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Antiplasmodial and antigonococcal bioactivity of *Tabernaemontana elegans* Stapf. (Apocynaceae) stem bark fractions

by

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DECLARATION

I, **Victor Nhlakanipho Nzama**, declare that the thesis, which I hereby submit for the degree Master of Science in Medicinal Plant Science at the University of Pretoria, is my work and has not previously been submitted by me for a degree in this or any other tertiary institution.

Signature : Victor Nhlakanipho Nzama

Date : 09 – 11 – 2023

DEDICATION

This thesis is dedicated to my grandparents Mdubane and Ntombifuthi Magubane, my mother Fikile, my aunts CJay and Samkelisiwe, and my uncle Nkosinathi. Thank you for your love and for always believing in me. I could never have asked for a greater family.

Lastly, to a boy who grew up in Umlazi Township, Durban. He was always fascinated by the wonders of the natural world. Little did he know that one day he would become a science teacher and a scientist studying medicinal plants. I am proud of the boy you were and of the man you have grown into. I dedicate this thesis to my younger self.

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ABSTRACT

Malaria and gonorrhoea are severe infectious diseases in sub-Saharan Africa (SSA), with pregnant women predisposed to health risks due to a co-infection with both diseases. These infectious diseases are associated with poor pregnancy outcomes owing to growth retardation, preterm birth, and miscarriages. The treatment of such ailments is with drugs, although drug availability and accessibility differ across economic and social levels, especially in some parts of SSA where access to primary healthcare services is still a challenge. The emergence of pathogen resistance has made it difficult to treat malaria and gonorrhoea. Therapeutics with varied modes of action must be developed to destroy the evolving infectious agents. Medicinal plants are the most likely source of novel drug leads and are extensively used to develop drugs through ethnopharmacology-based means. Traditional medicine is used in the treatment of a wide range of diseases in many developing countries, and South Africa is no exception. Several cultural groups in the country use numerous plant species from the vast biodiversity for medicinal purposes.

Tabernaemontana elegans Stapf. (Apocynaceae) was evaluated for antiplasmodial, antigonococcal, and cytotoxic activities. The methanol crude extract of the stem bark was subjected to column chromatographic fractionation using a series of solvents with increasing polarity; hexane (H): dichloromethane (DCM) at ratios of 10:0 → 0:10 (v/v), ethyl acetate (EA): methanol (MeOH) at ratios of 10:0 → 0:10 (v/v), and distilled water (dH_2O). Thin layer chromatography (TLC) was used to analyze fractions, and those with similar chemical profiles were combined to yield 11 fractions (A – K).

To further separate the fractions into samples comprising of a smaller number of compounds, fraction F, G, I, and J was further subjected to column chromatography and fractionated with a volume of 100 ml each of DCM, DCM and MeOH (1:1, v/v), and MeOH. Analysis using TLC was applied and fractions with comparable chemical profiles were pooled together to yield 27 fractions in total. The fractions were assayed for *in vitro* antiplasmodial and antigonococcal activities against *Plasmodium falciparum* NF54 and *Neisseria gonorrhoeae* ATCC 19424, respectively. Cytotoxicity was carried out on rat skeletal muscle cell lines L6, and selectivity indices (SI) were determined. The fractions were then subjected to proton nuclear magnetic resonance (^{1}H NMR) and gas chromatography-mass spectrometry (GC-MS) analyses to tentatively identify classes of compounds and phytoconstituents for the observed antiplasmodial and antigonococcal biological activities and to dereplicate known antiplasmodial compounds. Metabolomics using GC-MS was employed to discriminate between selective and non-selective antiplasmodial fractions.

Of the 27 assayed fractions, J_4 and J_5 had significant antiplasmodial activity at IC_{50} of 0.20 µg/ml and 0.10 µg/ml, respectively. The SI for J_4 and J_5 were 118 and 165, respectively. Compared to the methanol crude extract of the stem bark of *T. elegans* from which they were fractionated, J_4 and J_5 demonstrated remarkably higher antiplasmodial efficacy against the NF54 strain of *P. falciparum*. The crude extract was active at an IC_{50} of 0.31 µg/ml with an SI of 79. Other fractions that showed significant activity were J_6 and J_7 at IC_{50} of 0.32 µg/ml and 0.96 µg/ml, respectively. The SI of J_6 was 163 and that of J_7 was too high to be determined. Fractions J_4 – J_7 were all non-toxic to the rat skeletal muscle cells. Fractionation of the crude extract enhanced the antiplasmodial activity and SI

values. Since the four fractions are consecutive, the antiplasmodial activity observed could be due to the same compound(s). Intermediately polar fractions displayed the highest antiplasmodial activity compared to relatively non-polar or polar ones.

After stacking and analyzing the chromatograms of ^{1}H NMR spectra of fractions J_4 – J_7 for similar chemical shifts, their chemical profiles were identified to reflect in the 0.5 – 1 ppm region. This section is represented by the class of compounds known as aliphatic hydrocarbons, and may have contributed to the antiplasmodial activity and non-toxicity of the fractions of *T. elegans.* Analysis of the GC-MS data for fractions J_4 – J_7 revealed the presence of dodecane, pentadecane, and heptadecane phytoconstituents. All three compounds are alkanes (aliphatic hydrocarbon constituents). Dodecane and heptadecane were isolated from a plant with established antiplasmodial activity. Heptadecane has been identified as potentially effective against *P. falciparum* NF54. The antiplasmodial activity of the fractions of *T. elegans* can be correlated with the results of the ^{1}H NMR and GC-MS data analyses.

Metabolomics using GC-MS was performed on 25 fractions. The unsupervised Principal Component Analysis model could not distinguish between selective and non-selective antiplasmodial fractions. This resulted in applying the Supervised Orthogonal Projections to Latent Structures-Discriminant Analysis (OPLS-DA). The OPLS-DA model successfully discriminated between selective and non-selective antiplasmodial fractions, with R^2 and Q^2 values of 0.472 and 0.396, respectively. The model was, however, demonstrated to be an unreliable and non-robust prediction tool for unknown antiplasmodial samples, with T-test P = 0.592. According to the contribution plot generated from the OPLS-DA model, icosane and hexadecane were identified to have greatly contributed to the observed

variations. Alkanes icosane and hexadecane were detected in medicinal plants with antiplasmodial efficacy. The dereplication of antiplasmodial compounds is aided by ^{1}H NMR and GC-MS, which work in tandem. In the exploration of novel compounds with varied action modes, priority should be placed on isolating and characterizing formerly unstudied phytoconstituents with activity. Compound dereplication is important since it avoids the unnecessary isolation of phytoconstituents previously isolated.

Fractions I_1 and I_4 had noteworthy antigonococcal activity with a minimum inhibitory concentration (MIC) of 0.78 mg/ml among the 17 fractions assayed. The two fractions showed reduced efficacy against *N. gonorrhoeae* ATCC 19424 when compared to the methanol crude extract from which they were fractionated. The crude extract was active at a MIC of 0.20 mg/ml. Fractionation of the crude extract diminished the antigonococcal activity. Relatively non-polar fractions exhibited the highest antigonococcal activity than intermediately polar or polar fractions. The aliphatic hydrocarbon class of compounds could have contributed to the antigonococcal efficacy since they have established antibacterial activity.

The fractions of the methanol crude extract of the stem bark of *T. elegans* are great candidates for malarial and gonococcal drugs as shown in this study. Further research should focus on the isolation, purification, and characterization of compounds derived from active fractions that are responsible for the observed antiplasmodial and antigonococcal biological activities.

LIST OF ABBREVIATIONS

ACT	Artemisinin Combination Therapy	MeOH	Methanol
AIDS	Acquired Immunodeficiency Syndrome	MIC	Minimum Inhibitory Concentration
CC	Column Chromatography	OPLS-DA	Orthogonal Projections to Latent Structures – Discriminant Analysis
CFU	Colony Forming Units	PCA	Principal Component Analysis
DCM	Dichloromethane	SI	Selective index
dH_2O	Distilled Water	STI	Sexually Transmitted Infections
GC – MS	Gas Chromatography-Mass Spectrometry	TLC	Thin Layer Chromatography
H	Hexane		
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid		
INT	Iodonitrotetrazolium		
IC_{50}	Half Maximal Inhibitory Concentration		
HIV	Human Immunodeficiency Virus		
^1H NMR	Proton Nuclear Magnetic Resonance		
MDA	Multivariate Data Analysis		

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CHAPTER 1
General introduction

1.1. Problem statement

The challenges of infectious diseases and the subsequent mortality caused by these diseases are apparent in both developed and developing countries around the world (Bloom and Cadarette, 2019; World Health Organization (WHO), 2020), with developing countries bearing the brunt of the burden. Persistent poverty and disadvantaged economies in Africa heighten the load of infectious diseases, particularly in the sub-Saharan region (Wang et al., 2021). Malaria and sexually transmitted infections (STIs) are common in sub-Saharan Africa (SSA), with pregnant women being notably vulnerable to serious health implications due to co-infection. The diseases result in infant mortality, premature birth, low birth weight (LBW), and miscarriages (Lingani et al., 2021). The region has the greatest neonatal mortality rate in the world (WHO, 2022). One of the leading causes of neonatal death is LBW (a birth weight < 2.5 kg) caused by intrauterine growth retardation, preterm birth, or both (Katz et al., 2013). Intrauterine infections that are responsible for the occurrence of LBW have been linked to malaria and sexually transmitted infections (Slyker et al., 2014). While malaria is strongly associated with intrauterine growth retardation, untreated STIs are related to prematurity (Rogerson et al., 2018). Preventing and managing malaria and STIs is imperative, particularly for expectant mothers (Amimo et al., 2020).

A great deal of the global malaria burden (cases and deaths) occurs in Africa, with *Plasmodium falciparum* as the most dominant species in the continent (Hassett and Roepe, 2021; WHO, 2022). The use of artemisinin monotherapy in the treatment of uncomplicated *P. falciparum* malaria has been discontinued. Artemisinin-based combination therapies (ACTs), which combine artemisinin and a companion drug, are the conventional treatment for uncomplicated malaria. However, malarial drug resistance has been reported in *P. falciparum* to ACTs in some parts of Africa like Burkina Faso and Angola (Romphosri et al., 2020). The success of ACTs is hampered by partner drug resistance (Suresh and Haldar, 2018). This warranted the inclusion of a second partner drug in triple artemisinin-based combination therapy, demonstrating how ACTs have to an extent become futile in the treatment of malaria.

Excess exposure to antibiotics, mainly owing to their misuse and overuse in healthcare facilities, is one of the primary contributors to the development of drug-resistant bacteria (Sepulveda and Wilson, 2019). Antibiotic resistance occurs when bacteria develop tolerance to a drug intended to destroy them. This signifies that the drug will no longer be effective in treating that bacterial infection, as in the case of using some antibiotics to treat gonorrhoea caused by *Neisseria gonorrhoeae*. Challenges in treating gonorrhoea with azithromycin, ciprofloxacin, tetracycline, and penicillin have been observed since 2006 (Da Costa-Lourenco et al., 2018).

The issue of drug resistance extends the burden of malaria and gonorrhoea. It impedes the health sector by limiting treatment options, raising treatment costs, prolonging hospital stays, and increasing the likelihood of incapacitation of the patient and mortality (WHO, 2020). There is also a possibility of adverse effects as health professionals adopt harsher,

potentially toxic treatments. This necessitates the ongoing development of new effective chemotherapeutic regimens.

Plants have a long history of use as medicines due to their accessibility, affordability, and relative safety. Regardless of this, ethnopharmacological studies for most of these plants are still limited. The need for bioprospecting medicinal plants for novel antiplasmodial and antigonococcal pharmaceuticals with multifunctional biological capabilities to treat malaria and gonorrhoea is at a record-high level. South Africa is considered a biodiversity hotspot since it is home to several plant species, accounting for 10% of all species in the world (Sobiecki, 2014). Plants have been documented in various cultural groups' *materia medica* across the country (Van Wyk, 2011; De Wet et al., 2013; Xego et al., 2016), and have been employed in traditional medicine to treat a variety of diseases such as malaria and gonorrhoea.

African traditional medicine systems are marked by the oral practice of sharing information about the use of medicinal plants from multiple cultural knowledge systems from one generation to the next and as a result, they are barely recorded. Establishing existing information through ethnomedicinal documentation and ascertaining the therapeutic applications of plants through scientific evaluation is a matter of great importance. This will prevent the information from disappearing along with its holders as they age.

Many commercially available drugs are created from secondary metabolites produced by different medicinal plants. Even with the success of drug discovery from medicinal plants, future efforts encounter numerous hurdles. The drug discovery process is predicted to

take 10 – 15 years and can cost up to US$ 2.6 billion (Derep, 2022). A fair amount of this investment of time and money is spent on the countless leads that are abandoned during the process. Only one out of every ten drug candidates will complete clinical trials and regulatory approval (Mullard, 2016). Additional obstacles include the acquisition and verification of plant materials, the deployment of large-scale biological assays, and the expansion of the production of bioactive lead compounds. The challenge of continuous isolation of known compounds with confirmed bioactivity further hinders the process. Analytical techniques used in metabolomics such as proton nuclear magnetic resonance (^{1}H NMR) and gas chromatography-mass spectrometry (GC-MS) can be powerful in accelerating the speed of drug discovery from medicinal plants.

1.2. Aim of the study

The study aimed to investigate the antiplasmodial, antigonococcal, and cytotoxic activities of fractions from the methanol crude extract of the stem bark of *Tabernaemontana elegans*.

1.3. Research questions of the study

- Can fractionation of the methanol crude extract of the stem bark of *T. elegans* enhance the antiplasmodial and antigonococcal activities?
- Can GC-MS metabolomics discriminate between selective and non-selective antiplasmodial fractions of *T. elegans*?

1.4. Hypotheses of the study

- Fractionation of the methanol crude extract of the stem bark of *T. elegans* will yield enhanced antiplasmodial and antigonococcal active fractions.
- Metabolomics approaches using GC-MS will distinguish between selective and non-selective antiplasmodial fractions of *T. elegans*.

1.5. Objectives of the study

- To fractionate the methanol crude extract of the stem bark of *T. elegans* using various solvent systems.
- To assay the fractions for antiplasmodial, antigonococcal, and cytotoxic activities.
- To conduct ^{1}H NMR analysis to identify the classes of compounds responsible for the antiplasmodial and antigonococcal activities.
- To dereplicate established antiplasmodial phytoconstituents from unknown ones.
- To perform GC-MS metabolomics to distinguish between selective and non-selective antiplasmodial fractions.

1.6. Structure of the thesis

This thesis is written in the format of the South African Journal of Botany.

Chapter 1: The problem statement highlighting the burden of malaria and STIs, and the motivation for the current study, are described in this chapter. The aim, research questions, hypotheses, objectives of the study, and structure of the thesis are also covered.

Chapter 2: A brief review of the literature on malaria and sexually transmitted infections (with a focus on gonorrhoea) is provided. This chapter also covers the taxonomy, description, flowering season, distribution, ethnomedicinal uses, phytochemistry, and pharmacology of *T. elegans*, as well as the possibility of metabolomics to aid in the discovery of novel plant chemicals.

Chapter 3: The antiplasmodial, antigonococcal, and cytotoxic activities of fractions of the stem bark of *T. elegans*, together with ^{1}H NMR analysis and GC-MS metabolomics, are discussed in this chapter.

Chapter 4: This chapter presents the current study's general conclusion, challenges, and prospects.

Chapter 5: This chapter contains supplemental information about the study.

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CHAPTER 2
Literature review

2.1. Introduction

Infectious diseases caused by pathogenic microorganisms, such as parasites and bacteria, have become a global pandemic. The alarming rate of the number of newly acquired infections is on the rise, owing to pharmacological ineffectiveness in treating infectious diseases. *Plasmodium falciparum* and *Neisseria gonorrhoeae* cause a substantial burden globally, particularly in sub-Saharan Africa (SSA). Drug resistance to pathogens has prompted scientists to seek other therapies such as herbal remedies in the treatment of infectious diseases.

A review of the literature on malaria and sexually transmitted infections with a focus on gonorrhoea is presented in this chapter. The taxonomy, description, flowering season, distribution, ethnomedicinal uses, phytochemistry, and pharmacology of *Tabernaemontana elegans* Stapf. (Apocynaceae) are also discussed, as well as the potential of plant metabolomics to aid in the discovery and development of novel drugs.

2.2. The global burden of malaria

Human malaria is caused by five main protozoan parasite species; *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*. The distribution of *Plasmodium* species varies according to geography. *Plasmodium falciparum*, which is found in SSA, South East Asia, and South America, causes the most severe form of malaria and has the greatest mortality rate (World Health Organization (WHO), 2022).

In South America, *P. vivax* outnumbers *P. falciparum,* and both species are prevalent in South East Asia (Autino et al., 2012). *Plasmodium malariae* is found in all major areas of the world where malaria is endemic, and its distribution overlaps that of *P. falciparum*, and its highest transmission is across SSA. *Plasmodium ovale* has a far more restricted range, with endemic transmission in Africa, eastern Indonesia, and the Philippines (WHO, 2022). Nearly all countries in South East Asia have reported the presence of *P. knowlesi* (Yusof et al., 2014).

Most malaria-endemic countries are in tropical regions (Figure 2.1). These regions have hotter, wetter, and more humid weather than countries in the middle latitudes/temperate zones and the polar areas. With two distinct seasons throughout the year – the wet/rainy season, which see most of the rainfall, and the dry season – most tropical regions see year-round temperatures of 18°C or higher (Climate Science, 2022). *Plasmodium falciparum* cannot complete its development cycle at temperatures below 20°C and hence cannot be transmitted (Centers for Disease Control and Prevention, 2020). The presence of high temperatures, rainfall, and humidity in these areas promotes the survival of the mosquitoes of the *Anopheles* genus which are the vectors of the *Plasmodium* species, and allows the parasites to flourish (Traoré et al., 2020). Africa is the most tropical region because the equator, as well as the Tropics of Cancer and Capricorn, pass through the centre of the continent (World Meteorological Organization, 2020). Countries in SSA reside inside this tropical zone, allowing mosquitoes and parasites to thrive.

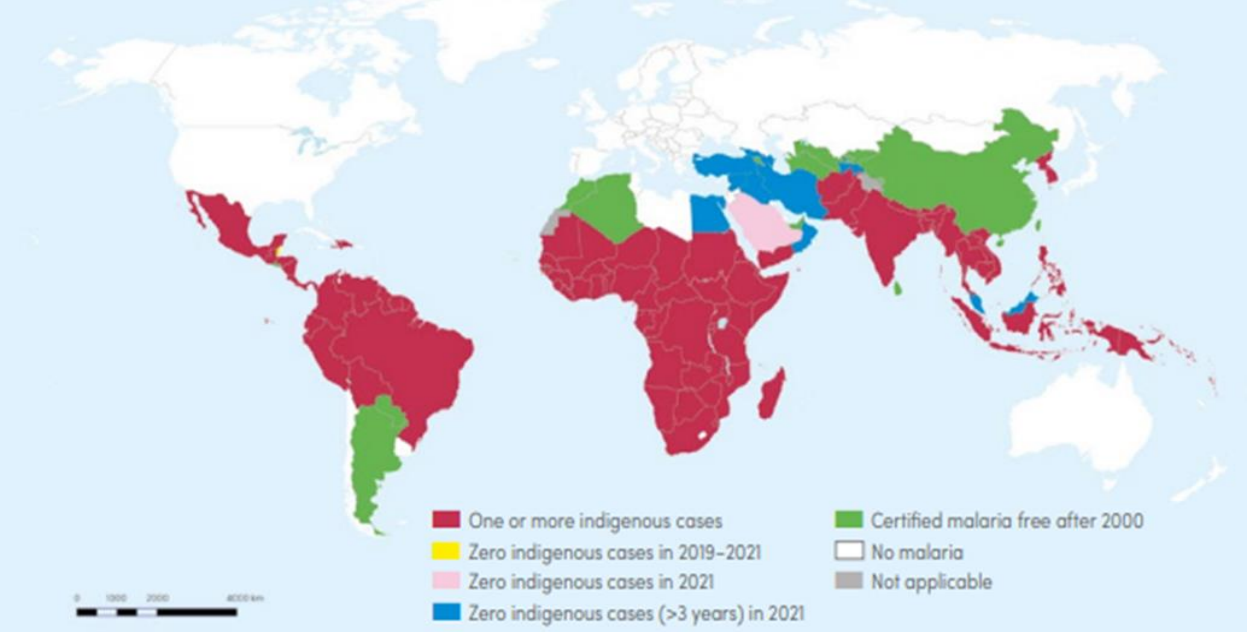


Figure 2.1. Global malaria distribution map showing the affected countries and the current number of cases (WHO, 2022). All countries in sub-Saharan Africa had at least one or more reported cases of malaria in 2021.

Malaria appears to have a complicated relationship with climate change. The impacts of climate change will increase the likelihood of malaria transmission in formerly malarial areas, in places where the disease has been suppressed, and in new, previously non-malarial locations (Ryan et al., 2020). Increases in temperature, precipitation, and humidity may result in the *Anopheles* mosquito populations at higher altitudes, which might culminate in an upsurge in malaria transmission in previously non-malarial areas (Zermoglio et al., 2019; Ryan et al., 2020). In lower altitudes where malaria is already a challenge, warmer temperatures will disturb the parasite's life cycle and allow it to develop quicker, boosting transmission and influencing the disease load (Nissan et al., 2021).

Community capacity to prevent and treat malaria influences the number of cases and deaths (Adeola et al., 2016). The Democratic Republic of the Congo, Niger, Nigeria, and the United Republic of Tanzania were responsible for slightly more than half of all global malaria cases and deaths in 2021 (WHO, 2022). It is hardly astonishing that countries in SSA are struck the hardest given that the region is home to 17 of the world's 28 most resource-scarce countries (Institute for Economics and Peace, 2020). Governments in these nations simply do not have the financial means for malaria education, prevention, and treatment efforts. Malaria control initiatives have been hampered by a lack of resources and socioeconomic instability, especially in African countries.

Ensuring that essential medicines are readily available, affordable, safe, effective, and of high quality poses a significant challenge in Africa (Yenet et al., 2023). The availability of medicines, which is the second most expensive resource after human necessities and makes up 20 – 60% of the national budgets of some countries, is still far below the WHO-defined target in the continent (WHO, 2020). In most African countries, medicine availability does not exceed 65% of the population, and the costs of purchasing medicines are prohibitive (Zuma and Modiba, 2019; WHO, 2020; Oridanigo et al., 2021). The inability to afford an appropriate variety of necessary medicines adds significantly to the malaria burden.

Malaria disease has the potential to affect approximately 50% of the human global population, with 247 million cases and 619 000 deaths currently recorded, and SSA accounts for nine out of ten of those cases and deaths (WHO, 2022). While *Plasmodium* species have varied geographical ranges, there is substantial overlap. Thus, individuals can be infected with many species at the same time, especially among returned travelers

(Lalloo, 2020). Falciparum malaria is caused by *P*. *falciparum*. Malaria induced by *Plasmodium* species other than *P. falciparum* is known as non-falciparum malaria. Mono-infections with one of the *Plasmodium* species are the most common, though mixed infections within one individual can occur and the interaction between contemporaneous species may affect the course and outcome of the disease (Kotepui et al., 2020). Mixed infections, in which more than one *Plasmodium* species is present in the blood or liver stage of infection at the same time, can have different clinical presentations since different species have distinct medical manifestations and therefore require different treatments. *Plasmodium malariae* and *P. ovale* often occur with *P. falciparum*. In low-transmission areas of SSA, it is anticipated that a person will experience one to three episodes of malaria infection per year (Rono et al., 2015). During the COVID-19 pandemic, an estimated 13.4 million additional cases and 63 000 deaths were linked to disruptions in key malaria services between 2019 and 2021 worldwide (WHO, 2022). Although malaria-related mortality decreased marginally in 2021 (WHO, 2022), efforts to eliminate and eradicate the disease are still required.

A higher risk of malaria infection exists in infants, expectant mothers, and people with human immunodeficiency virus (HIV) or acquired immunodeficiency syndrome (AIDS). Children between the ages of six months to five years have lost their maternal immunity and have not developed specific immunity to infections (Schumacher and Spinelli, 2012). In SSA, children under the age of five accounted for nearly 80% of all the global malaria deaths currently reported (WHO, 2022), especially in hyperendemic areas where *P. falciparum* attacks in children can occur every four to six weeks over many years (Ndungu et al., 2015). Transmission in SSA is frequently possible year-round because of

local weather conditions. Women are exposed to malaria during their first or second pregnancy since they lack adequate immunity to the parasites' surface proteins expressed on the surface of infected red blood cells, allowing infected cells to hide in the placenta throughout the pregnancy (Lennartz et al., 2019; Wang et al., 2021). White blood cells in the body are attacked by HIV, which weakens the immune system and increases susceptibility to disease in humans (WHO, 2023). Acquired immunodeficiency syndrome is the most advanced form of HIV where a person's immune system is suppressed, causing health decline. Symptoms of malaria include a high temperature, weariness, headache, muscle aching, nausea, abdominal discomfort, and profuse sweating. In more severe cases and cases of lengthy sickness without treatment, brain tissue injury, lung edema, kidney failure, severe anemia, yellow skin coloring, and low blood sugar might occur (Bittaye et al., 2022; WHO, 2022).

Malaria incidence is often lower in urban regions than in rural areas (Wilson et al., 2015). Differences in malaria prevalence between urban and rural locations are explained in part by fewer possibilities for mosquito vector reproduction and lower blood feeding on people in urban areas. Due to the scarcity of adequate breeding sites, urban settings are typically regarded as less favourable for *Anopheles* species. Furthermore, urban housing designs tend to minimize adult mosquito access to people at night, lowering transmission whereas in rural areas, houses are usually badly constructed, allowing vectors to enter easily (Gachelin et al., 2018). Since water serves as breeding grounds for mosquitoes, distances from bodies of water correspond with decreased malaria prevalence, as seen in urban settings (Target Malaria, 2022). Mosquitoes would be more prevalent in human settlements near to the water than in those further away. Hospitals and clinics are

sparsely located in rural areas. Access to primary healthcare services could considerably reduce the burden of malaria, and even a small increase in travel time to healthcare facilities could double the likelihood of hospitalization due to malaria infection (Lowassa et al., 2012). Some individuals with malaria are less motivated to treat the disease because of the increased travel time and distance to healthcare facilities in rural areas (Kavita et al., 2014), thereby further exacerbating the malaria burden.

2.2.1. Malaria in South Africa

The principal locations for malaria transmission in South Africa are the northern and north-eastern border regions (Figure 2.2). Along these border regions are the provinces of Limpopo, Mpumalanga, and KwaZulu-Natal. Malaria threatens about 10% of the South African population (National Department of Health (NDOH), 2018). Transmissions follow a seasonal pattern, with peaks from November to April during the rainy season (Burke et al., 2018). *Plasmodium falciparum* is the cause of almost all infections in the country with very small contributions from *P. ovale, P. malaiae*, and *P. vivax* (Maharaj et al., 2013). The primary vectors of malaria in South Africa are *Anopheles arabiensis* and *A. funestus* (Dahan-Moss et al., 2020), while *A. merus* and *A. vaneedeni* may act as secondary carriers (Burke et al., 2017; Mbokazi et al., 2018).

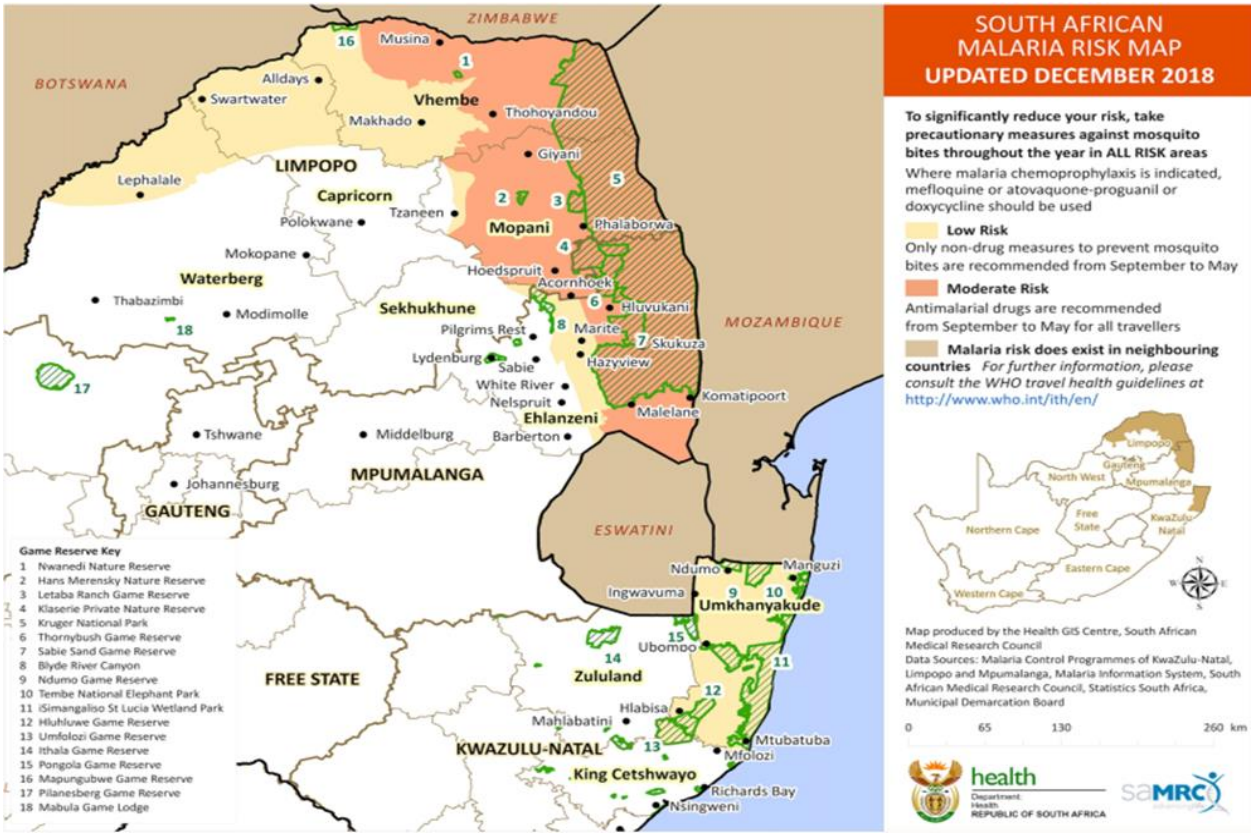


Figure 2.2. Malaria risk map of South Africa showing the endemic provinces of Limpopo, Mpumalanga, and KwaZulu-Natal with districts/municipalities (NDOH, 2018).

Much of the burden in the malarious provinces is caused by imported cases from neighbouring countries. Limpopo shares borders with Zimbabwe, Mpumalanga with Eswatini and Mozambique, and KwaZulu-Natal with Eswatini and Mozambique. For example, most malaria cases in KwaZulu-Natal and Mpumalanga were imported from adjacent countries in recent years between 2012 and 2017, and accounted for 72% and 82% of total cases in KwaZulu-Natal and Mpumalanga, respectively (NDOH, 2018). Since cross-border migration plays a significant role in transmission in South Africa, achieving national malaria elimination can benefit the country's regional efforts as well as global health security.

Even though the number of malaria cases has decreased, there have been significant fluctuations (Figure 2.3 and Figure 2.4) (NDOH, 2018; Aide et al., 2019). At least two major malaria outbreaks have occurred in South Africa in the last two decades. According to Maharaj et al. (2012), KwaZulu-Natal experienced the majority of the first outbreak in the early 2000s, which resulted in over 60 000 cases reported. In 2017, more than 28 000 cases were confirmed nationwide, primarily in Limpopo, accounting for most new malaria transmissions (Ravhuhali et al., 2017; WHO, 2020). In 2023, malaria cases significantly increased in Gauteng and malaria-endemic provinces (National Institute for Communicable Diseases, 2023). The majority of these cases are severe due to late presentation or detection. Some patients with malaria are undiagnosed and therefore untreated. Consequently, the disease quickly progresses to a serious illness with a potentially fatal outcome. Out of the three malarious provinces, only KwaZulu-Natal is on course to reach malaria elimination in the next few years (Maharaj et al., 2019, Tsoka-Gwegweni, 2022).

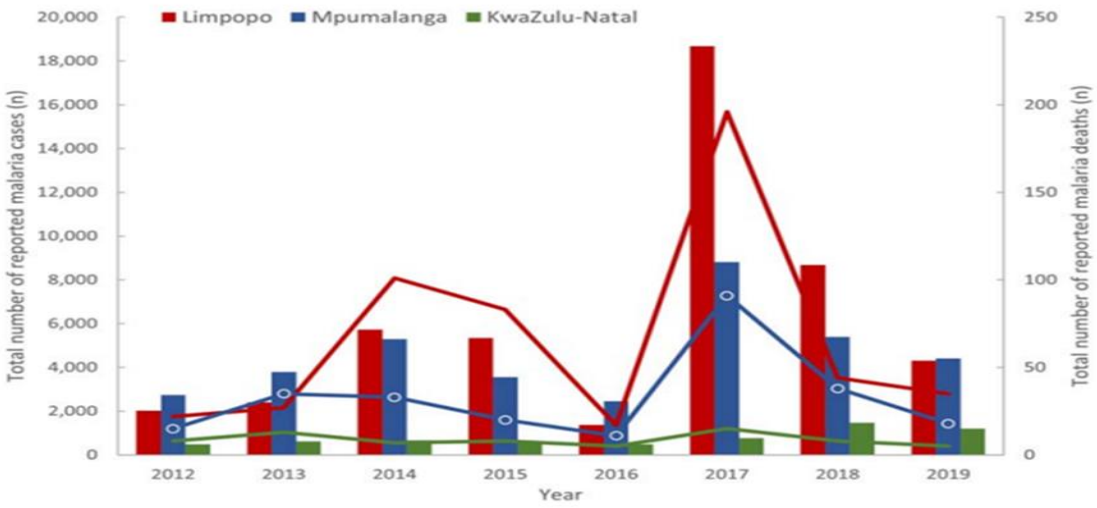


Figure 2.3. Malaria cases (bars) and deaths (lines) in South Africa's malaria-endemic provinces of Limpopo, Mpumalanga, and KwaZulu-Natal from 2012 to 2019 (NDOH, 2018). The year 2017 had the greatest number of cases and deaths.

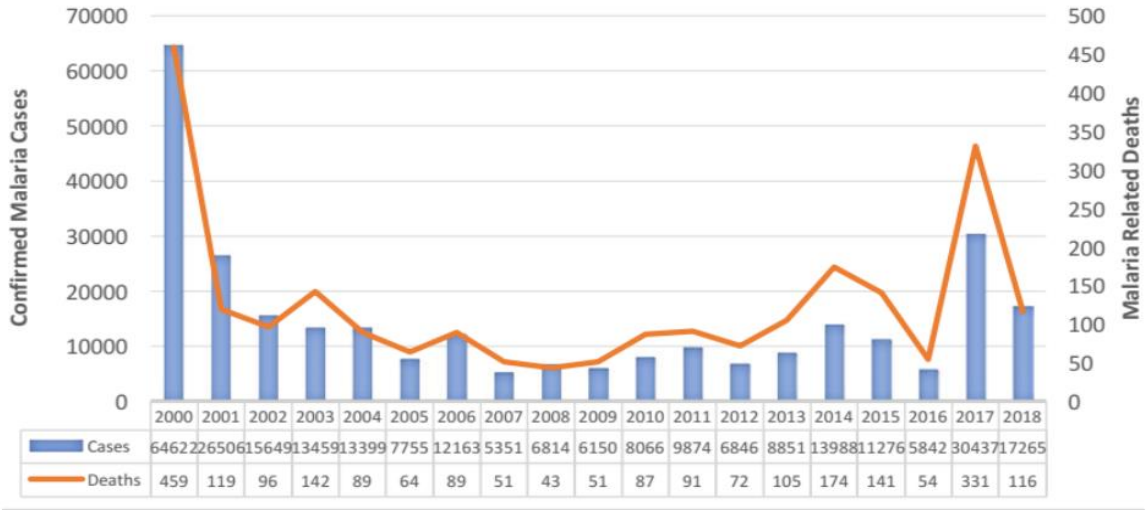


Figure 2.4. Malaria reported cases (bars) and deaths (line) in South Africa between 2000 and 2018 (Aide et al., 2019). The most cases and deaths occurred in the years 2000 and 2017.

South Africa has made significant progress in combating the malaria burden within its borders. The extensive history of the country's effective malaria control has led to a low incidence, resulting in extremely low cases (<1 case/1000 population at risk) (WHO, 2017). Accordingly, South Africa was designated by the WHO for malaria elimination, as described in the malaria strategic plan of the country for the period 2019 – 2023 (WHO, 2018, NDOH, 2019). Regrettably, given the prevalence of COVID-19, this objective might be unattainable.

The COVID-19 pandemic arrived in South Africa in March 2020 (Giandhari et al., 2020), causing widespread disruption to regional, provincial, and national malaria control efforts (Brooke et al., 2020). This complicated malaria detection and diagnosis, further impeding elimination initiatives (Wilairatana et al., 2021). As delayed health-seeking behaviour is common, most malaria cases in the country are detected on symptomatic presentation in public sector hospitals and clinics (Brooke et al., 2020). Individuals infected with the SARS-COV-2 virus, the agent responsible for the coronavirus disease, manifest similar symptoms as those with malaria. Fever, chills, fatigue, headache, as well as joint and muscle pain, are medical features of both malaria and COVID-19 in the early stages, and more severe presentations of malaria include respiratory distress and metabolic acidosis, which may be confused with COVID-19 pneumonia (Dittrich et al., 2020). Unquestionably, COVID-19 still has a high index of suspicion, which could explain the recent sudden rise of malaria cases in Gauteng and the malarious provinces since patients infected with the latter disease are misdiagnosed hence the late presentation and/or detection. The South African government should make compulsory that healthcare facilities screen patients for

both malaria and COVID-19 when presenting with symptoms that may point to one or the other disease to rule out misdiagnosis.

