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NMR-based metabolomic study of medicinal plants used against malaria and the isolation of bioactive alkaloids

by

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DECLARATION

I, Mahwahwatse Johanna Bapela declare that the thesis/dissertation, which I hereby submit for the degree Doctor of Philosophy at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other institution.

Signature:.....

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PREFACE

A section of the research study presented in this thesis has been published in a peer-reviewed journal. Parts of this research have also been presented at conferences.

Scientific publication in peer-reviewed journal:

Bapela, M.J., Meyer, J.J.M., Kaiser, M., 2014. *In vitro* antiplasmodial screening of ethnopharmacologically selected South African plant species used for the treatment of malaria. Journal of Ethnopharmacology 156: 370–373.

Scientific publications in preparation:

Bapela, M.J., Meyer, J.J.M., Kaiser, M., 2015. Antileishmanial activity of selected South African indigenous plant species.

Bapela, M.J., Meyer, J.J.M., Kaiser, M., Senejoux, F., 2015. Antiplasmodial constituents isolated from *Vangueria infausta* subsp. *infausta* (Rubiaceae).

Conferences:

Bapela, M.J., Meyer, J.J.M., Heyman, H.M**.,** 2013. Metabolite fingerprinting of plants with antiplasmodial activity. South African Association of Botanists (SAAB), Drakensberg, KwaZulu-Natal, South Africa. Oral presentation.

Bapela, M.J., Meyer, J.J.M., Heyman, H.M**.,** 2014. Isolation of antiplasmodial constituents from traditionally used plants by means on NMR-based metabolomics and chromatography. Fanie de Meillon Postgraduate Symposium, Department of Plant Science, University of Pretoria. Oral presentation.
Awarded: Best PhD presentation

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Bapela, M.J., Meyer, J.J.M., Heyman, H.M**.,** 2014. Isolation of antiplasmodial constituents from traditionally used plants by means on NMR-based metabolomics and chromatography. South African Association of Botanists (SAAB), University of Venda, Tshipise Forever Resort, South Africa. Oral presentation.

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ABSTRACT

Despite the significant advances achieved in lessening the burden of malaria and other tropical diseases in recent years, protozoal infections remain a major cause of mortality in many developing countries, with malaria accounting for a large proportion of the recorded mortality. The main aim of this study was to bioprospect indigenous plant species for novel antiplasmodial plant products by means of NMR-based metabolomics.

In this study, an ethnobotanical criterion was followed in collecting twenty indigenous plant species used to treat malaria or its symptoms by Vha-Venda people living in Mutale Municipality of Limpopo Province, South Africa. Plant samples were extracted in dichloromethane:50% methanol (1:1), separated into polar and non-polar fractions, and tested on *Trypanosoma brucei rhodesiense*, *T. cruzi*, *Leishmania donovani* and *Plasmodium falciparum*. Plant extracts were correspondingly subjected to an antiproliferative bioassay against mammalian skeletal myoblast cells.

The current study is the first scientific account on the significant antileishmanial efficacy (IC_{50} ≤ 5 µg/ml) of *Bridelia mollis* (Phyllanthaceae), *Vangueria infausta* subsp. *infausta* (Rubiaceae), *Syzygium cordatum* (Myrtaceae) and *Xylopia parviflora* (Annonaceae), as well as high antitrypanosomal activity (IC_{50} = 3.45 µg/ml) of *Albizia versicolor* (Fabaceae). Ten plant extracts exhibited significant *in vitro* antiplasmodial activity (IC_{50} ≤ 5 µg/ml), with *Tabernaemontana elegans* (IC_{50} = 0.331 µg/m and IC_{50} = 0.834 µg/m) and *V. infausta* subsp. *infausta* (IC_{50} = 1.84 µg/ml) being the best samples. This is the first scientific report to document significant antiplasmodial activity of extracts from *T. elegans.* The findings of this study substantiate the rationale for adopting an ethnopharmacological approach when bioprospecting medicinal plants for antiplasmodial compounds.

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Dichloromethane extracts were subjected to ^{1}H NMR-based metabolomic analysis, where each crude extract was reconstituted in $CDCl_3$, analysed on a Varian 600 MHz spectrometer and the acquired ^{1}H NMR spectra were then analysed collectively using multivariate data analysis (MDA). Principal Component Analysis (PCA) could not separate the analysed profiles according to the detected antiplasmodial bioactivity. Application of supervised Orthogonal Projections to Latent Structures–Discriminant Analysis (OPLS-DA) on the ^{1}H NMR profiles resulted in a discrimination pattern that could be correlated to the observed antimalarial bioactivity. A contribution plot generated from the OPLS-DA scoring plot illustrated the classes of compounds responsible for the observed grouping.

Further phytochemical analyses were conducted on lipophilic extracts of *T. elegans* and *V. infausta* subsp. *infausta*. These best candidates were fractionated, purified and identified based on conventional chromatographic and spectroscopic techniques. Two known indole alkaloids isolated from *T. elegans*, were identified as dregamine and tabernaemontanine. The antiplasmodial activity of these acyl indole alkaloids has previously been established and ranges from moderate to good. Three compounds were isolated from *V. infausta* subsp. *infausta* of which two were identified as friedelin (IC_{50} = 3.01 µg/ml) and morindolide (IC_{50} = 18.5 µg/ml). While these compounds have been previously identified, this is the first account of their occurrence in the genus *Vangueria* and their antiplasmodial activity. An unidentified compound with significant antiplasmodial activity (IC_{50} = 0.143 µg/ml) was also isolated. The study demonstrated the potential of discovering novel antiplasmodial scaffolds from medicinal plants.

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LIST OF ABBREVIATIONS

ACTs: Artemisinin-based Combination Therapies

CC: Column Chromatography

^{13}C NMR: Carbon Nuclear Magnetic Resonance

COSY: Correlated Spectroscopy

HMBC: Heteronuclear Multiple Bond Coherence

^{1}H NMR: Proton Nuclear Magnetic Resonance

HSQC: Heteronuclear Single Quantum Coherence

MDA: Multivariate Data Analysis

NOESY: Nuclear Overhauser Effect Spectroscopy

OPLS-DA: Orthogonal Projections to Latent Structures–Discriminant Analysis

PCA: Principal Component Analysis

SIMCA: Soft Independent Modelling of Class Analogy

TLC: Thin Layer Chromatography

WHO: World Health Organization

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1.1. Problem statement

While the prevalence of malaria has significantly declined in developed countries, clinical incidences of the disease have been increasing in regions of greater endemicity. Presently, malaria transmission occurs in 108 countries causing an estimated 655 000 deaths annually, most of which are in the sub-Saharan Africa (Murray et al., 2012; World Health Organization (WHO), 2013). Despite decades of global eradication efforts coupled with the technological advances in modern medicine, malaria remains a public health problem affecting many people. The challenges encountered in malaria prevention, control and elimination programmes include differences in parasite, vector, human, economic, social as well as environmental factors (Mills et al., 2008). How each determinant modulates the risk for increased parasitaemia is rather complex and continues to be the main constraint to scaling-up malaria control and strengthening of health systems in the affected countries (Kiszewski and Teklehaimanot, 2004). Aiming at elimination and the eventual eradication of malaria further implies the need for effective surveillance strategies to monitor progress, which could be challenging for underesourced health systems found in most endemic areas.

Malaria is widespread in tropical and subtropical regions, which are climatic zones characterized by warm and humid environmental conditions. The vegetation and climatic conditions of these areas provide an ideal breeding zone for the proliferation of *Anopheles* species and hence increased resistance to pesticides. It is estimated that 40% of the earth's population live in malaria-endangered areas and the figure is projected to increase as a result of global climate change (Patz and Olson, 2006; Stresman, 2010). In South Africa, malaria is mainly restricted to KwaZulu Natal, Limpopo and Mpumalanga provinces and the transmission season is

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from October to May (Khosa et al., 2013). Temperature is particularly critical as temperatures below 20 ^{0}C inhibit malaria parasites to complete their life cycle and can therefore interrupt the transmission between hosts (Cohen et al., 2008). Parasites, which include *Plasmodium* spp., may be quicker at adapting to climatic changes than the animals they live on since they are smaller and grow more rapidly (Lefevre et al., 2013). In addition to the modifying factors associated with global warming, population migration and changes in land use may possibly increase the current transmission rate of malaria (Adimi et al., 2010).

The imminent threat of increased prevalence and distribution due to environmental changes are likely to exacerbate the adverse effects of malaria in endemic countries. More than two-thirds of global malaria cases occur in the low-income countries of the world, thereby imposing substantial socio-economic burdens on the affected populations (Stratton et al., 2008). Micro-economic assessments on the burden of malaria have found a negative correlation between the disease and economic development in the affected countries. The expenses on treatment and the loss of productivity as a result of malaria-related morbidity and mortality may represent a considerable portion of the annual income of poor households (Malaney, et al., 2004; Filauri et al., 2011). Additionally, most malaria endemic areas are characterised by poor public health infrastructure with ineffective health care systems. This is especially the case in sub-Saharan Africa where 90% of the estimated annual global malaria deaths occur (WHO, 2013). The current socio-economic settings of the affected areas present an operational challenge to effectively implement the strategies of malaria eradication campaigns.

An unprecedented progress in malaria prevention and control has been achieved during the last ten years (Murray et al., 2012). This has been partly due to

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the increased scaling-up of vector control interventions around the world, particularly in sub-Saharan Africa. According to WHO (2013), the most effective and extensively applied systems of control for malaria-carrying insects are indoor residual spraying (IRS) and long-lasting insecticidal nets (LLINs). However, there is substantial evidence of insecticide resistance among *Anopheles* mosquitoes, which has been documented in 64 countries around the world (Ranson et al., 2011; WHO, 2013). Of the four classes of insecticides currently used in the control of malaria carrying mosquitoes, pyrethroids are of particular cause for concern. Pyrethroids are the only recommended class that are used in LLINs and losing them to resistance will limit the available options for vector control (White et al., 2014). Innovative strategies are desperately needed to maximize the longevity of the pyrethroid insecticides and to also develop novel insecticides for malaria control.

The malaria threat is as old as humanity, yet no effective vaccine offering a high level of protection has been introduced into clinical practice (Tanabe et al., 2010). Various vaccines have reached the state of clinical trials yet most demonstrated insufficient immunogenicity. The search for a malaria vaccine has been plagued by a number of practical limitations. Obstacles central to all malaria vaccine development efforts include, poor understanding of antimalarial immunity, inducing a protective immune response to the genetically diverse strains of *Plasmodium* species and establishing a vaccine correlate of protection (Lymbery and Thompson, 2012; Ouattara and Laurens, 2014). Extensive genetic diversity in *Plasmodium* populations is the main problem for the development of an effective vaccine against the parasite, since antigenic diversity limits the efficacy of acquired protective immunity to malaria (Olasehinde et al., 2012). Furthermore, the parasitic

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surface proteins and metabolic pathways keep changing during these different stages, which help the parasite to evade the immune clearance by hosts.

Chemotherapy and chemoprevention of malaria are dependent on a few and not chemically diverse number of therapeutic agents. The variable efficacy of the drugs between strains or species, their toxicity and the required treatment regimens are undermining the control approach to the disease (Winstanley, 2000; Schlitzer, 2008). Control of malaria has also been weakened by the increasing resistance of *Plasmodium* species to the commercially available antimalarial drugs. Modern treatment regimens are dispensed in the form of artemisinin-based combination therapies (ACTs) (WHO, 2013; Watsierah and Ouma, 2014). Emerging evidence on resistance against artemisinin derivatives are appearing in some regions of South-East Asia, where patients are taking longer periods to clear their fever and parasitaemia (Dondorp et al., 2009; Witkowski et al., 2010). Given that artemisinins have very short half-lives, this loss of potency would render the more slowly eliminated drugs of the ACTs more vulnerable to development of resistance. The reduced susceptibility of *P. falciparum* to artemisinin derivatives highlights the urgency of the problem and is an indication that new classes of antimalarials are desperately needed.

The main challenge in malaria chemotherapy is to discover safe and selective therapeutic agents with potency that will not be compromised by plasmodial resistance and with more convenient dosing regimens (The MalERA Group, 2011; White et al., 2014). Plants have been the basis of the current antimalarial drugs and continue to be the promising source of novel molecular templates for malaria therapy. In spite of the concerted effort and advanced analytical technologies directed at exploring plant products, fewer novel antiplasmodial scaffolds have

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emerged from such studies. The isolation, chemical characterisation and quantification of plant metabolites involve a multidisciplinary approach, based on chromatographic and spectroscopic techniques. Although hyphenated analytical platforms show the potential to tackle some of the challenges inherent in the analysis and characterisation of plant products, they are technically complex and expensive (Wilson and Brinkman, 2003; Wu et al., 2013). Similarly, notwithstanding the advances in technology and an understanding of biological systems, drug discovery remains a lengthy, difficult and costly process. It is an endeavour that requires capital, long-term funding commitment as well as innovation in order to increase and diversify the current antimalarial drug portfolio.

1.2. Hypotheses

The study aims at testing the following hypotheses:

a) Some of the ethnopharmacologically selected plant species will exhibit significant *in vitro* antiplasmodial activity.

b) Metabolomics can be used to determine major classes of compounds responsible for the antiplasmodial activity.

1.3. Objectives

The specific objectives of the study are to:

a) Validate the antiplasmodial activity of selected plant species.

b) Conduct a metabolomic study on the plant extracts.

c) Isolate and identify antiplasmodial compounds.

d) Confirm the antiplasmodial activity of the isolated compounds.

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1.4. Structure of the thesis

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

Chapter 1: A problem statement highlighting some of the major challenges and practical limitations encountered in the control and elimination of malaria is presented in this chapter. The tested hypotheses, objectives for the study and the structure of the thesis are stated in this chapter.

Chapter 2: This chapter outlines a concise review on the complexity of the life cycle of *Plasmodium* spp. and the antimalarial drugs currently used in the treatment and control of the disease. It also presents a review on the potential of ethnopharmacology and metabolomics in the discovery and development of novel antiplasmodial phytotherapeutic agents.

Chapter 3: A comprehensive study on the *in vitro* antiprotozoal screening of twenty ethnopharmacologically selected indigenous plant species is described in this chapter.

Chapter 4: In this chapter, an NMR-based metabolomics study conducted on the screened plant species is detailed.

Chapter 5: This chapter describes the isolation and identification of bioactive indole alkaloids from *Tabernaemontana elegans* Stapf. (Apocynaceae).

Chapter 6: The bioassay-guided fractionation, isolation and identification of bioactive constituents of *Vangueria infausta* subsp. *infausta* Burch. (Rubiaceae) are presented in this chapter.

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Chapter 7: General interpretations based on the findings of the study are presented in this chapter. Knowledge gaps and suggestions for future research are also highlighted.

Chapter 8: Acknowledgements and credits are given to those who made commendable contributions towards the research undertaken.

Chapter 9: Supplemental data are provided in this chapter.

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2.1. Introduction

The past decade has seen a significant decrease in the clinical episodes of protozoal infections, especially malaria incidences. World Health Organization (WHO) estimates that between the years 2000 and 2010, the global malaria occurrences declined by 17% while malaria-specific mortality rates decreased by 26% (Cotter et al., 2013; WHO, 2013). These accomplishments have been driven by several factors, including increased funding, effective vector control, strengthening of endemic health systems, efficient treatment regimens as well as continued surveillance (WHO, 2013; White et al., 2014). The global malaria agenda has presently broadened its focus from mere control and management of clinical cases to elimination and the eventual eradication of the disease (Feachem et al., 2010). Artemisinin-based combination therapies (ACTs) are currently the most effective treatments available for malaria, despite the emergence of plasmodial resistance in some regions of South-East Asia (Dondorp et al., 2009; Witkowski et al., 2010). In light of the development of resistance and the limitations experienced with the development of malaria vaccine, more tools are needed to overcome the challenges encountered in malaria eradication and to circumvent the emerging resistance.

Antimalarial drugs have always been part of the indispensible tools used at all phases of malaria elimination along the path towards eradication (The MalERA Group, 2011). Currently, the malaria research agenda for global eradication has identified several high priority research areas that need to be addressed in an effort to eliminate the disease. One such area is the optimization of ACTs and other available antimalarial drugs in an attempt to maximize their useful lifespan (Duparc et al., 2012). Another area of major focus is the continuation of research and development of new antimalarial drugs with novel mechanisms of action. Natural

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plant products and their derivatives have been the major foundation for the current malaria therapy and its chemoprevention. It is common knowledge that plants synthesize and accumulate hundreds of thousands of diverse metabolites endowed with distinct chemical properties, which may serve as leads in drug development (Verpoorte, 1998). Bioprospecting plants could yield novel molecular scaffolds that could form the basis for the next generation of antiplasmodial therapies. This chapter presents an overview of protozoal infections and the life cycle of *Plasmodium* parasites as a backdrop to challenges faced in the chemotherapy of malaria. In addition, ethnopharmacology as the suitable approach to bioprospect for antimalarial plant products and metabolomics as a technique for optimizing the recovery as well as the characterization of bioactive compounds are succinctly discussed.

2.2. Protozoal infections

Vector-borne infectious diseases are a worldwide health problem, particularly in most developing countries located in tropical regions. They include leishmaniasis, Chagas disease (American trypanosomiasis), sleeping sickness (human African trypanosomiasis) and malaria, which have been shown to impose a negative impact on the socio-economic status of the affected people. Leishmaniasis is caused by protozoan species of the genus *Leishmania*, which are transmitted by female sandflies belonging to the genera *Phlebotomus* and *Lutzomyia* (Desjeux, 2004). Depending on the causative species and the immune response of the host, the infection can clinically manifest as cutaneous, mucocutaneous, diffuse and visceral leishmaniasis (Alvar et al., 2012). Visceral leishmaniasis which is caused by *L. donovani* is the most deadly form of the disease and intermittent epidemics of the disease have been linked to high morbidity as well as mortality in some East African

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countries (Antinori et al., 2012). Globally, there is an estimated 1.3 million new cases annually and over 20 000 deaths occur as a direct result of the disease (Alvar et al., 2012). The chemotherapy of leishmaniasis is strongly reliant on pentavalent antimonials, miltefosine, amphothericin B and paromomycin. However, increasing treatment failure rates, high cost and significant side effects are the major drawbacks compromising their effectiveness.

The etiological agents responsible for the cause of Chagas' disease, human African trypanosomiasis are *Trypanosoma cruzi* and *T. brucei* spp. respectively. These protozoan parasites are characterized by complex life cycles alternating between insects and mammalian hosts (Rassi et al., 2012). Although Chagas disease is endemic to Latin America the disease is currently expanding to the United States of America, Canada as well as many European and some Western Pacific countries. Nearly 7 million people worldwide are infected with Chagas disease and about 12 000 people die each year as a result of the disease (WHO, 2013). Human African trypanosomiasis occurs in 36 sub-Saharan Africa countries. These are areas where the tsetse flies that transmit the disease are considered to be endemic. The disease infects about 5000 people on an annual basis and may be fatal if not treated. Therapeutic options for these *Trypanosoma* infections include benznidazole, nifurtimox, suramin, pentamidine, melarsoprol and eflornithine, however, the emergence of drug resistant strains is limiting their efficacy (Mishina et al., 2007, Jimenez et al., 2014). Furthermore, some of the chemotherapeutics that are still effective suffer from problems related to high costs, toxicity, prolonged treatment schedules, variable responses between strains and non-compliance by patients (Machado et al., 2010; Olasehinde, 2012). Of all these parasitic diseases, malaria

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remains the most fatal and accounts for a larger percentage of the recorded mortality resulting from protozoal infections.

Malaria is transmitted in 108 countries causing an estimated 216 million clinical cases and 655 000 deaths annually (Murray et al., 2012; WHO, 2013). About 90% of global malaria deaths occur in sub-Saharan Africa, mainly in children younger than 5 years (Figure 2.1.). Malaria is a vector-borne disease caused by protozoan parasites of the *Plasmodium* genus and transmitted by female *Anopheles* mosquitoes. Only five plasmodial protists, *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*, are infectious to humans. These species vary in geographical distribution, microscopic morphology, clinical presentation and their susceptibly to antimalarial drugs.

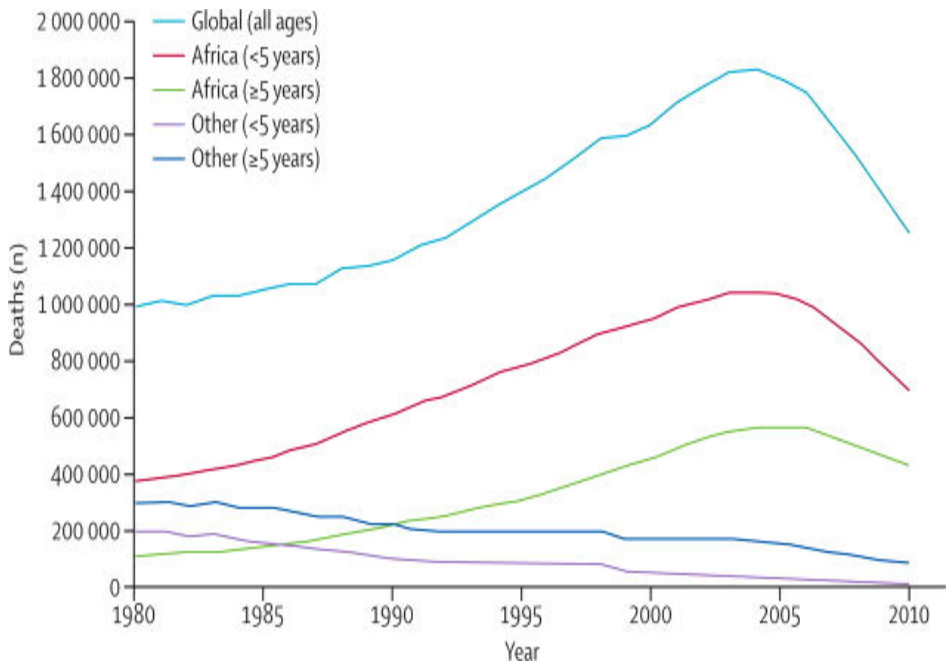

Figure 2.1. Trends in global malaria deaths (Murray et al., 2012)

Of all these parasitic species, *P. falciparum* which predominates in Africa is the most virulent and causes the vast majority of deaths (Greenwood et al., 2008).

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Infection by *P. falciparum* usually results in an uncomplicated disease; however, in some cases the disease becomes severe and may lead to death. *P. vivax* is the most geographically widespread human malaria parasite species and is common in tropical areas outside Africa. Although vivax malaria is considered to be benign, with a very low case-fatality ratio, it can lead to morbidity, severe disease and death as a result of splenomegaly (Cornejo and Escalante, 2006).

P. ovale is known to cause tertian malaria in humans and is very limited in its range, found mainly in West Africa, Philippines and eastern Indonesia (Mueller et al., 2007). *P. malariae* has low global prevalence, milder clinical manifestations and is widespread throughout sub-Saharan Africa, South-East Asia, Indonesia, western Pacific and in areas of the Amazon Basin of South America (Mueller et al., 2007). The fifth species, *P. knowlesi* is a primate malaria parasite endemic in some South-East Asian areas and can cause severe malaria in humans (Singh and Daneshvar, 2013). These five *Plasmodium* species are transmitted by more than 30 *Anopheles* mosquitoes with diverse breeding and feeding habits resulting in different disease spectra in various population groups (Sinka et al., 2011). Humans often harbour multiple *Plasmodium* species but the clinical implications of mixed infections remain unclear.

2.3. The life cycle of *Plasmodium* parasites

The *Plasmodium* parasite exhibits a complex, invasive multistage life cycle occurring within the vector mosquitoes and the vertebrate hosts. Infection of the human host with a malaria parasite begins when an infected *Anopheles* mosquito inoculates microscopic motile sporozoites during a blood meal (Schlitzer, 2008). Injected sporozoites are carried by the circulatory system to the liver where they

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invade hepatocytes and then undergo exoerythrocytic schizogony (Figure 2.2.). This asymptomatic liver stage of infection yields merozoites that are liberated into the bloodstream (Greenwood et al., 2008). Hypnozoites are parasites of *P. vivax* and *P. ovale* that go through a latent period instead of immediate asexual multiplication. On the contrary, *P malariae* does not form hypnozoites but it can persist for decades as an asymptomatic blood stage infection. Unlike *P. vivax* which binds to the Duffy antigen to invade reticulocytes, *P. falciparum* use multiple pathways to infect erythrocytes, further complicating the effort to develop anti-invasion vaccines (Baum et al., 2005). Merozoites invade erythrocytes, enlarge and develop into trophozoites.

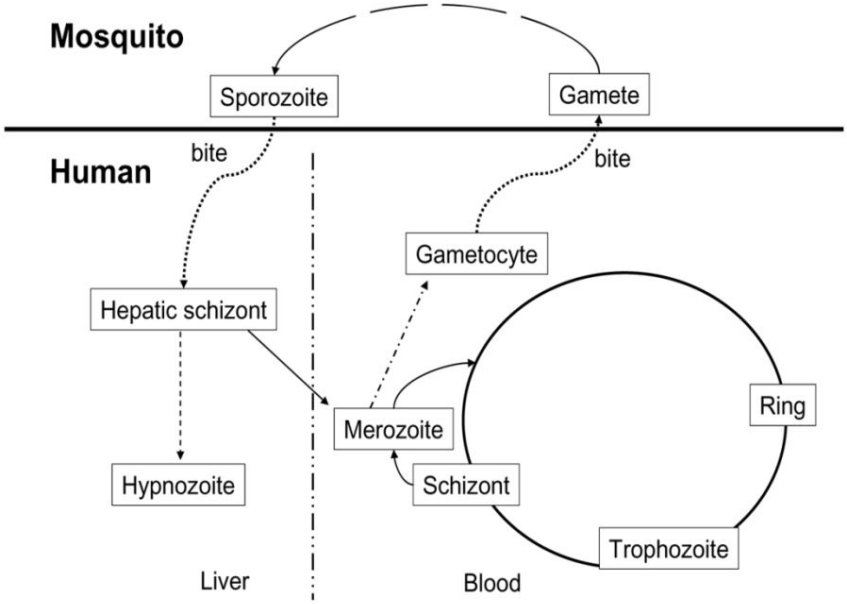

Figure 2.2. The life-cycle of *Plasmodium falciparum* (Schlitzer, 2008)

The early trophozoite stage is referred to as the ring form whereas the late form is known as the schizont. Merozoites bud from schizonts and initiate another round of blood stage replicative cycle. The blood stages of infection include asexual forms of the parasite that undergo repeated cycles of mitotic multiplication. They also include male and female sexual forms, called micro- and macrogametocytes,

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respectively, which await ingestion by mosquitoes before developing further (Sinden, 1982). The asexual stage is responsible for the pathogenesis of the disease. Intermittent fever results from the ingestion of host cytoplasm, proteolysis of haemoglobin, lysis of infected erythrocytes and a suite of host reactions. *P. falciparum* often exhibit continuous fever, higher levels of parasitaemia and sequestration of infected erythrocytes in tissues, which explain its increased virulence, compared to the other *Plasmodium* species (Greenwood et al., 2008). Sexual stage parasites are non-pathogenic but transmissible to the *Anopheles* vector. Ingestion of the gametocytes by the mosquito vector induces gametogenesis in the mid-gut of the mosquito. Microgametes fertilize macrogametes and form zygotes, which develop into ookinetes that penetrate the intestinal walls of the mid-gut and develop into oocysts (Schlitzer, 2008). Asexual replication occurs within the oocyst and produces sporozoites. The sporozoites migrate to invade the salivary glands, thereby completing the life cycle.

Each of the developmental stages represents a potential target at which the life cycle can be interrupted with vaccines, antivectors and drugs. Nevertheless, the complex life cycles of the five malaria species infecting humans exhibit diverse challenges. All these stages are characterized by unique shapes, distinct structures and many protein complements. *Plasmodium* species have approximately 16 chromosomes, yet their specialized proteins help the parasites to invade, multiply and grow within multiple cell types of their hosts (Gardner et al., 2002). Their surface proteins and metabolic pathways keep changing during these different stages, enabling the parasites to evade the immune clearance while also creating problems for the development of drugs and vaccines (Lymbery and Thompson, 2012). *P. vivax*, *P. ovale* and *P. malariae* can persist for years without causing symptoms and

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at low levels that cannot be detected by standard diagnostic methods. They can however, still propagate the transmission of malaria. Apart from its persistence, *P. vivax* is less responsive to control interventions than *P. falciparum* infections because of its wider ecological range and the ability to produce infectious gametocytes soon after its emergence from the liver (Greenwood et al., 2008). In contrast to the other *Plasmodium* species, the appearance of gametocytes in peripheral circulation is delayed in *P. falciparum* making recrudescence common in clinically cured individuals (Abdul-Ghani et al., 2014). Most of these malaria parasites attach and enter host cells via several pathways and their species-specific characteristics present varied complications to chemotherapy.

2.4. Chemotherapy of malaria

Antimalarial drugs are used to prevent the infection caused by *Plasmodium* species, treat the disease, eliminate latent parasites and prevent the transmission of malaria. The major purpose of antimalarial treatment in severe malaria is to prevent death whereas in uncomplicated malaria is to cure and to avoid the development of severe disease (White et al., 2014). While insecticides are aptly credited for much of the success in reducing the burden of malaria, treatment and prevention with drugs have always been the essential components of all successful malaria elimination programmes. Similarly, while many diseases have been eradicated primarily through the use of vaccines it is very unlikely that malaria could be eradicated with a vaccine alone without the integration of antivector methods and drugs (The MalERA Group, 2011). Antimalarial drugs possess selective actions at different stages of the parasite life cycle. The ideal target profile for malaria eradication is a coformulated drug combination that can be administered in a single dose and result in radical cure of all

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life cycle stages of the five *Plasmodium* species infecting humans (Gelb, 2007). However, the desirable drug might have limitations such as targeting only one or two parasite species, the priorities being *P. falciparum* and *P. vivax.* Given the differences in the life cycle stages of the five *Plasmodium* species, it is reasonable to assume that drugs which target *P. falciparum* will be generally effective against *P. malariae*, and those targeting *P. vivax* may be efficacious against *P. ovale* and *P. knowlesi* (The MalERA Group, 2011).

