Antiplasmodial/Antipyretic activity of some Zulu medicinal plants

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DECLARATION

The work described in this dissertation was conducted in the Department of Biochemistry and Microbiology, Faculty of Science and Agriculture, University of Zululand, under the supervision of Prof. A.R. Opoku, Dr. A. Shonhai and Prof. O.A Oyedeji, The School of Chemistry, Westville Campus, University of KwaZulu-Natal under the supervision of Prof. F.O. Shode, and Division of pharmacology, University of Cape Town, under the supervision of Prof. P. Smith.

This study represents the original work by the author. Where use was made of the work of others, it has been duly acknowledged in the text.

I declare the above statement to be true.

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DEDICATION

This work is dedicated to my darling mother Nethengwe Gladys Maluta.

“And my God will meet all your needs according to his glorious riches in Christ Jesus”. Philippians 4:19

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ABSTRACT

Malaria is one of the major diseases that have partially paralysed the world's health presently. There is, therefore, an urgent need to identify new antimalarial drugs as the *plasmodium* species continues to gain resistance to presently used drugs. In developing communities where malaria is prevalent, people depend strongly on traditional medicine as a source of inexpensive treatment for this disease. *Gardenia thunbergia* T.A Sprague, *Siphonochilus aethiopicus* (Schweif.) B.L Burtt, *Schotia brachypetala* Sond., *Acorus calamus* L., *Withania somnifera* (L) Dunal in DC., *Elaeodendron transvalense* (Burtt Davy) R.H. Archer, *Hypoxis hemerocallidea* Fisch., C.A. Mey.&Ave-Lall., *Vernonia adoensis* Sch. Bip. Ex Walp. and *Acanthospermum australe* (Loefl.) Kuntze) are some of the medicinal plants commonly used by Zulu traditional healers in South Africa to treat malaria.

Aim

The study aims to determine the phytochemicals present in the plants, larvicidal, antioxidant, *in vivo* antipyretic and *in vitro* antiplasmodial activities as well as the cytotoxicity of the nine plants. The study also aims to isolate and purify the active compound in the most active plant extract.

Material and methods

Plants obtained from the *muti* market were botanically identified and were screened for phytochemicals; the appropriate portions of each plant were separately extracted

into dichloromethane, methanol and water solvents. Larvicidal activity against *Culex quinquefascitus* larvae was determined by incubating the larvae with the plant extracts for 24 hours, where after percentage mortality was calculated.

The antioxidant activity of the methanol extracts of the plants was determined by measuring the decrease in the colour of an oxidative system in the presence of the plants extracts. The various antioxidant activities investigated included the free radical (DPPH, ABTS, super oxide, nitric oxide and hydroxyl) scavenging activity, Fe^{2+} chelating, reducing power, and total antioxidant capacity.

Antipyretic activity was determined by treating different groups of pyretic rats with different concentrations of the plants extracts (100 mg/kg, 500 mg/kg and 1000 mg/kg). The pyretic condition was induced by subcutaneous injection of 12% brewer's yeast. Temperatures before and after treatment were also compared. The antimalarial activity of the plants extracts were also screened against the chloroquine sensitive *plasmodium falciparum* D10 strain. Tests were done in triplicate for three concentrations (20 µg/ml, 10 µg/ml and 5 µg/ml). The active extracts were screened for cytotoxicity using the MTT assay. The most active *in vitro* antiplasmodial extract was subjected to isolation, purification and characterization using chromatographic and spectrometric techniques-IR, GC-MS, ^{1}H-NMR, and ^{13}C-NMR.

Results

Phytochemical screening revealed the presence of saponins, terpernoids, flavonoids, anthroquenones, cardiac glycosides; alkaloids (the major active constituents of most antimalarial drugs) were also observed in *A. australe* which, amongst others,

exhibited the most *in vitro* antiplasmodial activity. The plant extracts either killed or reduced spontaneous movement in *Culex quinquefascitus* larvae after 24 hours following treatment. Methanol extracts exhibited antioxidant (DPPH, ABTS scavenging, Fe^{2+} chelating) activity, albeit to varying degree of efficiency. The dichloromethane and methanol extracts significantly ($p \leq 0.05$) reduced pyrexia with activity increasing in a concentration dependent manner. The antiplasmodial activity against chloroquine sensitive strain of *Plasmodium falciparum* (D10) showed that the methanol extracts of *G. thunbergia, V. adoensis* and the dichloromethane extracts of *E. transvalense,* and *W. somnifera* were active (IC_{50} of 1.04-5.07µg/ml).

Although *A. australe* exhibited high *in vitro* antiplasmodial activity, the major compounds (Sitosterol and Stigmasterol) present in the extract did not exhibit any observable antiplasmodial activity.

Conclusion

The results support the use of some of these plants in folk medicine and suggest that these plants contained constituents that could be developed as potent antimalarial drugs (mosquito larvicide, anti-fever and anti-plasmodial).

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LIST OF ABBREVIATIONS USED

AA	Ascorbic acid
ABTS	2,2’-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)
ACTs	Artemisinin based combination therapies
ADS	Antioxidant Defense System
ANOVA	One-way analysis of variance
ASChl	S-acetyle-thiocholine iodide
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
CC	Column chromatography
CMC	Carboxymethylcellulose sodium salt
COSY	Correlation spectroscopy
CQS	Chloroquine sensitive
DEPT	Distortionless enhancement by polarization transfer
DMSO	Dimethyl sulfoxide
DPPH	1,1’-diphenyl-2-pictyhydrazyl
DTNB	2-nitrobenzoic acid
EDTA	Ethylenediaminetetra-acetic acid
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectroscopy
HEK293	Human kidney cell line
HepG2	Human hepatocellular carcinoma cell lines
HMBC	Heteronuclear multiple bond correlation

HPLC	High performance liquid chromatography
HSQC	Heteronuclear multiple quantum coherence
IC_{50}	Inhibition concentration with 50%
IR	Infra-red
IRS	Indoor residual spraying
LC_{50}	Lethal concentration with 50% inhibition
LLINs	Long-lasting insecticide-treated mosquito nets
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MS	Mass spectroscopy
NAD^+	Nicotinamide adenine dinucleotide
NBT	Nitro blue tetrazolium salt
NMR	Nuclear magnetic resonance
NOESY	Nuclear overhauser enhancement spectroscopy
PB	Phosphate buffer
PBS	Phosphate buffer saline
PF	Potassium ferricyanide
ROS	Reactive oxygen species
TBA	2-thiobarbituric acid
TCA	Trichloroacetic acid
TLC	Thin layer chromatography
UN	United Nations
UV	Ultraviolet light
WHO	World Health Organization

CONTRIBUTION TO KNOWLEDGE

(See appendix F for details)

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CHAPTER 1

INTRODUCTION

Malaria, caused by parasites of the genus *Plasmodium* (*P. falciparum, P. vivax, P.ovale, P. malariae* and *P. knowlesi*) is one of most life-threatening diseases, with over 300 million cases and one million deaths per year (WHO, 2008). Pillay *et al.*, (2008) suggest that the large number of malaria death cases in Sub-Saharan Africa is caused by *Plasmodium falciparum,* which is the most dangerous of the human malaria parasites. It was once believed that the disease came from fetid marshes, hence the name mal aria (bad air).