2.2.2. Life cycle of *Plasmodium falciparum*

The *Plasmodium* parasite has a complex life cycle involving two organisms; the female *Anopheles* mosquitoes and humans (Figure 2.5). Mosquitoes are the insect vectors of the parasites while humans are the vertebrate hosts. The parasite has two life stages; sexual and asexual. The sexual stage happens in mosquitoes while the asexual stage occurs in humans. All adult *Anopheles* mosquitoes ingest plant nectar to obtain glucose, which provides enough sustenance for both males and females to survive. The female mosquitoes, on the other hand, also consume blood meals from animals to receive the nutrients, including proteins, necessary for egg production (Mitchell and Catteruccia, 2017). Blood nutrients are absorbed into the eggs, which form in the ovaries 2 – 3 days following the blood feeding. When a mosquito bites a human carrying *Plasmodium* gametocytes, the production of eggs and parasite maturation occur concurrently (Costa et al., 2018).

Human malaria infection begins with a bite of an infected mosquito, injecting the sporozoites into the bloodstream of the human (Amoah et al., 2020). Around two weeks before erythrocyte penetration, the sporozoites mature into schizonts (*P. falciparum*) or liver hypnozoites (*P. ovale* and *P. vivax*) (Ashley et al., 2018). When sporozoites invade hepatocytes, a process known as sporozoite traversal occurs. The sporozoites mature to create schizonts at the end of the liver cycle (Ashley et al., 2018). Once the schizonts reach maturity, the erythrocytes rupture, and merozoites are released. This stage causes

symptoms such as fever, cold, and chills in infected humans (Malaria Vaccine Initiative, 2021). Merozoites develop into gametocytes in the blood of the human host.

The female *Anopheles* mosquitoes become infected after ingesting a blood meal from a human bearing *Plasmodium* gametocytes, the sexual-stage parasite cells responsible for malaria transmission. After the ingested gametocytes reach the mosquito's gut epithelium, male and female gametocytes mature, followed by fertilization, which leads to the production of ookinetes. The resulting ookinetes infiltrate the gut epithelium and mature into oocysts, which undergo mitosis and grow into sporozoites in the haemolymph. About 10 – 14 days after the infected blood meal, the sporozoites enter the mosquito's salivary glands. The mosquito then becomes infectious and will inject *Plasmodium* parasites into humans with its saliva during subsequent bites for the rest of its life (Schneider and Reece, 2021). Ingestion of an infected blood meal will result in malaria transmission only if the parasites pass through bottlenecks in the gut and salivary glands and the mosquito bites humans after the extrinsic incubation period, which is the time required for parasites to become infectious (Chandler et al., 2015).

Anopheles mosquito Human

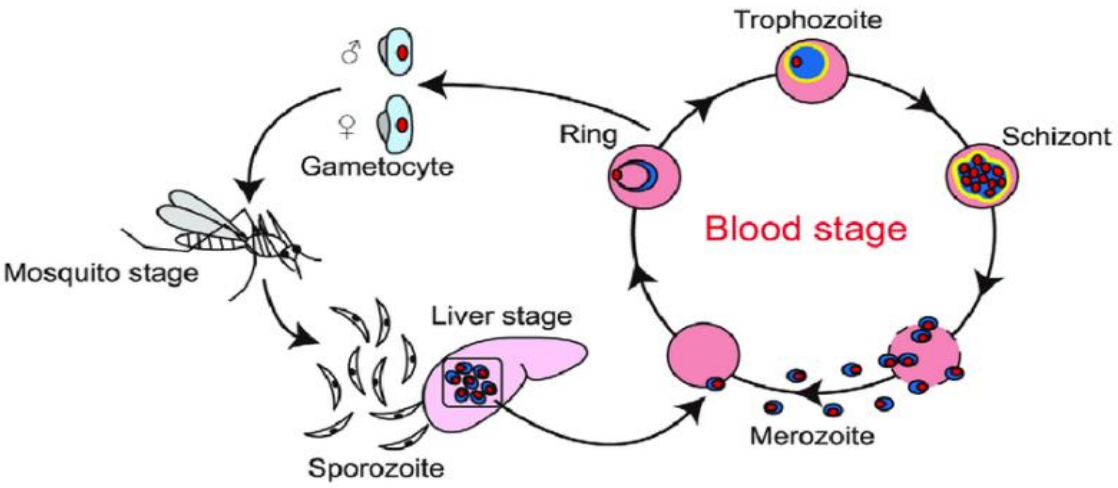
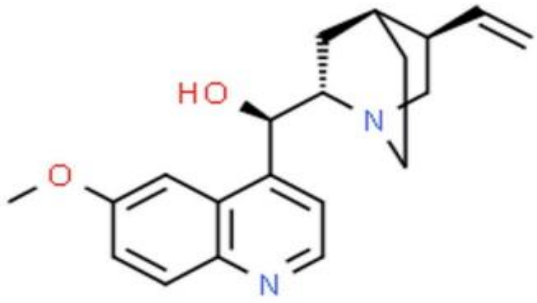


Figure 2.5. Life cycle of *Plasmodium falciparum* showing the relationship between the sexual and asexual stages in the *Anopheles* mosquito and human, respectively (Enomoto et al., 2012).

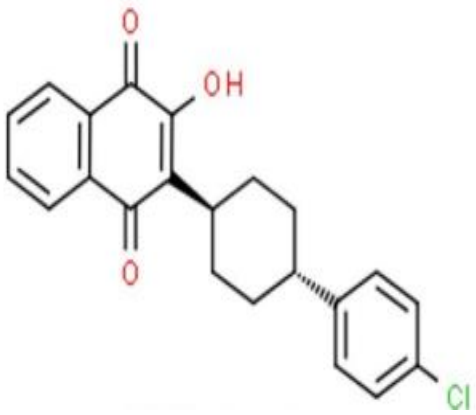
2.2.3. Malaria chemotherapy

Antimalarial drugs are critical chemotherapies in the control and elimination of malaria. These drugs have been used to treat patients with symptomatic malaria and continue to be used for malaria prevention and treatment. Malarial therapies primarily work by eliminating the erythrocytic stages of parasites that cause human sickness. *Plasmodium falciparum* has developed resistance to all the first-generation antimalarials (used as monotherapy drugs), and the majority are no longer used clinically (Cowell and Winzeler, 2019). Quinine (**1**) (Figure 2.6), isolated from the stem bark of *Cinchona calisaya* Wedd. (Rubiaceae), was the first prophylaxis used for malaria and has been used since the 1600s (Gisselmann et al., 2018). The treatment of malaria with quinine was the first effective use of a chemical compound to treat an infectious disease (Jacoby, 2005).

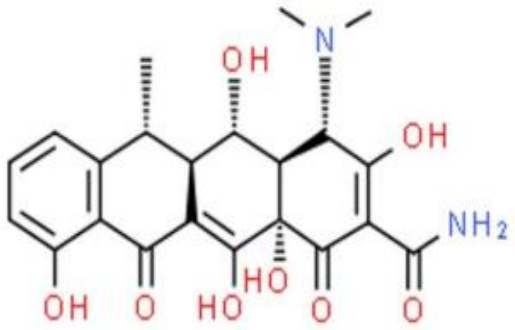
Quinine functions as a schizocide and destroys the gametocytes of *P. malariae* and *P. vivax* (Achan et al. 2011). Since it is quite hard to tolerate, the use of quinine is best limited to the treatment of severe malaria. Atovaquone (**2**), doxycycline (**3**), and proguanil (**4**) (Figure 2.6) are antimalarial drugs that are effective against malaria caused by *P. falciparum* but have poor efficacy against infections by *P. malariae* and *P. ovale* (Gaillard et al., 2018).



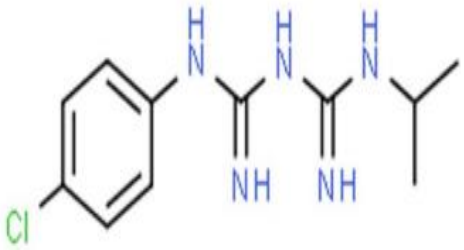
(1) Quinine



(2) Atovaquone



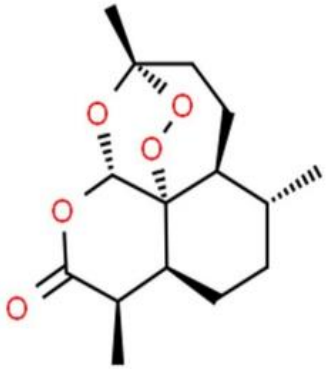
(3) Doxycycline



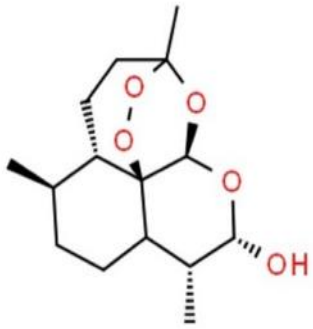
(4) Proguanil

Figure 2.6. Antimalarial drugs used as treatment for malaria caused by different *Plasmodium* species (Mabuza, 2022).

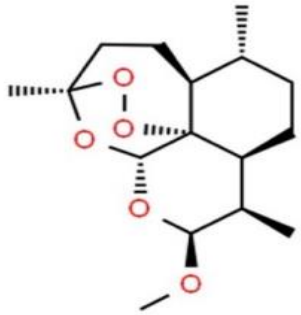
Since no single drug has been identified or developed that can destroy all stages of the *Plasmodium* life cycle, more than one class of drugs are often administered at the same time to combat malaria infection synergistically. Furthermore, dual therapy is recommended to improve therapeutic efficacy, prevent drug resistance, and shorten treatment time (Denford et al., 2014). Most current drugs rely on diverse molecular targets to block transmission at various stages of the parasite's life cycle (Batista et al., 2020). Artemisinin (**5**) (Figure 2.7), derived from the plant *Artemisia annua* L. (Asteraceae), is the preferred malaria treatment. Dihydroartemisinin (**6**), artemether (**7**), arteether (**8**), artesunic acid (**9**), and sodium artesunate (**10**) (Figure 2.7) are common artemisinin derivatives used to treat malaria (Patel et al., 2021). Both artemisinin and its derivatives are employed in artemisinin-based combination therapies (ACTs). These drugs function via a two-step process. Artemisinin is activated by intraparasitic heme-iron, which catalyzes endoperoxide cleavage. The free radical that forms destroys the parasites by alkylating and poisoning one or more important malarial protein(s) (Meshnick, 1998). The short half-life and low bioavailability of artemisinin and its derivatives make them ineffective as a single antimalarial drug. Parasite recrudescence is possible with the brief duration of action by artemisinin and its derivatives. Recrudescence occurs in all *Plasmodium* species when in the blood stage, the parasites are not entirely destroyed and then increase in number when drug concentrations in the blood drop. Recrudescent malaria infections are more contagious and difficult to treat than primary infections since the parasites are no longer responsive to drug treatment and have become resistant.



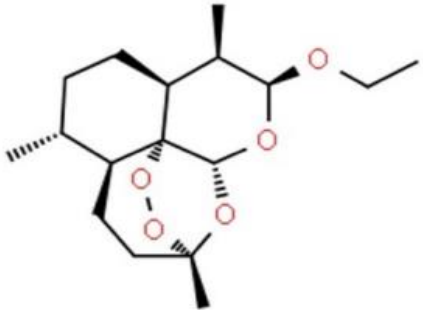
(5) Artemisinin



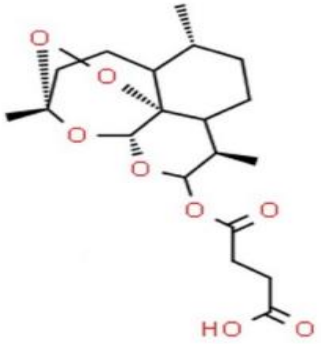
(6) Dihydroartemisinin



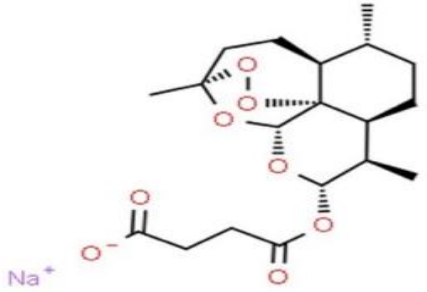
(7) Artemether



(8) Arteether



(9) Artesunic acid



(10) Sodium artesunate

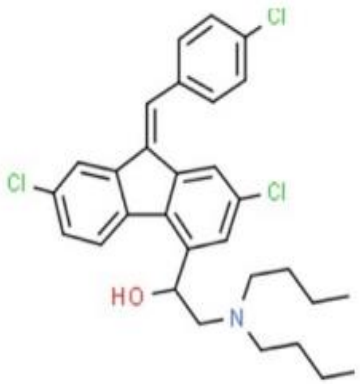
Figure 2.7. Chemical structures of the antimalarial drug artemisinin and its derivatives (Mabuza, 2022).

The drug regimens for treating the two most common malaria parasites, *P. falciparum* and *P. vivax*, differ. With the prevalence of resistance to previous drugs, ACTs are currently indicated for the treatment of uncomplicated falciparum malaria in nearly all malaria-endemic countries (WHO, 2022). Although there were initial delays, countries in SSA switched to ACTs for malaria treatment in 2004 (Nanyunja et al., 2011), and these ACTs have mostly remained successful in the treatment of uncomplicated *P. falciparum* malaria in African countries (Tindana et al., 2022). Uncomplicated malaria occurs when a person has symptoms but no sign of severe infection or organ failure. However, if not treated or if a person has low immunity, severe malaria can develop. General malaise, headaches, and vomiting are more advanced symptoms that develop later during the sexual blood stages of malaria if untreated (Bartoloni and Zammarchi, 2012). The two ACTs recommended by the WHO (2020) are artemether-lumefantrine (AL) and artesunate-amodiaquine, which belong to the sesquiterpene lactone endoperoxides class of available antimalarial drugs. The two-drug combinations are the existing primary treatment options in most African countries. The erythrocytic stages of non-falciparum malaria parasites can be treated with ACTs as well. Replacing failing or failed monotherapies with effective drugs, such as ACTs, has lowered the mortality and morbidity due to malaria (WHO, 2022).

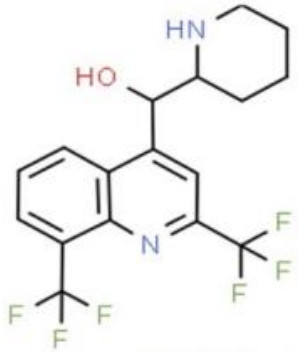
A fixed-dose mixture of two active drugs that act in different ways is used in artemisinin-based combination therapies. This is a combination of a fast-acting artemisinin derivative and another structurally unrelated and slow-acting drug (Phyo and Seidlein, 2017). Lumefantrine (**11**), mefloquine (**12**), amodiaquine (**13**), pyrimethamine (**14**), primaquine (**15**), and chlorproguanil (**16**) (Figure 2.8) are common artemisinin companion partner

drugs. During the first three days of treatment, the artemisinin compound functions to decrease the number of parasites, and the function of the companion partner is to eradicate any residual parasites (WHO, 2022).

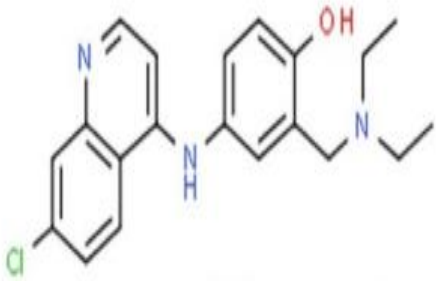
Understanding and preventing the spread of antimalarial resistance is a critical issue, particularly for artemisinin and its companion drugs. *Plasmodium falciparum* parasites that are resistant to antimalarials such as chloroquine, amodiaquine, or piperaquine have mutations in the *P. falciparum* chloroquine resistance transporter (Si et al., 2023). The chloroquine resistance transporter is a key contributor to multidrug resistance and is also essential for the survival of the *Plasmodium* parasites. This transporter is found on the digestive vacuole membrane of the parasites and can transport these drugs out of this organelle thus preventing the pharmaceuticals from binding to the heme-iron and thereby inhibiting their detoxification (Siddiqui et al., 2021).



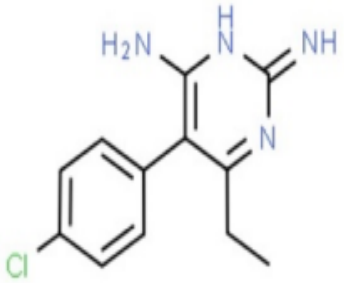
(11) Lumefantrine



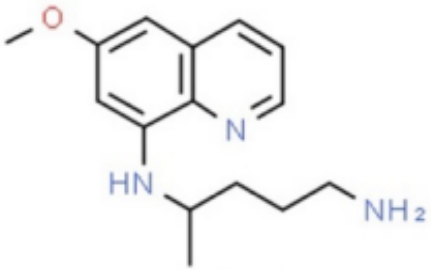
(12) Mefloquine



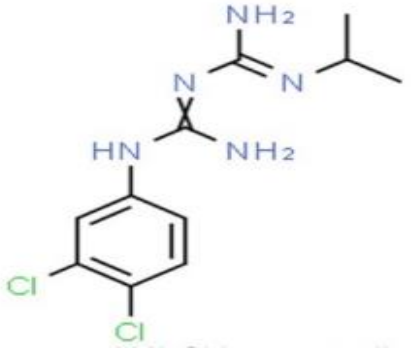
(13) Amodiaquine



(14) Pyrimethamine



(15) Primaquine



(16) Chlorproguanil

Figure 2.8. Molecular structures of partner drugs used in artemisinin-based combination therapies for malaria treatment (Mabuza, 2022).

Even though ACTs treatment failures have not been established in Africa, partial artemisinin resistance has been documented (Balikagala et al., 2021; Uwimana et al., 2021). *Pfkelch13 561H* mutations were found to be associated with artemisinin partial resistance in Rwanda (Uwimana et al., 2020), and this mutation and the *Pfkelch13 469Y* mutation signify delayed parasite clearance in Uganda (Balikagala et al., 2021). Recently, there have been reports of probable AL failure in Burkina Faso and Angola, which could be attributed to partial lumefantrine resistance (Dimbu et al., 2021; Gansane et al., 2021). The need for novel drugs with diverse antimalarial properties is at the highest level ever.

2.3. Sexually Transmitted Infections

Sexually transmitted infections (STIs) or venereal diseases have become a major global public concern, with a daily average of one million cases (Nthulane et al., 2020). Annually, the four curable STIs are recorded at approximately 374 million infections worldwide (WHO, 2022) affecting both males and females of all ages. Chlamydia, gonorrhoea, syphilis, and trichomoniasis are the most prevalent infections caused by *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Treponema pallidum*, and *Trichomonas vaginalis*, respectively. Furthermore, around 500 million people in the world are infected with genital herpes (WHO, 2019). Co-infection of STIs and HIV is common, mostly due to comparable transmission routes and risk factors, which are typically tied to sexual behaviours. At the end of 2022, 39 million people globally were living with HIV (WHO, 2023). This is a significant challenge for the resource-constrained SSA, which bears more than 70% of the worldwide burden of HIV infections, with women mainly affected (Kharsany and Karim, 2016). Nearly one in every twenty-five adults is infected with HIV

in SSA, making up more than two-thirds of all HIV-positive people worldwide (WHO, 2023). Figure 2.9 depicts the current estimates of global new episodes of chlamydia, gonorrhoea, trichomoniasis, and syphilis, with the bottom right of the figure showing the latest WHO estimates of new cases of these STIs worldwide (Rowley et al., 2019, WHO, 2023).

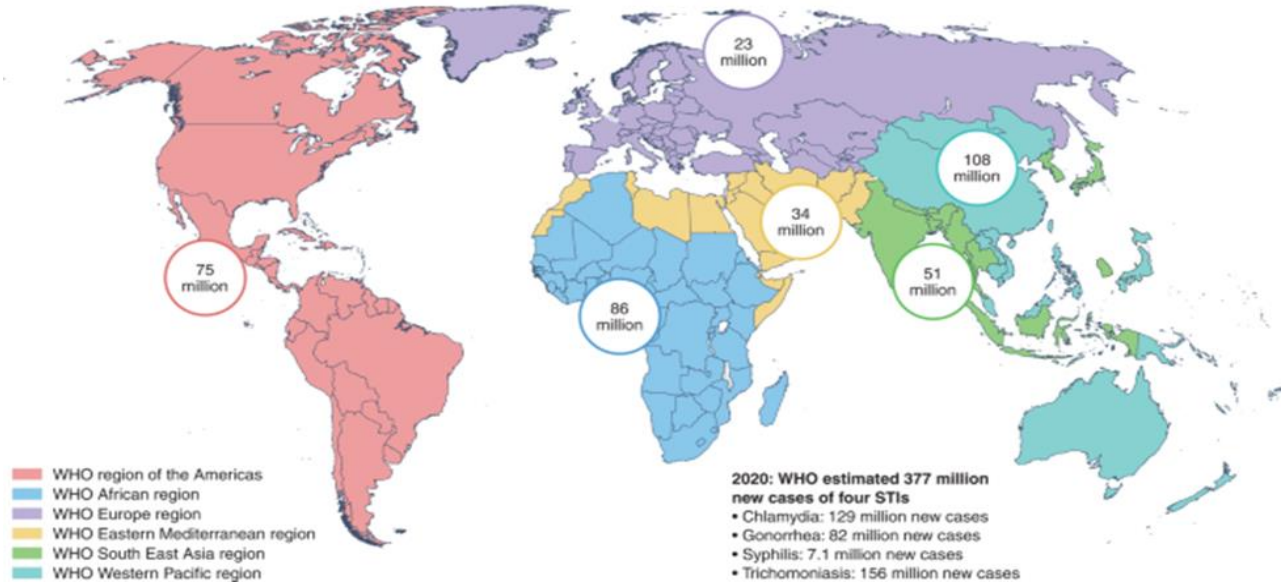


Figure 2.9. Cases of sexually transmitted infections reported in 2016 (Rowley et al., 2019). According to the World Health Organization (2023) estimates, trichomoniasis had the greatest number of new cases in 2020, followed by chlamydia, gonorrhoea, and syphilis.

Sexual contact is the most frequent route for STIs to spread. Primary transmission is by anal, oral, and vaginal intercourses. Secondary transmission occurs through maternofetal contact, contaminated needles, and blood transfusions (Shaukat and Kazmi, 2015; Kharsany et al., 2020). The female anatomy contributes to the higher prevalence of STIs in women than in men. The urogenital structure of a woman is more exposed and

sensitive to STIs because the vaginal mucosa is thin, delicate, and easily penetrated by infectious agents (Centers for Disease Control and Prevention, 2011). Treatment for STIs usually consists of antibiotics and/or antiviral drugs depending on the infection. Untreated STIs are associated with significant long-term morbidity and mortality. This includes infertility, premature birth, ectopic pregnancies, spontaneous abortion, and stillbirth (Moodley et al., 2015; Wynn et al., 2020).

The increased risk of contracting or transmitting HIV is associated with STIs that cause genital tract inflammation such as gonorrhoea, herpes, and syphilis (Wand et al., 2020). This is due to a concentration of 'activated' immune cells in the affected area because of the body's response to assist in fighting the infection. It is then much easier for HIV to infect and multiply in the 'activated' immune cells. Chlamydia and gonorrhoea infections can cause pelvic inflammatory disease, which can accelerate the progression of ovarian cancer if left untreated (Shen et al., 2016; Tien et al., 2020). The prevalence of gonorrhoea among prenatal women in SSA can be as high as 21.1% (Abdelrahim et al., 2017). Although most STIs are usually not fatal, they result in a substantial burden of disease because of negative health, economic, and social implications.

Based on the most recent statistics in South Africa, the total number of people living with HIV is estimated to be around 8.5 million, with an approximate prevalence rate of 14% among the population and the prevalence for adults aged 15 to 49 years was projected to be 20% in 2022 (Statistics South Africa, 2022). Average cases of gonorrhoea are roughly 2 million each year in the country (Kularatne et al., 2018). The occurrence of

gonorrhoea can be as high as 6.9% among pregnant women in South Africa (Medina-Marino et al., 2020).

2.3.1. Overview of gonococcal infection

Gonorrhoea, caused by the Gram-negative bacterium *Neisseria gonorrhoeae*, is the second most common bacterial STI and causes significant morbidity. The WHO (2023) estimates that 82.4 million people were newly infected with gonorrhoea in 2020, most in Africa and the West Pacific. *Chlamydia trachomatis* is present in 10 – 40% of gonorrhoea patients (Lim et al., 2015; Trecker et al., 2015). Typically, the pathogen is transmitted through unprotected vaginal, anal, and oral sexual intercourses. The disease can also be passed from mother to child during pregnancy and childbirth (Rowley et al., 2019). Gonococcal infections include urogenital, anorectal, pharyngeal, and conjunctival infections. The disease may result in epididymitis, prostatitis, and urethritis in males whereas cervicitis, pelvic inflammatory disease, infertility, ectopic pregnancy, and maternal death, as well as first-trimester abortion, have been reported in females (Tshikalange et al., 2016; WHO, 2021). If untreated, gonorrhoea increases HIV transmission by fivefold (WHO, 2021).

Uncomplicated gonococcal infections, which include any urogenital, anogenital, or pharyngeal infection caused by *N. gonorrhoeae* but do not result in bacteremia, are far more common (Unemo et al., 2019). In men, uncomplicated gonococcal infections often show as urethritis, with symptoms of urethral discharge and dysuria. Gonorrhoea is frequently asymptomatic in women. Less than half of infected women report non-specific symptoms such as abnormal vaginal discharge, dysuria, lower abdomen discomfort, and

dyspareunia (Erasmus et al., 2012). The most prevalent clinical manifestations are vaginal discharge and cervical friability caused by mucopurulent cervicitis. Rectal infections in men and women are mostly asymptomatic, with patients occasionally complaining of rectal and anal pain or discharge. Pharyngeal infections are mostly asymptomatic and can cause a moderate sore throat and pharyngitis (WHO, 2016).

2.3.2. Gonococcal infection mechanism

In the initial stages of gonorrhoea infection, *N. gonorrhoeae* adheres to the epithelial cells of the human host via type IV pili. Following the adherence, *N. gonorrhoeae* duplicates and produces microcolonies, possibly biofilms (Anderson and Byerly, 2016), and competes with existing microbiota. The processes of invasion and transcytosis occur when the bacteria enter the epithelium. Right through the initial stages of infection, *N. gonorrhoeae* releases fragments of peptidoglycans, lipooligosaccharides (LOS), and outer membrane vesicles (OMV) (Mavrogiorgos et al., 2014; Zhou et al., 2014). The fragments activate Toll-like receptor (TLR) and nucleotide-binding oligomerization domain-containing protein (NOD) signaling in epithelial cells, macrophages, and dendritic cells (DCs) (Mavrogiorgos et al., 2014). These innate immune signaling pathways release pro-inflammatory cytokines and chemokines. This attracts a high number of polymorphonuclear leukocytes, or neutrophils to the infection site, where they interact and phagocytize *N. gonorrhoeae*. The influx of neutrophils results in the formation of a purulent exudate, which facilitates transmission. Figure 2.10 shows the summary of gonorrhoea infection (Quillin and Seifert, 2018).

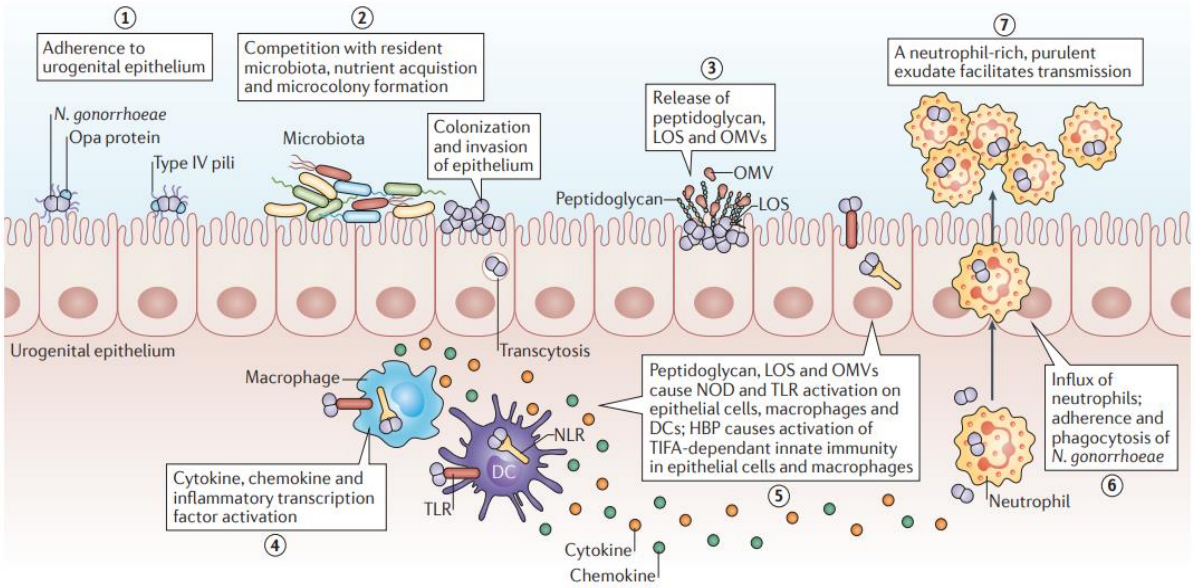


Figure 2.10. Outline of the pathogenesis of gonococcal infection caused by the Gram-negative *Neisseria gonorrhoeae* (Quillin and Seifert, 2018).

2.3.4. Treatment options for gonococcal infection

Antibiotic resistance in treating gonorrhoea has risen over the last 50 years, rendering many early-generation cephalosporins ineffective (Wi et al., 2017; Unemo et al., 2019; WHO, 2021). *Neisseria gonorrhoeae* shows resistance to several antibiotics meant to destroy it. Most recently, the gonococcus has evolved resistance to third-generation extended-spectrum cephalosporins, known as the last line of defense antibiotics, resulting in *N. gonorrhoeae* becoming a multidrug-resistant pathogen (Unemo et al., 2019). The bacterium has mostly used all known resistance mechanisms, including antimicrobial inactivation, antimicrobial target modification, increased export (e.g., via efflux pumps such as MtrCDE), and decreased uptake (e.g., via porins such as PorB). Mechanisms that alter gonococcal cell permeability are particularly problematic because

they reduce susceptibility to a wide spectrum of antimicrobials with distinct modes of action, such as penicillins, cephalosporins, tetracyclines, and macrolides (Goire et al., 2014; Unemo et al., 2014). This widespread resistance has been achieved through the accumulation of AMR determinants, the majority of which do not appear to significantly affect the biological fitness of the bacterium (Figure 2.11).

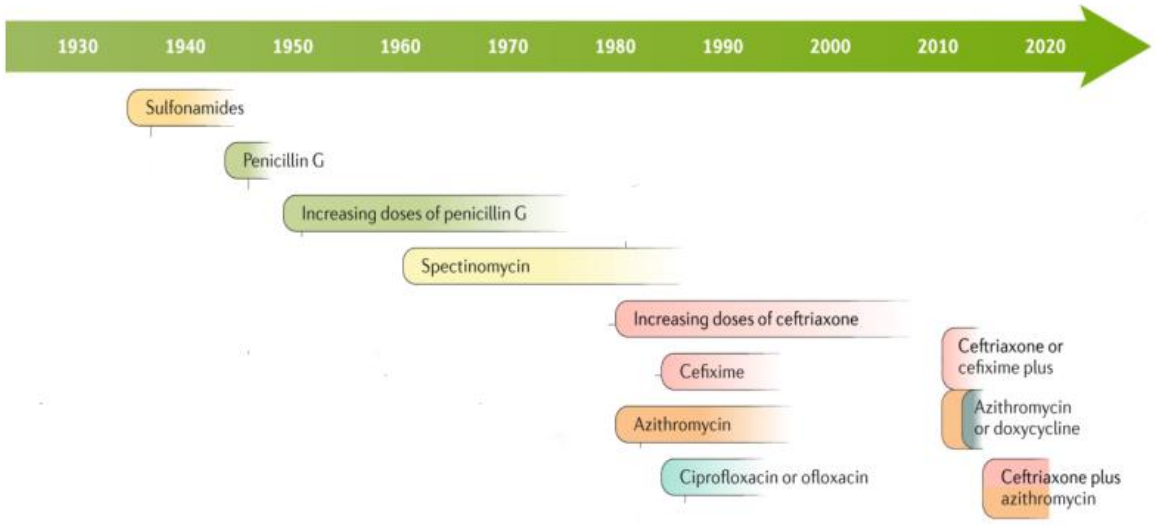
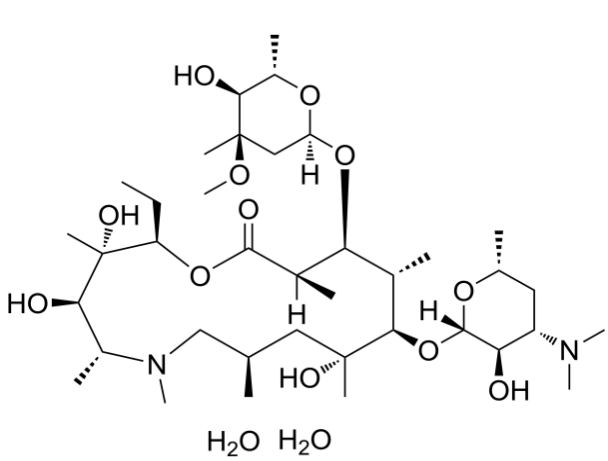


Figure 2.11. Treatment recommendations for gonococcal infections and the establishment of antimicrobial resistance of *Neisseria gonorrhoeae* (Unemo et al., 2019). Each bar represents a gonococcal therapy, and the length of the bar denotes the period elapsed between when the therapy was first used and the onset of clinical and/or *in vitro* resistance, which threatened the efficacy of that specific antimicrobial therapy.

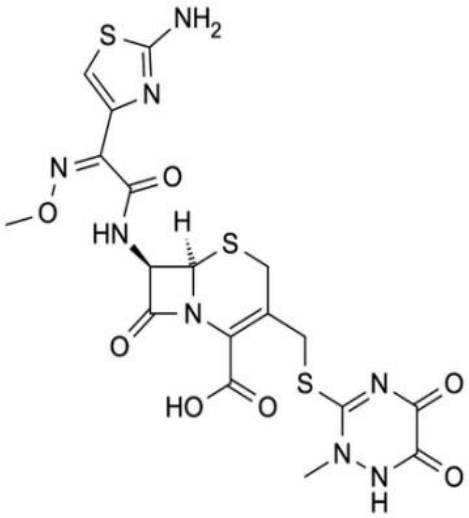
The WHO (2017) published a list of bacteria that require immediate attention with new antibiotics. The bacteria were categorized as critical, high, or medium priority according to their antibiotic resistance. *Neisseria gonorrhoeae* was classified in the high priority category. The outer membrane of Gram-negative bacteria is the primary cause of

antibiotic resistance. Most antibiotics must pass through the outer membrane to reach their targets; for example, hydrophobic drugs can use a diffusion pathway; hydrophilic antibiotics use porins; and vancomycin cannot cross the outer membrane due to its structure, which prevents it from using any of these passages (Miller, 2016; Exner et al., 2017). Any change in the hydrophobic characteristics of the outer membrane of Gram-negative bacteria, as well as modifications in porins and other factors, might result in resistance, making Gram-negative bacteria more resistant to antibiotics (Gupta and Datta, 2019).

Treatment guidelines for specific gonococcal infections for single and combination therapies are provided by the WHO (2016). Azithromycin and ceftriaxone (Figure 2.12) are the current dual antimicrobial regimens, and they appear to be successful in counteracting the mechanisms displayed by *N. gonorrhoeae* in resisting treatment. Nonetheless, gonococci susceptibility to the dual regimens has decreased globally. Concomitant resistance to either azithromycin or ceftriaxone has been identified in several countries, and dual antimicrobial regimens are not affordable in many less-resourced settings, particularly in SSA (Unemo and Shafer, 2015; Whiley et al., 2015).



Azithromycin



Ceftriaxone

Figure 2.12. Antibiotics used as dual antimicrobial treatment for gonorrhoea (Menzel, 2017).

There has been no confirmation of gonorrhoea treatment failure with dual antimicrobial therapy. Even so, treatment failures with these regimens are more likely to be a matter of when, and not if, they will emerge. Consequently, innovative low-cost antimicrobials for monotherapy or, at the absolute least, inclusion in dual gonorrhoea treatment regimens, which may be required for all newly developed antimicrobials, are critical. While not as effective as antibiotics by themselves, when combined with antibiotics, plant compounds can assist in overcoming antibiotic resistance in bacteria. Studies have revealed the synergistic effects of combining phytopharmaceuticals and antibiotics in the management of resistant bacterial infections (Haroun and Al-Kayali, 2016; Stefanović, 2018).

2.4. Malaria and Sexually Transmitted Infections co-infection

Co-infection, in which an individual is infected with both malaria and STI at the same time, is a serious public health concern that can have serious consequences for the affected person's health. Malaria and STI co-infection can occur in people who live in areas like SSA where both diseases are endemic. Syndemics arise when two or more co-existing infections have a deleterious interaction, and when co-infection occurs, the total outcome is worse than any of the individual infections as in the case of malaria and STIs which both adversely impact pregnancy. The co-infection of malaria and STI is a common occurrence in SSA (Lingani et al., 2021). Research suggests that traditional medicine usage during pregnancy is frequent in the region (Ahmed et al., 2018). The practice is, however, overshaded by secrecy especially when it is intended for serious diseases such as malaria and HIV/AIDS (Ozioma and Chinwe, 2019). Pregnant women are to an extent aware that using herbal medicine may have detrimental effects hence their anonymity.

