MIC values of 0.20 mg/mL for each extract.

The anti-gonococcal activity of *F. abutilifolia* has not been reported in literature, however, immense literature on the phytochemical profile of the stem bark exists. A naturally occurring phytosterol, known as β-sitosterol, has been isolated from the stem bark (Aliba et al., 2018). This compound is usually found within the plasma membrane of plant cells and has been associated with a wide range of biological effects, including antimicrobial activity (Babu and Jayaraman, 2020; Nweze et al., 2019; Sen et al., 2012). The stem bark also has a high phenolic

content and is particularly rich in flavonoids, saponins and tannins (Aliba et al., 2018; Djankou et al., 2020; Lawal et al., 2019). Flavonoids are associated with antibacterial activity and are effective against a wide range of microorganisms, including *N. gonorrhoea* (Kuete, 2010). Tannins are also associated with antibacterial properties, as they bind to proteins to interfere with enzyme activity (Ukwubile, 2014b; Yusiati et al., 2018). Thus, the activity displayed by the polar extracts of *F. abutilifolia* can be attributed to the abovementioned phytochemicals.

Table 3.1. The anti-gonococcal and anti-inflammatory activity of crude extracts prepared from the stem bark of *F. abutilifolia*.

Crude extract	**Anti-gonococcal activity MIC (mg/mL)**	**Anti-inflammatory activity IC_{50} ± SEM (µg/mL)**
Dichloromethane	6.25	> 166.70*
Absolute methanol	**0.20**	**18.33 ± 0.70**
50% Methanol	**0.20**	**23.82 ± 0.90**
Decoction	**0.20**	**25.58 ± 0.41**
Ciprofloxacin	< 0.01	-
Quercetin	-	15.13 ± 0.19

MIC – minimum inhibitory concentration; $n = 3$

IC_{50} – half maximal inhibitory concentration; $n = 3$

>166.70; activity greater than highest tested concentration.

< 0.01; activity shown at all concentrations less than 0.01 mg/mL

*; means are statistically significant ($p < 0.05$)

3.3.3 Anti-inflammatory Activity

The anti-inflammatory assay, as described above, was conducted to investigate the inhibitory potential of crude extracts produced from the stem bark of *F. abutilifolia*, against the enzyme 15-LOX. This oxidative enzyme plays a vital role in the inflammatory cascade, where it is involved in the biosynthesis of a variety of inflammatory mediators (Sadeghian and Jabbari, 2015). Several studies have revealed that a wide range of phytochemicals effectively inhibits the COX/LOX

isoenzymes (Elisha et al., 2016). Thus, the ability of a plant extract to inhibit 15-LOX can be used to determine the anti-inflammatory potential of an extract.

The anti-inflammatory activity of each crude extract was determined over six different concentrations (166.70, 83.30, 41.70, 20.80, 10.40, and 5.20 µg/mL). The optical densities were measured at 234 nm using a spectrophotometer and used to calculate the percentage inhibition of the enzyme and the mean IC_{50} value for each extract. Quercetin, a naturally occurring flavonoid, was used as the positive control, as it is a known inhibitor of the COX/LOX isoenzymes (Li et al., 2016).

The results of the anti-inflammatory assay are displayed in **Table 3.1**. According to Lawal et al., (2019), a plant extract with an IC_{50} value lower than 83.00 µg/mL is considered to be a good inhibitor of 15-LOX. The absolute methanol extract showed significant inhibitory activity and had a low IC_{50} value of 18.33 ± 0.70 µg/mL, which was comparable to that of the positive control which displayed an IC_{50} value of 15.13 ± 0.19 µg/mL. Significant activity was also observed for the 50% methanol extract and the decoction, with IC_{50} values of 23.82 ± 0.90 and 25.58 ± 0.41 µg/mL respectively. The dichloromethane extract showed poor activity, with an IC_{50} value greater than 83.00 µg/mL.

The anti-inflammatory activity of *F. abutilifolia* has been reported in a previous study, where an acetone extract prepared using the stem bark moderately inhibited 15-LOX, at 41.70 µg/mL (Lawal et al., 2019). IC_{50} values, however, could not be obtained during the study, as the extract showed strong absorptivity at the highest tested concentrations (Lawal et al., 2019). In the current study, IC_{50} values were obtained by expanding the range of test concentrations used by Lawal et al., (2019), to include much lower test concentrations. The extracts were also prepared with methanol and distilled water, instead of acetone, therefore the differences in results could also be attributed to differences in the phytochemical profiles of the extracts.

The inhibitory effects of the polar extracts against 15-LOX can be attributed to the presence of phytochemicals such as phenolic compounds and flavonoids, which are both associated with anti-inflammatory activity (Pendota et al., 2017). Flavonoids, for example, are known to alleviate inflammation by targeting the different stages of the COX/LOX pathway (Elisha et al., 2016). In addition to these compounds, β-sitosterol and triterpenes such as lupeol acetate, α-amyrin acetate and β-amyrin acetate have also been detected in the stem bark of *F. abutilifolia* (Aliba et al., 2018; Djankou et al., 2020). According to several studies, all four of these phytochemicals possess anti-inflammatory properties (Babu and Jayaraman, 2020; Holanda Pinto et al., 2008; Lucetti et al., 2010)

Lastly, the differences in activity between the dichloromethane extract and the polar extracts could be due to differences in the phytochemical profiles of these extracts as dichloromethane is a non-polar solvent. The results obtained in this study indicate that polar extracts produced from the stem bark of *F. abutilifolia* have significant activity against 15-LOX, thus indicating their anti-inflammatory potential and validating the traditional usage of this plant for the treatment and management of inflammatory diseases.

3.3.4 Conclusion

The anti-gonococcal and anti-inflammatory activity of *F. abutilifolia* was confirmed through the results obtained from this study. The polar extracts (absolute methanol, 50% methanol, and water) displayed significant activity against *N. gonorrhoea* and 15-LOX, whilst the dichloromethane extract did not display significant activity.

This study provides a scientific rationale for the traditional use of *F. abutilifolia* in the treatment and management of gonorrhoea and associated inflammatory diseases. The results from this study also provide a lead for potential compounds

that could be incorporated into current treatment plans or used to develop new and effective drugs against gonorrhoea and inflammation. As a follow-up to this study, the absolute methanol and 50% methanol (methanol: water, 1:1, v/v) crude extracts were subjected to bioassay-guided fractionation, to distinguish between active and non-active fractions through metabolomics analysis using proton nuclear magnetic resonance (^{1}H-NMR), followed by tentative identification of the classes of compounds present in these fractions, through gas chromatography (GC) coupled to time-of-flight mass spectrometry (ToF-MS).

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CHAPTER 4

Bioassay-Guided Fractionation and Metabolomics Analysis of the Anti-Gonococcal and Anti-Inflammatory Constituents of *Ficus abutilifolia* (Miq.) Miq.

4.1 Introduction

For many centuries, medicinal plants have been used to cure and manage life-threatening conditions (Mbuni et al., 2020). These plants are used to produce herbal remedies that possess immense therapeutic potential (Naquvi et al., 2020). The success of these remedies has captured the attention of the pharmaceutical industry, as they contain bioactive metabolites that provide insight into alternative chemical entities with novel modes of action, that could be useful for modern drug development (Dawurung et al., 2021; Nthulane et al., 2020).

Ficus abutilifolia, more commonly known as the large-leaved rock fig, is an indigenous South African plant, with a wide range of ethnobotanical applications (Danmalam et al., 2012). In South Africa, for example, traditional healers use the stem bark to prepare a decoction, that is used as a treatment for inflammation, infertility and multiple sexually transmitted infections (STIs), including gonorrhoea (Chauke et al., 2015). In addition to STI treatment, in countries such as Nigeria and Cameroon, the plant is also used as a strengthening tonic for men, to remove skin warts and to treat a variety of conditions ranging from mild fever to more severe illnesses such as typhoid, epilepsy, jaundice, dysentery, malaria, hypertension and rheumatism (Aliba et al., 2018; Danmalam et al., 2012; Taiwo et al., 2016).

Several pharmacological studies have provided a scientific rationale for some of the ethnobotanical uses of *F. abutilifolia*. For example, investigations carried out on the root bark have indicated that the plant has anti-epileptic activity (Danmalam et al., 2012), whilst investigations carried out on the leaves have

shown that the plant has antimicrobial effects against a broad range of bacteria, including *Salmonella typhi*, the causative agent of typhoid (Taiwo et al., 2016). Studies have also shown that the stem bark has a high phenolic content, which contributes to the antioxidant potential of the plant (Lawal et al., 2019). Finally, investigations carried out in **Chapter 3**, confirmed that the stem bark possesses significant anti-gonococcal and anti-inflammatory activity, thus supporting the use of this plant in the treatment of gonorrhoea and associated inflammatory conditions.

As observed in **Chapter 3** and throughout literature, *F. abutilifolia* is a medicinally significant species with proven pharmacological activity. This activity is undoubtedly due to the presence of bioactive phytochemicals in the leaves, stem and root bark of the plant. Phytochemicals are complex secondary metabolites, that are synthesized by plants in response to adverse environmental conditions (Mahomoodally, 2013). These compounds are not necessary for survival, however, they may play a protective role against external stressors such as microorganisms (Fongang et al., 2021; Wink, 2018). These compounds also possess protective or curative properties and are effective against microorganisms that cause disease within human populations (Ahamad et al., 2020). Therefore, bioactive phytochemicals play a vital role in the pharmaceutical industry, where they are used to develop new drugs (Dawurung et al., 2021; Lee et al., 2020).

Once the biological activity of an extract has been established, the next step in the process would be to isolate and identify the phytochemicals responsible for the desired activity. Isolation, however, is a very challenging and arduous procedure, that is further complicated by the complex nature of these organic molecules (Sasidharan et al., 2011). Despite the complexity of these molecules, separation or purification can be achieved using various chromatographic techniques (Naquvi et al., 2020). For example, thin-layer (TLC) and column chromatography (CC), are solvent-based separation techniques that are often used

in conjunction, to isolate or purify bioactive phytochemicals (Naquvi et al., 2020; Sasidharan et al., 2011). Once the desired metabolites have been separated based on chemical similarities or purified, the next step in the process would be to determine the structure and identity of these metabolites. This can be achieved through the coupled application of metabolomics-based analytical techniques, such as nuclear magnetic resonance (NMR) spectroscopy and gas chromatography (GC) coupled to time-of-flight mass spectrometry (ToF-MS) (Tugizimana et al., 2013). Together, both of these high-throughput analytical techniques facilitate the rapid characterisation of bioactive phytochemicals.

Therefore, in this study, a bioassay-guided approach was used to fractionate crude extracts (absolute methanol and 50% methanol) obtained from the stem bark of *F. abutilifolia*, followed by the untargeted analysis of these fractions using ^{1}H-NMR-based metabolomics, to distinguish chemical differences between the active and non-active fractions. Subsequently, GC-ToF-MS analysis was also used to tentatively identify the classes of compounds present in the active fractions.

4.2 Materials and Methods

4.2.1 Collection of Plant Material

As previously discussed in **Chapter 3**, fresh plant material was collected from Venda in Limpopo, South Africa, and identified at the H. G. W. J. Schweickerdt Herbarium at the University of Pretoria (voucher number: TE0211).

4.2.2 Extraction

The fresh plant material was cleaned, dried and ground into a fine powder, using a Janke and Kunkel grinder (IKA Labortechnik, Germany), before sequential extraction with dichloromethane, absolute methanol and 50% methanol (methanol: water, 1:1, v/v). A decoction was also prepared by boiling the dried plant material in water, to mimic the preparation method followed by traditional healers (Fongang et al., 2021; Sasidharan et al., 2011).

4.2.2.1 Sequential Extraction with Organic Solvents

Dried plant material (700 g) was first extracted in 2 L of dichloromethane with continuous agitation (Labcon, South Africa) for 3 hours. The extract was filtered in a Buchner funnel (Buchi Vac V-500, South Africa), through Whatman No.3 filter paper. The retained filtrate was stored at 4°C, whilst the residue was extracted further using the same amount of fresh solvent, with continuous agitation for 3 hours, followed by an overnight repeat. The filtrates from each extraction were combined (6 L), and the extraction procedure was repeated with absolute methanol and then finally 50% methanol (methanol: water, 1:1, v/v). The three filtrates were concentrated using a Heidolph Hei-Vap Rotary Evaporator (Labotech, South Africa), and the dichloromethane and absolute methanol extracts were dried in a laminar flow (VividAir, South Africa). The remaining water in the 50% methanol extract was removed using a VirTis Manuel freeze dryer (United Scientific, South Africa).

4.2.2.2 Water Extraction (Decoction)

The decoction was prepared by boiling 300g of the dry plant material in 3 L of distilled water for 30 minutes. The extract was cooled and filtered through a Buchner funnel (Buchi Vac V-500, South Africa) using Whatman No.3 filter paper, and the filtrate was frozen overnight at -80°C and dried in a VirTis Manuel freeze dryer (United Scientific, South Africa).

Following extraction, the crude extracts were subjected to preliminary *in vitro* screening against *Neisseria gonorrhoea* and 15-lipoxygenase (15-LOX). Based on bioactivity, the absolute methanol (MIC=0.20 mg/mL; IC_{50} 18.33 ± 0.70 µg/mL) and 50% methanol (MIC=0.20 mg/mL; IC_{50} 23.82 ± 0.90 µg/mL) extracts were then selected for fractionation, as outlined in **Figure 4.1.**

4.2.3 Determination of Bioactivity

4.2.3.1 Anti-Gonococcal Screening

The microdilution assay was used to determine minimum inhibitory concentration (MIC) values for the extracts, as outlined in **Chapter 3**. Briefly, stock solutions (10 mg/mL) were prepared by dissolving each extract in 10% dimethyl sulfoxide (DMSO). Sterile Mueller-Hinton (MH) broth (100 µL) was loaded into a microtiter plate, followed by 100 µL of extract, in triplicate. The extracts were serially diluted down the microtiter plate, starting with a concentration of 2.50 mg/mL, and ending with 0.02 mg/mL. Finally, the wells were inoculated with 100 µL of the bacterial solution and incubated under anaerobic conditions (37°C for 24 hours) with CO_2 supplementation. Ciprofloxacin (0.2 mg/mL) was included as the positive control, whilst the negative controls included a solution of sterile MH broth and 10% DMSO. Bacterial growth was determined using PrestoBlue® cell viability reagent (Kowalska-Krochmal and Dudek-Wicher, 2021).

4.2.3.2 Anti-inflammatory Activity

Extracts were screened for inhibitory effects against 15-LOX, as previously outlined in **Chapter 3**. Briefly, this was achieved by monitoring the effect of each extract on product formation, using a DU® 720 General purpose UV/Vis Spectrophotometer (Labotec, South Africa) at a wavelength of 234 nm. Both enzyme and substrate solutions were prepared using a 0.2M borate buffer solution (pH 9.00). Stock solutions were prepared for serial dilution, by dissolving 10 mg of extract in 1 mL DMSO. Experimental samples were analysed in triplicate, and absorbance values were recorded at 30-second intervals, over 5 minutes, for each sample. Quercetin was included as the positive control, whilst the negative control included a solution of DMSO (Li et al., 2016; Lyckander and Malterud, 1996). The experimental procedure was repeated 3 times for each concentration of extract, and the inhibitory activity was determined by calculating enzyme inhibition (%) from the changes in absorbance values over 5 minutes.

4.2.4 Bioassay-Guided Fractionation of the Stem Bark of *Ficus abutilifolia* (Miq.) Miq.

4.2.4.1 Fractionation of the Absolute Methanol Crude Stem Bark Extracts

The absolute methanol crude extract was selected for silica gel chromatography, based on bioactivity against *N. gonorrhoea* and 15-LOX. The column was packed with a slurry of silica gel prepared in absolute chloroform. The extract (20 g) was reconstituted in methanol and loaded into the column, which was eluted in increments of 1000 mL of solvent, starting with absolute chloroform, followed by a mixture of chloroform and ethyl acetate (9.5:0.5). The polarity of the solvent system was gradually increased by reducing the chloroform content and increasing the ethyl acetate content by 5% until 100% ethyl acetate was reached. At this point, methanol was introduced and the polarity of each solvent system

increased by 10% until 100% methanol was reached. Thereafter, the solvent system was changed to a mixture of methanol and distilled water (5:5), followed by pure distilled water. The collected fractions were subjected to thin-layer chromatography (TLC), and fractions with similar chemical profiles were pooled together, resulting in a total of 12 fractions (A1-A12).

4.2.4.2 Fractionation of the Crude 50% Methanol Stem Bark Extract

The crude 50% methanol (methanol: water, 1:1, v/v) extract was also selected for silica gel chromatography, based on desirable bioactivity. The extract (20 g) was reconstituted in methanol, loaded into the column and eluted in increments of 1000 mL, starting with absolute chloroform, followed by a mixture of chloroform, ethyl acetate and methanol (7:2:1). The polarity of the solvent system was gradually increased until a ratio of (1:5:4) was reached. At this point, the solvent system was changed to a mixture of ethyl acetate, methanol and distilled water (7:2:1). Once again, the polarity of the solvent system was gradually increased until a ratio of (1:5:4) was attained. Thereafter, a mixture of methanol and distilled water was introduced in a ratio of (6:4), followed by (5:5), and then finally distilled water. The collected fractions were subjected to thin-layer chromatography, and fractions with similar chemical profiles were pooled together, to obtain a total of 12 fractions (B1-B12).

4.2.4.3 Fractionation of B4-B7 on Silica Gel

The 24 fractions that were obtained from the absolute methanol and 50% methanol extracts, were subjected to thin-layer chromatography and further screening against *N. gonorrhoea* and 15-LOX. Following this assessment, fractions with similar chemical profiles and significant bioactivity (B4-B7) were recombined (5 g), and further chromatographed on silica gel starting with absolute chloroform (500 mL), followed by a mixture of chloroform, ethyl acetate and methanol (7:2:1). The polarity of the solvent system was gradually increased

by reducing the chloroform content and increasing the ethyl acetate and methanol content by 5% until a ratio of (1:5:4) was attained. Thereafter, the solvent system was changed to a mixture of ethyl acetate, methanol and distilled water (7:2:1). Once again, the polarity of the solvent system was gradually increased until a ratio of (1:5:4) was reached, then a mixture of methanol and distilled water was introduced in a ratio of (6:4), followed by (5:5), and then finally distilled water. Once again, the collected fractions were analysed using thin-layer chromatography, and those with similar chemical profiles were recombined to produce 17 sub-fractions (C1-C17).

4.2.4.4 Fractionation of D3 and E3 on Sephadex LH-20

The recovered sub-fractions (C1-C17) were subjected to thin-layer chromatography, followed by further screening against *N. gonorrhoea*. Due to the limited amount of plant material, sub-fractions with similar chemical profiles and MIC values were recombined, resulting in a total of 6 sub-fractions (D1-D6). Sub-fraction D3 (0.25 g) was selected for re-chromatography on Sephadex LH-20 (Sigma, USA), which had been conditioned with absolute methanol. The column was eluted in increments of 200 mL, starting with a mixture of methanol and chloroform (9:1), followed by (8:2), (5:5), (2:8) and finally 100% chloroform. Once again, the collected sub-fractions were analysed using thin-layer chromatography and pooled together based on chemical similarities (E1-E7).

Finally, E3 (0.11g) was selected for fractionation on Sephadex LH-20, which had been previously conditioned with chloroform. The column was eluted in increments of 200 mL, starting with a mixture of chloroform and methanol (9:1). The polarity of the solvent system was gradually increased until a ratio of (2:8) was reached. Collected fractions were analysed through thin layer chromatography, resulting in a total of 8 sub-fractions (F1-F8).

A logical schematic representation of the fractionation procedure followed is shown in **Figure 4.1.**

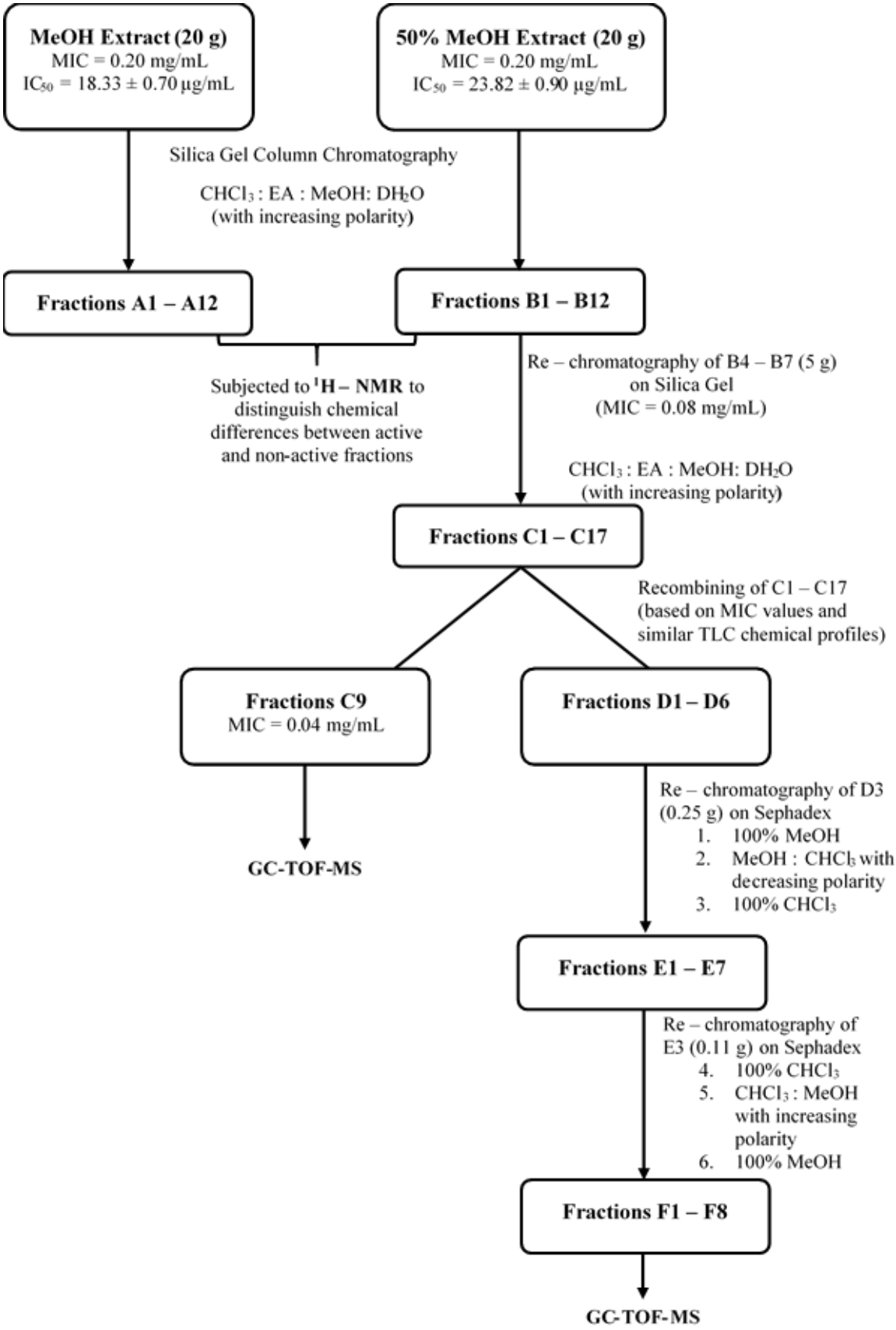


Figure 4.1. A logical schematic representation of the procedure used to fractionate *F. abutilifolia*.