A limited number of antimalarials are currently in clinical use and they fall into seven different pharmacological classes. They include 4-aminoquinolines, aryl-amino alcohols, 8-aminoquinolines, antifolates, hydroxynaphthoquinones, antibiotics and artemisinins (Schlitzer, 2008; Rudrapal, 2011). Classification of these antimalarial drugs into groups is based on the differences in their pharmacophores, mechanisms of action and selective actions on the developmental stages of the parasites. 4-Aminoquinolines, chloroquine (Figure 2.3.) and its derivative amodiaquine, are quinoline-based molecules containing a primary amine group in the 4-position (Elderfield et al., 1946). They accumulate in vacuoles of susceptible parasites thereby interfering with the parasite's detoxification pathway (Witkowski et al., 2010). Aryl-amino alcohols include quinine, mefloquine, halofantrine as well as lumefantrine, and their antimalarial activity seem to require the presence of an aromatic portion and an amino alcohol portion (Figure 2.3.). Like the 4-aminoquinolines, aryl-amino alcohols are assumed to act primarily on the erythrocyte stage of the malaria parasite by inhibiting the formation of hemozoin (Anderson et al., 2006). 8-Aminoquinolines are derived quinoline molecules with an amine group at the 8-position of quinoline. Primaquine is the only 8-aminoquinoline used in malaria therapy and studies have shown that it interferes with the parasite's DNA

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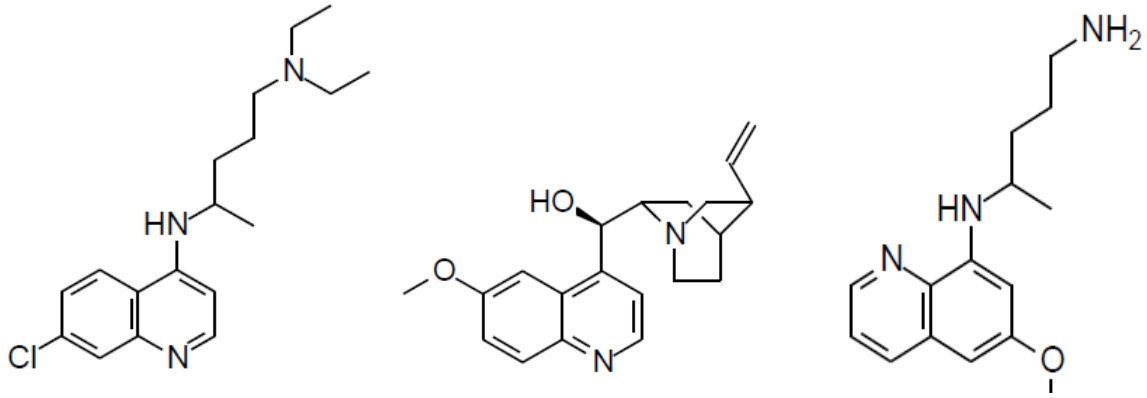
structure and disrupts its mitochondrial membranes (Miller et. al., 2013). It is also the only available drug that can prevent transmission of mature gametocytes although it can cause intravascular hemolysis in patients with glucose-6-phosphate dehydrogenase deficiency (Sinha et al., 2014). The precise mechanisms of action of the abovementioned quinoline based antimalarial drugs are not yet fully understood and still under investigations.



chloroquine **quinine** **primaquine**

Figure 2.3. Chemical structures of some quinoline based antimalarials

Antifolates include two classes of antimalarial drugs that interfere with folate metabolism, a pathway essential for DNA replication and protein synthesis of malaria parasite. They specifically target two enzymes involved in the biosynthesis of tetrahydrofolate, the dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR) (Rudrapal, 2011). Sulphonamides, sulphadoxine (Figure 2.4.), sulphones, dapsone and sulphalene act as competitive inhibitors against DHPS thereby preventing the formation of dihydropteroate. The second class includes proguanil, chloroproguanil, cycloguanil and pyrimethamine, which inhibit DHFR, consequently preventing the reduction of dihydrofolate (DHF) to tetrahydrofolic acid (THF) (Nzila et al., 2005; Müller and Hyde, 2013). Combination of both classes of antifolates, proved to have a synergistic effect against malaria, though clinical resistance developed

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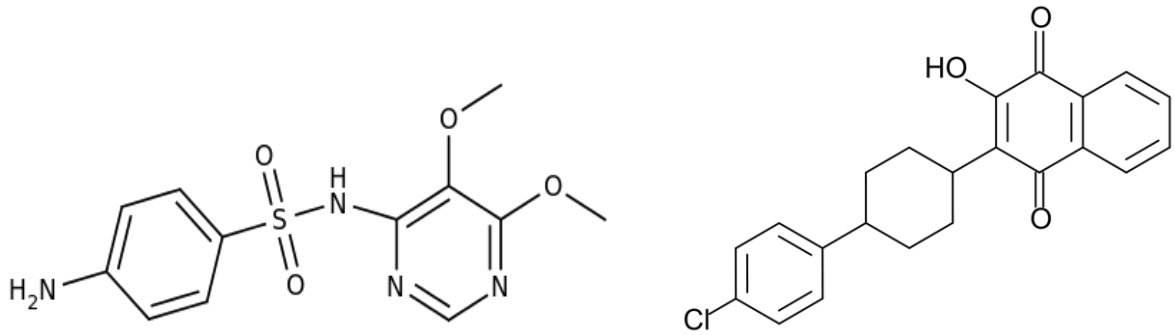
quickly compromising their use in many malaria endemic areas (Heinberg and Kirkman, 2015). Atovaquone is a hydroxynaphthoquinone that is used in combination with proguanil for prophylaxis and therapy of asymptomatic malaria (Figure 2.4.). It is active against all *Plasmodium* species, inhibiting the exoerythrocytic stage in humans and the oocyst development in the mosquito. It acts by inhibiting the mitochondrial electron transport chain thereby leading to a breakdown of the mitochondrial membrane potential (Barton et al., 2010). The prevalence of mutations of the cytochrome *b* gene has resulted in therapy failure of atovaquone (Sutherland et al., 2008).



sulphadoxine **atovaquone**

Figure 2.4. Structures of sulphadoxine and atovaquone

Certain antibiotics, such as doxycyclin, clindamycin, tetracycline and chloramphenicol play a major role in the treatment and prophylaxis of malaria. Antibiotics display an antimalarial effect by inhibiting mitochondrial protein synthesis and blocking the biosynthetic pathway of nucleic acids in *Plasmodium* species (Rudrapal, 2011). Monotherapy of antibiotics for malaria treatment is ineffective, with fever and parasite-clearance taking longer than with classical antimalarials. To increase their therapeutic effect, they are used in combination with faster acting

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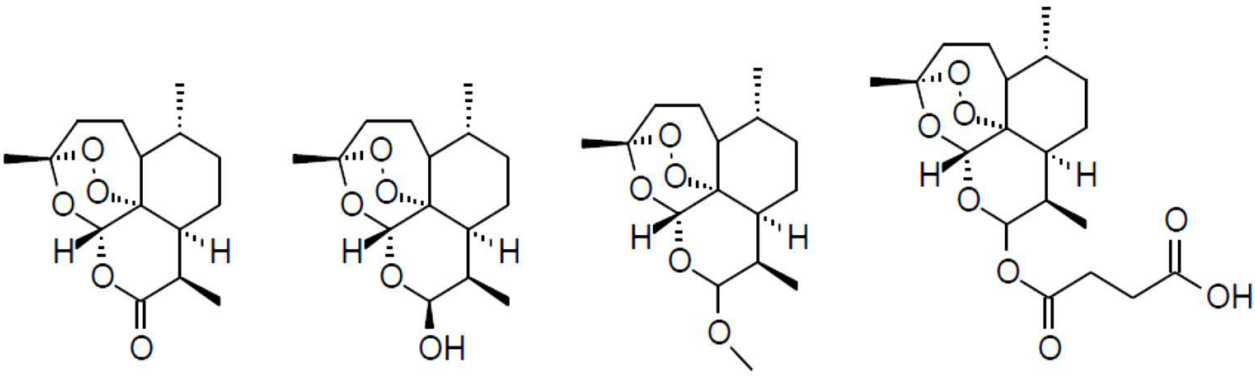
drugs and to date no resistance against antibiotics has been reported (Pradel and Schlitzer, 2010).

Artemisinin (Figure 2.5.) is the active constituent of *Artemisia annua* and is a sesquiterpene lactone consisting of three isoprene units bound to cyclic organic peroxide esters (Maude et al., 2010). While the endoperoxide bond is now known to be necessary for antimalarial activity, the exact mechanism of action is still a topic of debate (O'Neill et al., 2010; Paddon and Keasling, 2014). The relatively poor pharmacokinetics of artemisinin had resulted in its semi-synthetic and synthetic structural variations of clinical use. They include dihydroartemisinin, artemether, arteether, artesunate and artelinic acid. Artemisinins display rapid parasite clearance, are active against all species of malaria that invade humans, are potent blood schizontocide and they inhibit the early gametocytic stage of the parasite (Ding et al., 2010). The advantage of their short half-lives is that selection for drug-resistant parasites is reduced; nevertheless, there is also a higher associated risk of recrudescence when these drugs are administered as monotherapeutic regimens (Eastman and Fidock, 2009). Recrudescence is an indication of treatment failure and has been thought to arise in response to the selection of drug-resistant parasites.



artemisinin **dihydroartemisinin** **arteether** **artesunate**

Figure 2.5. Artemisinin and its derivatives

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In an attempt to reduce the occurrence of recrudescence and therefore the development of resistance, WHO advocates the use of ACTs, which provide symptomatic relief, prevent severe disease and reduce mortality rates (WHO, 2013). The ACTs combine a derivative of artemisinin with a longer lasting partner drug that continues to reduce the parasite biomass after the short-lived artemisinin has dropped below therapeutic levels (Nosten and White 2007). Recommended ACTs that are currently under clinical use include artemether-lumefantrine (AL), artesunate-mefloquine (ASMQ), artesunate-amodiaquine (ASAQ), artesunate-sulfadoxine-pyrimethamine (AS-SP) and dihydroartemisinin-piperaquine (DHA-PQ) (Ashley and White, 2005; Banek et al., 2013). Reports of delayed responses to some ACTs as well as supporting *in vitro* drug-susceptibility data, suggest the possibility of clinical artemisinin resistance along the Thai–Cambodian border (Noedl et al., 2008; Dondorp et al., 2009). Significant failure rates of these combinations have also been observed in African countries where resistance to one drug has been previously encountered. Since artemisinins have very short half-lives, this loss of potency will render the partner drugs more vulnerable to resistance. These findings present a significant challenge to efforts aimed at preventing, controlling and eliminating malaria in recent years.

Antimalarial drug resistance is the persistence or recurrence of *Plasmodium* parasites after appropriate drug treatment. Most of the chromosomal mutations that confer antimalarial drug resistance occur in nature, are spontaneous, were found to be independent of the kind of drug used and are not necessarily a result of treatment failure (White, 2004; Koenderink et al., 2010). These chromosomal mutations are selected by different concentrations of antimalarial drugs that are sufficient to reduce susceptible parasites but not adequate to inhibit multiplication of mutants (Abay,

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2013). Presently, antimalarial drug resistance has been documented in *P. falciparum*, *P. vivax* and *P. malariae* strains (WHO, 2013). Resistance to antimalarials presents several survival advantages to the malaria parasite. They include recrudescence, proliferation of resistant parasites and enhancement of the transmission of gametocytes harbouring the resistant genes (Abdul-Ghani et al., 2014).

Recrudescent infections as a result of resistant strains of the parasite tend to have higher gametocyte carriage rates with higher infectivity than primary infections. Gametocytes are therefore an important mechanism for resistance and drugs that could block transmission by eliminating the gametocytes are much needed to counteract resistance (Craft, 2008). Antimalarial drug resistance is complicated by cross-resistance, which mostly occurs among groups of drugs that belong to a similar chemical family or which have the same mode of action. Furthermore, multiple drug resistance of *P. falciparum* has been observed when the parasite is resistant to more than two operating antimalarial compounds of different chemical classes and mechanisms of action (Sinha et al., 2014). The prevalence of resistance to the well-established antimalarial drugs has resulted in the expansion of antimalarial drug discovery efforts.

There is a growing global collaboration between academic institutions, non-profit organizations as well as pharmaceutical companies, in an attempt to develop new antimalarials including compounds with novel properties (Gelb, 2007). Several new antimalarial agents are currently in advanced stages of clinical studies. They include mainly those compounds that were resurrected from previous antimalarial drug discovery programs or structural analogues of existing antimalarials. Ferroquine, AQ-13, tafenoquine, OZ439, artemisone and sevuparin are some of the

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compounds developed based on existing chemical scaffolds but with improved pharmacological properties (Figure 2.6.) (Held et al., 2014). The advantage of this approach is that the safety and pharmacokinetic profiles of the modified compounds are well established, however, cross-resistance remains a threat.

Whole-cell screening is an invaluable strategy that is used to select hit molecules from compound libraries irrespective of their mode of action. Cellular screens have led to the identification of KAE609 (spiroindolone) and KAF156 (imidazolopiperazine) (Figure 2.6.) as new classes of antimalarial compounds with promising pharmacokinetic profiles (Held et al., 2014). Other drug candidates that are currently in clinical trials have been detected by molecular target-based approaches. They include methylene blue, fosmidomycin, DSM265 and SAR97276 (albitiazolium bromide) (Figure 2.6.). Although the global pipeline of new medicines for the control and elimination of malaria is broadening, there are still several gaps in the current portfolio (Anthony et al., 2012). Simpler single-dose regimens, that can prevent transmission, inhibit relapse and with better safety profiles than current medicines are still needed.

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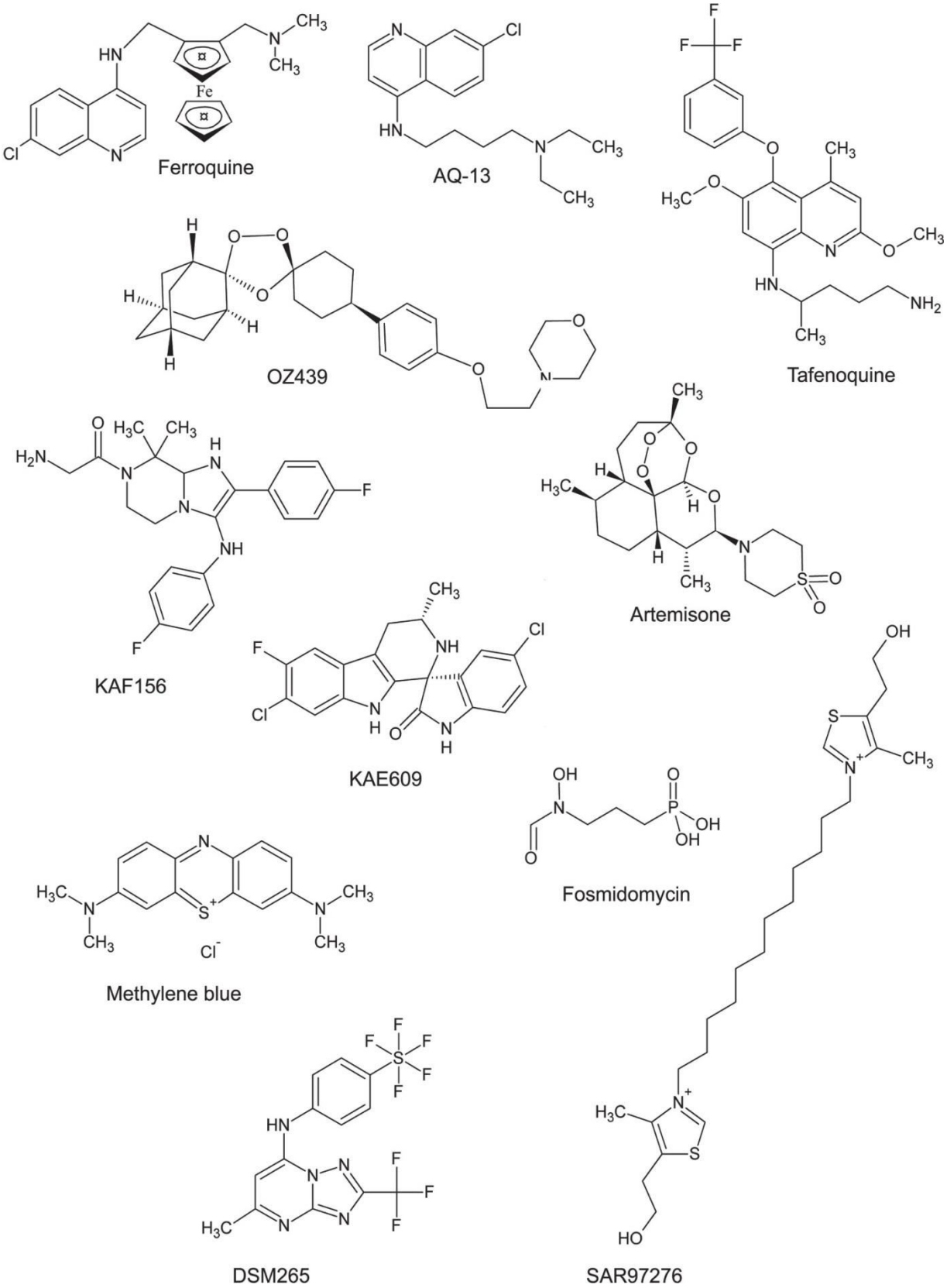

Figure 2.6. Chemical structures of compounds in clinical development (Held et al., 2014)

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2.5. Ethnopharmacology as an approach to bioprospect for antimalarial plant products

The history of people-plant interactions can be dated back to the evolution of the human race. Prior to the advent of modern science, indigenous people were largely dependent on plants for their well-being and survival (Rates, 2001). Higher plants are endowed with numerous and diverse arrays of secondary metabolites, which increase their fitness under harsh environmental conditions and against harmful microorganisms (Verpoorte, 1998). In a similar manner, their chemodiversity has always been exploited by the human race to fight off various pathogens that threatened their existence (Seigler, 1998). Alongside their cultural diversification, indigenous people acquired a wealth of information on the curative potential of plants growing within their immediate environments and this became a communally shared knowledge. The traditional knowledge was then passed on orally from generation to generation and plants continued to be the major resource of therapeutic formulations to most human beings (Cox and Balick, 1994). Studies on traditional knowledge systems continue to play a major role in the progression of ethnopharmacological research.

Ethnopharmacology is premised on the documentation of indigenous medicinal knowledge and subsequent scientific studies on the pharmacological effects of ethnomedicines (Etkin and Elisabetsky, 2005). The main goal of ethnopharmacology in ethnobotany is to bioprospect for novel plant-based chemotherapeutic agents from existing indigenous remedies (Leonti and Casu, 2013). Although ethnobotanical effectiveness is a combination of pharmacology and cultural meaning, there is still the potential for discovering novel compounds from indigenous knowledge (Reyes-Garcia, 2010). The use of an ethnomedicinal

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approach in the search for antiplasmodial scaffolds from indigenous plant species could possibly yield novel antimalarial plant products. Most of the currently effective standard antimalarial drugs have been derived from medicinal plant products or from structures modelled on plant lead compounds (Newman and Cragg, 2012). Quinine discovered from *Cinchona* bark and artemisinin isolated from *Artemisia annua* have continued to stimulate interest in bioprospecting medicinal plants as potential sources of novel antimalarial scaffolds (Klayman, 1985; McHale, 1986).

Screening plants based on ethnopharmacological data seems to increase the likelihood of finding novel compounds due to their long history of safe use and may therefore increase the prospects of finding new antiplasmodial plant leads with better pharmacokinetic profiles. This is also based on the immense anecdotal evidence of their efficacy by the indigenous people, particularly when the same herbal preparation is used by different ethnic groups with the same results (Pillay et al., 2008). In recent years, many publications have reported on the effectiveness of extracts and isolated compounds from plant species where their selection was based on an ethnopharmacological approach (Oliveira et al., 2009; Zimmermann et al., 2012; Ntie-Kang et. al., 2014). Most of these data together tend to justify the usage of an ethnopharmacological approach when bioprospecting for antimalarial plant products.

Despite the rise of combinatorial chemistry as a key technique with increased efficiency in total synthesis of new chemical entities, natural products still play a significant role as the starting materials for drug discovery (Gyllenhaal et al., 2012; Cragg and Newman, 2013). The contemporary significance of the traditional knowledge has become even more important nowadays due to the less number of drug candidates emerging from combinatorial chemistry libraries. Approximately 64%

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of the currently used drugs are naturally derived or semisynthetic derivatives of natural products (Newman and Cragg, 2012). In general, natural products exhibit higher binding affinities for specific receptor systems and their bioactivity is often selective when compared to totally synthetic compounds. The main structural differences between natural and combinatorial molecules result from the introduced properties which enhance the efficiency of combinatorial synthesis. They include the number of chiral centres, prevalence of aromatic moieties, complex ring systems, degree of saturation as well as ratios of different heteroatoms (Stahura et al., 2000). The failure of combinatorial libraries to generate preclinical drug candidates at anticipated rates has resulted in shifting focus to modification of active natural product skeletons as leads to novel agents (Szychowski et al., 2014). This may probably yield more novel compounds with desirable pharmacokinetic profiles.

Much of nature's plant biodiversity remains to be explored in search of new bioactive antiplasmodial scaffolds. To continue to be competitive and relevant with other drug discovery methods ethnopharmacological research needs to continually improve the speed of the screening, isolation and structure elucidation processes as well as addressing the suitability of screens for natural product extracts (Harvey, 2008). It is now apparent that selection, collection, extraction and biological evaluation are no longer barriers to the flow of samples through the discovery pipeline. The major impediments to drug discovery are the bioactivity-directed fractionation, isolation and structure elucidation of the biologically active principles. These steps are labour intensive, as one can only focus on a limited number of active samples at a time and they also require the expertise of a phytochemist (Cragg and Newman, 2013). Isolation of relatively few compounds and their

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subsequent screening represent the culmination of most ethnopharmacological investigation of plants.

Numerous alternative approaches are being explored in an attempt to increase the efficiency through which novel compounds can be discovered from natural products. Techniques for extraction of medicinal plants that improve the matrix effects, yield and quality of extracts with reduced operational time are becoming standards for many laboratories. They include non-conventional extraction methods such as ultrasound-assisted extraction, enzyme digestion, pulsed electric field, microwave heating, pressurized liquid and supercritical fluids (Azmir et. al., 2013). Innovations in analytical instrumentations have always paralleled advances in natural product chemistry. The advent of modern hyphenated analytical techniques such as LC-MS, GC-MS, LC-NMR, LC-FTIR, LC-NMR-MS, etc., afford enhanced speed, sensitivity and enable an active hit to be rapidly assessed (Sarker and Nahar, 2012; Wu et al., 2013). For example, LC-NMR has found application in the analysis of plant extracts, where metabolites are separated and enter the NMR instrument directly from the HPLC column so that no isolation and sample manipulation is necessary (Zimmermann et al., 2012). Tedious isolation of known compounds can be avoided and selective isolation of novel chemical entities could be undertaken.

Combining integrated techniques with on-line biochemical screening leads to simultaneous generation of both chemical and bioactivity information on natural plant products (Brusotti et al., 2014). Recent developments in these analytical tools coupled with statistical and mathematical modelling provide the basis for extraction of biological information from data with multiple variables (Patel et al., 2010). These hybrid techniques are currently being applied in analyses of crude extracts or fractions from various natural sources, as well as isolation and on-line detection of

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natural products. Their potential in chemotaxonomic studies, chemical fingerprinting, quality control of herbal products, dereplication of natural products, and metabolomic studies has been demonstrated (Sarker and Nahar, 2012). However, the high cost of these techniques and the technicality associated with their operations may preclude their broader use in routine drug discovery and development processes.

2.6. The potential of NMR-based metabolomics in bioprospecting for antimalarial plant products

Every plant species has a specific set of secondary metabolites that may differ considerably in their concentration, chemical nature and solubility, both between plant species and different parts of a plant. Drug discovery is constrained by the lack of suitable techniques that can optimize the recovery, global analysis as well as the identification of low molecular weight plant metabolites in complex matrices. Metabolomics is the detailed analysis of the entire complement of low molecular weight metabolites present within a cell, tissue or organism (Verpoorte et al., 2007). The eventual goal of metabolomics is to qualitatively and quantitatively measure all metabolites in an organism. Metabolomics is capable of generating a comprehensive profile of small metabolites and has the capacity for high-throughput analysis required for screening large numbers of samples (Schripsema, 2010). Large datasets could be produced thereby enabling the possibility to simultaneously evaluate similarities and differences among samples. Therefore, instead of analysing individual metabolites separately, a suitable approach is to have a general view of all metabolites given any metabolomic data. Global analysis of low weight metabolites in plant extracts could then allow for identification of the few chemical features

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against a large and complex background of metabolites that may possibly be associated with bioactivity (Worley and Powers, 2013).

Metabolomics with the use of NMR spectroscopy can serve as a valuable tool for rapid discovery and development of phytotherapeutics. NMR-based metabolomics has the advantage of having simple sample preparation, being fast, reproducible, non-selective and non-destructive when compared to the other techniques (Kim et al., 2010). This analytical platform has the capacity of providing global information of single or different plant samples in one analysis and can also allow for structural characterization or dereplication of individual compounds in complex plant extracts (Shyur and Yang, 2008). The technique is solely based on physical characteristics with limited experimental factors that may influence the positions of signals and is also amenable to plant extracts. Other analytical platforms which are mainly chemically based often involve analyte derivatization and variable chromatographic requirements between different plant samples thereby making reproducibility and standardization very difficult to attain (Sarker and Nahar, 2012).

However, there are clear limitations associated with NMR-based metabolomics. They include low sensitivity, overlapping signals and the representation of chemical compounds as complex signal groups (Schripsema, 2010). This could obstruct a direct observation of single chemical entities when compared to well-separated chromatographic peaks. Experimentation at high magnetic field strengths gives a new potential to overcome limitations associated with NMR spectrometers operating at lower frequencies. Recent improvements in NMR hardware achieve increased spectral dispersion, reduced coupling-associated distortion and enhanced signal-to-noise ratio (Kim et al., 2010; Sheridan et al., 2012). For complex spectra, better signal resolution may be accomplished by means

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of 2D NMR spectroscopy. NMR spectroscopy has also been shown to be inadequate for the analysis of volatiles and lipids. Nevertheless, the integration of data derived from more than one analytical platform could provide more reliable information and wider metabolite coverage on the composition of the analysed samples.

The majority of data sets these days are multivariate due to the availability of instrumentation, complexity of systems as well as the processes being investigated. NMR spectroscopy datasets are represented as complex matrices with several hundreds of proton signals in response to the diversity of secondary metabolites contained in each sample (Hendriks et al., 2011). As a result, multivariate statistical approaches are used in an attempt to derive biological meaning from the datasets. The most commonly used pattern recognition techniques are the Principal Component Analysis (PCA) and Orthogonal Projection Least Square (OPLS) (Lindon et al., 2001). PCA is an unsupervised chemometric method which forms the basis of Multivariate Data Analysis (MDA) and is used to reduce the metabolomic data into few dimensions that can be readily understood and evaluated. It reveals how the observations are naturally clustered on the basis of their variables in metabolomic data. In the absence of a distinct discrimination pattern, subsequent OPLS analyses with additional information on the datasets are used to determine similarities and differences between predetermined groups. The application of chemometric methods can provide simultaneous evaluation of the most influential variables without the identification of the metabolites (Worley and Powers, 2013). This feature allow for the selection of the most focused technique and optimization of the experimental condition for isolation and identification of bioactive compounds (Heyman et al., 2015).

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In the past decade the application of NMR spectroscopy for both targeted and global metabolomics in the analysis of complex plant extracts and traditional medicines has been well-established. Studies that highlight the potential of NMR-based metabolomics include quality control of herbal remedies, identification and quantification of compounds, detection of impurities as well as adulterants, determination of species' origin and the correlation of a plant's metabolome with biological activity (Bailey et al., 2004; Choi et al., 2004; Van der Kooy et al., 2008; Heyman and Meyer, 2012; Heyman et al., 2015). The main challenge for metabolomics is not only to elucidate unknown chemical structures, but also to develop standard protocols which remain a barrier for comparing results among laboratories and to reproducing metabolomics experiments (Alvarez-Sanchez, 2010). Given the excellent reproducibility acquired with NMR spectroscopy, the increasing use of NMR for metabolomic studies and the efforts towards the establishment public NMR data platforms, NMR-based metabolomics remains a feasible alternative approach for investigating the bioactivity of plant extracts.

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CHAPTER 3: IN VITRO SCREENING OF ETHNOPHARMACOLOGICALLY SELECTED SOUTH AFRICAN PLANT SPECIES USED AGAINST MALARIA

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3.1. Introduction

Despite the scientific developments made in contemporary medicine, vector-borne infectious diseases remain the main public health problem affecting many people in tropical and subtropical regions (Murray et al., 2012). The etiological agents responsible for the cause of Chagas disease, African trypanosomiasis, leishmaniasis and malaria are *Trypanosoma cruzi*, *T. brucei* spp., *Leishmania* spp. and *Plasmodium* spp., respectively (Osorio et al., 2008). It is estimated that tropical parasitic diseases cause over 3 million deaths annually, with malaria accounting for a large proportion of the recorded mortality (World Health Organization, 2013). Chemotherapy is still one of the fundamental measures used in the control and treatment of protozoal diseases in spite of the emergence of drug resistant strains. Additionally, some of the indispensable drugs that are still effective suffer from problems related to toxicity, prolonged treatment schedules, variable responses between strains and non-compliance by patients (Stratton et al., 2008; Olasehinde, 2012). These factors combined with the absence of effective vaccines highlight the need for new chemotherapeutic agents with novel modes of action that may possibly alleviate the burden of tropical diseases in endemic countries.

In light of the limiting factors associated with chemotherapy, the majority of people living in disease endemic areas use herbal remedies prepared from native plant species as alternatives to antiparasitic drugs (Gathwira et al., 2007). Screening plants based on ethnopharmacological approaches could increase the prospects of finding bioactive therapeutic plant leads due to the anecdotal evidence of their efficacy and a long history of safe use (Bourdy, et al., 2008). South Africa, with its rich biodiversity and cultural diversity, could serve as a resource base of therapeutic plant leads with new mechanisms of action. Studies conducted on South African

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medicinal plant species have confirmed their potential in antimalarial drug discovery (Pillay et al., 2008; De Villiers et al., 2010; Mokoka et al., 2012). In our continued search for novel antimalarial plant products (Prozesky et al., 2001; Tetyana et al., 2002; Adelekan et al., 2008), twenty indigenous plant species used to treat malaria and/or malarial symptoms by Vha-Venda people, were evaluated for their antiplasmodial activity. As part of our collaborative research with the Swiss Tropical and Public Health Institute and to evaluate their specificity for *P. falciparum*, the collected plant species were also tested against *Trypanosoma cruzi*, *T. brucei* and *Leishmania donovani*.

3.2. Materials and methods

3.2.1. Plant collection

The selection of medicinal plants investigated in this study was based on informal interviews with Vha-Venda people living in Mutale Municipality of Limpopo Province, which is one of the malaria transmission areas in South Africa (Khosa et al., 2013). An ethnobotanical survey and a chemotaxomic approach were followed to select and collect indigenous plant species used to treat malaria and its symptoms by Vha-Venda people. The data was gathered and then compared to published literature. In cases where the locally used plant species was not documented in the ethnopharmacological data, the plant was not harvested. Similarly, if plants were documented in literature and not used locally, they were not collected for this study. Plant samples from the selected twenty species (Table 3.1.) were collected at various locations in Mutale Municipality, Limpopo Province. Voucher specimens of the harvested plant species were identified and deposited at the H.G.W.J. Schweickerdt Herbarium of the University of Pretoria.

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Table 3.1. Plant species evaluated for antiprotozoal activity and their ethnomedicinal uses against malaria

Plant species	**Venda name**	**Voucher number**	***Ethnomedicinal uses**	**Collected plant part**
Albizia versicolor Welw. ex Oliv. (Fabaceae)	Mutambapfunda, muvhambangoma	120322	Root and stem bark are used as ingredients to prepare a polyherbal decoction taken against malaria	Roots and Stem bark
Anthocleista grandiflora Gilg. (Loganiaceae)	Mueneene	120323	A decoction of the stem bark and leaves is administered in cases of malaria	Stem bark and leaves
Bridelia mollis Hutch. (Phyllanthaceae)	Mukumbakumba	120324	Root infusion from a closely related plant species, *B. micrantha*, is used against malaria-related fevers	Roots
Capparis tomentosa Lam. (Capparidaceae)	Gwambadzi, Moubadali	120325	Root decoction is drunk as an antipyretic in the treatment of malaria	Roots
Clematis brachiata Thunb. (Ranunculaceae)	Tshiumbeumbe	120326	Hot root decoction is used for steaming or taken orally for malaria and colds	Roots
Clerodendrum glabrum E. Mey. (Verbenaceae)	Umnukalembeba	120327	Leaf infusion is taken as a remedy for fevers associated with malaria	Leaves
Cussonia spicata Thunb. (Araliaceae)	Musenzhe	120328	A root infusion made from a handful of roots are used as emetics for fevers	Root bark
Dichrostachys cinerea Wight et Arn. (Fabaceae)	Murenzhe	120329	Crushed roots are soaked in water and administered in cases of febrifuge	Roots
Diospyros mespiliformis Hochst. ex A.DC. (Ebenaceae)	Musuma	120330	Root decoction is used to alleviate febrile symptoms	Roots
Pappea capensis Eckl. & Zeyh. (Sapindaceae)	Tshikavhavhe, Tshikwakwashi	120331	Branches are boiled and taken as tea for malaria	Twigs

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Parinari curatellifolia Planch. Ex Benth. (Rosaceae)	Muvhula	120332	Stem bark soaked together with other plant species are used for the treatment of malaria	Stem bark
Pyrenacantha grandiflora Baill. (Icacinaceae)	Bwere, Mbengelele	120333	A decoction prepared from powdered roots is used for malaria	Roots
Rauvolfia caffra Sond. (Apocynaceae)	Munadzi	120334	Used as a substitute for *T. elegans* (of the same family) to treat malaria and fevers	Stem bark
Senna petersiana (Bolle) Lock. (Fabaceae)	Munembenembe	120335	Leaf infusion are taken as tea for malaria	Leaves
Syzygium cordatum Hochst. (Myrtaceae)	Mutu	120336	Leaf infusions administered for febrifuge and headaches related to malaria	Leaves
Tabernaemontana elegans Stapf. (Apocynaceae)	Muhatu	120337	Stem bark and root decoctions are used for febrifuge and malaria	Stem bark
Vangueria infausta Burch. subsp. *Infausta* (Rubiaceae)	Muzwilu	120338	Infusions made from the roots and leaves is taken orally to treat malaria	Roots
Ximenia americana Linn. (Olacaceae)	Muthanzwa	120339	Root infusions are taken for febrifuge and ground root powder is applied topically for febrile headaches	Roots
Ximenia caffra Sond. (Olacaceae)	Mutshili	120340	Powdered leaves and twigs are used for fevers and febrifuge	Leaves
Xylopia parviflora (A.Rich.) Benth. Oliv. (Annonaceae)	Muvhulavhusika	120341	Hot root decoctions are used as emetics for fevers	Roots

* (Watt and Breyer-Brandwijk, 1962; Arnold and Gulumamian, 1984; Mabogo, 1990; Bandeira et al., 2001)

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3.2.2. Extraction of plant samples

About 20 g of ground plant material was weighed and transferred into a glass beaker. A volume of 300 ml from each of dichloromethane (DCM) and 50% methanol was added to the plant material. The extraction solvent system, DCM: 50% methanol (1:1) was used to extract the compounds from the biosamples (Choi et al., 2004). The mixture was homogenized for 10 min using a blender, sonicated for 10 min in an Ultrasonic waterbath (Labotec) and then filtered. The filtrate was transferred to a separating funnel and yielded two layers of different polarities, which were then separated. Residual plant material was collected and the extraction procedure was repeated. Non-polar fractions were concentrated under vacuum at 30 ºC. Methanol in the polar fractions was vaporized with a rotary evaporator at 40 ºC and the resulting aqueous extracts were freeze-dried using a bench top manifold freeze dryer (Virtis). Dichloromethane and aqueous crude extracts were analysed independently. *Ximenia americana* could not separate into polar and non-polar extracts and therefore the total number of the analysed extracts was forty-three.

3.2.3. Antiprotozoal bioassays

In vitro screening for antiprotozoal activity was conducted on 43 crude plant extracts. The acquired plant extracts were tested against *Trypanosoma brucei rhodesiense* (STIB 900), *T. cruzi* (Tulahuen C4), *Leishmania donovani* (MHOM-ET-67/L82) and *Plasmodium falciparum* (NF54) strains. Each extract was dissolved in 10% DMSO to afford a stock solution with a concentration of 10 mg/ml. All tests were performed in 96-well microtiter plates. Tests were conducted in duplicates and repeated twice.

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3.2.3.1. *In vitro* antitrypanosomal assay

Trypanosoma brucei rhodesiense (STIB 900) strain was cultivated under axenic conditions following the method of Baltz et al. (1985). The growth medium, Minimum Essential Medium (MEM), consisted of 0.2 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 0.5 mM hypoxanthine and 15% heat-inactivated equestrian serum. *In vitro* antitrypanosomal assay was performed on the cultured bloodstream forms of *T. b. rhodesiense.* The Alamar Blue solution was used as an indicator in order to determine the sensitivity of trypomastigote forms to the plant extracts (Raz et al., 1997). Fluorescence was measured after incubation using a Spectramax Gemini XS microplate fluorescence reader (Molecular Devices Cooperation). The 50% inhibitory concentration (IC_{50}) values were calculated by linear interpolation from the dose inhibition curves using Softmax Pro Software (Molecular Devices Cooperation). Melarsoprol was used as a standard drug.