In South Africa, malaria transmission is seasonal showing a significant inter-annual variation in the number of malaria cases with the greatest number of cases occurring between October and May. In the year 2005, it was reported that the annual number of malaria cases was approximately 7755, while in 2006 it was 12,098 (Department of Health, 2007). In South Africa transmission is currently constrained to the low-altitude regions of KwaZulu-Natal, Mpumalanga and Limpopo; three provinces in the North-eastern part of the country (along the border with Mozambique and Swaziland), (Pillay *et al.*, 2008).

Despite exhaustive efforts to control malaria, the disease remains to be one of the greatest health difficulties confronting Africa. Drugs are available for *falciparum* malaria, however, the parasite's increasing resistance to traditionally used antimalarial drugs such as chloroquine and artermisinin-based combination therapies (ACT) enthused search for alternative antimalarial drugs (Afonso *et al.*, 2006). In addition, the disease is well-associated with poverty and underdeveloped communities. Poor people cannot afford mosquito nets and insecticides as a method of prevention of mosquito bites.

Historically, the majority of antimalarial drugs have been derived from plants (Newman *et al.*, 2003). The first used antimalarial drug was quinine originating from the bark of the tree *Cinchona calisaya*. Thus, investigating South African plants as a source of new antimalarial drugs could be a lead to discovering effective antimalarial drugs.

Many plants are used by traditional healers in South Africa to treat malaria (Pillay *et al.*, 2008; Xu *et al.*, 2011; Bero and Quetin-Leclercq Joëlle, 2010). Ethnobotanical survey of traditional healers around Kwa-Zulu Natal (SA) revealed that, in addition to the plants already studied (Clarckson *et al.*, 2004; Pillay *et al*., 2008), *Gardenia thunbergia, Siphonochilus aethiopicus, Schotia brachypetala, Acorus calamus, Withania somnifera, Elaeodendron transvalense, Hypoxis hemerocallidea, Vernonia adoensis* and *Acanthospermum australe* are among the many plants used by Zulu traditional healers to manage malaria. Though these plants are commonly used by traditional healers, their medicinal uses have not yet been scientifically validated. This study aims to investigate the *in vitro* antiplasmodial, larvicidal and *in vivo* antipyretic activity of the extracts of these plants. An attempt will be made to identify and characterise the active constituents responsible for the antimalarial activity in the plant extracts.

CHAPTER 2

LITERATURE REVIEW

Malaria is an incapacitating blood-borne disease caused by a parasite of the genus *Plasmodium (P. falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi),* which is widely spread by the bite of infected female *Anopheles* mosquitoe. It has been reported that about 40% of the world's population living in the most undeveloped countries are at risk of malaria (Maregesi *et al.,* 2009, Xu *et al*., 2011). Over 300 million new cases and 1 million deaths are reported annually (Thomas *et al*., 2010) of which, children under the age of five are the major contributors (WHO, 2008). Malaria often leads to miscarriage in pregnant women at the early stage of pregnancy (Bulter, 1997; Raimi, 2010).

Human blood sucking *Anopheles* are more likely to transmit malaria and the most common, and widely known are *A. funestus* and *A. gambiae*.

There are over 3,000 species of mosquito of which, approximately 100 are vectors of human malaria but of all these, only 30-40 transmit the parasites of the genus *Plasmodium*. The female *anopheles* mosquito is responsible for transmitting malaria; she requires blood to nurture her eggs. Different species such as *culex* and *aedes* mosquitoes transmit other diseases such as filariasis, yellow fever, and many others (Oxfam, 2006; Pohlit *et al*., 2011). Although the *anopheles* mosquito is mainly known as the malaria vector, it has been reported that it also carries arboviruses, including West Nile Virus and Japanese Encephalitis amongst many other (Lehrer, 2010). An association of brain tumour incidence and malaria has been observed (Lehrer, 2010).

2.3 Plasmodium, the species

Plasmodium falciparum is the most dangerous of the *plasmodium* species and responsible for most malaria deaths, especially in Africa. The nucleus, mitochondrion, apicoplast and the microtubules of *Plasmodium* sporozoites are linked to the parasite pellicle via long tethering proteins. The apicoplast is an essential plastid, similar to a chloroplast; however, it is not photosynthetic. The apicoplast seem to be involved in the metabolism of fatty acids, isoprenoids, and heme (Gardener *et al.*, 2002). It is estimated that 551, or roughly 10%, of the predicted nuclear-encoded proteins are targeted to the apicoplast (Gardener *et al.*, 2002). This has become an interesting study of research for new antimalrial drugs since humans do not harbor apicoplasts. *Plasmodium* lacks mitochondrial pyruvate dehydrogenase (McMillan *et al.*, 2005 and Foth *et al.,* 2005) and the hydrogen ion translocating NADH dehydrogenase (Complex I, NDH1-nicotinamide dinucleotide 1). Nishimoto *et al.*, (2008) revealed that *Plasmodium* species have two or three distinct SSU rRNA (18S rRNA) molecules encoded within the genome. These are expressed in the asexual stages, sexual stage and in the oocyte. It is not quite clear why the gene is duplicated.

This species digests the red blood cells haemoglobin changing the adhesive properties of the cell it occupies. Infection by *P. falciparum* can result in a condition known as cerebral malaria, which is caused by sticking of the red blood cells to the capillaries in the brain (Holding and Snow, 2001). *Plasmodium ovale* is a rare infection but can cause relapses (12-17 days incubation). Although *Plasmodium vivax* (12-17 days incubation period) is the most geographically widespread it produces less severe symptoms (Mendis *et al*., 2001). Fatality rate for *P. ovale* and

P. vivax is low provided effective medicine is given (WHO, 2006). On the other hand, *Plasmodium malariae* (18-40 days incubation period) manifests itself like any other malaria infection but can also stay dormant in the blood for a very long period without ever showing any irrefutable manifestation. *Plasmodium knowlesi* causes malaria in apes, but can also infect human (WHO, 2006).

2.2 The Malaria cycle

Two hosts are involved in the life cycle of malaria; female *Anopheles* mosquitoes as the primary host and humans as the secondary host. The parasite is transmitted from person to person through the bite of an infected female *Anophele* mosquito. The infected (ingested the parasite from a previously infected human) female *Anapheles* mosquitoes carry *Plasmodium* sporozoites in their salivary glands. Once ingested by the primary host, the parasite gametocytes taken up in the blood will further differentiate into male or female gametes and then fuse in the mosquito's gut (the parasite begins to reproduce sexually i.e. by merging the parasite's sex cells). Ookinete that penetrates the gut lining are then produced and these produce an oocyst in the gut wall which ruptures to release sporozoites. Sporozoites are stored in the salivary glands of the mosquito where it mixes with the primary host's saliva to be released into the human host blood stream during their next blood meal (Fujioka and Aikawa, 2002).