4.2.5 ^{1}H-NMR Analysis of the Crude Methanol and 50% Methanol Fractions

Following silica gel chromatography, fractions A1-A12 and B1-B12 were subjected to ^{1}H-NMR spectroscopy, to distinguish chemical differences between the active and non-active fractions. Samples (5 mg/mL) were prepared for analysis by dissolving plant material in deuterated DMSO (DMSO-d6), with tetramethylsilane (TMS) as the internal standard. Each sample was then transferred into a 5 mm NMR tube and analysed at 400 MHz, using a Bruker AVIII-400 instrument (Bruker, Rheinstetten, Germany) at a temperature of 30°C (64 scans), with assistance by Dr Mamoalosi Selepe (Chemistry Department, University of Pretoria) (Aro et al., 2019).

4.2.6 GC-ToF-MS Analysis of Active Fractions

Based on TLC analysis, sub-fractions C9 (MIC 0.04 mg/mL) and F1-F8 were subjected to further analysis using GC-ToF-MS, to identify potential compounds or classes of compounds responsible for bioactivity. Samples were dissolved in methanol to a final concentration of 1 mg/mL and transferred into glass vials for analysis. Analysis was conducted using a LECO Pegasus® 4D GCxToF-MS instrument (LECO Africa (Pty) Ltd., Kempton Park, South Africa), with assistance by Dr Yvette Naudé (Chemistry Department, University of Pretoria) (Wooding et al., 2020). Each sample (1 µL) was injected into the splitless mode with the injector temperature set at 250 °C. The GC column used consisted of Rxi-1 MS, 30 m x 0.25 mm ID x 0.25 µm film thickness (Restek, Bellefonte, PA, USA). The oven temperature was programmed to 40 °C for 3 min and increased at 8 °C min^{-1} to a final temperature of 300 °C and held for 5 min. UHP helium (Afrox, South Africa) was used as a carrier gas at a constant flow rate of 1 mL min^{-1}. The ion source temperature was set to 230 °C, with an electron energy of 70 eV in the electron ionisation mode (EI+). Spectral data was acquired at a rate

of 10 spectra s^{-1}, with the detector voltage set at 1750 V. Tentative identification of compounds was achieved based on a comparison of sample mass spectra to that of the NIST14 library (version 2.2).

4.2.7 Multivariate Data Analysis

Following column chromatography, the 24 fractions obtained from the absolute methanol and 50% methanol crude extracts were subjected to ^{1}H-NMR spectroscopy. Out of the 24 fractions, 20 spectra (A1-A12, B1-B7 and B12) were selected for further processing and analysis using MestReNova software (Mestrelab Research S. L., Spain). To ensure that the signals remained symmetrical, the spectra were subjected to automatic phasing, followed by Whittaker smoother baseline correction. Next, the spectra were normalised to the DMSO-d6 peak (2.50 ppm) and referenced to the internal standard, TMS (0 ppm). Finally, the spectra were reduced into regions of equal bin width (0.02 ppm), and resonances associated with residual water were excluded from all spectra (3.30 – 3.40 ppm).

Following processing in MestReNova, the data set was exported to SIMCA 14.1 software (Umetrics, Umeå, Sweden), for multivariate data analysis. The data set was subjected to Pareto scaling and the extracts were assigned classes, according to their bioactivity. Extracts with MIC values less than or equal to 0.63 mg/mL were grouped as active, whilst extracts with MIC values greater than 0.63 mg/mL were grouped as non-active.

Multivariate data analysis was carried out using unsupervised principle model analysis, to identify outliers in the data set and to observe trends such as clustering or separations (Ali et al., 2012). This was followed by a supervised model (OPLS-DA), which was to explore the chemical differences between the active and non-active data sets, and to generate a contribution plot thereof (Ali et al., 2012).

4.3 Results and Discussion

4.3.1 Bioassay-Guided Fractionation of *F. abutilifolia*

In **Chapter 3**, crude extracts prepared from the stem bark of *F. abutilifolia* were subjected to *in vitro* preliminary screening for bioactivity. Extracts with significant anti-gonococcal and anti-inflammatory activity (the absolute methanol and 50% methanol crude extracts), were selected for bioassay-guided fractionation. These extracts were subjected to silica gel column chromatography, which resulted in a total of 24 fractions, that were further assessed for activity against gonorrhoea and 15-LOX. The results from this assessment are displayed in **Table 4.1.**

Table 4.1. The anti-gonococcal and anti-inflammatory activity of column fractions obtained from the crude stem bark extracts of *F. abutilifolia*.

Fractions	Anti-gonococcal activity MIC (mg/mL)		Anti-inflammatory activity IC_{50} ± SEM (µg/mL)	
	MeOH (A)	50% MeOH (B)	MeOH (A)	50% MeOH (B)
1	> 2.50	> 2.50	115.2 ± 0.04	> 166.70
2	> 2.50	0.63	98.62 ± 0.04	36.00 ± 0.05
3	> 2.50	0.63	97.59 ± 0.05	34.65 ± 0.06
4	> 2.50	**0.08**	32.62 ± 0.03	**9.15 ± 0.03**
5	0.32	**0.08**	8.61 ± 0.08	**8.43 ± 0.03**
6	0.32	**0.08**	8.80 ± 0.02	**5.26 ± 0.05**
7	0.63	**0.08**	30.34 ± 0.03	**8.68 ± 0.03**
8	0.63	0.16	16.41 ± 0.02	14.98 ± 0.04
9	1.25	2.50	84.45 ± 0.03	49.69 ± 0.10
10	2.50	2.50	71.84 ± 0.34	> 166.70
11	2.50	> 2.50	52.92 ± 0.12	> 166.70
12	> 2.50	> 2.50	> 166.70	64.79 ± 0.20
Crude extracts	0.08	0.08	18.33 ± 0.70	23.82 ± 0.90
Ciprofloxacin	< 0.01	< 0.01	-	-
Quercetin	-	-	15.13 ± 0.19	15.13 ± 0.19

MIC – minimum inhibitory concentration (n = 3)

IC_{50} – half maximal inhibitory concentration (n = 3)

MeOH (A) – fractions obtained from the absolute methanol crude extract

50% MeOH (B) – fractions obtained from the 50% methanol crude extract

>166.70; activity greater than highest tested concentration.

< 0.01; activity shown at all concentrations less than 0.01 mg/mL

*; means are statistically significant (P < 0.05)

Fractions B4-B7 displayed significant activity against *N. gonorrhoea*, with low MIC values of 0.08 mg/mL reported for each fraction. Low IC_{50} values ranging from 5.26 ± 0.05 to 9.15 ± 0.03 µg/mL were also obtained for these fractions, thus indicating an increase in bioactivity following fractionation. These fractions were recombined (5 g), as they showed similar chemical profiles, and re-chromatographed on silica gel, yielding a total of 17 sub-fractions (C1-C17),

which due to time constraints and for simplicity, were subjected to further testing against *N. gonorrhoea* only. The results from this assessment are displayed in **Table 4.2.**

Table 4.2. The anti-gonococcal activity of sub-fractions C1-C17.

Sub-fraction	Anti-gonococcal activity MIC (mg/mL)	
1	0.63	**D1**
2	0.63	
3	0.16	**D2**
4	0.16	
5	0.16	
6	0.08	**D3**
7	0.08	
8	0.08	
9	**0.04**	
10	0.08	**D4**
11	0.08	
12	0.08	
13	0.16	**D5**
14	0.08	
15	0.08	
16	0.31	**D6**
17	> 2.50	
Parent extract (B4-B7)	0.08	
Crude extract	0.08	
Ciprofloxacin	< 0.01	

MIC – minimum inhibitory concentration (n = 3)

< 0.01; activity shown at all concentrations less than 0.01 mg/mL

Sub-fraction C9 displayed significant anti-gonococcal activity with a low MIC value of 0.04 mg/mL. This value was significantly lower than the MIC value obtained for the crude extract (0.08 mg/mL), thus indicating an increase in bioactivity following fractionation. Sub-fractions C6, C7, C8, C10, C11, C12, C14 and C15 also showed considerable activity, with a low MIC value of 0.08 mg/mL reported for each sub-fraction.

The 17 sub-fractions were further analysed through thin-layer chromatography. Due to the limited amount of plant material available, sub-fractions with similar chemical profiles and MIC values were recombined to obtain D1-D6, to facilitate further separation through gel filtration chromatography. Based on TLC analysis, sub-fraction D3 (0.25 g) was prioritised for gel filtration on Sephadex LH-20, which resulted in 7 sub-fractions (E1-E7). Following TLC analysis, sub-fraction E3 was selected for re-chromatography on Sephadex LH-20, which resulted in another 8 sub-fractions (F1-F8). Due the limited amount of plant material available, sub-fractions E1-E7 could not be tested for bioactivity, however, sub-fractions C9 (MIC 0.04 mg/ml) and F1-F8 were subjected to GC-ToF-MS analysis, to identify the potential compounds or classes of compounds present in these samples.

4.3.2 ^{1}H-NMR-based Metabolomics

Crude extracts from the stem bark of *F. abutilifolia* were screened for bioactivity against *N. gonorrhoea* and 15-LOX, as reported in **Chapter 3**. Following this preliminary assessment, two of these extracts (absolute methanol and 50% methanol) were selected for bioassay-guided fractionation, which yielded a total of 24 fractions. These fractions were further assessed for bioactivity (**Table 4.1**) and eventually subjected to ^{1}H-NMR spectroscopy. Out of the 24 fractions, only 20 (A1-A12, B1-B7 and B12) were selected for ^{1}H-NMR-based metabolomics

analysis. Fractions B8-B11 were excluded from analysis, as the internal standard (TMS) could not be detected in these samples. TMS is an extremely volatile solvent, therefore it is likely that it evaporated from these samples before the spectra were recorded (Becker, 2000).

Spectral data obtained from ^{1}H-NMR spectroscopy were saved and imported to MestReNova (Mestrelab Research S. L., Spain), where spectra were processed and assigned classes based on anti-gonococcal activity for simplicity, as there was no clear correlation between fractions with significant anti-gonococcal and anti-inflammatory activity. Ten of the fractions were grouped as active (MIC ≤ 0.63 mg/mL), whilst the other ten were grouped as non-active (MIC > 0.63 mg/mL). Visual inspection of the spectra generated for the plant extracts indicated that the active fractions had chemical profiles that differed significantly from that of the non-active fractions. Prominent signals were detected especially in the aromatic region (6.00 – 9.50 ppm) for the active fractions (**Figure 4.2**), whilst fewer to no signals were detected for some of the non-active fractions in the specified region (**Figure 4.3**).

To reduce the dimensionality of the data set and to enable visual exploration of the trends between samples, the spectral data was subjected to multivariate analysis (Ali et al., 2012). An unsupervised principle component analysis (PCA) model was generated from the spectral data, resulting in the PCA score plot shown in **Figure 4.4**. The active fractions separated from the non-active fractions, thus indicating chemical variability between these fractions. Furthermore, clustering was also observed between the active fractions, suggesting that large proportion of chemical similarities exist between these fractions. Separation along PC1 and PC2 was also observed between some of the non-active fractions, suggesting that some of these fractions contained a few metabolites with similar functional groups to those found in the active fractions. Permutation testing was carried out to determine the goodness of fit (R^2) and the predictability (Q^2) of the

PCA model (Ali et al., 2012). The R^2 and Q^2 values were 0.866 and 0.701 respectively, thus indicating that 87% of the variation observed in the data set could be explained by the model, with a high predictability of 70%.

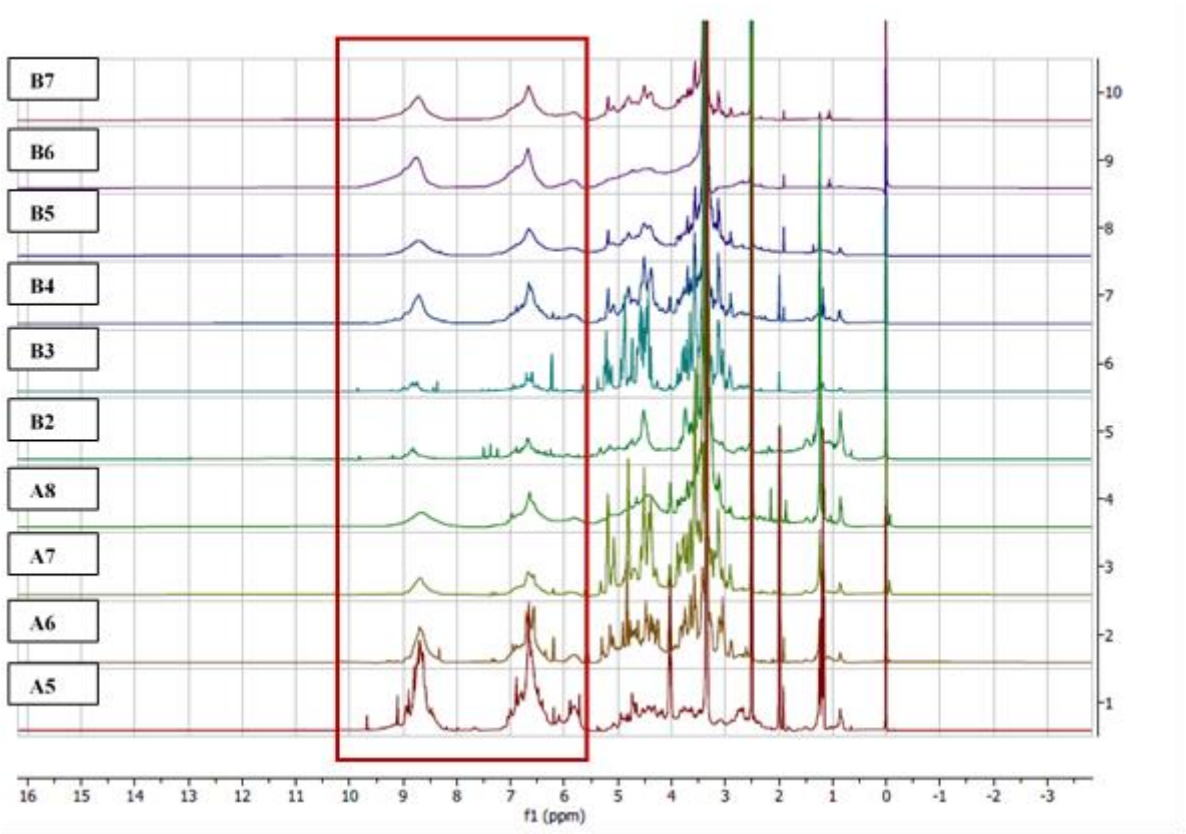


Figure 4.2. Stacked ^{1}H-NMR spectra of the active fractions.

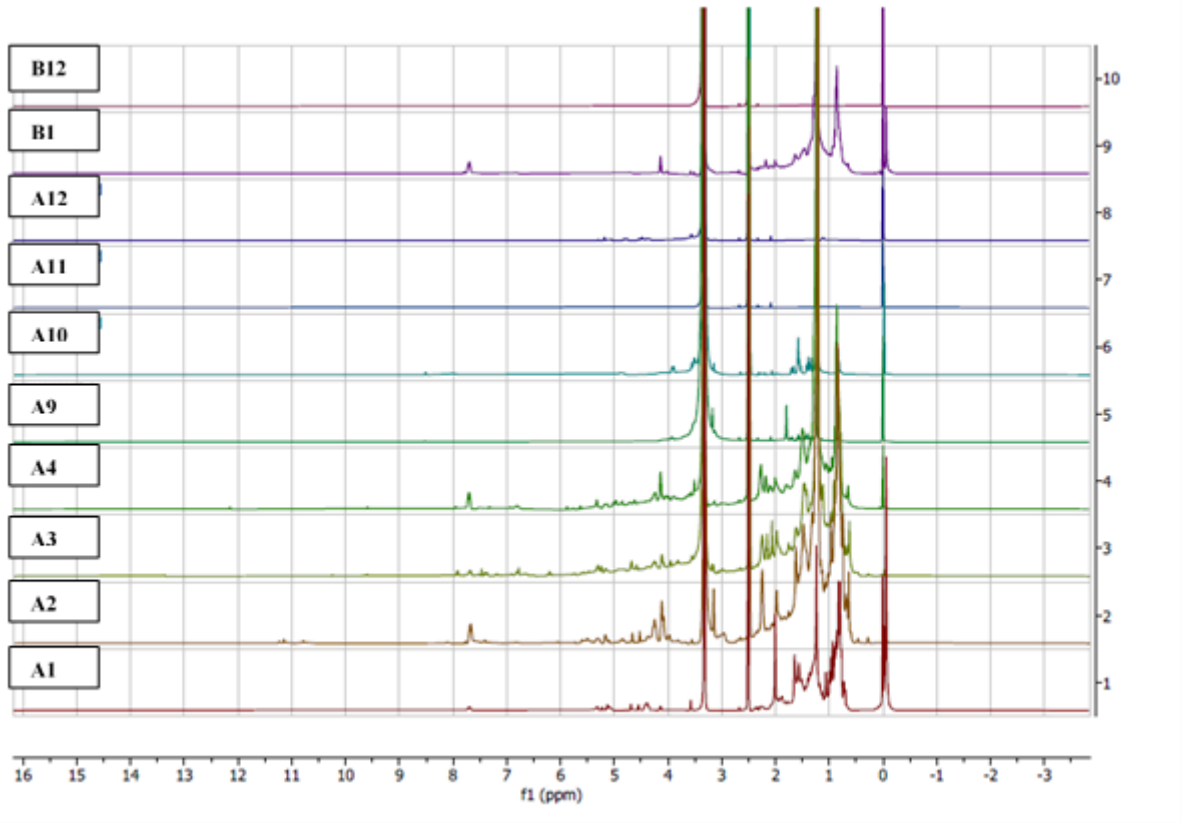


Figure 4.3. Stacked ^{1}H-NMR spectra of the non-active fractions.

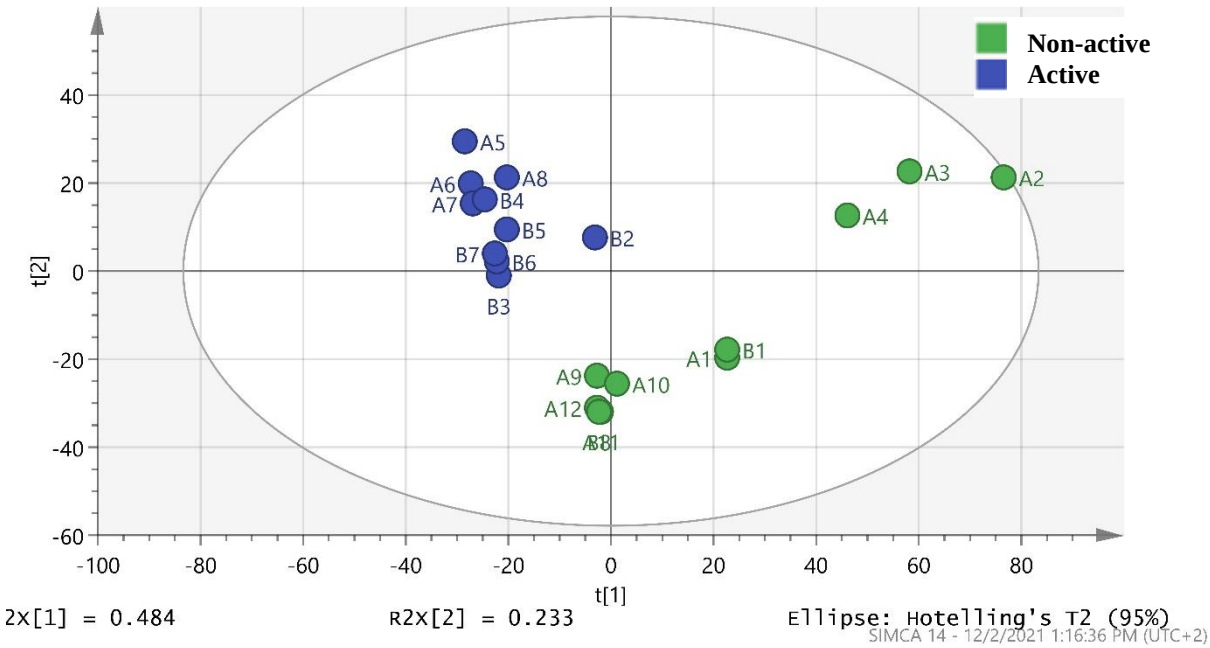


Figure 4.4. A principal component analysis (PCA) score plot generated from ^{1}H-NMR spectra of active (blue) and non-active (green) stem bark extracts of *F. abutilifolia*.

Next, a supervised model was applied to determine the correlation between the chemical composition and the bioactivity of the fractions. This was achieved by assigning classes to the fractions (class 1: active versus class 2: non-active), before subjecting the spectral data to orthogonal partial least squares-discriminant analysis (OPLS-DA). The model separated the components based on their chemical profiles and bioactivity, resulting in a score scatter plot that showed clear separation between the active and non-active fractions (**Figure 4.5.**), suggesting that there is a significant difference in the chemical composition of these groups of fractions (active versus non-active). Once again, clustering was also observed amongst the active fractions, suggesting that these fractions contain similar phytochemicals that contribute towards bioactivity. Permutation testing produced high R^2 (0.713) and Q^2 (0.914) values that validated the OPLS-DA model.

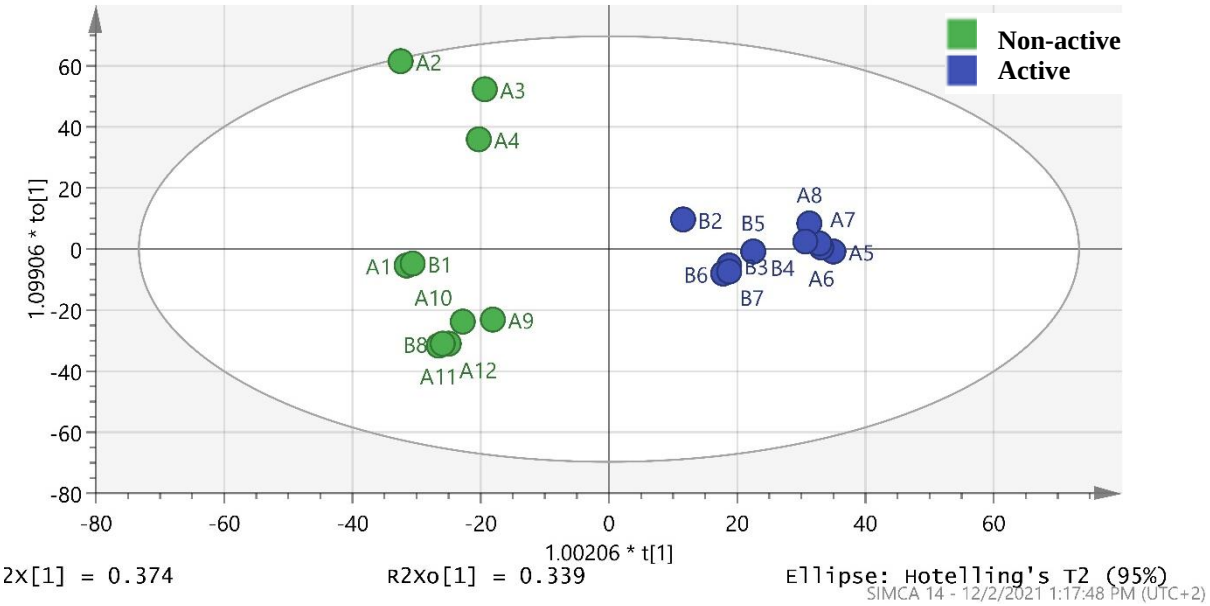


Figure 4.5. An orthogonal partial least squares-discriminant analysis (OPLS-DA) score plot generated from ^{1}H-NMR spectra of active (blue) and non-active (green) stem bark extracts of *F. abutilifolia*.