Amastigote forms of *Trypanosoma cruzi* (Tulahuen C4) expressing β-galactosidase (Lac Z) gene were cultured as described by Buckner et al., (1996). Seeded rat skeletal myoblasts (L-6 cells) grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS) and 2 mm L-glutamine served as the host cells for transfected parasites. After incubation with plant extracts and a positive control (benznidazole), the substrate chlorophenol red β-D-galactopyranoside (CPRG) was added to all wells. The colorimetric reaction developed was measured photometrically at 540 nm with a VersaMax microplate reader (Molecular Devices Cooperation) and IC_{50} values were calculated by linear regression from sigmoidal dose inhibition curves (Mokoka et al., 2011).

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3.2.3.2. *In vitro* antileishmanial assay

The inhibitory effects of the plant extracts against axenically grown amastigote forms of *Leishmania donovani* (MHOM-ET-67/L82) was carried out following the resazurin assay protocol (Adams et al., 2009). Amastigotes were cultivated in Schneider's medium (SM) supplemented with 10% heat-inactivated FBS under an atmosphere of 5% CO_2 in air. Plates were incubated for 48 h and resazurin solution was then added to each well in order to assess the viability of *Leishmania* parasites. The absorbance was read on a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation) with an excitation and emission wavelength of 536 nm and 588 nm, respectively. The IC_{50} values were calculated by linear regression based on dose response curves. Miltefosine was used as positive control.

3.2.3.3. *In vitro* antiplasmodial assay

In vitro activity against erythrocytic stages of *P. falciparum* was determined using a [^{3}H]hypoxanthine incorporation assay (Desjardins et al., 1979; Matile and Pink, 1990). The test organism was the chloroquine sensitive NF54 strain and the standard drug was chloroquine (Sigma C6628) (Ponnudurai et al., 1981). Dissolved plant extracts were added to parasite cultures and then incubated in RPMI 1640 medium without hypoxanthine. The medium was supplemented with HEPES (5.94 g/l), $NaHCO_3$ (2.1 g/l), neomycin (100 U/ml), AlbumaxR (5 g/l) and washed human red cells A^+ at 2.5% haematocrit (0.3% parasitaemia). Serial drug dilutions of eleven 3-fold dilution steps covering a range from 100 to 0.002 µg/ml were prepared. The 96-well plates were incubated in a humidified atmosphere at 37 °C, 4% CO_2, 3% O_2 and 93% N_2. After 48 h, 50 µl of [^{3}H]hypoxanthine was added to each well.

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The plates were incubated for a further 24 h under the same conditions and then harvested with a Betaplate™ cell harvester (Wallac). Red blood cells were transferred onto a glass fibre filters and washed with distilled water. The dried filters were inserted into a plastic foil with 10 ml of scintillation fluid and counted in a Betaplate™ liquid scintillation counter (Wallac). IC_{50} values were calculated from sigmoidal inhibition curves by linear regression using Microsoft Excel (Huber and Koella, 1993).

3.2.4. Cytotoxicity assay

Crude plant extracts were subjected to an antiproliferative bioassay in an attempt to determine their potential lethality or safe therapeutic application against mammalian cells. Assays were performed in 96-well microtiter plates, each well containing 100 µl of RPMI 1640 medium supplemented with 1% L-glutamine (200 mM), 10% fetal bovine serum and 4000 rat skeletal myoblast L6 cell line (Ahmed et al., 1994). Serial drug dilutions of eleven 3-fold dilution steps covering a range from 100 to 0.002 µg/ml were prepared. After 70 hours of incubation the plates were inspected under an inverted microscope to assure growth of the controls and sterile conditions. Alamar Blue (10 µl) was then added to each well and the plates were incubated for another 2 hours.

The plates were then read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. The IC_{50} values were calculated by linear regression from the sigmoidal dose inhibition curves using SoftmaxPro software (Molecular Devices Cooperation) (Huber and Koella, 1993). Podophyllotoxin was used as control.

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3.3. Results and discussion

In vitro antiprotozoal screening was conducted on twenty indigenous plant species used traditionally by Venda people to treat malaria and / or its symptoms. Plant extracts were tested against *Trypanosoma brucei rhodesiense* (STIB 900), *T. cruzi* (Tulahuen C4), *Leishmania donovani* (MHOM-ET-67/L82) and *Plasmodium falciparum* (NF54) strains, which are infectious parasites of human importance. Selectivity index (SI) values were calculated by dividing the IC_{50} value obtained for the L-6 rat skeletal myoblast cells by the IC_{50} value of the protozoal species under study. For the purpose of this study, a plant extract was considered to be a potential hit for drug discovery when the concentration that inhibits 50% of parasite growth (IC_{50}) was ≤ 5 µg/ml and an SI value of ≥ 10 was established. It is generally acceptable that the antiprotozoal efficacy of a given plant extract is not due to the *in vitro* cytotoxicity when the SI ≥ 10, therefore displaying selectivity against the parasitic species under investigation (Vonthron-Senecheau et al., 2003). Plant extracts that showed no significant antiprotozoal activity (IC_{50} > 5 µg/ml) under the described conditions of the cell-based assays conducted are not discussed. The IC_{50} and SI values of all the plant extracts are shown in Appendix (Table 9.1.).

Table 3.2. shows the IC_{50} values of plant extracts that exhibited high antitrypanosomal and antileishmanial activity as well as their corresponding Selective Index (SI) values. Of all the 43 plant extracts assayed, only 9% of the extracts showed high antileishmanial activity against *L. donovani* and one extract demonstrated a significant antitrypanosomal activity on *T. b. rhodesiense*. Dichloromethane root extract of *Bridelia mollis* exhibited the highest leishmanicidal activity (IC_{50} = 1.92 µg/ml; SI = 27). To our knowledge, no significant antileishmanial property of *B. mollis* has been reported and there is no substantial data documented

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on its phytochemical constituents. Nonetheless, methylene chloride leaf extract of *B. ferruginea* from Ivory Coast exhibited moderate antileishmanial activity (IC_{50} = 25 µg/ml) (Okpekon et al., 2004). Further phytochemical studies conducted on other members of the genus *Bridelia* led to the isolation as well as identification of a diverse number of polyphenols, triterpenes, glycosides and lignans, which may possibly be attributed to the observed antileishmanial activity (Ngueyem et al., 2009).

Lipophilic extracts of *Vangueria infausta* subsp. *infausta*, *Syzygium cordatum* and *Xylopia parviflora* displayed high inhibitory effects on the growth of amastigote forms *L. donovani* with IC_{50} values of 4.51, 4.95 and 5.01 µg/ml, respectively. Their SI values ranged from 10 to 13, therefore displaying selectivity of the assayed extracts against the parasitic species investigated. While phytochemical analyses of *V. infausta* subsp. *infausta* revealed the presence of terpenoids and glycosides, literature information about the inhibitory effects of the plant species and other members of the same genus on *L. donovani* is still lacking (Abeer, 2011). Detailed biochemical investigations on the antileishmanial activity of chemical constituents from *V. infausta* subsp. *infausta* are underway and may lead to identification of their antileishmanial principles.

There are no previous scientific reports that account on the significant leishmanicidal activity of *S. cordatum* and *X. parviflora*. Nevertheless, essential oils extracted from aerial parts of a sister species, *S. cumini*, showed significant antileishmanial activity when tested against *L. amozonensis* promastigotes (Dias et al., 2013). Previous chemical investigations of *X. parviflora* led to the isolation and identification of numerous bioactive components such as isoquinoline alkaloids, acetogenins, terpenes and essential oils, some of which are known to have antiprotozoal properties (Nishiyama et al., 2006). Additional chemical studies are

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needed to determine the specific secondary metabolites which could be attributed to the detected leishmanicidal efficacy of *X. parviflora* and their relative mode of action thereof. Polar root extract of *Albizia versicolor* showed a significant antitrypanosomal activity (IC_{50} = 3.45 µg/ml and SI = 12) against the *T. b. rhodesiense* strain tested. No plant extract could significantly inhibit the proliferation of the aflagellated forms of *T. cruzi* in the assay conducted.

Table 3.2. The IC_{50} values of plant extracts that displayed high antitrypanosomal and antileishmanial activity together with their cytotoxicity and selectivity index (SI) values. Data shown represent mean values of two independent experiments run in duplicate.

Plant species	[a]Extraction solvent	[b]Antitrypanosomal activity (µg/ml)	[c]Antileishmanial activity (µg/ml)	[d]Cytotoxicity (µg/ml)	[e]Selectivity Index
B. mollis	DCM	ND	1.92	51.4	27
S. cordatum	DCM	ND	4.95	65.7	13
V. infausta subsp. *infausta*	DCM	ND	4.51	45.7	10
X. parviflora	DCM	ND	5.01	51.5	10
A. versicolor	50% MeOH	3.48	ND	42.0	12
Melarsoprol		0.005			
Miltefosine			0.191		
Podophyllotoxin				0.007	

[a]Extraction solvents: DCM = Dichloromethane, 50% MeOH = Methanol: Distilled water (1:1)
[b]*Trypanosoma brucei rhodesiense* strain (STIB 900)
[c]*Leishmania donovani* strain (MHOM-ET-67/L82)
[d]Rat skeletal myoblast L6 cell line
ND: not determined

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Despite the lack of reports on the antitrypanosomal activity of *A. versicolor,* related species from the same genus are well documented for their efficacy against *T. b. rhodesiense*. Lipophilic extracts of *A. gummifera* root bark showed a remarkable *in vitro* antitrypanosomal activity (IC_{50} = 0.07 µg/ml) (Freiburghaus et al., 1996), whereas methanol extracts of *A. zygia* stem bark showed a strong activity (IC_{50} = 0.2 µg/ml) against the same parasite (Lenta et al., 2007). However, Atindehou et al., (2004) found a weak antiparasitic activity (IC_{50} = 9 µg/ml) from ethanol extract of *A. lebbeck* root bark, which is about threefold more than found in this study. The observed inconsistency at the genus level could be ascribed to the interspecific chemical variation and the different geographical locations at which the plant species were collected. The evident lack of notable antitrypanosomal and antileishmanial activities in this study may be attributed to the preference for plant species used traditionally to treat malaria and its symptoms in South Africa.

The IC_{50} and selectivity index (SI) values of plant extracts that demonstrated significant antiplasmodial activity ($IC_{50} \leq 5$ µg/ml) when tested against chloroquine-sensitive strain (NF54) of *P. falciparum* are shown in Table 3.3. Among the 10 promising antimalarial plant extracts, *Tabernaemontana elegans* was the best candidate, as both the non-polar and polar extracts from its stem bark displayed the highest antiplasmodial activity at concentrations of IC_{50} = 0.331 and IC_{50} = 0.834 µg/ml, respectively. With respective SI values of 14 and 46, these extracts were considered to be not toxic to rat myoblast L6 cells.

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Table 3.3. The IC_{50} values (µg/ml) of plant extracts that exhibited high antiplasmodial activity, their antiproliferative activity and selectivity index (SI) values. Data shown represent mean values of two independent experiments run in duplicate.

Plant species	Extraction solvent	Antiplasmodial activity (*Pf*-NF54)	Cytotoxicity (L6-cells)	Selectivity Index
A. versicolor	DCM	2.12	55.1	26
B. mollis	DCM	3.06	51.4	17
C. tomentosa	DCM	2.19	40.8	19
C. spicata	DCM	3.25	47.8	15
D. cinerea	DCM	2.10	51.6	25
R. caffra	DCM	2.13	26.9	13
T. elegans	DCM	0.331	4.68	14
T. elegans	MeOH:H_2O	0.834	38.2	46
V. infausta subsp. *infausta*	DCM	1.84	45.7	25
X. parviflora	DCM	2.19	51.5	24
Chloroquine		0.003		
Podophyllotoxin			0.007	

DCM: Dichloromethane, MeOH: Methanol, H_2O: Distilled water
Pf-NF54: *Plasmodium falciparum* NF54 strain
L6-cells: Rat skeletal myoblast L6 cell line

In addition to its antileishmanial properties, *V. infausta* subsp. *infausta* showed a high inhibitory effect (IC_{50} = 1.84 µg/ml, SI = 25) against the growth of the *P. falciparum* strain tested. The chloroquine-sensitive strain of *P. falciparum* was also susceptible to lipophilic extracts of *A. versicolor*, *Capparis tomentosa*, *Dichrostachys cinerea*, *Rauvolfia caffra* and *Xylopia parviflora* at concentrations

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ranging from 2.10 to 2.19 µg/ml. These extracts were considered to be of safe therapeutic use when evaluated on rat skeletal myoblast cells, with SI values ranging between 12 and 26. The non-polar root extracts of *B. mollis* and *Cussonia spicata* demonstrated significant *in vitro* antiplasmodial activity (IC_{50} ~3 µg/ml) and selectivity for malaria parasite with SI values of 17 and 15, respectively.

Despite the wide ethnomedicinal use of *T. elegans* as an antimalarial remedy (Bandeira et al., 2001), this is the first study to document its significant antiplasmodial activity. Studies conducted by Ramalhete et al., (2008) revealed moderate or no significant activity of polar leaf extracts from the same plant species. However, studies conducted on indole alkaloids from a closely related species, *T. sessilifolia,* showed some good antiplasmodial activity (Girardot et al., 2012), which could explain the observed bioactivity. According to Mabogo (1990) infusions made from the roots and leaves of *V. infausta* subsp. *infausta* are administered orally to treat malaria. In a related study, chloroform root bark extract from *V. infausta* subsp. *infausta* significantly inhibited two strains of *P. falciparum* at IC_{50} values of 3.8 and 4.5 µg/ml (Abosi et al., 2006). Although many compounds have been isolated from *V. infausta* subsp. *infausta* their antiplasmodial activities are not reported.

Decoctions and infusions prepared from *Albizia versicolor*, *Capparis tomentosa, Dichrostachys cinerea*, *Rauvolfia caffra* and *Xylopia parviflora* are also widely used in African Traditional Medicine for the treatment of malaria and its symptoms, among other ethnomedical uses (Mabogo, 1990; Watt and Breyer-Brandwijk, 1962). *Albizia* species are well documented for their strong antimalarial activities as demonstrated in both *in vitro* as well as *in vivo* experiments (Rukunga et al., 2007; Samoylenko et al., 2009), which are attributable to spermine alkaloids, known to retain their significant activity subsequent to fractionation. While the

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antimalarial properties observed in this genus are commonly linked to spermine alkaloids, previous isolated constituents of *A. versicolor* include kaempferol glycosides and several triterpenes (Rukunga and Waterman, 2001), no spermine alkaloids have been identified from this plant species. It is not clear as to which of these classes of compounds are responsible for the observed antimalarial activity; therefore bioassay-guided fractionation may lead to the identification of the antiplasmodial compounds.

Clarkson et al., (2004) detected a weak antiplasmodial activity (IC_{50} = 38 µg/ml) in the dichloromethane root extract of *C. tomentosa*, which is relatively low compared to the results (IC_{50} = 2.19 µg/ml) found in this study. Phytochemical studies on *Capparis* species have revealed the presence of glucosinolates, alkaloids and phenolic compounds (Tlili et al., 2011). An oxindole, 3-hydroxy-3-methyl-4-methoxyoxindole has been isolated from a South African *C. tomentosa* (Dekker et al. 1987), yet reports on its antimalarial activity are still lacking. In contrast to the findings of this study, ethanol leaf extract of *D. cinerea* from Ivory Coast showed no activity at the highest concentration (5 µg/ml) tested (Atindehou, 2004). The major phytochemical constituents of *D. cinerea* are triterpenoids (Joshi and Sharma, 1974), which may be correlated to the activity found in this study.

The IC_{50} value obtained for *R. caffra* from the current study do not support the antiplasmodial activity (IC_{50} ≥ 10 µg/ml) reported previously for the same plant species (Clarkson et al., 2004; Mokoka et al., 2011). This intraspecific disparity could be best explained by the differences in plant parts tested, the extraction procedures, as well as geographical and seasonal variation. About 32 alkaloids were isolated from the stem bark of *R. caffra*, most of which have known pharmacological significance and may therefore clarify the ethnomedical uses of the plant (Nasser

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and Court, 1984). The genus *Xylopia* comprises many species, which are widely distributed in African tropical areas and endowed with many bioactive components. Boyom et al. (2011) reported on the potency of methanol leaf and stem extracts of *X. parviflora* from Cameroon. In agreement with the results obtained in this study, these extracts showed good *in vitro* antiplasmodial activity ($IC_{50} \leq 5$ µg/ml). Previous chemical investigations of *X. parviflora* led to the isolation and identification of numerous isoquinoline alkaloids (Nishiyama et al., 2006) endowed with antiprotozoal properties (Waechter et al., 1999).

In South Africa, *B. mollis* is traditionally used as an antiparasitic against intestinal worms, among other uses, while a closely related species, *B. micrantha* is used against malaria-related fevers (Watt and Breyer-Brandwijk, 1962; Mabogo, 1990). Literature data on the biological activity and phytochemical constituents of *B. mollis* is limited. Nonetheless, a moderate antiplasmodial activity ($IC_{50} = 6.9$ µg/ml) that was twice the one established in this study was found from aqueous extracts of *B. micrantha* (Clarkson et al., 2004). Polyphenolic compounds including friedelin, taraxerone, delphinidin, gallic and ellagic acid from *B. micrantha* (Pegel and Rogers, 1968) are well-known to have analgesic and anti-inflammatory effects (Ngueyem et al., 2009). It remains to be demonstrated, as to whether these compounds have a chemotaxonomic significance that may be used to explain the bioactivity of *B. mollis*.

Given the strong ethnopharmacological association with malaria, the genus *Cussonia* has been extensively studied for its antiplasmodial properties, and the polar bark extracts of *C. spicata* were reported to have a relatively weak activity ($IC_{50} \geq 10$ µg/ml) (Tetyana et al., 2002; Clarkson et al., 2004; De Villiers et al., 2010). Results achieved in this study are consistent with those reported for other members of the same family, when extracted with non-polar solvent ($IC_{50} \leq 5$ µg/ml) (Gessler

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et al., 1994; Clarkson et al., 2004). The inhibitory effects of *C. spicata* could be due to polyacetylenes, flavonoids and triterpenoid glycosides, which have been identified in various members of the genus (Srivastava and Jain, 1989; Grishkovets et al., 2005; Senn et al., 2007).

Although some of the plant species investigated in this study were previously screened for their antiprotozoal properties, their geographical location, plant parts harvested, the extraction solvent as well as parasite strain used were not always the same, which could explain the inconsistency with some reports. It is worth noting that significant antimalarial activity was mainly found in lipophilic plant extracts, which confirm earlier reports that dichloromethane extracts have a higher antiplasmodial activity as compared to methanol and aqueous extracts (Koch et al., 2005; Irungu et al., 2007). This trend could possibly be explained by the lack of polyphenols, polysaccharides and other polar molecules, which have been shown to have less antiplasmodial activity.

Several species that were strongly associated with malaria by Vha-Venda people and which are cited in ethnobotanical literature demonstrated less antimalarial activity. Their lack of *in vitro* antiplasmodial activity does not necessarily disqualify their use in herbal antimalarials. Thus, traditional remedies that are inactive against the *Plasmodium* asexual erythrocytic stage might be active against the hepatocyte phase that starts the infection of the vertebrate host, thereby preventing infection of red blood cells and its associated clinical manifestations (Carraz et al., 2006). Extracts from *Nauclea pobeguinii* (Rubiaceae) were found to be active in both rodent models and patients, despite none of the alkaloids showing *in vitro* activity (Mesia et al., 2010). Investigations into treatments for malaria should therefore be directed at targeting the various stages of *Plasmodium* life-cycle and

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other clinical symptoms related to the disease state (Rasoanaivo et al., 2011). It is therefore necessary to consider many factors that could be acting independently or jointly before any conclusions on the efficacy of medicinal plants can be drawn.

3.4. Conclusion

The findings of this study provide a measure of credibility to the ethnomedicinal use of the investigated plant species by Vha-Venda people and to the rationale of an ethnopharmacological approach when bioprospecting medicinal plants for antiplasmodial lead compounds. The present study has also highlighted some of South African medicinal plant species whose potential pharmacological activities are still scientifically less explored and the need to identify the molecular basis of their phytotherapeutic efficacy. Further phytochemical analyses are currently underway in an attempt to fractionate, isolate and identify the active constituents in extracts that demonstrated significant bioactivity.

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CHAPTER 4: NMR-BASED METABOLOMICS OF SELECTED ANTIPLASMODIAL PLANT SPECIES

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4.1. Introduction

Higher plants have been the major foundation of many chemotherapeutic agents that are currently in clinical use. Despite the rise of combinatorial chemistry as a key technique with increased efficiency in the synthesis of new chemical entities, natural products still play a significant role as the starting materials for drug discovery (Cragg and Newman, 2013). In general, natural products exhibit higher binding affinities for specific receptor systems and their bioactivity is often selective when compared to totally synthetic compounds (Stahura et al., 2000). However, bioprospecting medicinal plants for novel scaffolds remains a major challenge given that the presence and yield of secondary compounds may be affected by many factors such as physiological variations and environmental conditions (Figueiredo et al., 2008). Furthermore, there exists a considerable infra- and interspecies variation in the biochemical diversity and physicochemical properties of secondary metabolites in plants. Metabolomics with the use of NMR spectroscopy and multivariate data analysis has the potential to serve as a valuable tool for rapid discovery of phytotherapeutics. The technique is fast, reproducible, non-selective and exclusively based on physical characteristics, which makes it more amenable to plant extracts when compared to the chemically-based platforms (Sarker and Nahar, 2012).

One major focus of most metabolomic investigations relating to medicinal plants has been the quality control of plant samples that are used for herbal preparations. With conventional methods, quality assessment of medicinal plants involves the detection and identification of specific chemical markers which are associated with the intended bioactivity. Several studies have shown the potential of NMR-based metabolomics in chemical fingerprinting of herbal preparations thereby

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implying a global overview of all the plant constituents contained in such preparations (Schaneberg et al., 2003; Van der Kooy et al., 2008; Heyman and Meyer, 2012). The technique could therefore serve as a better authentication method for quality control of medicinal plants because it has the capacity to cover and characterise a wide range of metabolites in a plant sample. Other studies have demonstrated the ability of NMR spectroscopy coupled with multivariate analysis in predicting the bioactivity of the assessed plant extracts (Bailey et al., 2004; Heyman et al., 2015). In particular, Heyman et al., (2015) showed that metabolomic analysis could be used to discriminate bioactive fractions and to further identify compounds responsible for predicted activity in complex matrices without bioassay-guided fractionation and purification steps. Very few studies have explored the potential of NMR-based metabolomics in bioprospecting plant species belonging to different families for novel chemotherapeutic agents. The main objective of this study is to assess the robustness of NMR-based metabolomics in discriminating classes of secondary compounds that are responsible for antiplasmodial activity from diverse plant species with the eventual goal of developing a rapid throughput antiplasmodial screening technique.

4.2. Materials and methods

4.2.1. Plant material

Plant samples from the selected twenty species (Table 3.1., Chapter 3) were collected at various locations in Mutale Municipality, Limpopo Province. Voucher specimens of the harvested plant species were identified and deposited at the H.G.W.J. Schweickerdt Herbarium of the University of Pretoria.

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4.2.2. Extraction of plant samples

For each plant sample, 20 g of ground material was extracted in dichloromethane:50% methanol (1:1) (Choi et al., 2004). The mixture was homogenized using a blender, sonicated in an Ultrasonic waterbath (Labotec) and then filtered. The filtrate was transferred to a separating funnel and yielded two fractions of different polarities, which were then separated. Residual plant material was collected and the extraction procedure was repeated. Non-polar fractions were concentrated under vacuum at 30 ºC. Methanol in the polar fractions was vaporized with a rotary evaporator at 40 ºC and the resulting aqueous extracts were freeze-dried using a bench top manifold freeze dryer (Virtis). Dichloromethane and aqueous crude extracts were analysed independently, as previously described.

4.2.3. ^{1}H NMR spectroscopy of plant extracts

Given the apparent lack of antiplasmodial activity by the investigated polar plant extracts (Chapter 3), the metabolomic analysis was conducted on the dichloromethane plant extracts using ^{1}H NMR spectroscopy. 1D ^{1}H NMR spectra of the DCM extracts were acquired on a Varian 600 MHz spectrometer (CSIR, Pretoria, South Africa) operating at a proton NMR frequency of 600.13 MHz. Each crude DCM extract was reconstituted in deuterated chloroform ($CDCl_3$) at a concentration of 15mg/ml, with 0.1% tetramethylsilane (TMS) as the internal frequency lock (0.00 ppm). The dissolved plant extracts (600 µl) were transferred into 5 mm NMR tubes and then analysed on the spectrometer. Each spectrum was acquired with 64 scans per sample, a spectral width of 14 ppm, and the temperature was kept constant at 25°C. Prior to statistical analyses, all ^{1}H NMR spectra were referenced to TMS, manually phased and baseline corrected (Whittaker smoother).

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4.2.4. Multivariate data analysis (MDA)

The ^{1}H NMR spectra were reduced to ASCII files using MestReNova 8.1.1 (Mestrelab Research). Normalisation was done by scaling the spectral intensities to 0.1% of TMS and the region of 0.00 – 14.00 ppm was then integrated into bins of 0.04 ppm in width, resulting in 350 variables. The ASCII files generated were then imported into Microsoft Excel 2010 for secondary variable labelling after which the files were imported into SIMCA-P 13.0.0 (Umetrics, Umeå, Sweden). The acquired data was scaled (Pareto scaling) prior to Principal Component Analysis (PCA) and Orthogonal Projections to Latent Structures–Discriminant Analysis (OPLS-DA) algorithms. Pareto scaling is commonly used to reduce the influence of intense peaks so that large and small peaks are treated with equal emphasis in the MDA (Hendriks et al., 2011). A PCA model was constructed with the aim of detecting natural clustering patterns and for the identification of outliers. Further application of OPLS-DA established a model that could be used to differentiate selective (antiplasmodial and not toxic) (SI ≥ 10) from non-selective (SI < 10) antiplasmodial classes in the dataset analysed (Chapter 3). A contribution plot was then derived from the OPLS-DA scores plot in order to determine the spectral domains that are associated with the antiplasmodial metabolites of the analysed plant extracts.

4.3. Results and discussion

NMR-based metabolomic analysis was conducted on twenty one dichloromethane extracts obtained from plant species that are traditionally used against malaria (Chapter 3). The acquired ^{1}H NMR spectra were then subjected to Principal Component Analysis (PCA) in an attempt to investigate whether the descriptors are capable of separating the training set according to the observed

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antiplasmodial activity (Chapter 3). Sample *A03* (*Ximenia americana*) was excluded from dataset as it could not separate into non-polar and polar fractions and was therefore not completely soluble in $CDCl_3$. Two significant outliers were identified based on the application of Distance to Model X (DMod[X]), Hotelling's T2Range Plots (T2Range) as well as visual observations of the PCA scores plot. Outlier points *A04* (*Diospyros mespiliformis*) and *A19* (*Senna pertisiana*) were investigated and then excluded from the dataset (Figure 4.1.). The resulting PCA scores plot could not discriminate the analysed plant extracts according to the detected antiplasmodial bioactivity (Figure 4.2.). The observed lack of an apparent PCA clustering pattern could be attributed to the biochemical variation inherent in the analysed plant samples. The plant samples were harvested from sixteen different plant families which are characterized by diverse classes of secondary metabolites.

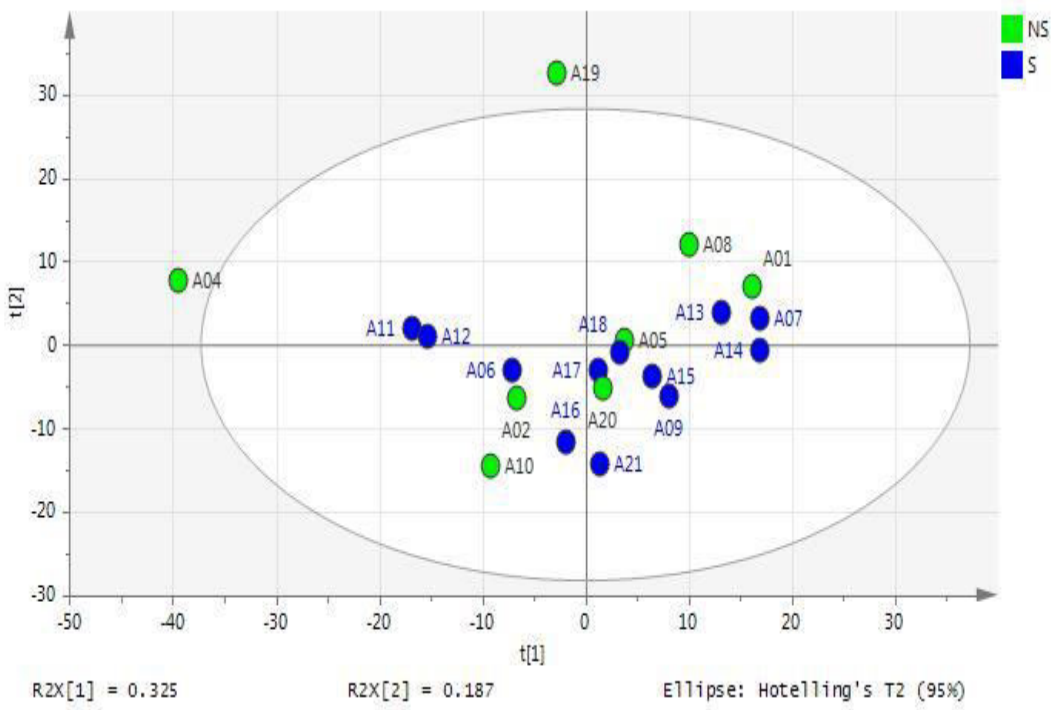

Figure 4.1. Principal component analysis scores plot of dichloromethane extracts showing outliers *A04* and *A19*. (S- selective and NS – not selective)

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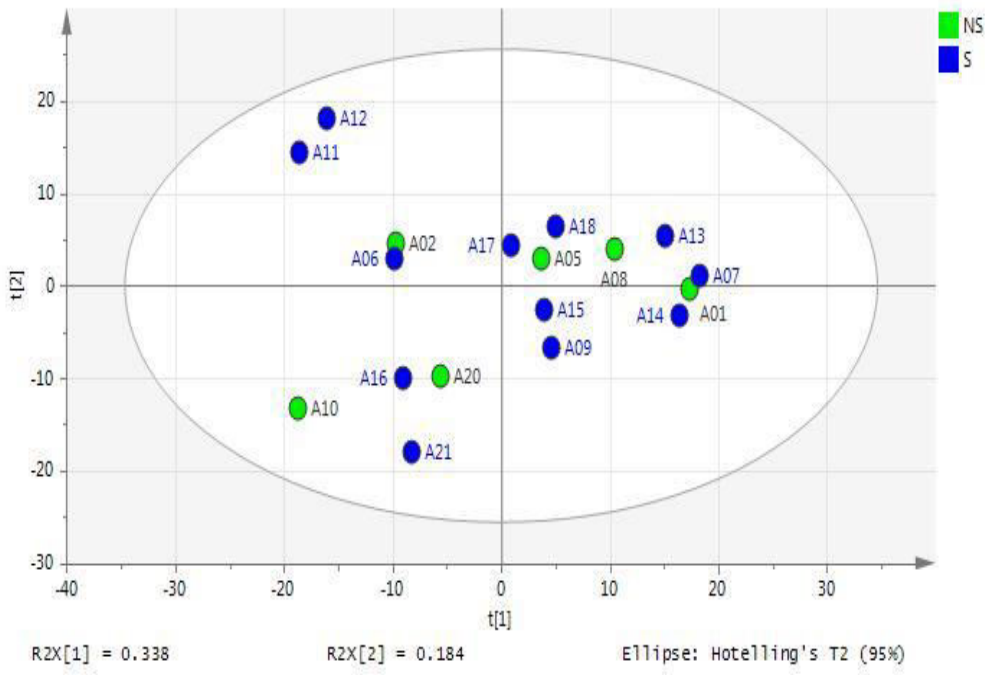

Figure 4.2. Principal component analysis scores plot of dichloromethane extracts after removing the outliers. (S- selective and NS – not selective)

Therefore, the observed antiplasmodial activity of the analysed plant extracts could be due to many chemically unrelated secondary compounds. It could also mean that most of the variability between the samples groups is due to inconsistencies in factors other than the biochemical diversity. The effects of factors such as geographical characteristics, climatic conditions, developmental stages and the plant parts harvested could be best controlled by increasing the biological replications in these types of investigations, which may be extremely costly. Owing to the lack of an inherent clustering by the PCA algorithm, a supervised OPLS-DA was then applied to the ^{1}H NMR data in an attempt to enhance the poor clustering obtained. The OPLS-DA scatter plot showed a clear discrimination between the selective (antiplasmodial and not toxic) and non-selective classes by principal component (PC) 1 (t[1]) while PC 2 (t[2]) indicated the variation of secondary metabolites within class (Figure 4.3.).