Malaria develops by means of two stages: the infection of the hepatic system or liver (exoerythrocytes stage) and the infection of the erythrocytes or red blood cells (erythrocytic stage). When infected female mosquitoes bite their next victim they

inject their saliva containing sporozoites into the victim's bloodstream. Sporozoites then enter into the hepatocytes in the liver where asexual division cycle takes place for 2 weeks, each sporozoite develops into a schizont (a structure that contains thousands of tiny merozoites) and thousands of merozoids are produced. Merozoites released from the liver enter the bloodstream and infect the victim's red blood cells, where each merozoite produces up to 20 merozoites thus beginning the erythrocytic stage of the life cycle. The parasite escapes from the liver undetected by wrapping itself in the cell membrane of the infected host liver cell (Sturm *et al.,* 2006).This accumulation of merozoites causes the red blood cells to burst releasing merozoites. As the number of parasites increase the symptoms start to show. Merozoites infecting the red blood cells go through different stages for 48 hours again forming schizonts filled with yet more merozoites (see figure 2.1). Several such escalation cycles occur. Thus, classical descriptions of fever conditions arise from concurrent waves of merozoites escaping and infecting red blood cells. Pathogenesis of malaria is a result of this cycle which occurs in red blood cells. When *Plasmodium falciparum* parasites stick to the walls of small blood vessels; it causes the small blood vessels to block; this decreases the supply of oxygen and blood to the tissues. All these factors are responsible for causing malaria (Miller *et al*., 2002).

Alternatively, some *P. vivax and P. ovale* sporozoites turn into hypnozoites, a form that can remain dormant in the liver for months and even years. The hypnozoites cause relapse in infected individuals by producing merozoites if they become activated again.

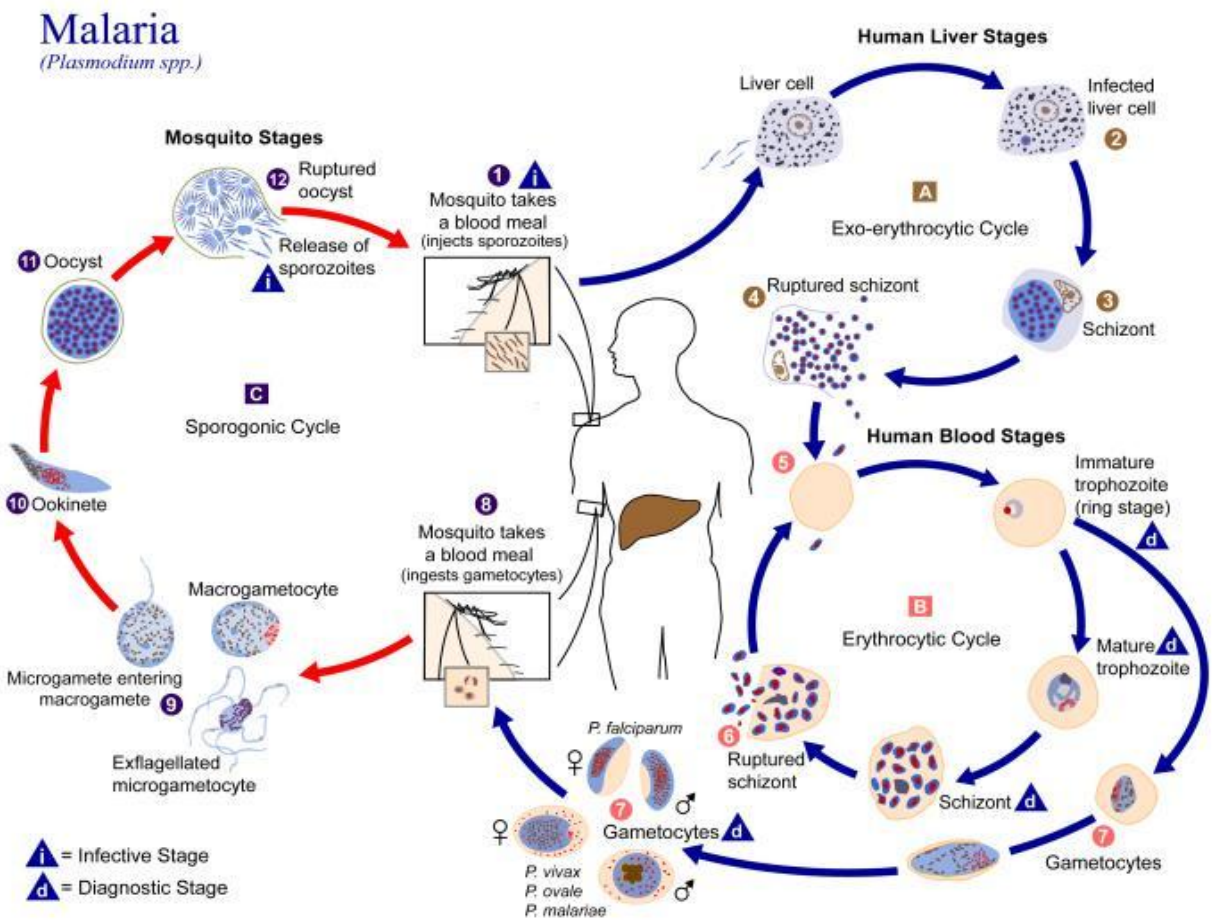


Figure 2.1 The life cycle of *Plasmodium falciparum* (adapted from http://upload.wikimedia.org/wikipedia/commons/0/0b/Plasmodium_lifecycle_PHIL_3405_lores.jpg)

2.9 Prevention of malaria

Malaria is preventable and curable given that proper precaution and treatment are taken into consideration. The United Nations (UN) placed forward a vision that is to halt malaria deaths by ensuring universal coverage of malaria interventions (WHO, World Malaria report, 2008; WHO, World Malaria report, 2010). There are many methods that are aimed at preventing malaria infection. Such methods include

indoor residual spraying (IRS), mosquito nets, long-lasting insecticide-treated mosquito nets (LLINs), and bedclothes (WHO, 2006; 2007; 2010).

Malaria can also be prevented by controlling malaria vector. The IRS helps to reduce the life span of the female mosquito as IRS will normally kill the mosquito before they can infect the next victim and thus human contact is reduced (WHO, 2010). Larvae control has also been of research interest lately, but this kind of vector control is, however, limited to the mosquito breeding sites.

Although several vaccines are under development, effective vaccine for malaria is not yet available (Rathore and Mccutchan, 2000; Graves and Gelband, 2006; Färnert *et al.*, 2009).

The survival of *Plasmodium falciparum* in the stressful environment it encounters in the human host has been attributed to the production of heat shock proteins (Shonhai, 2010) Heat shock proteins are a class of functionally related proteins involved in facilitating protein folding in cells of living organisms. They act as molecular chaperones. Through their action it has been proposed that heat shock proteins enable the parasite to become resilient to subsequent physiological threats, thereby enhancing parasite pathogenicity (Pavithra *et al*., 2004). Heat shock proteins are, therefore, becoming the molecular targets of newer antimalarial drugs (Banumathy *et al.*, 2003; Kumar *et al.*, 2003; Shonhai 2010).

2.10 Symptoms of malaria

The first symptoms of malaria are nonspecific and similar to the symptoms of a minor systemic viral illness. Symptoms of malaria include fever, shivering, vomiting, anaemia (caused by haemolysis), arthralgia (joint pain), hemoglobinurria, retinal

damage (Beare *et al*., 2006) and convulsion. Idro *et al*., (2005) revealed that children with malaria frequently exhibit abnormal posturing, a sign indicating severe brain damage. Though children are more vulnerable to direct brain damage, this neurologic damage is also manifested in adults resulting from cerebral malaria (Boivin, 2002; Holding and Snow, 2001). *P. falciparum* infection is a major cause of severe malaria (Trampuz *et al*., 2003) resulting in coma and death if left untreated. Victims of severe *P. falciparum* malaria are most likely to experience liver or kidney failure and problems related to the central nervous system. The parasite can remain inactive in the liver causing reappearance of symptoms for months or years later despite the fact that some of these infections cause fewer consequences or are not as life threatening.