Data from the OPLS-DA score plot was used to generate a contribution plot, which was used to determine the regions in the ^{1}H-NMR spectra responsible for the separation of the active and non-active fractions (**Figure 4.6**). The major bins representing the active fractions were regions between 2.50 – 5.39 ppm, 5.60 – 7.30 ppm and 8.10 – 9.40 ppm. These characteristic regions are often assigned to alcohols, vinylic, aromatic or phenolic compounds and amides. According to literature, a high total phenolic content (TPC) has been detected in acetone and methanol extracts prepared from the stem bark of *F. abutilifolia*, thus suggesting that bioactivity could possibly be linked to the presence of phenolic compounds (Aliba et al., 2018; Djankou et al., 2020; Lawal et al., 2019). Characteristic regions corresponding with alkyl, allylic, and benzylic (0.90 – 2.3 ppm) were observed in the non-active fractions, but were mostly absent for the active fractions. Minor peaks were also observed in the aromatic region for some of the non-active extracts (**Figure 4.6**).

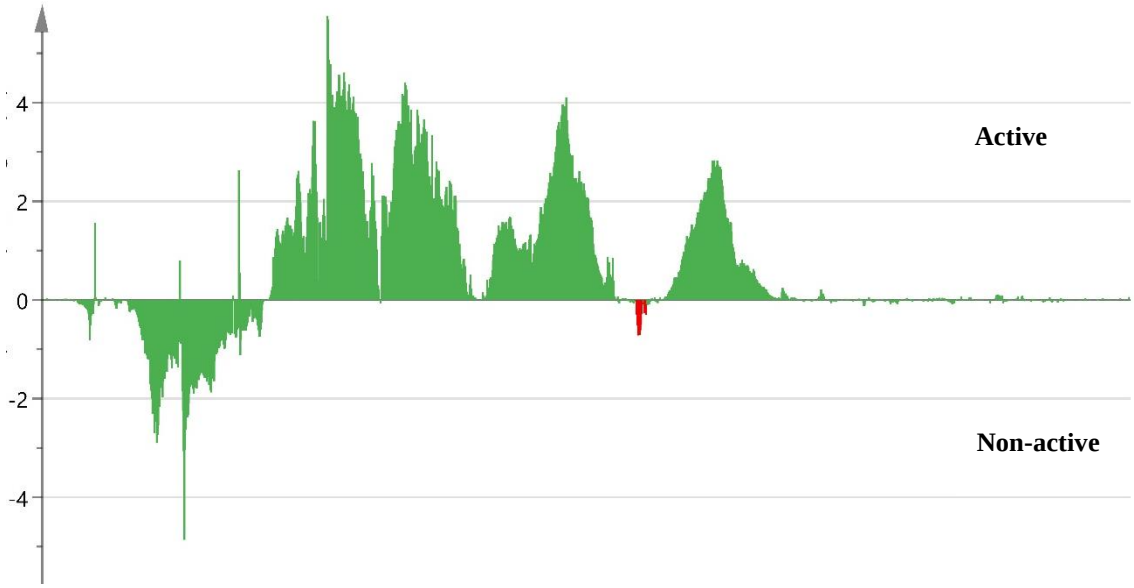


Figure 4.6. A contribution plot showing major regions in ^{1}H-NMR spectra bins responsible for class separation in the OPLS-DA score plot.
Bars projecting upwards represent active fractions, whilst bars projecting downwards represent non-active fractions. The highlighted (red) spectral region indicates minor peaks in the aromatic region of the non-active fractions.

4.3.3 GC-ToF-MS Analysis

To obtain a detailed profile of the secondary metabolites present in fractions C9 and F1-F8, the extracts were subjected to gas chromatography to time-of-flight mass spectrometric (GC-ToF-MS) analysis. This high-throughput analytical technique can be used to compare sample mass spectra to published spectral data retained in libraries such as NIST14, thus facilitating the tentative identification and quantitation of volatile secondary metabolites (Felipe et al., 2018; Kännaste et al., 2014). Area matches greater than or equal to 70% are often used to indicate the presence of a specific compound, however, it is also important to note that the identity of compounds obtained solely from mass spectral libraries are not always accurate, and should be subjected to further confirmation (Valdez et al., 2018; Wei et al., 2014).

Results obtained from GC-ToF-MS indicated that the fractions could possibly contain up to 160 volatile metabolites, however, none of the peaks yielded satisfactory mass spectral similarity to the compounds present in the library (**Appendix B**), thus the presence of these metabolites could not be confidently concluded. This could be due to the fact that the fractions were too polar for analysis using GC-ToF-MS, therefore further testing using alternative techniques such as liquid chromatography-mass spectrometry (LC-MS) may also be required.

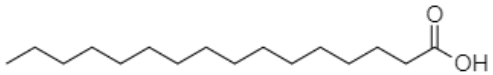
Interestingly, although mass spectral similarity was low, metabolites such as tetradecane, hexadecane and di-iso-octyl phthalate could be present in C9, which had a significantly low MIC value of 0.04 mg/mL (**Table 4.2**). These metabolites have been associated with broad antimicrobial activity (Banakar and Jayaraj, 2018; Khan and Javaid, 2019; Kumari et al., 2019), and have been detected in several *Ficus* species, including *F. religiosa* and *F. capensis* (Adeyemi et al., 2022; Nawaz et al., 2020). Several other metabolites with antimicrobial, anti-inflammatory or antioxidant properties may also be present in fractions F1-F8.

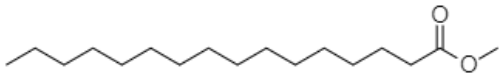
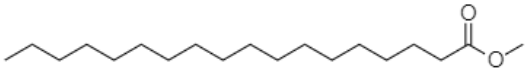
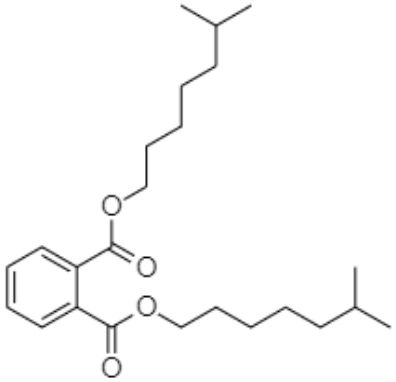
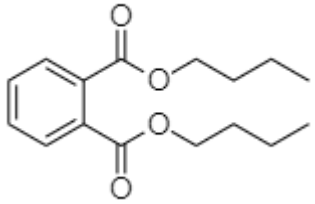
These metabolites include nonadecane, heneicosane, 1-nonadecene, palmitic acid, methyl palmitate, methyl stearate, dibutyl phthalate and 1,2-benzene dicarboxylic acid, bis(2-methyl propyl) ester, which have also been reported in several other *Ficus* species (Adeyemi et al., 2022; Nawaz et al., 2020). The biological properties associated with these metabolites are reported in **Table 4.3.**

Although the mass spectral similarities were low, to obtain a basic overview of the classes of compounds present in each fraction, the metabolites were grouped into their respective organic classes. As reported in **Table 4.4**, a total of 17 major classes were observed. A high proportion of these compounds included hydrocarbons, fatty acid esters and phthalate esters, which were detected in all 9 samples. These compounds, especially fatty acids and their esters, are associated with either anti-gonococcal, anti-inflammatory or broad antimicrobial properties (Agoramoorthy et al., 2007; Huang et al., 2010, 2021). An extremely high proportion of organosilicons, especially siloxanes, were also detected in all of the samples. Organosilicons, however, do not occur naturally in plants, therefore, their presence can be attributed to contamination that occurred during analysis, caused by septum bleed or methyl silicon column bleed (McMaster, 2008; Rood, 2004).

The detailed results and the spectra from the in-depth analysis of the 9 extracts are presented in **Appendix B**.

Table 4.3. The biological activity and structure of selected volatile secondary metabolites obtained from the stem bark of *F. abutilifolia* (Miq.) through GC-ToF-MS.

Secondary Metabolite	**Molecular Formula**	**Molecular Weight (g/mol)**	**Biological Activity**	**Structure of Metabolite**	**Reference**
Tetradecane	$C_{14}H_{30}$	198	Antioxidant; Antimicrobial; Anti-fungal		(Khan and Javaid, 2019); (Kavitha and Uduman, 2017); (Banakar and Jayaraj, 2018)
Hexadecane	$C_{16}H_{34}$	226	Antioxidant; Antimicrobial; Antifungal		(Banakar and Jayaraj, 2018)
Nonadecane	$C_{19}H_{40}$	268	Antioxidant Antimicrobial		(Kumari et al., 2019); (Banakar and Jayaraj, 2018)
Heneicosane	$C_{21}H_{44}$	296	Antimicrobial		(Vanitha et al., 2020)
1-Nonadecene	$C_{19}H_{38}$	266	Antimicrobial		(Khan and Javaid, 2019)
Palmitic acid	$C_{16}H_{32}O_2$	256	Antimicrobial; Anti-inflammatory		(Yff et al., 2002); (Lee et al., 2010); (Huang et al., 2010)

Methyl palmitate	$C_{17}H_{34}O_2$	270	Antimicrobial; Anti-inflammatory		(Agoramoorthy et al., 2007); (Saeed et al., 2012)
Methyl stearate	$C_{19}H_{38}O_2$	298	Antimicrobial		(Agoramoorthy et al., 2007)
Di-iso-octyl phthalate	$C_{24}H_{38}O_4$	390	Antimicrobial		(Kumari et al., 2019)
Dibutyl phthalate	$C_{16}H_{22}O_4$	278	Antimicrobial		(Khatiwora et al., 2013); (Shobi and Viswanathan, 2018); (Huang et al., 2021)

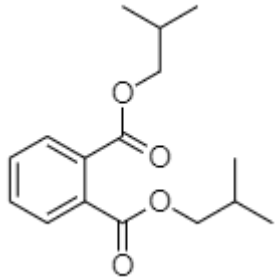
1,2-Benzene dicarboxylic acid, bis(2-methyl propyl) ester	$C_{16}H_{22}O_4$	278	Antioxidant; Antimicrobial		(Khan and Javaid, 2019); (Kumar et al., 2021)

Table 4.4. The proportion (%) of chemical compounds detected in stem bark extracts of *F. abutilifolia* through GC-ToF-MS.

Class of Compound	C9	F1	F2	F3	F4	F5	F6	F7	F8
Acetyl	-	-	-	-	0.1	0.1	0.2	0.2	-
Acid	0.6	-	0.9	0.7	0.4	-	0.7	1.1	-
Alcohol	4.6	-	1.9	-	2.4	-	-	0.4	0.5
Aldehyde	-	-	0.1	4.5	-	-	4.5	0.1	-
Alkaloid	-	0.5	0.2	0.1	-	0.5	0.1	0.1	-
Amide	-	-	4.0	37.4	0.3	2.2	37.4	0.3	-
Ester	2.0	6.4	5.4	5.6	5.1	10.2	5.6	0.5	4.0
Ether	-	-	0.8	0.2	-	-	0.2	-	-
Fatty Acid Ester	0.9	7.1	3.1	1.1	1.5	0.8	1.1	1.7	5.2
Furan	-	-	0.7	0.7	-	6.5	0.7	-	-
Hydrocarbon	58.0	78.2	52.5	41.2	43.1	48.1	41.0	71.0	72.3
Ketone	-	-	8.4	1.9	0.8	3.0	2.0	0.6	-
Organoiodide	-	-	-	-	-	-	-	0.4	-
Organosilicon	2.3	1.0	0.9	0.4	32.2	1.5	0.4	14.6	11.9
Phenolic	0.6	-	-	0.1	0.9	-	0.1	0.2	-
Phthalate Ester	2.8	-	1.7	0.3	1.6	14.2	0.3	3.6	3.2
Siloxane	28.3	6.8	18.5	5.9	10.0	12.7	5.9	5.6	3.0
Sugar	-	-	-	-	1.8	0.4	-	-	-
Sulphide	-	-	-	-	-	-	-	0.5	-

4.4 Conclusion

In this study, ^{1}H-NMR spectroscopy was used in conjunction with multivariate data analysis, to distinguish chemical differences between active (A5-A8; B2-B7) and non-active (A1-A4; A9-A12; B1-B2) fractions obtained from the crude stem bark extracts of *F. abutilifolia* (Moraceae). The computational PCA and OPLS-DA models both showed separation between these fractions, thus indicating that they differed significantly in their chemical profiles. Clustering observed between the active fractions in both models indicated that chemical similarities may exist between these fractions. This was supported through chemometrics analysis of the spectral data, which alluded to the presence of alcohols, amides, vinylic, aromatic and phenolic compounds in the active fractions.

For the tentative identification of compounds present in sub-fractions C9 and F1-F8, GC-ToF-MS analysis was used. Unfortunately, due to low mass spectral similarities caused by the polar nature of these fractions, the identity of the compounds present in these fractions could not be concluded with confidence. Therefore, further analysis using LC-MS, a technique more suited for polar samples, is required.

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CHAPTER 5

General Discussion, Conclusion and Recommendations for Future Studies

5.1 General Discussion

For centuries, medicinal plants have been used to produce remedies for the treatment of reproductive ailments, including sexually transmitted infections (STIs) (Mbuni et al., 2020; Mongalo et al., 2017). With rapidly increasing STI infection rates and antimicrobial resistance (AMR) on the rise, the search for novel therapeutic agents has become one of paramount importance (Nthulane et al., 2020). In response to this global dilemma, research has taken on a more natural approach, with a shift towards medicinal plants (Tariq et al., 2018). As a result, medicinal plants have become increasingly valuable to the pharmaceutical industry, as they are a primary source for novel drug leads, due to the chemically diverse nature of their secondary metabolites (Danmalam et al., 2017; Mahomoodally, 2013). However, despite the extensive use of medicinal plants throughout history, the biological efficacy and safety of the majority of these plants remains unknown (Seleteng-Kose et al., 2019). Therefore, it is essential for medicinal plants to undergo pharmacological evaluation, before they can be used to develop modern drugs (Ozioma and Chinwe, 2019).

The current study was conducted to gain insight into the anti-gonococcal and anti-inflammatory potential of crude extracts obtained from the stem bark of *Ficus abutilifolia*. The stem bark of this plant is used by traditional healers in the Venda region of Limpopo province, South Africa, to prepare decoctions for the treatment of gonorrhoea and inflammatory sequelae associated with this disease. Gonorrhoea is a serious public health issue, especially in South Africa, where annual infection rates exceed 2 million, and coinfection with HIV is very common (Kalichman et al., 2011; Kularatne et al., 2018; Mhlongo et al., 2010).

Pharmacological evaluation of this plant will therefore ensure that patients living in rural communities receive timely and effective treatment for this disease.

In this study, four crude extracts were prepared from the stem bark of *F. abutilifolia* and subjected to preliminary screening for bioactivity against *Neisseria gonorrhoea*, using the broth microdilution bioassay (**Chapter 3**). The polar extracts (absolute methanol, 50% methanol (methanol: water, 1:1, v/v), decoction) demonstrated moderate activity, with low MIC values of 0.20 mg/mL for each extract. Extracts were also screened for the ability to inhibit 15-lipoxygenase (15-LOX), an oxidative enzyme that plays a significant role in the inflammatory cascade, as discussed in **Chapter 2**. The polar extracts all demonstrated a dose-dependent response, with low IC_{50} values comparable to that of the positive control, quercetin (IC_{50}: 15.13 ± 0.19 µg/mL). Moderate anti-inflammatory activity has previously been reported for this plant (Lawal et al., 2019), however, this is the first report of activity against *N. gonorrhoea.*

Following the preliminary screening, the absolute methanol and 50% methanol (methanol: water, 1:1, v/v) extracts were subjected to bioassay-guided fractionation (**Chapter 4**). Silica gel chromatography produced a total of 24 fractions for both extracts. The extracts were subjected to ^{1}H-NMR spectroscopy, followed by multivariate data analysis. The PCA and OPLS-DA score plots both displayed clear separation between the active and non-active extracts, indicating that these extracts differed significantly in their chemical profiles. Grouping was also observed amongst the active extracts, indicating that these extracts had similar chemical profiles. The contribution plot and chemometrics data analysis detected prominent peaks associated with alcohols, amides, vinylic, aromatic and phenolic compounds for the active extracts, indicating that metabolites in these regions are probably responsible for the separation observed on the PCA and OPLS-DA score plots.

Further fractionation resulted in 9 fractions, that were all active against *N. gonorrhoea.* GC-ToF-MS analysis was used to tentatively identify the compounds present in these sub-fractions, however, due to low mass spectral similarities caused by the polar nature of these fractions, the identity of these compounds could not be established with certainty. Further research using other high through-put analytical techniques is therefore required.

5.2 Conclusion

Ficus abutilifolia is an important medicinal plant that is used by traditional healers in South Africa, to treat gonorrhoea and inflammatory conditions associated with this disease. The results obtained in this study therefore demonstrate that extracts prepared from the stem bark of this plant exhibit considerable activity against *N. gonorrhoea* and 15-LOX, thus providing clear scientific evidence to substantiate the traditional use of this plant. As this is the first report of anti-gonococcal activity in *F. abutilifolia*, these results may also help to discover new classes of compounds for the treatment of gonorrhoea and/or associated inflammatory conditions.

5.3 Recommendations for Future Studies

Considering that *F. abutilifolia* demonstrated significant activity against *N. gonorrhoea* and 15-LOX, it is also important to screen this plant for cytotoxic effects. Previously, Lawal et al (2019) reported that the plant was non-cytotoxic when tested against Vero cells (IC_{50} > 100 µg/mL), however, these results were for extracts that were prepared using acetone. Considering that phytochemical profiles differ considerably depending on the solvent used during extraction, it is therefore important to screen the extracts used in this study for cytotoxic effects, especially the decoction. This could also be followed by *in vivo* screening of the

plant extracts, to further assess the anti-gonococcal, anti-inflammatory and cytotoxic potential of the plant.

Bioassay-guided fractionation could also be used to dereplicate the anti-gonococcal and anti-inflammatory constituents of the plant, using high-throughput analytical techniques such as ^{13}C-NMR spectroscopy, high-performance liquid chromatography (HPLC) or ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLCQ-ToF-MS). These techniques could also assist in obtaining detailed chemical profiles, which could be used for synergistic studies.

Finally, targeted isolation or synthesis of the dereplicated active constituents could be used to further assess the bioactivity of the extracts, and to determine the mechanism of action for these constituents.

5.4 References

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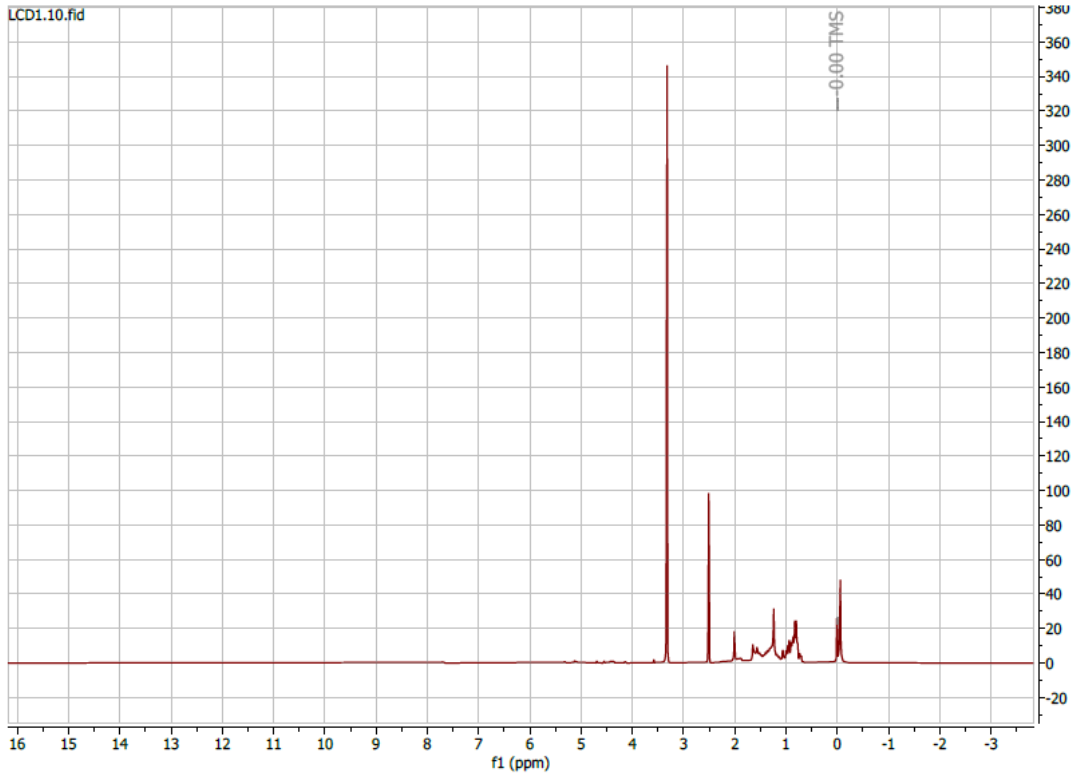
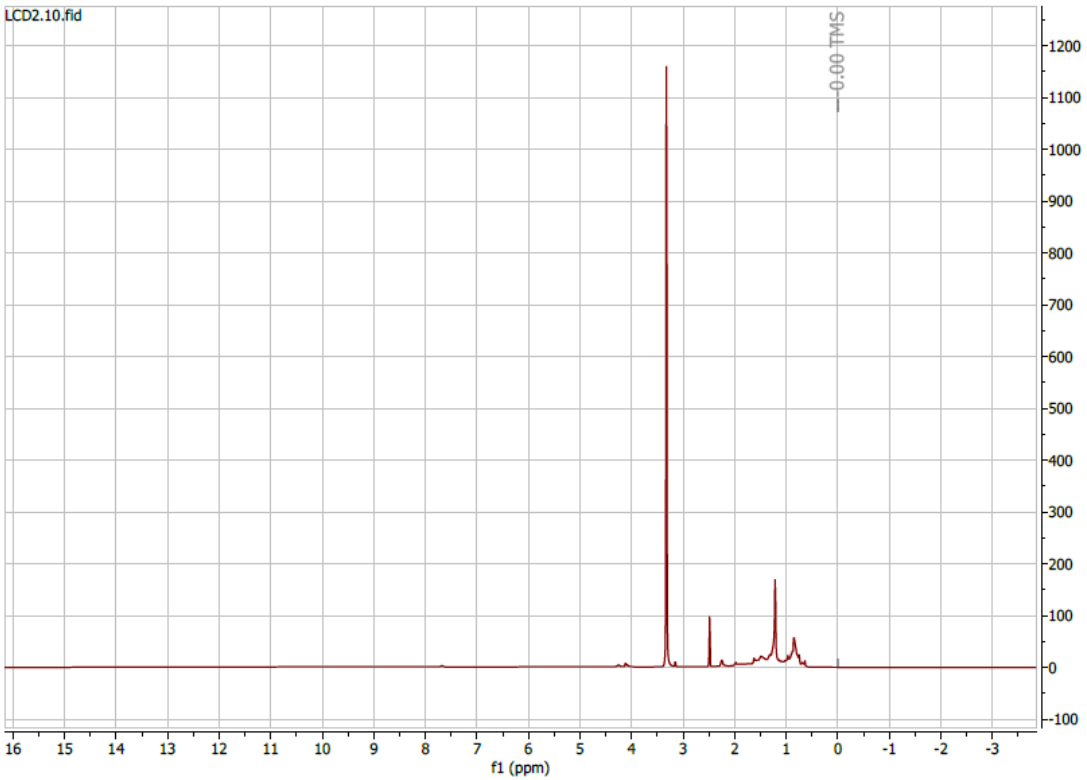
APPENDIX A

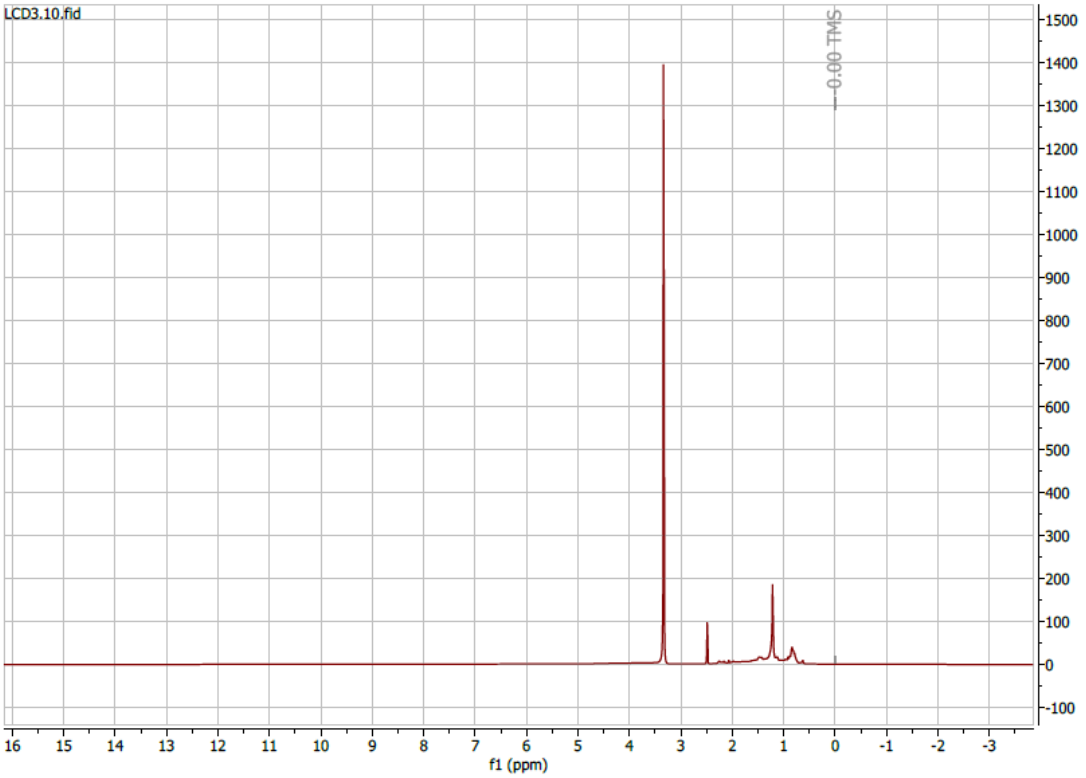
^{1}H-NMR spectra of fractions from *Ficus abutilifolia*.