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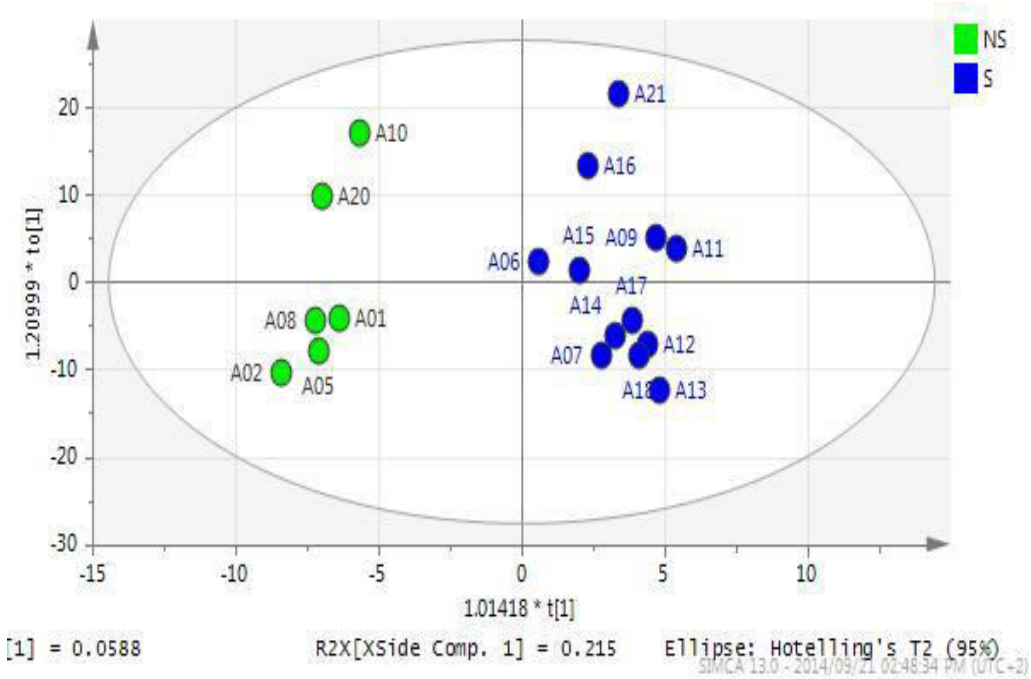

Figure 4.3. Score plot of OPLS-DA model based on the selective index of non-polar extracts classified into two groups: S- selective and NS – not selective (Observations: *N* = 18, Variables: *K* = 352 (*X* = 350, *Y* = 2))

The robustness of a model generated by the OPLS-DA algorithm is most commonly interpreted by means of R^2 and Q^2 values calculated by the supervised correlation with the secondary variable. The value of the cross-validated variance (R^2) indicates the amount of data explained by the model and gives a general overview of the fitness of the model whereas the variance (Q^2) value is a measure of the predictability of the model (Eriksson et al., 2006). The OPLS-DA model generated in this study was successful in discriminating selective from non-selective sample classes (R^2 = 0.95). However, the low value (Q^2 =0.09) calculated for the OPLS-DA model derived from the dataset showed that the model generated is not robust enough to predict the antiplasmodial activity of unknown samples (Bailey et al., 2004). Models that explain the data well and are not overfitted usually have R^2 and Q^2 values of ~0.5 and the disparate values between R^2 and Q^2 (R^2 >> Q^2)

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indicate that model is overfitting in supervised analyses (Hendriks et al., 2011). This observation is often encountered as a result of the nature of the datasets in MDA where the number of observations is significantly different from the total number of variables.

Ideally one aims to further interpret the OPLS-DA scoring plot in terms of the original variables used in the analysis, which in this study are represented by chemical shifts (ppm). To find out precisely which spectral regions of the ^{1}H NMR spectra are responsible for the observed separation between selective and non-selective plant extracts, a contribution plot was derived from the OPLS-DA scoring plot (Figure 4.4.). The chemical shifts of interest could then be correlated to classes of secondary metabolites found within the plant kingdom. The most prominent bars were tentatively identified as aliphatic (0.4 – 1.44 ppm), sugar-based (3.63 – 4.00 ppm) and aromatic (6.60 – 7.40 ppm) secondary metabolites however, their signals could not be precisely identified due to the complexity of the metabolite profile. Compounds in the aliphatic region appear to have played a significant role in this discrimination.

Although the major bars are significantly correlated to the observed antiplasmodial activity, the importance of the minor bars should not be overlooked as some secondary metabolites are restricted to certain plant families or genera and also accumulate in relatively trace amounts. Weaker signals possessing a discriminatory power could provide novel antiplasmodial scaffolds and should therefore be investigated in further characterization studies.

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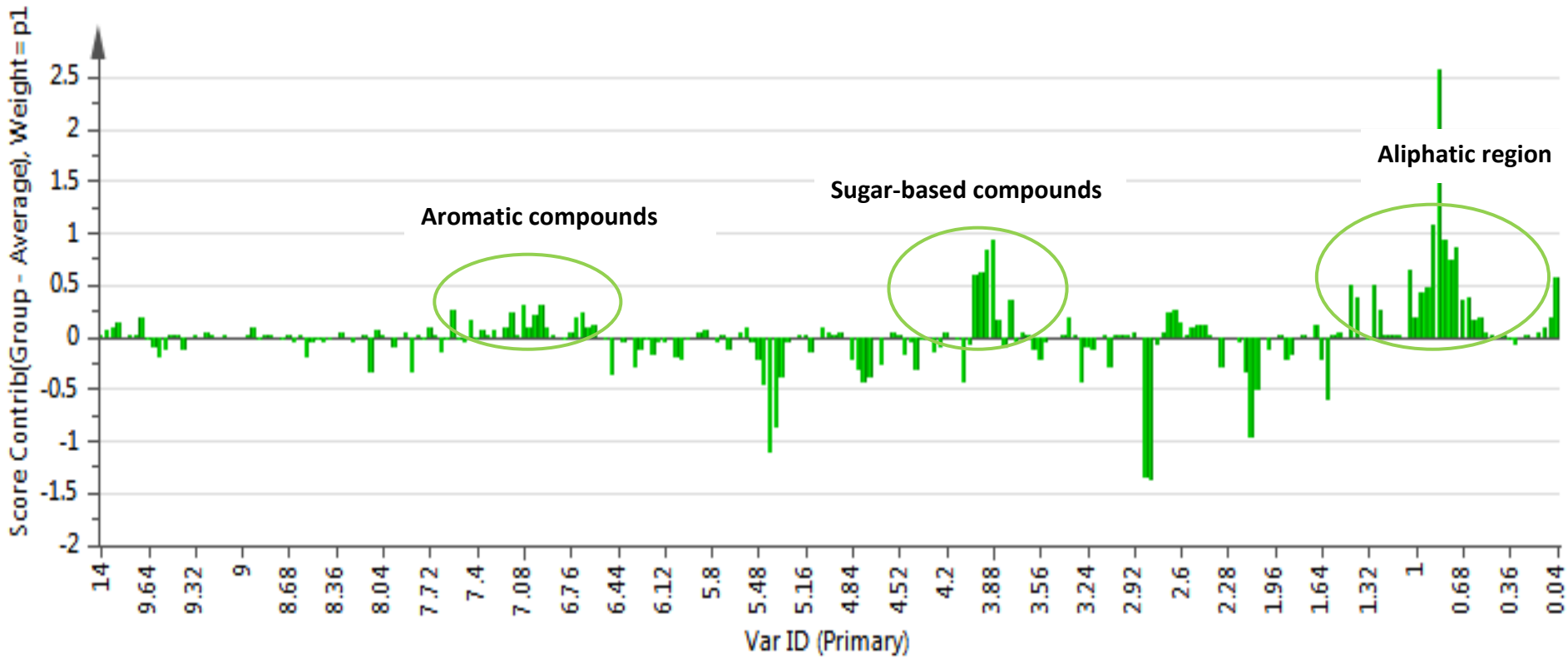

Figure 4.4. Contribution plot of non-polar plant extracts showing the comparison between selective and non-selective clusters.

(Selective – bars projecting upwards and Non-selective – bars projecting downwards)

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Two spectra of the most active plant extracts, *Tabernaemontana elegans* and *Vangueria infausta* subsp. *infausta*, were overlaid on the contribution plot in an attempt to visually assess their respective qualitative correlation to the global active profile (Figure 4.5.). The spectrum of *T. elegans* showed some prominent peaks in the aliphatic and the aromatic regions whereas major peaks of *V. infausta* subsp. *infausta* corresponded to aliphatic as well as sugar-based regions of the active profile. Indole alkaloids and terpenoids compounds have been previously isolated from *T. elegans*, which could explain the observation (Van Beek et al., 1984). Phytochemical analysis of *V. infausta* subsp. *infausta* indicated the presence of terpenoids and glycosides, which fit the profile (Abeer, 2011). Isolation and identification of antiplasmodial compounds from the active plant extracts could be used to further substantiate the bearing of the global active profile.

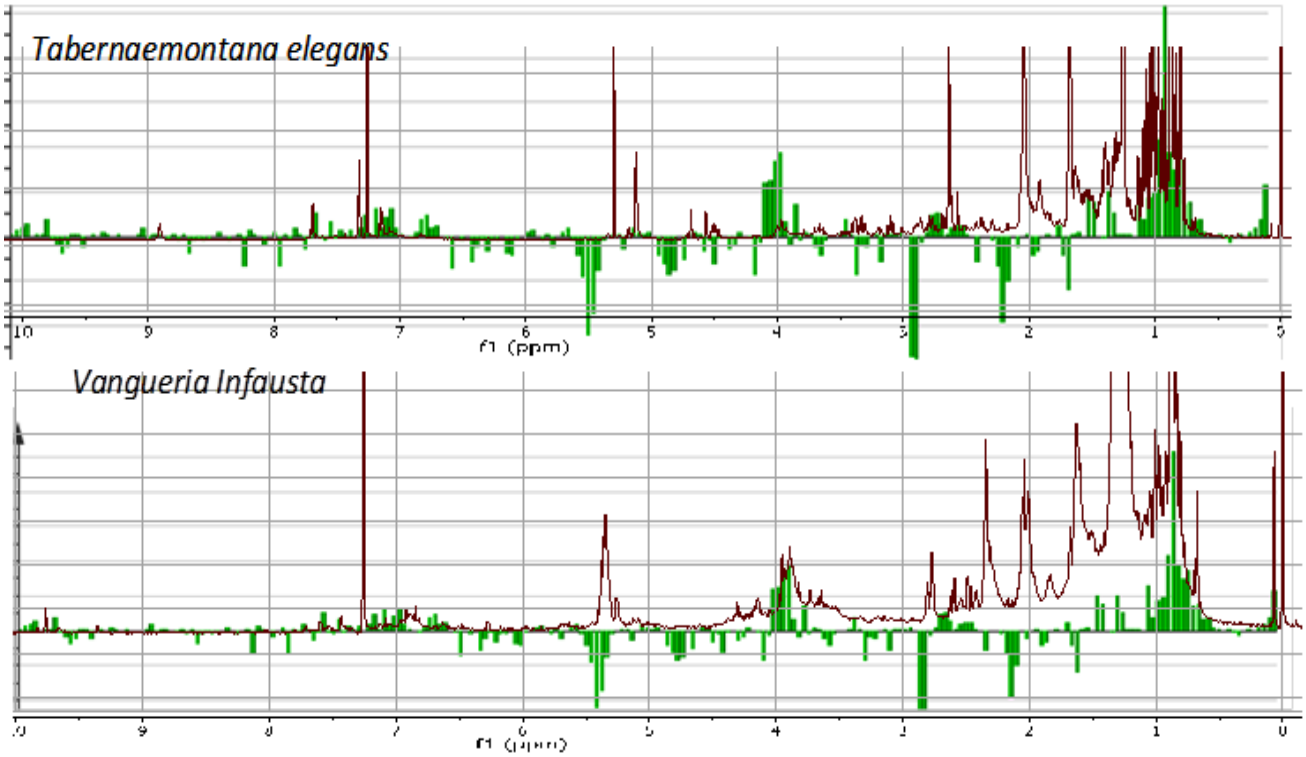

Figure 4.5. ^{1}H NMR spectra of *T. elegans* and *V. infausta* when compared to the active profile of the contribution plot

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4.4. Conclusion

The study illustrated the potential of NMR-based metabolomics in discriminating classes of compounds that may be attributed to antiplasmodial activity. Although the model generated lacked sufficient power to predict the antimalarial efficacy of unknown samples it yielded a global active profile for the plants investigated. Further studies should be carried out on a larger sample size and must also include a biological replication in a blind, new dataset for cross validation of the model. More efforts should also be directed at the identification all the metabolites responsible for the separation of samples.

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CHAPTER 5: INDOLE ALKALOIDS FROM TABERNAEMONTANA ELEGANS (STAPF.) WITH ANTIPLASMODIAL ACTIVITY

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5.1. Introduction

The genus *Tabernaemontana* (Apocynaceae) comprises of approximately 100 species, which are widely distributed throughout the tropical regions of the world. There are about 18 species occurring in Africa, of which only two, *Tabernaemontana elegans* (Stapf.) and *T. ventricosa* are indigenous to South Africa (Zhu et al., 1990). *Tabernaemontana elegans*, syn. *Conopharyngia elegans* (Stapf.) (Figure 5.1.), occurs as a deciduous tree, growing in bushveld and coastal forest, often along river edges at low altitudes (Van Wyk and Van Wyk, 1997; Coates Palgrave and Coates Palgrave, 2002).

Figure 5.1. *Tabernaemontana elegans* (Stapf.) (Apocynaceae) (http://www.ispotnature.org/)

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Southern African natives gather *T. elegans* for a wide variety of ethnomedicinal purposes including cancer, tuberculosis, stomachache, infertility and some venereal diseases (Watt and Breyer-Brandwijk, 1962; Arnold and Gulumian, 1984). In Zimbabwe, a root decoction prepared from *T. elegans* is taken orally as an aphrodisiac (Neuwinger, 1966). Its latex is used as a styptic whereas the roots are used for pulmonary complaints by the Tonga (Watt and Breyer-Brandwijk, 1962). Stem bark of *T. elegans* is commonly used in Mozambique for the treatment of malaria, while the root bark of a closely related species, *T. ventricosa*, is used as a febrifuge (Pooley, 1998; Bandeira, 2001).

Plant species belonging to the genus *Tabernaemontana* are known to synthesize and accumulate indole alkaloids of unusual skeletal types, which are endowed with novel bioactivities (Van Beek et al., 1984; Kam et al., 2003) (Figure 5.2.). Monomeric indole alkaloids of the corynanthean and ibogan types as well as bisindole alkaloids belonging to the corynanthean-ibogan class have previously been isolated from *T. elegans* (Gabetta et al., 1975; Bombardelli et al., 1976; Danieli et al., 1980; Van der Heijden et al., 1986). These indole alkaloids have displayed many pharmacological activities such as antibacterial, antileishmanial, antitumoral as well as showing hypoglycaemic, analgesic and cardiotonic properties (Van Beek et. al., 1984; Delorenzi et. al., 2001, Mansoor et. al., 2009). Published literature on the antiplasmodial activity of chemical constituents from *T. elegans* is still lacking.

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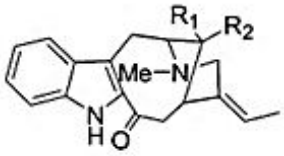
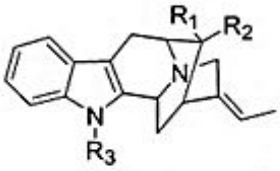
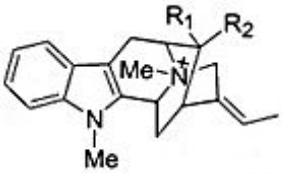


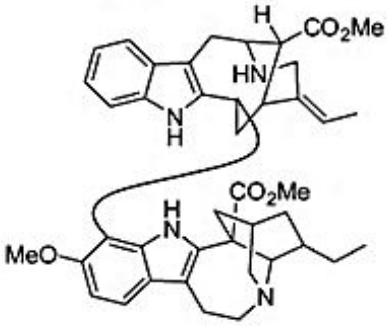
	R_1	R_2
(1) Affinine	CH_2OH	H
(7) Hystrixnine	H	CH_2OMe
(20) Vobasine	CO_2Me	H



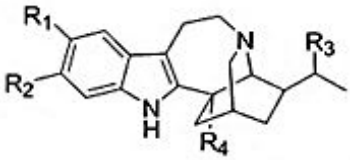
	R_1	R_2	R_3
(2) Affinisine	H	CH_2OH	Me
(18) Peryclyclivine	CO_2Me	H	H
(23) Voachalotine	CH_2OH	CO_2Me	Me



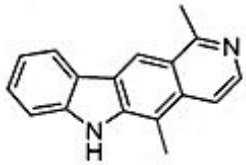
	R_1	R_2
(3) N_b-Methyl affinisine	H	CH_2OH
(16) N_b-Methyl voachalotine	CH_2OH	CO_2Me
(19) Normacusine B	H	CH_2OH



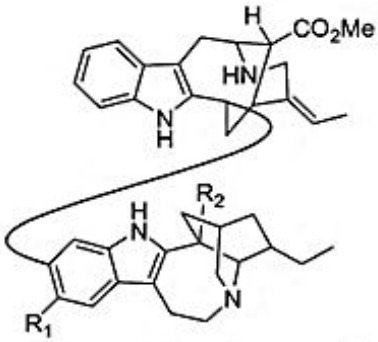
(4) Conodurine



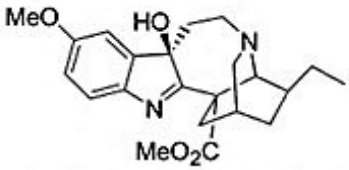
	R_1	R_2	R_3	R_4
(5) Coronaridine	H	H	H	CO_2Me
(6) Heyneanine	H	H	β-OH	CO_2Me
(8) Ibogamine	H	H	H	H
(9) Iboxygaine	OMe	H	β-OH	H
(10) Isovoacangine	H	OMe	H	CO_2Me
(11) 19-*epi*-Isovoacristine	H	OMe	α-OH	CO_2Me
(14) Voacangine	OMe	H	H	CO_2Me
(22) Conopharyngine	OMe	OMe	H	CO_2Me



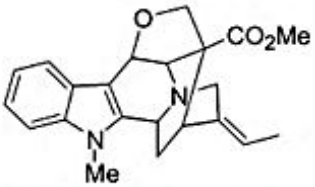
(12) Olivacine



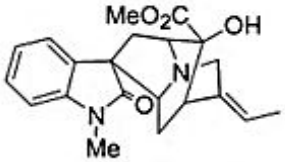
	R_1	R_2
(13) Voacamine	OMe	CO_2Me
(17) Tabernamine	H	H



(15) Voacangine hydroxyindolenine



(21) Dehydrovoachalotine



(24) Voachalotine oxindole

Figure 5.2. Alkaloids from *Tabernaemontana* species (Vieira et al., 2008)

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In our preliminary *in vitro* antiplasmodial screening of plant species used against malaria, the dichloromethane extract from the bark of *T. elegans* showed a high selective antiplasmodial activity (IC_{50} = 0.331 µg/ml) (Bapela et. al., 2014). This prompted further phytochemical investigation of the extracts in an attempt to identify the compounds responsible for the observed antiplasmodial activity. Additionally, given the documented bioactivity of indole alkaloids from Apocynaceae family, and as indicated by the fitness of the NMR spectrum of *T. elegans* to the active profile shown by the metabolomics study (Chapter 4), the present study targeted alkaloids. This chapter reports on the isolation of major indole alkaloids from the stem bark of *T. elegans*, their antiplasmodial activity as well as their cytotoxic properties.

5.2. Materials and methods

5.2.1. Plant material

Stem bark of *Tabernaemontana elegans* (Stapf.) was collected in Mutale Municipality, Limpopo Province of South Africa. An authenticated voucher specimen (120337) was deposited at the H.G.W.J. Schweickerdt Herbarium of the University of Pretoria.

5.2.2. Extraction and isolation

Ground stem bark (1 kg) of *T. elegans* was extracted in dichloromethane (DCM):50% methanol (MeOH) (1:1) solvent system, separated into polar and non-polar fractions and dried under vacuum (Choi et. al., 2004). The recovered DCM (37 g) extract was partitioned between DCM and 1% aqueous hydrochloric acid (HCl) yielding separate DCM and aqueous layers. An aqueous (acidic) layer was collected,

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basified to pH 9 with a solution of 10% NaOH and then extracted in DCM, resulting in a two-phase mixture, the neutral and the basic components. The basic / organic layer was collected and concentrated *in vacuo* to yield a dry crude alkaloidal fraction (3.64 g). For ^{1}H NMR analysis, 15 mg of the residual DCM extract and the tertiary alkaloidal fraction were dissolved in deuterated chloroform ($CDCl_3$) and then analysed on a Varian 200 MHz spectrometer.

The crude alkaloidal fraction was then subjected to column chromatography (CC) on silica gel, with hexane:ethyl acetate:methanol as an eluent mixture starting at 7:3:0.1 with increasing polarity up to 6.75:3:1.6 (approx. 2000 ml of each polarity step). Eluates were combined into 11 fractions (I – XI) based on qualitative thin layer chromatography (TLC) analysis. Compounds **2** (42 mg) and **3** (106 mg) were respectively recovered from fractions III and VI through recrystallization using the solvents MeOH and $CHCl_3$ (chloroform). Fractions II and IX were further chromatographed on Sephadex LH-20 (Sigma, USA) gel eluting with DCM:MeOH (1:1) to yield compound **1** (30 mg) and **4** (20 mg) respectively. All the four isolated compounds were reconstituted in deuterated chloroform ($CDCl_3$) and subjected to NMR analysis.

5.2.3. *In vitro* antiplasmodial and cytotoxicity screening

The residual DCM extract and the tertiary alkaloidal fraction were also subjected to an *in vitro* antiplasmodial screening in an effort to locate the bioactivity observed during the preliminary experiments (Chapter 3). Antiproliferative activity of the two fractions was also determined. The protocols followed were the same as those that are comprehensively detailed in Chapter 3 (3.2.3.3. and 3.2.3.4).

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5.3. Results and discussion

Stem bark of *Tabernaemontana elegans* (Stapf.) was extracted in dichloromethane (DCM):50% methanol (MeOH) (1:1), recovered and analysed separately. Recovered DCM extract of *T. elegans* (Stapf.) was then subjected to an acid-base extraction and yielded two fractions, the residual DCM extract and the tertiary alkaloidal fraction. The two fractions were then subjected to ^{1}H NMR analysis in an attempt to predict their antiplasmodial activity. Both fractions fitted well with the contribution plot profile that was previously generated during the metabolomics experiments (Figure 4.4.). The alkaloidal fraction showed major peaks that correspond to amines and aromatic rings. Peaks were also observed in the aliphatic region of the spectrum which may be attributed to terpenoids. The neutral fraction displayed prominent peaks mainly in the aliphatic region and could indicate the presence terpenes (Figure 5.2.).

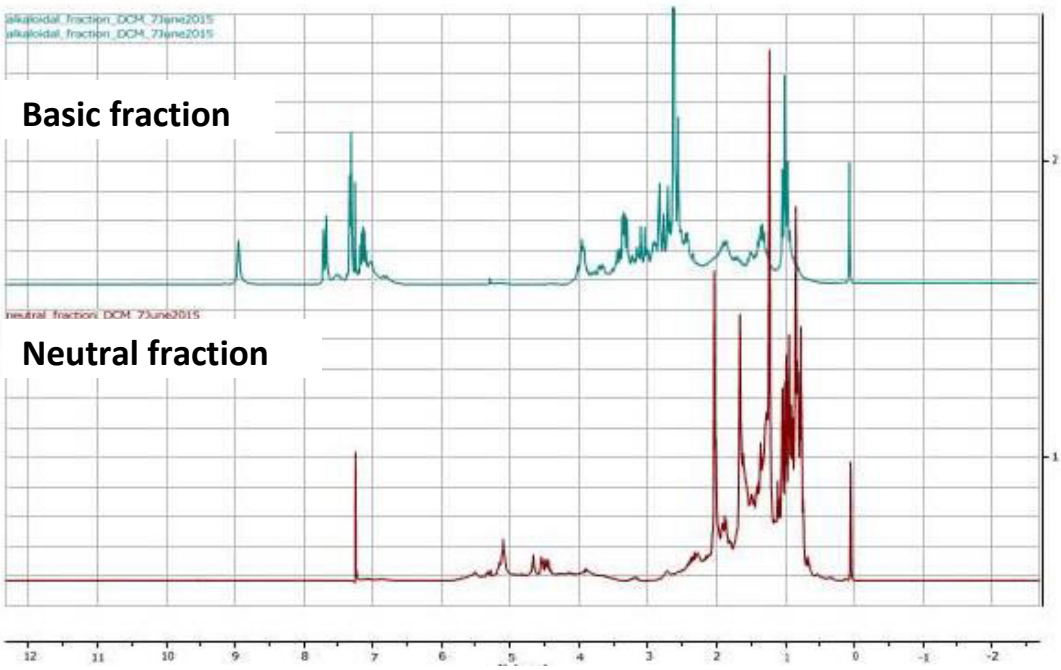

Figure 5.3. Stacked ^{1}H NMR spectra of the basic and neutral fractions

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The antiplasmodial activity and the cytotoxic effects of the two fractions are displayed in Table 5.1. As predicted, the alkaloidal fraction demonstrated significant antiplasmodial activity when compared to the neutral fraction. The observed bioactivity is attributable to the indole alkaloids, which are concentrated in the basic fraction. In an attempt to identify these indole alkaloids and to determine their respective antiplasmodial activities, the basic fraction was then subjected to column chromatography.

Table 5.1. *In vitro* antiplasmodial and cytotoxic activities of the basic and neutral fractions

	[a]Antiplasmodial activity (IC_{50}) µg/ml	[b]Cytotoxicity (IC_{50}) µg/ml	Selectivity Index (SI)
Basic fraction	0.06	2.87	47.8
Neutral fraction	6.05	25.1	4.1
Chloroquine	0.002	ND	ND
Podophyllotoxin	ND	0.005	ND

[a]Chloroquine resistant *Plasmodium falciparum* NF54 strain
[b]Rat skeletal muscle L-6 cell line

Further fractionation and purification of the alkaloidal fraction led to the isolation of four compounds (***1 - 4***) as shown on Figure 5.3. The ^{1}H NMR spectra of the isolated compounds shared many spectral features and displayed a biogenetic correlation to indole alkaloids (Figure 5.3.). Structural elucidation and configuration of the isolated compounds were based on the acquired spectroscopic data and facilitated by comparison with published ^{1}H and ^{13}C NMR data of the compounds (Ahond et. al., 1976; Van der Heijden et. al., 1986). Compounds ***2*** and ***3*** were

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identified as dregamine and tabernaemontanine, respectively (Appendix, Table 9.2. and 9.3.). The two corynanthean indole alkaloids are epimers differing only at one stereogenic centre (position 20), where the hydrogen atom is in the α position on tabernaemontanine and in the β position for dregamine (Figure 5.4). Compound ***1*** and ***4*** were not identified given that the spectra were acquired on a Varian 200 MHz spectrometer and therefore poorly resolved in the crowded spectral regions. Further studies on compounds ***1*** and ***4*** may include among others, measurements on a 600 MHz spectrometer and assignments of their ^{1}H and ^{13}C NMR spectra using two-dimensional experiments such as COSY, HSQC, HMBC and NOESY.

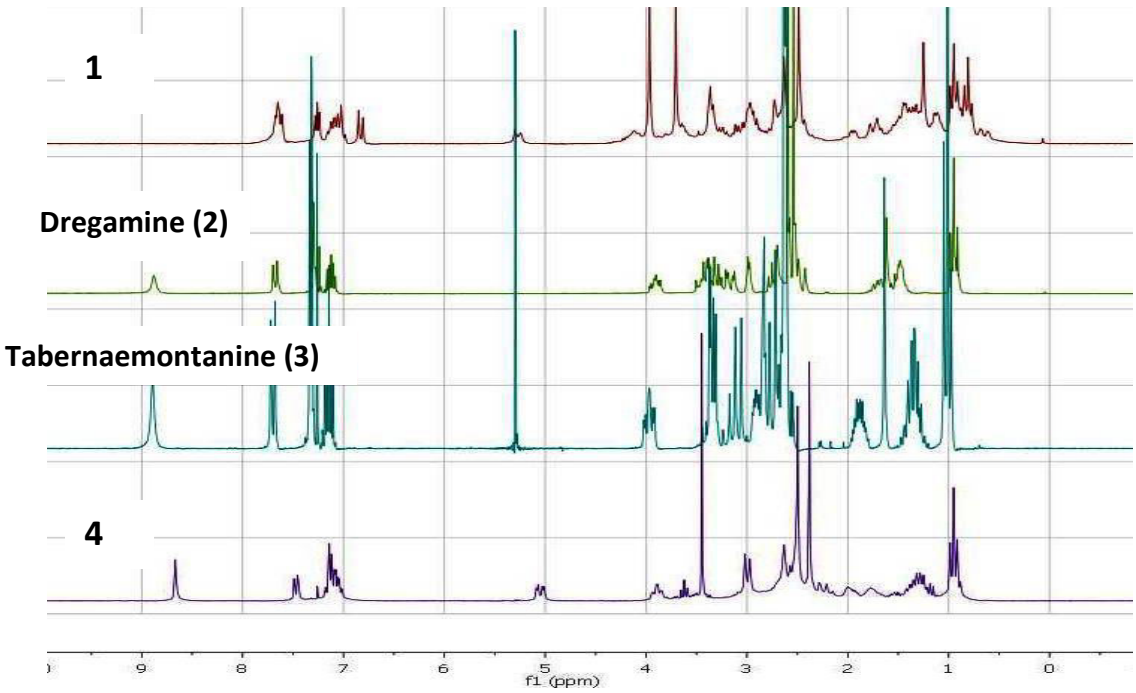

Figure 5.4. Stacked ^{1}H NMR spectra of the isolated indole alkaloids

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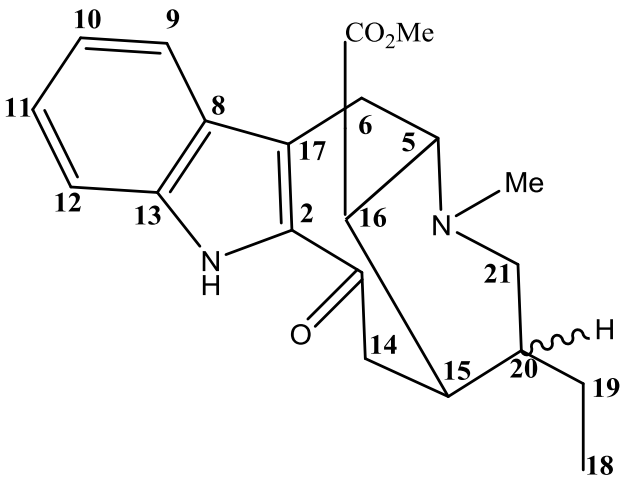

2. 20β-H **3.** 20α-H

Figure 5.5. Molecular structures of dregamine (**2**) and tabernaemontanine (**3**) isolated from *Tabernaemontana elegans* (Girardot et. al., (2012)

Chemotaxonomically significant secondary metabolites of *T. elegans* are the corynanthean monomeric indole alkaloids dregamine, tabernaemontanine and vobasine as well as the bisindole alkaloids conoduramine and tabernaelegantines (corynanthean-ibogan class) (Gabetta et al., 1975; Bombardelli et al., 1976; Danieli et al., 1980; Van der Heijden et al., 1986). There is no published data on the antiplasmodial activity of indole alkaloids isolated from *T. elegans*. Nevertheless, studies conducted by Girardot et al., (2012) on indole alkaloids isolated from *Muntafara sessilifolia* demonstrated significant antiplasmodial activities by dregamine and tabernaemontanine when tested against the chloroquine-resistant strain of *P. falciparum* (FcB1) (Table 5.2.). Tabernaemontanine exhibited a relatively higher selectivity index (SI = 8.4) compared to its isomer and may therefore be a potential antiplasmodial drug candidate. The antiplasmodial activity of dregamine (SI = 3.1) could be due to the compound exhibiting higher cytotoxicity at lower concentrations. The stereochemistry at C-20 seems to possess an effect on the antiplasmodial activity of these two acyl indole alkaloids.

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Table 5.2. *In vitro* antiplasmodial and cytotoxic activities of dregamine and tabernaemontanine (Girardot et al., 2012)

	[a]Antiplasmodial activity (IC_{50}) µM	[b]Cytotoxicity (IC_{50}) µM	Selectivity Index (SI)
Dregamine	62.0±2.4	195.8±8.6	3.1
Tabernaemontanine	12.0±0.8	100.7±9.1	8.4
Chloroquine	0.097±0.002	20.1±0.1	207

[a]Chloroquine resistant strain FcB1 of *Plasmodium falciparum*
[b]Rat skeletal muscle L-6 cell line

5.4. Conclusion

In this study, four indole alkaloids were isolated and only two could be absolutely configured. The antiplasmodial activity of the identified acyl indole alkaloids was previously confirmed and may be used to partially validate the traditional use of *T. elegans* for the treatment of malaria. Further studies should be aimed at the derivatization of the indole alkaloids and the elucidation of their modes of action.

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CHAPTER 6: ANTIMALARIAL CONSTITUENTS ISOLATED FROM VANGUERIA INFAUSTA (RUBIACEAE) SUBSPECIES INFAUSTA

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6.1. Introduction

Vangueria infausta subsp. *infausta* Burch. (Rubiaceae) grows as a shrub to small deciduous tree, often multistemmed and commonly found in wooded grassland, bushveld as well as coastal forest (Van Wyk and Van Wyk, 1997). The plant species is widely distributed in southern Africa and also native in East Africa as well as Madagascar (Coates Palgrave and Coates Palgrave, 2002). *V. infausta* is believed to possess evil powers and that it could cause cattle to bear only male offspring (Watt and Breyer-Brandwijk, 1962; Hutchings et. al., 1996). Despite this, different parts of *V. infausta* subsp. *infausta* are extensively used by southern African people in cases of pneumonia, chest complications, dental pain, menstrual troubles, genital swelling, among other ethnomedicinal uses (Watt and Breyer-Brandwijk, 1962; Arnold and Gulumian, 1984). Venda people use powdered or boiled root bark in a polyherbal mixture administered to enhance fertility in women while stems are traditionally used as protective charms in homestead fences (Mabogo, 1990). Infusions prepared from the roots and leaves of *V. infausta* subsp. *infausta* are administered orally to treat malaria (Pooley, 1998; Mabogo, 1990).