Fever (pyrexia) is a common medical sign reflected by an elevation of temperature above the normal range of 36.5–37.5 °C (98–100 °F) (Karakitsos and Karabinis, 2008). It is the major common symptom of malaria. This kind of fever is periodical occurring every two days in *P. vivax* and *P. ovale* infections lasting four to six hours. In *P. malariae* this emerges every three days (Malaria life cycle & pathogenesis, 2006). On the other hand *P. falciparum* can have recurrent fever every 36–48 hours.

2.11 Treatment of malaria

Malaria treatment depends on the severity of the disease and the type of malaria infection.

Until recently, the most used treatment for severe malaria was quinine (a natural white crystalline alkaloid that occurs naturally in the bark of the *cinchona* tree).

Although quinine drugs have been relied upon for years, their ineffectiveness has been attributed to the evolvement of the *Plasmodium* species. Quinine was nearly abandoned in favour of chloroquine. It was brought back on the market when the falciparum form of malaria became chloroquine resistant, but retained sensitivity to quinine (Yaniv and Bachrach, 2005). Artemisinin, also known as Qinghaosu, and its derivatives are a group of drugs that are known to possess the most rapid action of all current drugs against strains of *P. falciparum* and *P. vivax,* and are well tolerated (Douglas *et al*., 2010). Artsunate (a semi-synthetic derivative of artemisinin) has been shown to be superior to quinine in both children (Dondorp *et al*., 2010) and adults (Dondorp *et al*., 2005). The WHO, however, recommends combinations of antimalarials for the treatment of falciparum malaria (WHO, 2006) as there are concerns that artemisinin-based monotherapy compromise the therapeutic life cycle of artemisinin based combination therapies (ACTs) as this may favour the spread of its resistance (WHO, 2010).

Although medication is currently available and the number of ACTs procured has increased in every year since 2005, there is little information on the sufficient availability of antimalarial medicines in the public and private sector to meet the patients' needs (WHO, 2010). People living in poor communities, however, continue to meet challenges in the share of these antimalarial drugs and, therefore, depend wholly or partly on traditional medicine.

2.6 Traditional medicine

In developing countries traditional medicine still plays an important role in local health care systems (Xu *et al*., 2011). The WHO estimates that up to 80% of some Asian and African countries depend on traditional medicine for their health care

needs (Xu, 2011; WHO, 2008). It is difficult to assign one definition to the broad range of characteristics and elements of traditional medicine. The WHO thus defines traditional medicine as the health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral-based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illness or maintain well-being (WHO, 2008). Chan (2005) concludes that traditional healing is "holistic" in nature. It does not focus on symptoms or diseases. Instead, it deals with the total individual. Healing focuses on the person, not just the illness and healing does not follow written guidelines, that is, different people may receive the same medication or treatment even if they suffer from different diseases, in contrast to modern medicine where different people may receive different treatments even if they suffer from the same disease.

Plants have been a source of medicine throughout the history of medicine. For thousands of years natural compounds, mostly from plants, have been the manifestation of traditional medicine (Ginsburg and Deharo, 2011). Although there is no clear distinction as to when an herb stops to be a health food and when it becomes a medicine, many food plants are used for therapeutic purposes (Iwu, 1993). These include aromatic substances, most of which are phenols or their oxygen-substituted derivatives such as tannins. In many cases, these substances (particularly the alkaloids) serve as plant defence mechanisms against predation by microorganisms, insects, and herbivores.

Larvicidal activity of different medicinal plants has been reported: *Zingiber officinale* (Ginger) exhibited larvicidal activity against *Anisakis simplex* (Lin, 2010), *Striga hermonthica* and *Mitracarpus scarber* against *Culex quinquefasciatus* (Abdullahi *et*

al., 2011) and *Tithonia diversifolia* on *Anopheles gambiae*, *Aedes aegypti* and *Culex quinquefasciatus* (Oyewole *et al.*, 2008).

Many plants have been reported to have antimalarial activity. Such plants include: *Ormocarpum kirkii* (Xu *et al*., 2011; Maregesi *et al*., 2009), *Bidens pilosa* (Tobinaga *et al*., 2009), *Combretum adenogonium*, *Euphorbia titrucalli* (Maregesi *et al.,* 2009), *Vanguera infausta*, *Ozoroa engleri, Balanites maughamii*, *Ozooroa insingitis* and *Balanites aegyptiaca* (Pillay *et al.*, 2008). Literature abounds with many compounds with antimalarial activity that have been isolated and characterized from plants. Such compounds include methyl uguenenoate and furoquinoline alkaloids isolated from *Vepris uguenensis* (Cheplogoi *et al.*, 2008), 2'-hydroxy-3,4,4',5,6'-pentamethoxychalcone isolated from ground fruits of *Neuraputia magnifica* var. *magnifiifica* (Bero and Quentin-Leclercq, 2010), β-hydroxydihydrochalcone named (s)-ela- tadihydrochalcone isolated from the seedpods of *Tephrosia elata* (Bero and Quentin-Leclercq, 2010), an isoprenylated flavone, and atropeden A isolated from the bark of *Artocarpus champeden* (Bero and Quentin-Leclercq, 2010).

2.7 Interview with Zulu Traditional Healers

The ethno-pharmacological interview of Zulu traditional healers (see Appendix C) confirmed that plants used to treat malaria diseases may be used for other medicinal purposes (i.e. to treat other disease as well). It was also noted that almost every part of a plant has a medicinal purpose though often the leaves and roots are used in most plants. It was found that some of the plants used work in combination with other plants. The solvent used most when preparing the concoctions seemed to be water, since it is easy to access and it is harmless. A few of the medicines used by traditional healers have side effects if abused and very few are age restricted.

Several different plants were mentioned by the Traditional Healers for the management or treatment of malaria. Among the plants mentioned (by most traditional healers) were, *Gardenia thunbergia* T.A Sprague, *Siphonochilus aethiopicus* (Schweif.) B.L Burtt*, Schotia brachypetala* Sond., *Acorus calamus* L., *Withania somnifera* (L) Dunal in DC., *Elaeodendron transvalense* (Burtt Davy) R.H. Archer*, Hypoxis hemerocallidea* Fisch., C.A. Mey.&Ave-Lall., *Vernonia adoensis* Sch. Bip. Ex Walp. and *Acanthospermum australe* (Loefl.) Kuntze), (see figs. 2.2 to 2.9).

2.8 The Medicinal plants

2.8.1 *Siphonochilus aethiopicus* (Schweif.) B.L Burt (Zingiberaceae)

S. aethiopicus is a forest plant with aromatic rhizomatous root. It is also known as the wild ginger in English, and *indungulo* or *isiphephetho* in isiZulu. It belongs to the Zingiberaceae family. The generic name *Siphonochilus* is derived from the Greek *siphon* meaning tube, and *chilus* meaning lip in reference to the shape of the flower. The specific name *aethiopicus* means from Southern Africa (Hutchings, 1996).

Figure 2.2 The purple flowers and green leaves of the *Siphonochilus aethiopicus*. (www.plantzafrica.com/plantqrs/siphonaeth.htm)

Wild ginger is found in Mpumalanga, Northern Province, Swaziland and KwaZulu-Natal. Wild ginger (Fig 2.2) is a deciduous plant with large hairless leaves developing annually from a small, cone-shaped rhizome (Hutchings 1989); the flowers appear in early summer at ground level. The leaves are deciduous and sprout annually from the underground stem in spring; they may reach a height of up to 400 mm. The leaves are light green, lance shaped and borne on the end of stem-like leaf bases.