One of the primary causes of neonatal death is low birth weight (LBW). The presence of LBW has been linked to malaria and STIs in pregnant women. According to the WHO (2022), 2.4 million children died in their first month of birth in 2020 worldwide, with SSA having the highest neonatal mortality rate (27 deaths per 1 000 live births) and accounting for 43% of infant deaths. The region has approximately 1.2 million neonatal deaths yearly (Hug et al., 2019).

Studies have been conducted in SSA to determine the scope of malaria and STIs in pregnant women. A study by Chico et al. (2012) showed that malaria, syphilis, bacterial vaginosis, and chlamydial infection were all relatively common in pregnant women of

West Africa. In another study by Chaponda et al. (2016), 38% of Zambian pregnant women had malaria and at least one STI. Other studies have reported that syphilis-related adverse pregnancies were estimated at 205 900 in 2015 and that malaria was the cause of about 217 000 stillbirths (20% of all stillbirths) in 2017 (Kuznik et al., 2015; Moore et al., 2017). In a two-year observational study of 31 639 pregnant women in rural Burkina Faso, malaria, STIs, and their co-infection were diagnosed in 7 747, 1 269, and 388 pregnancies, respectively (Zango et al., 2020). Despite their increased susceptibility to infections, pregnant women are typically asymptomatic, severely limiting clinical detection and appropriate case management (Yimam et al., 2021).

2.5. Bioprospecting medicinal plants for the treatment of malaria and gonorrhoea

Plants as medicines are by far the largest common human usage of a natural resource. The global number of plant species is estimated to be around 380 000, of which 7.5% are used as medicinal plants (Chassagne et al., 2020). The numerous families, genera, and species of the plant kingdom account for diverse biological activities. Plants have produced a great variety of molecules through different biosynthetic routes, and these molecules are called primary and secondary metabolites. Primary metabolites are important for life processes as they contribute to plant growth and development by providing carbohydrates, lipids, and proteins (Zaynab et al., 2019). The biochemical pathways also lead to the production of relatively small molecules known as secondary metabolites.

Secondary metabolites are produced as a protective mechanism in response to pathogens, herbivory, and environmental stress, and do not seem to play an important role in plant growth and development. However, research has shown that secondary metabolites have significant roles in plants, such as protecting against ultraviolet radiation exposure, defense against diseases caused by viruses, fungi, bacteria, and other phytopathogens, and repelling herbivores (Guerriero et al., 2018). These secondary metabolites are the most intriguing in the treatment of diseases, and they are classified into three major groups; polyphenols, terpenes, and alkaloids (Guerriero et al., 2018; Yang et al., 2018). The concentration and type of secondary metabolites vary among plants and between organs of the same plant (Builders, 2018; Yang et al., 2018). The biological activities exerted by plants can be attributed to the presence of several secondary metabolites, and they can act independently, synergistically, or antagonistically to produce a pharmacological effect (Reshi et al., 2023). The activities of medicinal plants are dependent on secondary metabolites.

Two of the most effective antimalarial drugs quinine and artemisinin originated from traditional medicine and are derived from plants. The advantage of plants having biologically active antimalarial compounds, especially alkaloids and terpenoids, include their use in the preparation of traditional medicines against malaria and fever (Okello et al., 2019). Failures to treat gonorrhoea with extended-spectrum cephalosporins (Unemo et al., 2012; Unemo and Nicholas 2012), as well as resistance to nearly all other available drugs, scientists are now investigating medicinal plants as potential sources of new drugs to treat gonorrhoea (Unemo et al., 2014; Taiwo et al., 2016).

Medicinal plants have multifunctional biological properties to cure various diseases. With the vast biodiversity, South African medicinal plants are excellent candidates for antiplasmodial and antigonococcal research. Prior studies have yielded promising results for bioprospecting such plants for malaria and gonorrhoea treatments (Naidoo et al., 2013; Simelane et al., 2013; Bapela et al., 2014; Mulaudzi et al., 2015; Tshikalange et al., 2016; Mongalo et al., 2017, Bapela et al., 2019; Lawal et al., 2019; Mkatshane, 2019; Salomane, 2020; Dembetembe et al., 2020; Nthulane et al., 2020; Domingo, 2022; Mabuza, 2022). The research gaps revealed by these studies suggest that the isolation, purification, and characterization of compounds from active extracts or fractions are barely prioritized. Such procedures are important in the search for novel drugs derived from plants.

2.6. Plant metabolomics and analysis

Natural products are still an essential source of medicines and drug templates. They are compounds derived from natural sources, such as plants, and many natural compounds exhibit different pharmacological activities. This is most likely owing to the tremendous diversity of chemicals in natural products, which raises the likelihood of discovering structurally distinct lead compounds for various diseases (Gu et al., 2013). The Dictionary of Natural Products has so far cataloged about 340 000 natural compounds (Routledge, 2023). Given that each plant species produces numerous metabolites, robust approaches capable of assessing large quantities of these metabolites in the quickest time possible are valuable (Yuliana et al., 2011).

Natural product research and drug discovery have profited enormously from the development of cutting-edge plant metabolomics approaches, which have enabled extensive qualitative/quantitative investigation of plant metabolites (Salem et al., 2020). Plant metabolomics is an effective approach for accelerating the discovery of compounds in plants because it can systematically study small molecules present in a plant sample, including identification and quantification (Hegeman, 2010; Burgess et al., 2014; Lajis et al., 2017). Furthermore, it is useful for detecting and isolating low-quantity compounds in biological systems (Dayalan et al., 2018). Plant metabolomics is currently used mostly for source identification, authentication, processing method evaluation, and other quality control connections in phytomedicine (Barding et al., 2012).

Metabolomic techniques like proton nuclear magnetic resonance (^{1}H NMR), gas chromatography (GC), and mass spectrometry (MS) (GC-MS) complement each other when studying plant samples. The use of ^{1}H NMR spectroscopy for studying phytomedicines has drawbacks. It has limited sensitivity, and signals may overlap, thus it is best to use multiple analytical techniques for accuracy (Bhinderwala et al., 2018). The low sensitivity of ^{1}H NMR spectroscopy may impair the reliability of the acquired data, complicating the investigation of metabolites in plant samples. Nonetheless, these limitations can be solved through optimization and the use of GC-MS to compensate for the drawbacks of ^{1}H NMR spectroscopy (Emwas, 2015). Gas Chromatography-Mass Spectrometry is used to detect low molecular weight phytoconstituents in plant extracts or fractions since ^{1}H NMR cannot analyze volatile compounds. To examine plant crude extracts, GC combined with MS can be utilized in conjunction with another version of ^{1}H NMR known as 2D NMR (Guo et al., 2020).

Several studies have used metabolomics to identify bioactive compounds from medicinal plants with antiplasmodial and antigonococcal activities (Allman et al., 2016; Kamal et al., 2017; Bapela et al., 2019; Freire et al., 2019; Mkatshane, 2019; Alhadrami et al., 2021; Domingo, 2022; Mabuza, 2022). To comprehend the antiplasmodial and antigonococcal pharmacological efficacies of these plants and isolated compounds, various analytical techniques and metabolomics approaches have been used.

2.7. *Tabernaemontana elegans* as a source of antimalarial and antigonococcal drug leads

Tabernaemontana elegans (Stapf.) (also known as *Conopharyngia elegans* and *Leptopharyngia elegans*) is called a toad tree or bushveld toad tree in English, laeveldse paddaboom in Afrikaans, umkhadlu and umkhalwana in isiZulu, and muhatu in Tshivenda (South African National Biodiversity Institute (SANBI), 2019). The plant is a member of the Apocynaceae family. The family consists of approximately 250 genera and 2 000 species (Wong et al., 2014). Species of the Apocynaceae family include shrubs and trees. These plants are distinguished by their vivid flowers and opposing leaves. There are about 110 species of *Tabernaemontana* globally, of which around 18 occur in mainland Africa and 15 in Madagascar (Arnold et al., 2002). Only two species are indigenous to South Africa, *T. elegans* and *T. ventricose* (Zhu et al., 1990).

Species of the Apocynaceae family have traditionally been used to treat diabetes, fever, malaria, gastrointestinal illnesses, pain, and a variety of other ailments (Endress and Bruyns, 2000; Arnold et al., 2002; Wong et al., 2011; Mongalo and Makhafola, 2018; Islam and Lucky, 2019). Additionally, various species of this family have antiplasmodial and

anticancer properties (Wong et al., 2014). Several monoterpene indole alkaloids (MIAs) from the Apocynaceae family have been utilized to treat cancer, analgesia, inflammation, and spasticity. Vinblastine, vinorelbine, vincristine, and vindesine, for example, were used as anticancer drugs, whereas ajmalicine and ajmaline were used to treat cardiovascular diseases (Mondal et al., 2019). The therapeutic action of a plant can be determined to be exclusive to a particular genus or species. This is consistent with the idea that secondary metabolites in a particular plant are frequently taxonomically distinct (Zhu et al., 1990). The presence of alkaloids which are responsible for various biological activities distinguishes the *Tabernaemontana* genus taxonomically (Islam and Lucky, 2019).

2.7.1. Taxonomy, description, flowering season, and distribution of *Tabernaemontana elegans*

Tabernaemontana elegans belongs to the Plantae kingdom, Tracheophyta division, Eudicots class, Gentianales order, Apocynaceae family, *Tabernaemontana* genus, and *elegans* species.

Tabernaemontana elegans is recognizable by its huge, glossy leaves, fragrant white flowers, and large fruits that are produced in pairs. The plant is hairless and has milky sap in all parts. It is a semi-deciduous tree or shrub that reaches a height of 5 m and at times can reach a height of 12 m, found in evergreen river fringes at low altitudes and in coastal scrub forests (Coats Palgrave et al., 2003). The trunk is 50 – 300 mm thick, with corky, pale brown stem bark that has longitudinal fissures (Figure 2.13A) (Arnold et al., 2002). The corky stem bark provides some fire resistance to the plant.

Twigs are distinguished by large leaf scars which create transverse ridges. The leaves are opposite, leathery, and dark glossy green above and paler beneath. Leaf sizes range from 90 – 200 mm by 50 – 70 mm, and they are often 2 to 4 times as long as they are wide (Omino, 2002). Petioles range in length from 10 – 25 mm (Schmelzer and Gurib-Fakim, 2008). On the underside of the leaf, the elevated midrib and lateral nerves are especially prominent.

Each inflorescence is made up of several white, fragrant flowers that are held together by a 30 – 50 mm long peduncle (Omino, 2002; Schmelzer and Gurib-Fakim, 2008). A flower is up to 15 mm broad and carried on a pedicel 4 – 7 mm long (Figure 2.13B) (Arnold et al., 2002). The fruit grows in pairs and is subglobose in shape, with a green covering covered in light warts. Each fruit measures 60 – 70 mm long by 40 – 50 mm diameter and has two lateral and one dorsal ridge (Schmelzer and Gurib-Fakim, 2008). The fruit is leathery to woody, with a 5 – 15 mm thick wall (Arnold et al., 2002). When they reach maturity, they break apart along one side, frequently while still attached to the tree, revealing the yellowish pulp inside. Each fruit, with its green, warty skin, looks like a toad, hence the common name toad tree (Figure 2.13C). Numerous dark brown seeds 14 –17 mm in length by 7 – 9 mm broad are embedded in the yellow pulp (Schmelzer and Gurib-Fakim, 2008).

A **B** **C**

Figure 2.13. A pictorial view of *Tabernaemontana elegans* showing the stem bark, leaves, flowers, fruits, and seeds (Kerryn, 2018).

Different countries have the flowering season of *T. elegans* at various times of the year. In Kenya, the plant flowers from October to May, in Mozambique from April to September, and bears fruits from July to October in both countries (Omino, 2002). It flowers in South Africa from September to March, with a peak in November, and bears fruits from February to August (Arnold et al., 2002).

Tabernaemontana elegans is found in bushland, forest on coastal dunes, up to 1 km altitude, and occurs in the east and south of Africa (Arnold et al., 2002). It is distributed in Somalia, Kenya, Tanzania, Mozambique, Zimbabwe, Madagascar, Eswatini, and South Africa. The plant can be found in the provinces of Limpopo, Mpumalanga, and

KwaZulu-Natal in South Africa (SANBI, 2019). *Tabernaemontana elegans* is not threatened in any way.

2.7.2. Ethnomedicinal uses of *Tabernaemontana elegans*

Tabernaemontana elegans is used for a variety of ethnomedicinal purposes by southern Africans, including the treatment of cancer, bleeding, malaria, STIs, fever, and pulmonary diseases. The endocarps in the fruits are dried, pulverized, boiled in water, filtered, and taken orally to treat cancer (Arnold et al., 2002). To reduce fever, a decoction of the stem bark and roots is drunk (Watt and Breyer-Brandwijk, 1962). The Zulu people drink the root decoction for chest pains and pulmonary diseases and apply it to wounds and sores to promote healing (Watt and Breyer-Brandwijk, 1962; Arnold and Gulumian, 1984). An infusion of the roots is macerated and taken orally for stomach aches and lung ailments, and it is also said to be an aphrodisiac (Arnold and Gulumian, 1984). Burnt root powder is mixed with salt and water to use as a vaginal wash to treat menorrhagia and infertility (Arnold and Gulumian, 1984). A purgative is prepared from the maceration of the root (Watt and Breyer-Brandwijk, 1962). A styptic agent is made from the coagulated latex of roots mixed with petroleum jelly and applied to wounds to stop the bleeding (Arnold et al., 2002). Table 2.1 provides the traditional uses of *T. elegans* for malaria and STIs in southern Africa.

Table 2.1. Ethnomedicinal uses of *Tabernaemontana elegans* associated with malaria and sexually transmitted infections.

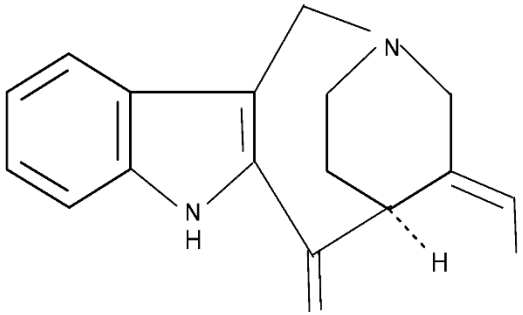
Plant part	Preparation method and administration	Traditional use	Reference
Fruits	Powdered fruits are boiled in water and taken orally.	Venereal diseases	Mabogo, 1990
Leaves	Dried leaves of *T. elegans* and *Ipomoea batatas* are ground into a powder. A decoction of the leaf powder is prepared. Two cups of this decoction are drunk three times a day.	Gonorrhoea	Arnold and Gulumian, 1984; Mabogo, 1990; de Wet et al., 2012
Stem bark	A decoction is made from stem bark and administered orally.	Malaria and fever	Watt and Breyer-Brandwijk, 1962; Pooley, 1998
	A handful of crushed leaves of *T. elegans*, *Euphorbia tirucalli*, *Ozoroa engleri*, and *Hypoxis hemerocallidea* corm are boiled in water. Two cups of this remedy are drunk twice a day.	Gonorrhoea	de Wet et al., 2012
Roots	The roots of *T. elegans* and *I. batatas* are boiled and drunk three times daily.	Venereal diseases	Arnold and Gulumian, 1984; Mabogo, 1990
	Boil in water a handful of chopped *Ximenia caffra* roots and half a handful of chopped *T. elegans* roots. Three cups of the concoction are drunk a day.	Gonorrhoea	de Wet et al., 2012
	The roots are soaked in hot water for a few minutes and administered orally. To treat fever, a decoction of the roots is made and drank.	Malaria and fever	Watt and Breyer-Brandwijk, 1962; Ngarivhume et al., 2015
	A vaginal wash is macerated by combining burnt root powder with salt and water.	Venereal diseases	Arnold et al., 2002

*One cup is approximately 250 ml.

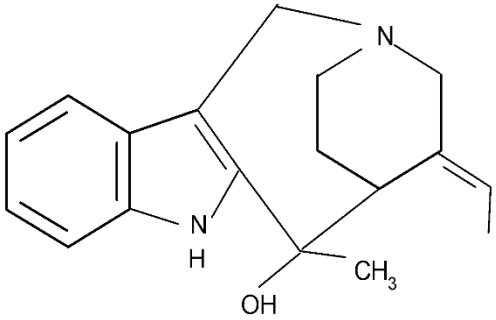
2.7.3. Phytochemistry of *Tabernaemontana elegans*

Alkaloids are basic nitrogenous natural secondary metabolites with a wide range of intriguing bioactivities. Many alkaloids are detected in plants (Mondal et al., 2019). It is worth noting that multipart alkaloids are found in a few numbers of plant families such as Apocynaceae, Loganiaceae, and Rubiaceae (Marinho et al., 2016). These families are closely connected taxonomically. Monoterpene indole alkaloids are thought to be derived from strictosidine, which is formed by the condensation of tryptophan with secologanin (C9 or C10) and can be split into linear one-carbon (1 C), three-carbon (3 C), and six-carbon (6 C) units (Mondal et al., 2019).

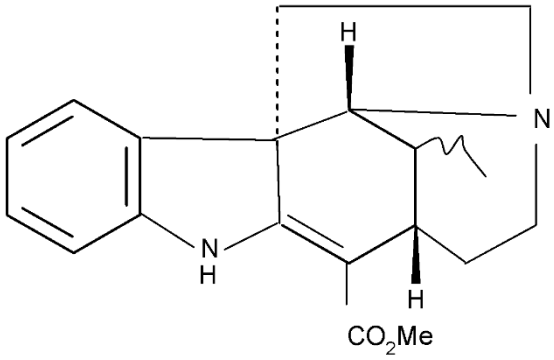
Alkaloids of the genus *Tabernaemontana* contain L-tryptophan-derived alkaloids called indole (Aniszewski, 2007). *Tabernaemontana* indole alkaloids are classified as corynanthean, ibogan, corynanthean-ibogan, vobasan, vobasinyl ibogan, or aspidspermatan (Schmelzer, 2006). To the extent that the current study could determine based on the literature that is accessible using conventional scientific search databases, about 32 indole alkaloids have been isolated from *T. elegans* (Figure 2.14). However, some of these compounds have not been tested for bioactivity. *Tabernaemontana elegans* produces and stores monoterpene indole and bisindole alkaloids. Monoterpene indole alkaloids are metabolites with a bicyclic structure consisting of a benzene ring fused to a five-membered pyrrole ring. Bisindole alkaloids are alkaloids that arise naturally and contain two indole nuclei with a tryptamine unit (Rahman et al., 2016).



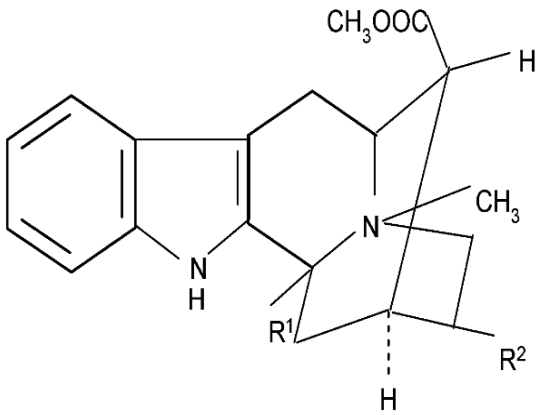
(1) Apparicine



(2) 16-*S*-hydroxy-16, 22-dihydro-apparicine



(3) Tubotaiwine



(4) Vobasine: R^1 = O, R^2 = CH-CH_3

(5) Vobasinol: R^1 = α-OH, R^2 = CH-CH_3

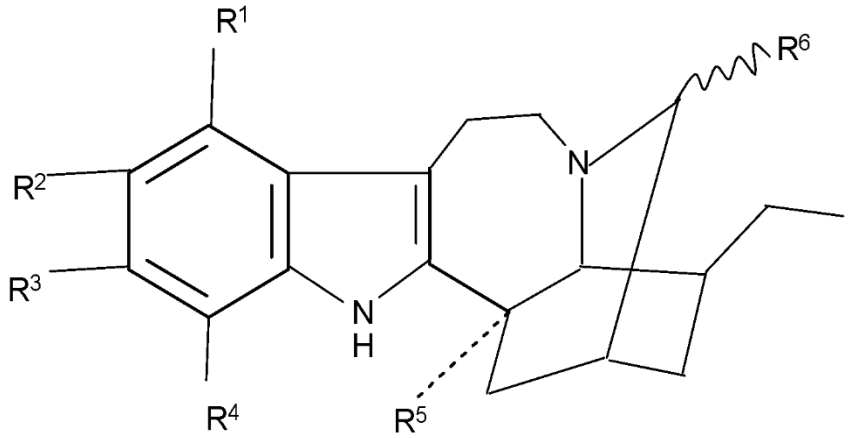
(6) Dregamine: R^1 = O, R^2 = α-C_2H_5

(7) Dregaminol: R^1 = α-OH, R^2 = α-C_2H_5

(8) Tabernaemontanine: R^1 = O, R^2 = β-C_2H_5

(9) Tabernaemontaninol: R^1 = α-OH, R^2 = β-C_2H_5

(10) Dregaminol-methylether: R^1 = OCH_3, R^2 = α-C_2H_5



(11) Isovoacangine: $R^1 = R^2 = R^4 = H$, $R^3 = OCH_3$, $R^5 = COOCH_3$, $R^6 = H$

(12) 3-*R*/*S*-hydroxy-conodurine: $R^1 = R^2 = H$, $R^3 = OCH_3$, R^4 = 3'-vobasinyl, R^5 = COOCH3, $R^6 = OH$

(13) Tabernaelegantine A: $R^1 = R^2 = H$, $R^3 = OCH_3$, R^4 = 3'-tabernaemontanyl, $R^5 = COOCH_3$, $R^6 = H$

(14) Tabernaelegantine B: $R^1 = H$, R^2 = 3'-tabernaemontanyl, $R^3 = OCH_3$, $R^4 = H$, $R^5 = COOCH_3$, R6 = H

(15) Tabernaelegantine C: $R^1 = R^2 = H$, $R^3 = OCH_3$, R^4 = 3'-dregaminyl, $R^5 = COOCH_3$, $R^6 = H$

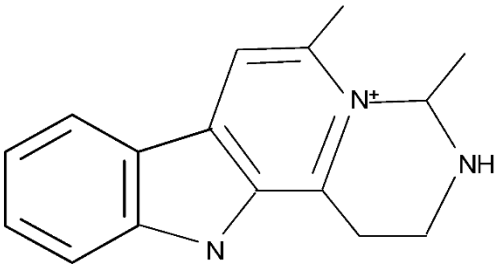
(16) Tabernaelegantine D: $R^1 = H$, R^2 = 3'-dregaminyl, $R^3 = OCH_3$, $R^4 = H$, $R^5 = COOCH_3$, $R^6 = H$

(17) 3-*R*/*S*-hydroxy-tabernaelegantine B: $R^1 = H$, R^2 = 3'-tabernaemontanyl, $R^3 = OCH_3$, $R^4 = H$, $R^5 = COOCH_3$, $R^6 = OH$

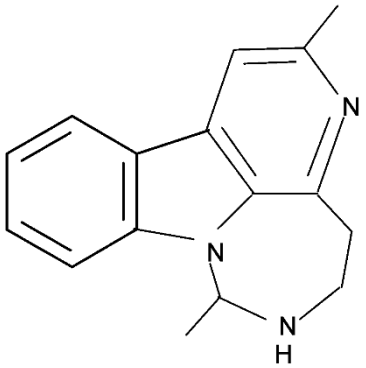
(18) 3-*R*/*S*-methoxy-tabernaelegantine C: $R^1 = R^2 = H$, $R^3 = OCH_3$, R^4 = 3'-dregaminyl, $R^5 = COOCH_3$, $R^6 = OCH_3$

(19) Conopharyngine: $R^1 = R^4 = R^6 = H$, $R^2 = R^3 = OCH_3$, $R^5 = COOCH_3$

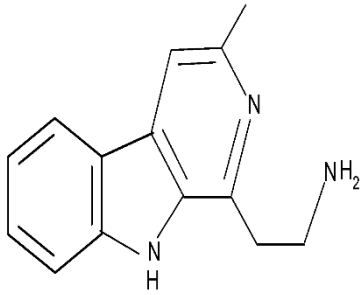
(20) Conoduramine: $R^1 = R^4 = R^6 = H$, R^2 = 3'-vobasinyl, $R^3 = OCH_3$, $R^5 = COOCH_3$



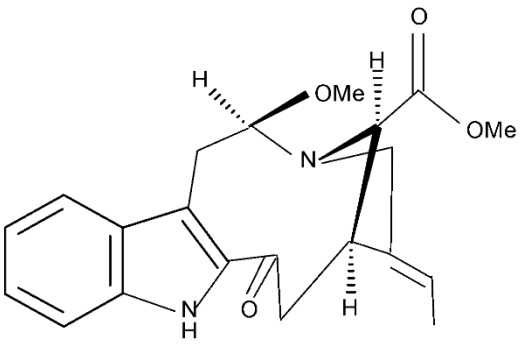
(21) Tabernine A



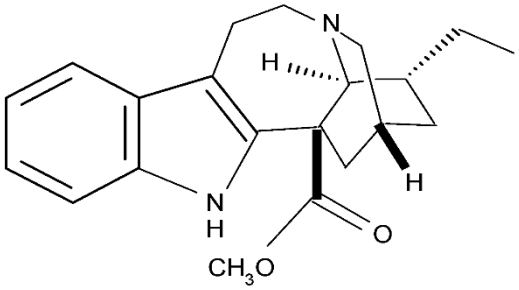
(22) Tabernine B



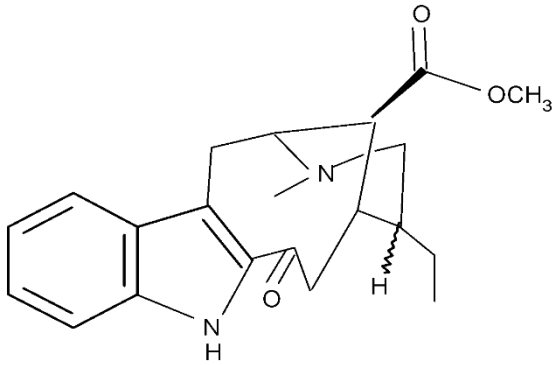
(23) Tabernine C



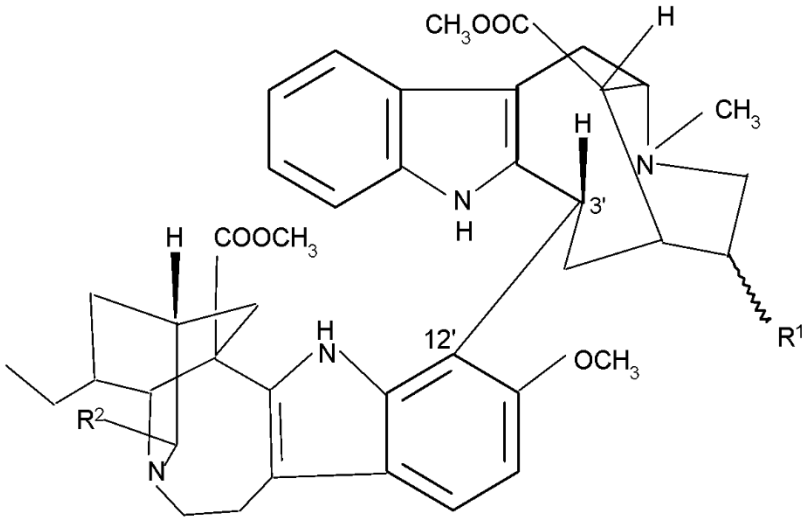
(24) Eleganine A



(25) Voacangine

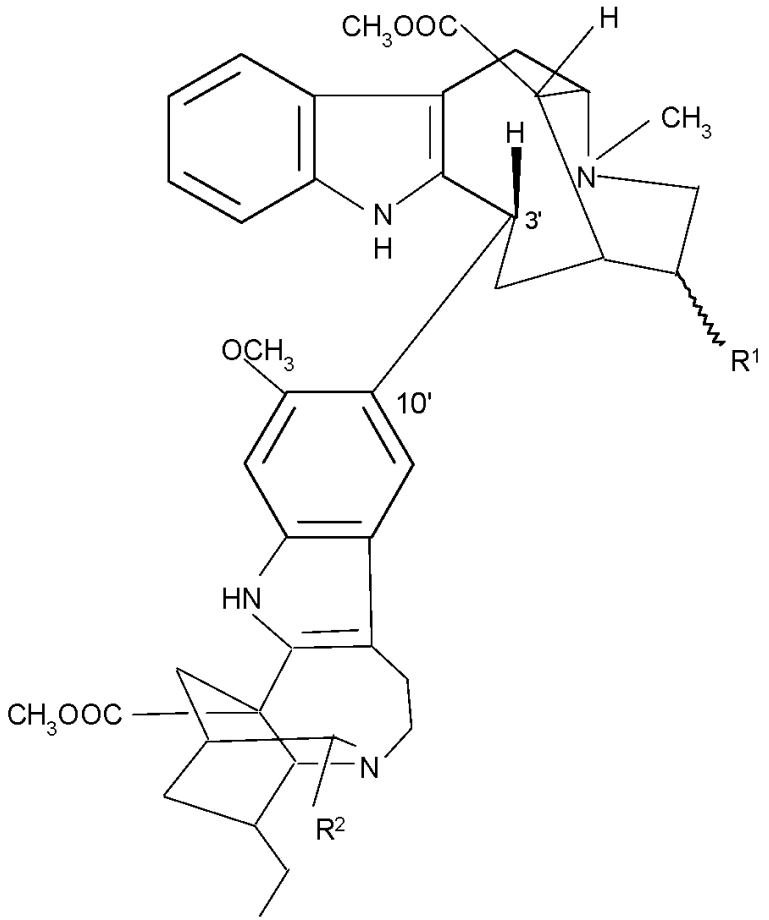


(26) 16-Epidregamine



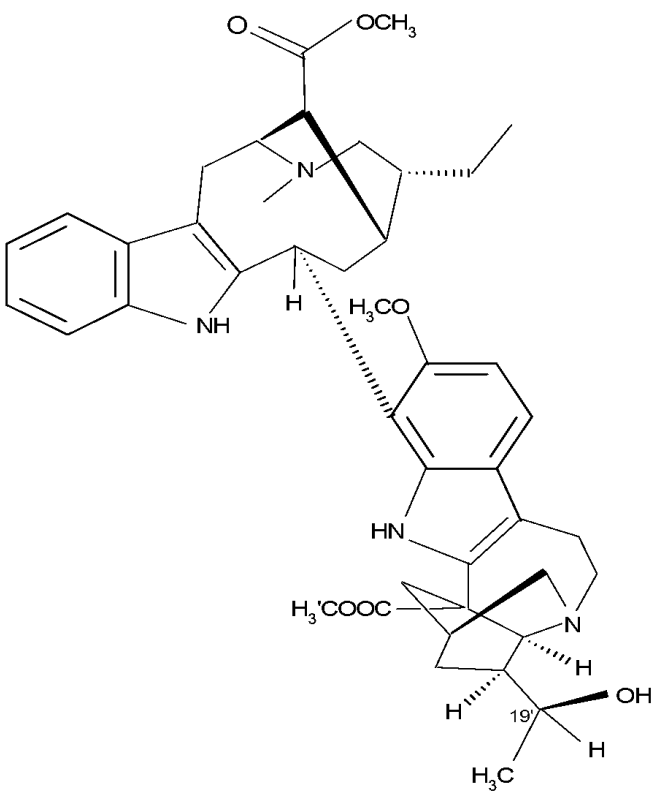
(27) Tabernaelegantinine A: R^1 = β - Et, R^2 = CH_2COCH_3

(28) Tabernaelegantinine C: R^1 = α – Et, R^2 = CN

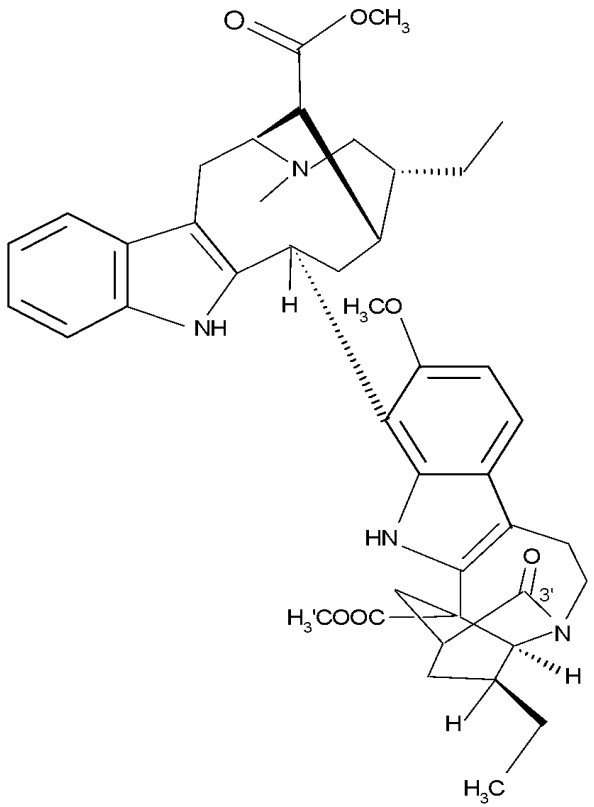


(29) Tabernaelegantinine B: R^1 = β – Et, R^2 = CH_2COCH_3

(30) Tabernaelegantinine D: R^1 = α – Et, R^2 = CN



(31) (19'*S*)-hydroxytabernaelegantine A



(32) 3'-Oxotabernaelegantine C

Figure 2.14. Indole alkaloids isolated from *Tabernaemontana elegans* (Van der Heijden et al., 1986; Gabetta et al., 1975; Bombardelli et al., 1976; Danieli et al., 1980, Mansoor et al., 2009; Mansoor et al., 2013; Paterna et al., 2016; Bapela et al., 2019). Molecular structures drawn by Mkatshane (2019).

2.7.4. Pharmacological activities of *Tabernaemontana elegans*

The pharmacological properties of *T. elegans* have been extensively studied, although there is still much work to be done. There has not been much research conducted on the plant to assess its antiplasmodial and antigonococcal properties. Different plant parts of *T. elegans* have been evaluated for various biological activities on various bacteria, fungi, cancer, and parasites.

2.7.4.1. Antibacterial activity of *Tabernaemontana elegans*

The aqueous root extract exhibited significant activity against *Staphylococcus aureus* and *Mycobacterium smegmatis* at a minimum inhibitory concentration (MIC) of 0.50 mg/ml and 1.00 mg/ml, respectively (Pallant and Steenkamp, 2008). The methanol root extract was not active against *Bacillus subtilis* and *S. aureus* at 64.00 mg/ml and 32.00 mg/ml, respectively (Pallant et al., 2012). Polar (methanol and water) root extracts demonstrated antibacterial efficacy against *Mycobacterium tuberculosis* H37Rv, *M. tuberculosis* H37Ra, *M. bovis* BCG, and *M. smegmatis* at MIC of 15.6 µg/ml, 31.2 µg/ml, 15.6 µg/ml, and 125 µg/ml, respectively (Luo et al., 2011). In another study, the methanol extracts of the leaves inhibited the growths of *Enterococcus faecalis* and *S. aureus* at 62.5 µg/ml and 12.5 µg/ml, respectively (Maduriera et al., 2012). The stem bark of *T. elegans* was tested for activity against bacteria usually seen to cause STIs in South Africa. In a study by Naidoo et al. (2013) the bacteria were *Ureaplasma urealyticum*, *Oligella ureolytica*, *Gardnerella vaginalis*, and *N. gonorrhoeae* ATCC 19424. The powdered stem bark of *T. elegans* was immersed in distilled water (aqueous extract) and a mixture of dichloromethane and methanol (1:1, v/v) (polar extract). The aqueous extract showed activity at 0.25 mg/ml,

8.00 mg/ml, 8.00 mg/ml, and 1.00 mg/ml against *U. urealyticum*, *O. ureolytica*, *G. vaginalis*, and *N. gonorrhoeae*, respectively, and the polar extract displayed activity at 1.00 mg/ml, 4.00 mg/ml, 0.25 mg/ml, and 1.00 mg/ml, respectively. This study by Naidoo et al. (2013) did not fractionate any of the extracts with significant results and tested the fractions for activity. The synergetic effects of the polar extracts of the roots of *T. elegans* and *Ximenia caffra* were tested and demonstrated significant activity against *U. urealyticum* at 0.63 mg/ml (Naidoo et al., 2013). The water extract of the stem bark, as well as the methanol and decoction extracts of the root, showed significant antigonococcal activity at 0.60 mg/ml, 0.10 mg/ml, and 0.80 mg/ml, respectively (Mkatshane, 2019).

The ethnomedicinal significance of using *T. elegans* for the treatment of gonorrhoea is established, and the phytoconstituents associated with the observed antigonococcal activity are yet unidentified and should be regarded for further studies. The use of *T. elegans* by indigenous southern Africans to treat chest pains and pulmonary diseases, wounds, and sores, as well as STIs, especially gonorrhoea, is supported by the abovementioned pharmacological studies.

2.7.4.2. Anticancer activity of *Tabernaemontana elegans*

Tabernine A (**21**), B (**22**), and C (**23**) isolated from the methanol extract of the leaves of *T. elegans* were tested for their ability to change multidrug resistance in mouse lymphoma cell lines. At high doses, tabernine A and C demonstrated moderate apoptosis (Mansoor et al., 2009). Indole alkaloids isolated from the roots were tested *in vitro* for cytotoxicity against HCT116 human colon cancer cells. Tabernaelegantine B (**14**) was more cytotoxic

than tabernaelegantine C (**15**) at 20.00 µM (Mansoor et al., 2013). The cytotoxic activities of isolated compounds tabernaelegantine A (**13**), tabernaelegantine D (**16**), (19'S)-hydroxytabernaelegantine A (**31**), and 3'-oxotabernaelegantine C (**32**) against HCT116 colon cancer cells and HepG2 liver carcinoma cells were investigated. Tabernaelegantine D, (19'S)-hydroxytabernaelegantine A and 3'-oxotabernaelegantine C were shown to be cytotoxic to HCT116 colon cancer cells, with IC_{50} values ranging from 8.40 – 10.00 µM, and none of the compounds were found to be cytotoxic to HepG2 liver carcinoma cells (Paterna et al., 2016).