The ethnopharmacological significance of treating malaria with *V. infausta* subsp. *infausta* has been partly validated in many studies but the chemical constituents responsible for the bioactivity are still not documented. Previous studies conducted on aqueous leaf and dichloromethane:methanol (1:1) fruit extracts from *V. infausta* demonstrated moderate antiplasmodial activity with IC_{50} values ranging from 10 to 23 µg/ml (Nundkumar and Ojewole, 2002; Clarkson et. al., 2004). In another study, chloroform root bark extract from *V. infausta* subsp. *infausta* significantly inhibited two strains of *P. falciparum* at IC_{50} of 3.8 and 4.5 µg/ml (Abosi et. al., 2006).

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Figure 6.1. *Vangueria infausta* subsp. *infausta* Burch. (Rubiaceae) (www.thegardenlady.org)

When subjected to *in vivo* tests, the same extract displayed a parasite suppression of 73.5% and a repository effect of 88.7% against *P. berghei* in mice. Subsequent phytochemical screening of the root chloroform extract revealed the presence of flavonoids, coumarins, tannins, terpenoids, anthraquinones and saponins. Further phytochemical studies on this plant reported on the isolation of flavonoids, their glycosides and polyketides (Mbukwa et. al., 2007; Abeer, 2011). Although many compounds have been isolated *from V. infausta* subsp. *infausta* their antiplasmodial activities are not reported. In our initial study, dichloromethane root extract of *V. infausta* subsp. *infausta* showed a marked inhibitory effect (IC_{50} = 1.84 µg/ml, SI = 25) against the growth of the intraerythrocytic forms of the *P. falciparum*

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strain tested (Chapter 3). These findings as well as the results obtained in the metabolomics study compelled further phytochemical studies on the DCM extract. This chapter reports on the fractionation and isolation of antiplasmodial compounds of dichloromethane root extract of *V. infausta* subsp. *infausta*.

6.2. Materials and methods

6.2.1. Plant material

Roots of *Vangueria infausta* Burch. subsp. *infausta* were collected in Mutale Municipality, Limpopo Province of South Africa. An authenticated voucher specimen (120338) was deposited at the H.G.W.J. Schweickerdt Herbarium of the University of Pretoria.

6.2.2. Plant extraction

Ground roots (3.00 kg) of *V. infausta* subsp. *infausta* were extracted in dichloromethane (DCM):50% methanol (MeOH) (1:1) solvent system (Choi et al., 2004). The aqueous and organic extracts were collected and dried separately, as previously reported.

6.2.3. Fractionation and isolation of compounds

Thin layer chromatography (TLC) was used to develop a method for the fractionation of the DCM extract. Concentrated DCM extract (3 g) was subjected to silica column chromatography (CC) eluting with hexane:ethyl acetate (EtOAc) (98:2 – 9:1), DCM: MeOH (98:2 – 96:4) and EtOAc:MeOH: H_2O (94:4:2 – 60:25:15) to yield 16 fractions (I – XVI). *In vitro* antiplasmodial screening was then conducted on the fractionated extracts. Bioactive fractions were subjected to column

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chromatography in an attempt to isolate antiplasmodial compounds. Fraction III was chromatographed on silica eluting with hexane:ethyl acetate (9:1) with increasing polarity to yield compound ***1*** (7 mg) through recrystallization. Compound ***2*** (4 mg) was isolated from fraction VIII through rechromatography of partially resolved fractions. Fraction XI was chromatographed on silica with ethyl acetate: methanol: water (90:7:3) as the mobile phase and yielded compound ***3*** (5 mg), which could not be identified and is still under investigation. ^{1}H NMR and ^{13}C NMR spectra of the isolated compounds were recorded in $CDCl_3$ or CD_3OD on a Bruker 400 MHz spectrometer. The configuration of the isolated compounds was based on comparison of the acquired spectroscopic data with published ^{1}H NMR and ^{13}C NMR spectral data of identified compounds (Appendix, Figures 9.5 – 9.14).

6.2.4. *In vitro* antiplasmodial and cytotoxicity screening

For bioassay-guided fractionation, 16 fractions acquired from the DCM extract were subjected to an *in vitro* antiplasmodial screening in an attempt to determine the fractions with concentrated activity. The antiplasmodial efficacy and cytotoxicity of the isolated compounds were also determined. The protocols followed were the same as those that are broadly described in Chapter 3 (3.2.3.3. and 3.2.3.4).

6.3. Results and discussion

Fractionation of the dichloromethane extract of *Vangueria infausta* subsp. *infausta* yielded 16 fractions (I – XVI), which were subsequently subjected to *in vitro* antiplasmodial screening against chloroquine sensitive NF54 strain of *P. falciparum*. Fractions III, VII, VIII, IX, X, XI and XII demonstrated significant antiplasmodial

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activity ($IC_{50} \leq 5$ µg/ml) (Appendix, Table 9.4.). However, their respective IC_{50} values were not significantly different from that of the crude extract, thus highlighting the complexity of the fractions. Compounds **1** and **2** were identified as friedelin and morindolide, respectively. Friedelin (**1)**, a pentacyclic, triterpenoid is very abundant in nature and has been previously isolated from several plant species (Lenta, et al., 2007; Mann et. al., 2011; Sousa et al., 2012) however; this is the first report on its presence in the genus *Vangueria* (Figure 6.2.).

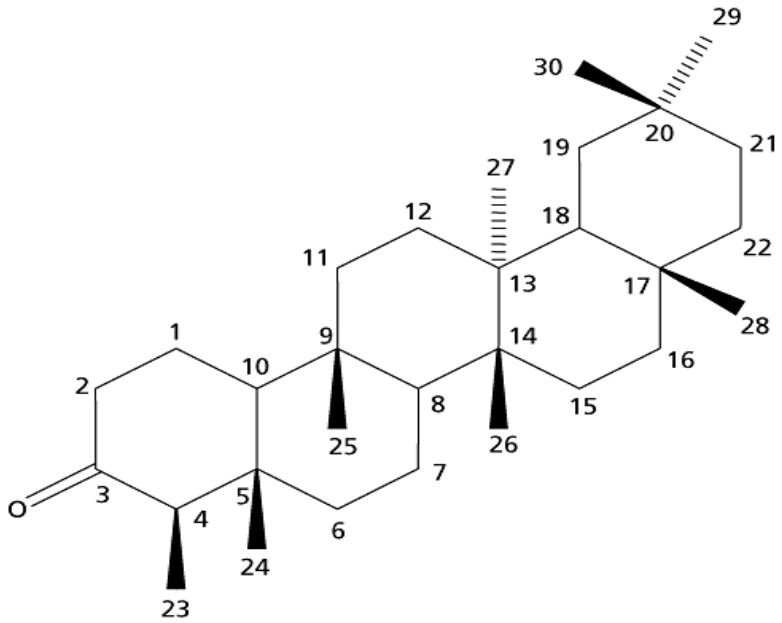

Figure 6.2. Molecular structure of friedelin

Most triterpenoids frequently occur together, are not restricted to any specific plant family and have exhibited good antiplasmodial activities in the micromolar ranges (Lenta et al., 2007; Ngouamegne et al., 2008). Friedelin has demonstrated moderate antiplasmodial activity (IC_{50} = 3.01 µg/ml) when tested against chloroquine-resistant strain (W2) of *P. falciparum* (Lenta, et al., 2007; Mann et. al., 2011). Antiplasmodial efficacy of the related fraction (III) was found to be 3.94 µg/ml, which may be mostly attributed to friedelin.

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Morindolide is a rare iridoid lactone with an undocumented antimalarial activity. Although the compound has been previously isolated from the Rubiaceae family (Yoshikawa et al., 1995), this is the first account of its presence in the genus *Vangueria* as well as its antiplasmodial activity (Figure 6.3.). In this study, morindolide demonstrated moderate antimalarial activity (IC_{50} = 18.5 µg/ml, SI = 3.32) and has shown to be toxic to the mammalian cells tested. Iridoid glucosides and related aglycones have showed a wide range of pharmacological activities including significant antiplasmodial activity (Tchimene et al., 2013; He et al., 2014). Additional studies on morindolide may include its derivatization, which could possibly ameliorate its toxicity while optimising its relatively low antiplasmodial activity.

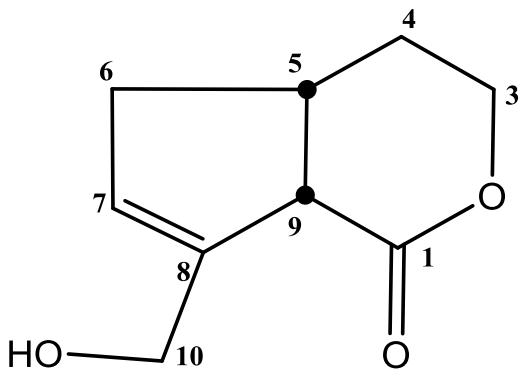

Figure 6.3. Molecular structure of morindolide

The unknown compound X exhibited a higher antiplasmodial activity (IC_{50} = 0.143 µg/ml, SI = 183) against the sensitive strain of *P. falciparum* when compared to morindolide (Table 6.1.). Preliminary spectroscopic analysis of the acquired spectra suggests a triterpene glycoside (Appendix, Figures 9.15 and 9.16). Further spectroscopic measurements are currently underway in an attempt to absolutely configure the molecular structure of the unidentified compound.

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Table 6.1. *In vitro* antiplasmodial and cytotoxic activities of morindolide and the unidentified compound X

	[a]Antiplasmodial activity (IC_{50}) µg/ml	[b]Cytotoxicity (IC_{50}) µg/ml	Selectivity Index (SI)
Morindolide	18.5	61.5	3.32
Compound X	0.143	26.1	182.5
Chloroquine	0.002	ND	ND
Podophyllotoxin	ND	0.005	ND

[a]Chloroquine resistant *Plasmodium falciparum* NF54 strain
[b]Rat skeletal muscle L-6 cell line

6.4. Conclusion

The study has demonstrated the potential of discovering novel antiplasmodial scaffolds from medicinal plants. Further phytochemical studies should be directed at making use of the current techniques in an effort to recover more compounds from roots of *V. infausta* subsp. *infausta* and to further derivatize them so as to increase their spectrum of activity.

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CHAPTER 7: GENERAL DISCUSSION

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7.1. General discussion

Malaria is caused by Apicomplexan pathogens of the genus *Plasmodium* and transmitted by *Anopheles* mosquitoes. The disease is killing nearly a million children each year in Africa alone (WHO, 2013). The malaria research agenda for eradication is based on the assumption that malaria eradication is not possible with existing tools, which include artemisinin-based combination treatments (ACTs), long-lasting insecticide-treated nets and insecticide spraying (The MalERA Drugs Consultative Group on Drugs, 2011). Although ACTs are highly effective, history has taught us that we must continue to search for new drugs in the event that artemisinin resistance develops. While cross-resistance has been observed in some endemic countries, many groups around the world are still working on molecular analogues of the artemisinin derivatives. There exists an urgent need for novel antimalarial chemotherapeutic agents that can circumvent the acquired resistance to artemisinin derivatives, treat severe malaria to reduce complications and kill gametocytes to block transmission.

The current study has demonstrated the significance of following an ethnopharmacological approach when bioprospecting for antiplasmodial scaffolds. It has also highlighted some of the research gaps and bottlenecks inherent in the field of ethnopharmacology. One of the problems associated with ethnopharmacological studies is the need for the standardization of research methods so as to capture the theoretical underpinnings of traditional systems. Published literature on ethnomedicinal studies that reflect both the therapeutic and cultural meaning of herbal remedies is still lacking. Several species that were strongly associated with malaria by Vha-Venda people and which are cited in ethnobotanical literature

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demonstrated less antimalarial activity. Their lack of *in vitro* antiplasmodial activity does not necessarily disqualify their use in herbal antimalarials. Investigations into treatments for malaria may concentrate on killing the *Plasmodium* spp. responsible for the disease but do not investigate anti-inflammatory or antipyretic activity that might help reduce the feverish state associated with the disease. There is a need for research studies that include a battery of tests for different activities that are related to the disease state (Rasoanaivo, 2011). Extracts may be more active than purified compounds particularly if the employment of a battery of tests shows the existence of a variety of active compounds and activities. This may also be reflected in a loss of activity in the subsequent *in vitro* tests when a crude extract is fractionated. Ethnopharmacological researchers are therefore faced with the challenge of scientifically demonstrating the polyvalence of traditional extracts particularly when they can be backed by *in vivo* or clinical studies.

The current availability of genome sequences for humans, *Anopheles* mosquitoes and *Plasmodium* parasites has raised hopes for new interventions against human malaria. However, the present screens and models used for the assessment of antimalarial efficacy do not effectively examine the whole range of asexual and sexual stages of the human malaria parasites (Abdul-Ghani et. al., 2014). For example, the asymptomatic hypnozoites stage of infection represents a source for future infections therefore our inability to efficiently screen these dormant parasites is a major limitation in developing tools for elimination of the disease. In addition, we rely mostly on animal models that act as surrogates for human malaria and therefore more humanised mouse models are needed for *in vivo* studies. Natural products display higher binding affinities for specific receptor systems and their

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bioactivity is often selective, which necessitate the use of sensitive and specific screening tools.

Many plant species have been screened against malaria and several antiplasmodial compounds have been isolated. There is a need for platforms that will allow for detection of known secondary metabolites in complex plant extracts and at early stages of experimentation. This study has highlighted the potential of NMR-based metabolomics in holistically discriminating classes of compounds that are associated with the observable antiplasmodial activity in the analysed plant extracts. These types of studies are still few and mostly serve to separate groups and do not lead to identification of the discriminating plant metabolites. Metabolomic analysis of crude extracts yield many unidentified chemical structures and the structural identification of potential metabolite candidates associated with bioactivity will be a major task for biological interpretation. The possibility of directly elucidating bioactive compounds in data sets of complex plant matrices has been demonstrated by 2D NMR spectroscopy (Kim et al., 2010). These techniques will help identify compounds that have already been tested for antimalarial properties and protocols aimed at targeting novel compounds can then be followed. However, one practical limitation with these studies is that their databases reflect high variability between instruments, among lab as well as in detection and quantification of results. This could limit the usability of metabolomic data for comparative analyses between studies.

Much of nature's plant biodiversity remains to be explored in our quest for new bioactive antiplasmodial scaffolds. Although most of the compounds isolated in this research study have been previously identified and in some cases tested for their antimalarial efficacy, the study demonstrated the potential of discovering new bioactive compounds from plant species. The study documented the first account of

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two secondary metabolites in the genus *Vangueria*, one with unknown antiplasmodial activity. Higher plants could still continue to serve as the major foundation of many chemotherapeutic agents; nevertheless, the main obstacle to the recovery of many secondary metabolites remains the physical and chemical properties that they are endowed with. Techniques that could effectively ameliorate the matrix effects of plant crude extracts during the fractionation and purification steps are needed.

Ultimately, the elimination of malaria requires advancements in drug development, vaccines and vectors. Until malaria elimination has been achieved, the treatment of the disease with drugs will remain fundamental for the prevention of complications and death from malaria. Novel antiplasmodial scaffolds in the development pipeline will always be needed as these parasites naturally acquire resistance to each new generation of antimalarial drugs.

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CHAPTER 8: ACKNOWLEDEMENTS

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Table 9.1. *In vitro* antiprotozoal screening results

Parasite:	***Strain:***	***Stage:***	***Reference drug:***
Trypanosoma brucei rhodesiense	STIB 900	trypomastigotes	Melarsoprol
T. cruzi	Tulahuen C4	amastigotes	Benznidazole
Leishmania donovani	MHOM-ET-67/L82	amastigotes	Miltefosine
Plasmodium falciparum	NF54	IEF	Chloroquine
Cytotoxicity	L6		Podophyllotoxin

Plant species /	**[a]Solvent**	**Antiprotozoal activity IC_{50} (µg/ml)**				**Cytotoxicity**	**Selectivity Index (SI)**			
Reference drugs		***T. b. rhodesiense***	***T. cruzi***	***L. donovani***	***P. falciparum***	**IC_{50} (µg/ml)**	**b**	**c**	**d**	**e**
Anthocleista grandiflora	DCM	49.9	69.2	12.1	8.69	55.6	1	1	5	6
A. grandiflora	50% MeOH	72.9	75.0	45.7	>50	70.1	1	1	2	ND
Albizia versicolor	DCM	14.3	57.4	33.0	7.08	72.1	5	1	2	10
A. versicolor	50% MeOH	5.32	22.5	69.4	27.3	52.3	10	2	1	2
A. versicolor	DCM	19.5	55.8	56.3	2.12	55.1	3	1	1	26
A. versicolor	50% MeOH	3.48	20.9	42.3	23.8	42.0	12	2	1	2
Bridelia mollis	DCM	47.7	40.3	1.92	3.06	51.4	1	1	27	17
B. mollis	50% MeOH	14.1	58.8	>100	28.5	49.6	4	1	ND	2
Capparis tomentosa	DCM	11.8	51.2	12.1	2.19	40.8	3	1	3	19
C. tomentosa	50% MeOH	>100	66.2	67.7	29.2	70.4	ND	1	1	2
Clematis brachiata	DCM	18.8	55.0	10.5	5.36	42.6	2	1	4	8
C. brachiata	50% MeOH	37.2	88.5	55.3	>50	72.3	2	1	1	ND
Clerodendrum glabrum	DCM	61.6	66.2	13.4	8.89	62.2	1	1	5	7
C. glabrum	50% MeOH	58.2	56.3	55.6	>50	72.7	1	1	1	ND
Cussonia spicata	DCM	19.1	52.7	8.15	3.25	47.8	3	1	6	15
C. spicata	50% MeOH	>100	84.4	>100	>50	69.1	ND	1	ND	ND
Dichrostachys cinerea	DCM	62.2	45.3	10.5	2.10	51.6	1	1	5	25
D. cinerea	50% MeOH	>100	60.5	92.9	>50	65.3	ND	1	1	ND
Diospyros mespiliformis	DCM	33.6	32.0	7.70	4.40	24.3	1	1	3	6
D. mespiliformis	50% MeOH	51.7	61.8	54.0	28.4	60.4	1	1	1	2

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Pappea capensis	DCM	30.3	39.1	8.36	5.47	54.0	2	1	6	10
P. capensis	50% MeOH	>100	54.6	76.8	24.8	55.2	ND	1	1	2
Parinari curatellifolia	DCM	53.7	55.3	13.6	6.99	57.6	1	1	4	8
P. curatellifolia	50% MeOH	70.8	61.1	>100	16.9	55.4	1	1	ND	3
Pyrenacantha grandiflora	DCM	12.7	1.83	5.60	5.82	0.52	0	0	0	0
P. grandiflora	50% MeOH	71.8	31.4	76.9	>50	10.5	0	0	0	ND
Rauvolfia caffra	DCM	17.2	45.9	19.6	2.13	26.9	2	1	1	13
R. caffra	50% MeOH	56.6	66.5	62.4	10.8	57.2	1	1	1	5
Senna pertisiana	DCM	44.9	59.5	15.7	22.5	59.3	1	1	4	3
S. pertisiana	50% MeOH	57.4	71.7	72.9	22.1	66.8	1	1	1	3
Syzygium cordatum	DCM	6.94	48.7	4.95	6.15	65.7	9	1	13	11
S. cordatum	50% MeOH	19.2	62.1	>100	10.4	53.8	3	1	ND	5
Tabernaemontana elegans	DCM	6.55	17.5	36.3	0.33	4.68	1	0	0	14
T. elegans	50% MeOH	22.7	52.3	>100	0.83	38.2	2	1	ND	46
Vangueria infausta	DCM	17.9	39.7	4.51	1.84	45.7	3	1	10	25
V. infausta	50% MeOH	83.4	73.6	>100	>50	71.5	1	1	ND	ND
Ximenia caffra	DCM	2.23	14.5	1.79	3.01	8.68	4	1	5	3
X. caffra	50% MeOH	65.7	78.9	42.6	>50	74.1	1	1	2	ND
[f]*Ximenia americana*	DCM:50% MeOH	62.8	68.3	>100	28.2	69.1	1	1	ND	2
Xylopia parviflora	DCM	11.8	48.6	5.01	2.19	51.5	4	1	10	24
X. parviflora	50% MeOH	72.3	65.3	>100	14.2	78.3	1	1	ND	6
Melarsoprol		0.005								
Benznidazole			0.511							
Miltefosine				0.191						
Chloroquine					0.003					
Podophyllotoxin						0.007				

[a]Solvents used for extraction: DCM = dichloromethane, MeOH = methanol

Selectivity Index (SI): b = IC_{50} for L6 cells/ IC_{50} for *Trypanosoma brucei rhodesiense*, c = IC_{50} for L6 cells/ IC_{50} for *Trypanosoma cruzi*, d = IC_{50} for L6 cells/ IC_{50} for *Leishmania donovani*, e = IC_{50} for L6 cells/ IC_{50} for *Plasmodium falciparum*

[f]*Ximenia americana* – did not result in two phases between the DCM and 50% MeOH

ND = not determine

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Table 9.2. Spectroscopic data of dregamine

^{13}C NMR spectroscopic data			^{1}H NMR spectroscopic data δ, mult; *J* [Hz]		
Position	[a]Compound 1	Dregamine (Ahond et al.,1976)	Position	[a]Compound 1	Dregamine (Van der Heijden et al., 1985)
2	133.8	133.8	5	3.95, ddd; 10.8, 3.5	3.97, ddd; 10.8, 3.5
3	190.7	190.7	6a	3.38, dd; 15, 8	3.36, dd; 15, 8
5	56.5	56.5	6b	3.33, dd; 14, 10	3.33, dd; 14, 10
6	20.1	20.1	9	7.0, dd; 8, 0.8	7.0, dd; 8, 0.8
7	120.1	120.1	10	7.15, ddd; 8.0, 5.0, 3	7.14, ddd; 8.0, 5.0, 3
8	128.7	128.8	11	7.33, m	7.33, m
9	120.5	120.5	12	7.33, m	7.33, m
10	120.0	120.0	14a	2.69, dd; 12, 6	2.68, dd; 12, 6
11	126.3	126.3	14b	2.58, dd; 12, 4	2.59, dd; 12, 4
12	11.8	111.8	15	2.90, m	2.90, m
13	136.2	136.4	16	2.83, dd; 3.5, 3.5	2.83, dd; 3.5, 3.5
14	39.2	39.1	18	1.01, t; 7	1.01, t; 7
15	30.5	30.5	19a	1.33, bq	1.33, bq
16	48.7	48.8	19b	1.33, bq	1.33, bq
18	11.3	11.3	20	1.89, m	1.89, m
19	22.3	23.3	21a	3.10, dd; 12, 12	3.10, dd; 12, 12
20	43.3	43.2	21b	2.74, d; 12	2.75, d; 12
21	48.5	48.5	NMe	2.64 or 2.62, s	2.64 or 2.62, s
C=O		170.9	COOMe	2.64 or 2.62, s	2.64 or 2.62, s
OMe	50.1	50.1	NH	8.5, bs	8.5, bs
NMe	42.3	42.3			

[a] Measured at 200 MHz in $CDCl_3$

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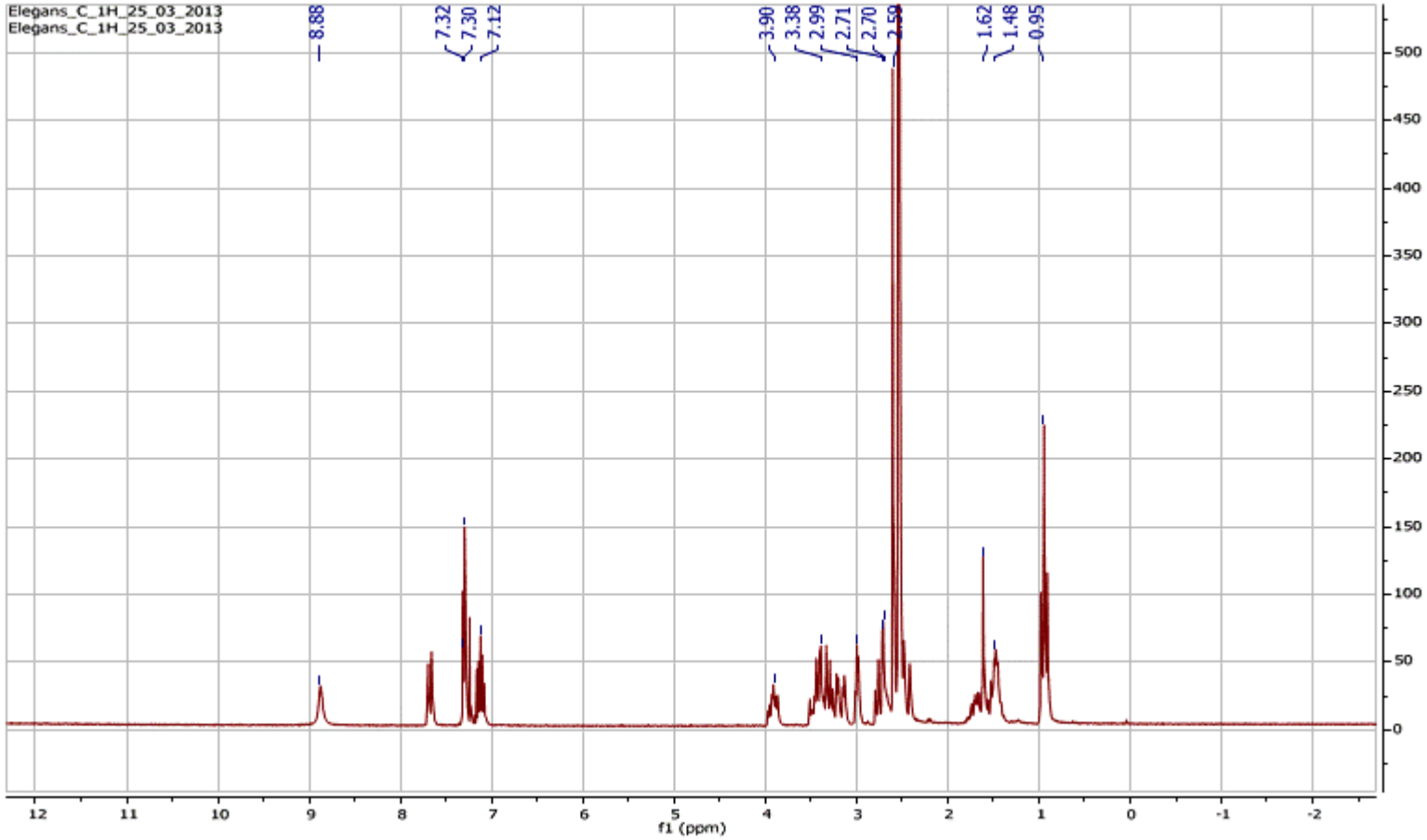

Figure 9.1. ^{1}H NMR spectrum of dregamine

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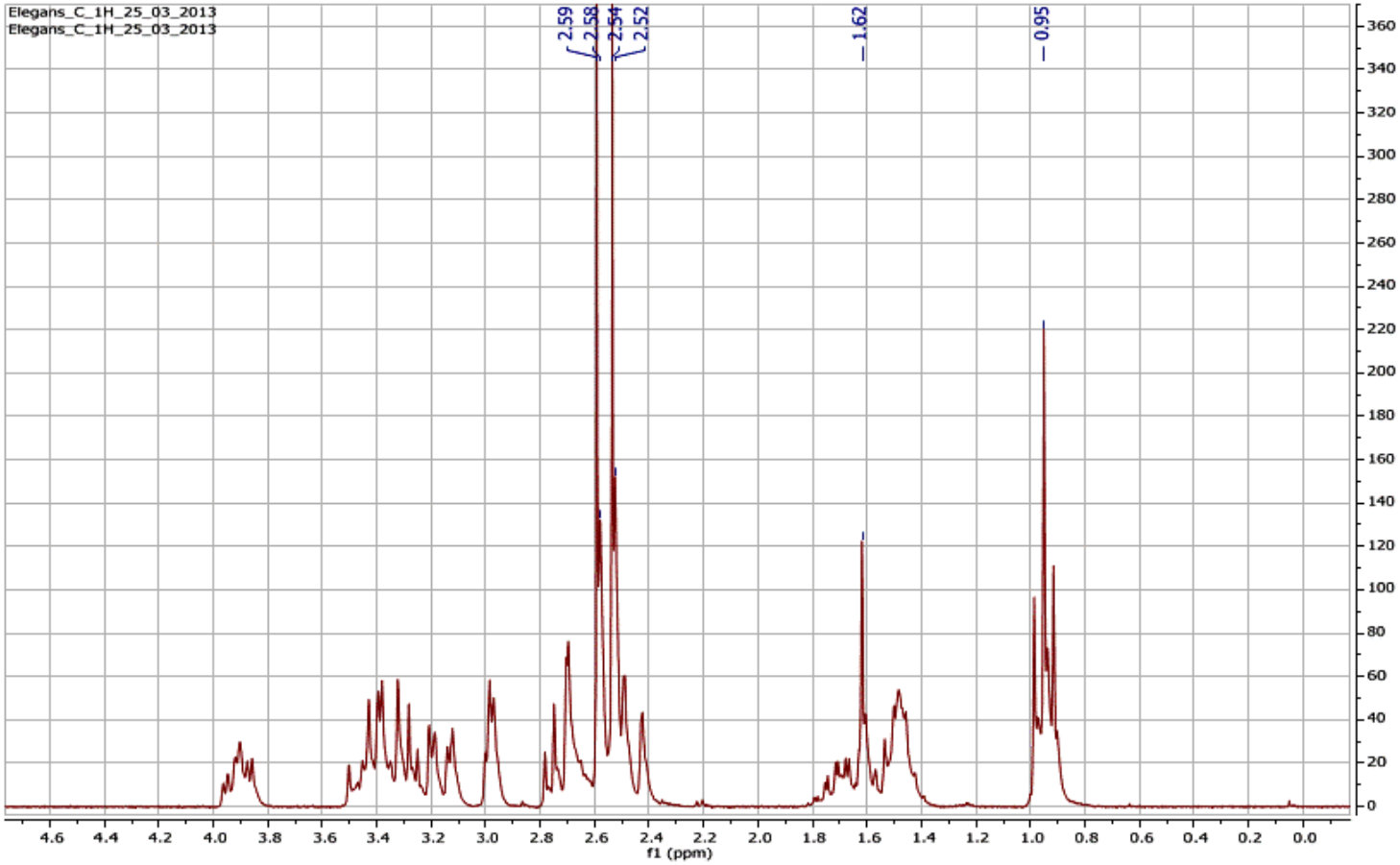

Figure 9.1.1. ^{1}H NMR spectrum of dregamine (0 ppm – 4.6 ppm)

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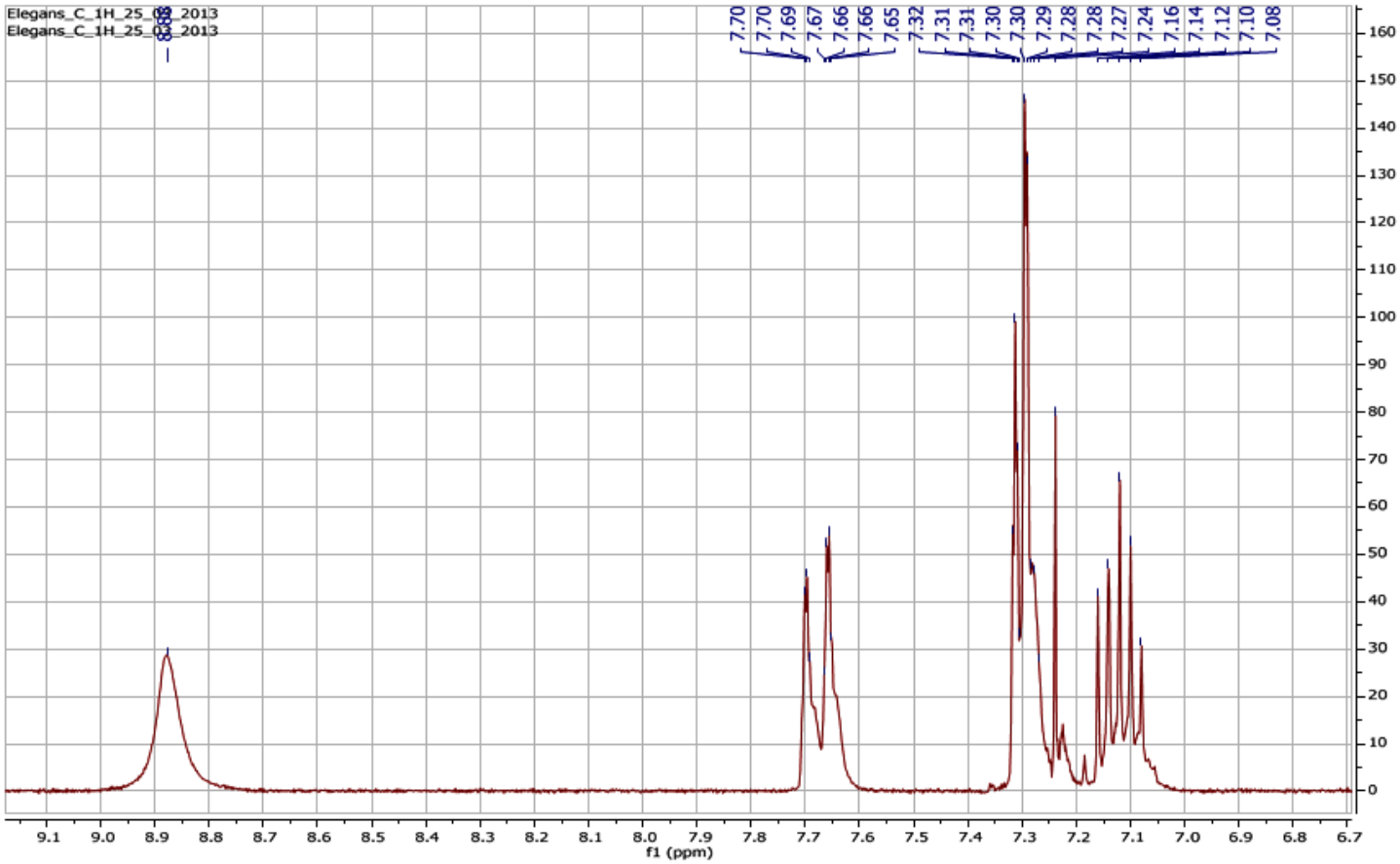