The highly aromatic roots have a variety of medicinal and traditional uses and the native South African people have cultivated this plant for many years. It is used by Zulu people as a protection against lightning and snakes (Hutchings, 1996). Fresh rhizomes are taken for colds, coughs, influenza, etc. (Watt and Breyer-Brandwijk 1962; Pujol, 1990; Hutchings, 1996). Traditional uses include the treatment of asthma, malaria and many other diseases (Crouch, 2000). Infusion of the rhizome and roots are used to treat epilepsy, hysteria and relieve dysmennorhoea. The Swazi use the plant for the treatment of malaria (Dyer, 1963). Cold rhizome infusions of

wild ginger are also administered to horses as prophylactics against horse sickness (Watt and Breyer-Brandwijk, 1962).

The conical rhizome and roots contain a high percentage of a sesquiterpenoid and monoterpernoids (Verotta & Rodgers 1997). Extracts of the rhizome have been demonstrated to be anti-inflammatory (prostaglandin-synthetase inhibition), bronchodilatory, smooth muscle relaxant, mild sedative, and anti-candidal (Verotta and Rodgers, 1997).

2.8.2 *Acorus calamus* L. (Acoraceae)

It is commonly known as Sweet-flag and *Ikalamuzi* in isiZulu. Its habitats are moist soils and shallow water in ditches, marshes, river edges and ponds.

Figure 2.3 Acorus calamus (www.hlasek.com)

Acorus calamus is a reed-like, perennial, aquatic plant with long, thin, sword-shaped leaves growing from a long creeping rhizome (Fig 2.3). Its flowers are compactly arranged on a long, fleshy axis surrounded by a large leaf-like spathe. *Acorus calamus* has been tested for numerous activities such as anticellular and

immunosuppressive activity (Mehrotra *et al.,* 2005). Its antimicrobial (Bhuvaneswari and Balasundaram, 2007; Asha and Ganjewala, 2009; Singh *et al.,* 2010) and antiinflammatory (Hyeri *et al*., 2008) activity has also been reported.

2.8.3 *Gardenia thunbergia* T.A Sprague (Rubiaceae)

Gardenia thunbergia is an evergreen shrub or a small tree with a smooth, whitish, usually straight main stem found in forest, occurring in the Eastern Cape, KwaZulu-Natal and Transkei in South Africa. It is also known as white gardenia, forest gardenia, wild gardenia (Eng.) and *umValasangweni* or *umKhwakhwane* in isiZulu. It belongs to the family of Rubiaceae, which includes plants such as coffee (Coffea arabica), quinine (Cinchona) and numerous ornamental trees and shrubs with showy flowers (Fig 2.4).

The leaves are smooth, shiny and clustered at the ends of branchlets. They are a glossy light green, hairless, softly to thinly leathery and conspicuously veined. The flowers are large, creamy white and extremely fragrant, particularly at night. *Gardenia* nectar contains fragrant essential oils which serve to attract insects for pollination. Often plants will become yellow (chlorotic) owing to a deficiency of one or more micronutrients usually iron (Bradshaw, 2003).

Traditional healers use the roots of *G. thunbergia* as a treatment for numerous ailments, including skin diseases, skin lesions caused by leprosy, and as an emetic against fever. The root bark is used as an emetic for biliousness and to treat gall bladder problems. The roots and leaves are used in various parts of Africa by traditional healers to treat syphilis, and the latex is used as a purgative.

Figure 2.4 The leaves and flowers of *Gardenia thunbergia* T.A Sprague (Rubiaceae)

http://en.wikipedia.org/wiki/file:*Gardenia thunbergia*385207587.jpg

The wood is heavy, dense, extraordinarily hard and a pleasing yellowish colour. It also has the unusual ability to bend without breaking, but its use is limited because it is difficult to find large pieces.

2.8.4 *Schotia brachypetala* Sond. (Fabaceae)

It is a medium to large tree with a wide-spreading, densely branched, rounded crown. Flowers are rich deep red, and are produced in masses (Fig 2.5). The seeds are edible after roasting. It belongs to a family, Fabaceae and subfamily, Caesalpinioideae. Its common names are the Weeping Boer-Bean, Huilboerboon, Tree Fuchsia, African Walnut. It is known as *Ihluze* in isiZulu.

(a)

(b)

(c)

Figure 2.5: *Schotia brachypetala's* wide-spreading, densely branched, rounded crown (a) Rich deep red flowers (b) Edible seeds (c).
(http://www.plantzafrica.com/plantqrs/schotiabrachy.htm)

Trees can reach a height of 20 m, but most commonly grow 5 to 10 m with a spread to 15 m. The bark is rough and brown or grey brown. The leaves are compound, with 4 to 6 pairs of leaflets. A decoction of the bark is taken to treat heartburn and hangovers. Bark and root mixtures are used to strengthen the body and purify the blood, to treat nervous heart conditions and diarrhoea, as well as for facial saunas. Study done by Ramalivhana *et al.*, (2010) revealed that *S. brachypetala* possess antimicrobial constituents against extended spectrum beta-lactamase producing *E.coli*, *E cloace* and *P. aeroginosa*. The seeds are edible after roasting, and although low in fat and protein they have high carbohydrate content.

2.8.5 *Withania somnifera* (L) Dunal in DC. (Solanaceae)

Withania somnifera (Fig 2.6) is commonly known as Winter cherry and *Ubuvimbha* in isiZulu. This medicinal plant is distributed throughout dry regions of India where it can also be cultivated.

Figure 2.6: *Withania somnifera* (L) Dunal in DC. (Solanaceae)

(drprabhattandon.wordpress.com)

It is about 150 cm in height, leaves ovate up to 10 cm long. It bears greenish or lurid yellow flowers and fruits which are orange coloured when mature. *W. somnifera* is claimed to have potent aphrodisiac rejuvenative and life prolonging properties. It is used to treat insomnia, skin problems and coughing; its immunomodulatory activity (Davis and Kuttan, 2000), and antiangiogenic (Mathur *et al*., 2006; Mohan *et al.,* 2004) activity has been previously studied.

2.8.6 *Acanthospermum australe* (Loefl.) Kuntze)

This is known as *Unsukumbili* in isizulu. The plant stem prostrate, often rooting at the nodes, 1-6 cm long**.** Leaves are rhombic-ovate to triangular, 1-3 cm wide, with conspicuous glands on both surfaces, margins irregularly serrate above the middle, base cuneate (Fig 2.7).

Figure 2.7: *Acanthospermum australe* (Loefl.) Kuntze). (luirig.altervista.org)

2.8.7 *Elaeodendron transvalense* (Burtt Davy) R.H. Archer (Celastraceae)

This plant (Fig 2.8) is commonly known as Transvaal Safronwood and *Ingwavuma* in isiZulu. It is found in deciduous woodland, along streams and on rocky hillsides.

Figure 2.8: *Elaeodendron transvalense* (Burtt Davy) R.H. Archer (Celastraceae) (www.plantzafrica.com)

It is a multi-branched small shrub tree, which is usually around 5 meters in height. Its leaves are about 50 mm long and 20 mm wide, with firm texture. The leaf margins are sometimes toothed. The bark is generally smooth and has a pale grey colour. *E. transvalense* is prescribed by traditional healers to people who suffer from HIV/Aids (Bessong and Obi, 2006).