Compounds of *T. elegans* appear to be toxic in nature, as revealed by these studies, validating the ethnomedicinal use to cure cancer. Many plant secondary metabolites including alkaloids and compounds that are poisonous have resulted in drugs or drug design templates. Plenty of these compounds influence cell division processes, which have given rise to drugs used to treat cancer (Rates et al., 2015). Pharmaceutical companies would have to ensure that the drug is solely hazardous to cancer cells and not to humans.

2.7.4.3. Antifungal activity of *Tabernaemontana elegans*

Aqueous root extract of *T. elegans* demonstrated significant antifungal efficacy against *Candida albicans* at <1.00 mg/ml (Steenkamp et al., 2007). Similarly, the aqueous and polar (dichloromethane and methanol) (1:1, v/v) extracts of the stem bark displayed significant activity against *C. albicans* at 0.38 mg/ml and 2.00 mg/ml, respectively (Naidoo et al., 2013). All polar and non-polar extracts from different parts of *T. elegans* were not active against *C. albicans* at the highest concentration used for testing which was 6.00

mg/ml (Mkatshane, 2019). These studies on the pharmacology of *T. elegans* support ethnomedicinal usage as a remedy to treat STIs, chest pains, and lung diseases.

2.7.4.4. Antiparasitic activity of *Tabernaemontana elegans*

The methanol extract of the leaves of *T. elegans* was not active for antiplasmodial activity at IC_{50} = 26.9 µg/ml (Ramalhete et al., 2008). The polar (dichloromethane, and methanol with distilled water) extracts of the stem bark displayed significant antiplasmodial activity against *P. falciparum* NF54 with IC_{50} of 0.33 µg/ml and 0.83 µg/ml, and SI of 14 and 46, respectively (Bapela et al., 2014). The study was the first to establish that *T. elegans* possesses significant antiplasmodial activity. The acetone extract of leaves was tested for inhibitory activity on egg hatching of *Haemonchus contortus*. The extract inhibited Vero cells by 47% and was not active at LC_{50} = 32.35 µg/ml (Fouche et al. 2016). The aqueous and polar (dichloromethane and methanol, 1:1) extracts of the stem bark were active at MIC >16.00 mg/ml and 1.00 mg/ml, respectively, against *T. vaginalis* (Naidoo et al., 2013). The methanol extract of the root demonstrated significant antiplasmodial activity against the *P. falciparum* NF54 at IC_{50} of 0.16 µg/ml and SI of 16 (Mkatshane, 2019).

Earlier studies on *T. elegans* phytochemicals revealed that the plant is high in MIAs (Pallant et al., 2012). These indole alkaloids are also present in other species of *Tabernaemontana*, of which about six have been tested for antiplasmodial activity. Dregamine (**6**), tabernaemontanine (**8**), tabernaelegantine A (**13**), B (**14**), and D (**16**), all islotaed from *Muntafara sessifolia* (also known as *Tabernaemontana sessifolia*) (Apocynaceae) exhibited significant activity against *P. falciparum* at concentrations of 62.0 µM, 12.0 µM, 13.3 µM, 2.7 µM, 1.20 µM, respectively, and tabernaelegantine B and

D were toxic to human and rat cells, confirming their cytotoxic effects against mammalian cells (Girardot et al., 2012). Dregamine and tabernaemontanine were also isolated from the stem bark of *T. elegans* by Bapela et al. (2019) but were not tested for antiplasmodial activity because they were already confirmed by Girardot et al. (2012) to possess such activity. Apparicine (**1**) isolated from *Aspidosperma olivaceum* (Apocynaceae) was not active at 41.0 µg/ml against *P. falciparum* (Chierrito et al., 2014). The studies carried out by Girardot et al. (2012) and Chierrito et al. (2014) lay the precedence of the possibility of results that will be obtained from compounds of *T. elegans*. The lack of any studies investigating the potential of compounds isolated from *T. elegans* as antimalarials represents a research gap. Conventional extraction and compound isolation processes could be the reason for the absence of studies. While these methods are beneficial, they are inefficient, laborious, time consuming, and there is no assurance of a pure compound in the end.

Although the ethnomedicinal relevance of utilizing *T. elegans* to treat malaria has been confirmed, the compounds responsible for the observed antiplasmodial activity remain limited because none of the isolated compounds from the plant have been assayed. *Tabernaemontana elegans* is used to cure malaria and fever in southern Africa, which is supported by ethnopharmacological studies.

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CHAPTER 3

Antiplasmodial, antigonococcal, and cytotoxic activities of fractions of the stem bark of *Tabernaemontana elegans*, and the application of GC-MS metabolomics to dereplicate antiplasmodial compounds

3.1. Introduction

Tabernaemontana elegans Stapf. (Apocynaceae) is an important medicinal plant species to indigenous southern Africans due to its widespread use in the treatment of numerous diseases. The Vhavenda utilizes the plant to treat malaria and gonorrhoea, among other ailments. Ethnopharmacological studies on the antiplasmodial and antigonococcal activities of *T. elegans* have mainly focused on crude extracts (Naidoo et al., 2013; Bapela et al., 2014; Mkatshane, 2019) leaving a research gap worth exploring. There is still considerable research to be conducted on the fractions of *T. elegans*.

The current study is a continuation of research undertaken by Mkatshane (2019) at our laboratory in the Department of Medicinal Plant Science at the University of Pretoria as part of our ongoing search for new antiplasmodial and antigonococcal drug leads. The study aimed to investigate the antiplasmodial, antigonococcal, and cytotoxic activities of fractions from the methanol crude extract of the stem bark of *T. elegans*. The preliminary study determined that the crude extract was efficacious against *Plasmodium falciparum* NF54 and *Neisseria gonorrhoeae* ATCC 19424, and was not toxic to rat skeletal muscle cell lines L6. The results from the study for antiplasmodial and antigonococcal activities were 0.31 µg/ml and 0.20 mg/ml, respectively. Accordingly, the crude extract was

selected for more research. Sections 3.2.2 and 3.2.3 were carried out by Mkatshane (2019).

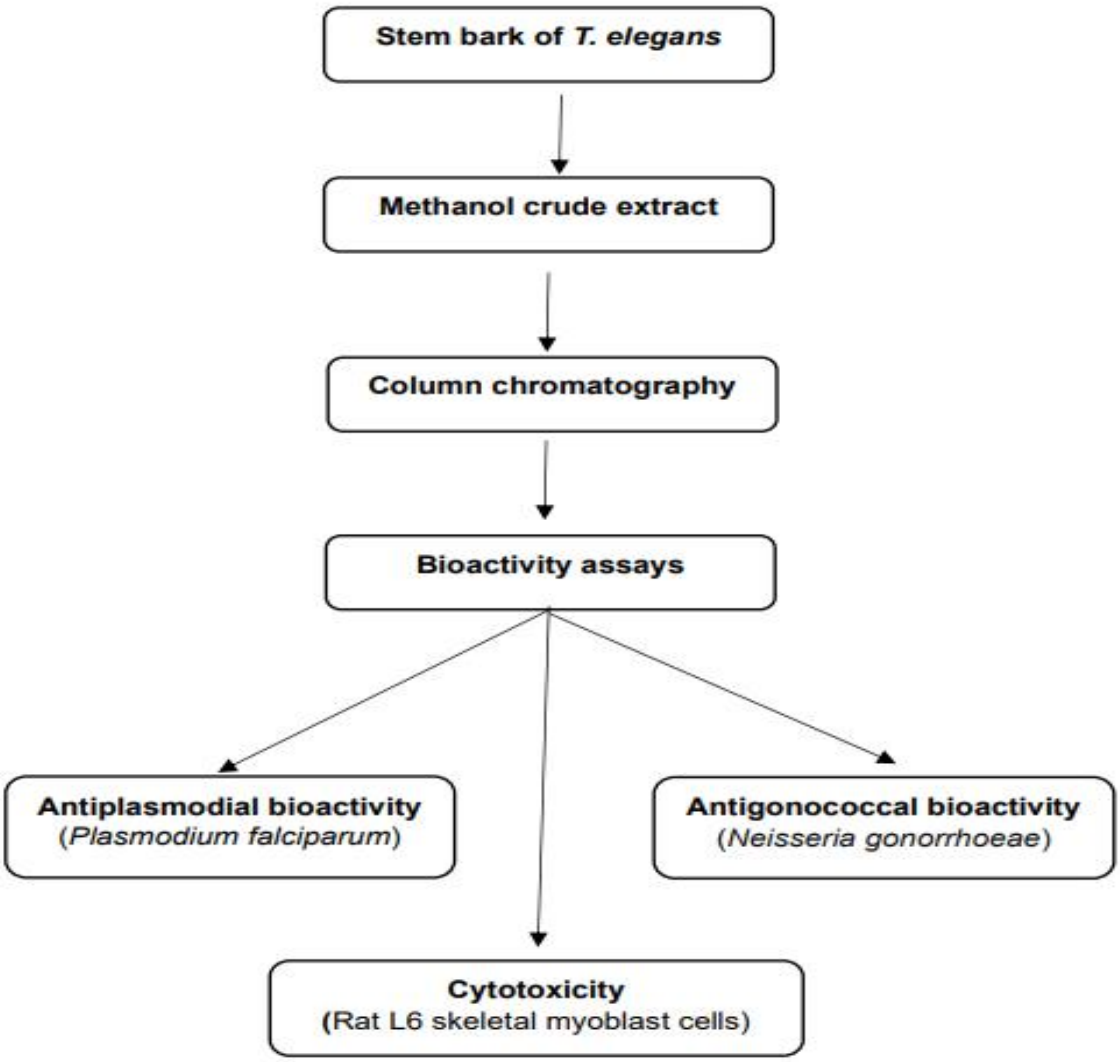


Figure 3.1. An overview of the techniques used to evaluate the biological activities of fractions of the stem bark of *Tabernaemontana elegans*.

3.2.1. Materials and methods

General

Five solvents were used in this study because of their wide polarity range; hexane (H), dichloromethane (DCM), ethyl acetate (EA), methanol (MeOH), and distilled water (dH_2O). The polarity of solvent systems was described in the following manner for this study; a mixture of DCM with H is relatively non-polar, a combination of EA with MeOH is intermediately polar, and dH_2O is polar.

At room temperature, the chemical profiles of the fractions were examined using silica-precoated Thin Layer Chromatography (TLC) plates (MERCK, silica gel 60 F254 0.2 mm thickness). Chemical spots on the TLC plates were observed under ultraviolet light with a spectrum ranging from 254 – 366 nm. The stationary phases for column chromatography were Sephadex and silica gel. The eluting solvents used were all of the analytical grades.

3.2.2. Collection of stem bark of *Tabernaemontana elegans*

The stem bark of *T. elegans* was collected based on the ethnomedicinal use to treat malaria and STIs by VhaVenda people in Mutale Municipality, Limpopo Province, South Africa. A voucher specimen (120337) was submitted to the H.G.W.J. Schweickerdt Herbarium at the University of Pretoria.

3.2.3. Extraction of the stem bark of *Tabernaemontana elegans*

The stem bark was air dried and ground to a powder in an Ultra Centrifugal Mill (ZM, 200, Retsch®, Germany). About 1 kg of powdered plant material was sequentially extracted

with hexane, dichloromethane, and methanol. Each extraction was performed three times with each solvent. The methanol crude extract was then vacuum filtered through Whatman No. 1 filter paper and dried in a laminar flow (VividAir, South Africa), yielding 14 g (2.30%) of crude extract.

The percentage yield of the crude extract was calculated using the equation below:

$$\text{Percentage yield} = \frac{\text{Mass of extract}}{\text{Mass of plant material}} \times 100\%$$

3.2.4. Column chromatography of the methanol crude extract of the stem bark of *Tabernaemontana elegans*

The methanol crude extract of 14 g was subjected to silica gel column chromatography. Selection of the crude extract was based on the significant antiplasmodial and antigonococcal efficacy demonstrated. The crude extract was fractionated with H: DCM, EtOAc: MeOH at a ratio of 10:0 → 0:10 (v/v), and MeOH: dH_2O at 1:1 (v/v). Thin Layer Chromatography was used to analyze all fractions that were collected. All fractions with similar chemical profiles were combined to yield 11 fractions (A – K). Each of the fraction F, G, I, and J were then subjected to Sephadex column chromatography to further partition them into simpler chemical fractions comprising of a reduced number of compounds. The solvents used for fractionation were 100 ml DCM, a 1:1 (v/v) ratio mixture of DCM and MeOH, and 100 ml MeOH. Thin Layer Chromatography was again utilized to analyze all the subsequent fractions collected. Fractions that shared chemical profile similarities were combined to yield a total of 27 fractions.

3.2.5. *In vitro* antiplasmodial activity of fractions of the stem bark of *Tabernaemontana elegans*

The [^{3}H] hypoxanthine incorporation assay was used for antiplasmodial screening of all the acquired 27 fractions (Desjardins et al., 1979; Matile and Pink, 1990). The parasite strain used was *P. falciparum* NF54, and the standard drug was chloroquine (Sigma C6628) (Ponnudurai et al., 1981). Before parasite culture and incubation in RPMI 1640 without hypoxanthine, all samples were dissolved in 10% dimethyl sulfoxide (DMSO). HEPES (5.94 g/l), $NaHCO_3$ (2.1 g/l), neomycin (100 U/ml), AlbumaxR (5 g/l), and human red blood cells A+ at 25% haematocrit (0.3% parasitaemia) were added to the RPMI 1640 growth medium. In 96 well plates, a serial drug dilution of eleven 3-fold dilution steps covering the range of 100 – 0.002 ug/ml was prepared, then incubated in a humidified environment at 37°C, 4% CO_2, 3% O_2, and 93% N_2. After an hour, 50 µl of [^{3}H] hypoxanthine was added to each well, and the plates were incubated for 24 hours before the cells were harvested using a Betaplate™ cell harvester (Wallac, Switzerland). Red blood cells were transferred to a glass fiber filter and rinsed with dH_2O. The filters were dried and then inserted into a plastic foil with 10 ml scintillation fluid and counted in a Betaplate™ liquid scintillation counter (Wallac, Switzerland). The sigmoidal inhibition curves were used to calculate the 50% inhibitory concentration (IC_{50}) values by linear regression using Microsoft Excel Office 365 (Huber and Koella, 1993).

3.2.6. *In vitro* cytotoxic activity of fractions from the stem bark of *Tabernaemontana elegans*

Cytotoxicity testing was performed on rat skeletal muscle cell lines L6 to determine the toxicity or safety of the fractions. The assay was performed on 96-well microtiter plates. Each well contained 100 ul of RPMI 1640 medium supplemented with 200 nM 1% L-glutamine, 10% fetal bovine serum, and 4 000 skeletal cells (Ahmed et al., 1994). A serial drug dilution of eleven 3-fold dilution steps spanning the range of 100 – 0.002 µg/ml was prepared. Podophyllotoxin was used as an experimental control. After an incubation period of 70 hours, the plates were observed under an inverted microscope to ensure the growth of the controls and sterile conditions. Following that, 10 g/ml Alamar Blue was added to each well, and the plates were incubated for another 2 hours. After the incubation, the plates were analyzed with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Corporation, United States of America) at 536 nm excitation wavelength and 588 nm emission wavelength. Using the SoftmaxPro software (Molecular Devices Corporation, United States of America), the IC_{50} values were calculated using linear regression from the sigmoidal curves (Huber and Koella, 1993).

3.2.7. *In vitro* antigonococcal activity of fractions of the stem bark of *Tabernaemontana elegans*

Culturing the bacteria

Agar plates containing chocolate blood agar were inoculated with *N. gonorrhoeae* ATCC 19424 and incubated for 24 hours at 37°C with CO_2 supplementation under anaerobic conditions (CO_2 Gen™ 2.5l; Thermo Scientific, South Africa). Mature bacterial colonies were transferred into 100 mL of Mueller-Hinton broth and subcultured with mild agitation for 24 hours at 37°C. The DU® 720 general-purpose UV/Vis spectrophotometer (Labotec, South Africa) was utilized to prepare the bacterial solution to an optical density of 1.5 x 10^8 colony-forming units (CFU)/ml (0.5 McFarland standard), at a wavelength of 600 nm.

Microdilution assay

The minimum inhibitory concentrations (MICs) of plant fractions against *N. gonorrhoeae* were determined using the microdilution method in 96 well microtiter plates, as described by Eloff (1998). Dimethyl sulfoxide (DMSO) (10%) was used to dissolve each fraction. Sterile Mueller-Hinton broth (100 µl) was pipetted into each of the wells in the microtiter plate. Fractions (100 µl) were then loaded in triplicate, into the first row of the microtiter plate, creating a two-fold dilution. After that, the fractions were serially diluted down the microtiter plate, starting at 12.50 mg/ml and ending at 0.10 mg/ml. The bacterial solution (100 µl) was then added to each well and cultured under anaerobic conditions at 37°C for 24 hours with CO_2 supplementation. The antibiotic ciprofloxacin (0.2 mg/ml) was used as a positive control in the experiment, whereas sterile Mueller-Hinton broth and 10% DMSO were used as negative controls. Bacterial growth was measured by adding 10µl of

PrestoBlue® Cell Viability Reagent (Thermo Scientific, South Africa) to each well before incubating at 37°C for 30 minutes. A pink colour showed microbial growth. PrestoBlue® Cell Viability Reagent 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). The MIC was defined as the lowest concentration of a fraction that visibly prevented the turbidity associated with microbial growth in a liquid medium. A blue colour indicated bacterial inhibition. Therefore, in this experiment, the MIC of each fraction was recorded as the lowest concentration of wells in the microtiter plate that remained blue. The biological assay was repeated three times for each fraction.

3.2.8. ^{1}H NMR spectroscopy of fractions of the stem bark of *Tabernaemontana elegans*

To tentatively identify the different classes of compounds in the fractions, ^{1}H NMR analysis was performed. These are identified by examining the chemical profiles represented by the different spectral chemical shifts for different fractions. Samples of 5 mg/ml each were prepared for analysis by dissolving the fraction in deuterated dimethyl sulfoxide (DMSO-d_6), with 0.1% tetramethylsilane (TMS) as the internal standard. Each sample was transferred into a 5mm NMR tube (Wilmad, South Africa) and analyzed at 400 MHz ^{1}H NMR spectrometer (Bruker, Germany) at a temperature of 30°C. Each spectrum had a spectral width of 14 ppm, and the captured scans were 512 per sample. The collected spectral data were manually phase-adjusted and automatically baseline-corrected using the Whittaker smoother (Eilers, 2003) and referenced to the DMSO-d_6 solvent peak (2.5000 ppm).

3.2.9. GC-MS of fractions of the stem bark of *Tabernaemontana elegans*

To identify the various phytoconstituents attributed to the observed antiplasmodial activity, the fractions were submitted to GC-MS analysis. All relatively non-polar fractions were reconstituted in dichloromethane, intermediate polar fractions in methane, and the polar fraction in water. Each sample was sonicated for 5 minutes at 25°C in an ultrasonic water bath (Labotec, South Africa) to a final concentration of 1 g/ml. All prepared samples were placed in short thread vials (Separations, South Africa). The GC-MS-QP2010SE (Shimadzu, Japan) machine was utilized for gas chromatography and mass spectrometry analysis, and an AOC-20i+s autosampler was used to inject the samples into the machine. The start temperature was set at 100°C, while the end temperature was set at 280°C. Every sample was injected into the splitless mode at 250°C with a 1 µl injection volume. The ion source and interphase temperatures were both adjusted to 250°C and the solvent cut time was 3.5 minutes. The detector voltage was set to 0.1 kV and was modified to produce a turning result. The timer programmed was to start at 4 minutes and end at 26 minutes. The acquisition mode was calibrated to the scan mode, which was set to run at a speed of 2 000. The mass ranged from 35 – 550 m/z. Compounds were identified provisionally by comparing their relative mass spectral data to those in the National Institute of Standards and Technology 11 and 14 (NIST 11 and 14) databases.

3.2.9.1. Multivariate Data Analysis of spectra of the stem bark of *Tabernaemontana elegans*

Spectral data from the GC-MS analysis were converted to ASCII files using MestreNova 8.1.1. (Mestrelab Research, United Kingdom). All the spectra were normalized and scaled to a concentration of 0.1% TMS. The range of 0.00 – 14.00 ppm was binned to 0.04 ppm, yielding 350 variables. ASCII files were imported into Microsoft Excel Version 356 before being opened in SIMCA-P 13.0.0 (Umetrics, Umea, Sweden) to visualize trends and clustering patterns of the selective and non-selective antiplasmodial fractions. To reduce peak interference, Pareto scaling was performed before Multivariate Data Analysis (MDA) (Hendriks et al., 2011). An unsupervised principal component model was used to identify outliers in the data set and to observe trends such as clustering. This was followed by a supervised model to investigate the chemical differences between the selective and non-selective data sets and construct permutation and contribution plots.

3.3. RESULTS AND DISCUSSION

ANTIPLASMODIAL ACTIVITIES AND CYTOTOXICITY

The results of the antiplasmodial activity and cytotoxicity of fractions of the stem bark of *T. elegans* are shown in Table 3.1. Results in bold show significant antiplasmodial activity. *In vitro* assays against *P. falciparum* NF54 and rat skeletal muscle cell lines L6 were performed on 27 fractions. The antiplasmodial activity was considered noteworthy if IC_{50} ≤ 1 µg/ml, good 1 µg/ml < IC_{50} ≤10 µg/ml, and not active IC_{50} > 10 µg/ml. The selectivity index (SI) values were calculated by dividing the values of the cytotoxicity IC_{50} by the antiplasmodial IC_{50}. In this current study, a fraction was considered to have significant antiplasmodial activity and a potential hit for drug discovery when the concentration of the fraction tested inhibited 50% of the growth of *P. falciparum* (i.e., IC_{50} value ≤ 5 µg/ml with SI ≥ 10). It was also considered that the antiplasmodial activity of a fraction is not due to the *in vitro* cytotoxicity when the SI ≥ 10 (Vonthron-Senecheau et al., 2003; Ndjakou et al., 2007). Therefore, the higher the SI, the more effective and safer a crude extract as well as a fraction and a compound are thought to be. Not determined (ND) denotes that the fraction is less toxic, and the cytotoxicity value is relatively too high to be used to determine SI.

Table 3.1.

Antiplasmodial activity and cytotoxicity of fractions of the stem bark of *Taberneamontana elegans* and selectivity index values. IC_{50} values are expressed as a mean value of two independent assays and were recorded in µg/ml.

Fraction	[1]Antiplasmodial bioactivity (IC_{50} (µg/ml))	[2]Cytotoxicity (IC_{50} µg/ml)	Selectivity index (SI)
Stem bark methanol extract	0.31 ± 0.01	24.3 ± 0.05	79
A	>100	>100	ND
B	>100	>100	ND
C	>100	48.6 ± 3.25	ND
D	13.3 ± 1.70	46.6 ± 2.55	4
E	>100	>100	ND
F_2	16.3 ± 0.57	>100	ND
F_3	>100	55.3 ± 0.50	ND
F_4	8.11 ± 1.59	55.0 ± 1.13	7
G_2	**2.31 ± 0.11**	**42.9 ± 10.96**	**19**
G_3	>100	>100	ND
G_4	24.9 ± 2.83	>100	ND
G_5	**3.95 ± 1.27**	**40.2 ± 5.02**	**10**
G_6	20.7 ± 1.06	>100	ND
H	5.77 ± 1.56	65.9 ± 27.7	11
I_1	**0.46 ± 0.04**	**18.2 ± 0.57**	**39**
I_2	**0.33 ± 0.01**	**17.7 ± 1.41**	**54**
I_3	**2.58 ± 0.16**	**41.8 ± 6.51**	**16**
I_4	**2.05 ± 0.67**	**44.4 ± 4.88**	**22**
J_1	5.72 ± 0.50	53.2 ± 0.00	9

J_3	10.6 ± 1.47	>100	ND
J_4	**0.20 ± 0.02**	**23.55 ± 5.16**	**118**
J_5	**0.10 ± 0.01**	**16.50 ± 3.11**	**165**
J_6	**0.32 ± 0.01**	**52.1 ± 2.90**	**163**
J_7	**0.96 ± 0.04**	**>100**	**ND**
J_8	20.4 ± 0.71	>100	ND
J_9	26.3 ± 2.90	>100	ND
K	38.1 ± 1.41	>100	ND
Chloroquine	0.004		
Podophyllotoxin		0.009	

[1]*P. falciparum* NF54 strain

[2]Rat skeletal muscle cell lines L6

ND: Not determined

Fractions G_1 and J_2 were not tested for biological activities because there was not enough quantity to conduct the assays. The positive control used was chloroquine and it demonstrated activity at 0.004 µg/ml. The SI value was not calculated. Out of the 27 fractions assayed for antiplasmodial activity and cytotoxicity, ten demonstrated significant activity against *P. falciparum* and non-toxic to mammalian cells. All these fractions had $IC_{50} \leq 5$ µg/ml and SI ≥ 10 or were not determined. The ten fractions were able to inhibit half of the growth of the parasite, and the inhibition was not attributable to *in vitro* cytotoxicity on rat skeletal muscle cells. The best candidates were J_4 and J_5, which had IC_{50} of 0.20 µg/ml and 0.10 µg/ml as well as SI of 118 and 165, respectively. Compared to the methanol crude extract of the stem bark of *T. elegans* from which they were fractionated, J_4 and J_5 demonstrated remarkably greater antiplasmodial efficacy against the NF54 strain of *P. falciparum* and were not toxic to mammalian cells. The crude extract

exhibited activity at IC_{50} of 0.31 µg/ml with SI of 79. Fractionation of the crude extract enhanced the antiplasmodial activity. The SI also increased in some fractions, indicating the relative safety of the samples. Other fractions that showed significant antiplasmodial activity were J_6 and J_7 with IC_{50} of 0.32 µg/ml and 0.94 µg/ml, respectively. The SI of J_6 was 163 and that of J_7 was too high to be determined. Although J_6 and J_7 had slightly less activity than the crude extract, the fractions showed significant activity. Fraction group $J_4 – J_7$ exhibited the most significant antiplasmodial activity. Another group of fractions that demonstrated significant antiplasmodial activity was $I_1 – I_4$. Fractions I_1 and I_2 displayed higher activity than the crude extract at IC_{50} of 0.46 µg/ml (SI of 39) and 0.33 µg/ml (SI of 54), respectively. This is important because it shows the presence of bioactive compounds in these fractions, which might be used to treat malaria and its related symptoms. Although most of the fractions did not display significant antiplasmodial activity against *P. falciparum* NF54, the lack of significant biological activity from these fractions cannot be completely ruled out as they could be active against other *Plasmodium* strains.

Interactions between multiple compounds can have antagonistic, non-interactive, or synergistic effects on biological activity. Although the interaction of various phytoconstituents in the crude extract produced significant results, the compounds that are essential antiplasmodial contributors may have had their activity partially obscured by the number of compounds present. The phytoconstituents in the crude extract appear to have had (to some extent) antagonistic effects on antiplasmodial activity because upon fractionation the activity greatly increased. Also, the compound(s) responsible for the observed activity was less in J_5 than in the crude extract, allowing for less antagonistic

effects among them. Plant compounds may potentially hide the effects or activities of other compounds in a sample (Sasidharan et al., 2011). This can happen for several reasons; complex chemical mixtures, antagonistic interactions, dose-dependent effects, and different mechanisms of action.

One of the ultimate goals of biological assays using plant material is to obtain pure compounds with significant activity. Of the methods employed to achieve that goal is the fractionation of a crude extract. As a result, fractionation of a crude extract reduces the number of compounds present in each sample. In the search for new antiplasmodial phytoconstituents, fractionation of plant samples is necessary, as demonstrated by the current study. The higher efficacy observed in the fractions could be due to compounds operating in smaller amounts in the fractions compared to the crude extract. Single compounds with improved biological activity are thus prioritized for drug discovery. The measured activity in fractions I_1 – I_4 and J_4 – J_7 supports this proposed hypothesis. Since they are consecutive, the observed antiplasmodial activity in the fractions of I_1 – I_4 and J_4 – J_7 could be due to the same compound(s). The activity and SI of J_5 increased slightly more than twice as much as that of the crude extract. The concentration of the active compound(s) in the fraction also plays a role in biological activity. In this case, the concentration of the compound(s) responsible for the antiplasmodial activity was reduced in J_5 than in the crude extract. This would mean that at a high concentration, some compounds do not exhibit the desired activity.

The findings of this study are consistent with other prior studies on the *in vitro* antiplasmodial activity of medicinal plants. In a study by Bapela et al. (2014), the stem bark of *T. elegans* was extracted with polar solvents. The DCM, and MeOH: dH_2O (1:1,

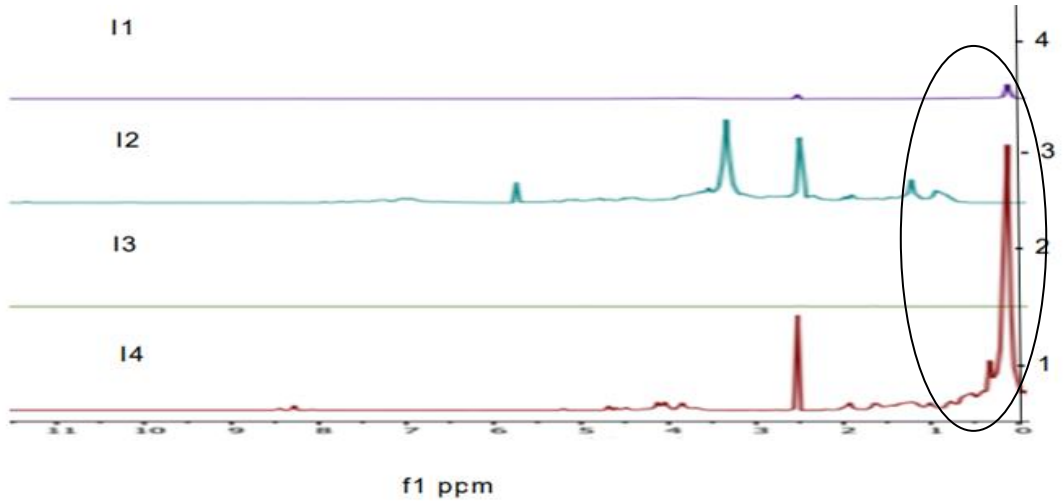
v/v) extracts were assayed for antiplasmodial activity on *P. falciparum* NF54 and cytotoxicity was carried out on rat skeletal cells L6. The DCM extract was active at IC_{50} of 0.33 µg/ml with SI of 14, while the MeOH: dH_2O extract was efficacious at IC_{50} of 0.83 µg/ml with SI of 46. The extracts demonstrated significant antiplasmodial activity. In the same study, an alkaloidal fraction of the stem bark of *T. elegans* displayed significant antiplasmodial efficacy at IC_{50} of 0.06 µg/ml with SI of 48. This was the first study to report significant activity of *T. elegans* against *P. falciparum*. In a study conducted by Mabuza (2022), the twigs of *Pappea capensis* were extracted with DCM and then fractionated the extract using H: DCM solvent system. Both the extract and fraction were tested against *P. falciparum* NF54. The extract was active at IC_{50} of 2.59 µg/ml with an SI of 21, while the fraction was active at IC_{50} of 0.91 µg/ml with an SI of 30. In the studies by Bapela et al. (2014), Mabuza (2022), as well as the current one, the antiplasmodial activity and SI of a crude extract was greatly enhanced by fractionation.

Extraction is one of the most important procedures in the development of plant formulations for pharmacological assays since it is necessary to obtain the desired phytoconstituents (Azmir et al., 2013). Inappropriate extraction processes may result in natural product degradation. The extraction and eluting solvents have a substantial function in acquiring the necessary compounds for biological activity. Modern techniques of extraction often use organic solvents with different polarities to take advantage of the different solubilities of phytoconstituents, but traditional approaches employ water as an extractant. Most of the solvents used in the studies by Bapela et al. (2014), Mabuza (2022), and the current one were mainly polar. Ivanov et al. (2022) showed that the extraction and eluting solvents have major effects on the biological activities of a

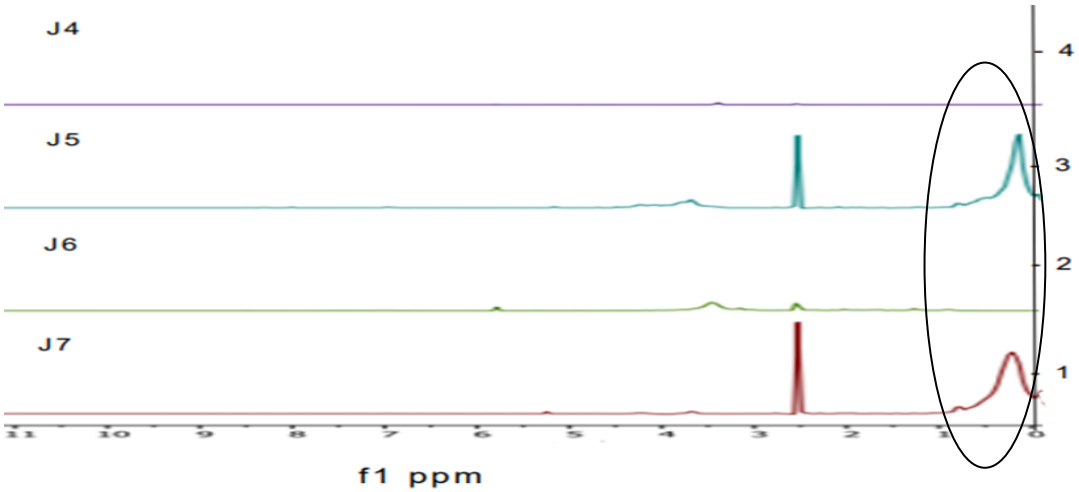
medicinal plant part. A general trend is that antiplasmodial activity increases with solvent polarity. In the current study, the solvents that can elute some antiplasmodial compound(s) from the crude extract appear to be polar. Fractions I_1 – I_4 were eluted with relatively non-polar (H and DCM) solvents. Fractions J_4 – J_7, on the other hand, were eluted with intermediately polar (EA and MeOH) solvents. All these fractions showed significant antiplasmodial activity, however, the intermediately polar fraction group displayed higher antiplasmodial activity. The results are most likely owing to the ability of ethyl acetate and methanol to elute more secondary metabolites with antiplasmodial activity (Chandrasekaran and Venkatesalu, 2004). Fraction K, the only polar (dH_2O) sample, exhibited antiplasmodial activity at IC_{50} of 38.1 µg/ml and the SI was too high to be determined. Apart from the fractions with IC_{50} >100, fraction K showed the lowest bioactivity. In this case, water was not a suitable eluting solvent. The evaluation of water fraction attempts to replicate traditional use, and when it is found to lack significant biological activity, it is quite discouraging. When extracting, fractionating, and purifying plant materials, much emphasis should be placed on the solvents to be utilized.

^{1}H NMR AND GC-MS ANALYSES

Nineteen fractions were subjected to ^{1}H NMR analysis to tentatively identify classes of compounds that are responsible for the antiplasmodial activity. The ^{1}H NMR spectra I_1 – I_4 and J_4 – J_7 were stacked and analyzed to observe their chemical shifts (Figure 3.2A and Figure 3.2B). The ^{1}H NMR spectral region of both fraction groups of 0.5 – 1 ppm depicts some similarities, pointing to a comparable class of compounds attributable to the observed antiplasmodial bioactivity. Aliphatic hydrocarbons, especially alkanes, were the most abundant constituents in the two fraction groups and may have mainly contributed to the observed antiplasmodial activity. It was subsequently validated by comparisons with other research that used ^{1}H NMR to evaluate aliphatic hydrocarbons, and the association was established (Vougioukalakis and Grubb, 2008). Antiplasmodial activity has been described for alkanes (Kgokong et al., 2008, Anjuwon et al., 2023, Séguin et al., 2023). Fraction J_5 was the most bioactive and had the most noticeable signal. The fraction would be the best candidate for dereplicating new antiplasmodial compounds.



A



B

Figure 3.2. ^{1}H NMR chromatograms of spectra of the most antiplasmodial active fractions of *Tabernaemontana elegans* showing two fraction groups; (A) I_1 – I_4 and (B) J_4 – J_7. Fractions I_4 and J_5 had the most prominent peaks in each fraction group.

From the analysis of the fractions J_4 – J_7 using GC-MS, the fractions shared identical phytoconstituents at 7.5 – 14.3 minutes (Figure 3.3). These were tentatively determined to be dodecane, pentadecane, and heptadecane (NIST, 2020). Dodecane and heptadecane were isolated from *Annona squamosa* (Annonaceae), a plant with known antiplasmodial properties (Sawant et al., 2023). Heptadecane was detected in a sample extracted from the twigs of *Pappea capensis* (Sapindaceae), and the sample demonstrated significant activity against *P. falciparum* NF54 at 0.91 µg/ml with SI of 30 (Mabuza, 2022). The antiplasmodial activity of the relevant compounds has not been investigated. However, their presence in antimalarial plants shows that they may have the needed antiplasmodial pharmacological effects. The antiplasmodial activity of dodecane, pentadecane, and heptadecane would need to be evaluated.