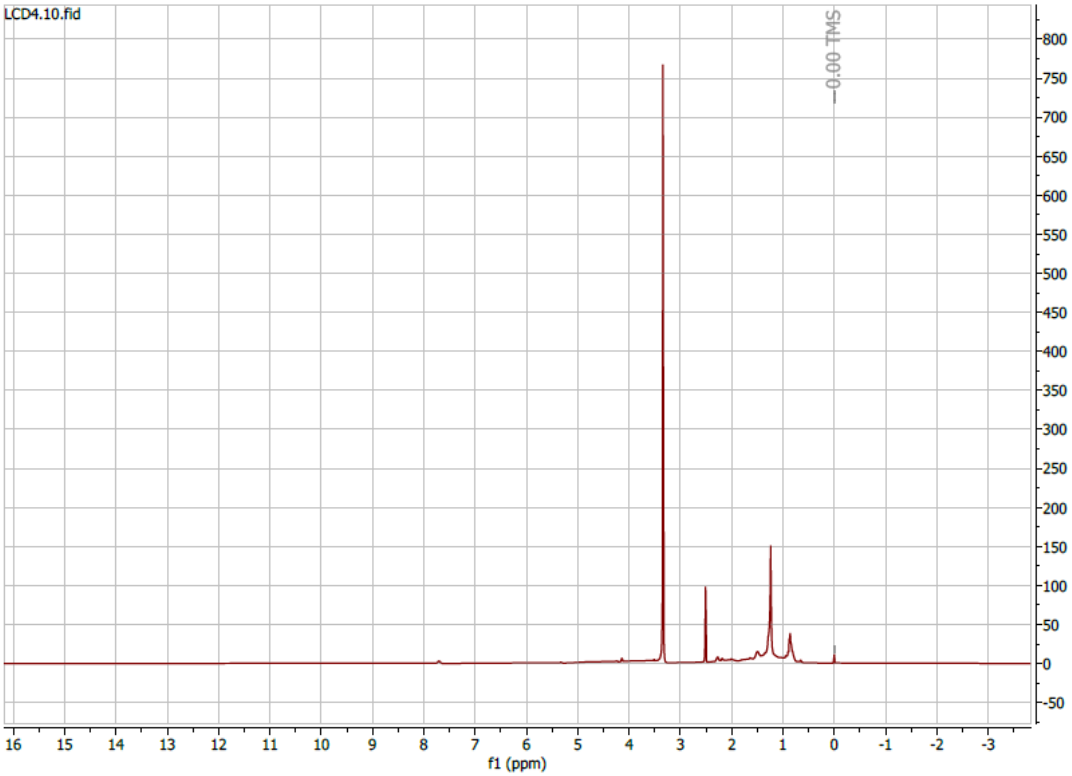
Appendix I: ^{1}H-NMR spectra of fraction A1.



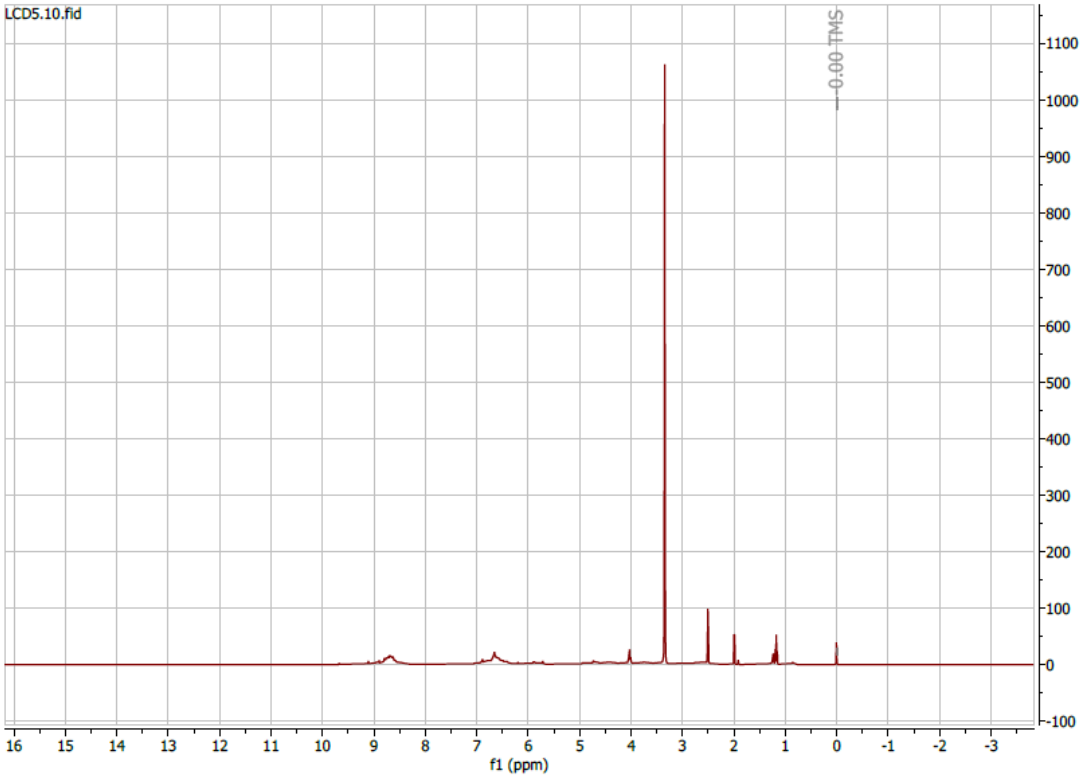
Appendix II: ^{1}H-NMR spectra of fraction A2.



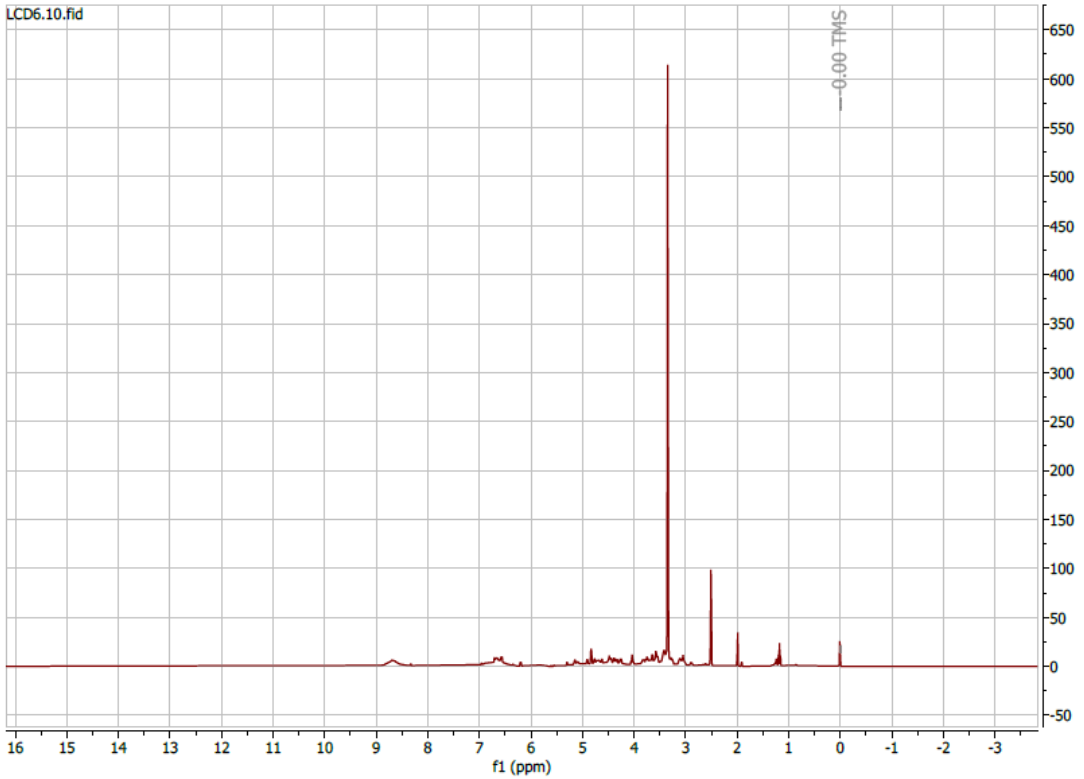
Appendix III: ^{1}H-NMR spectra of fraction A3.



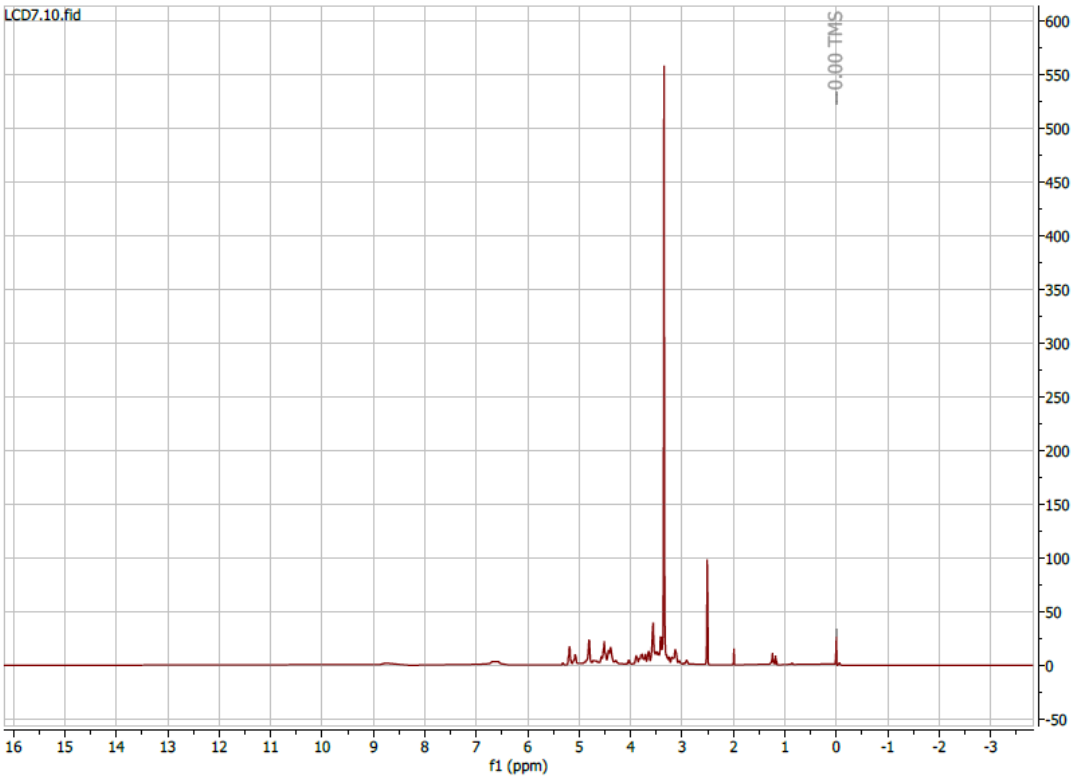
Appendix IV: ^{1}H-NMR spectra of fraction A4.



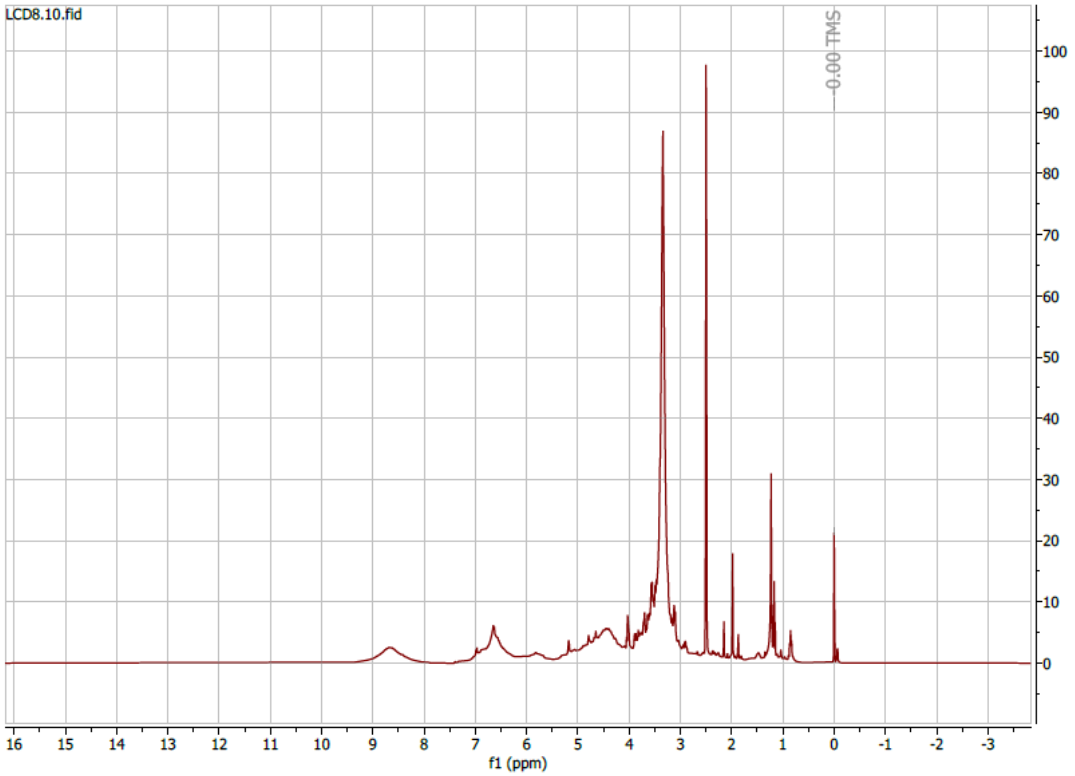
Appendix V: ^{1}H-NMR spectra of fraction A5.



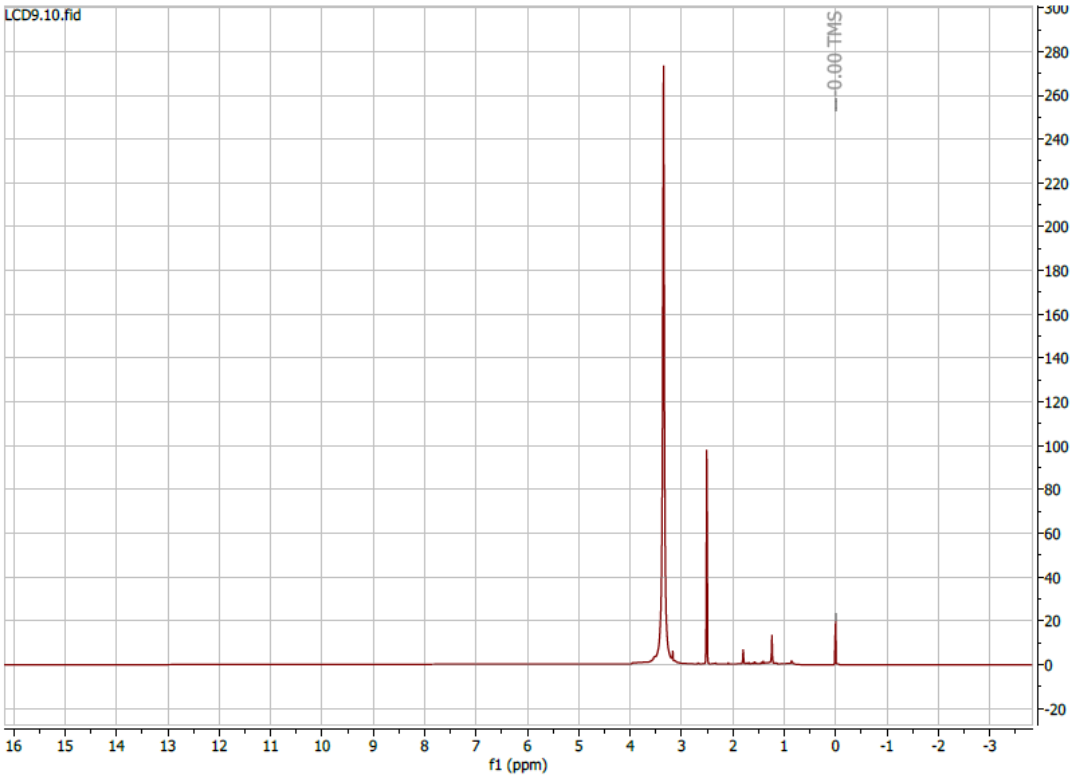
Appendix VI: ^{1}H-NMR spectra of fraction A6.



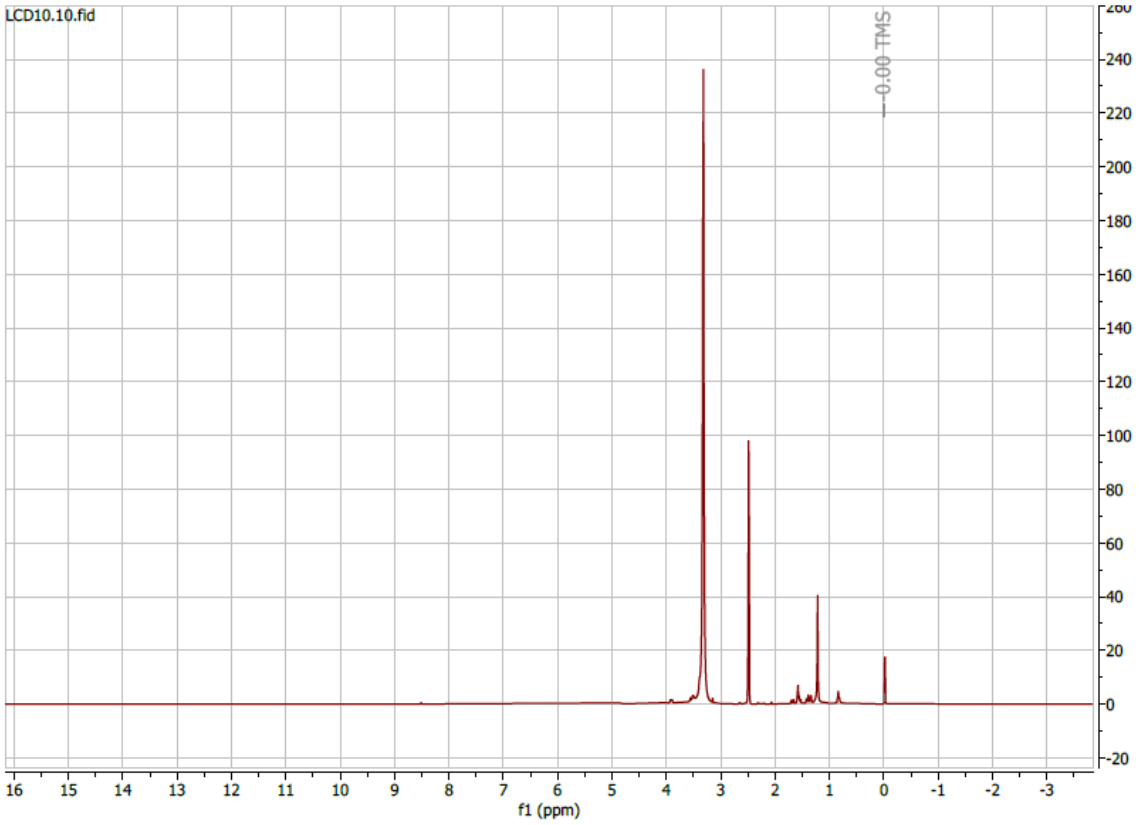
Appendix VII: ^{1}H-NMR spectra of fraction A7.



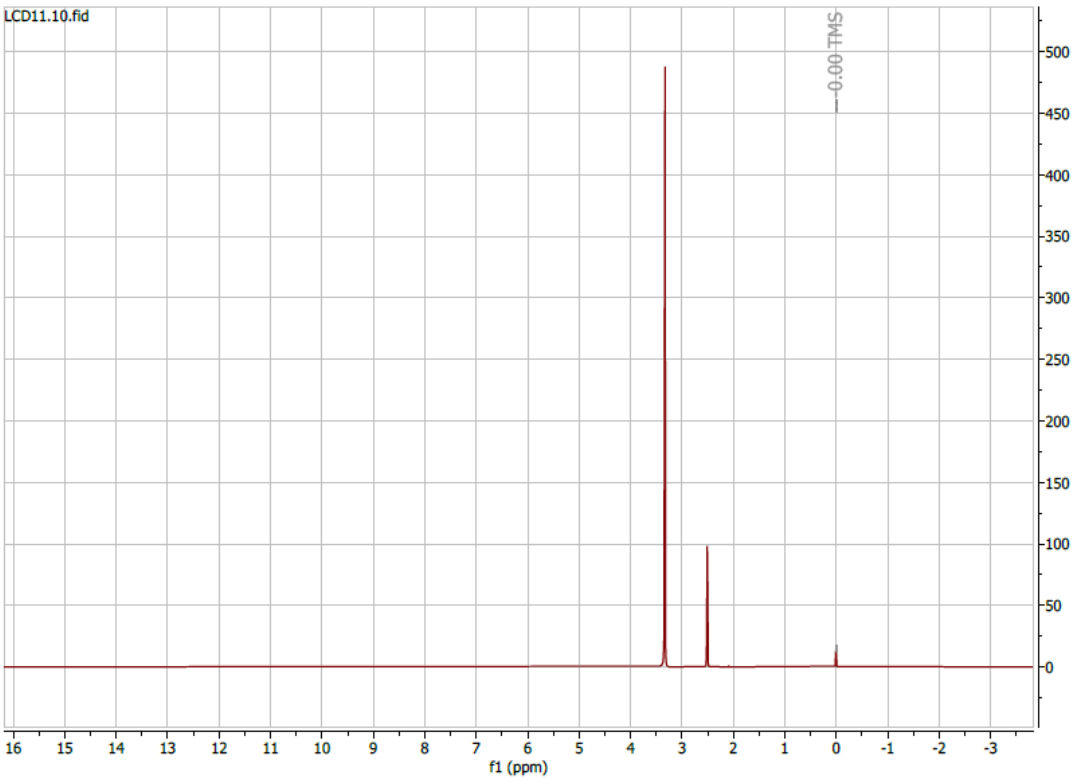
Appendix VIII: ^{1}H-NMR spectra of fraction A8.