Figure 9.1.2. ^{1}H NMR spectrum of dregamine (6.8 ppm – 9.1 ppm)

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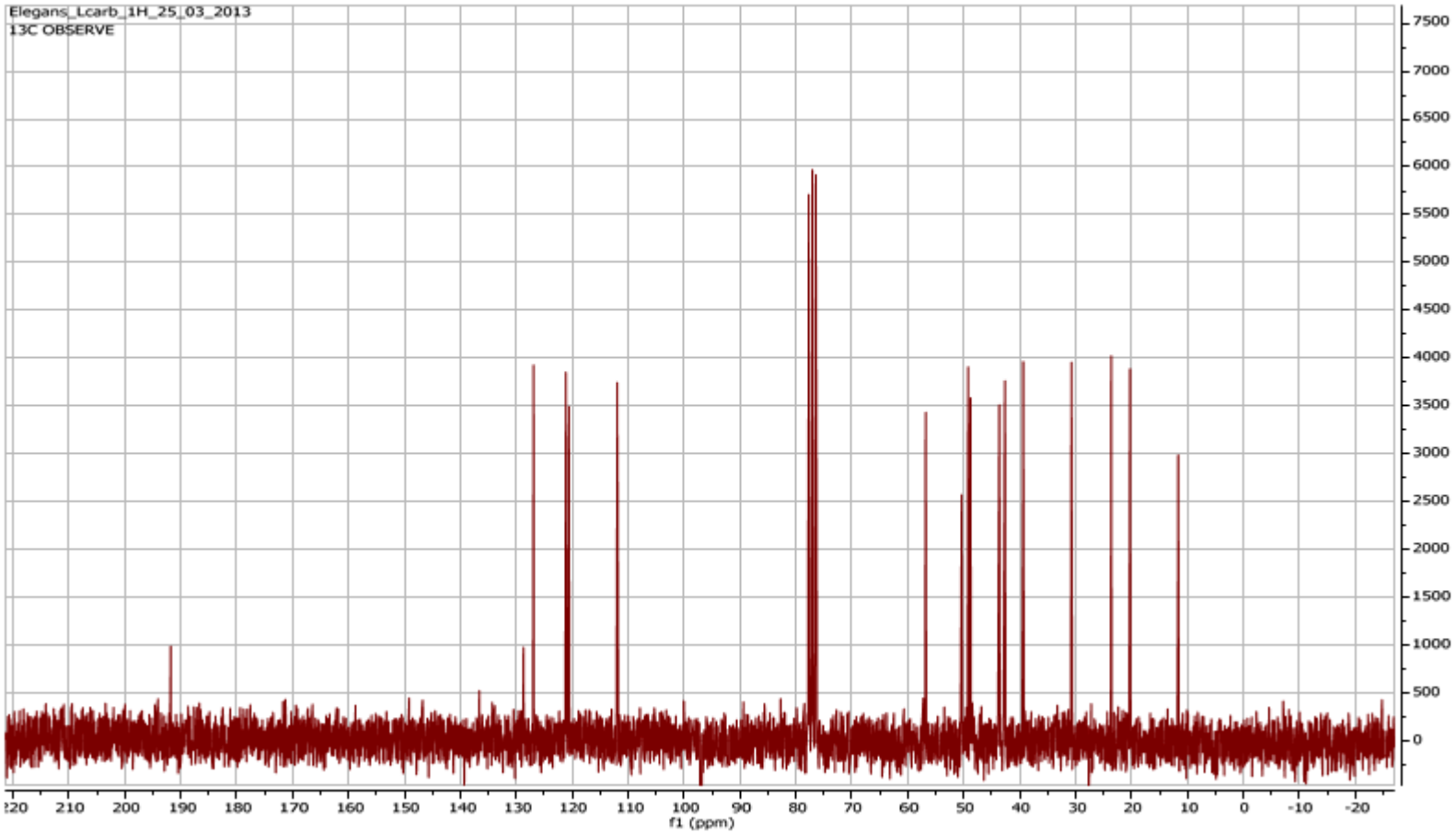

Figure 9.2. ^{13}C NMR spectrum of dregamine

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Table 9.3. Spectroscopic data of tabernaemontanine

^{13}C NMR spectroscopic data			^{1}H NMR spectroscopic data δ, mult; *J* [Hz]		
Position	[a]Compound 2	Tabernaemontanine (Ahond et al.,1976)	Position	[a]Compound 2	Tabernaemontanine (Van der Heijden et al., 1985)
2	133.6	133.7	5	3.94, ddd; 11, 8, 3.5	3.93, ddd; 11, 8, 3.5
3	190.5	190.5	6a	3.44, dd; 15, 11	3.44, dd; 15, 11
5	56.8	56.7	6b	3.32, dd; 15, 8	3.32, dd; 15, 8
6	18.4	18.4	9	7.71, dd; 8, 0.8	7.70, dd; 8, 0.8
7	120.5	120.5	10	7.14, m	7.14, ddd; 8.0, 5.0, 3.0
8	128.3	128.3	11	7.33, m	7.33, m
9	120.5	120.5[e]	12	7.33, m	7.33, m
10	119.9	119.9[e]	14	2.70, dd; 12, 7	2.70, dd; 12, 7
11	126.3	126.3	15	3.48, m	3.48, m
12	111.0	111.7	16	3.01, dd; 3.5, 3.5	3.01, dd; 3.5, 3.5
13	136.4	136.4	18	0.97, 7	0.97, 7
14	45.4	45.4	19	1.52, bq	1.52, bq
15	31.7	31.7	20	1.71, m	1.71, m
16	43.3	43.3	21a	3.19, dd; 13.4, 3.8	3.19, dd; 14, 2.5
18	12.6	12.6	21b	2.48, d; 14	2.48, d; 14
19	25.3	25.3	NMe		2.61 or 2.56, s
20	42.4	42.4	COOMe		2.61 or 2.56, s
21	46.4	46.4	NH	8.90 bs	8.85, bs
C=O	171.6	171.6			
OMe	50.2	50.1			
NMe	42.9	42.9			

[a] Measured at 200 MHz in $CDCl_3$

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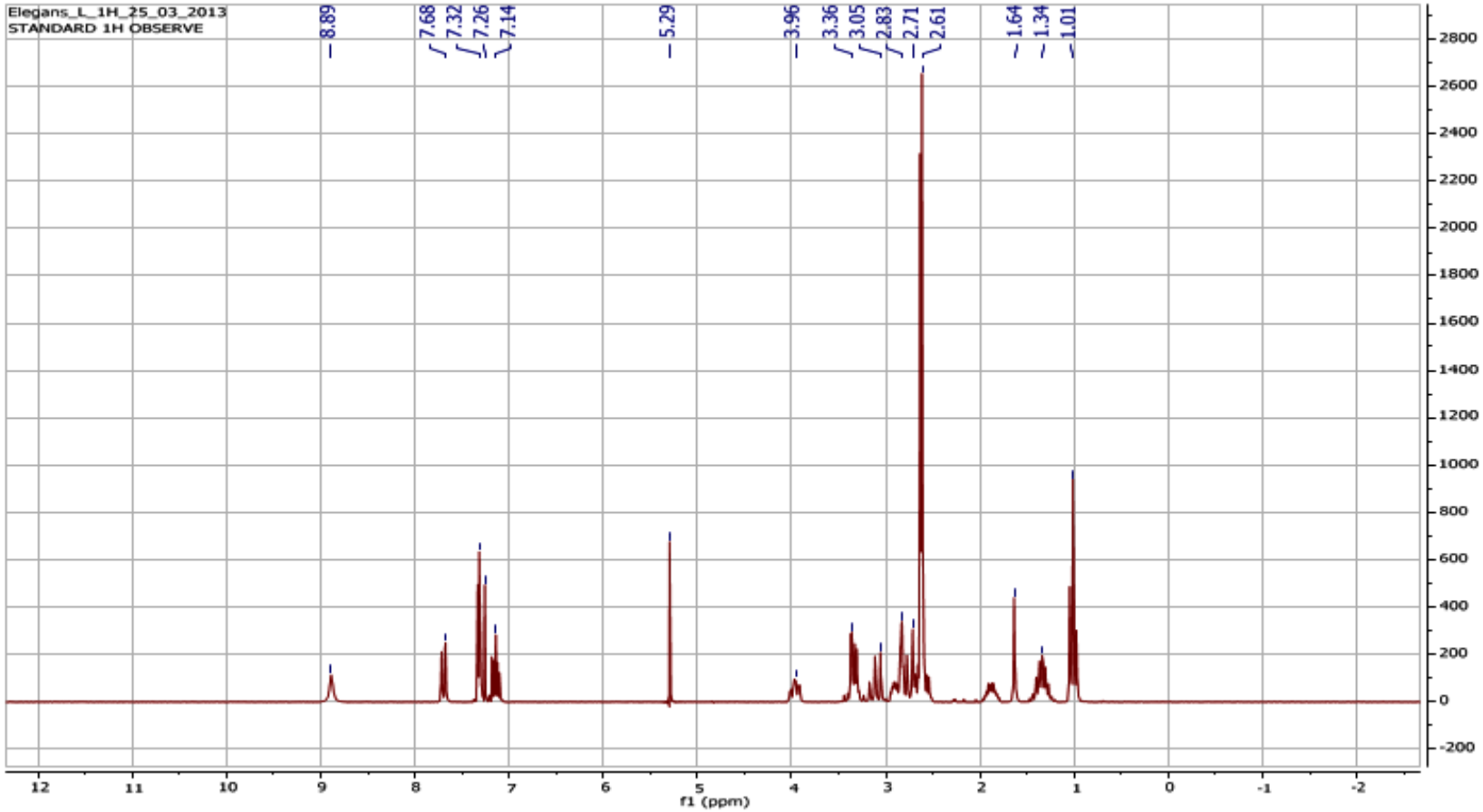

Figure 9.3. ^{1}H NMR spectrum of tabernaemontanine

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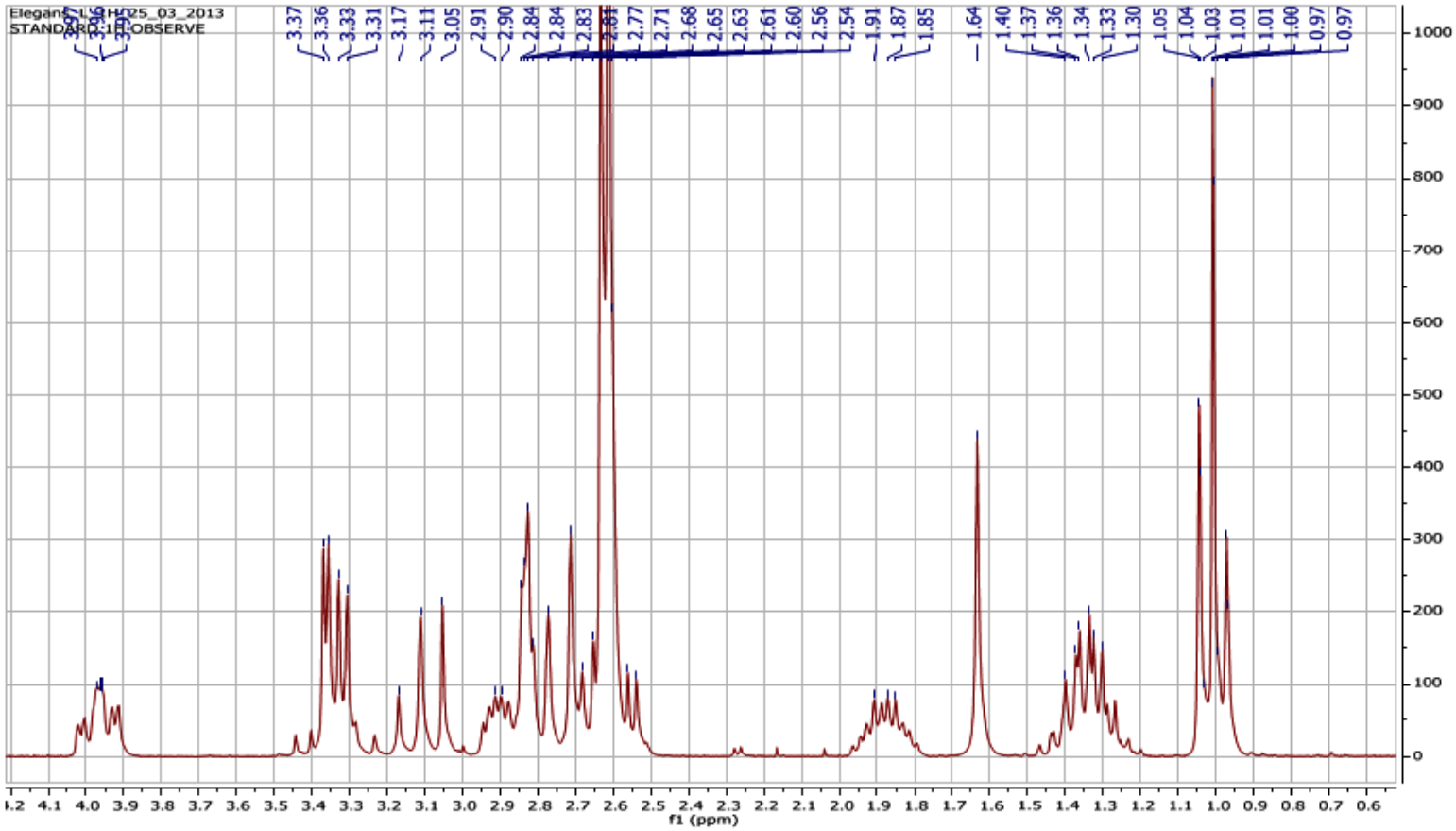

Figure 9.3.1. ^{1}H NMR spectrum of tabernaemontanine (0.6 ppm – 4.2 ppm)

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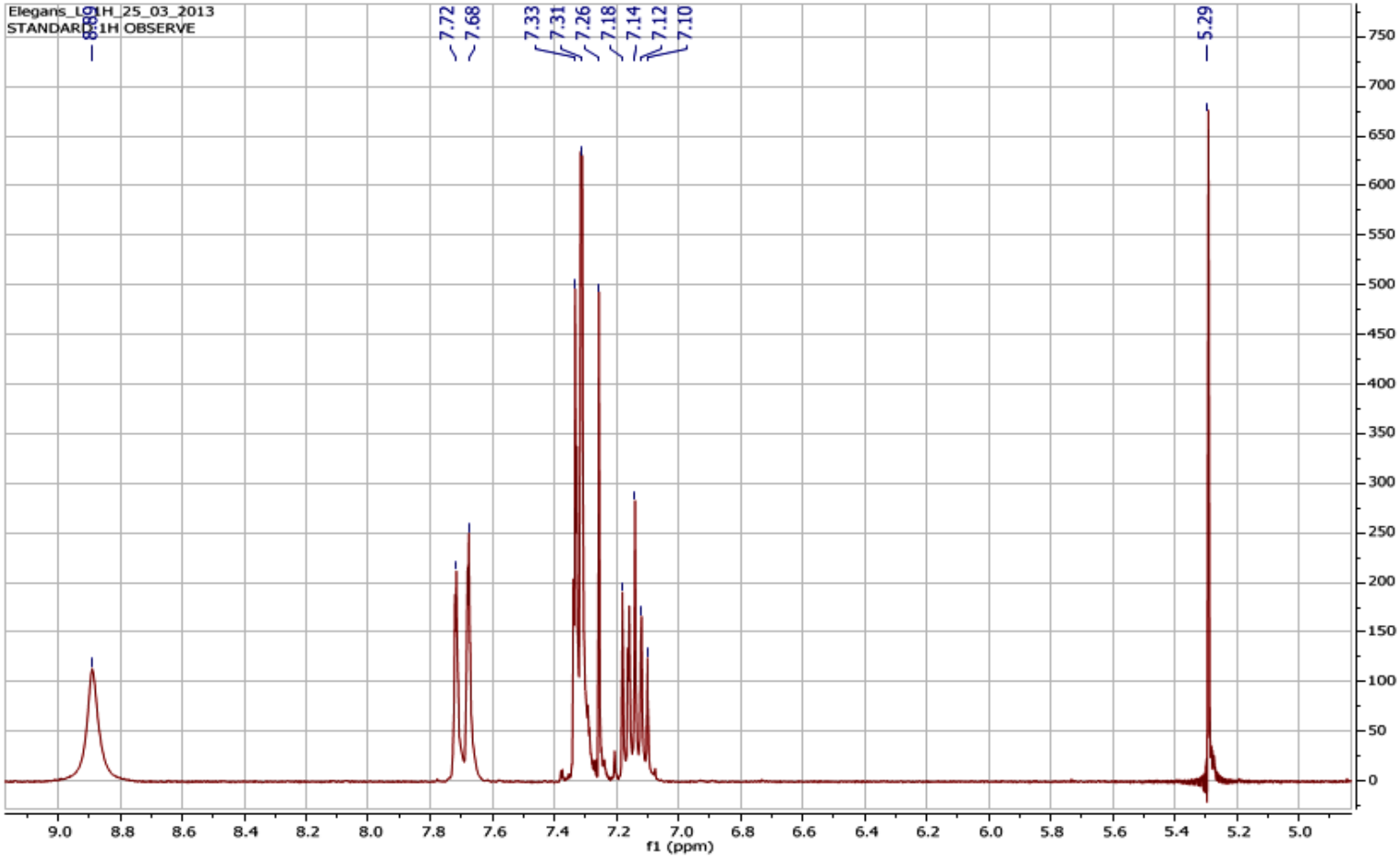

Figure 9.3.2. ^{1}H NMR spectrum of tabernaemontanine (5.0 ppm – 9.0 ppm)

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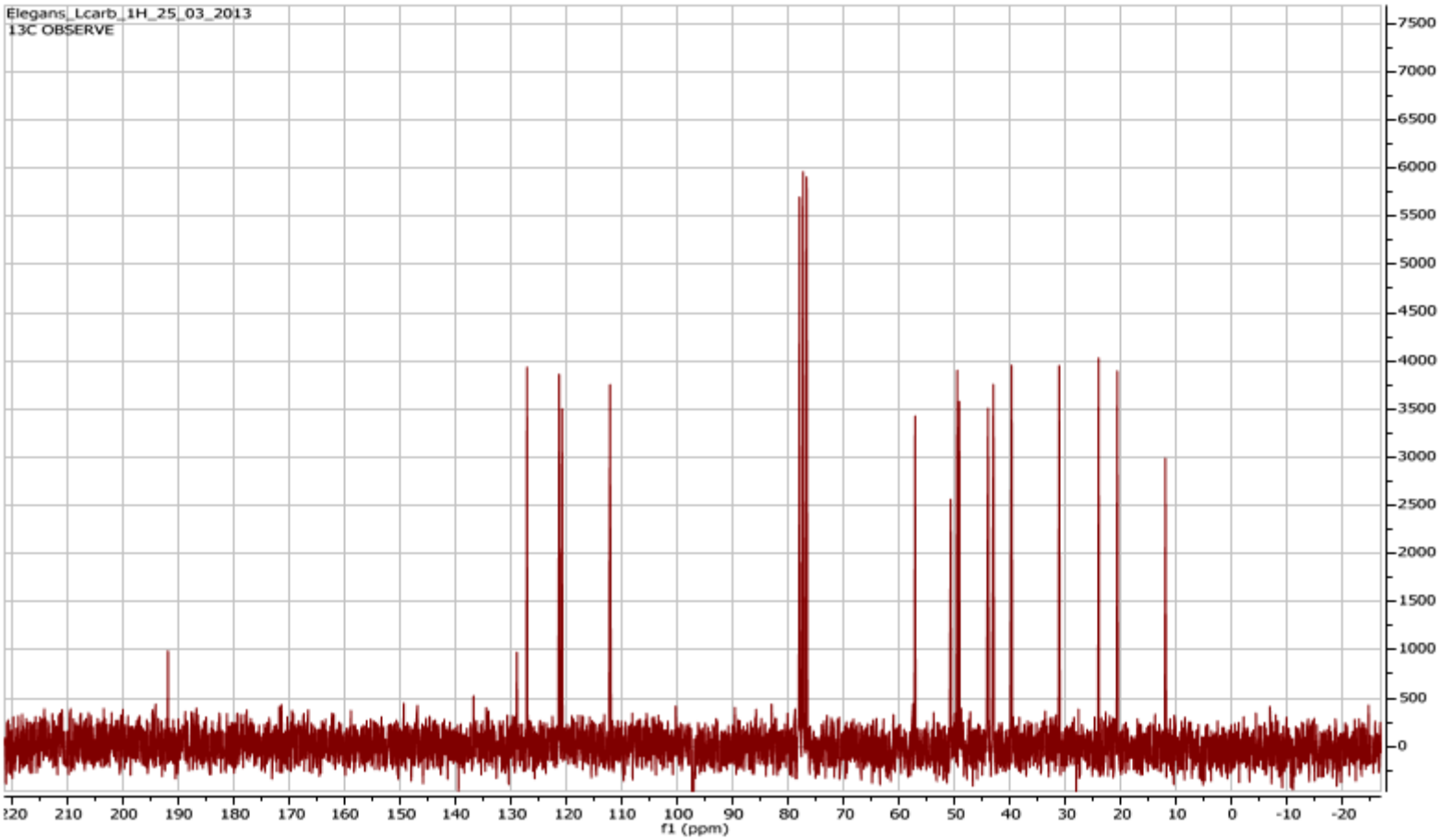

Figure 9.4. ^{13}C NMR spectrum of tabernaemontanine

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Table 9.4. *In vitro* antiplasmodial activity of fractions from *Vangueria infausta* subspecies *infausta*

Fraction	Amount (mg)	[1]IC_{50} (µg/ml)
I	26	49.7
II	38	30.6
III	110	3.94
IV	05	11.6
V	06	6.15
VI	207	12.6
VII	40	3.90
VIII	204	2.18
IX	697	2.13
X	78	2.34
XI	217	3.22
XII	210	4.12
XIII	76	15.9
XIV	99	9.63
XV	164	8.65
XVI	153	16.1
Extract		2.5
[2]Chloroquine		0.002

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Table 9.5. Spectroscopic data for friedelin

Position (H/C)	^{1}H NMR spectroscopic data δ, mult; *J* [Hz]		^{13}C NMR spectroscopic data	
	[a]Compound 2	[b]Friedelin	[a]Compound 2	[b]Friedelin
1	1.95, 1.71 ddd	1.95, 1.71 ddd	22.3	22.3
2	2.37, 2.27 ddd	2.37, 2.27 ddd	41.5	41.5
3	-	-	213.2	213.2
4	2.25 q	2.25 q	58.2	58.2
5	-	-	42.1	42.1
6	1.74, 1.28 d	1.73, 1.28 d	41.3	41.2
7	1.49, 1.36 m	1.49, 1.36 m	18.2	18.2
8	1.38 dd	1.38 dd	53.1	53.1
9	-	-	37.4	37.4
10	1.53 m	1.53 m	59.5	59.5
11	1.45, 1.26 m	1.45, 1.26 m	35.6	35.6
12	1.33, 1.32 m	1.33, 1.33 m	30.5	30.5
13	-	-	39.7	39.7
14	-	-	38.3	38.3
15	1.47, 1.27 m	1.47, 1.27 m	32.4	32.4
16	1.58, 1.35 m	1.58, 1.35 m	36.0	36.0
17	-	-	30	30
18	1.56 m	1.56 m	42.8	42.8
19	1.37, 1.22 m	1.37, 1.21 m	35.3	35.3
20	-	-	28.2	28.2
21	1.50, 1.31 m	1.50, 1.31 m	32.8	32.8
22	1.51, 0.95 m	1.50, 0.94 m	39.2	39.2
23	0.88 d	0.88 d	6.9	6.8
24	0.73 s	0.71 s	14.6	14.6
25	0.87 s	0.86 s	17.9	17.9
26	1.01 s	1.00 s	20.2	20.2
27	1.05 s	1.04 s	18.6	18.6
28	1.18 s	1.17 s	32.1	32.1
29	1.00 s	0.99 s	35.0	35.0
30	0.94 s	0.94 s	31.8	31.8

[a] isolated compound (Measured at 400 MHz in $CDCl_3$)

[b]Friedelin data from literature (Mann et. al., 2011)

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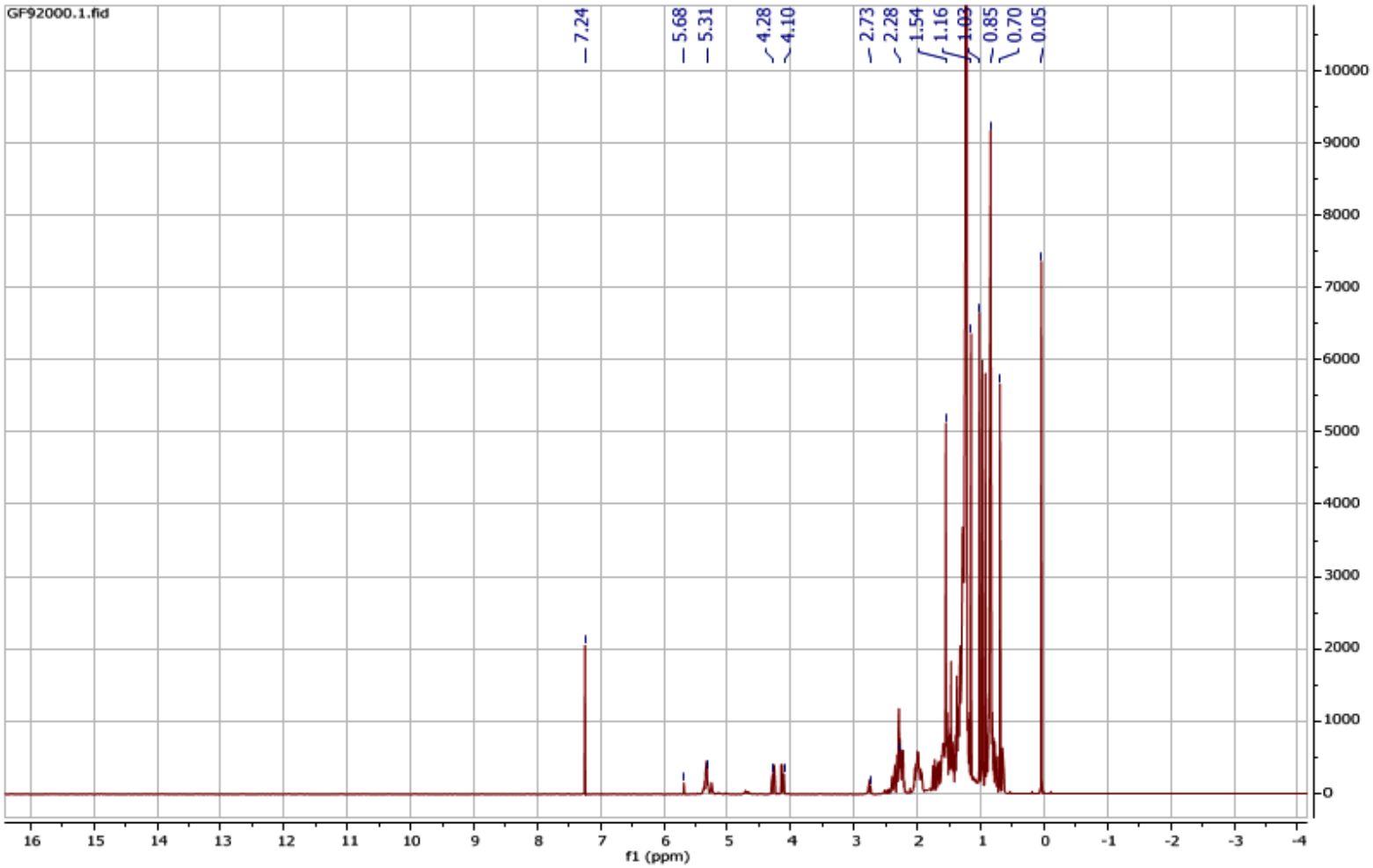

Figure 9.5. ^{1}H NMR spectrum of friedelin

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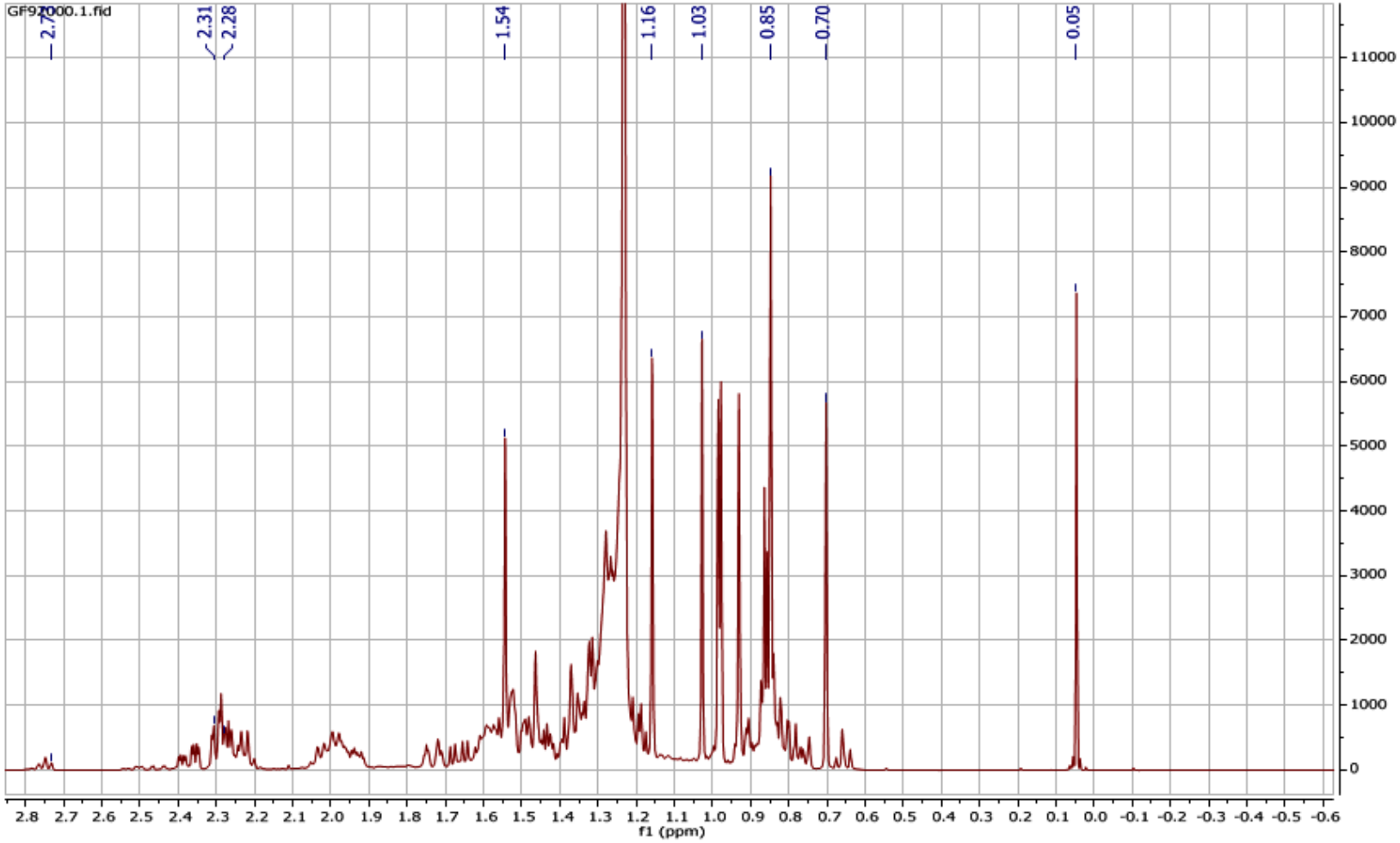

Figure 9.5.1. ^{1}H NMR spectrum of friedelin (0.0 ppm – 2.8 ppm)