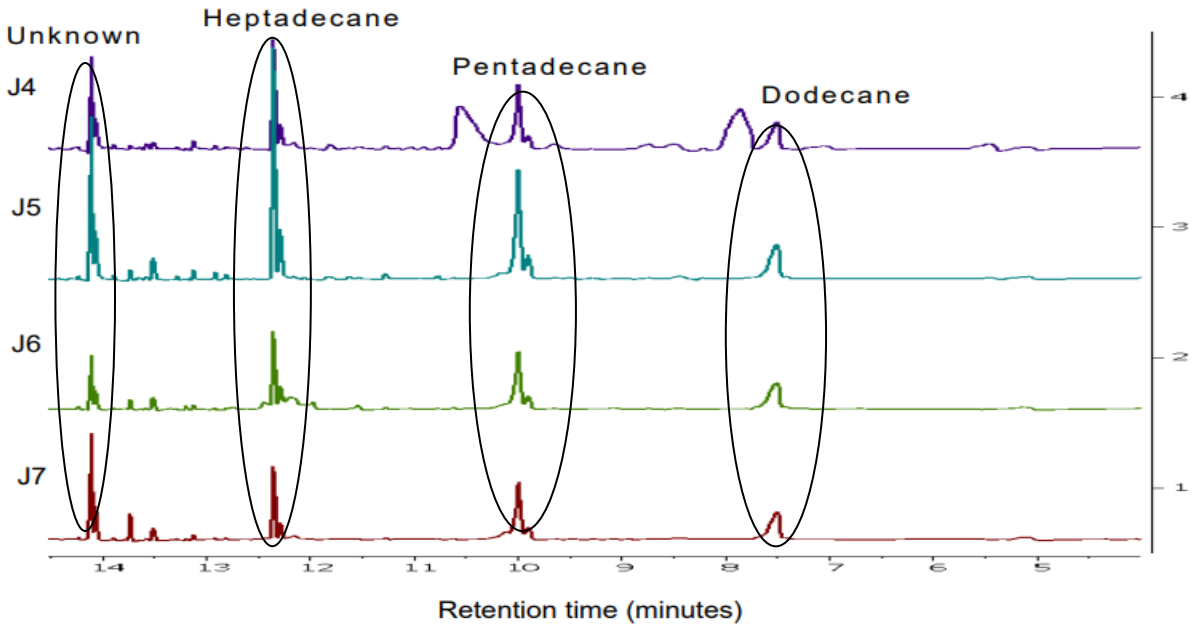


Figure 3.3. Stacked GC-MS chromatogram of spectra of the most antiplasmodial active fraction group J_4 – J_7 of *Tabernaemontana elegans* showing heptadecane, pentadecane, and dodecane.

MULTIVARIATE DATA ANALYSIS

Metabolomics was only performed on antiplasmodial fractions because there were more of these than antigonococcal fractions, which were 17. The fractions obtained from the methanol crude extract of the stem bark of *T. elegans* were grouped into selective and non-selective based on their antiplasmodial activity. Metabolomics using GC-MS was conducted on 25 fractions. Fractions G_1 and J_2 were excluded because there was not enough quantity to conduct the analysis. The fractions were subjected to unsupervised Principal Component Analysis (PCA) to assess if the model would be capable of separating the fractions based on the observed antiplasmodial bioactivity. The Distance to Model X (DMod[X]), Hotelling's T2Range plot, and visualizing the PCA score plot were applied. The resulting plot showed outliers E and J_1 which were studied and not excluded in the analysis. The resulting PCA score plot was unable to discriminate between selective and non-selective antiplasmodial fractions (Figure 3.4). The lack of clustering of the PCA score plot based on the observed bioactivity could have resulted from the diverse nature of the compounds present in the fractions given that clustering in this case should be due to the respective chemistry of the individual compounds.

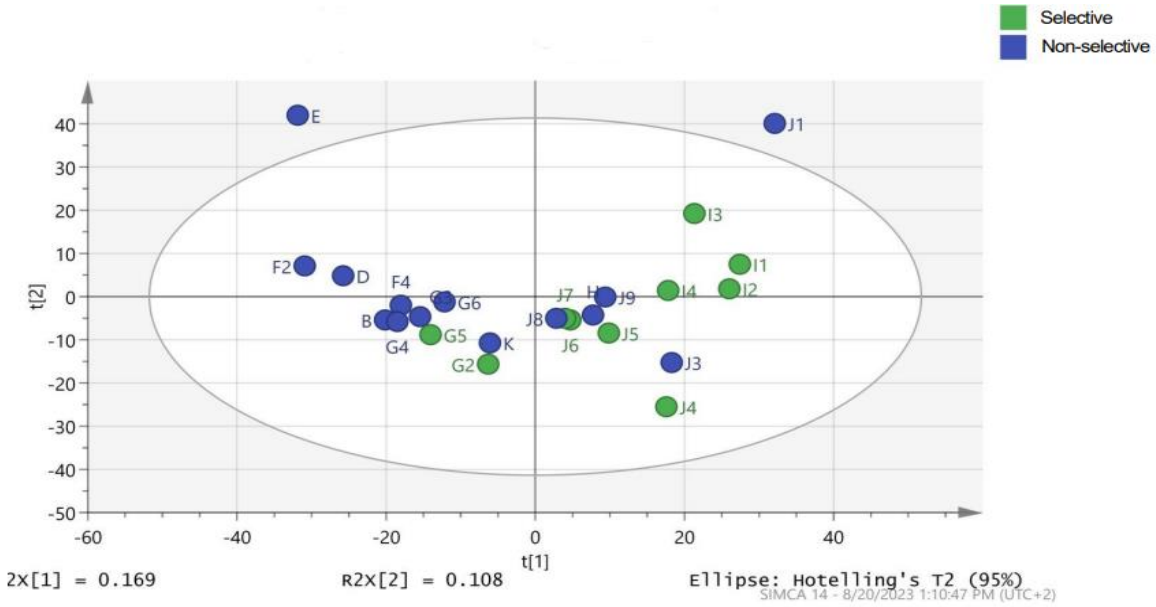


Figure 3.4. Principal Component Analysis score plot of fractions of the stem bark of *Tabernaemontana elegans* from the GC-MS data based on the selective indices of the fractions with two groupings; selective and non-selective. The plot shows outliers E and J_1.

The absence of clustering shown in the PCA score plot based on observed antiplasmodial bioactivity led to the application of the supervised Orthogonal Projections to Latent Structures-Discriminant Analysis (OPLS-DA) to the GC-MS dataset. The algorithm was supplied with the selective and non-selective classes. The OPLS-DA score plot was able to discriminate between the antiplasmodial classes using Principal Component (PC) 1 (t[1]), while PC2 (t[2]) displayed varying secondary metabolites within a class (Figure 3.5). Upon visualization, the previously observed outliers E and J_1 now correspond to the reported antiplasmodial bioactivity and are grouped in the non-selective class.

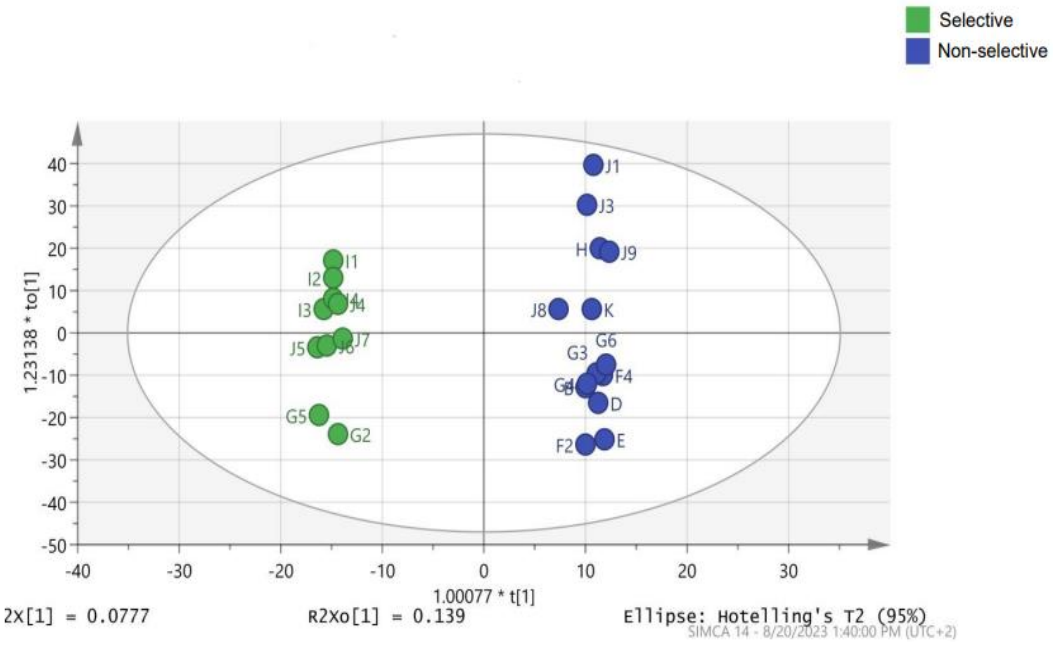


Figure 3.5. Score plot of OPLS-DA model of fractions of the stem bark of *Tabernaemontana elegans* from the GC-MS data based on the selective indices of fractions classified into two groups; selective and non-selective.

The OPLS-DA model generated is interpreted by the variables R^2 and Q^2. The cross-validation variance value is represented by R^2 and provides a general indication of the fitness of the model while Q^2 is the variance and measures the model's ability to predict antiplasmodial bioactivity (Eriksson et al., 2013). The R^2 and Q^2 values were 0.472 and 0.396, respectively. The model generated from the GC-MS dataset was successful in discriminating selective from non-selective antiplasmodial classes since the R^2 value was 0.472. Nonetheless, the low Q^2 value of 0.395 showed that the model was not robust enough to predict the antiplasmodial activity of unknown samples. Manipulating GC-MS data for metabolomic studies is rather challenging since all the spectra lack a reference or solvent peak, and therefore cannot be aligned to a single peak present in all the

samples. Models can interpret the data well and are not considered to be overfitted when the R^2 and Q^2 are approximately values of 0.5. Values of R^2 and Q^2 above 0.5 are regarded as good biological samples. The disparate values between R^2 and Q^2 (R^2 >> Q^2) define the model as one that is overfitted (Hendricks et al., 2011). Overfitting generates bias because the training data set is related to the model. It limits the model's applicability to the data set in issue and excludes it from other data sets. To avoid overfitting for future studies, data augmentation, cross-validation, feature selection data, and regularization can be applied. A robust and non-overfitted model should also have a T-test *P*-value of less than 0.05. The model was validated further using CV-ANOVA and permutation score plots (Figure 3.6). The CV-ANOVA score plot obtained investigated the variance in the cross-validation of the Y variable residuals, whilst the permutation plot assessed the degree of risk that a model is spurious (Eriksson et al., 2013). With R^2 and Q^2 values below 0.5, the OPLS-DA model was at a substantial risk of overfitting. The low Q^2 value hampered its ability to forecast antiplasmodial bioactivity. The model has a major chance of overfitting with a T-test $P = 0.592$, irrespective of the clear grouping based on observed bioactivity.

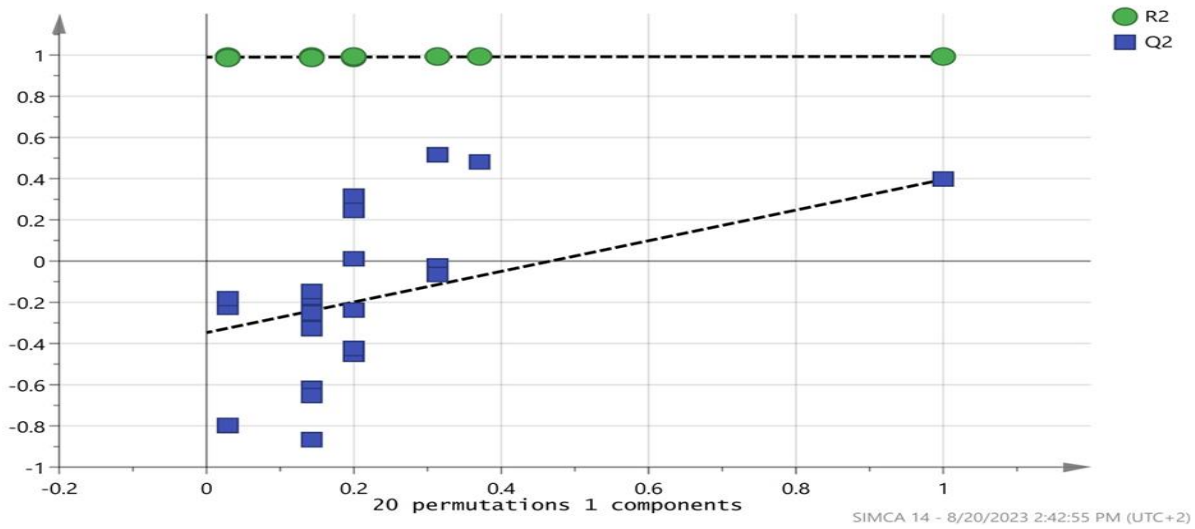


Figure 3.6. Permutation score plot validating the OPLS-DA generated GC-MS data with R^2 and Q^2 values of 0.472 and 0.396, respectively.

The GC-MS-derived OPLS-DA data was used to generate a contribution plot of different phytoconstituents suggested to influence the antiplasmodial bioactivity (Figure 3.7). Using GC-MS spectrum libraries (NIST 11 and 14), it was discovered that icosane and hexadecane contributed considerably to antiplasmodial activity. Icosane and hexadecane are alkanes and were detected in medicinal plants with established antiplasmodial efficacy (Nahrevanian et al., 2010; Iyekowa and Edema, 2017). Despite its low prediction, the OPLS-DA analysis remains a strong technique for identifying phytoconstituents. Unknown compounds can also be easily discovered and targeted for additional antiplasmodial and cytotoxicity studies utilizing various optimal methods. To discover novel antiplasmodial compounds, all unknown compounds with notable peaks should be prioritized for isolation and characterization.

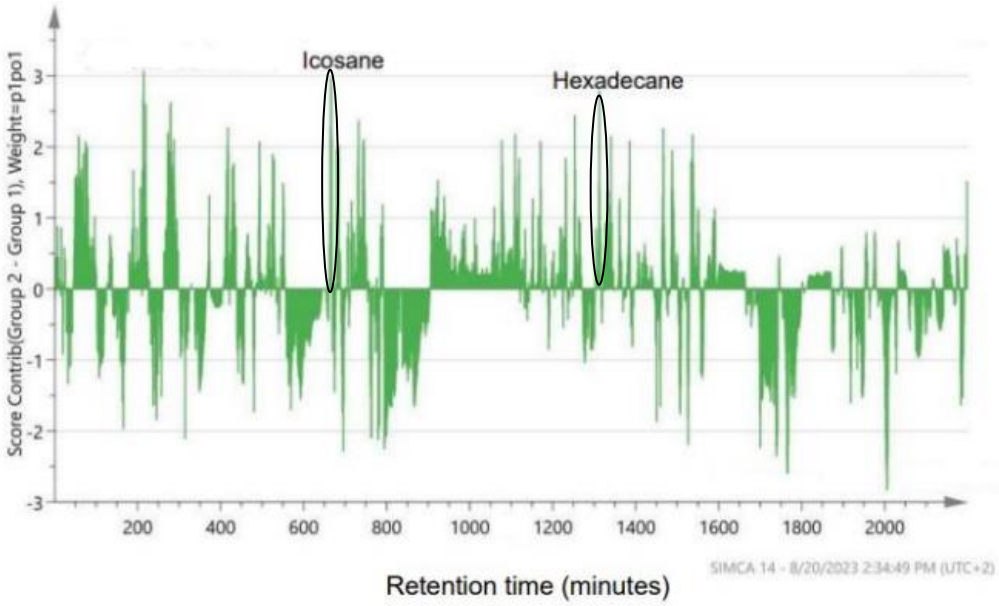


Figure 3.7. Contribution plot generated from the OPLS-DA model of the GC-MS data of the fractions of *Tabernaemontana elegans* showing the comparison between selective (bars projecting upwards) and non-selective (bars projecting downwards) phytoconstituents. The plot shows icosane and hexadecane compounds that contributed to the antiplasmodial activity.

ANTIGONOCOCCAL ACTIVITY

In vitro antigonococcal screening against *N. gonorrhoeae* ATCC 19424 was conducted on fractions acquired from the fractionation of the methanol extract of the stem bark of *T. elegans*. The MIC values of fractions are presented in Table 3.2. Results in bold show noteworthy antigonococcal activity.

Table 3.2.

Antigonococcal activity of fractions of the stem bark of *Tabernaemontana elegans*.

Fraction	[1]Antigonococcal bioactivity (MIC (mg/ml))
Stem bark methanol extract	0.20
B	12.5
D	12.5
E	12.5
H	6.25
I_1	**0.78**
I_2	3.13
I_3	6.25
I_4	**0.78**
J_1	12.5
J_3	6.25
J_4	6.25
J_5	12.5
J_6	12.5
J_7	>12.5
J_8	>12.5

J_9	12.5
K	12.5
Ciprofloxacin	<0.01

[1]*N. gonorrhoeae* ATCC 19424

Only 17 fractions were tested for antigonococcal activity since there was not enough quantity of the other samples to conduct the assay. The fractions were tested at concentrations ranging from 12.50 mg/ml to 0.10 mg/ml. In the current study, the antimicrobial activity of fractions was assessed as follows; fractions with MIC ≤ 1.00 mg/ml are considered to have noteworthy activity and should be prioritized for further studies. The positive control in the experiment which was the ciprofloxacin exhibited activity at <0.01 mg/ml, whereas the negative control of sterile Mueller-Hinton broth and 10% DMSO did not show any biological activity. Fractions I_1 and I_4 from the 17 tested exhibited noteworthy activity. The two fractions showed activity at a MIC of 0.78 mg/ml against *N. gonorrhoeae*. In this case, the antigonococcal activity observed in the fractions was less than that in the methanol crude extract which was 0.20 mg/ml. Column chromatographic fractionation of the crude extract did not increase antigonococcal activity. The lack of noteworthy antigonococcal activity demonstrated by most fractions of *T. elegans* does not imply the absence of bioactive phytoconstituents. The bioactive compounds may be present in trace levels in the fractions as compared to the crude extract, and their concentration was reduced thus the diminished activity. It is also worth noting that Gram-negative bacteria, as in the case of *N. gonorrhoeae*, are often impervious to antibacterial compounds because of lipopolysaccharides on their outer membrane (Gupta and Datta, 2019). This might explain why there are so few active fractions against *N. gonorrhoeae*.

Similar results were observed in a study by Ginovyan et al. (2017). The DCM extract was efficacious to *Staphylococcus aureus* and *Candida albicans* at 1.00 mg/ml and 0.25 mg/ml, respectively. After fractionation of the DCM extract, the activity against *S. aureus* and *C. albicans* diminished to 2.50 mg/ml and >6.00 mg/ml, respectively. In a study by Domingo (2022), the methanol crude extract of the stem bark of *Ficus abutilifolia* (Moraceae) demonstrated noteworthy antigonococcal activity at 0.20 mg/ml against *N. gonorrhoeae* ATCC 19424. Upon fractionation of the extract, the highest MIC value of fractions was 0.32 mg/ml, and five fractions did not show activity at the highest concentration tested, which was 2.50 mg/ml. The studies by Ginovyan et al. (2017), Domingo (2022), and the current one, fractionation of the crude extract reduced biological activity.

The ^{1}H NMR spectra of fractions I_1 and I_4 were stacked as shown in Figure 3.2A. The most noticeable chemical shifts shared were in the 0.5 – 1 ppm range, indicating that the samples had an abundance of aliphatic hydrocarbons. The antibacterial activity of alkanes has been demonstrated (Rouis-Soussi et al., 2014; Muhammad et al., 2018; Vanitha et al., 2020), which may account for the antigonococcal activity observed in the study.

3.4. Conclusion

The findings of the study lend credence to the Vhavenda people's ethnomedicinal use of *T. elegans*, as well as the reason for using an ethnopharmacological approach when bioprospecting medicinal plants for antiplasmodial and antigonococcal lead compounds. The main goal of ethnopharmacology is to find novel plant-derived compounds, based on

the indigenous use of medicinal plants, which can be developed into new pharmaceuticals. The current study also pointed out the need for South African medicinal plant species to be investigated for pharmacological properties, in addition to the necessity to determine the basis of their phytotherapeutic efficacy. The value of ^{1}H NMR and GC-MS analyses and metabolomics in identifying the class of compounds and phytoconstituents that may have contributed to the observed activity while also distinguishing groups of compounds that may be associated with antiplasmodial activity were also advantageous in the study.

As a prediction instrument for unknown antiplasmodial samples, the GC-MS OPLS-DA model was demonstrated to be unreliable and non-robust and therefore cannot be utilized to screen for antiplasmodial activity at high throughput. Regrettably, the model also cannot be used to investigate the antiplasmodial activity of other plant species. However, for identifying compounds from plants in complex samples, GC-MS analysis proved to be an invaluable instrument. It is an efficient tool for dereplicating known compounds with proven antiplasmodial efficacy from the unknown. Although plant metabolomics is still an emerging research field, it has made substantial advances in drug discovery. Other analytical techniques such as liquid chromatography and supercritical fluid chromatography could improve the outcome of the observed data. Developing new methods for referencing GC-MS spectra to a common standard could also increase the model's goodness of fit, improving its odds of being a trustworthy predictor of antiplasmodial activity (Mabuza, 2022). The ability of GC-MS to identify compounds of interest is appropriate in bioprospecting drug leads for antimalaria targets. This approach is an essential way of propelling plant metabolomics research to new

heights. This study proved the feasibility of employing these two strategies to focus on unknown notable antiplasmodial phytoconstituents by dereplicating known antiplasmodial compounds.

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CHAPTER 4
General conclusion, challenges, and prospects of the study

4.1. General conclusion

The current study highlights the importance of traditional medicine and the indigenous knowledge of the Vhavenda in the treatment of infectious diseases. The findings of the study provide an understanding of the ethnomedicinal use of *Tabernaemontana elegans* and show the potential of exploring different secondary metabolites of this medicinal plant in manufacturing new antimalarials and antibiotics with various mechanisms of action in the treatment of malaria and gonorrhoea.

The study aimed to explore the antiplasmodial, antigonococcal, and cytotoxic activities of fractions from the methanol crude extract of the stem bark of *T. elegans*. The intermediately polar fractions demonstrated the highest antiplasmodial activity while remaining non-toxic to mammalian cells. The relatively non-polar fractions exhibited the most antigonococcal activity. The only polar fraction did not show any significant or noteworthy biological activity. The desired activity is usually found in an isolated compound rather than a fraction. More research is required to isolate and identify compounds responsible for the observed biological activities. A great number of medicinal plants have been screened and tested for their antiplasmodial and antigonococcal activities, and compounds have been isolated. Little to no effort has been made to produce drugs from most of these phytoconstituents. The difficulties in obtaining a drug from a plant include spending a significant amount of time and money on innumerable leads that are discarded during the process and thus may be viewed as a non-viable path to embark on. The process of drug discovery and development from plants can be

mundane and laborious. Nevertheless, advances in biotechnology have provided new platforms that allow the detection of secondary metabolites from medicinal plants, especially in the initial stages of experiments.

Analysis of the chromatogram of the proton nuclear magnetic resonance (^{1}H NMR) spectra was able to provisionally determine the class of compounds responsible for the observed antiplasmodial and antigonococcal activities to be alkanes. The clustering of fractions was in accordance with the observed antiplasmodial activity when the Orthogonal Projections to Latent Structures-Discriminant Analysis (OPLS-DA) algorithm was applied to the gas chromatography-mass spectrometry (GC-MS) dataset, and was confirmed by the permutation score plot. Employing plant analysis and metabolomics in studies proves to be a powerful tool as it expedites the process of drug discovery from medicinal plants.

The research questions of the current study were; (1) Can fractionation of the methanol crude extract of the stem bark of *T. elegans* enhance the antiplasmodial and antigonococcal activities? (2) Can GC-MS metabolomics discriminate between selective and non-selective antiplasmodial fractions of *T. elegans*? The hypotheses of the study were; (1) Fractionation of the methanol crude extract of the stem bark of *T. elegans* will yield enhanced antiplasmodial and antigonococcal active fractions. (2) Metabolomics approaches using GC-MS will distinguish between selective and non-selective antiplasmodial fractions of *T. elegans*.

Fractionation of the methanol crude extract improved antiplasmodial activity against *Plasmodium falciparum* NF54, however, failed to enhance antigonococcal activity against *Neisseria gonorrhoeae* ATCC 19424. The Principal Component Analysis model generated from the GC-MS data was unable to distinguish between selective and non-selective antiplasmodial fractions. The OPLS-DA was then applied to the GC-MS dataset and the score plot could differentiate between selective and non-selective antiplasmodial fractions of *T. elegans*. The former statement relating to the enhanced antiplasmodial activity of the first hypothesis is accepted, while the latter statement about increased antigonococcal activity is rejected. The second hypothesis is accepted since it holds true to the results.

4.2. Challenges and prospects

A major challenge when conducting this study was a shortage of the methanol crude extract. Since the study was a continuation of previous research, there was only 14 g crude extract which proved to be insufficient. Some of the crude extract was lost during the column chromatography, antiplasmodial and cytotoxic assays as well as GC-MS analysis, leaving a small amount of it. As a result, not all the fractions could be tested for antigonococcal activity. More plant material will be collected and extracted in the future to enhance the yield of the crude extract which will also increase the amounts of plant material in the fractions, and perform more bioassays.

The fractions of the stem bark of *Tabernaemontana elegans* demonstrated significant antiplasmodial activity, prospects should prioritize the isolation, purification, and characterization of compounds from active fractions that are responsible for the biological

activity observed in the study. This is the most efficient method for studying phytoconstituents that have never been studied before, increasing the chances of discovering new compounds with novel mechanisms of action that could lead to new antimalarial and antigonococcal drugs.