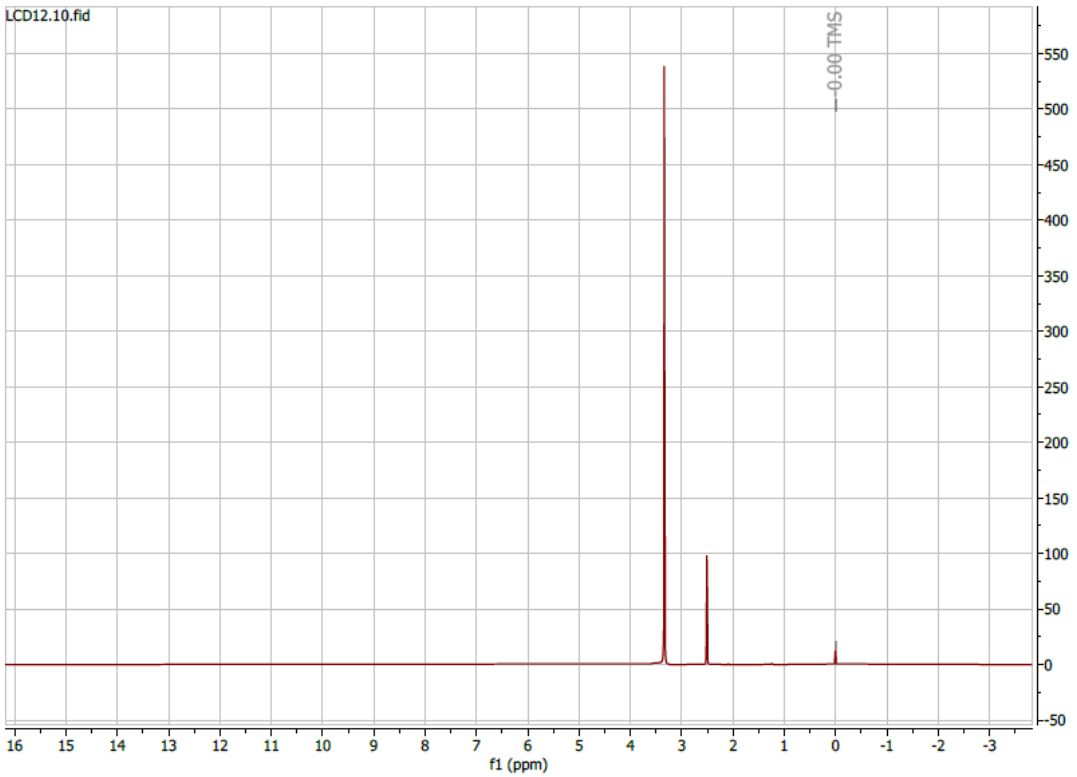
Appendix IX: ^{1}H-NMR spectra of fraction A9.



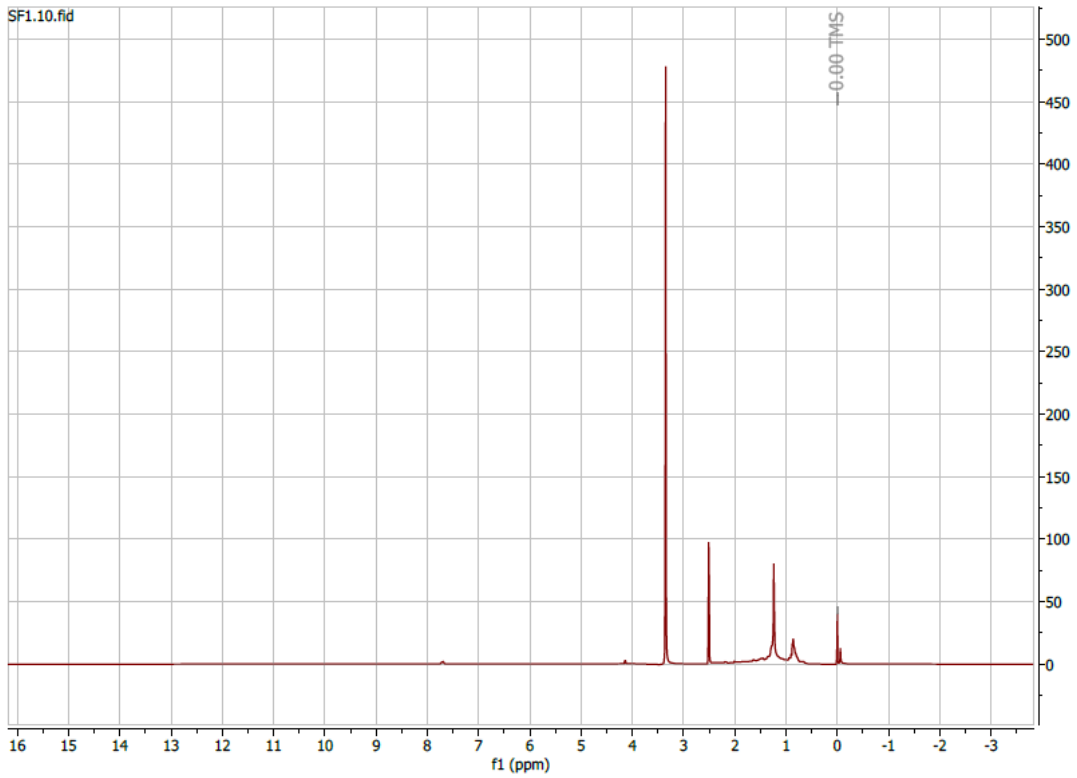
Appendix X: ^{1}H-NMR spectra of fraction A10.



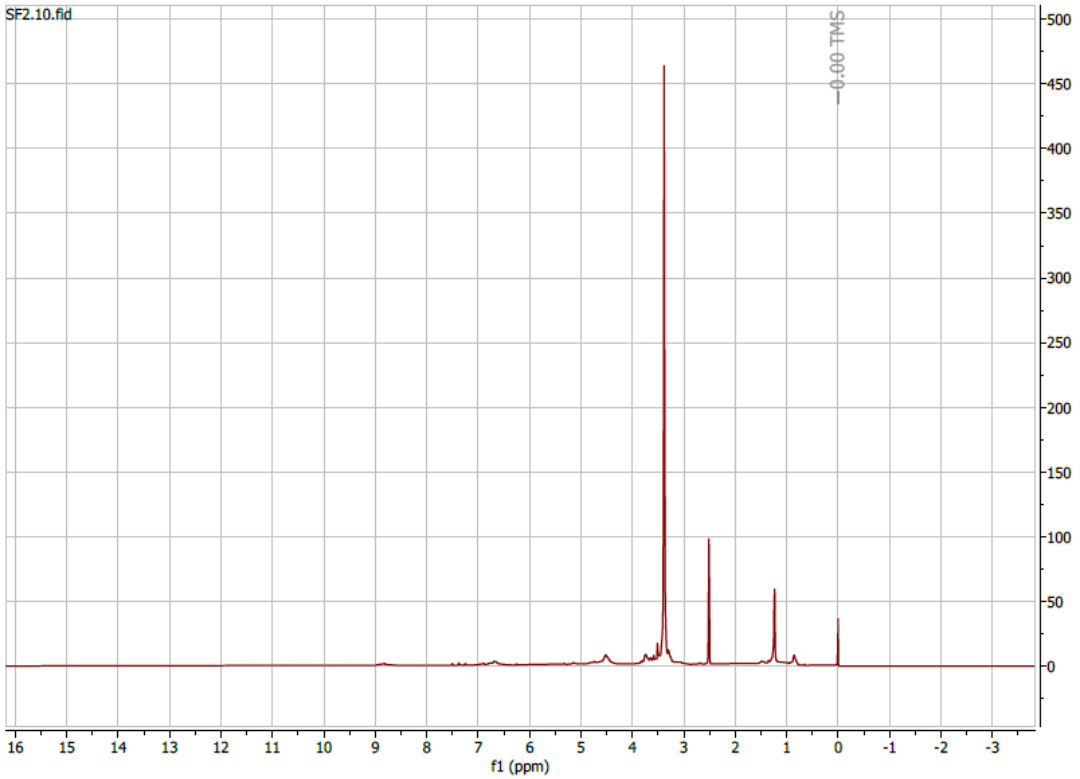
Appendix XI: ^{1}H-NMR spectra of fraction A11.



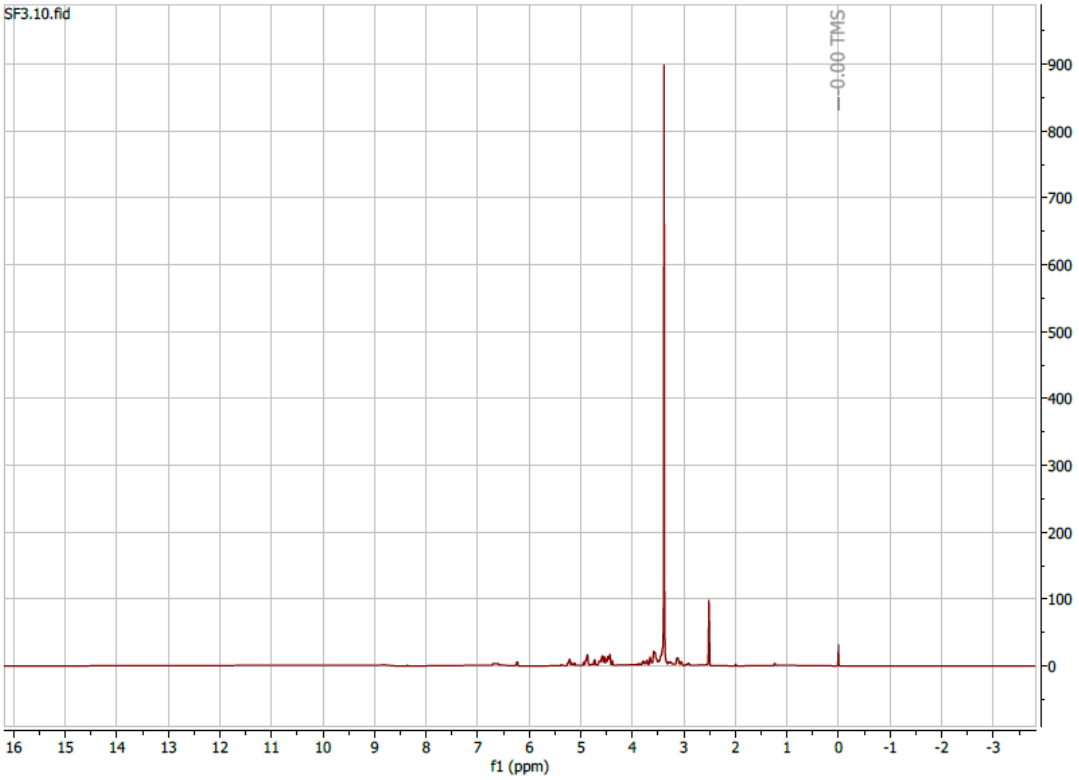
Appendix XII: ^{1}H-NMR spectra of fraction A12.



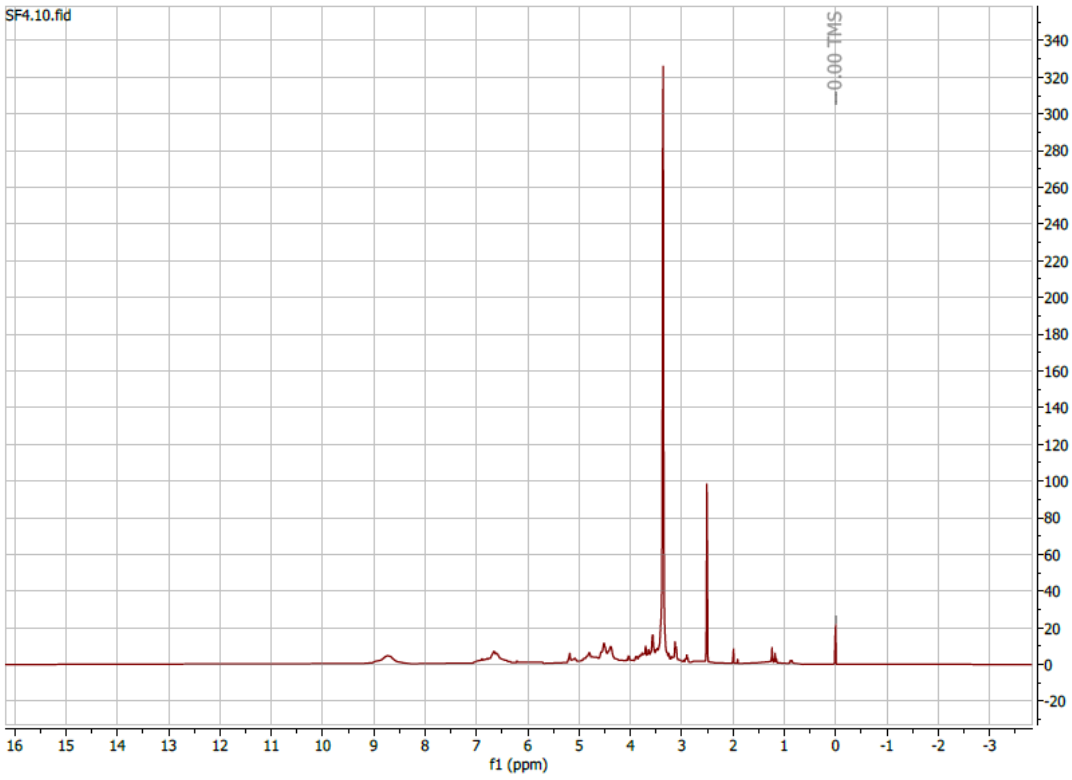
Appendix XIII: ^{1}H-NMR spectra of fraction B1.



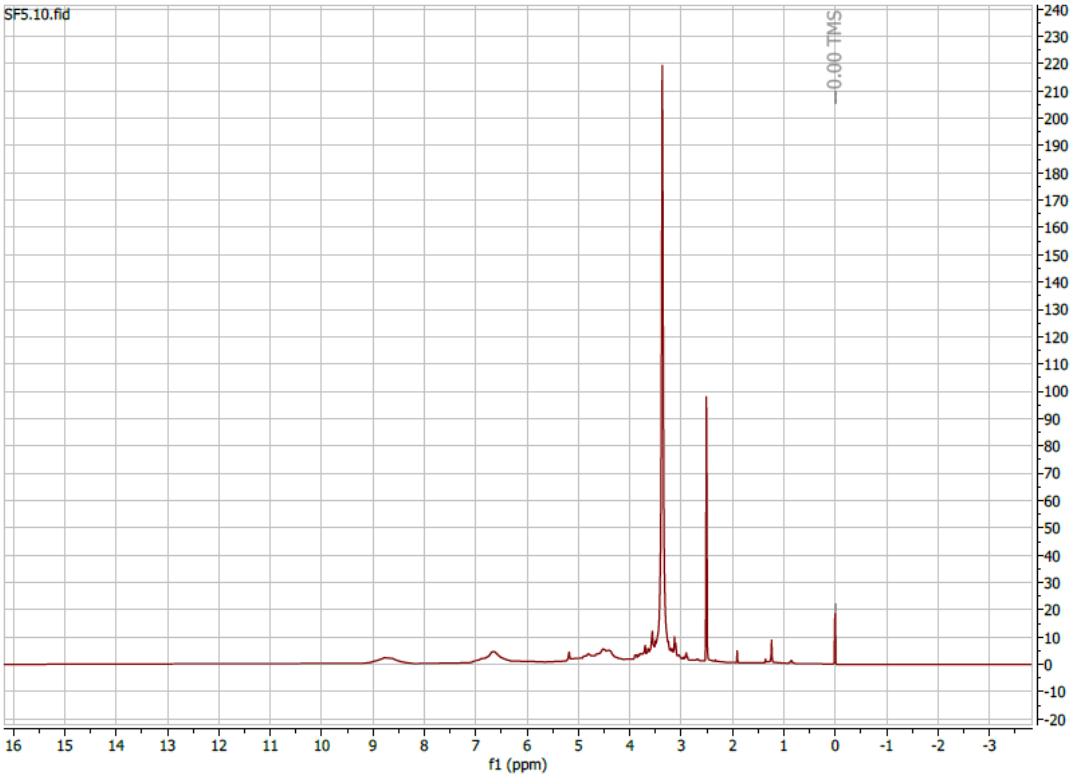
Appendix XIV: ^{1}H-NMR spectra of fraction B2.



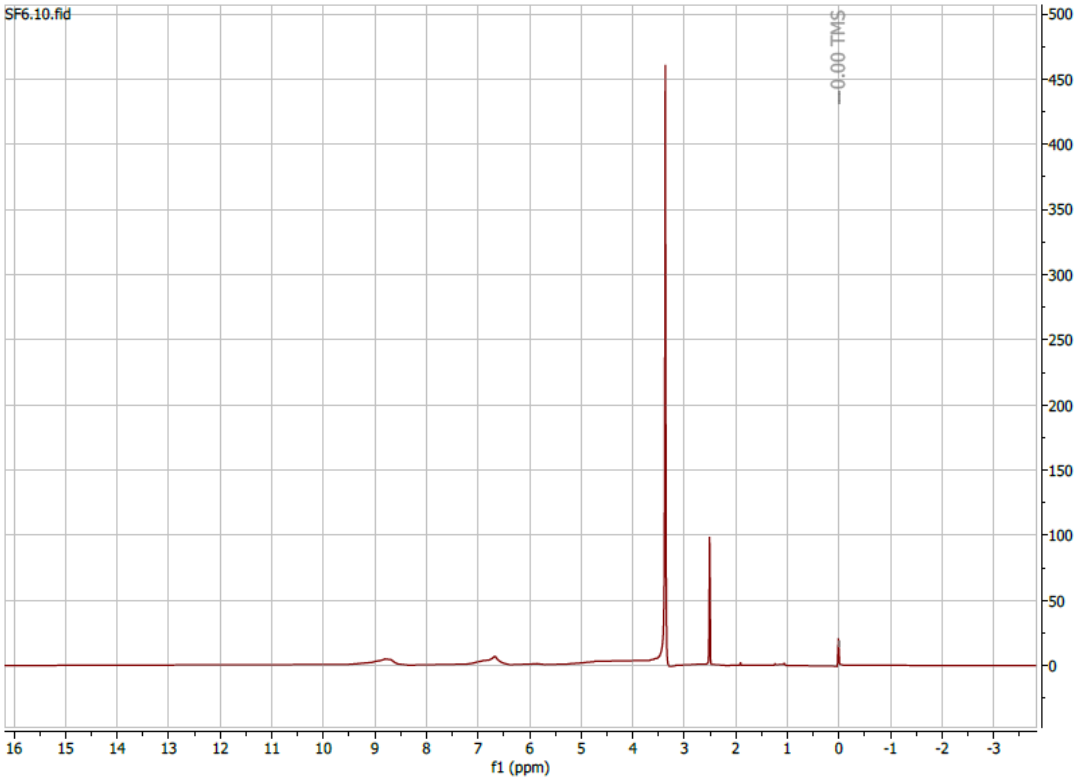
Appendix XV: ^{1}H-NMR spectra of fraction B3.



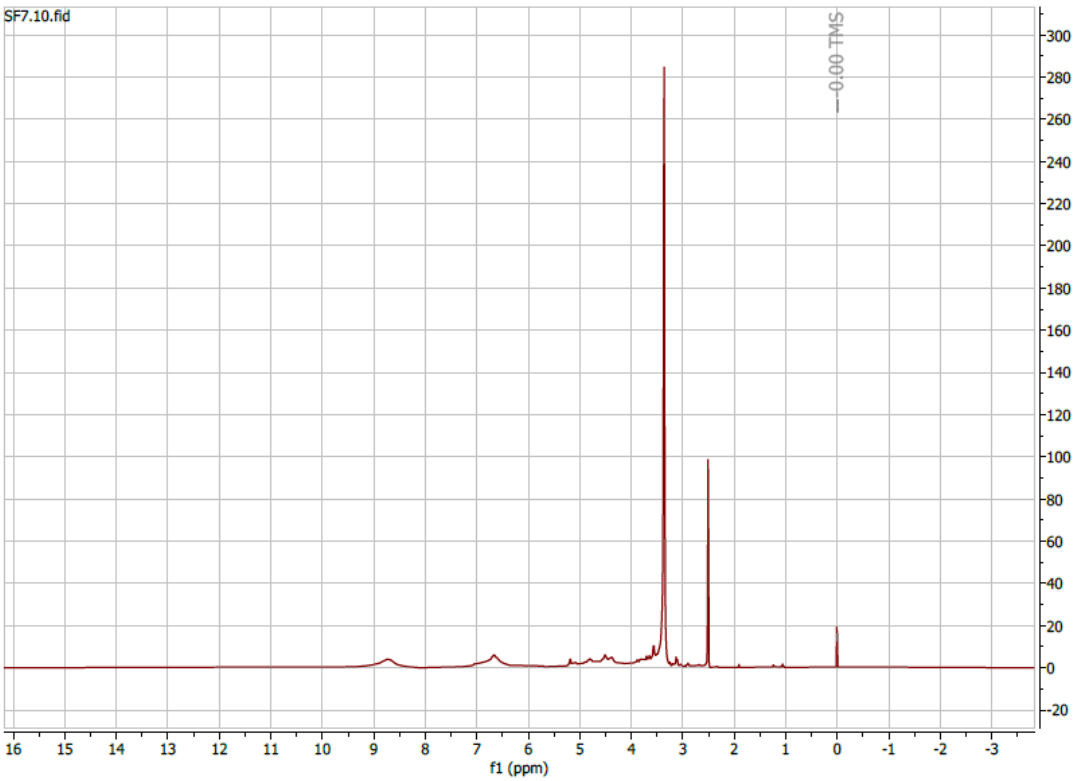
Appendix XVI: ^{1}H-NMR spectra of fraction B4.