2.8.8 *Hypoxis hemerocallidea* Fisch., C.A. Mey.&Ave-Lall

Figure 2.9: *Hypoxis hemerocallidea* Fisch., C.A. Mey.&Ave-Lall. (www.plantzafrica.com)

Hypoxis hemerocallidea (Fig 2.9) is commonly known as *inkomfe* in isiZulu. Leaves of *Hypoxis* projects directly from the apex of the rootstock and the arrangements differ from species to species. Leaves range from linear to broadly lance-shaped and are usually hairy (Singh, 1999; Singh, 2007; Sathekge, 2010).

The rootstocks of *Hypoxis hemerocallidea* are traditionally used to treat a wide variety of ailments such as type 2, diabetes, pimples and wounds. (Musabayane *et al*., 2005). *Hypoxis* was previously placed in Amaryllidaceae and Liliaceae families based on the similarity in appearance to members within these families. It was later discovered that the aerial parts of *hypoxis* are covered with soft hair and this feature distinguishes them from Amaryllidaceae (Betto *et al*., 1992; Sathekge, 2010). Sitosterol or phytosterol are compounds that have been previously isolated from *Hypoxis*, which is an immuno-enhancer (Nair *et al*., 2008).

2.8.9 *Vernonia adoensis* Sch. Bip. Ex Walp

Figure 2.10: *Vernonia adoensis* Sch. Bip. ex Walp (Bronberg plants)

It is a shrub that grows up to 2 m high**.** It is found mainly in the savanna, from Senegal to Nigeria, and extending across Africa to Ethiopia. It is known as *Inyathelo* in isiZulu.

The leaves (Fig 2.10) are used in Kenya by the Masai crushed in cold water and applied to cattle sores caused by ticks (Dalziel, 1937). The root is prepared in Northern Nigeria into a bitter medicine and is used as a digestive and appetiser. In Southern Nigeria the root is used as a chew-stick (Dalziel, 1937). Root-infusion is taken for stomach-pains, and for tuberculosis, and fresh roots sliced and cooked with milk and flour for gonorrhea. The Sukuma cut up and soak the fresh roots for a short time in water, which is used to wash white blotches, which feel a little ‘soapy’, on the skin of children. (Dalziel, 1937).

2.9 Free Radicals

Relationship between free radical and malaria

In a stable molecule, the core is surrounded by pairs of negatively charged electrons. Removal of one electron of a pair, a process called oxidation, will cause the molecule to become unstable and destructive. The name of this new molecule is "free radical" (Pawlak, 1998). These free radicals are highly reactive and seek stability through electron pairing with biological macromolecules in healthy human cells. If not regulated properly, excess reactive oxygene species (ROS) can damage cell's lipids, protein or DNA and disturb normal function (Bibhabasu *et al*., 2008).

Free radicals and other ROS are compounds found naturally in the body. Increased levels of ROS have been implicated in the pathology of several human diseases, including atherosclerosis, malaria, cancer, and neurodegenerative diseases (Okezei, 1998). Oxidative stress is initiated by free radicals resulting in fever as a signal of the body's invasion and also other diseases (Keller *et al*., 2005; Guidia *et al.,* 2006, Bibhabasu *et al*., 2008).

Of all infectious agents, malaria parasites have the most intensely observed association with free radicals (Clark, 1988); parasites responsible for malaria infection are very vulnerable to oxidant stress during the part of their life cycle when they populate erythrocytes. It has been reported that anti-malarial drugs invoke oxidative stress in the parasite, consequently inducing heat shock proteins that act as a second line of defence against thermal stress damage (Akide-Ndunge *et al.*, 2009).

Humans are equipped with a defense system that controls (ROS) and mends oxidative damage. This defense system is referred to collectively as the Antioxidant Defense System (ADS) and acts to regulate oxidative reactions. The ADS includes enzymes (superoxide dismutase, catalase) and other compounds (Vitamin C, vitamin E, glutathione etc.) that help to prevent the start of oxidative damage and/or control its spread (Bibhabasu *et al*., 2008, Okezei,1998). Antioxidants are either endogenous (made by the body) or exogenous (consumed) (Guidia *et al.,* 2006). Plants are a major source of antioxidants; the antioxidant activity of some Zulu medicinal plants have been reported (Opoku *et al.,* 2002, 2007, Simelane *et al.,* 2010).

2.10 Review of methods

2.10.1 Phytochemical screening

Plant-based chemicals (phytochemicals) are believed to play an important role in human health. A phytochemical is a naturally occurring bioactive compound present in plants. Common phytochemicals include alkaloids, tannins, terpenoids, flavonoids and many others. Plant growth and development are affected by genetic and environment factors. Furthermore, ecological factors, particularly light and temperature are known to influence the active agents in plants (Burbott and Loomis, 1967; Clark *et al.,* 1980). Phenolic compounds are attracting interest in the field of food, chemistry and medicine due to their effective antioxidant activity. Research has revealed biological activities of polyphenolics mostly as antioxidants (Panovska *et al.,* 2005; Adedapo *et al.,* 2008, Pawanjit and Saroj, 2010).

Phytochemical screening is an important step for drug discovery and also for discovering the actual value for traditional medicines. Several different methods are used to screen phytochemicals in order to know the phytochemical composition of the plant material. Such methods used only act as guides and are not specific therefore false positives results are likely to occur. The presence and absence of a phytochemical can be tested by the addition of appropriate chemical reagent to the plant material. For example, alkaloids give a precipitate with heavy metal iodides and give a coloured precipitate in the presence of Mayer's and Dragendroff's reagent whilst saponins present in plant materials are tested by frothing (Trease and Evans, 1983).

2.10.2 Extraction

Plant materials can be dried using different methods. Some methods used are sun drying, air drying and oven dry. Temperature plays an important role when drying plant material as it affects the quality and effectiveness of some sensitive components (heat-labile) of the material (Mendonça-Filho, 2006).

Solvents used to extract the plant material are of importance as a successful extraction also relies on a good and suitable solvent system choice depending on its intended use. Sequential extraction can be done as well as separate extraction depending on the desired outcome. Separate extraction allows independent extraction of nonpolar and polar components whereas sequential extraction involves the use of different solvents sequentially that yield fractions of plant components. The synergistic effects of an extract is often reduced following sequential extraction.

2.10.3 Isolation, purification and structure elucidation

Standardisation of the plant material and herbal preparations is meant to guarantee their therapeutic value and it is a result of the investigations on biologically active components. An extensive number of methods are used to investigate plant material, namely macroscopic, microscopic, biological and chemical methods. Chemical investigations of the plant material is focused on the quantitative analysis of the active composites, determination of the substance groups, isolation of the substances from the plant material for further identification, structural analysis of the isolated unknown compounds (Waksmundzka-Hajnos *et al*., 2008) and many more unmentioned aims. Among many other methods used in achieving chemical investigations aims, column chromatography (CC) (focused on isolating and the purity of the material), thin layer chromatography (TLC) (qualitative chemical analysis), nuclear magnetic resonance (NMR) (focussed on the identification of the material) and gas chromatography mass spectrometry (GC-MS) (focused on the molecular mass, formula and fragmentation pattern) are commonly used.