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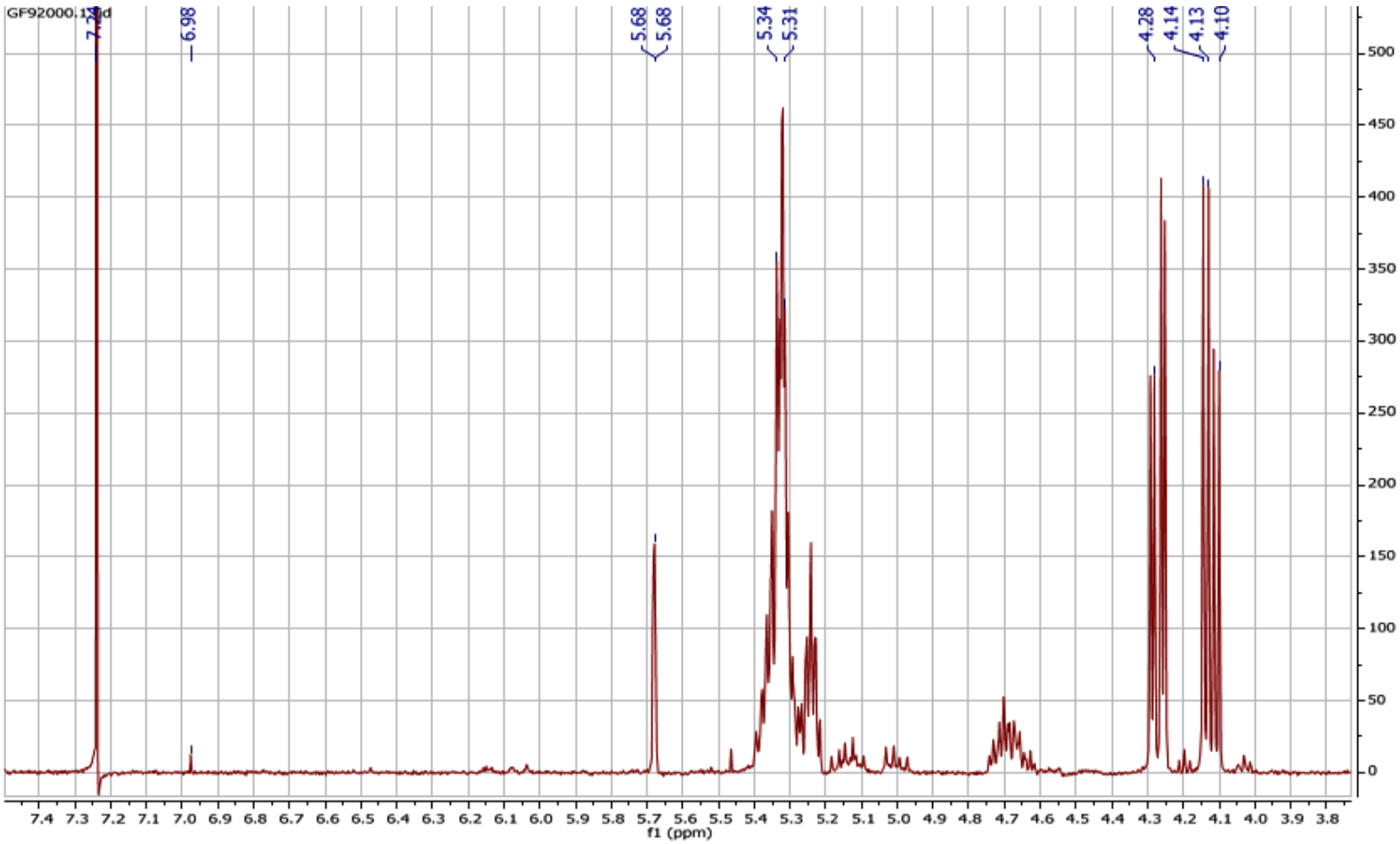

Figure 9.5.2. ^{1}H NMR spectrum of friedelin (3.8 ppm – 7.4 ppm)

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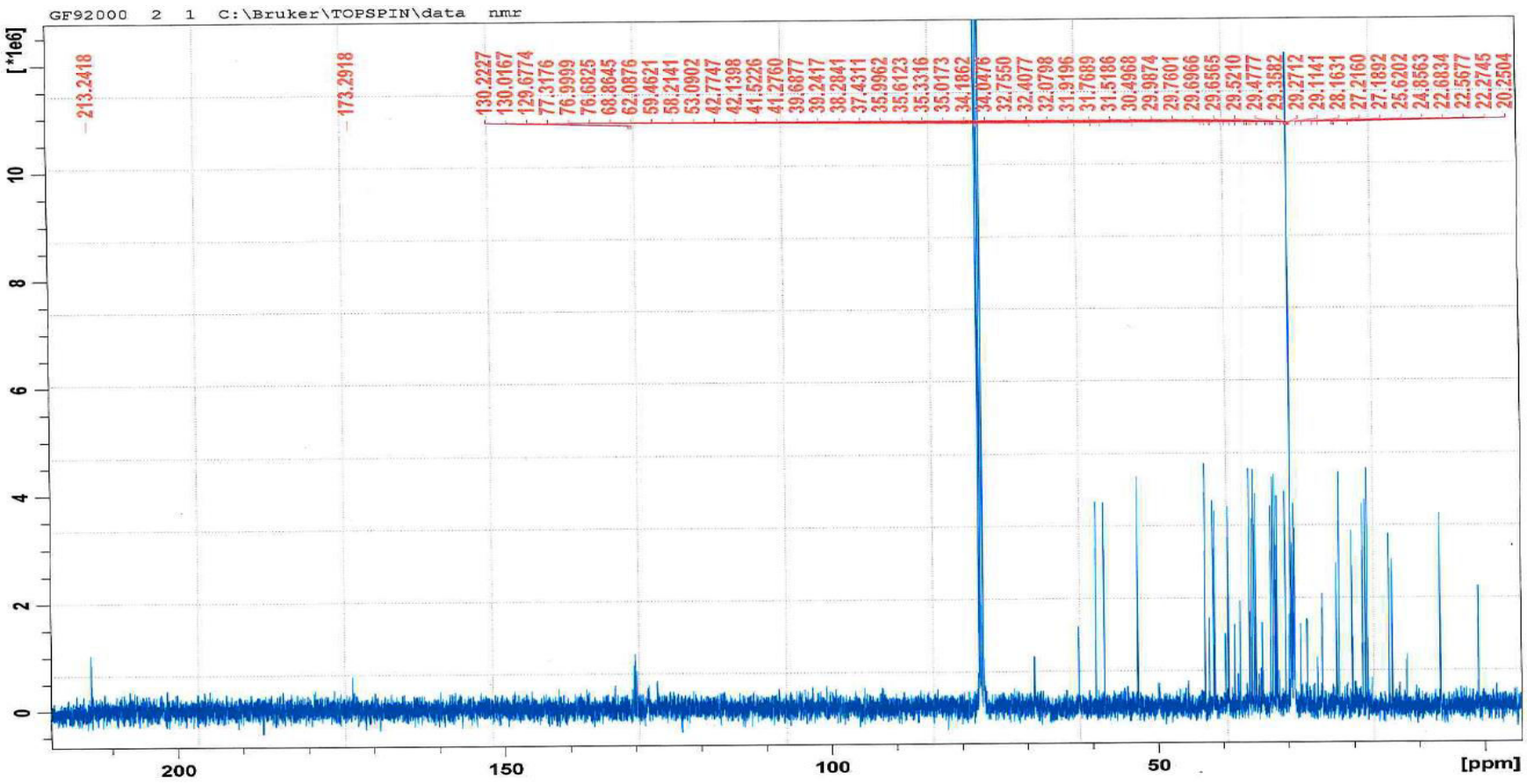

Figure 9.6. ^{13}C NMR spectrum of friedelin

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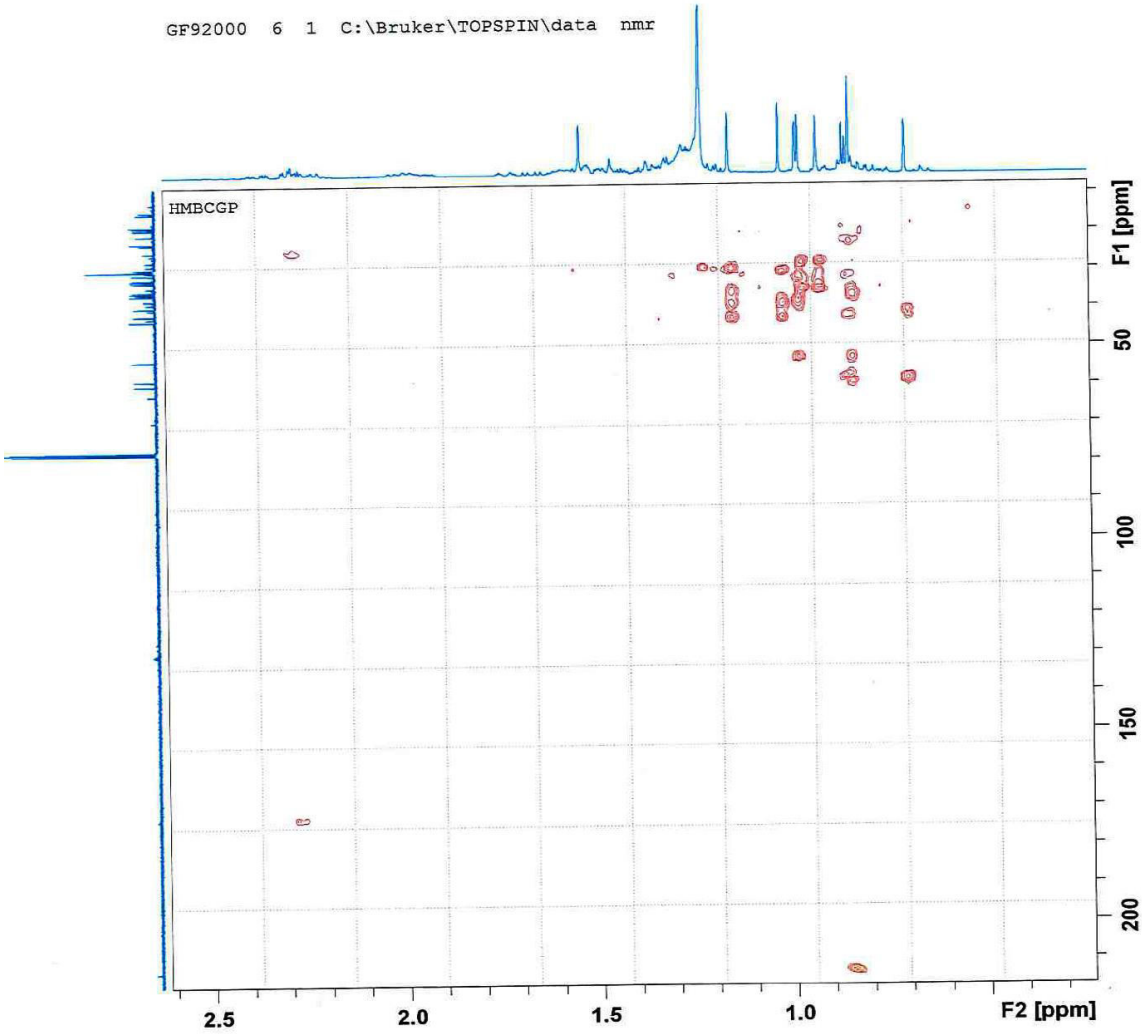

Figure 9.7. HMBC spectrum of friedelin

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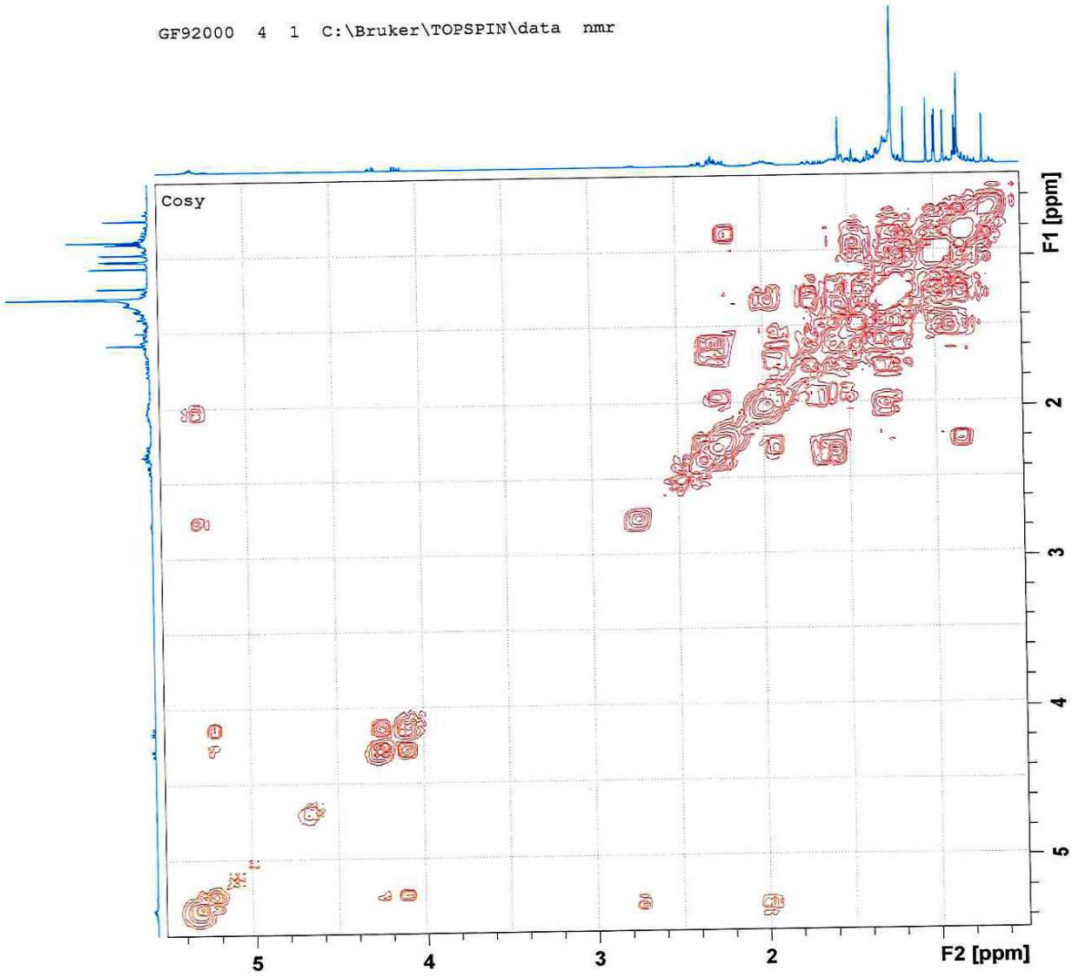

Figure 9.8. H-H COSY spectrum of friedelin

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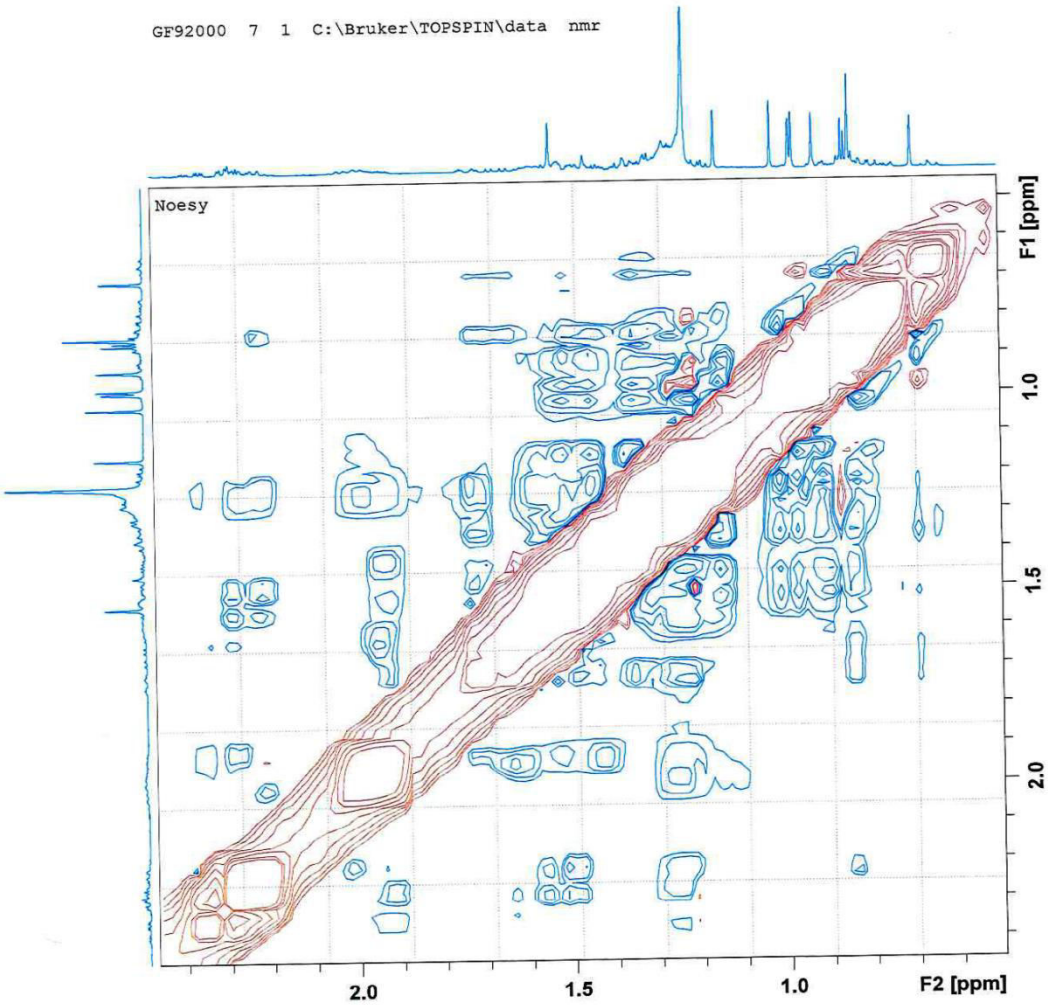

Figure 9.9. NOESY spectrum of friedelin

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Table 9.6. Spectroscopic data for morindolide

Position (H/C)	^{1}H NMR spectroscopic data δ, mult; *J* [Hz]		^{13}C NMR spectroscopic data	
	[a]Compound 1	[b]Morindolide	[a]Compound 1	[b]Morindolide
1	4.26 m	4.27 m	173.2	173.1
3	4.42 m	4.42 m	67.8	67.6
4	2.02 m	2.01 m	29.0	29.2
5	2.86 m	2.88 m	34.9	34.7
6	2.12 m	2.12 m	39.0	38.8
7	5.76 s	5.78 s	129.3	129.2
8	-	-	140.3	140.0
9	3.72 d	3.72 d	50.7	50.5
10	4.28 s	4.27 s	60.9	60.6

[a]Isolated compound (Measured at 400 MHz in $CDCl_3$)

[b]Morindolide data from the literature (Yoshikawa et al., 1995)

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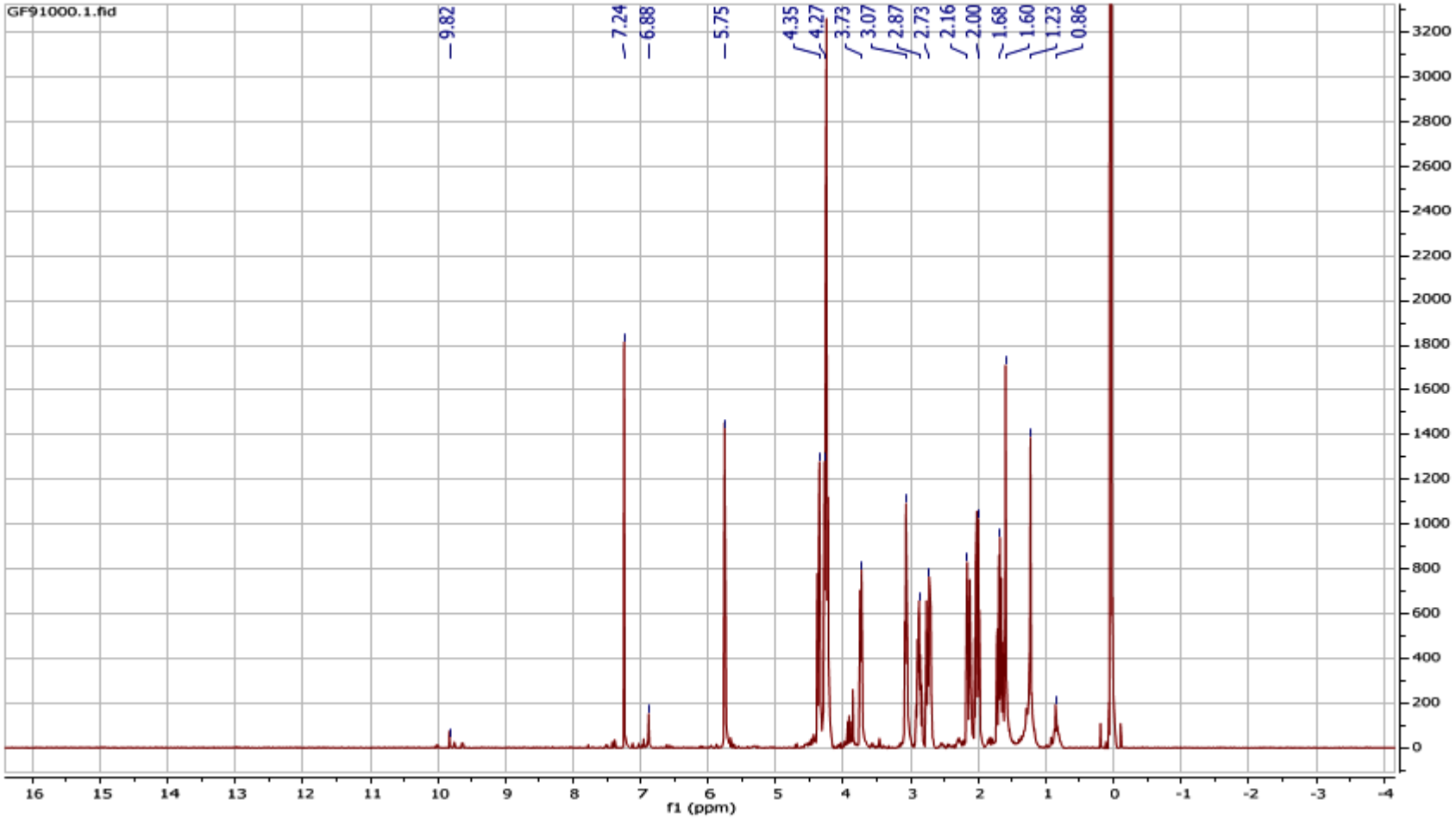

Figure 9.10. ^{1}H NMR spectrum of morindolide

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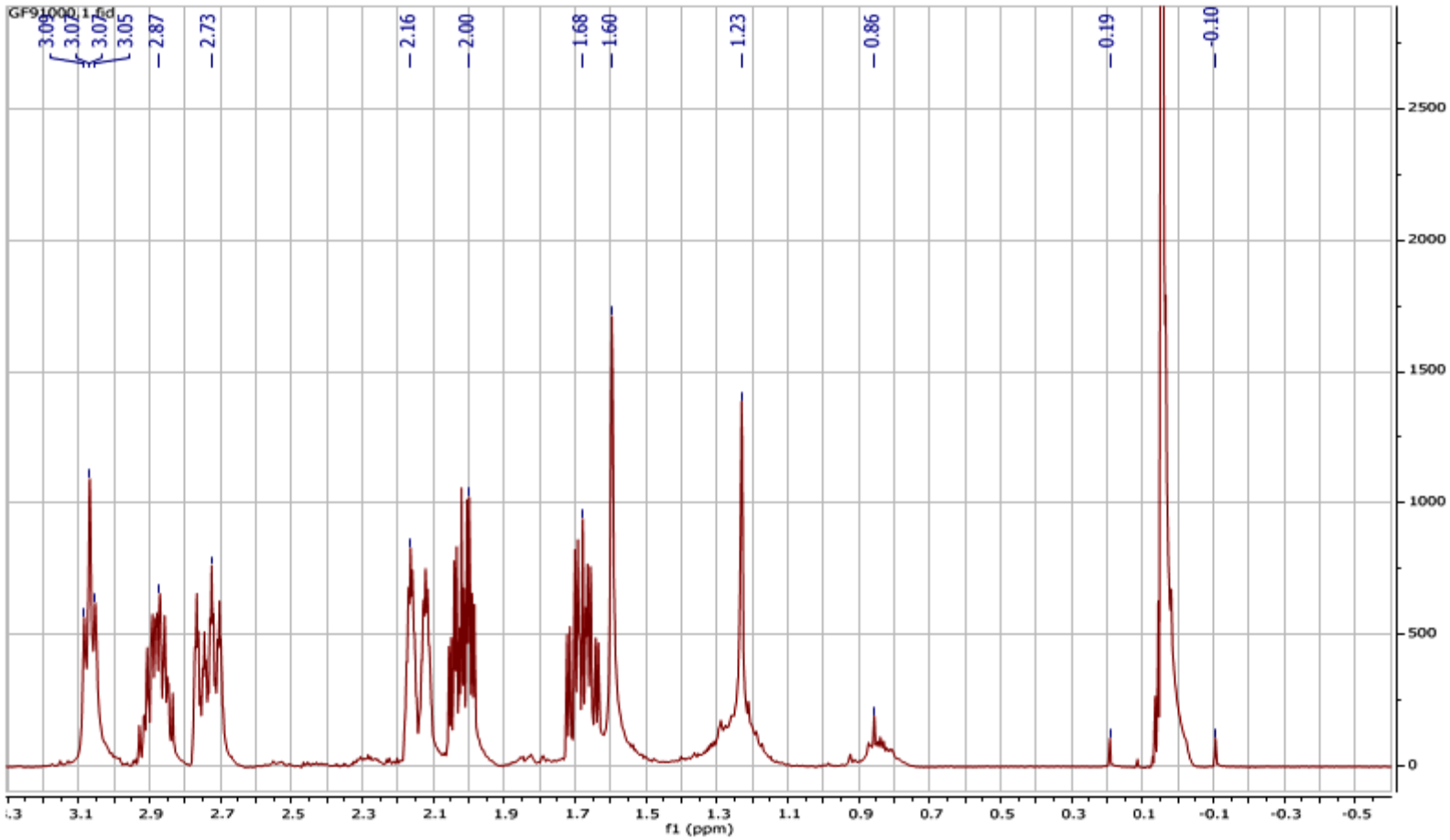

Figure 9.10.1. ^{1}H NMR spectrum of morindolide (0.0 ppm – 3.3 ppm)

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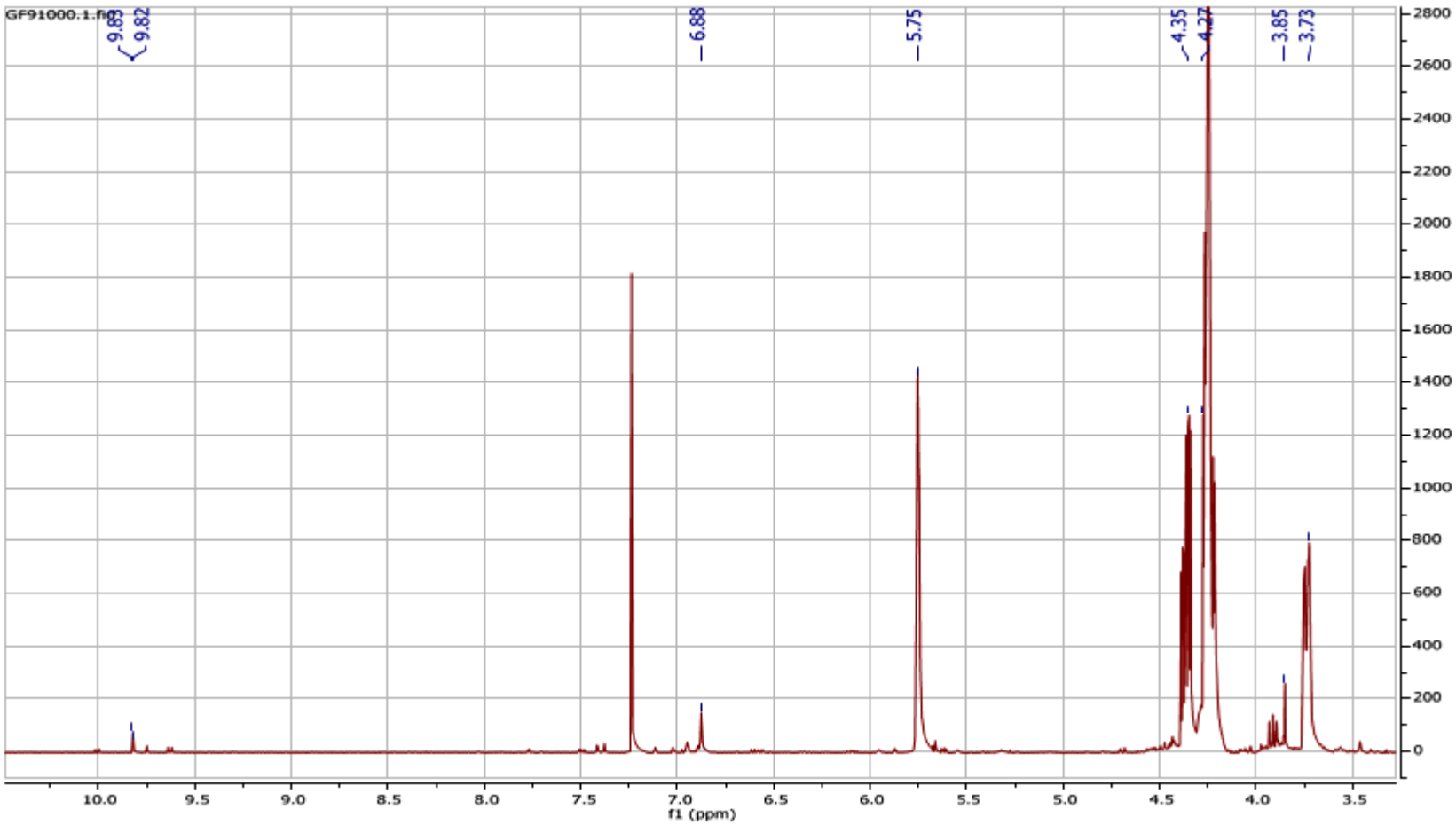

Figure 9.10.2. ^{1}H NMR spectrum of morindolide (3.5. ppm – 10.0 ppm)

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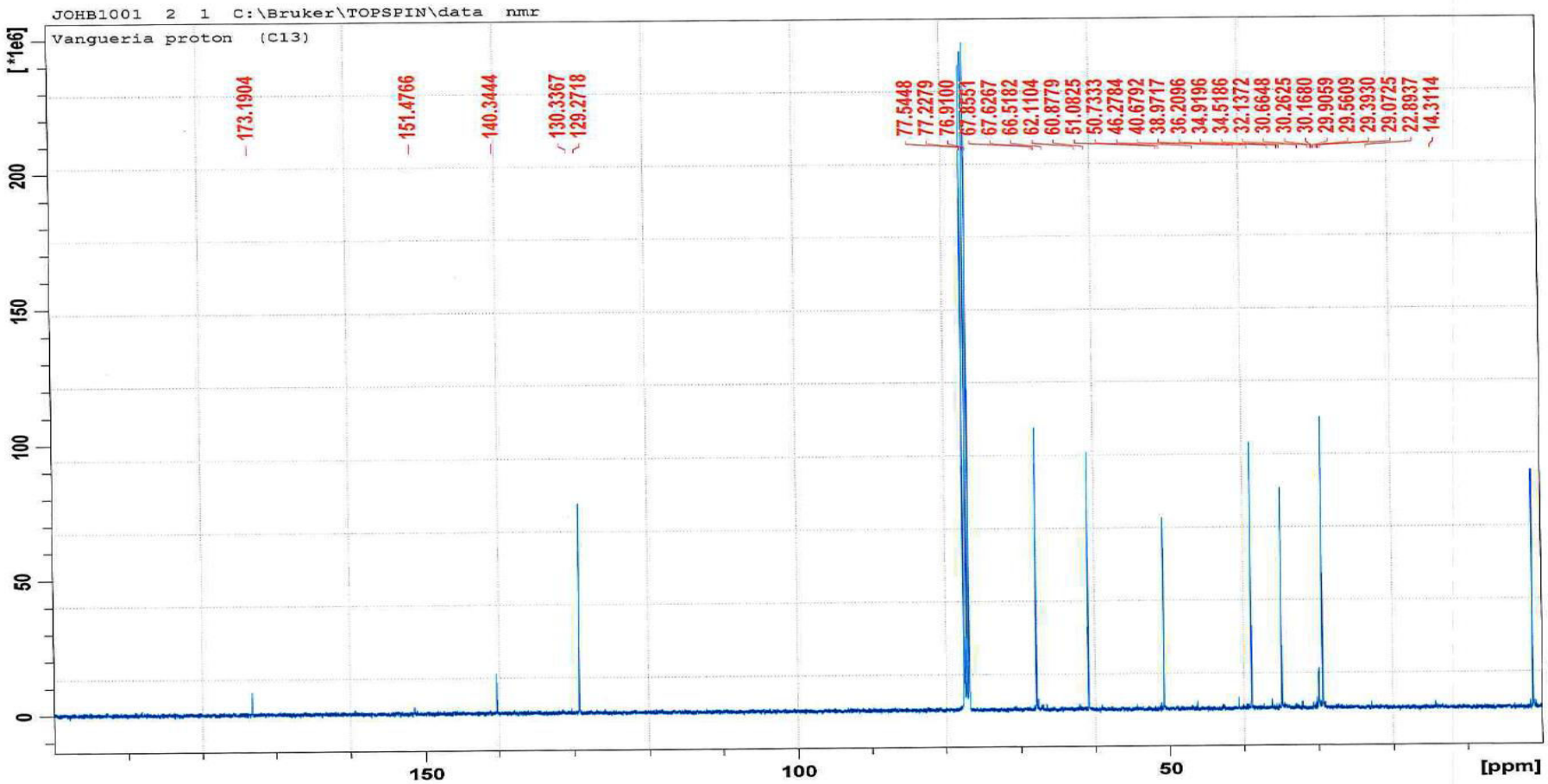