CHAPTER 5
Supplemental information

APPENDIX A

GC-MS chromatograms of fractions of the stem bark of *Tabernaemontana elegans*

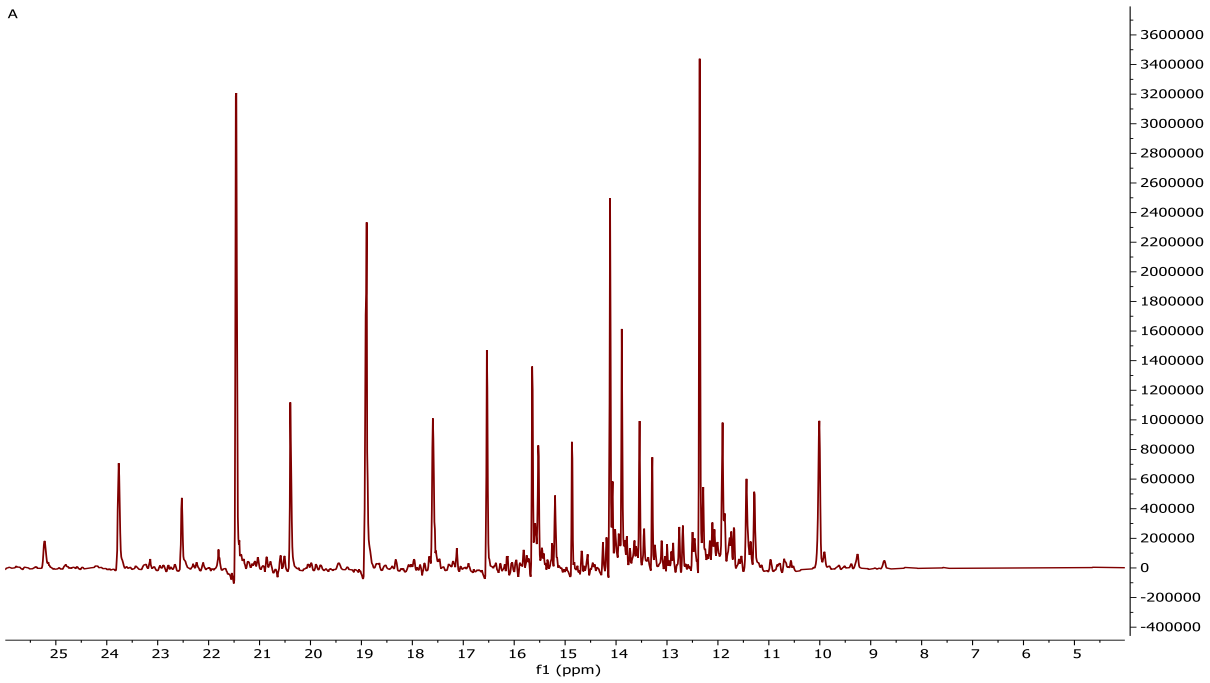

Figure 5.1. GC-MS spectrum of A

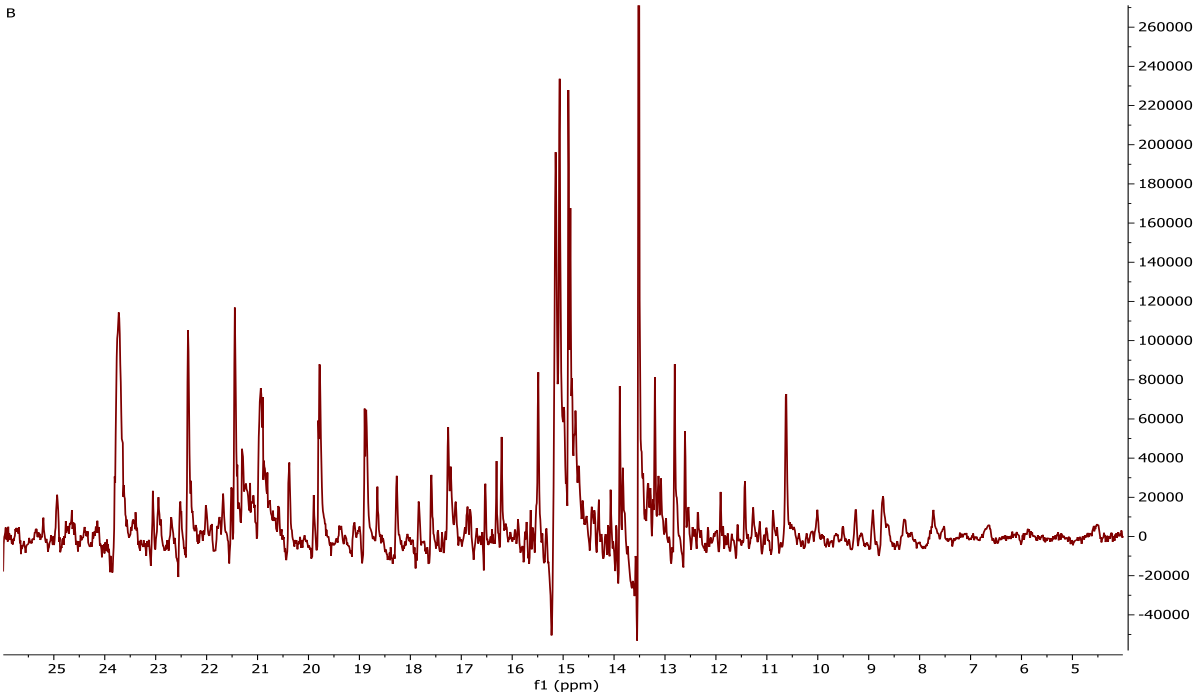

Figure 5.2. GC-MS spectrum of B

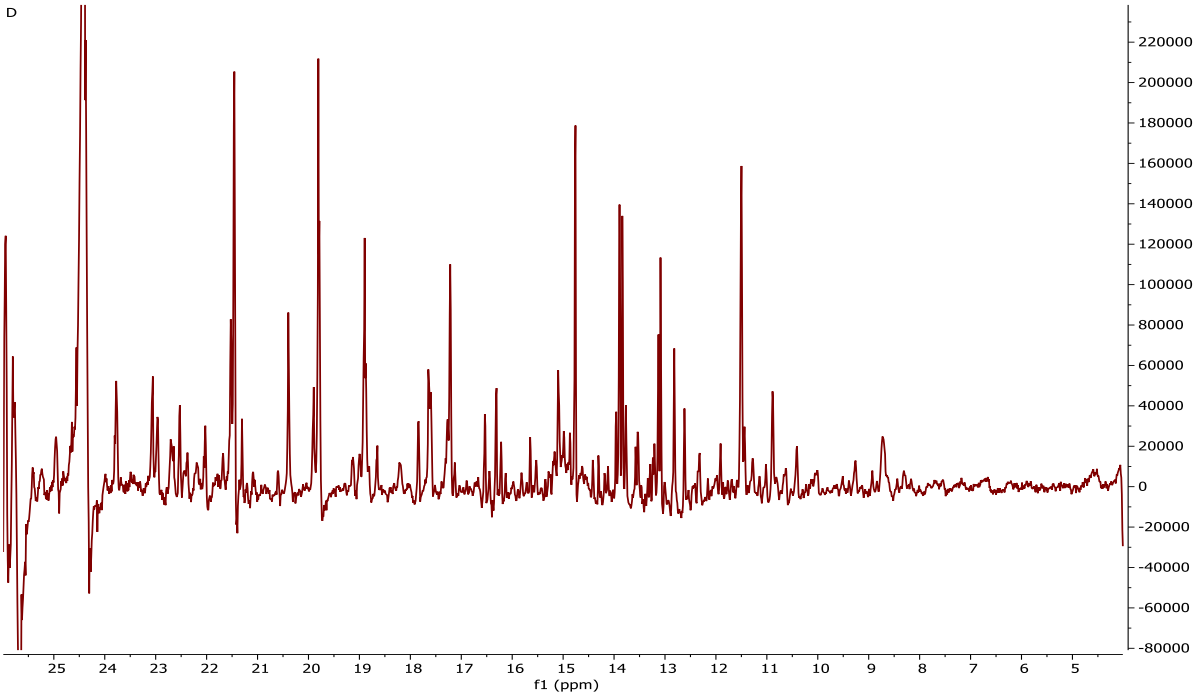

Figure 5.3. GC-MS spectrum of C

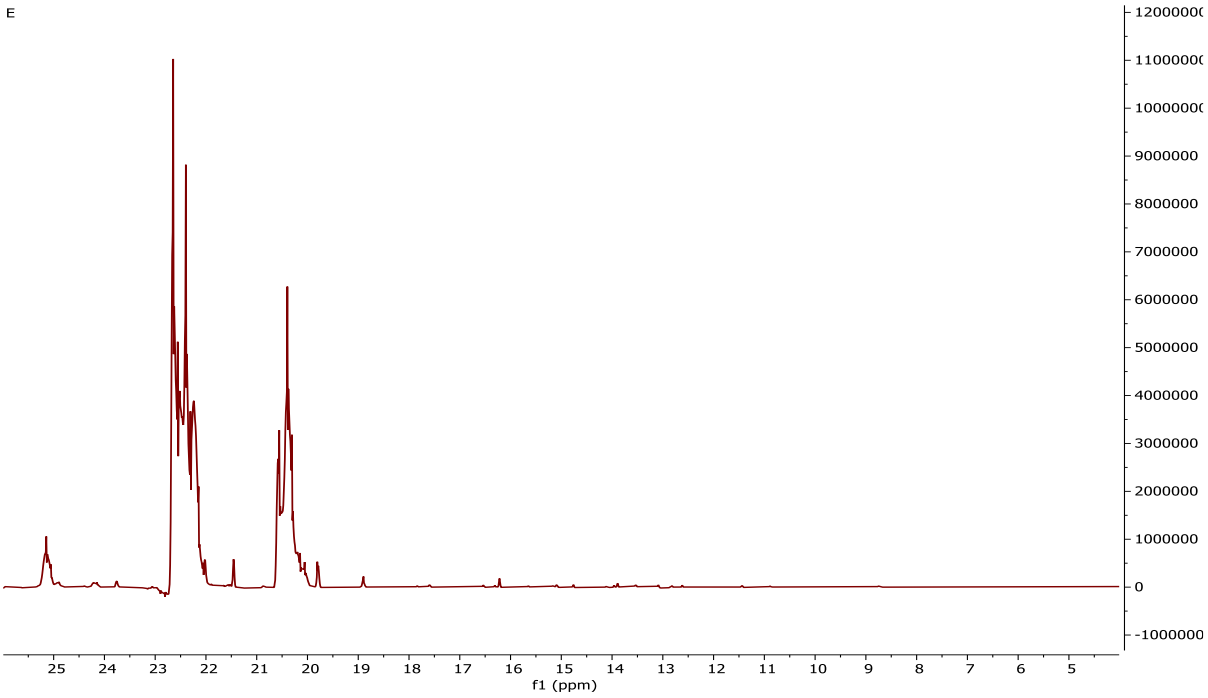

Figure 5.4. GC-MS spectrum of E

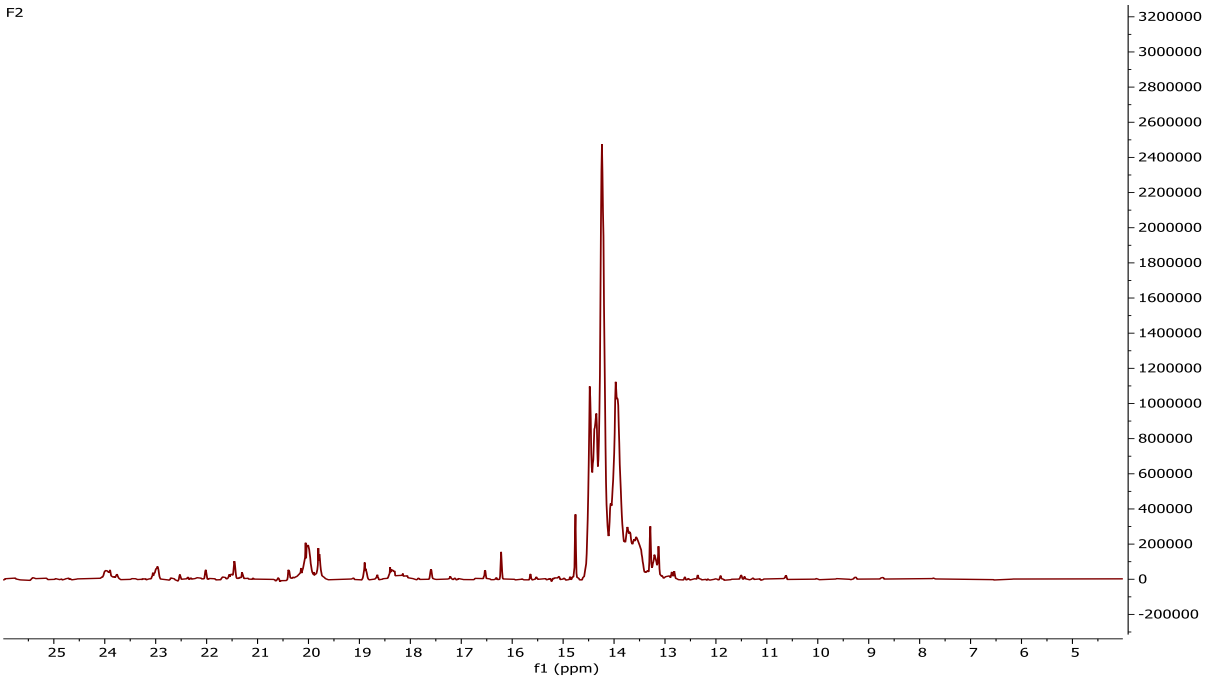

Figure 5.5. GC-MS spectrum of F_2

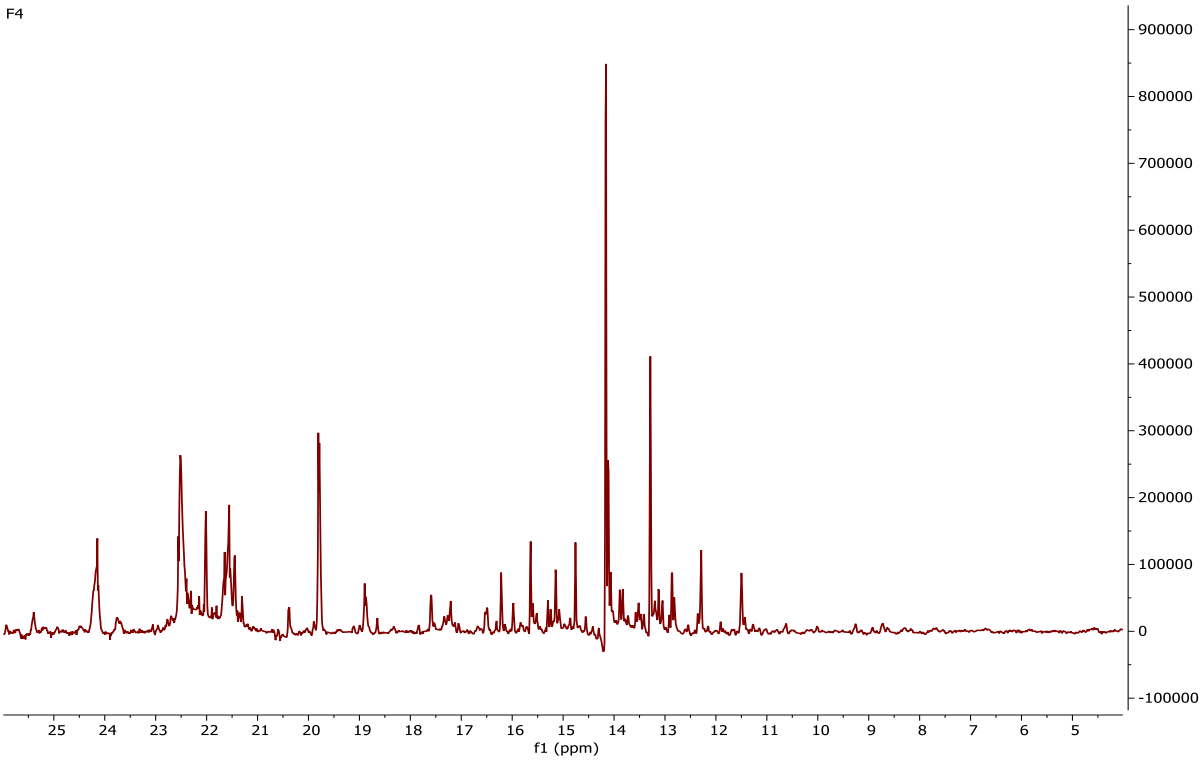

Figure 5.6. GC-MS spectrum of F_4

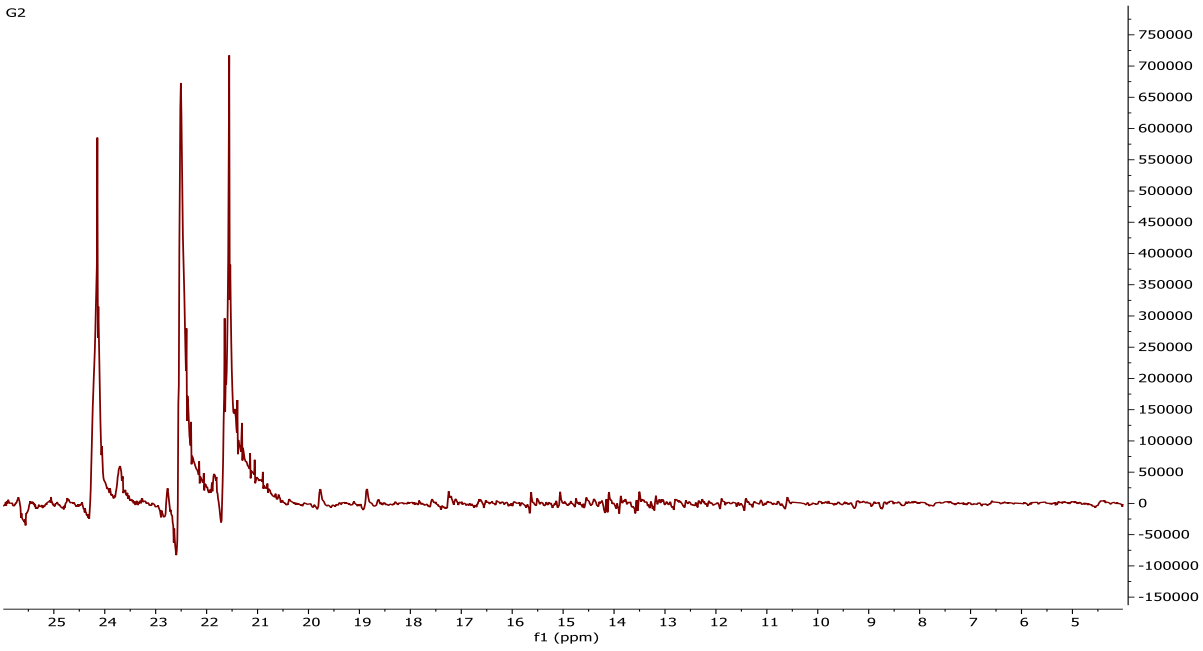

Figure 5.7. GC-MS spectrum of G_2

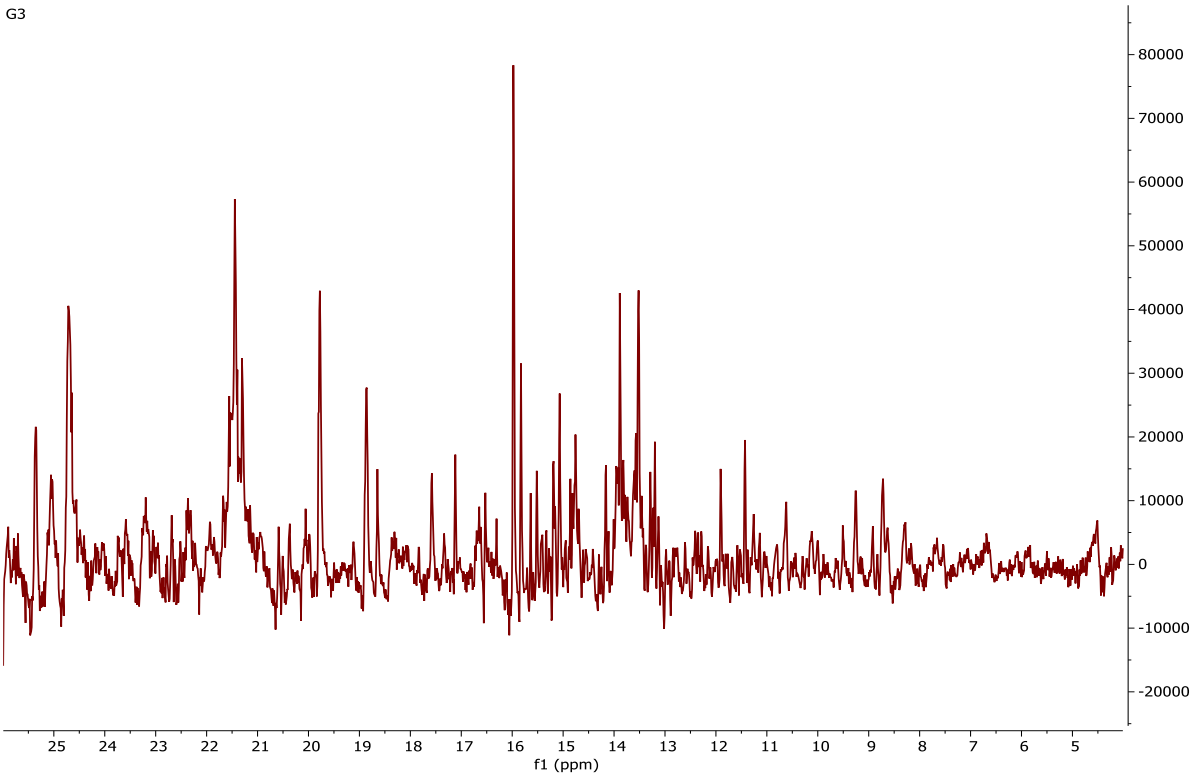

Figure 5.8. GC-MS spectrum of G_3

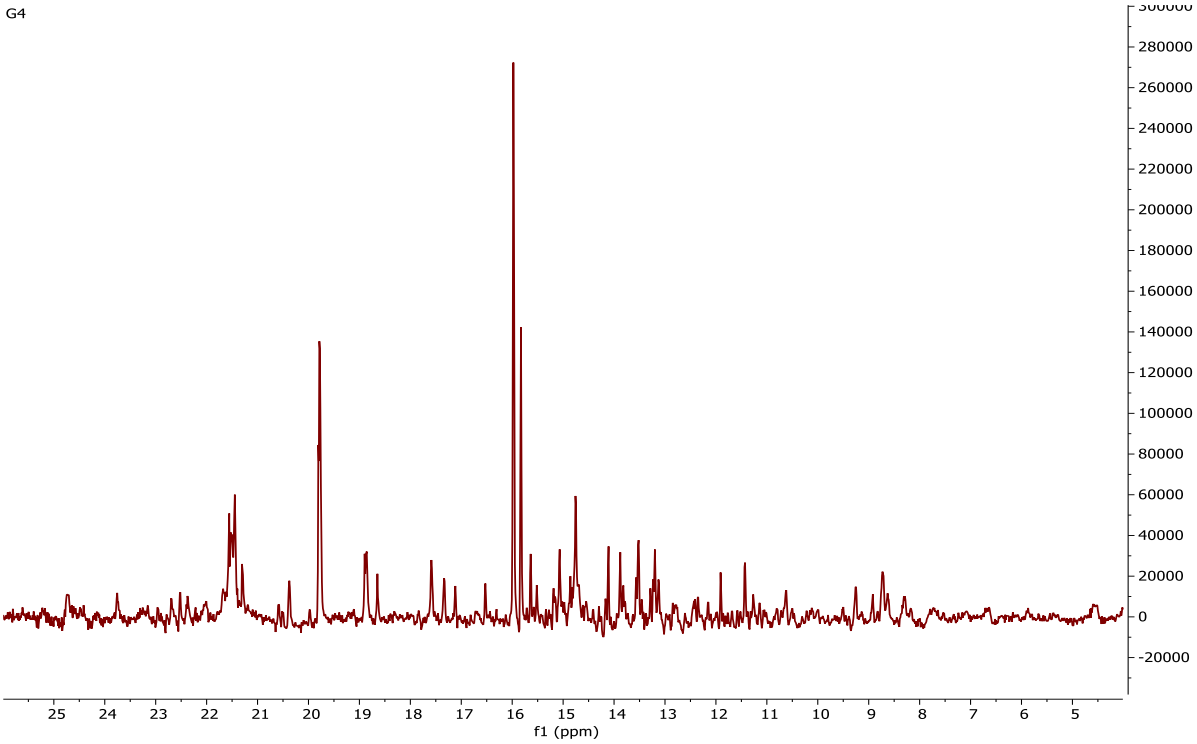

Figure 5.9. GC-MS spectrum of G_4

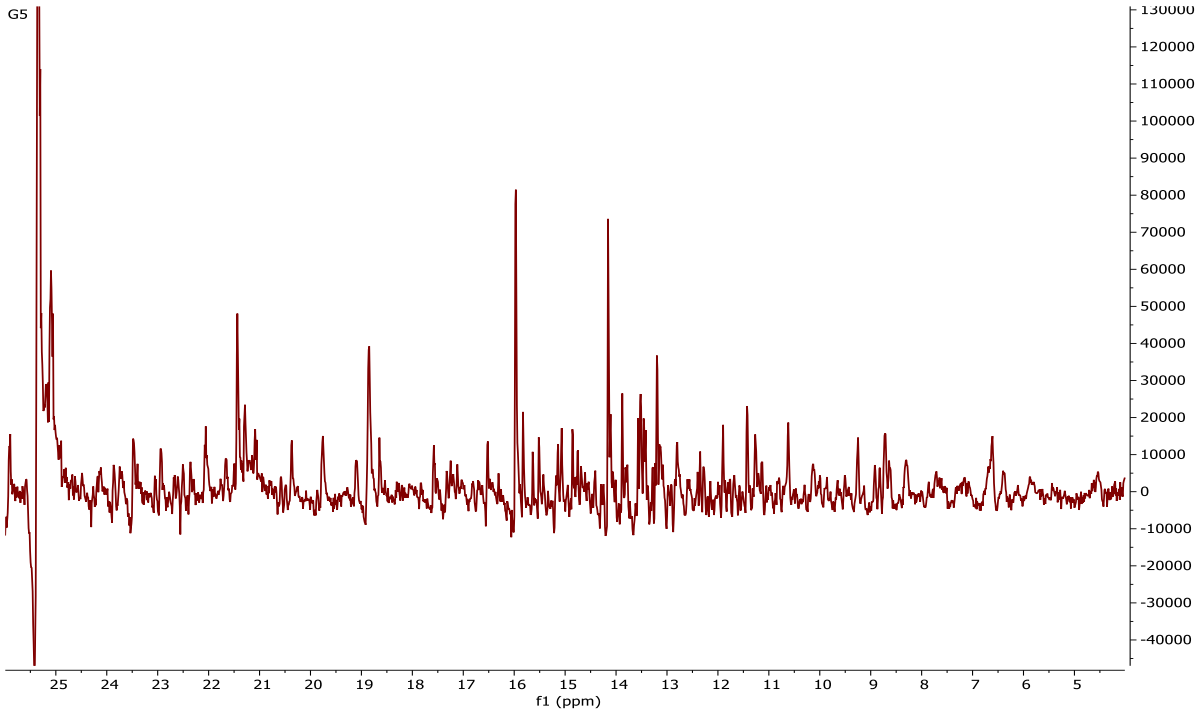

Figure 5.10. GC-MS spectrum of G_5

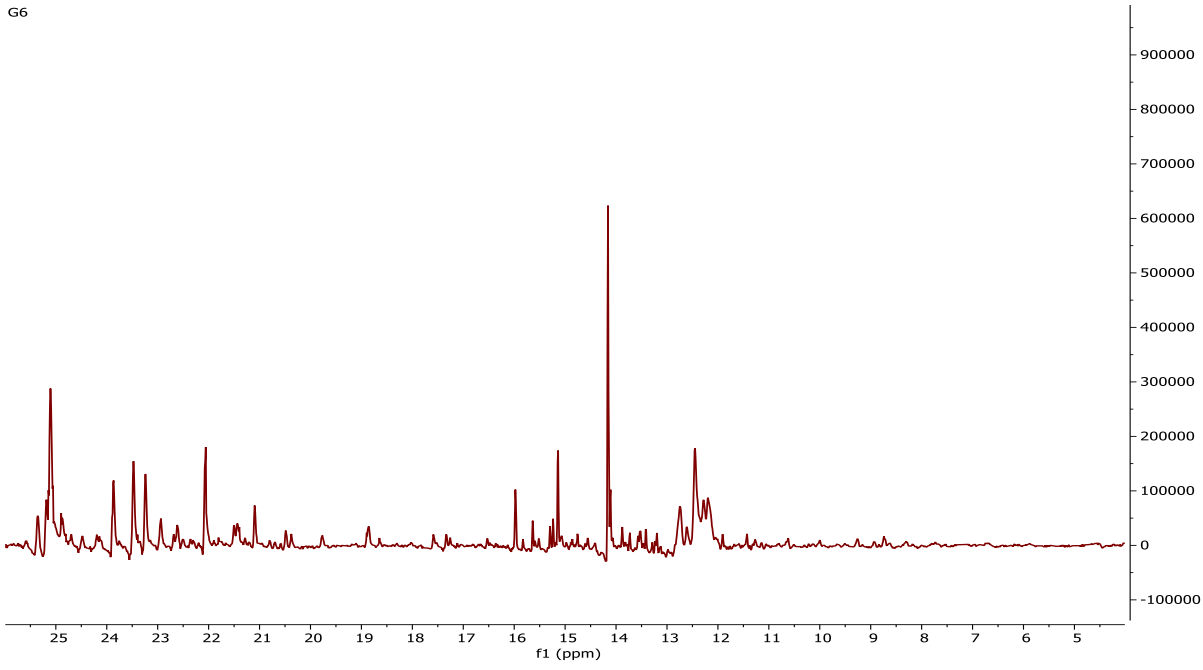

Figure 5.11. GC-MS spectrum of G_6

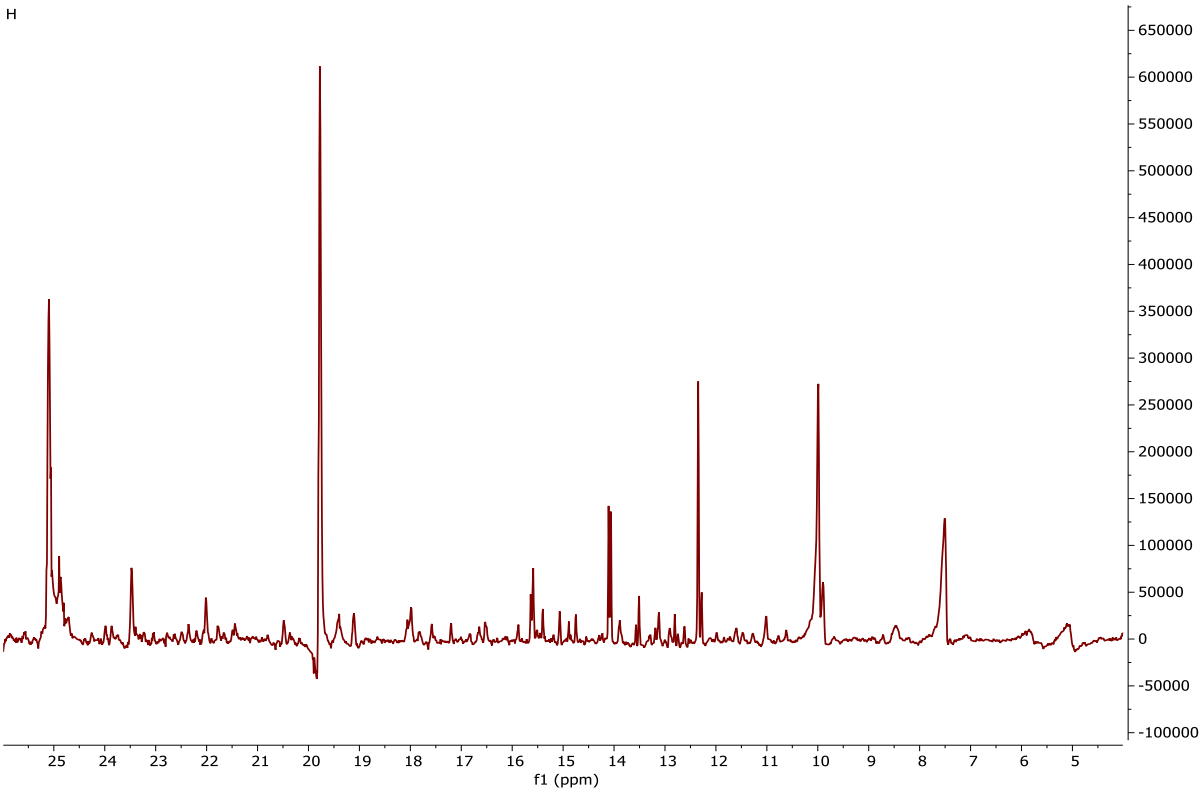

Figure 5.12. GC-MS spectrum of H

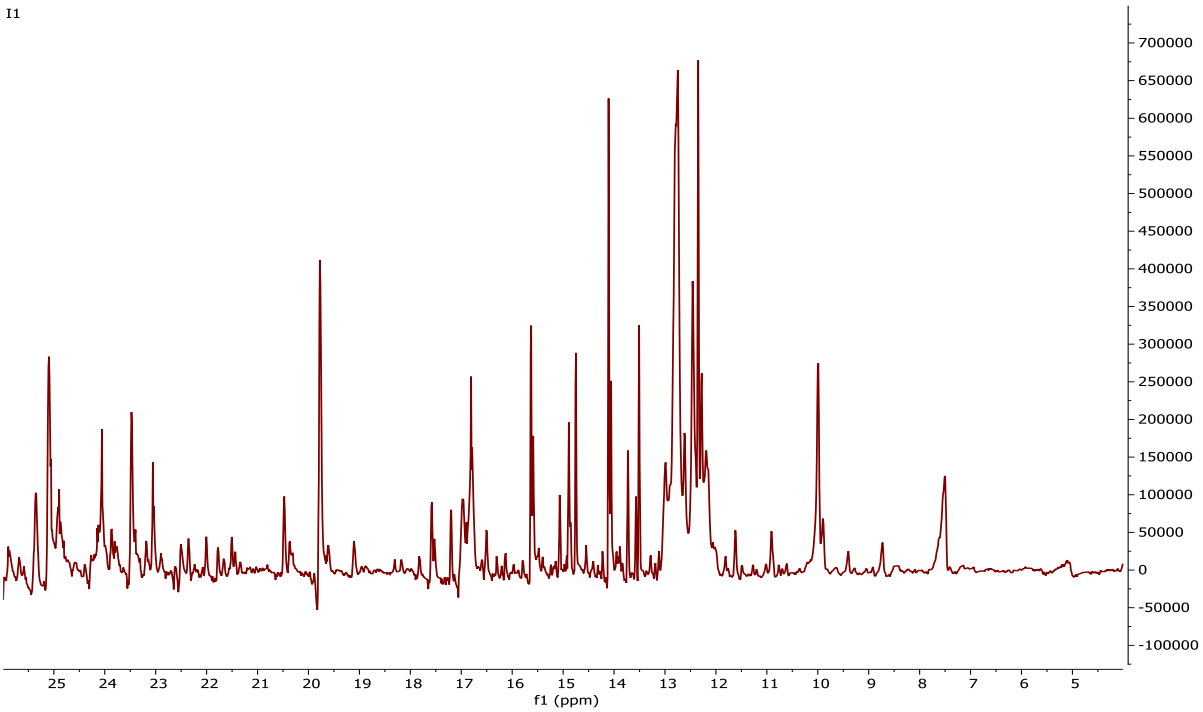