2.10.3.1 Isolation and purification

Chromatographic analysis plays an important role among many other chemical methods of plant material examination. Chromatographic methods comprise an integral part of the medicinal plant analysis because of its abundant advantages such as their specificity and a possibility to use them for qualitative and quantitative analysis (Waksmundzka-Hajnos *et al*., 2008). It is widely used for isolation and purification of chemical compositions of samples. Column chromatography (CC) and thin layer chromatography (TLC) are among many other methods used to

isolate and purify plant components. Column chromatography is usually used for preparative applications for sample purifications on scales from micrograms up to kilograms. Depending on the amount of sample different sizes of columns can be applied. This method is advantageous because of its low cost and disposability of the stationary phase used in the process.

Generally two methods are used to prepare a column, namely the dry method and the wet method. The dry method involves filling the column with dry stationary phase powder as the first step, followed by the addition of mobile phase which is flushed through the column until it is completely wet. From this point full observation of the column is encouraged as it column is not supposed to dry up until it is completely ran. The wet method involves preparation of slurry by thoroughly mixing the adsorbent powder with the solvent to be used for elution. This is carefully poured into the column avoiding air bubbles. A solution of the organic material (plant material) is placed (pipette, pour) on top of the stationary phase which is topped with a small amount of sand forming a thin layer or with cotton or glass wool to avoid disturbance of the organic layer by the velocity of newly added eluent. Components are retained differently by the stationary phase and separate from each other as the columns is running. The eluate is collected in series of fractions as the column runs.

TLC is a chromatographic technique widely used for qualitative analysis of organic compounds, isolation of the individual compounds, preparative-scale isolation and quantitative analysis. Sorbents applied in TLC have different surface characteristics and, therefore, different physiological properties. TLC allows each plate to be used only once serving as an advantage, thereby allowing simpler sample preparation methods when compared with techniques such as GC and HPLC, in which multiple

samples and standards must be applied to the column sequence. Multiple samples can be analysed at the same time on a single TLC plate reducing the solvent volume used per sample and time consumed (Waksmundzka-Hajnos *et al*., 2008). Apart from many other uses, TLC is also used to determine the proper solvent system for separation of compounds in the column chromatography. Fluorescent silica gel pre-coated aluminium sheets are commonly used as adsorbent (stationary face). The plate is spotted with the sample solution and placed in a chromatogram chamber to allow the solvent to move up the adsorbent by capillary action up to about a centimetre from the bottom to the top end of the plate. Ultraviolet (UV) light can be used to view certain spots.

2.10.3.2 Structure elucidation

Structure elucidation of compounds can be achieved by the GC-MS, UV, IR and NMR. All these methods can be used co-operatively to provide different information about the structure of the compound obtained, thus, helping to identify the compound. NMR provides information on the number and type of hydrogen and carbon atoms present (Van de Ven, 1995). The molecular mass, molecular formula and fragmentation pattern is determined by the use of MS. UV gives information about the chromophores present in the compound whereas IR helps to identify functional groups present in the compound being identified.

2.10.4 Cytotoxicity

For several years plant extracts have been known to cure human ailments and different methods have been employed to derive different benefits they have to offer to therapeutics. Although medicinal plants contain certain active constituents, just isolating the compound alone is not sufficient without determining the toxic effect of the compounds on human cells. Treating cells with a cytotoxic compound can result in a variety of cell fates. Cells may lose their membrane integrity and die rapidly as a result of cell lyses. Therefore, there is a need to test isolated compounds for cytotoxicity before they can be considered as a potent drug.

2.11 Objective and Scope of the work

Plasmodium falciparum has shown and continues to show resistance to modern drugs used. Drug-resistant *Plasmodium falciparum* malaria is one of the most challenging factors to human health worldwide especially in developing or under developed countries of Africa. Research is ongoing with the aim to discover a sustainable and more effective and affordable drug. Malaria control requires an integrated approach comprising prevention including vector control and treatment with effective antimalarials. Elimination of malaria is the ultimate malaria goal for many researchers.

2.11.1 Objective

The aim of this study was to investigate the antimalarial and antipyretic activity of *Gardenia thunbergia, Siphonochilus aethiopicus, Schotia brachypetala, Acorus*

calamus, Withania somnifera, Elaeodendron transvalense, Hypoxis hemerocallidea, Vernonia adoensis and *Acanthospermum australe.* An attempt will be made to extract and partially identify the active components present and responsible for the activity of the plant showing the most antimalarial activity.

2.11.2 Scope of the work

The research study considered the following topics:

- Collection and identification of the plants.
- Phytochemicals screening of the plants material.
- Separate extraction of the plants materials with dichloromethane, methanol and distilled water.
- Determination of antioxidant activity of the plants extracts.
- Determination of antipyretic activity of the plants extracts.
- Determination of *in vitro* antiplasmodial activity of the plants extracts.
- Determination of cytotoxicity of the active plants extracts.
- Determination of larvicidal activity of the plants extracts.
- Isolation and characterisation of active compounds from the extract that showed reliable *in vitro* antiplasmodial activity.
- Determination of *in vitro* antiplasmodial activity of the identified compound.

CHAPTER 3

MATERIAL AND METHODS

This chapter gives a brief description of the various materials and methods used in this study. The details of the reagents preparation and methods are given in the Appendix A and Appendix B, respectively.

3.1 Materials

3.1.1 Equipment

Spectrophotometer (Spekol 1300), Rotary evaporator (Heidolph—Laborota 4000), Platform shaker (labcon), Grinding mill (IKA), Oral Canola, Homogenizer (TKA T10 basic)–were all obtained from Polychem supplies.

BiotekELx 808 UI plate reader-(Biotek Instrument supplies)

96-well microtitre plates- (Sigma)

TLC Plates (Silica gel 60 TLC aluminium sheets 20 cm x 20 cm, F_{254})-(Merck)

Eppendorf centrifuge 5804 R, Micropipettes (Eppendorf AG)

Infra-red (IR) spectrophotometer (Perkin-Elmer 100 FTIR)

Violet (UV)-visible spectrophotometer- Varian- Cary 50 Utra

Gas chromatography Mass spectrophotometry (GC-MS), 7890A GC system coupled with agilent technologies 5975C VLMSD with triple-axis detector-Agilent technologies.

3.1.2 Animals

Sprague-Dawley rats (Animal House, Dept. of Biochemistry and Microbiology, Zululand University)

3.1.3 Chemicals and Reagents (See Appendix A for preparation details)

All the chemicals used including the solvents were of the analytical grade.

Reagents supplied by Merck: Chloroform, Calcium chloride, Ferrous chloride, Glacial acetic acid, n-butanol, Trichloroacetic acid (TCA), Hexane analytical, Ethyl acetate, Citric acid, Sodium Hydroxide, Ethylene diamine tetra-acetic acid (EDTA), Silica gel 60 0.063-0.2mm (70-230 mesh ASTM), Acid-washed sand.