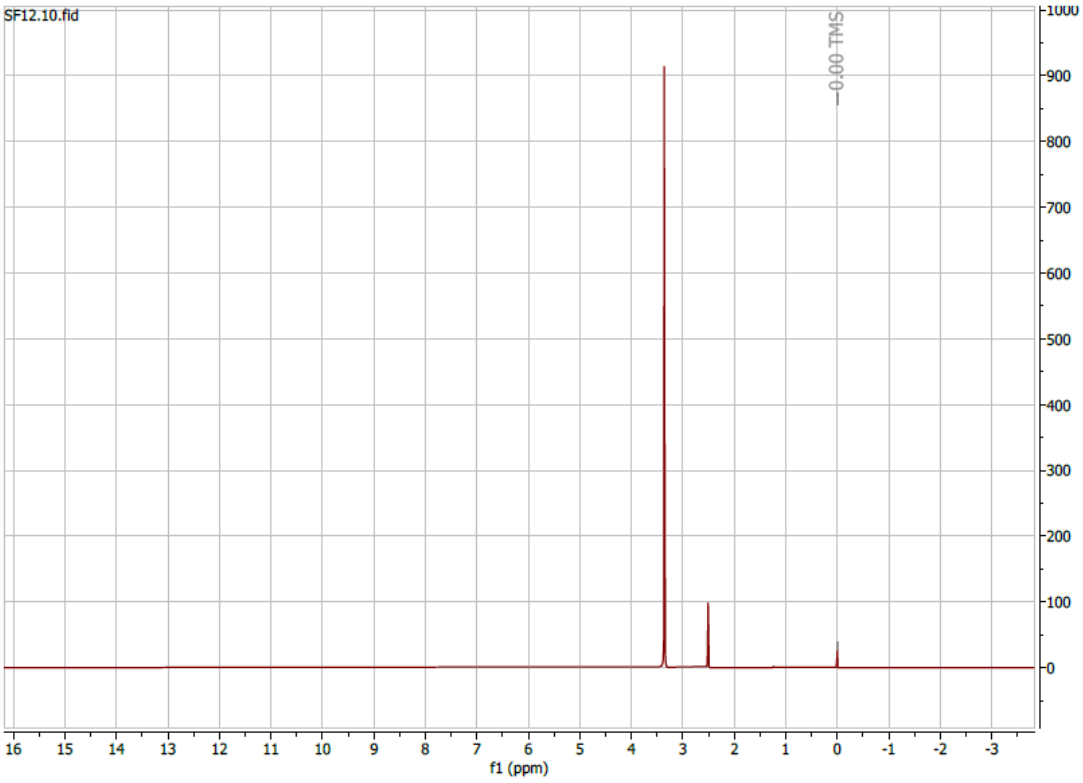
Appendix XVII: ^{1}H-NMR spectra of fraction B5.



Appendix XVIII: ^{1}H-NMR spectra of fraction B6.



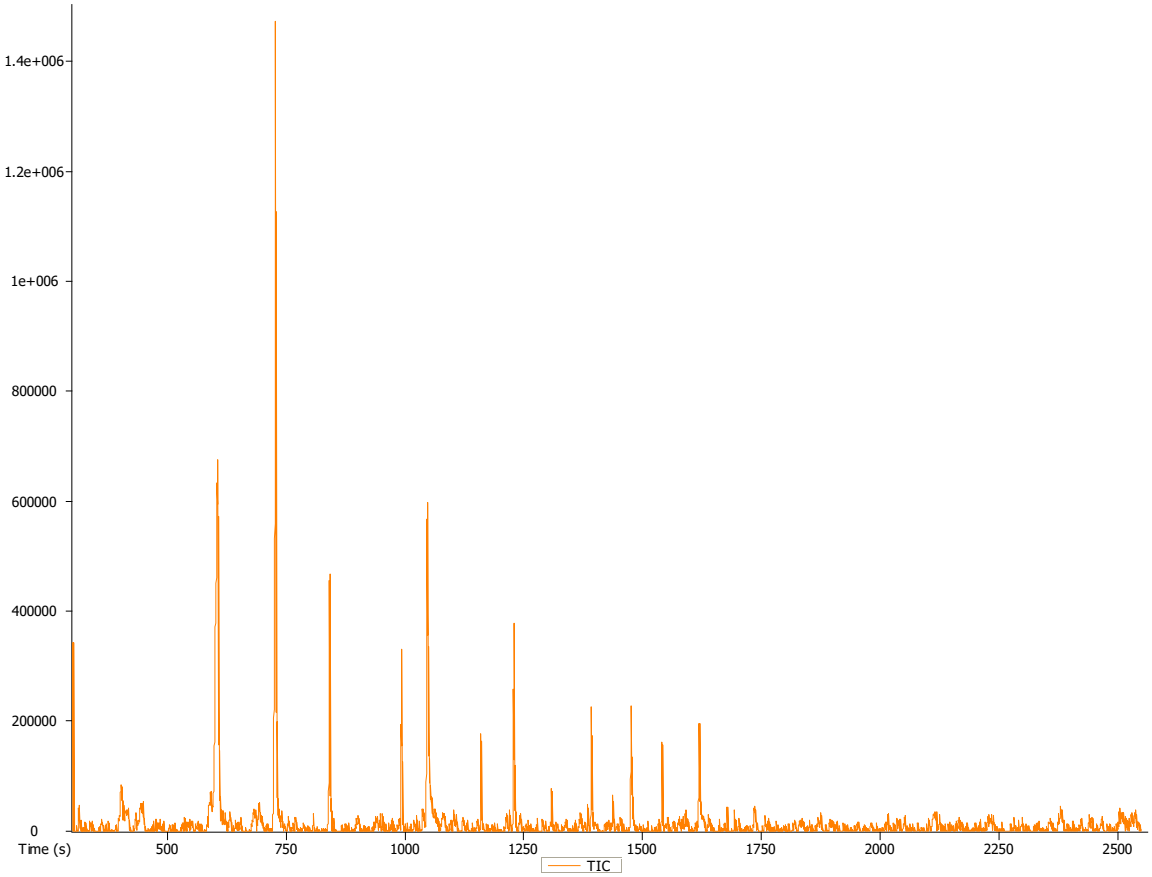
Appendix XIX: ^{1}H-NMR spectra of fraction B7.



Appendix XX: ^{1}H-NMR spectra of fraction B12.

APPENDIX B

GC-ToF-MS spectra and compounds identified in active fractions of *Ficus abutilifolia.*

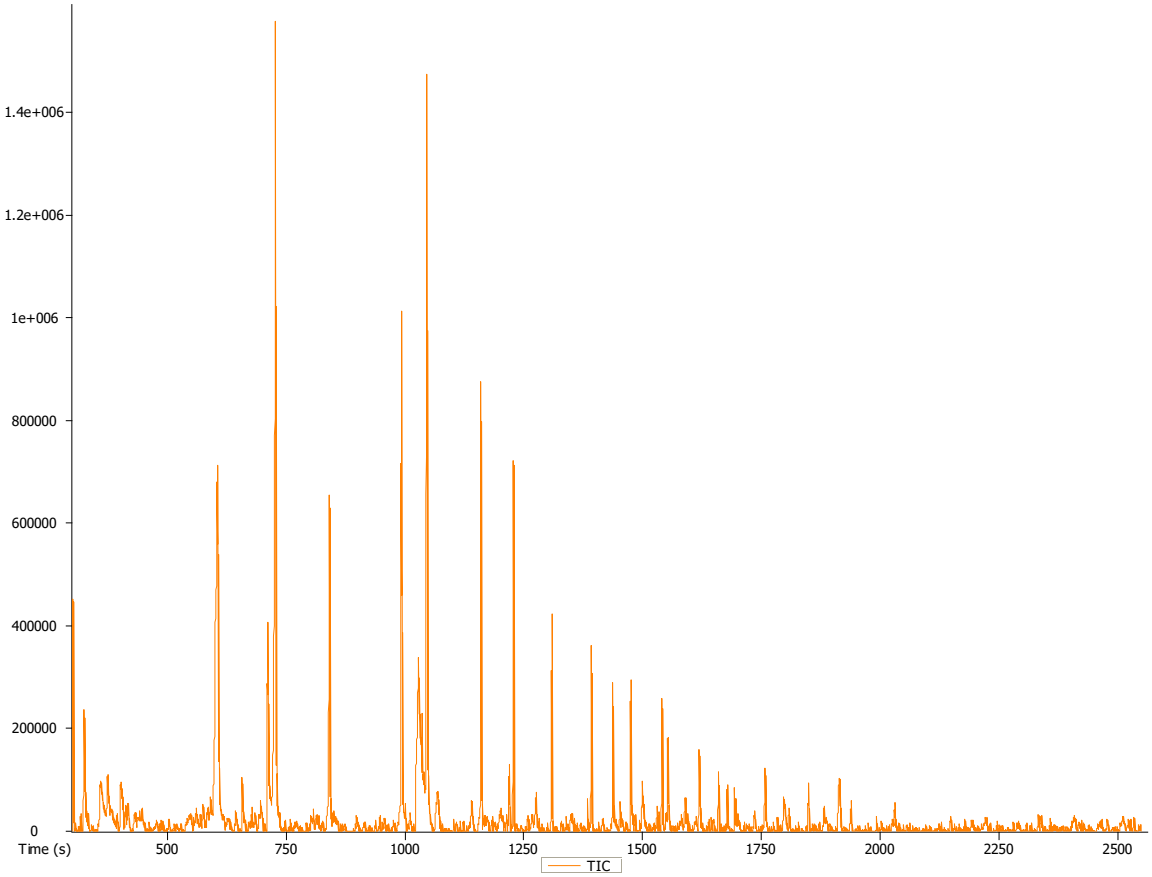


Appendix XXI: Representative Total Ion Chromatograph (TIC) of fraction F1.

Table I: Peak Report TIC (Fraction F1)

Peak	Name	Weight	Formula	Retention Time (s)	Area %
1	Methyl isovalerate	116	C6H12O2	301	2.8744
2	Butanoic acid, 2-methyl-, methyl ester	116	C6H12O2	302.4	2.8744
3	Tetramethyl silicate	152	C4H12O4Si	306.6	0.000239
4	Butanoic acid, 2-methyl-, methyl ester	116	C6H12O2	309.1	0.062234
5	Tetramethyl silicate	152	C4H12O4Si	314.6	0.49106
6	Benzene, 1,3-dimethyl-	106	C8H10	392.7	0.20487
7	p-Xylene	106	C8H10	403.4	2.1851
8	o-Xylene	106	C8H10	448.2	1.0819
9	Decane	142	C10H22	606.3	25.013
10	Undecane	156	C11H24	728.1	23.658
11	Cyclopentasiloxane, decamethyl-	370	C10H30O5Si5	807.8	0.35479
12	Undecane	156	C11H24	841.6	5.9411
13	Cyclohexasiloxane, dodecamethyl-	444	C12H36O6Si6	993.4	2.9279
14	Tetradecane	198	C14H30	1047.4	10.373
15	Trisiloxane, 1,1,1,5,5,5-hexamethyl-3,3-bis[(trimethylsilyl)oxy]-	384	C12H36O4Si5	1160.5	1.9386

16	Hexadecane	226	C16H34	1229.4	5.1066
17	Hexasiloxane, tetradecamethyl-	458	C14H42O5Si6	1309.4	0.86373
18	Heneicosane	296	C21H44	1392.9	2.8928
19	1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane	444	C13H40O5Si6	1437.9	0.75651
20	Pentadecanoic acid, 14-methyl-, methyl ester	270	C17H34O2	1476.3	3.927
21	Heptadecane, 2-methyl-	254	C18H38	1541.4	1.7038
22	Malonic acid, bis(2-trimethylsilylethyl ester	304	C13H28O4Si2	1553.6	0.38572
23	Theobromine, TBDMS derivative	294	C13H22N4O2Si	1590.7	0.4504
24	Methyl stearate	298	C19H38O2	1619.9	3.1928
25	Silane, tetramethyl-	88	C4H12Si	1660.1	0.26312
26	Silane, (2-methoxyethyl)trimethyl-	132	C6H16OSi	1693.8	0.27352
27	Trimethylsilyl 3-methyl-4-[(trimethylsilyl)oxy]benzoate	296	C14H24O3Si2	1697.4	0.20354

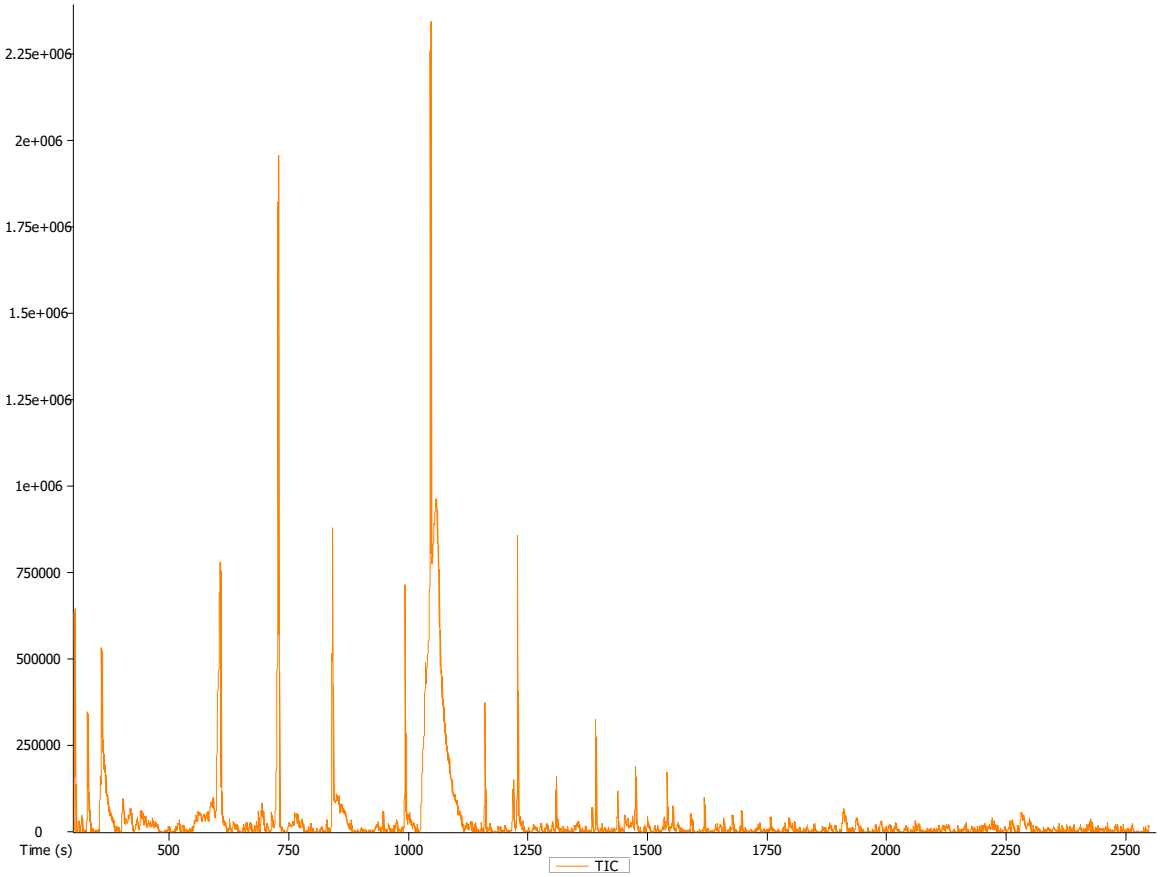


Appendix XXII: Representative Total Ion Chromatograph (TIC) of fraction F2.

Table II: Peak Report TIC (Fraction F2).

Peak	Name	Weight	Formula	Retention Time (s)	Area %
1	Butanoic acid, 2-methyl-, methyl ester	116	C6H12O2	302.3	2.1033
2	3-Hexen-2-one	98	C6H10O	317.8	0.16363
3	3-Penten-2-one, 4-methyl-	98	C6H10O	325.2	1.9471
4	2-Pentanone, 4-hydroxy-4-methyl-	116	C6H12O2	359.3	0.95694
5	3-Furaldehyde	96	C5H4O2	361.6	0.12016
6	2-Pentanone, 4-hydroxy-4-methyl-	116	C6H12O2	374.1	0.97856
7	Ethylbenzene	106	C8H10	392.9	0.30021
8	o-Xylene	106	C8H10	403.4	1.5
9	Ethylbenzene	106	C8H10	446.9	0.58264
10	Pyridine, 2,4,6-trimethyl-	121	C8H11N	575.5	0.31843
11	Decane	142	C10H22	605.9	13.5
12	N-(3-Oxobutyl)-2-methylazetidine	141	C8H15NO	657.4	0.88494
13	5-Methylfurfuryl acetate	154	C8H10O3	696.8	0.73343
14	4-Piperidinone, 2,2,6,6-tetramethyl-	155	C9H17NO	711.7	2.7895
15	3-Fluorobenzoic acid, tridec-2-ynyl ester	318	C20H27FO2	716	0.52383
16	Undecane	156	C11H24	728	15.266

17	Cyclopentasiloxane, decamethyl-	370	C10H30O5Si5	807.6	0.22238
18	Undecane	156	C11H24	841.4	4.8156
19	Cyclohexasiloxane, dodecamethyl-	444	C12H36O6Si6	993.3	6.3271
20	1-(3,6,6-Trimethyl-1,6,7,7a-tetrahydrocyclopenta[c]pyran-1-yl)ethanone	206	C13H18O2	1028.9	5.9486
21	2-Tridecene, (E)-	182	C13H26	1036	1.5303
22	Tetradecane	198	C14H30	1046.5	7.7067
23	1,6,6-Trimethyl-7-(3-oxobut-1-enyl)-3,8-dioxatricyclo[5.1.0.0(2,4)]octan-5-one	236	C13H16O4	1064.9	0.34759
24	Trisiloxane, 1,1,1,5,5,5-hexamethyl-3,3-bis[(trimethylsilyl)oxy]-	384	C12H36O4Si5	1160.5	4.473
25	Hexadecane	226	C16H34	1228.7	3.5227
26	Octane, 1,1'-oxybis-	242	C16H34O	1276.5	0.79297
27	Hexasiloxane, tetradecamethyl-	458	C14H42O5Si6	1309.3	2.1342
28	Nonadecane	268	C19H40	1392.4	1.8515
29	1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane	444	C13H40O5Si6	1438	1.5824
30	Trimethylsilyl 3-methyl-4-[(trimethylsilyl)oxy]benzoate	296	C14H24O3Si2	1475	1.9778
31	Hexadecanoic acid, methyl ester	270	C17H34O2	1475.6	1.9778
32	Dibutyl phthalate	278	C16H22O4	1484.9	0.23669
33	n-Hexadecanoic acid	256	C16H32O2	1499.6	0.8982
34	Heptadecane, 2-methyl-	254	C18H38	1541.3	1.373
35	1,1,1,3,5,5,7,7,7-Nonamethyl-3-(trimethylsiloxy)tetrasiloxane	384	C12H36O4Si5	1553.6	1.2663
36	Cyclopropane, 2-methylene-1-pentyl-1-trimethylsilyl-	196	C12H24Si	1590.8	0.55346
37	Tridecanoic acid, methyl ester	228	C14H28O2	1619.6	1.1007
38	Hexasiloxane, tetradecamethyl-	458	C14H42O5Si6	1660.2	0.60074
39	Heptacosane	380	C27H56	1678.6	0.53619
40	Silane, (2-methoxyethyl)trimethyl-	132	C6H16OSi	1693.9	0.43399
41	Trimethylsilyl 3-methyl-4-[(trimethylsilyl)oxy]benzoate	296	C14H24O3Si2	1697.5	0.3028
42	3-Hydroxyphenylacetic acid, 2TMS derivative	296	C14H24O3Si2	1700.6	0.20891
43	1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane	444	C13H40O5Si6	1757.9	0.94017
44	Di-trimethylsilyl peroxide	178	C6H18O2Si2	1788.3	0.086462
45	Trimethylsilyl 3-methyl-4-[(trimethylsilyl)oxy]benzoate	296	C14H24O3Si2	1797.3	0.53296
46	Dithioerythritol, O,O',S,S'-tetrakis(trimethylsilyl)-	442	C16H42O2S2Si4	1800	0.50361
47	Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-	430	C12H38O5Si6	1849.5	0.49088
48	Theobromine, TBDMS derivative	294	C13H22N4O2Si	1893.3	0.15812
49	Diisooctyl phthalate	390	C24H38O4	1914	1.4662
50	Hexasiloxane, tetradecamethyl-	458	C14H42O5Si6	1939.1	0.43116

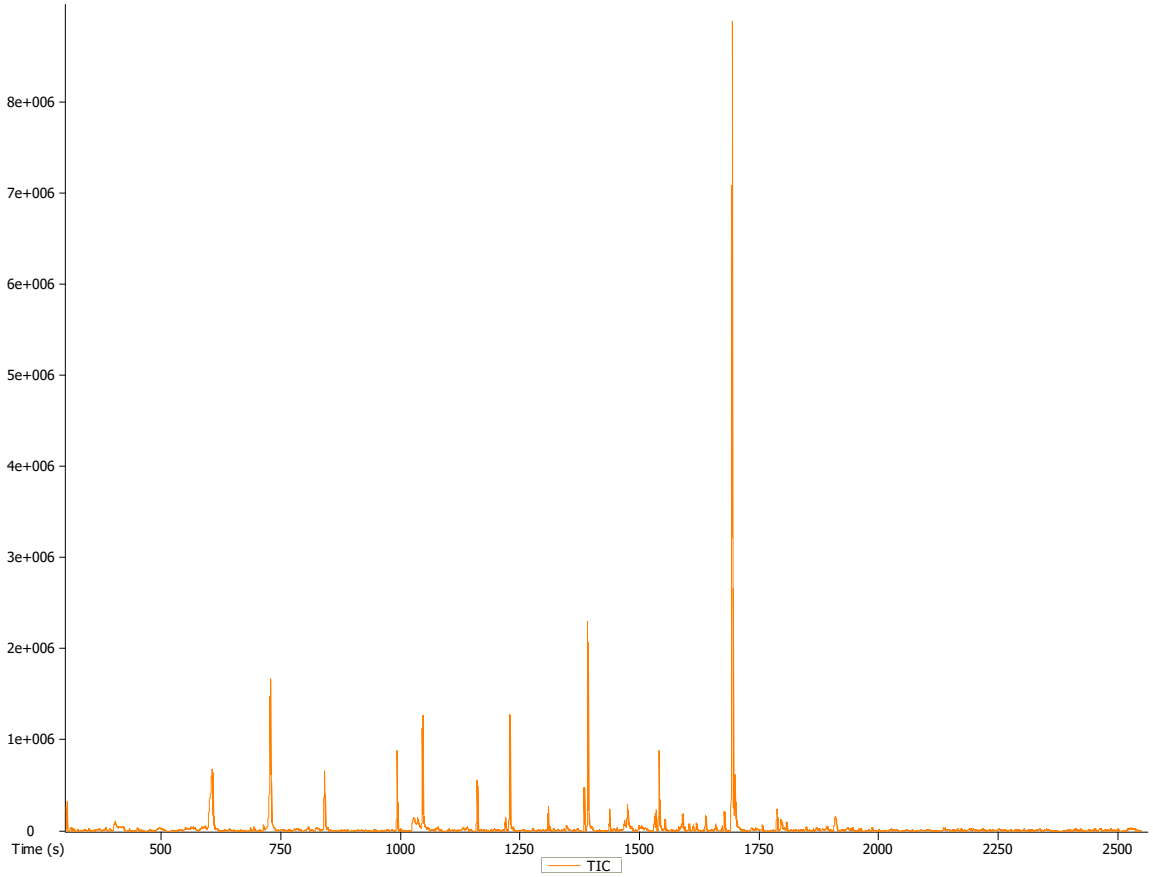


Appendix XXIII: Representative Total Ion Chromatograph (TIC) of fraction F3.

Table III: Peak Report TIC (Fraction F3).