Figure 5.13. GC-MS spectrum of I_1

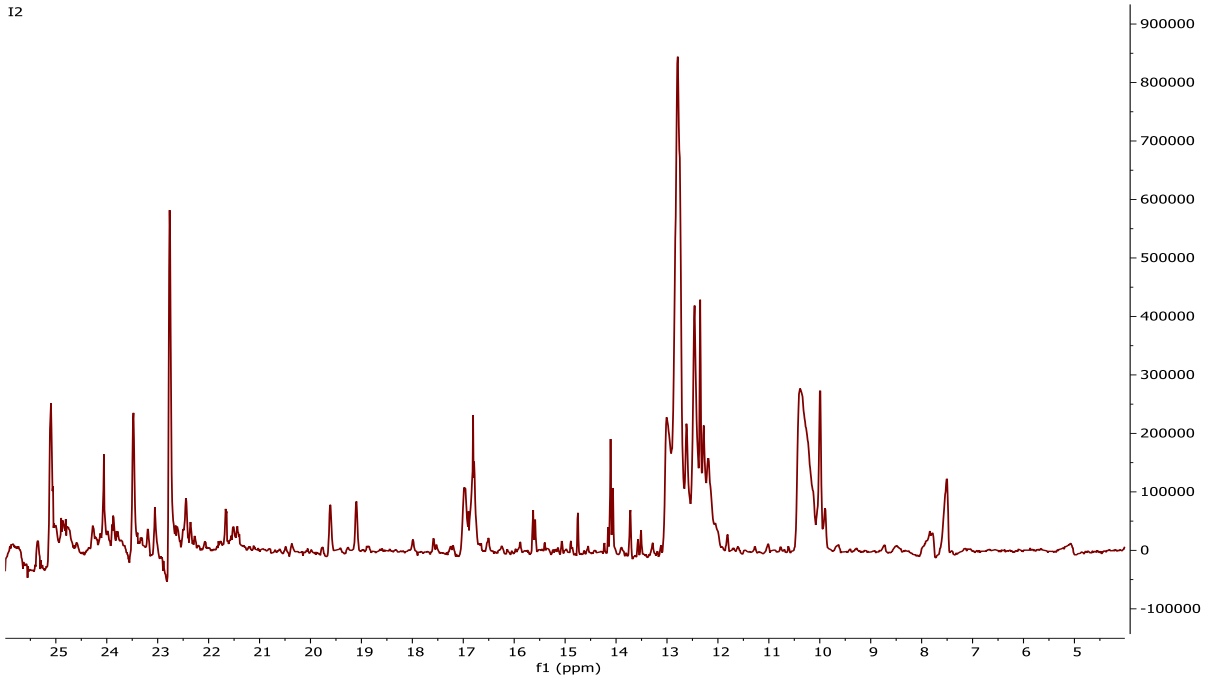

Figure 5.14. GC-MS spectrum of I_2

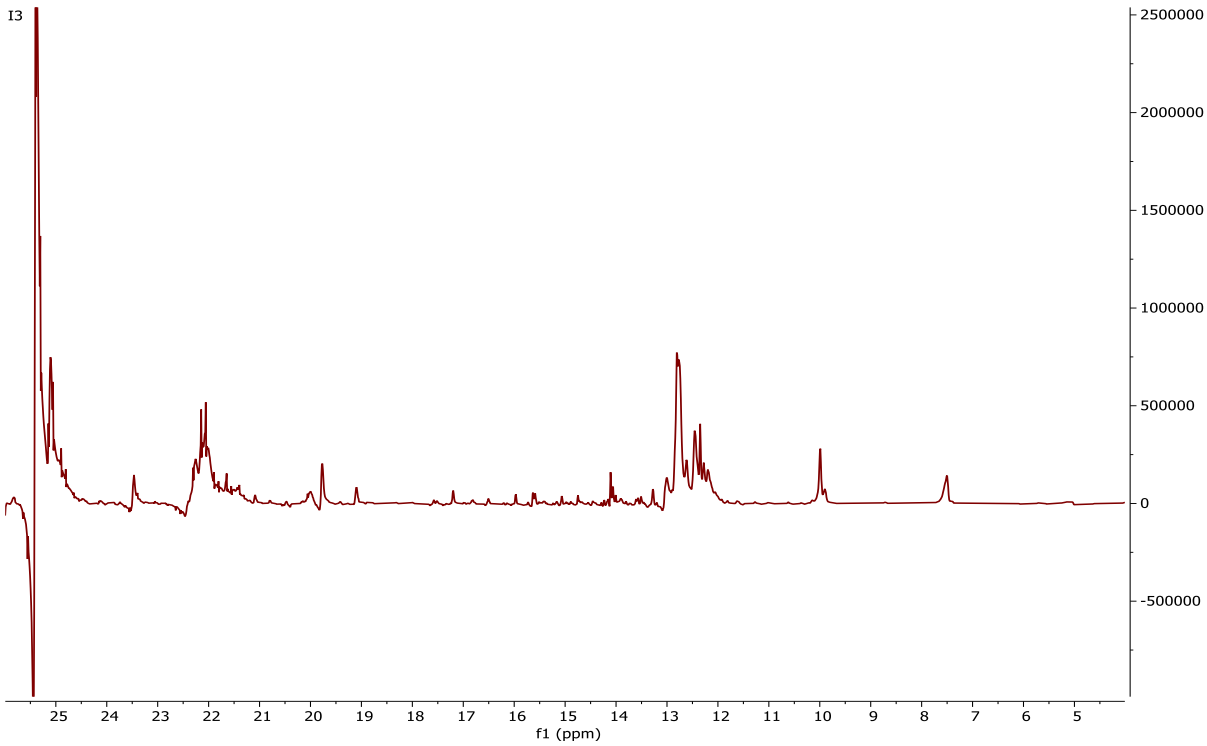

Figure 5.15. GC-MS spectrum of I_3

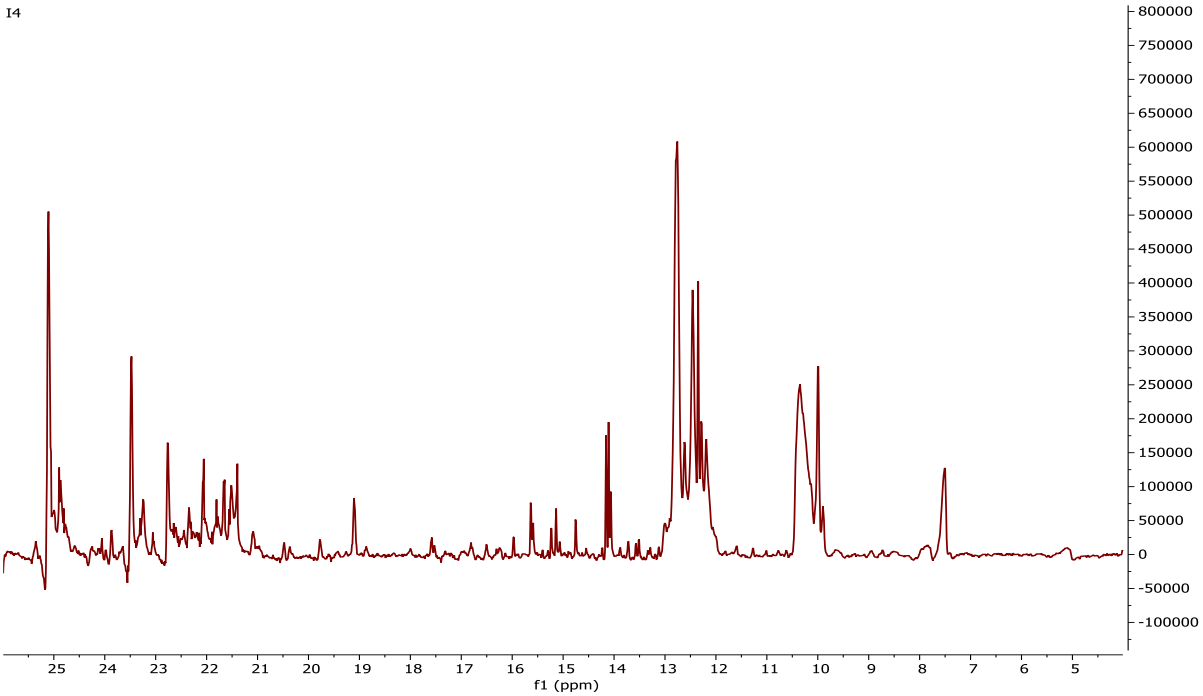

Figure 5.16. GC-MS spectrum of I_4

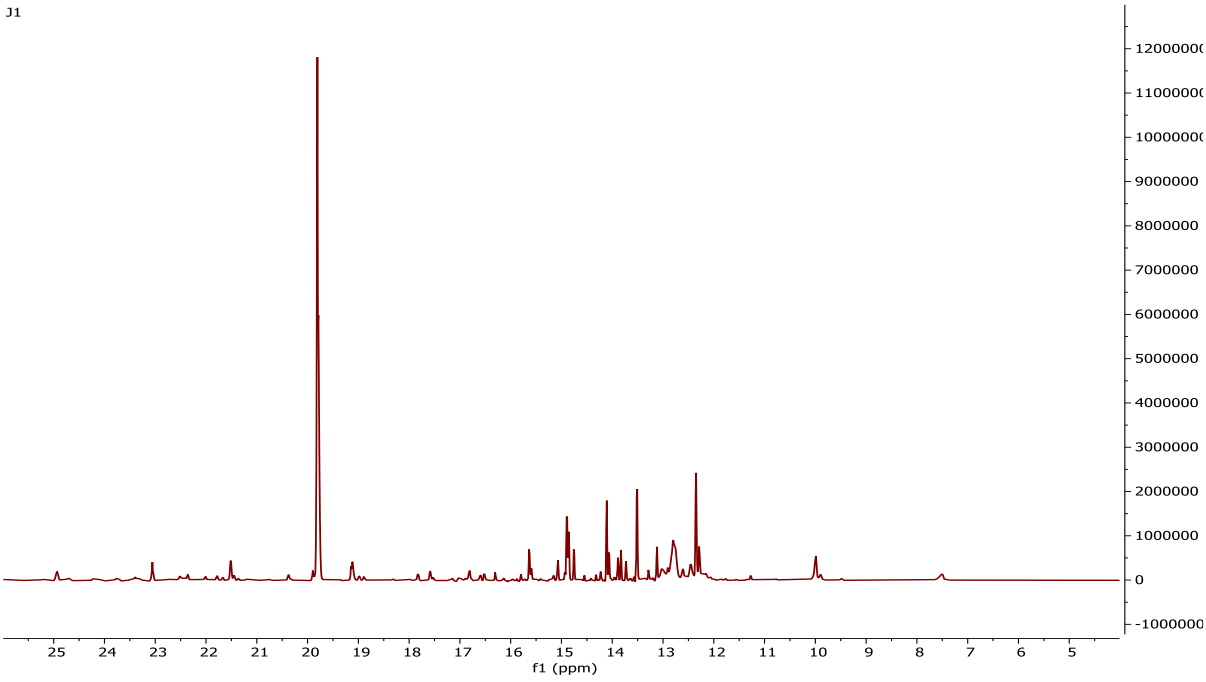

Figure 5.17. GC-MS spectrum of J_1

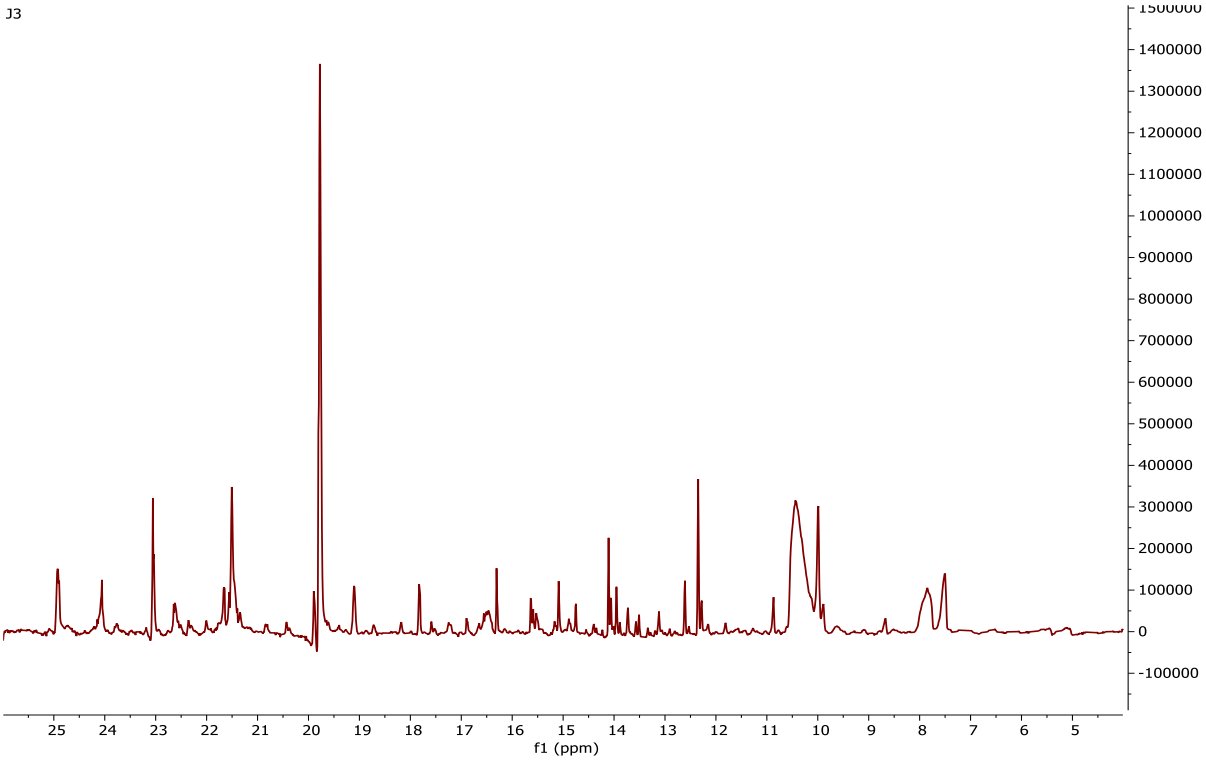

Figure 5.18. GC-MS spectrum of J_3

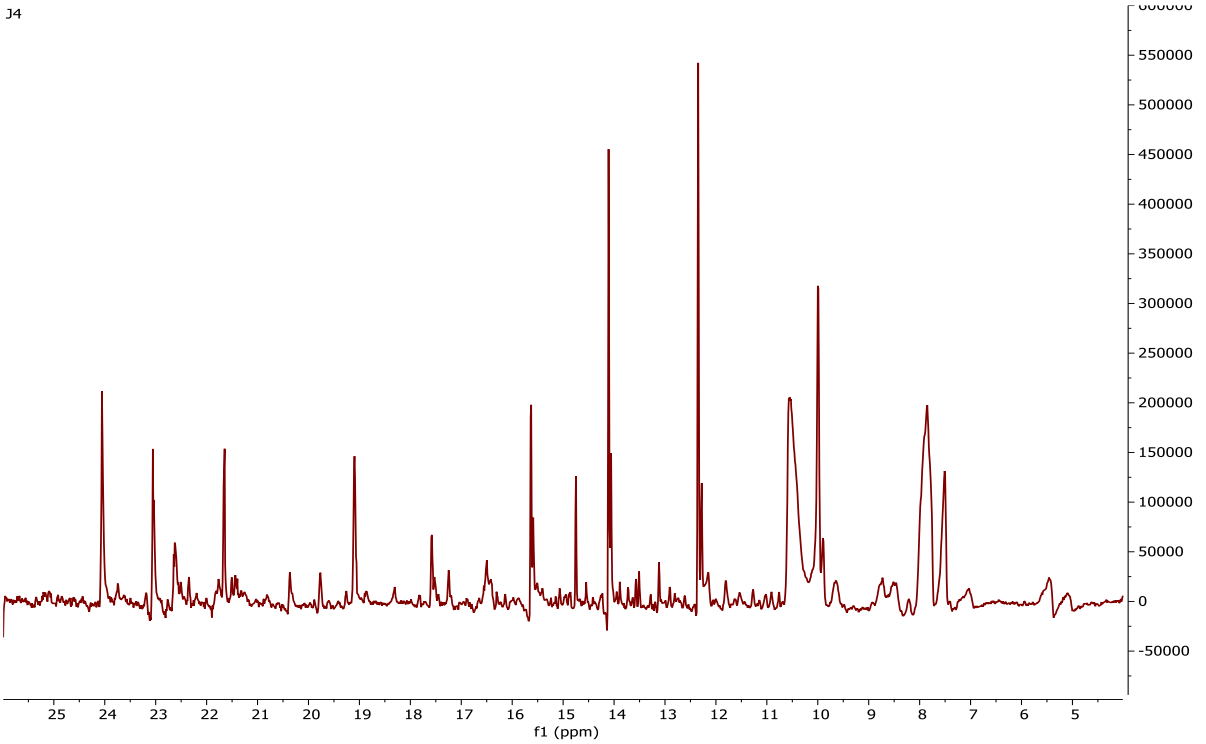

Figure 5.19. GC-MS spectrum of J_4

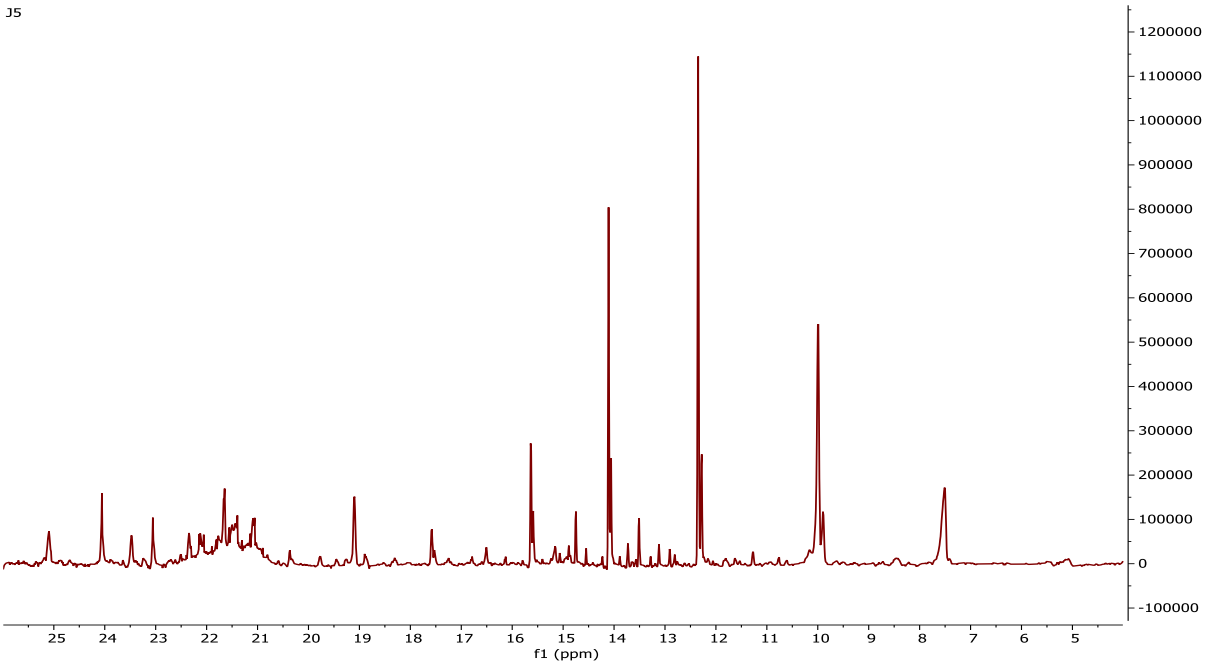

Figure 5.20. GC-MS spectrum of J_5

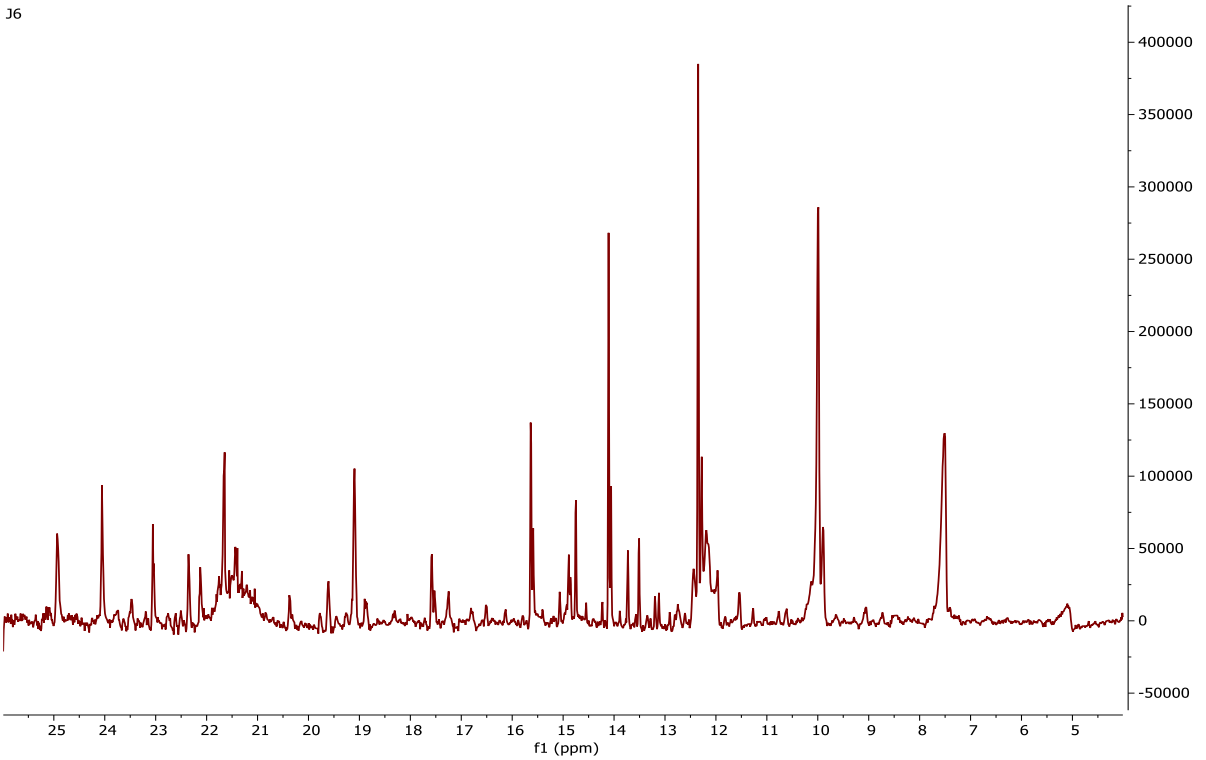

Figure 5.21. GC-MS spectrum of J_6

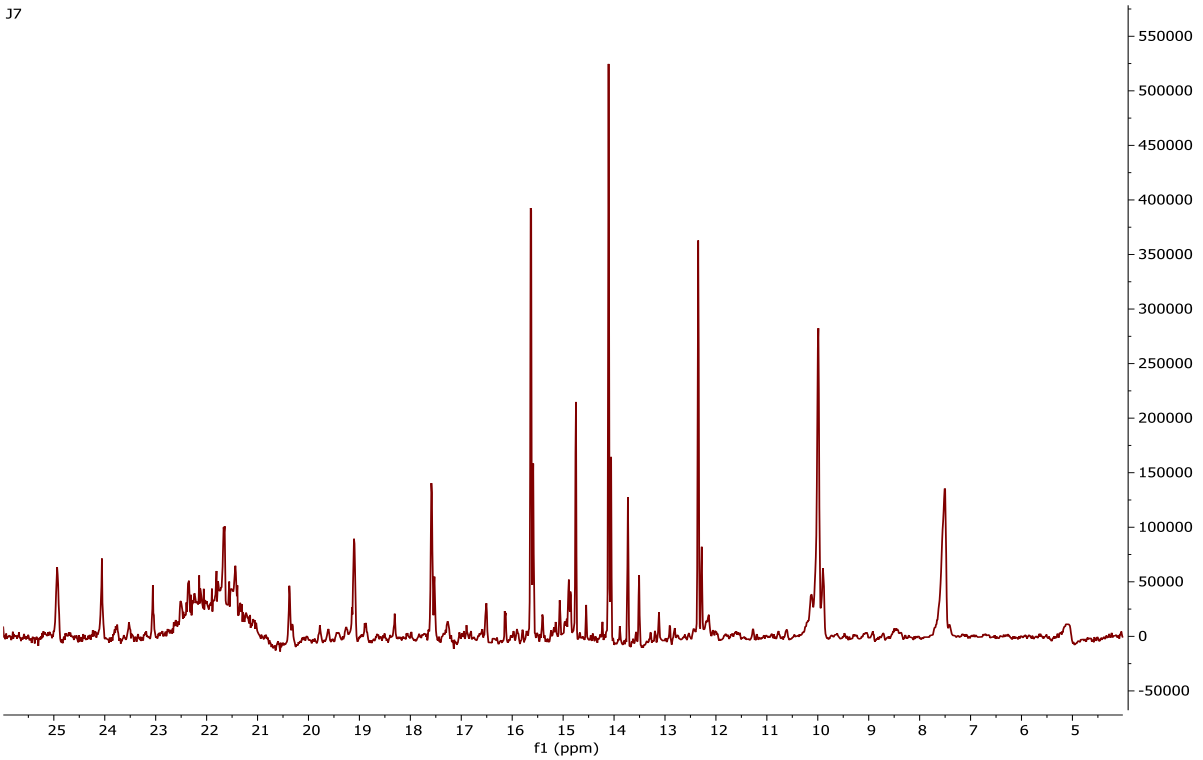

Figure 5.22. GC-MS spectrum of J_7

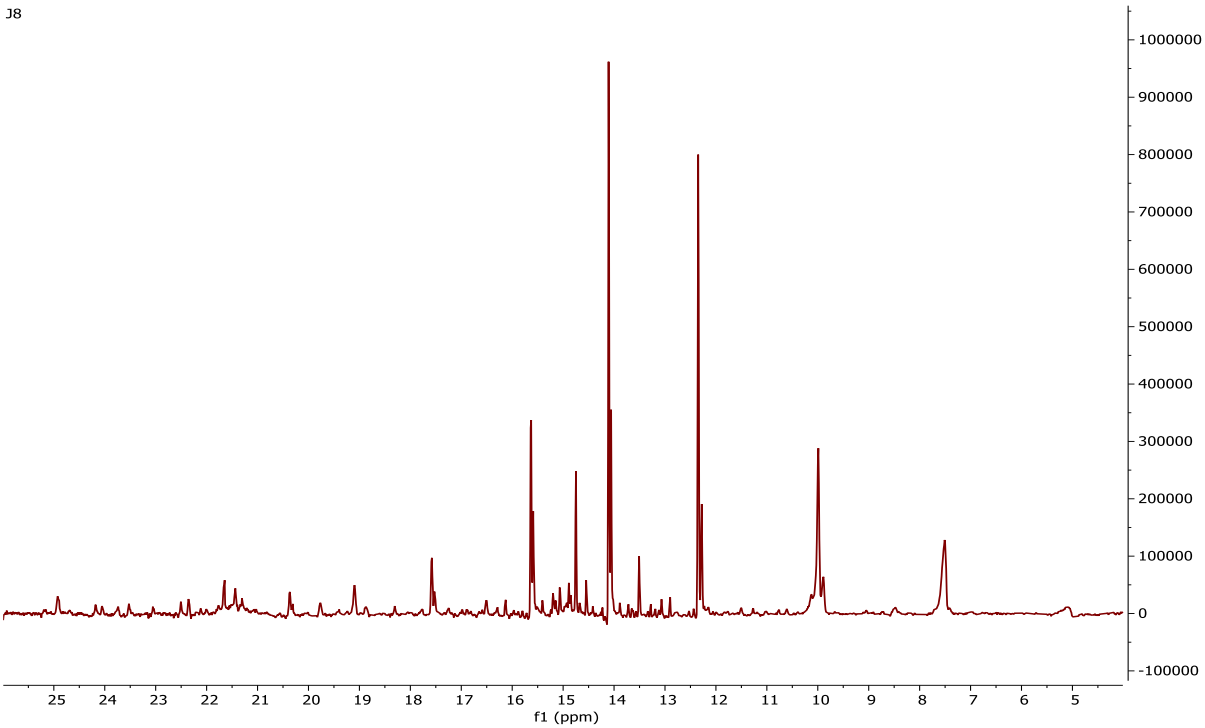

Figure 5.23. GC-MS spectrum of J_8

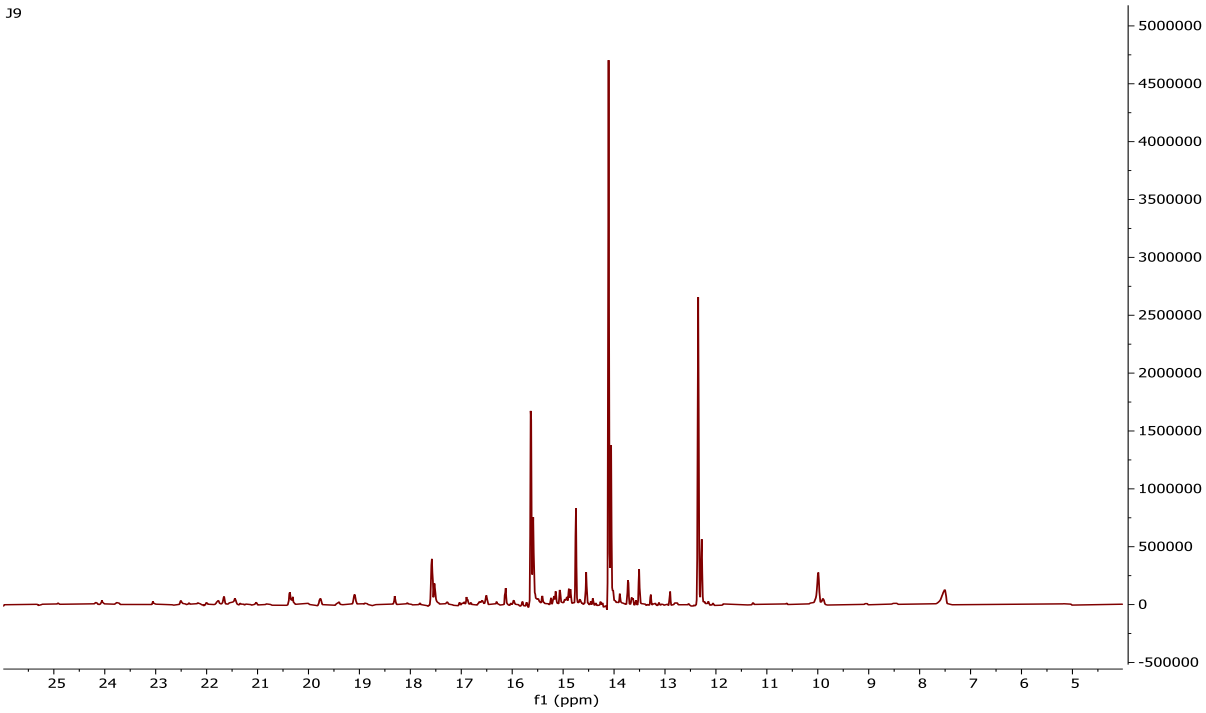

Figure 5.24. GC-MS spectrum of J_9

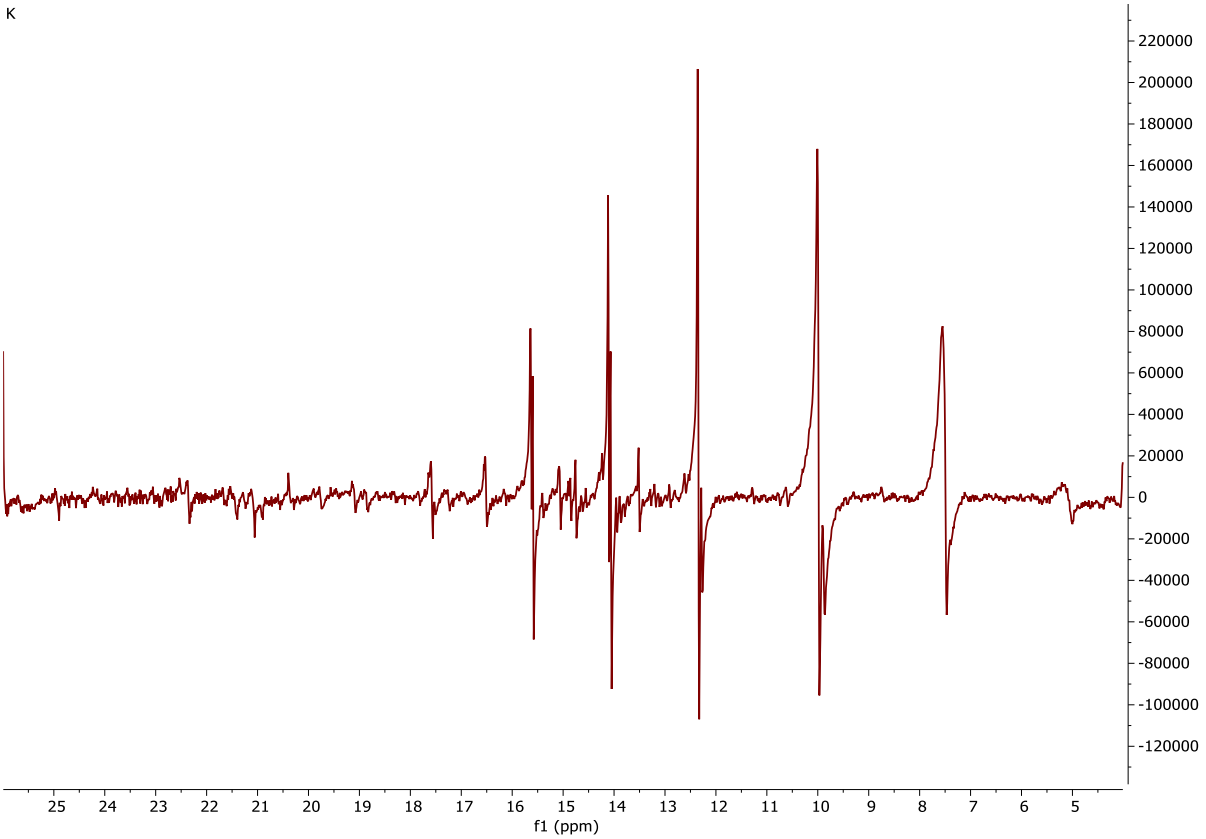

Figure 5.25. GC-MS spectrum of K

APPENDIX B

^{1}H NMR chromatograms of fractions of the stem bark of *Tabernaemontana elegans*