Figure 9.11. ^{13}C NMR spectrum of morindolide

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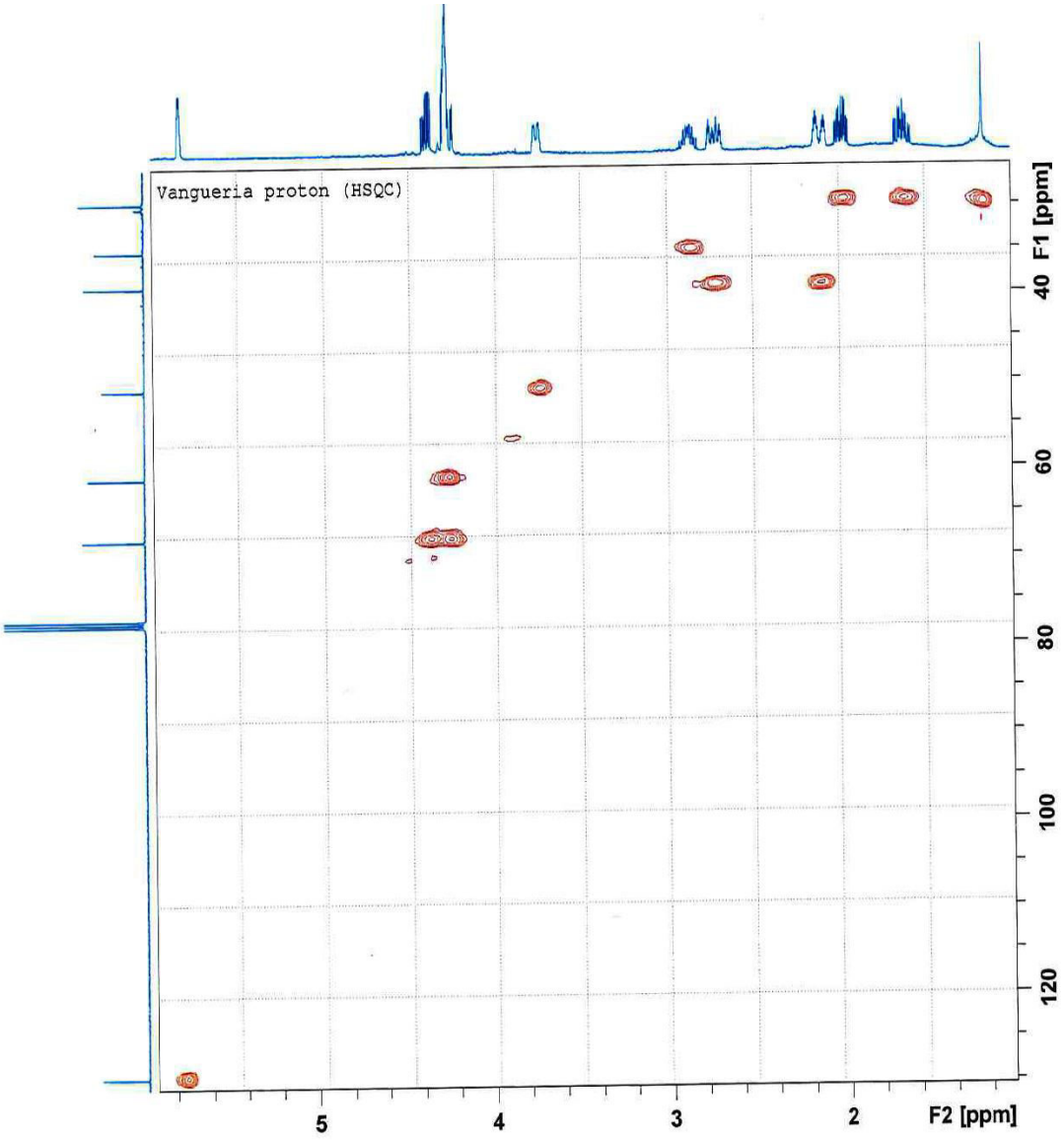

Figure 9.12. HSQC spectrum of morindolide

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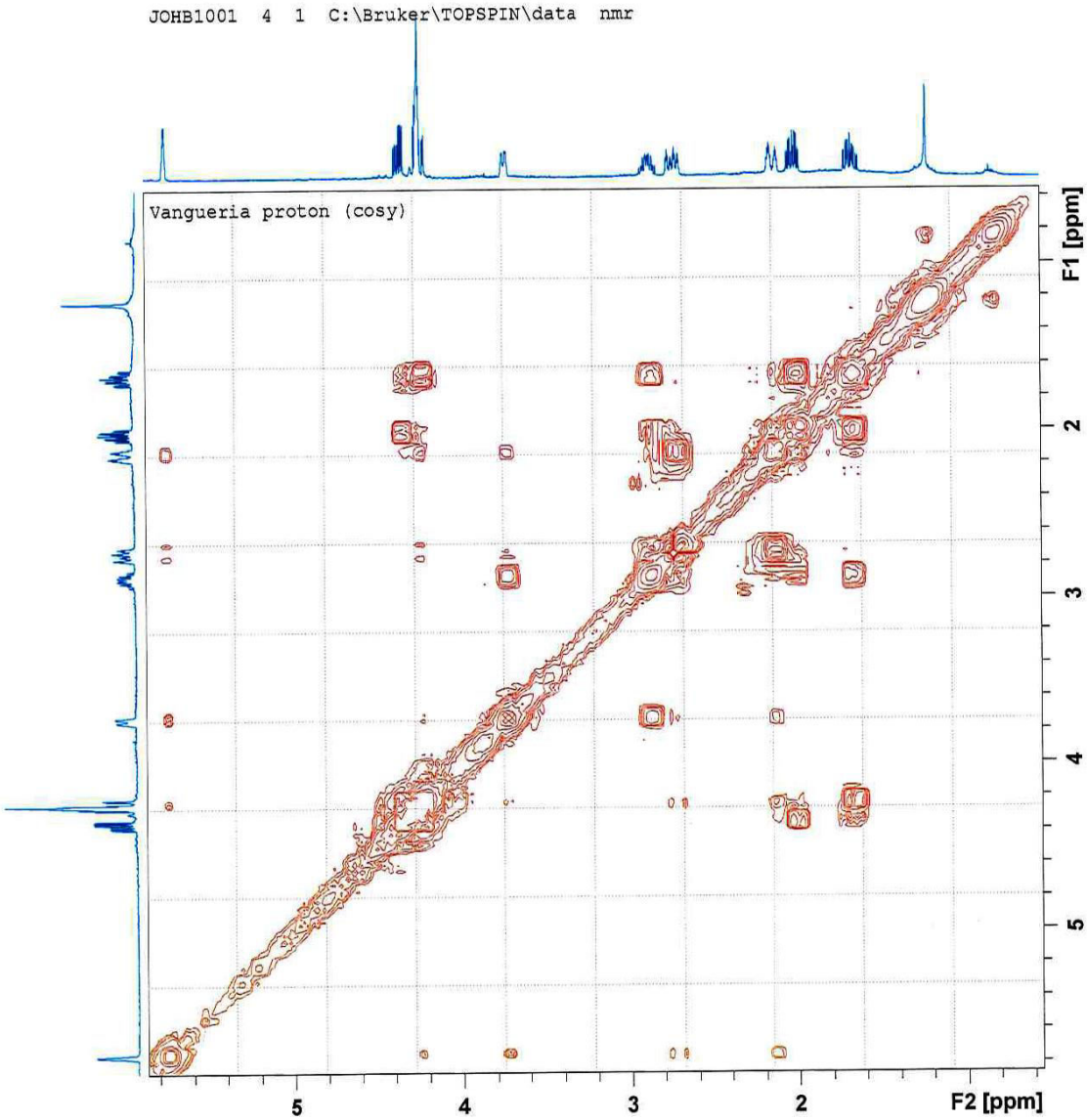

Figure 9.13. H-H COSY spectrum of morindolide

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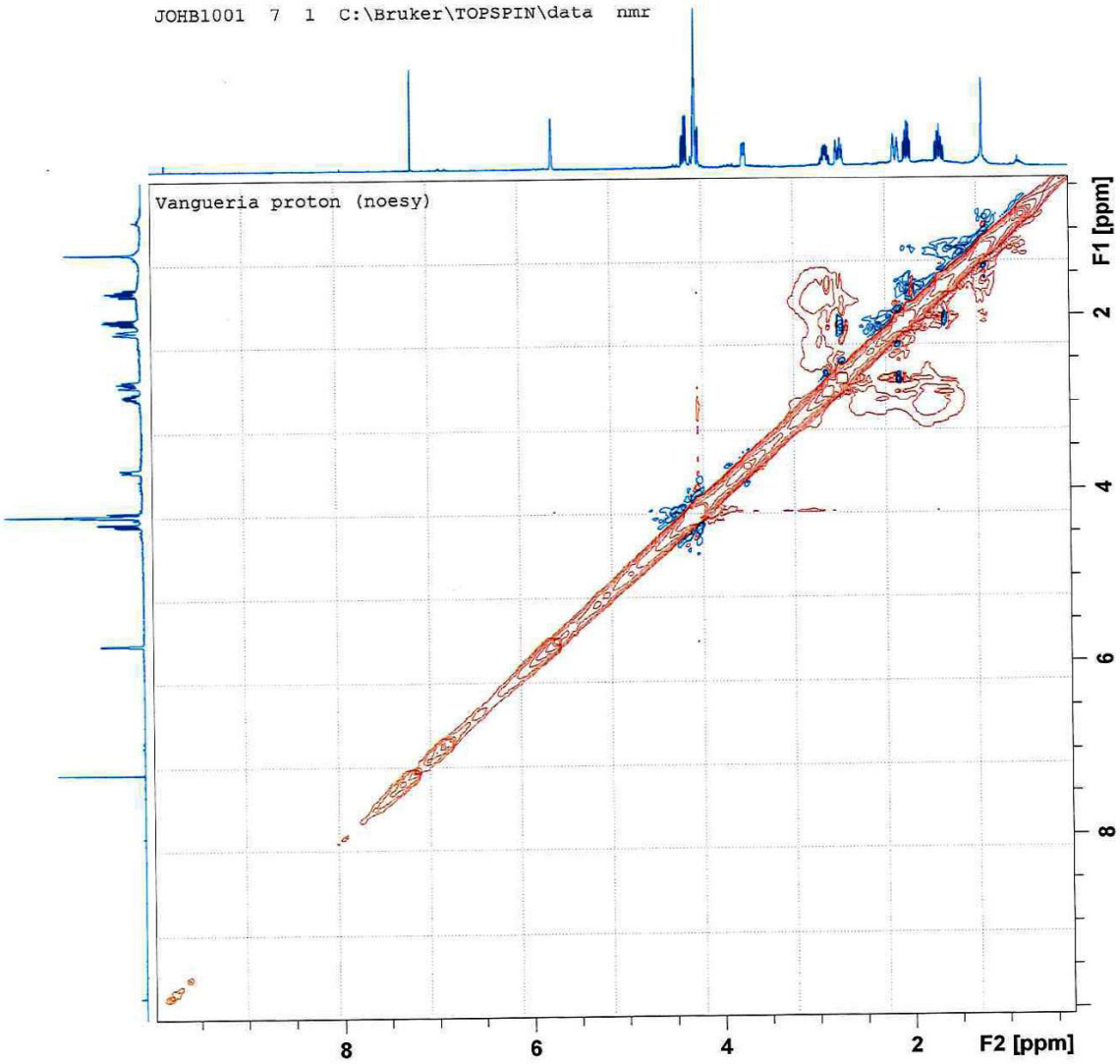

Figure 9.14. NOESY spectrum of morindolide

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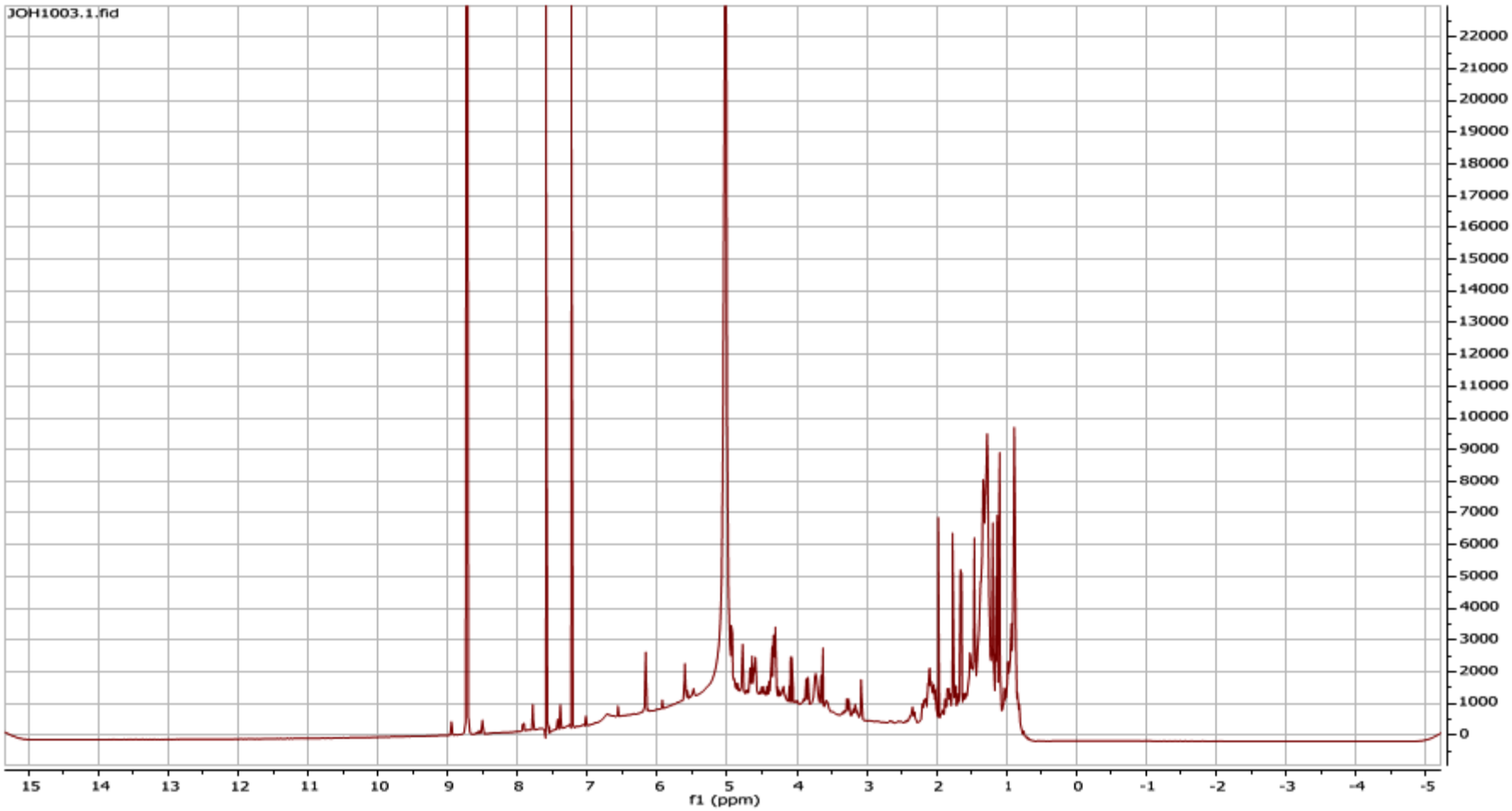

Figure 9.15. ^{1}H NMR spectrum of compound X

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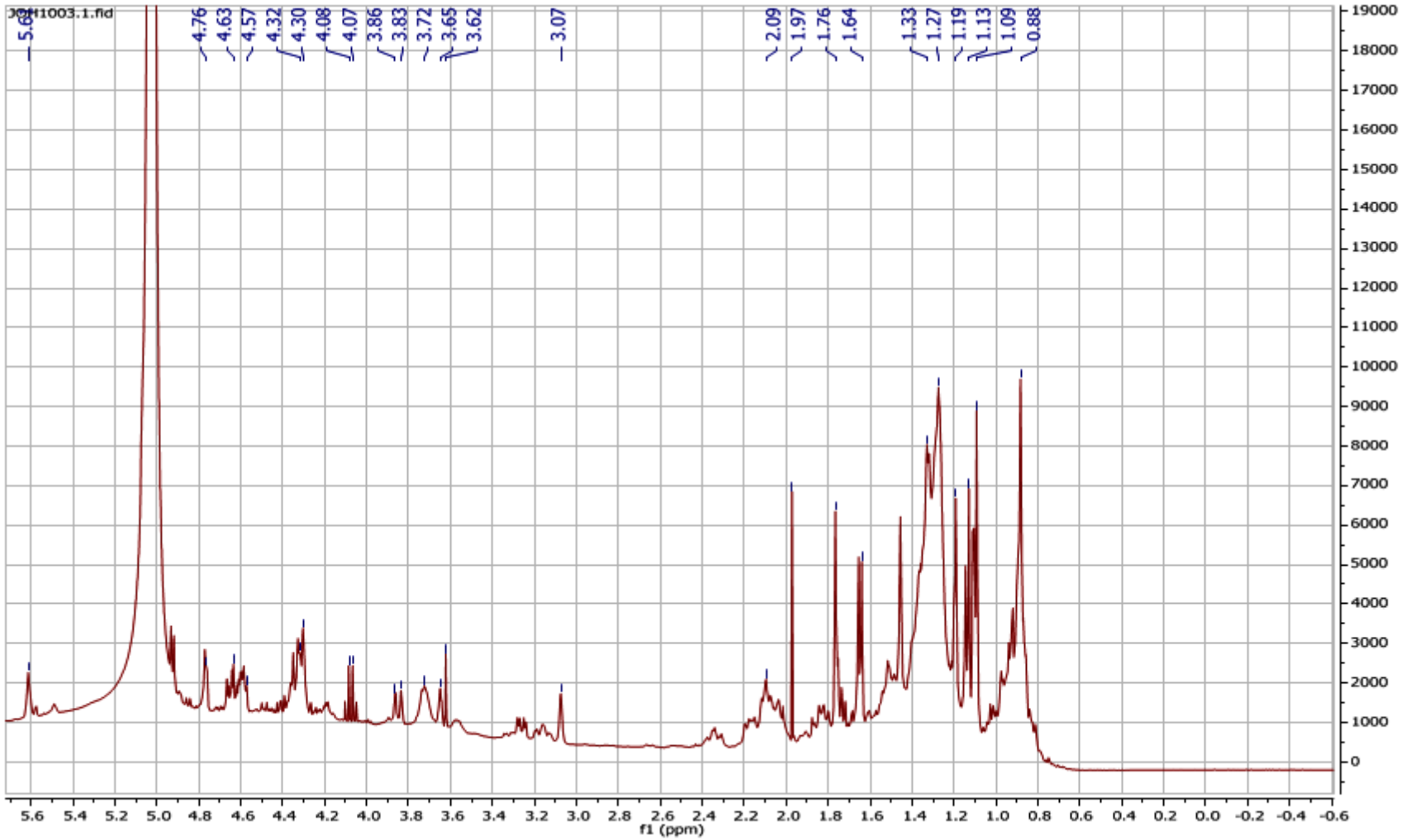

Figure 9.15.1. ^{1}H NMR spectrum of compound X (0.0 ppm – 5.7 ppm)

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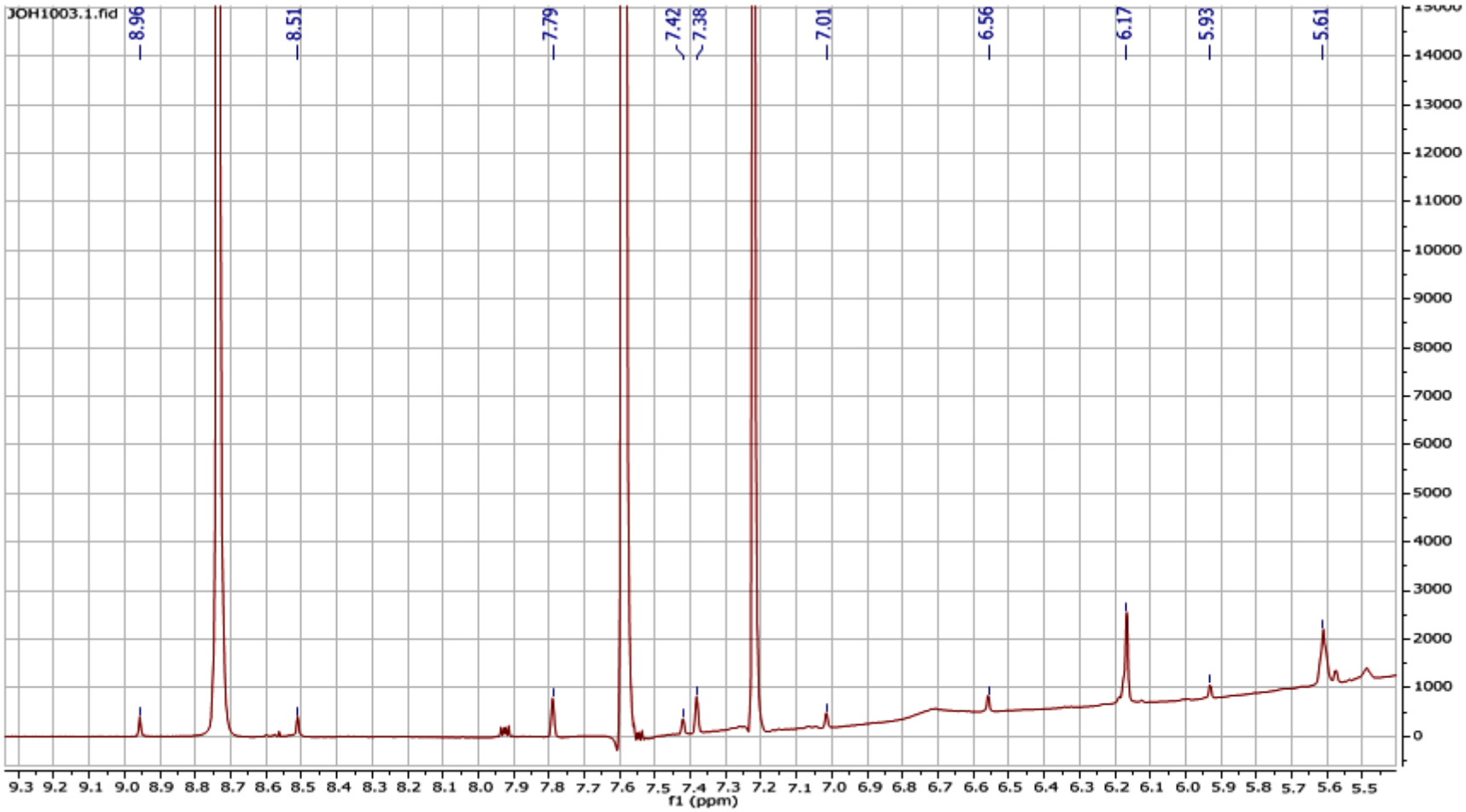

Figure 9.15.2. ^{1}H NMR spectrum of compound X (5.5 ppm – 9.3 ppm)

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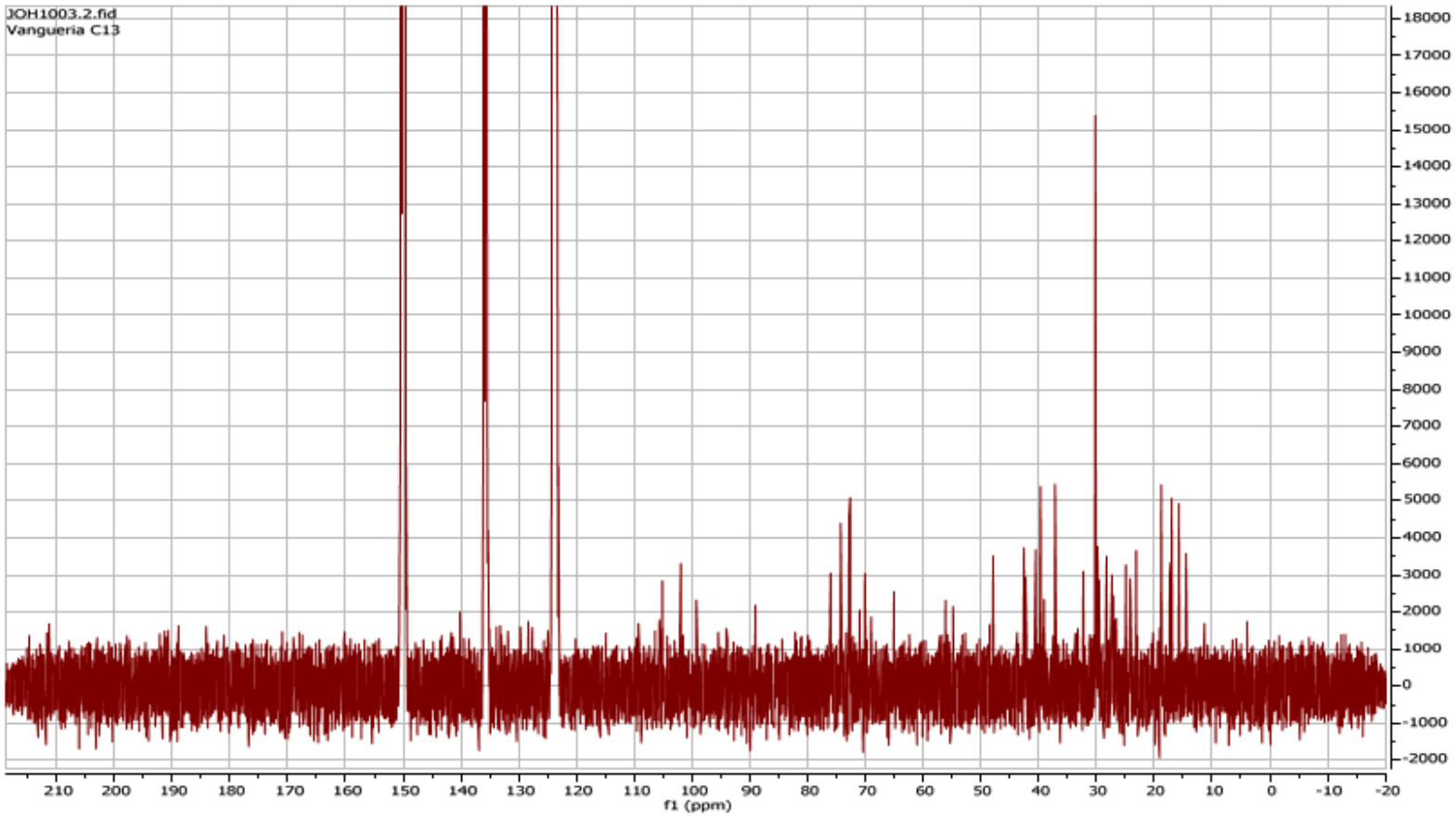

Figure 9.16. ^{13}C NMR spectrum of compound X

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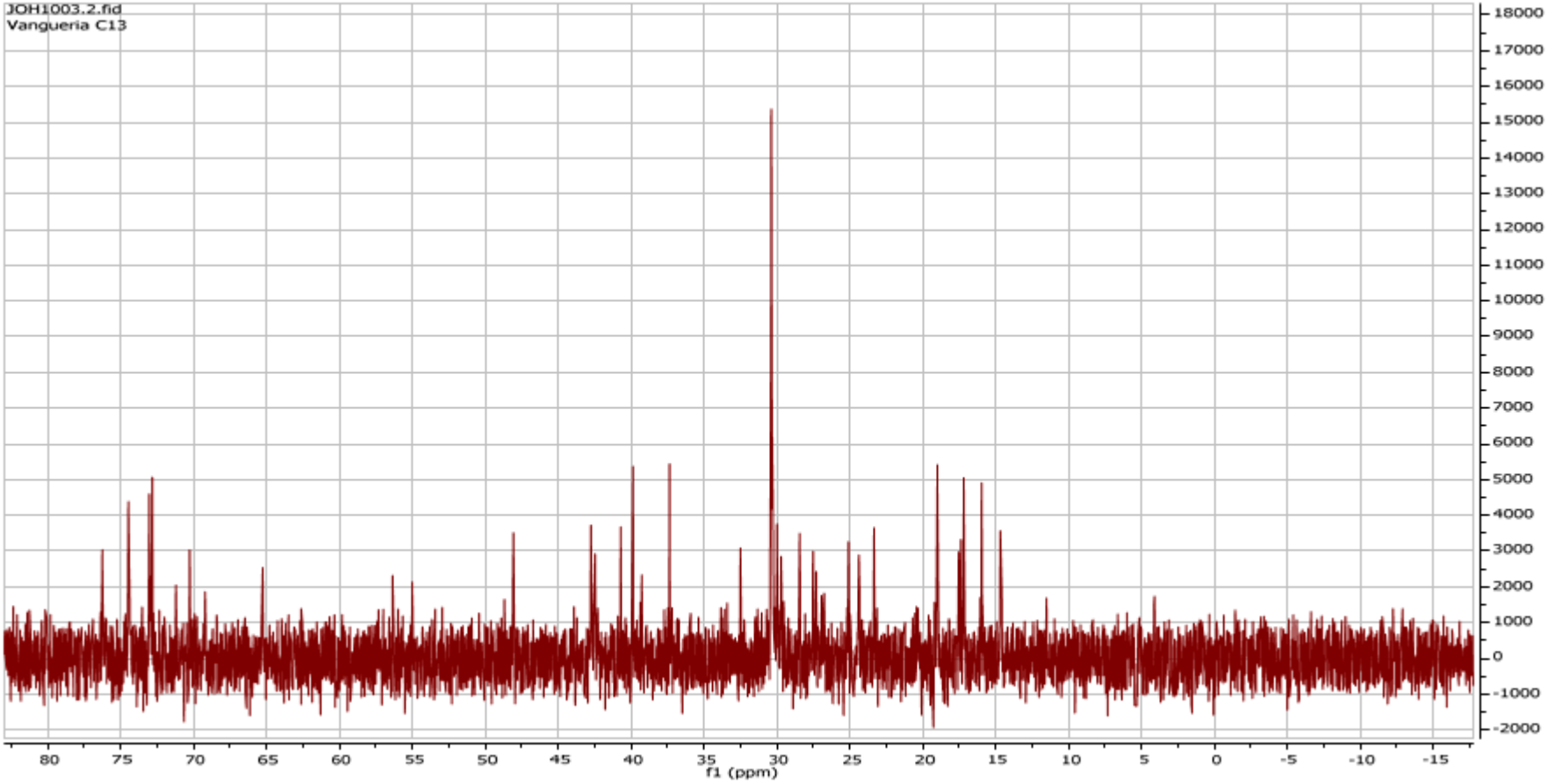

Figure 9.16.1. ^{13}C NMR spectrum of compound X (0 ppm – 80 ppm)

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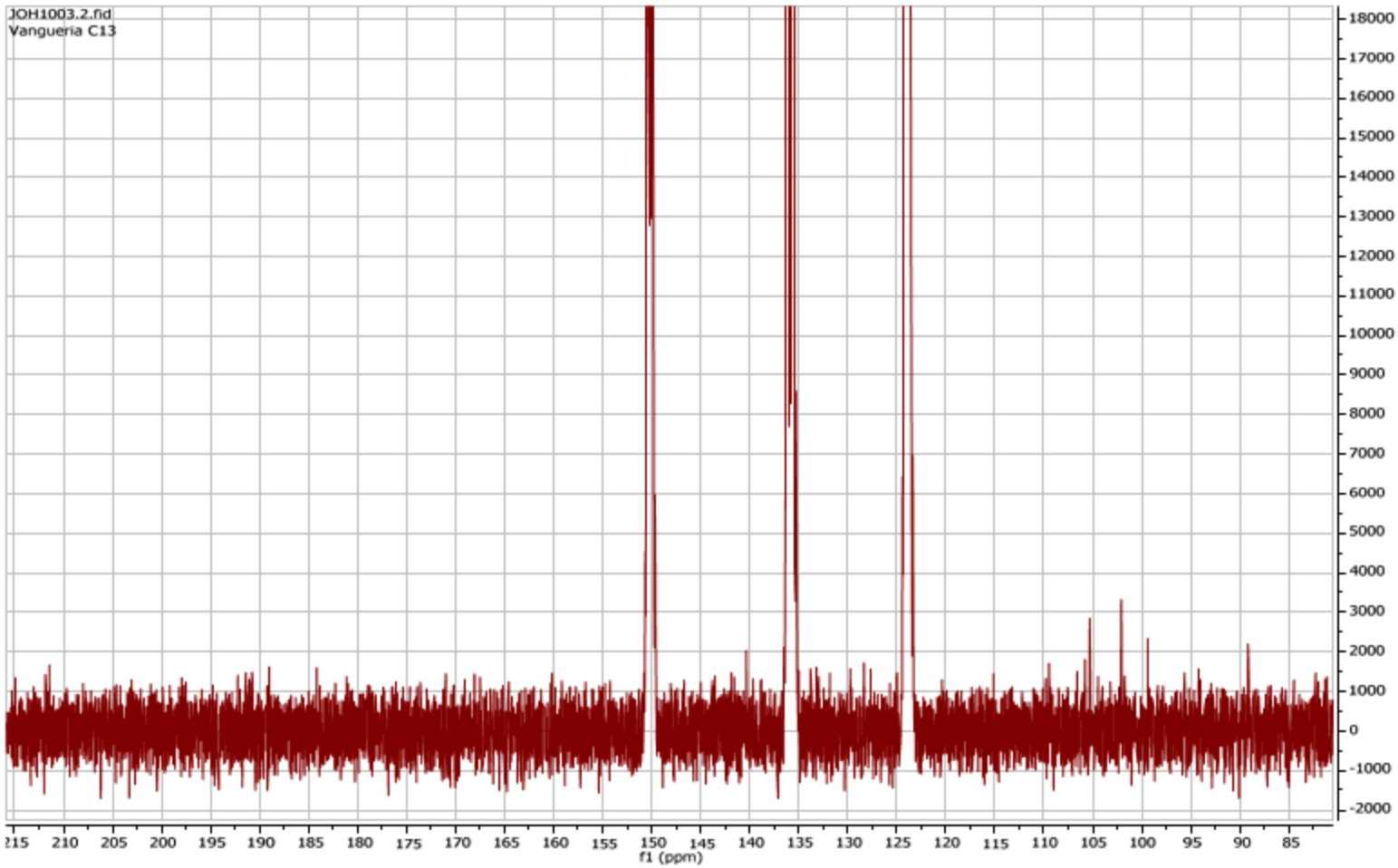

Figure 9.16.2. ^{13}C NMR spectrum of compound X (85 ppm – 215 ppm)

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