Peak	Name	Weight	Formula	Retention Time (s)	Area %
1	Pentanoic acid, 2,4-dimethyl-, methyl ester	144	C8H16O2	302.5	2.1118
2	Vinyl butyrate	114	C6H10O2	305.1	0.054117
3	Butanoic acid, 2-methyl-, methyl ester	116	C6H12O2	312.1	0.13356
4	Propanenitrile, 3,3'-oxybis-	124	C6H8N2O	316.8	0.23735
5	Tetramethyl silicate	152	C4H12O4Si	317.6	0.23735
6	3-Penten-2-one, 4-methyl-	98	C6H10O	329.4	1.9096
7	Maleic anhydride	98	C4H2O3	355.5	0.43
8	Methyl oxalate	118	C4H6O4	355.9	0.43879
9	Furfural	96	C5H4O2	358.2	4.5173
10	Methyl (+-)-2-hydroxy-3-butenoate	116	C5H8O3	364.8	2.1636
11	Benzene, 1,3-dimethyl-	106	C8H10	392.7	0.030837
12	p-Xylene	106	C8H10	403.7	0.86433
13	1,4-Dioxane, 2,3-dimethoxy-	148	C6H12O4	440.2	0.22561

14	o-Xylene	106	C8H10	449.7	0.34968
15	Decane	142	C10H22	607	9.5244
16	5-Methylfurfuryl acetate	154	C8H10O3	696.7	0.26608
17	4-Piperidinone, 2,2,6,6-tetramethyl-	155	C9H17NO	713.5	0.33681
18	Undecane	156	C11H24	728.2	10.423
19	Cyclopentasiloxane, decamethyl-	370	C10H30O5Si5	807.8	0.033927
20	Catechol	110	C6H6O2	833.3	0.039411
21	Undecane	156	C11H24	841.5	3.4252
22	Cyclohexasiloxane, dodecamethyl-	444	C12H36O6Si6	993.4	2.758
23	Silane, [(1,1-dimethyl-2-propenyl)oxy]dimethyl-	144	C7H16OSi	1010.5	0.10228
24	3-Tetradecene, (E)-	196	C14H28	1036	1.5356
25	Tetradecane	198	C14H30	1046.5	9.1325
26	Propanamide, 3-cyclopentyl-N-methyl-	155	C9H17NO	1058.4	37.018
27	Trisiloxane, 1,1,1,5,5,5-hexamethyl-3,3-bis[(trimethylsilyl)oxy]-	384	C12H36O4Si5	1160.5	1.2933
28	7-Hexadecene, (Z)-	224	C16H32	1219.7	0.73719
29	Hexadecane	226	C16H34	1228.7	3.0154
30	Hexasiloxane, tetradecamethyl-	458	C14H42O5Si6	1309.3	0.5487
31	Eicosane	282	C20H42	1392.4	1.2251
32	1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane	444	C13H40O5Si6	1438	0.47832
33	2,6-Dihydroxybenzoic acid, 3TMS derivative	370	C16H30O4Si3	1475.1	0.70814
34	Hexadecanoic acid, methyl ester	270	C17H34O2	1475.6	0.70814
35	Phthalic acid, cyclobutyl isobutyl ester	276	C16H20O4	1484.8	0.025955
36	Heptadecane, 2-methyl-	254	C18H38	1541.3	0.69589
37	1,1,1,3,5,5,7,7,7-Nonamethyl-3-(trimethylsiloxy)tetrasiloxane	384	C12H36O4Si5	1553.7	0.39833
38	Trimethylsilyl 3-methyl-4-[(trimethylsilyl)oxy]benzoate	296	C14H24O3Si2	1590.7	0.25715
39	Tridecanoic acid, methyl ester	228	C14H28O2	1619.5	0.41633
40	Hexasiloxane, tetradecamethyl-	458	C14H42O5Si6	1660.2	0.14938
41	1-Butene, 3,3-bis[[(tert-butyldimethylsilyl)oxy]methyl]-4-[(2-methoxyethoxy)methoxy]-	448	C22H48O5Si2	1693.8	0.058425
42	Trimethylsilyl 3-methyl-4-[(trimethylsilyl)oxy]benzoate	296	C14H24O3Si2	1697.4	0.21373
43	1,1,1,3,5,7,7,7-Octamethyl-3,5-bis(trimethylsiloxy)tetrasiloxane	458	C14H42O5Si6	1757.6	0.21293
44	Trimethylsilyl 3-methyl-4-[(trimethylsilyl)oxy]benzoate	296	C14H24O3Si2	1796.7	0.16293
45	Theobromine, TBDMS derivative	294	C13H22N4O2Si	1892.1	0.050246
46	Diisooctyl phthalate	390	C24H38O4	1909.7	0.31039
47	Methanol, [4-(1,1-dimethylethyl)phenoxy]-, acetate	222	C13H18O3	2312	0.034943



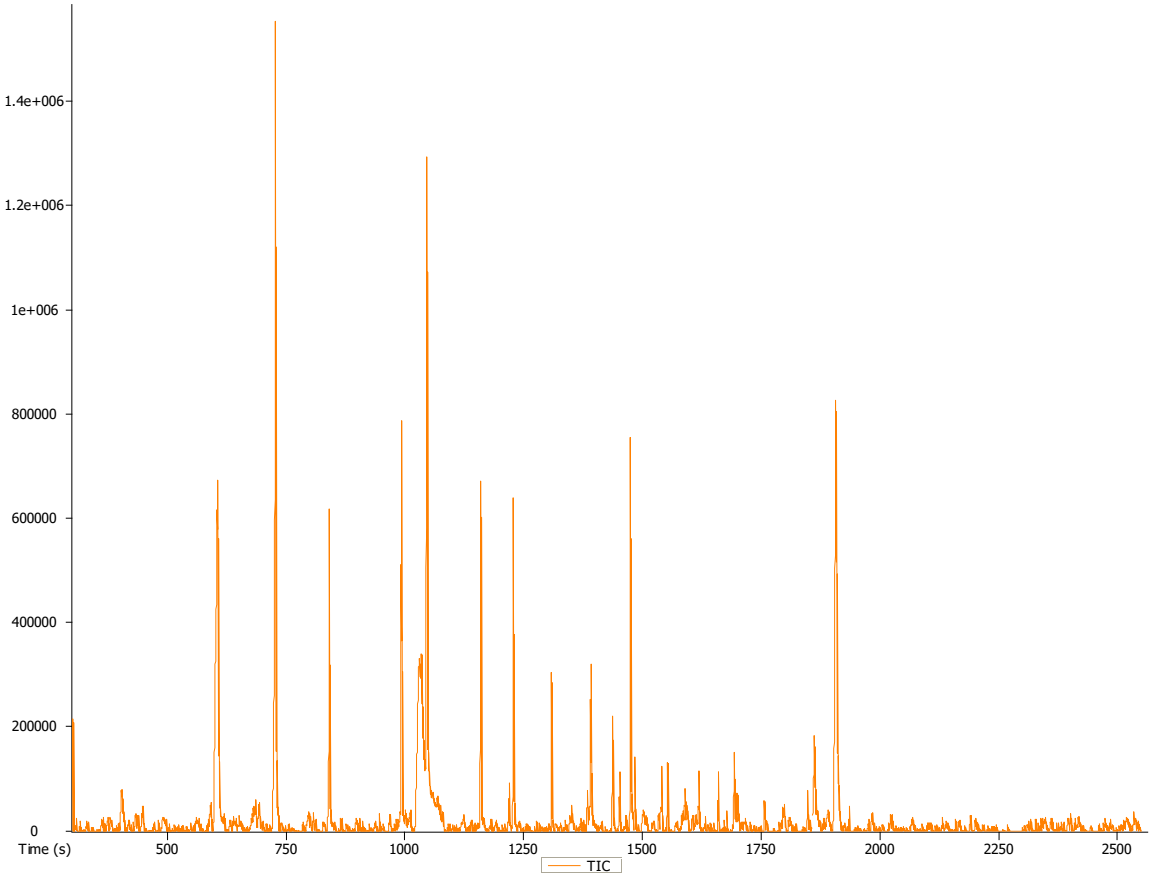
Appendix XXIV: Representative Total Ion Chromatograph (TIC) of fraction F4.

Table IV: Peak Report TIC (Fraction F4).

Peak	Name	Weight	Formula	Retention Time (s)	Area %
1	Phenol, 4-(2-phenylethyl)-	198	C14H14O	301.1	0.86752
2	Methyl isovalerate	116	C6H12O2	301.4	0.86752
3	Butanoic acid, 2-methyl-, methyl ester	116	C6H12O2	302.7	0.86752
4	Butanoic acid, 2-methyl-, methyl ester	116	C6H12O2	311.8	0.19888
5	Tetramethyl silicate	152	C4H12O4Si	317.1	0.061952
6	3-Penten-2-one, 4-methyl-	98	C6H10O	330.6	0.038477
7	Ethylbenzene	106	C8H10	393	0.12029
8	p-Xylene	106	C8H10	403.6	0.81573
9	3-Methylbenzyl alcohol, TBDMS derivative	236	C14H24OSi	433.7	0.05323
10	o-Xylene	106	C8H10	449.4	0.13376
11	Benzyl alcohol, TBDMS derivative	222	C13H22OSi	451	0.13376
12	Decane	142	C10H22	606.5	7.5613
13	4-Piperidinone, 2,2,6,6-tetramethyl-	155	C9H17NO	713.4	0.25067
14	Undecane	156	C11H24	728.3	8.3319
15	Cyclopentasiloxane, decamethyl-	370	C10H30O5Si5	807.7	0.22913

16	Tetradecane	198	C14H30	841.6	2.4441
17	Cyclohexasiloxane, dodecamethyl-	444	C12H36O6Si6	993.4	2.5167
18	Sucrose	342	C12H22O11	1027.4	1.7927
19	6-Tridecene	182	C13H26	1036.2	0.73099
20	Tetradecane	198	C14H30	1046.7	4.4244
21	Trisiloxane, 1,1,1,5,5,5-hexamethyl-3,3-bis[(trimethylsilyl)oxy]-	384	C12H36O4Si5	1160.4	1.5641
22	Cetene	224	C16H32	1219.8	0.54414
23	Hexadecane	226	C16H34	1228.7	3.8845
24	Hexasiloxane, tetradecamethyl-	458	C14H42O5Si6	1309.3	0.78978
25	Trimethylsilyl 3-methyl-4-[(trimethylsilyl)oxy]benzoate	296	C14H24O3Si2	1347.9	0.2702
26	1,3-Dioxolane, 2-pentadecyl-	284	C18H36O2	1351.9	0.097563
27	5-Eicosene, (E)-	280	C20H40	1384.7	1.3685
28	Nonadecane	268	C19H40	1392.4	7.4233
29	Tridecane, 4-methyl-	198	C14H30	1437.5	0.67638
30	1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane	444	C13H40O5Si6	1437.9	0.67638
31	3-Butoxy-1,1,1,5,5,5-hexamethyl-3-(trimethylsiloxy)trisiloxane	368	C13H36O4Si4	1468.7	0.55264
32	Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-	430	C12H38O5Si6	1474.9	1.1434
33	Dodecanoic acid, methyl ester	214	C13H26O2	1475.6	1.1434
34	3,4-Dihydroxymandelic acid, 4TMS derivative	472	C20H40O5Si4	1478.5	0.37253
35	Pentanedioic acid, 2-oxo-, dimethyl ester	174	C7H10O5	1484.6	0.24516
36	Dibutyl phthalate	278	C16H22O4	1484.9	0.25831
37	2-Tetradecanone	212	C14H28O	1531.3	0.49763
38	1-Docosene	308	C22H44	1534.7	0.71727
39	Heptadecane, 2-methyl-	254	C18H38	1541.3	2.8912
40	1,1,1,3,5,5,7,7,7-Nonamethyl-3-(trimethylsiloxy)tetrasiloxane	384	C12H36O4Si5	1553.6	0.38469
41	Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-	430	C12H38O5Si6	1586	0.12655
42	Methanol, TMS derivative	104	C4H12OSi	1587.7	0.075922
43	Ethyl 4-hydroxyphenylacetate, TBDMS derivative	294	C16H26O3Si	1590.7	0.90421
44	Methanol, TBDMS derivative	146	C7H18OSi	1590.9	0.90421
45	1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane	444	C13H40O5Si6	1594.5	0.159
46	Ethyl(dimethyl)methoxysilane	118	C5H14OSi	1603.9	0.27632
47	5-Ethyl-4-tridecanone	226	C15H30O	1612.7	0.27328
48	Methyl stearate	298	C19H38O2	1619.5	0.3229
49	2-Buten-1-ol, (Z)-, TBDMS derivative	186	C10H22OSi	1622.4	0.049741
50	Methanol, TBDMS derivative	146	C7H18OSi	1638.2	0.60624
51	Allyl alcohol, TBDMS derivative	172	C9H20OSi	1639.2	0.60624
52	Hexasiloxane, tetradecamethyl-	458	C14H42O5Si6	1660.1	0.27985
53	Heptacosane	380	C27H56	1678.4	0.68731
54	Ethyl(dimethyl)methoxysilane	118	C5H14OSi	1694	28.078

55	Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-	430	C12H38O5Si6	1697.2	0.52944
56	Ethanol, 2-(trimethylsilyl)-	118	C5H14OSi	1700.5	2.8996
57	1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane	444	C13H40O5Si6	1757.4	0.28621
58	Di-trimethylsilyl peroxide	178	C6H18O2Si2	1788	0.72609
59	Phthalic acid, heptyl tridec-2-yn-1-yl ester	442	C28H42O4	1795.9	0.49468
60	Trimethylsilyl 3-methyl-4-[(trimethylsilyl)oxy]benzoate	296	C14H24O3Si2	1796.7	0.49468
61	Trimethylsilyl 2-(trimethylsilyloxy)propaneperoxoate	250	C9H22O4Si2	1799.2	0.35234
62	Heptacosane	380	C27H56	1807.8	0.37874
63	1,1,3,3-Tetramethyl-1,3-di-n-propyldisiloxane	218	C10H26OSi2	1808.5	0.37874
64	Hexasiloxane, tetradecamethyl-	458	C14H42O5Si6	1848.7	0.21616
65	1-(1-Adamantyl)-2-dichloromethyldimethylsilyloxyethane	320	C15H26Cl2OSi	1891.5	0.11315
66	Trimethylsilyl 2-(trimethylsilyloxy)propaneperoxoate	250	C9H22O4Si2	1893.2	0.2768
67	Diisooctyl phthalate	390	C24H38O4	1910	1.2986
68	Hexasiloxane, tetradecamethyl-	458	C14H42O5Si6	1936.9	0.098973
69	Methyl 6,6,8,8-tetramethyl-3-oxo-2,5,7,9-tetraoxa-6,8-disilaundecan-11-oate	310	C10H22O7Si2	1986.7	0.16479



Appendix XXV: Representative Total Ion Chromatograph (TIC) of fraction F5.

Table V: Peak Report TIC (Fraction F5).

Peak	Name	Weight	Formula	Retention Time (s)	Area %
1	Butanoic acid, 2-methyl-, methyl ester	116	C6H12O2	302.2	0.72122
2	Butanoic acid, 2-methyl-, methyl ester	116	C6H12O2	309.5	0.18293
3	Tetramethyl silicate	152	C4H12O4Si	314.8	0.019902
4	Benzene, 1,3-dimethyl-	106	C8H10	393	0
5	p-Xylene	106	C8H10	404.8	1.166
6	Benzene, 1,3-dimethyl-	106	C8H10	448.7	0.5593
7	Decane	142	C10H22	606.3	11.774
8	Undecane	156	C11H24	728.1	12.783
9	Undecane	156	C11H24	841.5	3.7993
10	Cyclohexasiloxane, dodecamethyl-	444	C12H36O6Si6	993.4	4.0601
11	1-(3,6,6-Trimethyl-1,6,7,7a-tetrahydrocyclopenta[c]pyran-1-yl)ethanone	206	C13H18O2	1029.1	2.2905

12	Methyl-à-d-ribofuranoside	164	C6H12O5	1031.5	6.5212
13	7-Tetradecene	196	C14H28	1036.1	4.6133
14	Tetradecane	198	C14H30	1046.7	7.0057
15	á-D-Glucopyranose, 1,6-anhydro-	162	C6H10O5	1069.8	0.41838
16	Trisiloxane, 1,1,1,5,5,5-hexamethyl-3,3-bis[(trimethylsilyl)oxy]-	384	C12H36O4Si5	1160.5	3.3245
17	Hexadecane	226	C16H34	1228.8	3.5168
18	Hexasiloxane, tetradecamethyl-	458	C14H42O5Si6	1309.4	1.4826
19	Eicosane	282	C20H42	1392.4	2.224
20	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	278	C16H22O4	1414.7	0.097916
21	1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane	444	C13H40O5Si6	1437.9	1.0973
22	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	276	C17H24O3	1452.8	0.65863
23	Unknown 1	296	C14H24O3Si2	1475	4.3323
24	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	292	C18H28O3	1475.4	4.3323
25	Dibutyl phthalate	278	C16H22O4	1484.4	0.71494
26	Eicosane	282	C20H42	1541.2	0.63313
27	1,1,1,3,5,5,7,7,7-Nonamethyl-3-(trimethylsiloxy)tetrasiloxane	384	C12H36O4Si5	1553.5	0.75181
28	Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-	430	C12H38O5Si6	1586	0.2875
29	Monomethyl phthalate, TBDMS derivative	294	C15H22O4Si	1590.7	0.68436
30	Methyl stearate	298	C19H38O2	1619.4	0.82168
31	2,2-Diisopropyl-1,3-dioxolane	158	C9H18O2	1639.2	0.069349
32	Hexasiloxane, tetradecamethyl-	458	C14H42O5Si6	1660	0.6053
33	Silane, (2-methoxyethyl)trimethyl-	132	C6H16OSi	1693.7	0.77245
34	Trimethylsilyl 3-methyl-4-[(trimethylsilyl)oxy]benzoate	296	C14H24O3Si2	1697.1	0.33485
35	Ethanol, 2-(trimethylsilyl)-	118	C5H14OSi	1700.5	0.68504
36	Phthalic acid, 8-bromoctyl hexyl ester	440	C22H33BrO4	1701.1	0.68504
37	1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane	444	C13H40O5Si6	1757.2	0.38451
38	Phthalic acid, methyl 4-(2-phenylprop-2-yl)phenyl ester	374	C24H22O4	1787.7	0.089297

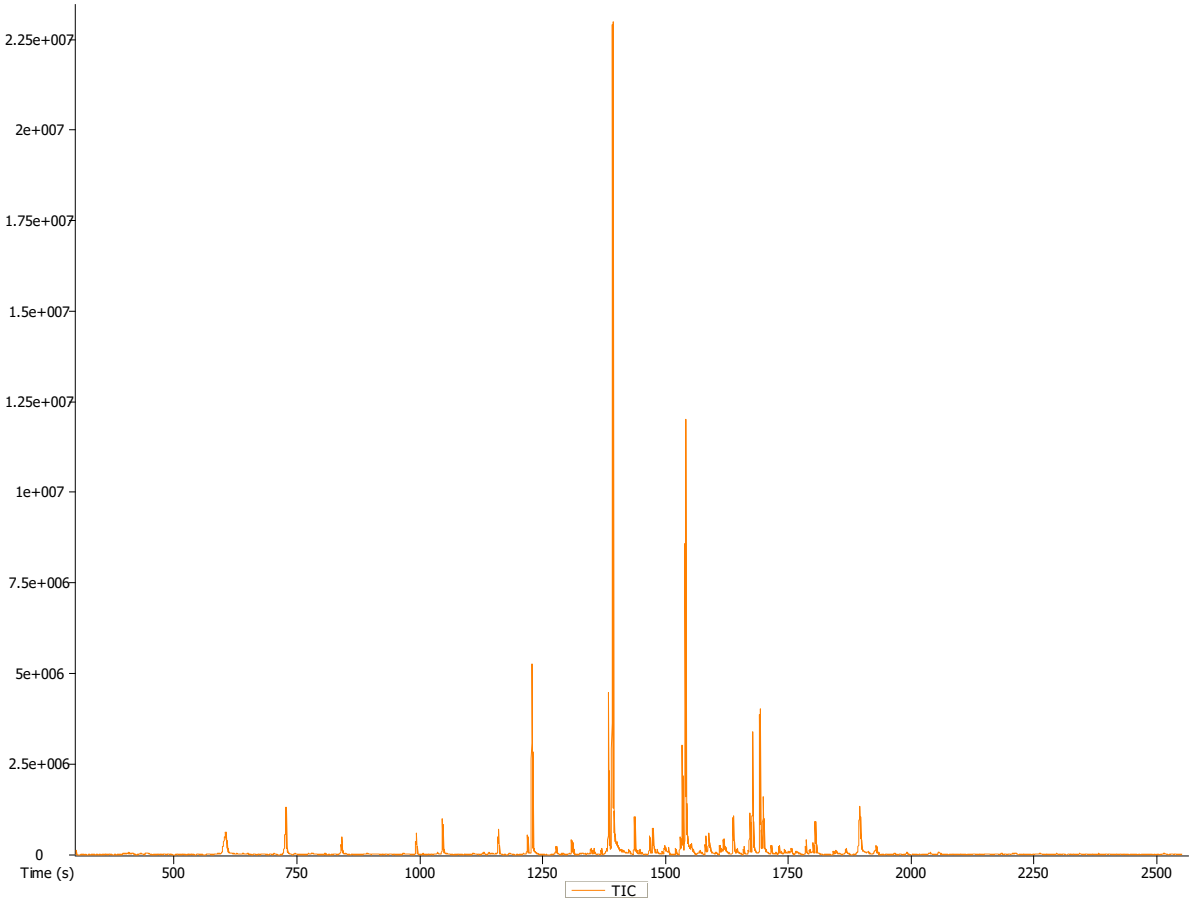
39	Trimethylsilyl 3-methyl-4-[(trimethylsilyl)oxy]benzoate	296	C14H24O3Si2	1796.2	0.28575
40	Hexasiloxane, tetradecamethyl-	458	C14H42O5Si6	1848.4	0.46515
41	1,4-Nonadiene, 2-nitro-, (Z)-	169	C9H15NO2	1862.4	2.1881
42	Theobromine, TBDMS derivative	294	C13H22N4O2Si	1890.7	0.44923
43	Diisooctyl phthalate	390	C24H38O4	1907.4	11.889
44	Hexasiloxane, tetradecamethyl-	458	C14H42O5Si6	1935.8	0.22302

Appendix XXVI: Representative Total Ion Chromatograph (TIC) of fraction F6.

Table VI: Peak Report TIC (Fraction F6).

Peak	Name	Weight	Formula	Retension Time (s)	Area %
1	Methyl isovalerate	116	C6H12O2	302.1	0.38102
2	Butanoic acid, 2-methyl-, methyl ester	116	C6H12O2	303.4	0.38102
3	Tetramethyl silicate	152	C4H12O4Si	307.8	7.47E-06
4	Tetramethyl silicate	152	C4H12O4Si	315.5	0.037085
5	Ethylbenzene	106	C8H10	393.2	0.014495
6	p-Xylene	106	C8H10	403.2	0.13506
7	o-Xylene	106	C8H10	448.3	0.078737
8	Decane	142	C10H22	606.4	3.0748
9	Undecane	156	C11H24	728.2	2.7033
10	Undecane	156	C11H24	841.7	0.6548
11	Cyclohexasiloxane, dodecamethyl-	444	C12H36O6Si6	993.4	0.33642
12	Tetradecane	198	C14H30	1047.7	1.2796

13	1,1,1,3,5,5,7,7,7-Nonamethyl-3-(trimethylsiloxy)tetrasiloxane	384	C12H36O4Si5	1160.5	0.21947
14	Hexadecane	226	C16H34	1229.9	0.45262
15	Hexasiloxane, tetradecamethyl-	458	C14H42O5Si6	1309.3	0.080974
16	Nonadecane	268	C19H40	1392.9	0.25147
17	1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane	444	C13H40O5Si6	1437.9	0.07946
18	Tridecanoic acid, methyl ester	228	C14H28O2	1476.6	0.11809
19	Phthalic acid, heptyl tridec-2-yn-1-yl ester	442	C28H42O4	1485.4	0.005308
20	Eicosane	282	C20H42	1541.4	0.14838
21	Silane, tetramethyl-	88	C4H12Si	1553.5	0.048504
22	Thiophene, 2-(trimethylsilyl)-5-[(trimethylsilyl)ethynyl]-	252	C12H20SSi2	1590.5	0.045068
23	Methyl stearate	298	C19H38O2	1619.6	0.1484
24	Hexasiloxane, tetradecamethyl-	458	C14H42O5Si6	1659.9	0.035383
25	Methanol, TBDMS derivative	146	C7H18OSi	1693.1	0.14242
26	1-(3-Nitrophenyl)ethanone oxime, (Z)-, TMS derivative	252	C11H16N2O3Si	1700.3	0.069522
27	Phthalic acid, methyl 4-(2-phenylprop-2-yl)phenyl ester	374	C24H22O4	1786.6	0
28	Diisooctyl phthalate	390	C24H38O4	1863.9	0.035663
29	Diisooctyl phthalate	390	C24H38O4	1900.8	44.302
30	3-Octene, (Z)-	112	C8H16	1901.3	44.741



Appendix XXVII: Representative Total Ion Chromatograph (TIC) of fraction F7.