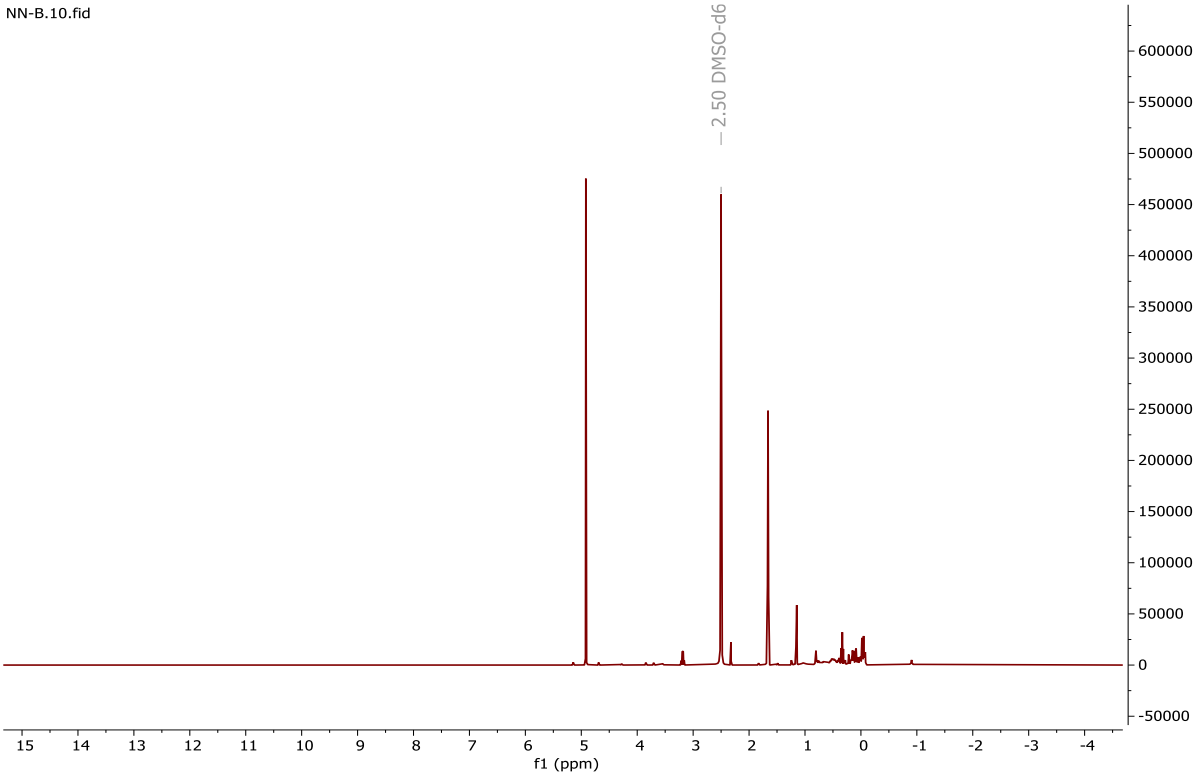

Figure 5.26. ^{1}H NMR spectrum of B

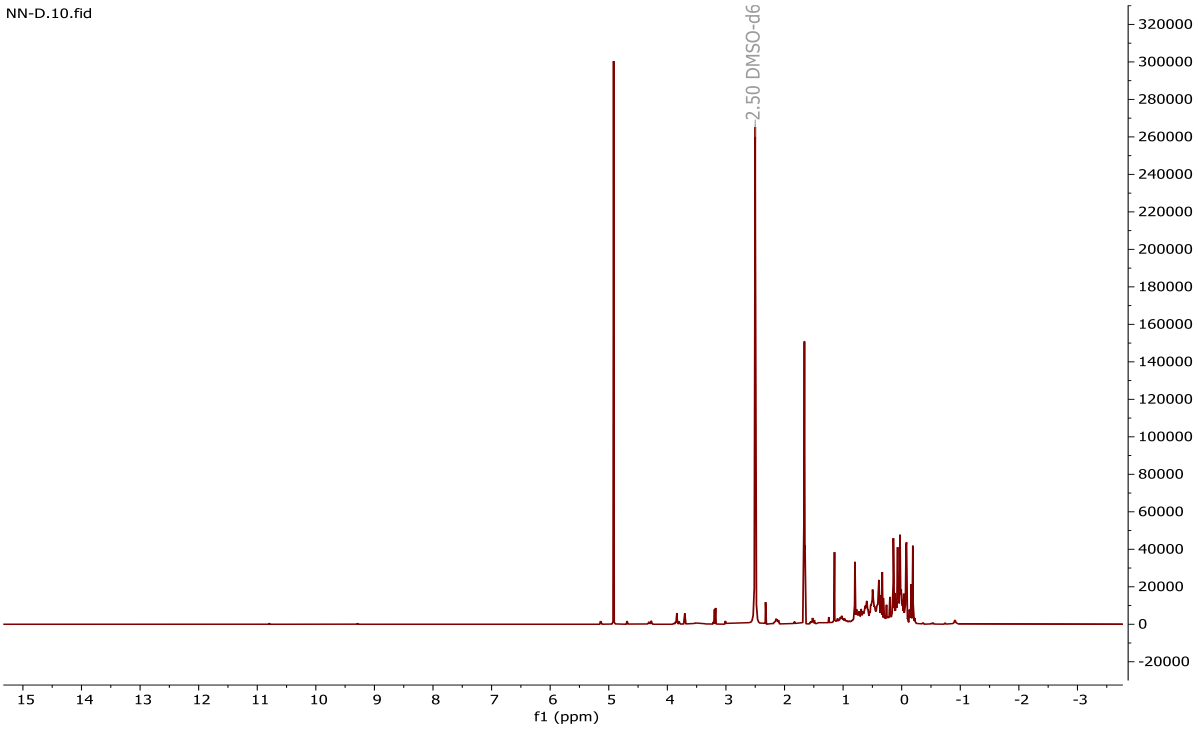

Figure 5.27. ^{1}H NMR spectrum of D

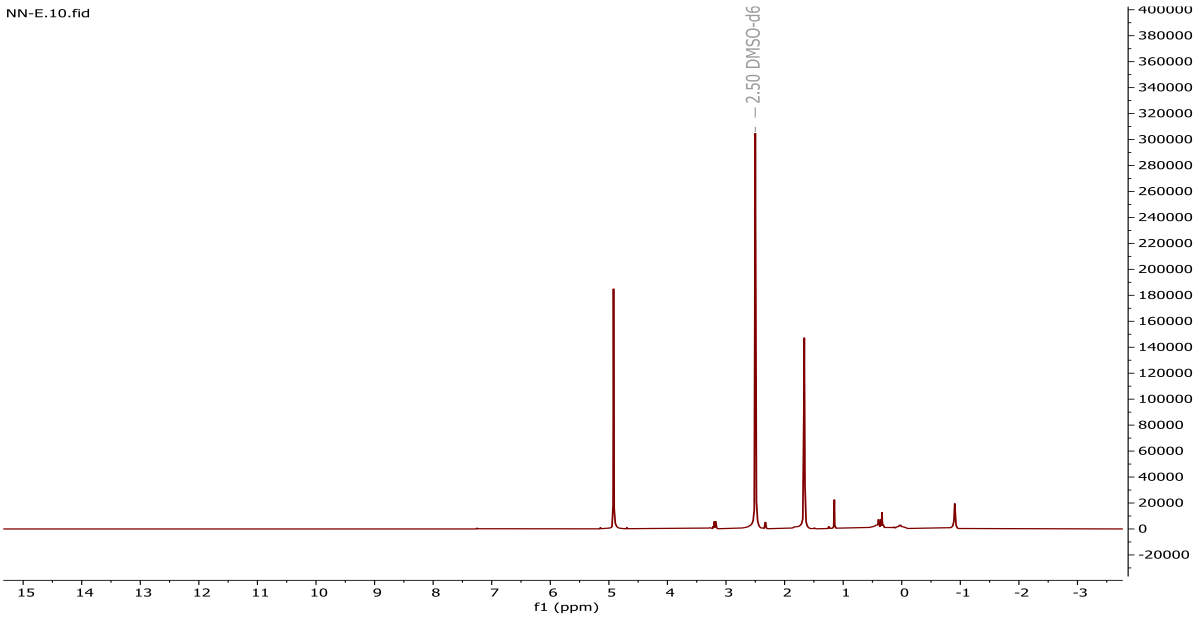

Figure 5.28. ^{1}H NMR spectrum of E

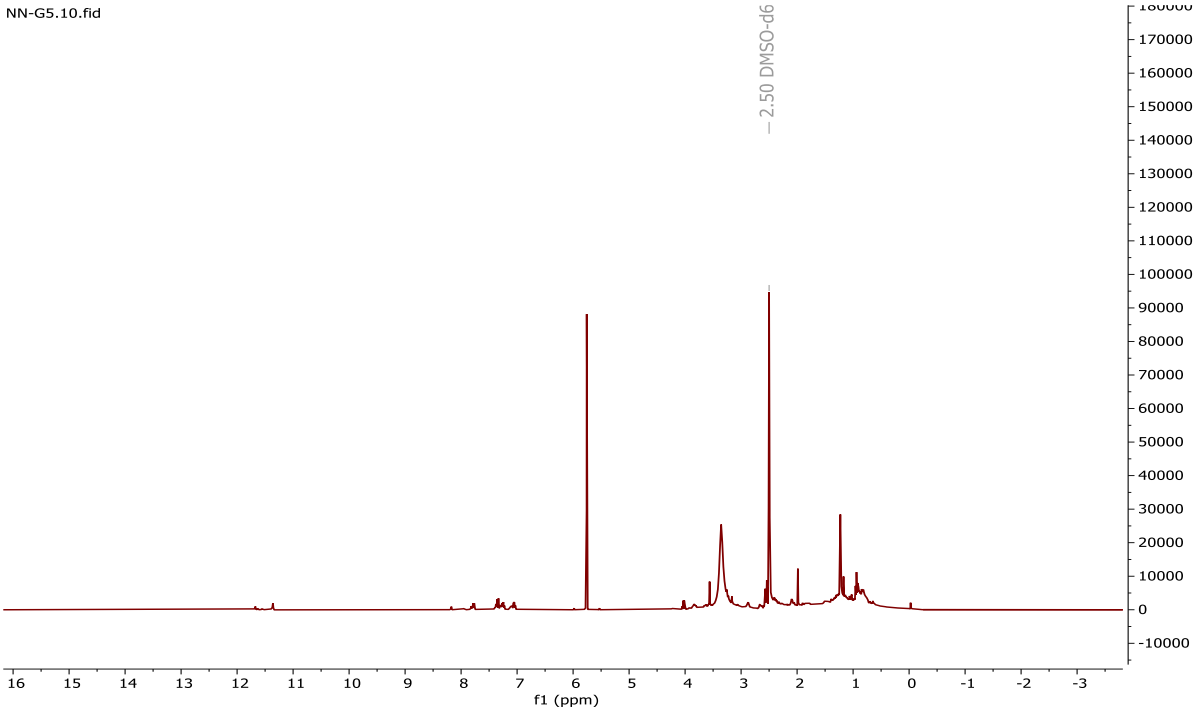

Figure 5.29. ^{1}H NMR spectrum of G_5

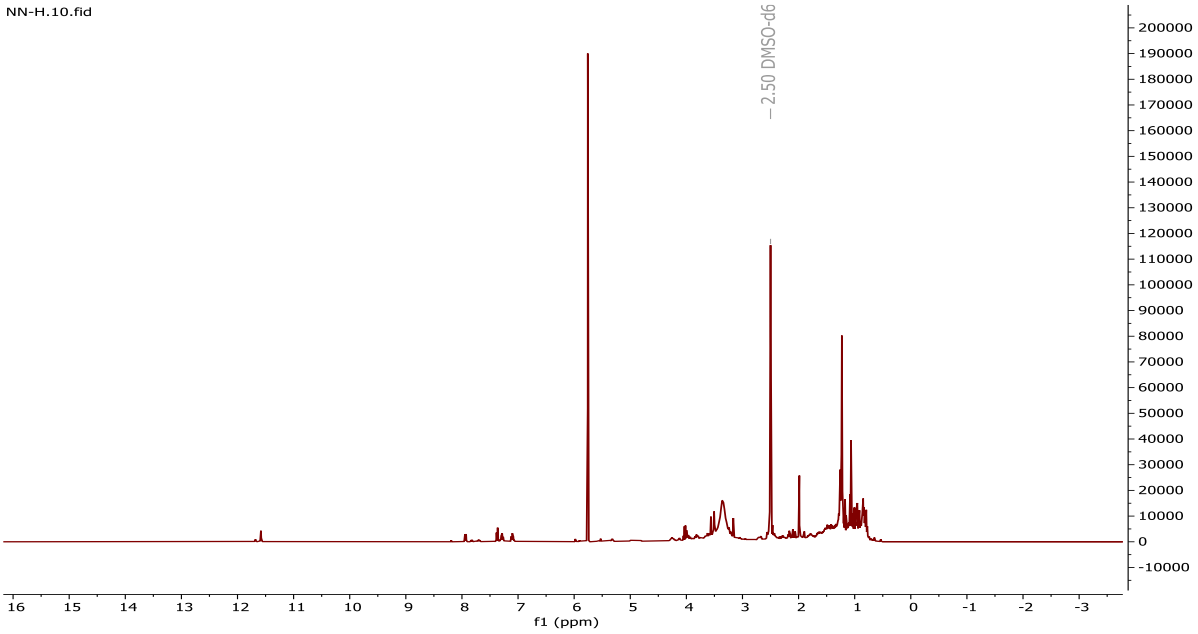

Figure 5.30. ^{1}H NMR spectrum of H

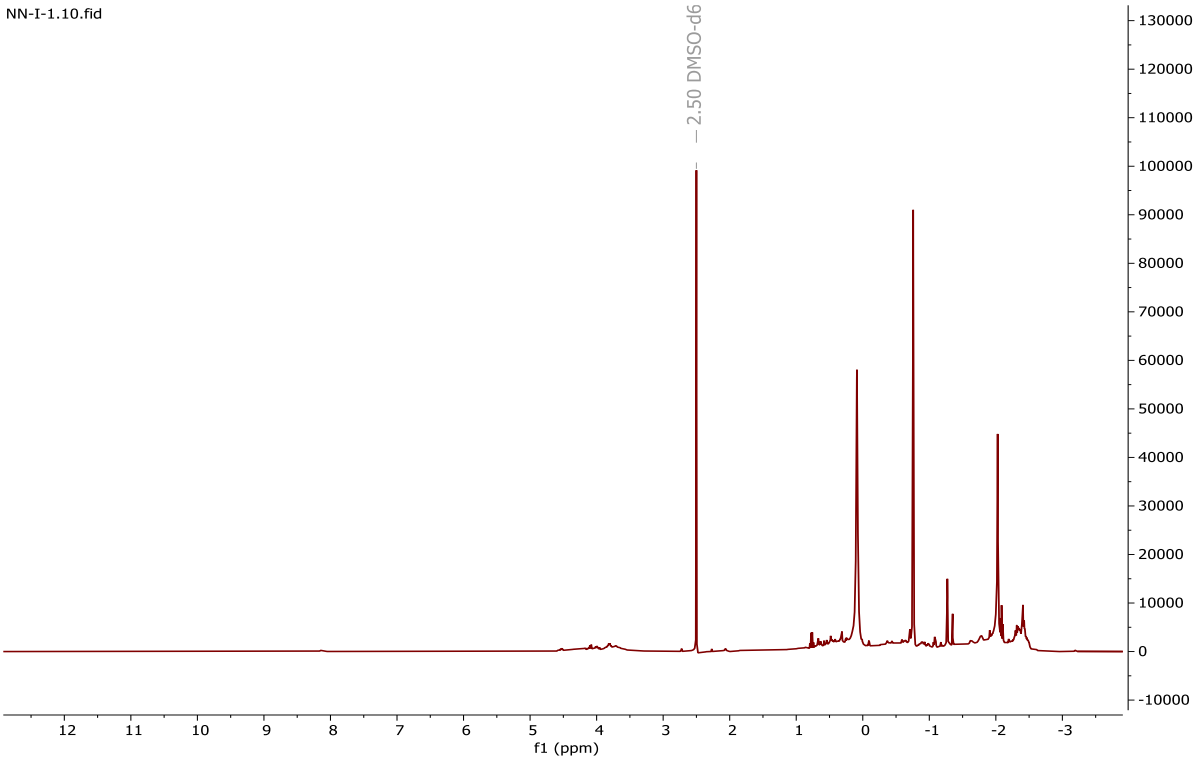

Figure 5.31. ^{1}H NMR spectrum of I_1

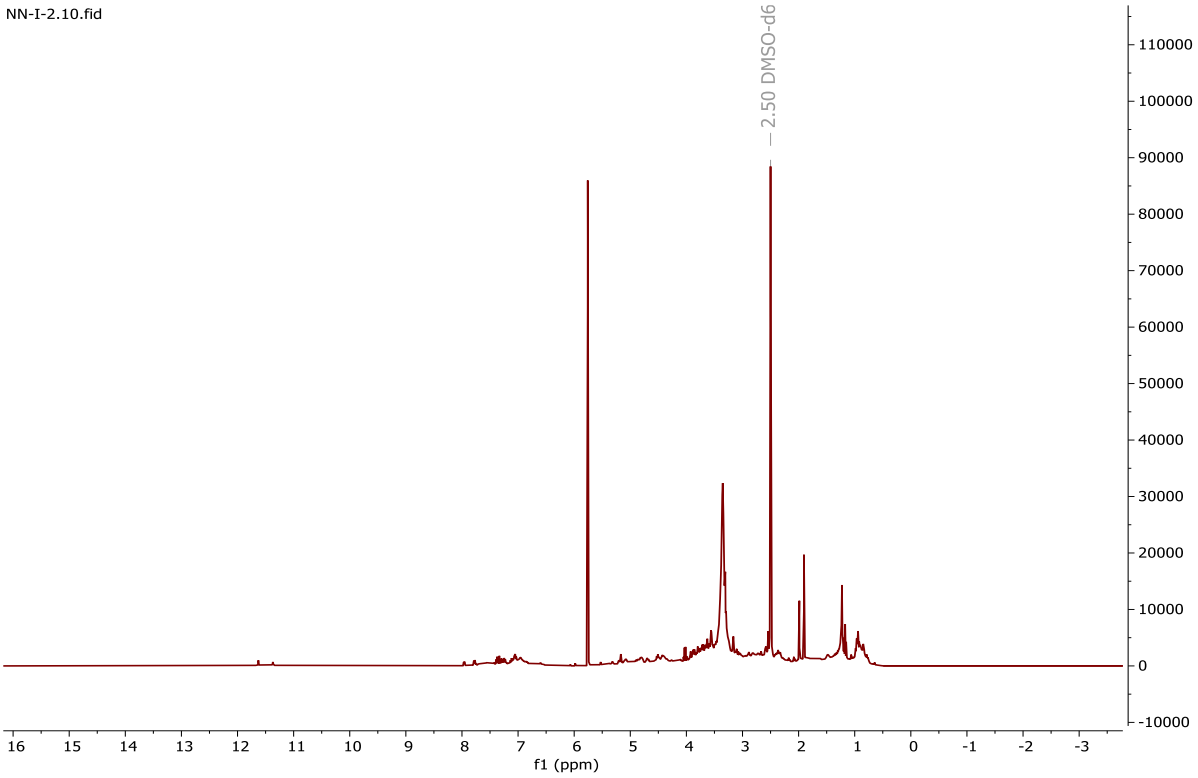

Figure 5.32. ^{1}H NMR spectrum of I_2

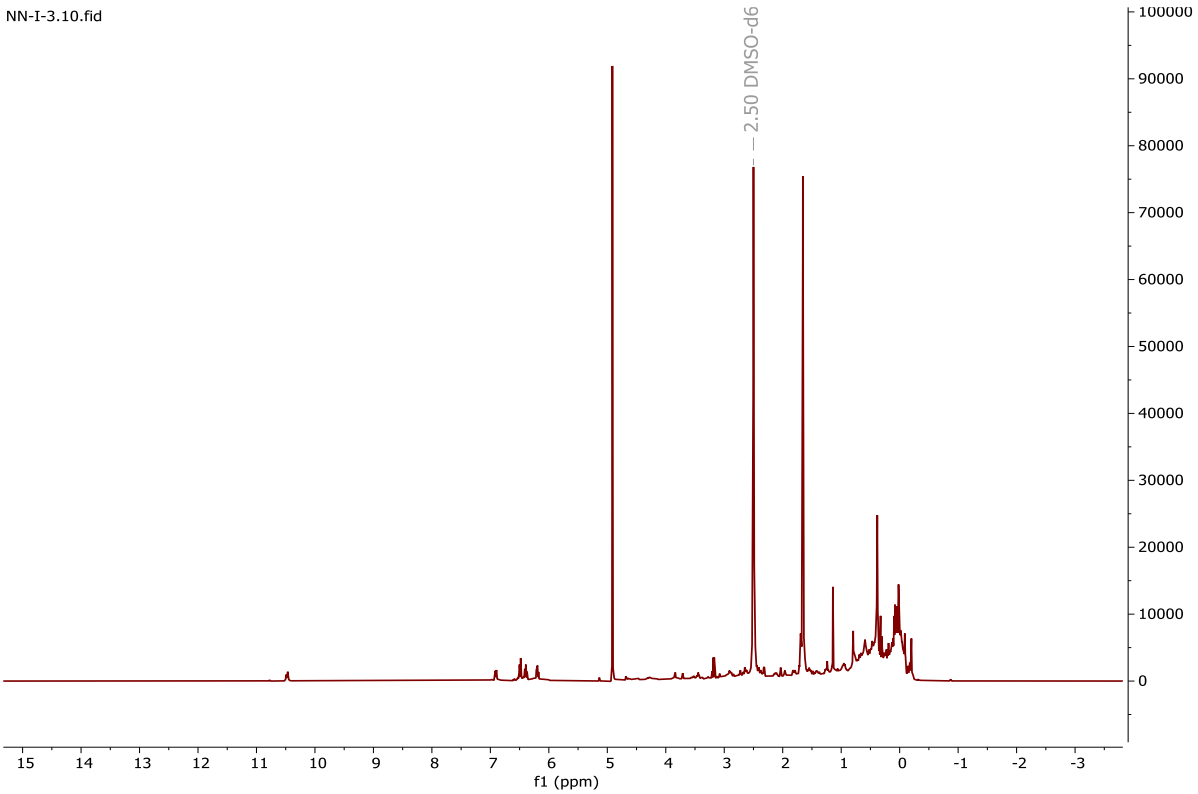

Figure 5.33. ^{1}H NMR spectrum of I_3

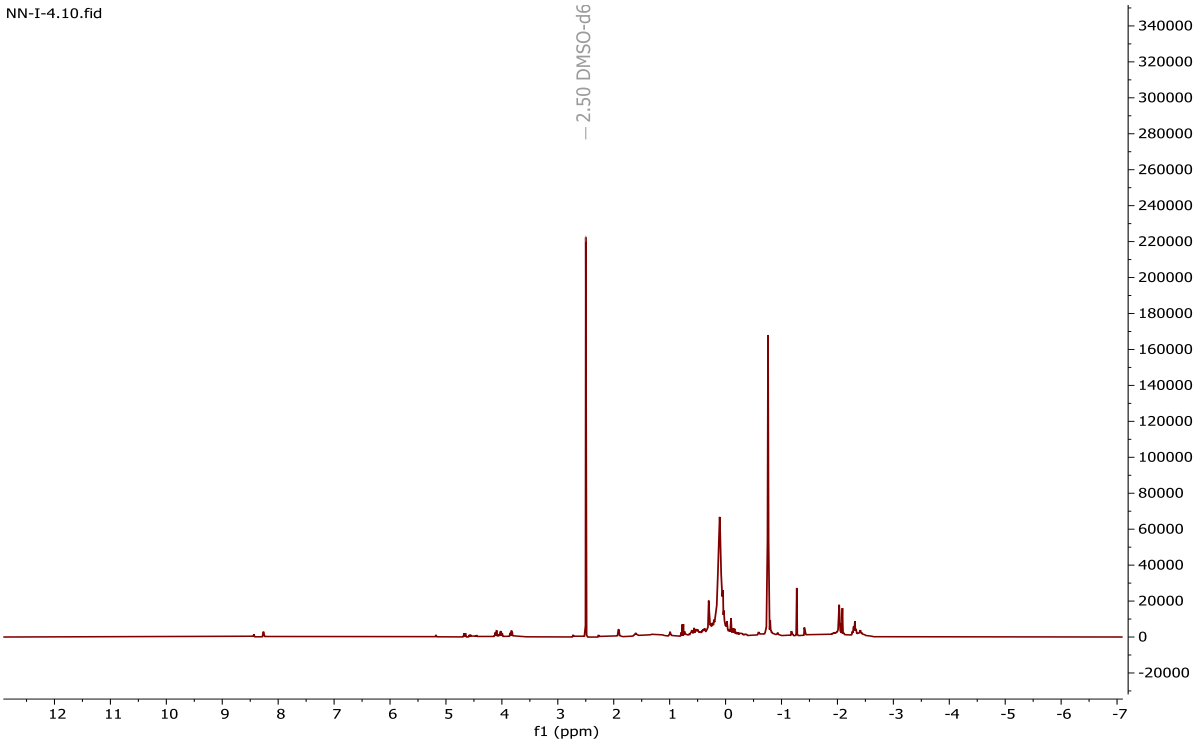

Figure 5.34. ^{1}H NMR spectrum of I_4

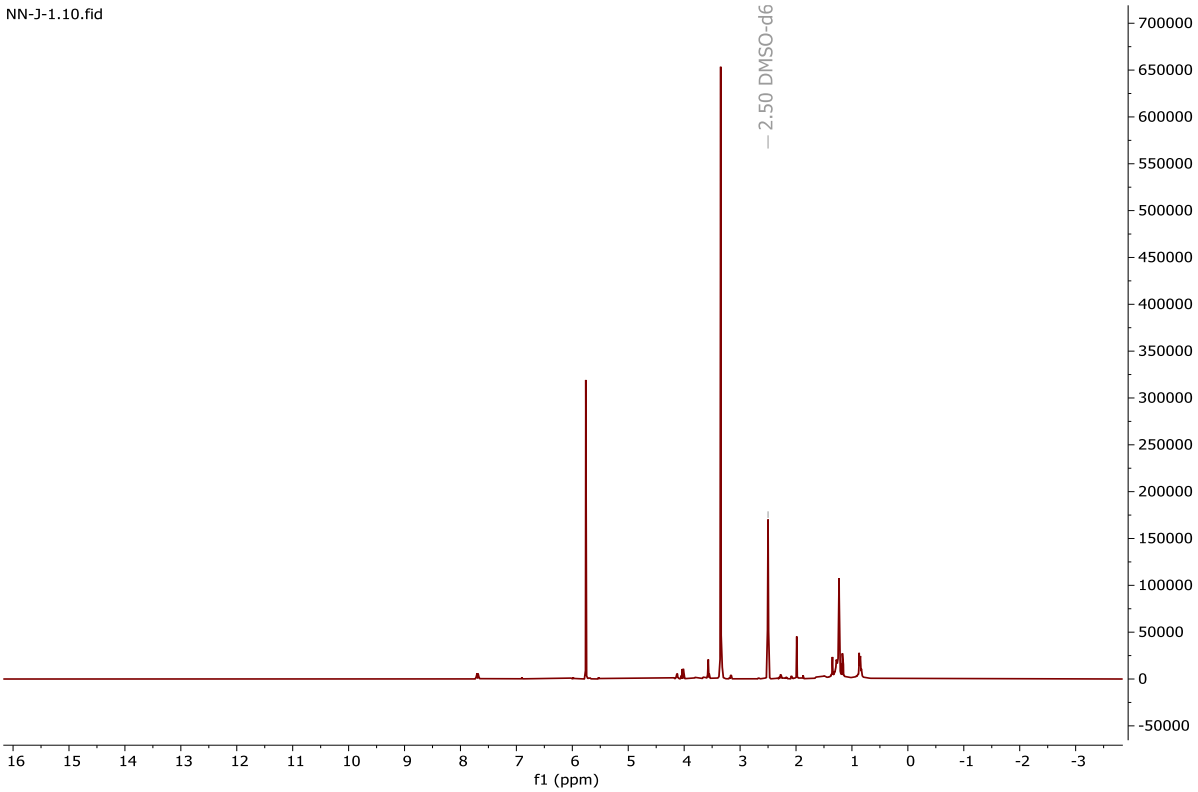

Figure 5.35. ^{1}H NMR spectrum of J_1

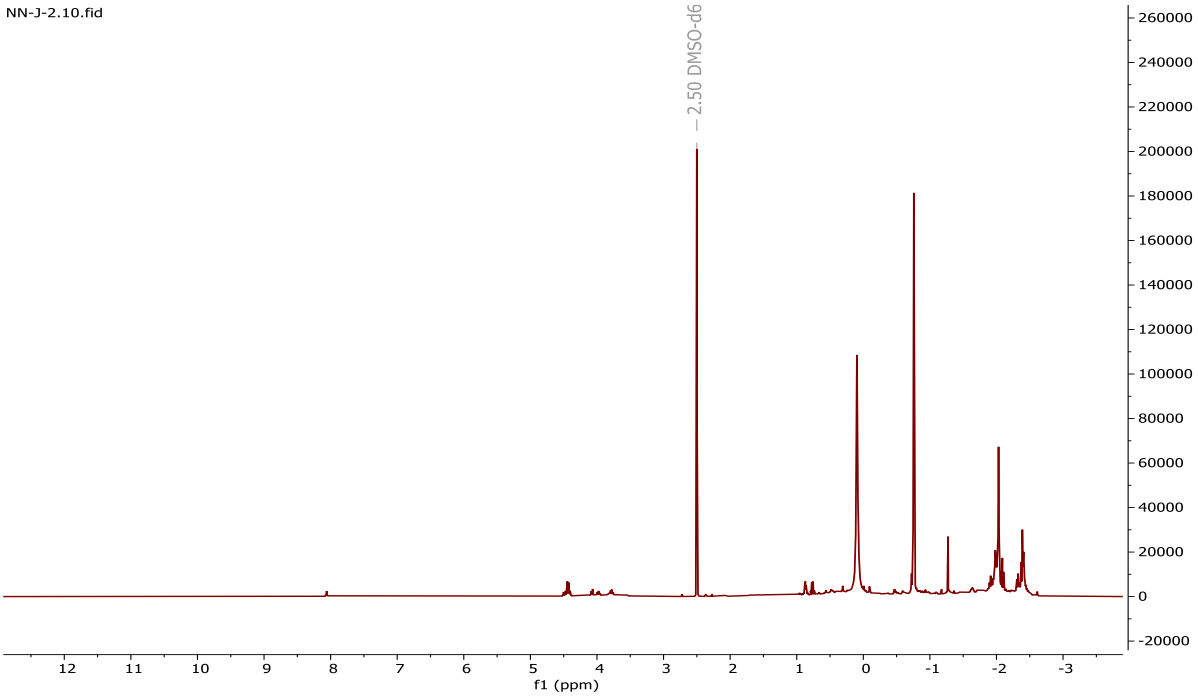

Figure 5.35. ^{1}H NMR spectrum of J_2

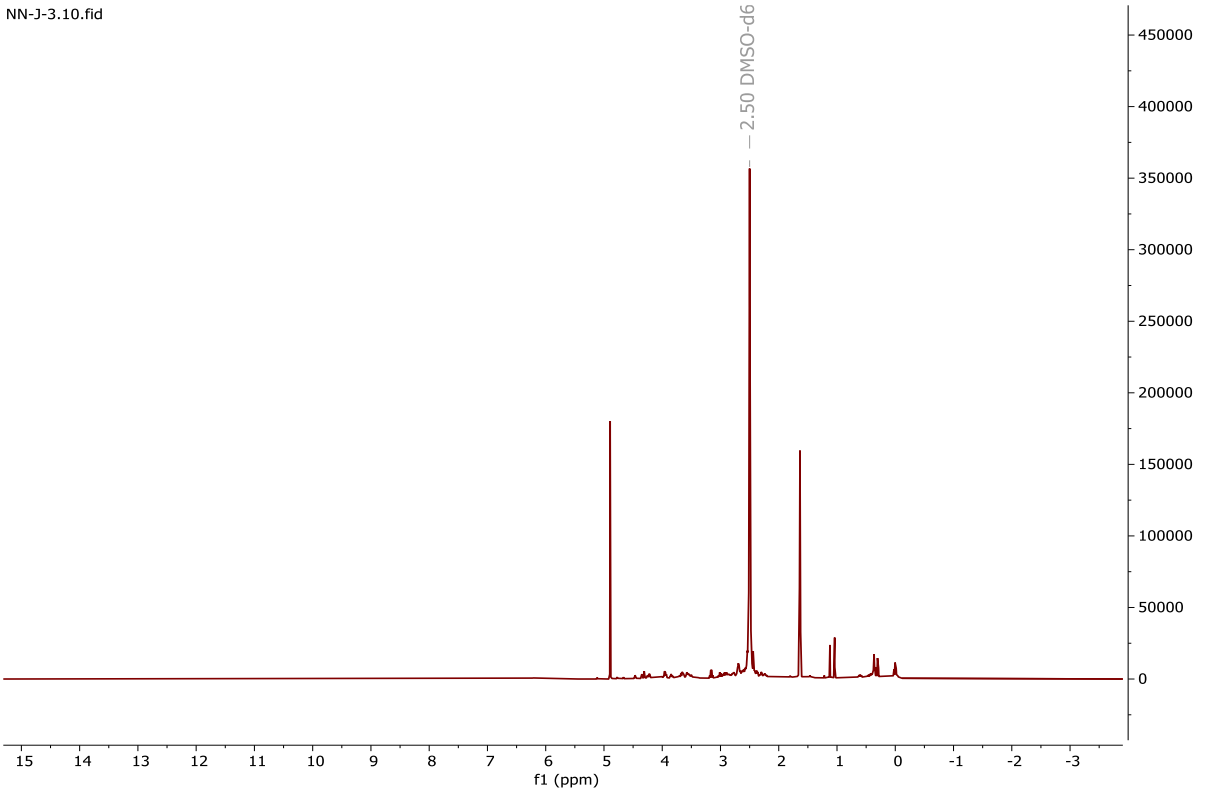

Figure 5.36. ^{1}H NMR spectrum of J_3

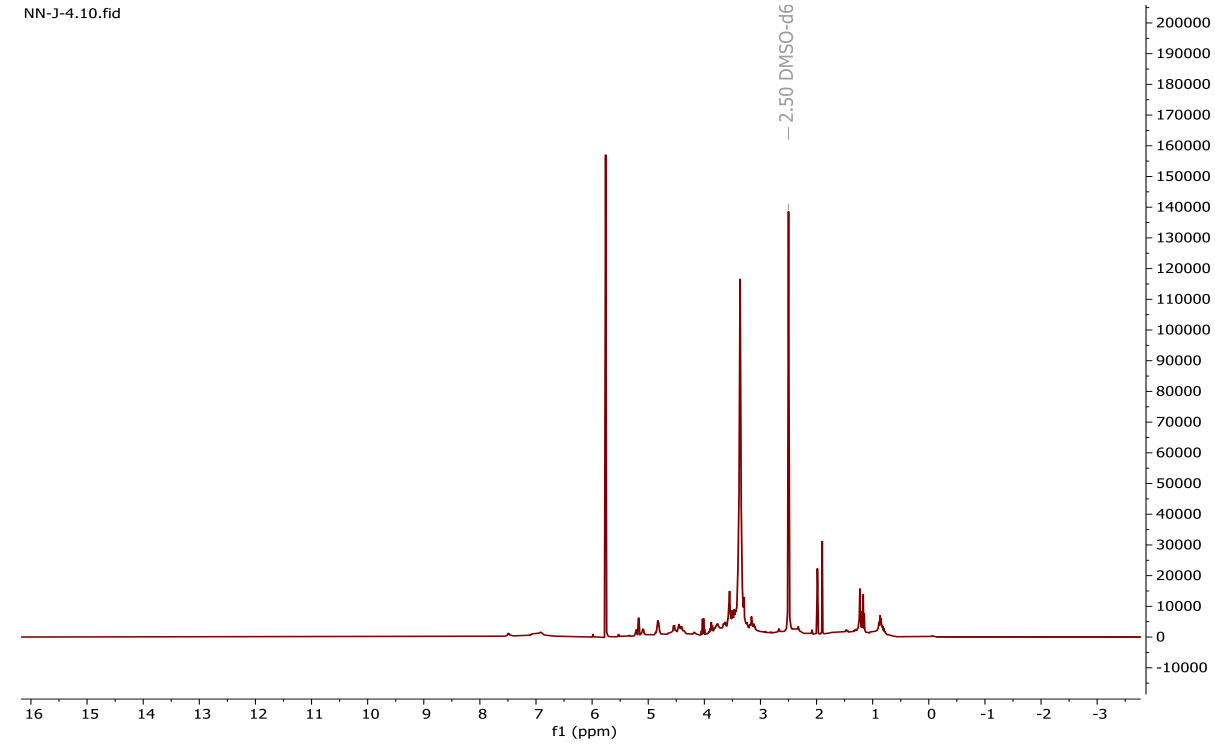

Figure 5.37. ^{1}H NMR spectrum of J_4

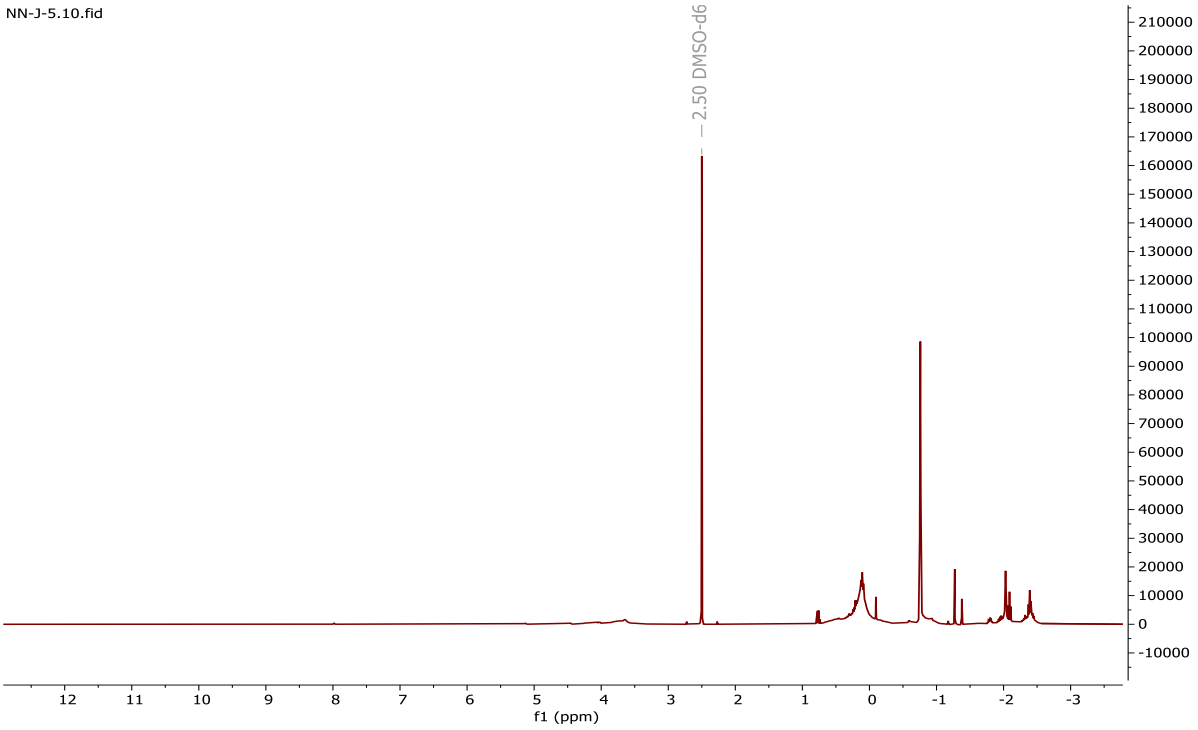

Figure 5.38. ^{1}H NMR spectrum of J_5

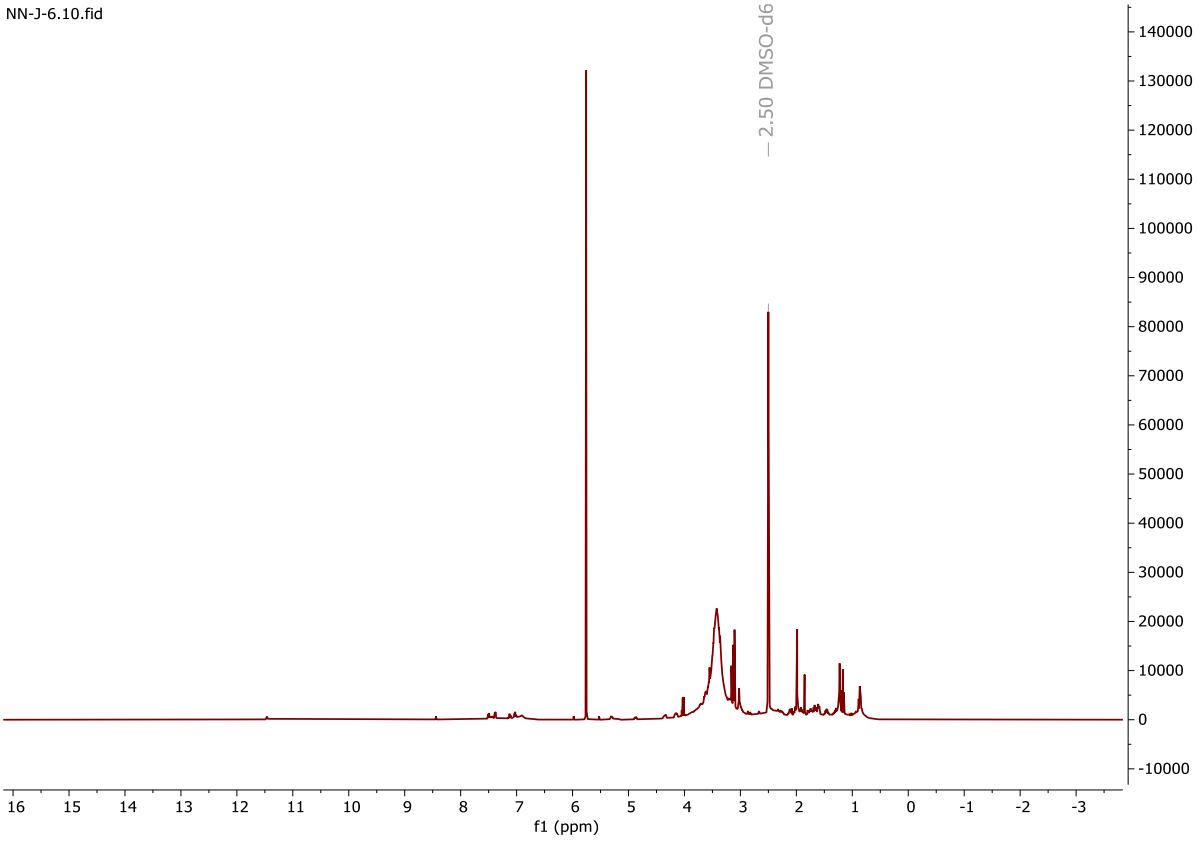

Figure 5.39. ^{1}H NMR spectrum of J_6

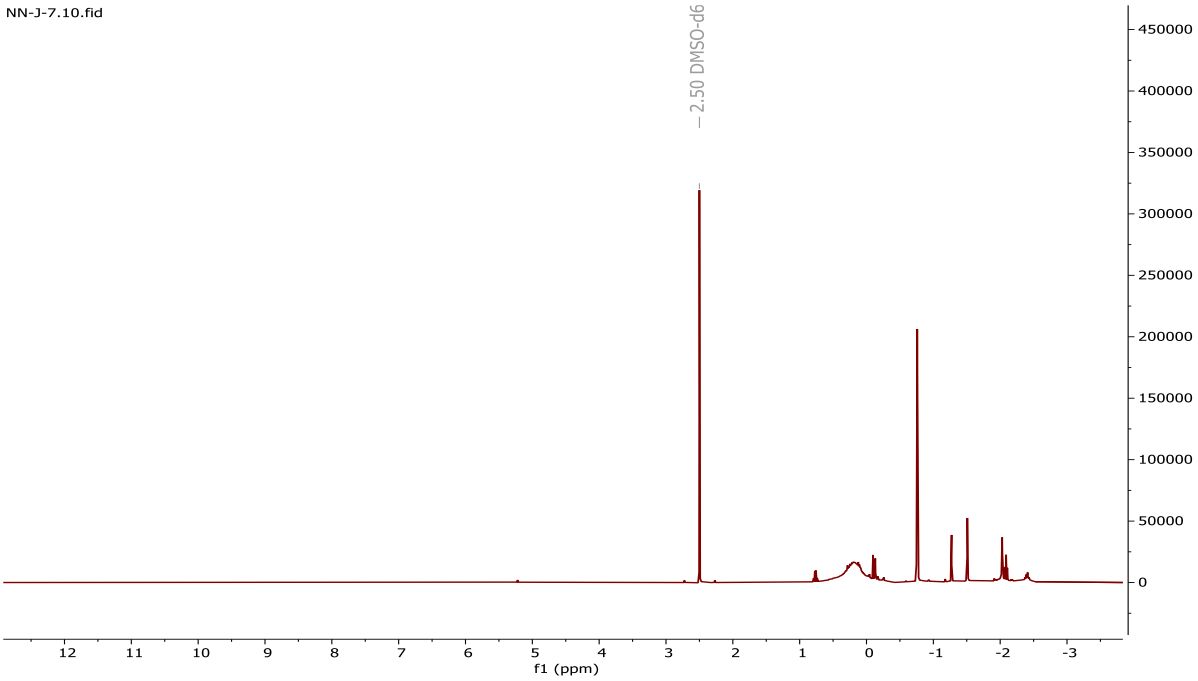

Figure 5.40. ^{1}H NMR spectrum of J_7

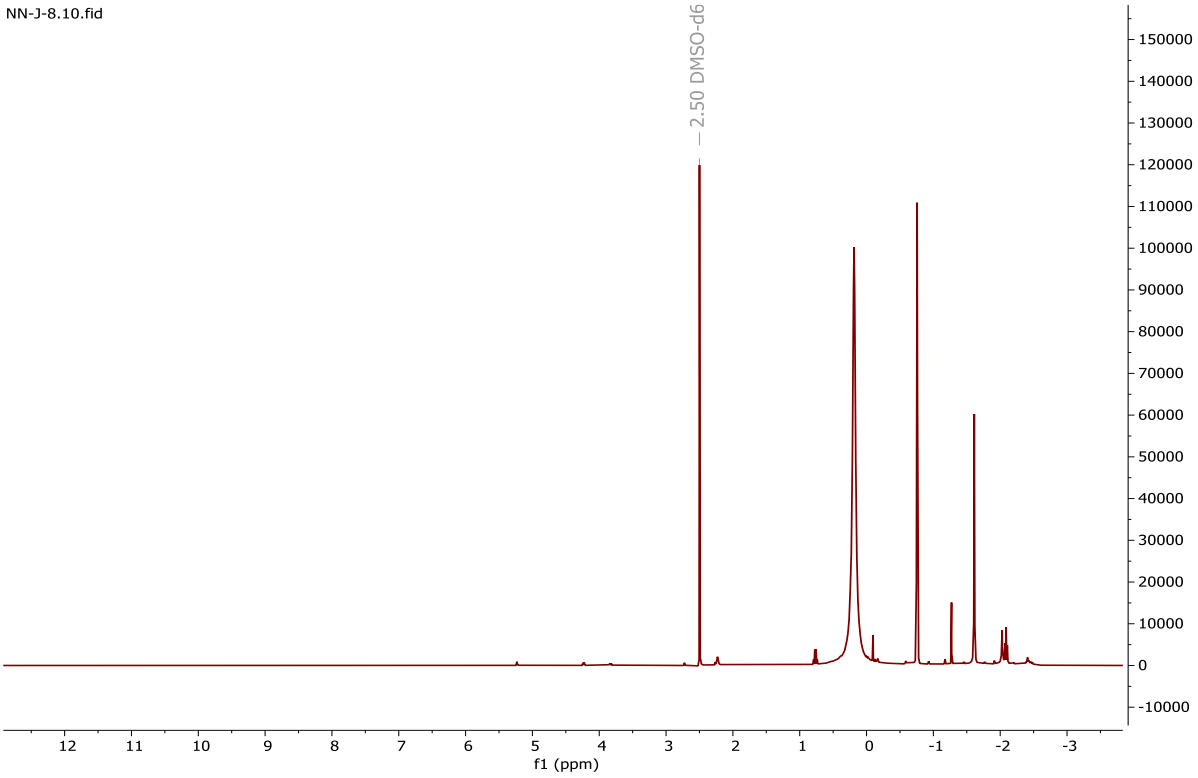

Figure 5.41. ^{1}H NMR spectrum of J_8

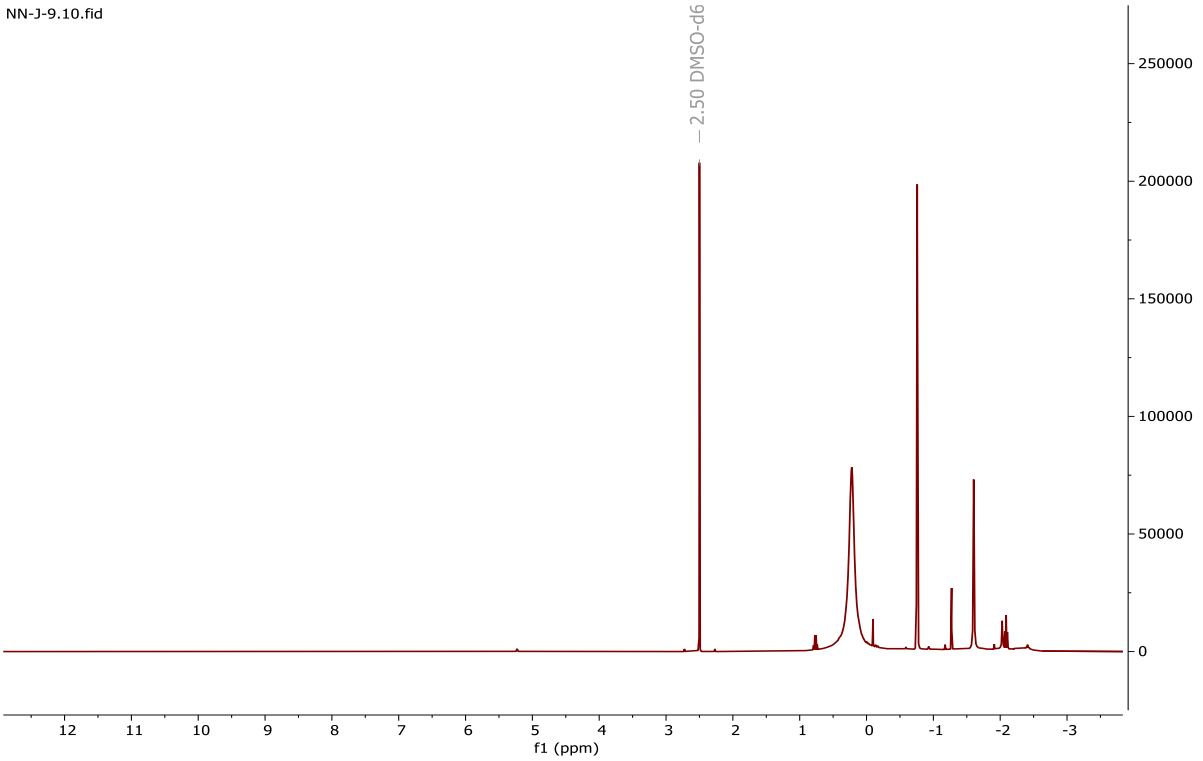

Figure 5.42. ^{1}H NMR spectrum of J_9

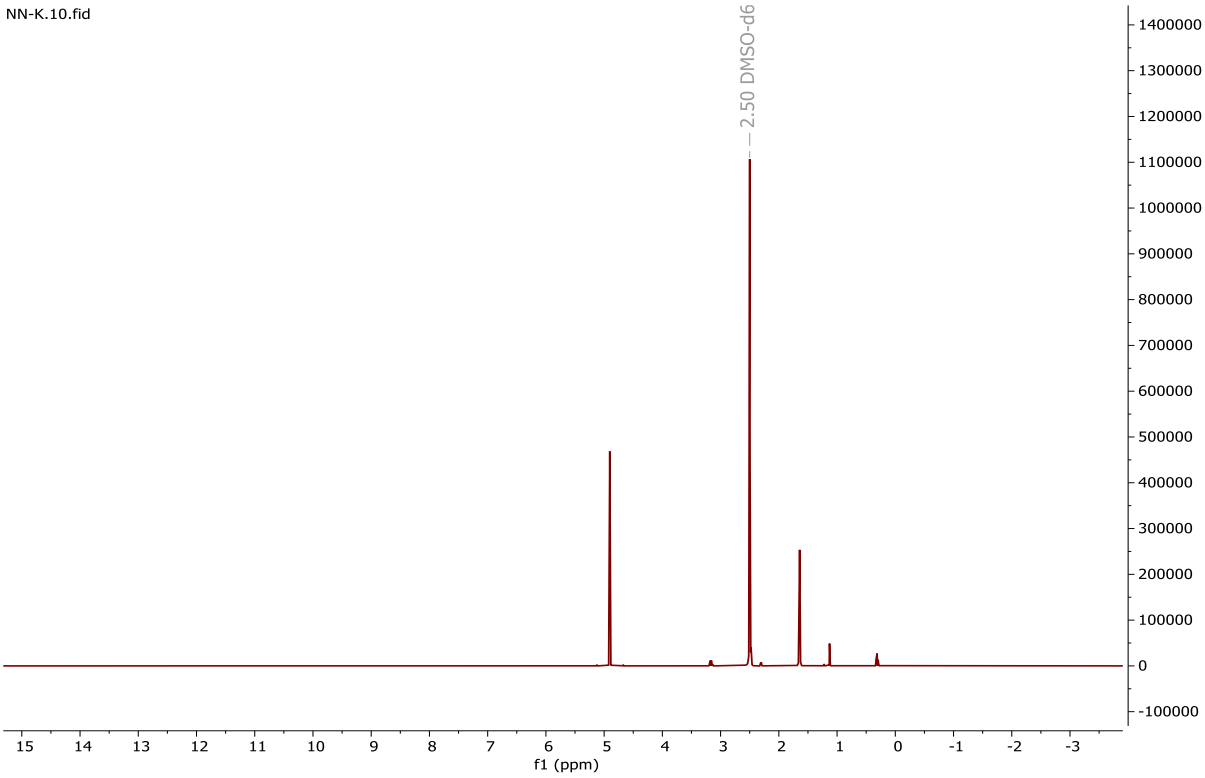

Figure 5.43. ^{1}H NMR spectrum of K