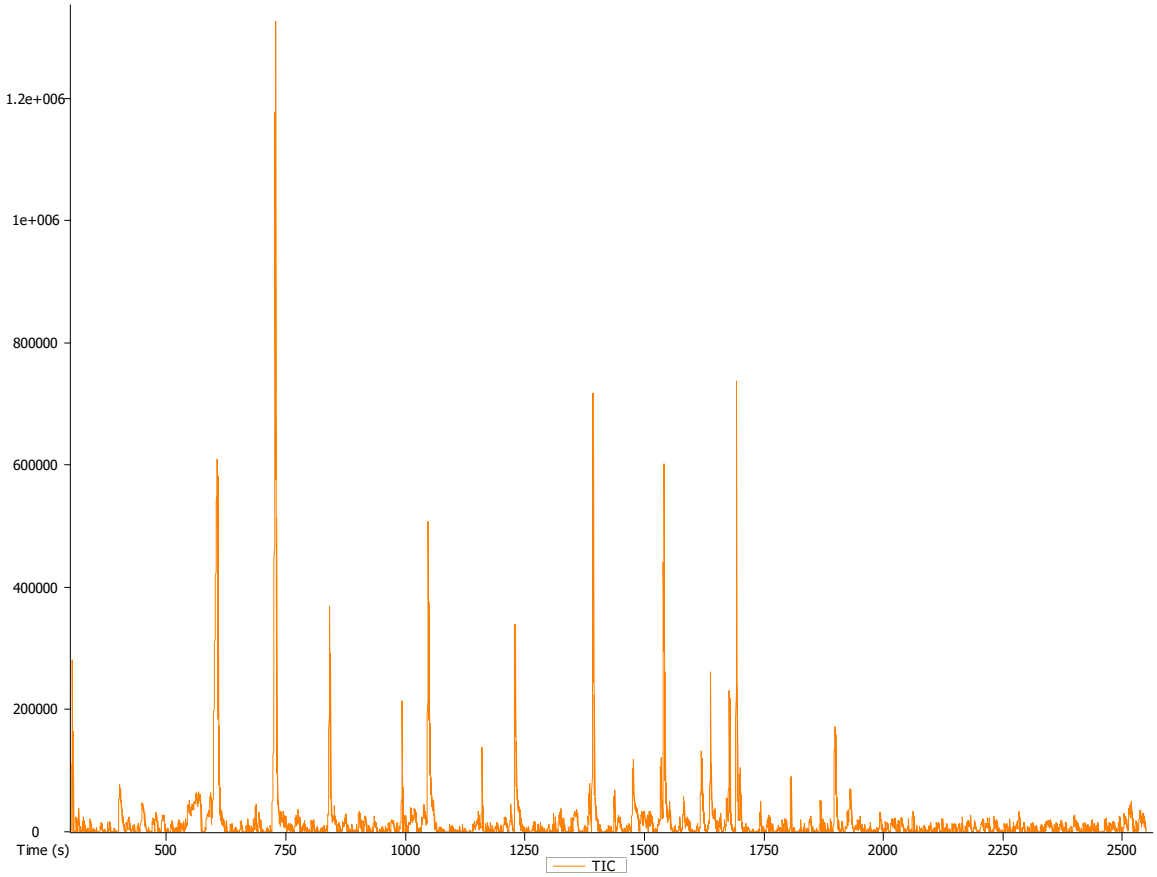
Table VII: Peak Report TIC (Fraction F7).

Peak	Name	Weight	Formula	Retention Time (s)	Area %
1	Butanoic acid, 2-methyl-, methyl ester	116	C6H12O2	307.3	0.001917
2	o-Xylene	106	C8H10	405.6	0.064798
3	Ethylbenzene	106	C8H10	433.2	0.022084
4	Oxime-, methoxy-phenyl-_	151	C8H9NO2	442.3	0.087096
5	o-Xylene	106	C8H10	447	0.054503
6	Silane, diethoxydimethoxy-	180	C6H16O4Si	449.1	0.042518
7	Decane	142	C10H22	606	2.3863
8	Undecane	156	C11H24	728.1	2.2769
9	Undecane	156	C11H24	841.6	0.6709
10	Cyclohexasiloxane, dodecamethyl-	444	C12H36O6Si6	993.4	0.5614
11	Tetradecane	198	C14H30	1046.8	1.1032
12	Methcathinone	163	C10H13NO	1095.5	0.01753
13	2,4-Di-tert-butylphenol	206	C14H22O	1130.4	0.099475

14	Pentanal, 2,4-dimethyl-	114	C7H14O	1140.9	0.084162
15	Trisiloxane, 1,1,1,5,5,5-hexamethyl-3,3-bis[(trimethylsilyl)oxy]-	384	C12H36O4Si5	1160.5	0.698
16	Cetene	224	C16H32	1219.7	0.47721
17	Hexadecane	226	C16H34	1228.8	5.416
18	Hexadecane, 4-methyl-	240	C17H36	1278.5	0.25863
19	Hexasiloxane, tetradecamethyl-	458	C14H42O5Si6	1309.3	0.38634
20	Nonadecane	268	C19H40	1312.7	0.3426
21	Heptadecane, 2,6-dimethyl-	268	C19H40	1349.5	0.27844
22	Heptadecane, 2-methyl-	254	C18H38	1355	0.186
23	Heptadecane, 3-methyl-	254	C18H38	1370.2	0.17381
24	Tridecane, 3-methylene-	196	C14H28	1380.1	0.12666
25	Cetene	224	C16H32	1384.8	4.0166
26	Nonadecane	268	C19H40	1392.8	23.394
27	Isopropyl myristate	270	C17H34O2	1401.5	0.12224
28	Heptadecane, 2,3-dimethyl-	268	C19H40	1410.2	0.063733
29	Undecane, 2-methyl-	170	C12H26	1425.8	0.1304
30	Octadecane, 4-methyl-	268	C19H40	1437.5	1.0712
31	1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane	444	C13H40O5Si6	1437.9	1.0712
32	Sulfurous acid, hexyl pentadecyl ester	376	C21H44O3S	1441.5	0.19697
33	2-Dodecene, 4-methyl-	182	C13H26	1447.1	0.11667
34	Nonadecane, 2-methyl-	282	C20H42	1447.3	0.11667
35	Nonadecane	268	C19H40	1468.5	0.52396
36	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	292	C18H28O3	1474.9	1.0881
37	Pentadecanoic acid, 14-methyl-, methyl ester	270	C17H34O2	1475.2	1.0881
38	Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-	430	C12H38O5Si6	1478.3	0.15513
39	1,2-Benzenedicarboxylic acid, diundecyl ester	474	C30H50O4	1484	0.16082
40	Nonadecane, 9-methyl-	282	C20H42	1499	0.25209
41	Octane, 3-ethyl-	142	C10H22	1501.1	0.19507
42	Diethylcyanamide	98	C5H10N2	1504.1	0.10179
43	Heptadecane, 2-methyl-	254	C18H38	1506.6	0.20108
44	Eicosane, 2-methyl-	296	C21H44	1521.3	0.15443
45	2-Tetradecanone	212	C14H28O	1530.8	0.43051
46	1-Docosene	308	C22H44	1534.6	2.8525

47	Heneicosane	296	C21H44	1541.3	12.543
48	Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-	430	C12H38O5Si6	1553.5	0.16941
49	Tetradecane, 1-iodo-	324	C14H29I	1556.7	0.087493
50	2,3-Dimethyldecane	170	C12H26	1570.5	0.15951
51	Decane, 2,4,6-trimethyl-	184	C13H28	1574	0.094961
52	Octadecane, 4-methyl-	268	C19H40	1582.3	0.50528
53	Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-	430	C12H38O5Si6	1585.7	0.054987
54	Methyl cis-2-trimethylsilyl-cyclopropane-1-carboxylate	172	C8H16O2Si	1587.4	1.0945
55	1-Nonadecene	266	C19H38	1588.4	1.1101
56	Monomethyl phthalate, TMS derivative	252	C12H16O4Si	1590.4	0.33019
57	Octane, 3,4-dimethyl-	142	C10H22	1591.6	0.21259
58	Silane, trimethyl[2-methylene-1-(4-pentenyl)cyclopropyl]-	194	C12H22Si	1593.8	0.075093
59	Silanol, allyldimethyl-	116	C5H12OSi	1600.6	0.005091
60	Allyl(methoxy)dimethylsilane	130	C6H14OSi	1603.5	0.066287
61	1-Iodo-2-methylundecane	296	C12H25I	1610.9	0.28464
62	Cyclododecanol	184	C12H24O	1614.6	0.23685
63	Dodecane, 1,1-dimethoxy-	230	C14H30O2	1615.3	0.23685
64	Methyl stearate	298	C19H38O2	1618.6	0.51195
65	2-Buten-1-ol, (Z)-, TBDMS derivative	186	C10H22OSi	1621.9	0.3879
66	Nonadecane, 9-methyl-	282	C20H42	1637.9	1.3394
67	Ethyl(dimethyl)allyloxysilane	144	C7H16OSi	1638.6	1.347
68	Dotriacontane	450	C32H66	1645.8	0.29673
69	Silanol, trimethyl-	90	C3H10OSi	1647.6	0.29673
70	Hexasiloxane, tetradecamethyl-	458	C14H42O5Si6	1659.9	0.27473
71	Tridecane, 3-methylene-	196	C14H28	1669	0.095976
72	1-Docosene	308	C22H44	1672.3	1.1341
73	Heptacosane	380	C27H56	1677.9	3.5597
74	Ethyl(dimethyl)methoxysilane	118	C5H14OSi	1692.9	3.8187
75	Trimethylsilyl 3-methyl-4-[(trimethylsilyl)oxy]benzoate	296	C14H24O3Si2	1696.6	0.067035
76	Silanol, allyldimethyl-	116	C5H12OSi	1699.2	2.065
77	Trimethylsilyl 3-methyl-4-[(trimethylsilyl)oxy]benzoate	296	C14H24O3Si2	1700	2.065
78	Undecane, 2-methyl-	170	C12H26	1704.5	0.18635
79	Octadecane, 4-methyl-	268	C19H40	1716.1	0.24343

80	Dodecyl acrylate	240	C15H28O2	1731.4	0.29924
81	1-Pentamethyldisilyloxyhexadecane	372	C21H48OSi2	1736.1	0.094227
82	Heptyl N,N-diisopropylphosphoramidocyanidate	288	C14H29N2O2P	1741.1	0.14625
83	Heptacosane	380	C27H56	1742.5	0.15842
84	1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane	444	C13H40O5Si6	1756.9	0.24541
85	Sulfurous acid, hexyl tridecyl ester	348	C19H40O3S	1766.8	0.14916
86	Heptadecane, 2,6-dimethyl-	268	C19H40	1770	0.11933
87	Di-trimethylsilyl peroxide	178	C6H18O2Si2	1786.3	0.41666
88	Phthalic acid, octyl tridec-2-yn-1-yl ester	456	C29H44O4	1793.6	0.20076
89	Trimethylsilyl 3-methyl-4-[(trimethylsilyl)oxy]benzoate	296	C14H24O3Si2	1795.3	0.20393
90	Trimethylsilyl 3-methyl-4-[(trimethylsilyl)oxy]benzoate	296	C14H24O3Si2	1798	0.070117
91	1-Docosene	308	C22H44	1801.1	0.34287
92	Heptacosane	380	C27H56	1805.6	1.0903
93	1,1,3,3-Tetramethyl-1,3-di-n-propyldisiloxane	218	C10H26OSi2	1806.6	1.0903
94	Sulfurous acid, 2-pentyl undecyl ester	306	C16H34O3S	1842.2	0.10368
95	Hexasiloxane, tetradecamethyl-	458	C14H42O5Si6	1847.4	0.13125
96	Tetracosane	338	C24H50	1867.6	0.2847
97	Sulfurous acid, decyl 2-propyl ester	264	C13H28O3S	1889.9	0.076868
98	Di-trimethylsilyl peroxide	178	C6H18O2Si2	1894.6	2.907
99	Diisooctyl phthalate	390	C24H38O4	1895.6	3.0598
100	Tetracosane	338	C24H50	1929.2	0.31533
101	Hexasiloxane, tetradecamethyl-	458	C14H42O5Si6	1933.4	0.080129
102	Acetic acid, [(2,4,6-triethylbenzoyl)thio]-	280	C15H20O3S	1976.2	0.016091
103	Dotriacontane	450	C32H66	1992.2	0.090721
104	Hexasiloxane, tetradecamethyl-	458	C14H42O5Si6	2017	0.030654

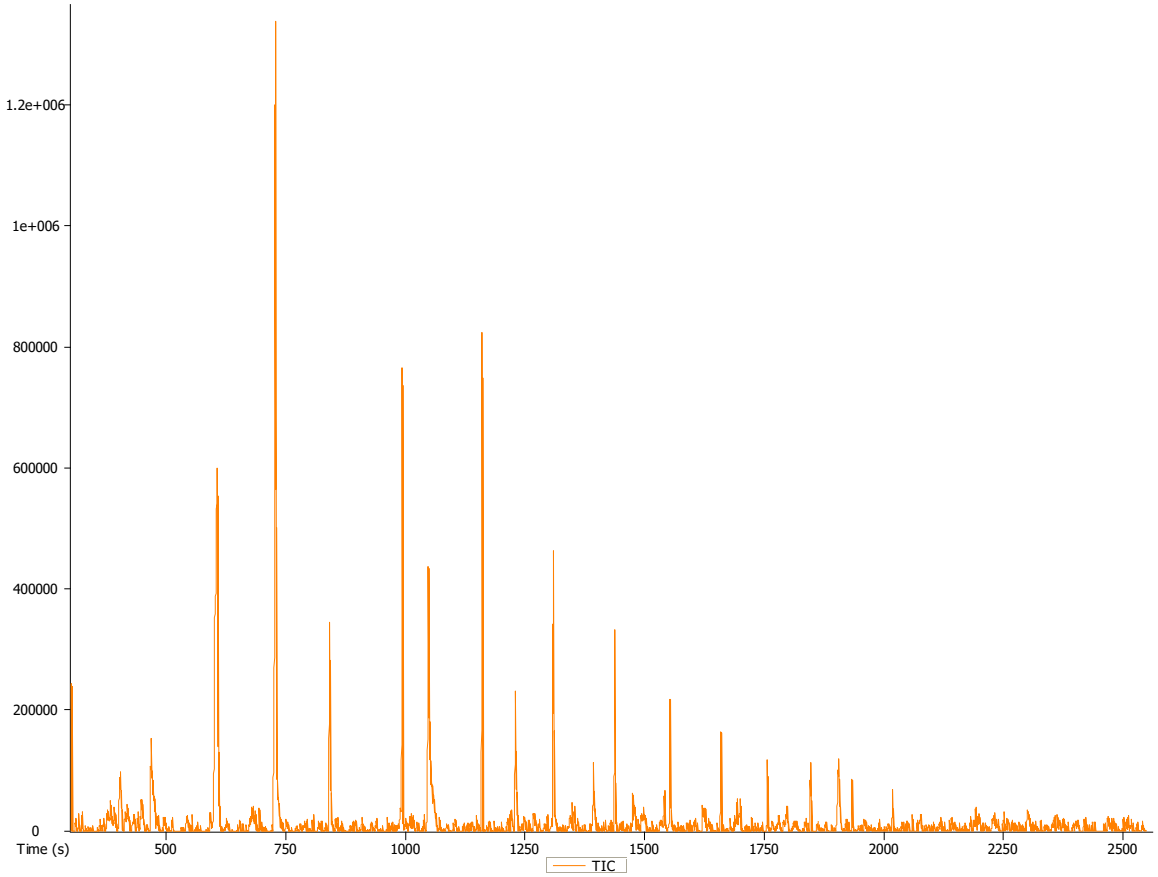


Appendix XXVIII: Representative Total Ion Chromatograph (TIC) of fraction F8.

Table VIII: Peak Report TIC (Fraction F8).

Peak	Name	Weight	Formula	Retention Time (s)	Area %
1	Methyl isovalerate	116	C6H12O2	302.1	1.8962
2	Butanoic acid, 2-methyl-, methyl ester	116	C6H12O2	303.4	1.8962
3	Butanoic acid, 2-methyl-, methyl ester	116	C6H12O2	311.1	0.17034
4	Tetramethyl silicate	152	C4H12O4Si	316.6	0.25811
5	Benzene, 1,3-dimethyl-	106	C8H10	392.3	0
6	p-Xylene	106	C8H10	403.4	1.6729
7	3-Methylbenzyl alcohol, TBDMS derivative	236	C14H24OSi	433.4	0.056137
8	p-Xylene	106	C8H10	449.3	0.52661
9	Benzyloxydimethyloctylsilane	278	C17H30OSi	451.2	0.60547
10	Decane	142	C10H22	606.6	17.705
11	Undecane	156	C11H24	728.3	16.487
12	Undecane	156	C11H24	841.8	4.0806

13	Cyclohexasiloxane, dodecamethyl-	444	C12H36O6Si6	993.4	1.7434
14	Tetradecane	198	C14H30	1047.8	8.1702
15	Trisiloxane, 1,1,1,5,5,5-hexamethyl-3,3-bis[(trimethylsilyl)oxy]-	384	C12H36O4Si5	1160.5	1.0784
16	Hexadecane	226	C16H34	1229.5	4.6091
17	1,1,1,3,5,7,7,7-Octamethyl-3,5-bis(trimethylsiloxy)tetrasiloxane	458	C14H42O5Si6	1309.3	0.20204
18	5-Eicosene, (E)-	280	C20H40	1385.4	0.71254
19	Nonadecane	268	C19H40	1392.8	7.3073
20	3-Pentanol, 2,4-dimethyl-	116	C7H16O	1437.9	0.49141
21	Dodecanoic acid, methyl ester	214	C13H26O2	1476.5	2.1429
22	1-Docosene	308	C22H44	1534.9	1.319
23	Heneicosane	296	C21H44	1541.3	5.9637
24	Silane, trimethyl[2-methylene-1-(4-pentenyl)cyclopropyl]-	194	C12H22Si	1553.5	0.44621
25	Methyl tetradecanoate	242	C15H30O2	1619.6	2.3765
26	Succinic acid, 2,4-dimethylpent-3-yl ethyl ester	244	C13H24O4	1622.2	0.69218
27	Ethyl(dimethyl)allyloxysilane	144	C7H16OSi	1638.8	3.0393
28	Heptadecane, 2-methyl-	254	C18H38	1678.1	2.4156
29	Ethyl(dimethyl)methoxysilane	118	C5H14OSi	1693.2	5.9896
30	Ethanol, 2-(trimethylsilyl)-	118	C5H14OSi	1700.3	1.4303
31	Di-trimethylsilyl peroxide	178	C6H18O2Si2	1786.6	0.021931
32	Phthalic acid, (2-chlorocyclohexyl)methyl octyl ester	408	C23H33ClO4	1794.5	0.1593
33	Tetracosane	338	C24H50	1806.3	0.73529
34	Heptacosane	380	C27H56	1868.7	0.54893
35	Diisooctyl phthalate	390	C24H38O4	1899.4	3.0504



Appendix XXIX: Representative Total Ion Chromatograph (TIC) of fraction C9.

Table IX: Peak Report TIC (Fraction C9).

Peak	Name	Weight	Formula	Retention Time (s)	Area %
1	Butanoic acid, 2-methyl-, methyl ester	116	C6H12O2	302.3	1.8545
2	Tetramethyl silicate	152	C4H12O4Si	306.9	5.76E-06
3	Butanoic acid, 2-methyl-, methyl ester	116	C6H12O2	311.3	0.17678
4	Tetramethyl silicate	152	C4H12O4Si	316.6	0.25309
5	Ethylbenzene	106	C8H10	392.6	0.34239
6	o-Xylene	106	C8H10	403.4	2.2459
7	3-Methylbenzyl alcohol, TBDMS derivative	236	C14H24OSi	433.4	0.23602
8	o-Xylene	106	C8H10	449.3	0.72139
9	Benzyl alcohol, TBDMS derivative	222	C13H22OSi	450.6	0.72139
10	Ethanol, 2-butoxy-	118	C6H14O2	467.8	3.8594
11	Decane	142	C10H22	606.6	18.238
12	Undecane	156	C11H24	728.3	18.866

13	Cyclopentasiloxane, decamethyl-	370	C10H30O5Si5	807.7	0.18333
14	Tetradecane	198	C14H30	841.8	3.8626
15	Cyclohexasiloxane, dodecamethyl-	444	C12H36O6Si6	993.4	6.7864
16	Tetradecane	198	C14H30	1047.8	8.0542
17	Trisiloxane, 1,1,1,5,5,5-hexamethyl-3,3-bis[(trimethylsilyl)oxy]-	384	C12H36O4Si5	1160.5	6.5849
18	Hexadecane	226	C16H34	1229.9	3.3746
19	Hexasiloxane, tetradecamethyl-	458	C14H42O5Si6	1309.3	4.5703
20	Heptadecane, 2-methyl-	254	C18H38	1393.4	1.7111
21	1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane	444	C13H40O5Si6	1437.9	2.8447
22	2,6-Dihydroxybenzoic acid, 3TMS derivative	370	C16H30O4Si3	1475.1	0.63309
23	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	292	C18H28O3	1475.8	0.63309
24	Tridecanoic acid, methyl ester	228	C14H28O2	1478.7	0.3268
25	Eicosane	282	C20H42	1541.9	0.89249
26	Trisiloxane, 1,1,1,5,5,5-hexamethyl-3,3-bis[(trimethylsilyl)oxy]-	384	C12H36O4Si5	1553.5	2.1854
27	Monomethyl phthalate, TBDMS derivative	294	C15H22O4Si	1590.7	0.13354
28	Monomethyl phthalate, TMS derivative	252	C12H16O4Si	1594.7	0.1338
29	Hexadecanoic acid, 15-methyl-, methyl ester	284	C18H36O2	1621.5	0.5283
30	Hexasiloxane, tetradecamethyl-	458	C14H42O5Si6	1659.9	1.4194
31	Methanol, TBDMS derivative	146	C7H18OSi	1693.9	0.48001
32	Trimethylsilyl 3-methyl-4-[(trimethylsilyl)oxy]benzoate	296	C14H24O3Si2	1700.1	0.65767
33	1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane	444	C13H40O5Si6	1757	1.0694
34	Hexasiloxane, tetradecamethyl-	458	C14H42O5Si6	1847.7	1.0458
35	Diisooctyl phthalate	390	C24H38O4	1905.3	2.8086
36	Hexasiloxane, tetradecamethyl-	458	C14H42O5Si6	1934.3	0.84411
37	Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-	430	C12H38O5Si6	2018.8	0.72142