Reagents supplied by Sigma-Aldrich: Methanol, 4-Acetamedophenol, Pyridine, Carboxymethylcellulose sodium salt (CMC), Trizma HCl, Ferric chloride, Citric acid, Sulphanilic acid, Ascorbic acid, Potassium ferricyanide, Potassium persulfate, Potassium chloride, Sodium nitropruside, 2-Thiobarbituric acid, Bovine serum albumin (BSA), Nitro blue tetrazolium salt (NBT), 1.1–diphenyl-2-pictyhyolrazyl (DPPH), 2.2'- Azino-bis (3-ethylbenzothiazoline-6-sulfonic (ABTS), Xanthine oxidase, Xanthine, Copper chloride, Glutathione (GSH), O-phthalaldehyde, Gallic Acid, Folinciocalteu phenol reagent, Naphthylethylenediaminedihydrochloride, Naphthylamine, Ammonium molybdate, Catechin, Vanillin, Iron sulphate, Tetraethylazanium, Hydrogen peroxide, Nicotinamide, Nicotinamide Adenine Dinucleotide (NAD^+), Hydrochloric acid (HCl), Phenazineethylphosphate, Tetrazolium bromide, Alcohol dehydrogenase, Quercetin, Silver Chloride, 5.5'-

Dithiobis (2-nitrobenzoic acid (DTNB)), S-acetyl-thiocholine iodide (ASChl), Ferrous ammonium chloride, Butylatedhydroxytoluene (BHT), Trolox, Dichloromethane (DCM), 2-thiobarbituric acid (TBA). Dragenorff reagent, Mayers reagent.

Other Sources: Diethyl ether (NT laboratory supplies), Sodium dihydrogen phosphate (Lab. consumables and chemical supplies), Sodium hydrogen phosphate (Lab consumables and chemical supplies), Potassium dihydrogen phosphate (Lab. consumables and chemical supplies), Potassium hydrogen phosphate (Lab Consumables and chemical supplies), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide].

3.2 Methods (See appendix B for details)

3.2.1 Plants collection and identification:

The plants (*Gardenia thunbergia* T.A Sprague, *Siphonochilus aethiopicus* (Schweif.) B.L Burtt*, Schotia brachypetala* Sond., *Acorus calamus* L., *Withania somnifera* (L) Dunal in DC., *Elaeodendron transvalense* (Burtt Davy) R.H. Archer*, Hypoxis hemerocallidea* Fisch., C.A. Mey.& Ave-Lall, *Vernonia adoensis* Sch. Bip. Ex Walp.and *Acanthospermum australe* (Loefl.) Kuntze) were collected from the *muthi* market at Empangeni, KwaZulu-Natal, South Africa. The plants were identified by the staff of the Botany Department University of Zululand.

3.2.2 Preparation of plant Extracts:

The parts of the plants used were washed and dried at 50^{o}C for 24 hours. They were separately ground to powder (2 mm mesh) and stored in brown bottles. Extraction (1:5 w/v) was separately done using three different solvents (dichloromethane,

methanol and distilled water) by incubating the mixture on a platform shaker for 24 hours (160 rpm, room temperature). The mixtures were filtered through Whatman's No.1 paper and the organic filtrates evaporated to dryness using a rotary evaporator at 40^{o} C; the water filtrates were freeze-dried. All the extracts were reconstituted in their respective solvents and stored in bottles and kept in the fridge (4^{o} C). The percentage yield of each plant extract was calculated.

3.2.3 Phytochemical Screening:

The powdered plants materials were screened qualitatively and quantitatively for phytochemicals. Preliminary default evidence of the presence of various phytochemicals (anthraquinones (Borntrager's test), cardiac glycosides (Liebermann's test, Salkowski test, Keller-Kiliani test), flavonoids, alkaloids (Lead acetate test, Ferric Chloride test, Sodium Hydroxide test), saponins, tannins, terpenoids, (Salkowski test)) in qualitative screening was observed as turbidity or formation of precipitates and colour changes upon the different corresponding test using standard methods described by Harborne, (1973), Odebiyi and Sofowara, (1978) and Sofowara, (1984).

3.2.3.1 Total Phenolic content (Gallic acid equivalent)

Phenolic content of the plant material was determined by the Folin-Ciocalteu reagent method (Kähkönen *et al*., 1999). Different concentrations of gallic acid (0.01- 0.1 mg/ml) and 0.5 mg/ml of the plant powder were prepared in diethyl ether. After the diethyl ether had been allowed to evaporate, the residue was dissolved in 1.5 ml Folin-Ciocalteu reagent (10%) and 1.2 ml sodium carbonate (7.5%) was added into the solution. It was allowed to stand in the dark for 30 minutes, and absorbance was

read at 765 nm. Total phenol content was estimated as gallic acid equivalent and expressed as mg/g dry plant material.

3.2.3.2 Flavonoids content (Quercetin equivalent)

Various concentrations (0.01- 0.1 mg/ml) of quercetin and 0.5 mg/ml of each plant extract were prepared in diethyl ether according to the method reported by Ordon-Ez *et al.,* (2006). The diethyl ether was evaporated off and 0.5 ml of 2% $AlCl_3$ (in 80% ethanol) was added. It was allowed to stand at room temperature for an hour; a yellow colour indicated the presence of flavonoid. The absorbance was read at 420 nm. Flavonoid was estimated as quercetin equivalent and expressed as mg/g dry plant material.

3.2.3.3 Proanthocyanidin content (Catechin equivalent)

Proanthocyanidin was estimated (Sun and Chen, 1998) by a method similar to those described above for total phenol and flavonoids. Vanillin (3 ml of 4%) and 1.5 ml of 1% HCl were added to the diethyl ether residue. It was allowed to stand for 15 minutes at room temperature and absorbance was read at 500 nm.

Proanthocyanidin was estimated as catechin equivalent and expressed as mg/g dry plant material.

3.2.4 *In vitro* Antioxidant activity

3.2.4.1 DPPH radical scavenging assay

DPPH solution (2ml, 2mg% in methanol) was added into each corresponding test tube containing separately 2 ml of each extract (0-5 mg/100 ml methanol). The

mixture was left to stand for 30 minutes (Brad-Williams, 1995) and the absorption was read at 517 nm. Discoloration of DPPH was taken as an indication of scavenging activity. The percentage scavenging activity was calculated (see section 3.2.4.9).

3.2.4.2 ABTS radical scavenging assay

The method of Re *et al.,* (1999) was used to estimate ABTS radical scavenging activity. ABTS solution (1ml) was added into each corresponding test tube containing 1 ml of each extract concentration (0-5 mg/ml). The mixture was left to stand for 6 minutes. Absorption was read at 734 nm. Discoloration of ABTS was an indication of scavenging activity; % scavenging activity was then calculated (see section 3.2.4.9).

3.2.4.3 Superoxide anion scavenging activity (SO)

The method of Nagai *et al*., (2001) was used to measure the superoxide anion scavenging activity of the plants extracts. Methanolic solution of different concentrations of the extracts (0.02 ml) was added to a reaction mixture of 0.48 ml sodium carbonate buffer (pH 10.5, 0.05 M), 0.02 ml of 0,75 mM NBT, 0.02 ml of 0.15% BSA, 0.02 ml of 3 mM EDTA, 0.02 ml of 3 mM xanthine. The mixture was incubated for 20 min at 25^{o}C. After incubation 6mM CuCl was added and absorbance was read at 560 nm and the % inhibition calculated (see section 3.2.4.9).

3.2.4.4 Hydroxyl radical scavenging assay (OH)

This was assayed as described by Chung *et al*., (1997). Methanolic extracts (0.2 ml) was added to a reaction mixture of 200µl H_2O_2 (10 mM), 1.0 ml phosphate buffer (0.1 M, pH 7.4), 0.2 ml FeSO4.H_2O (10mM), 0.2 ml EDTA (10 mM) and 0.2 ml 2-

deoxyribose (10 mM). The mixture was incubated at 37^{o}C for 4 hours. TBA (1 ml, 1%) and TCA (1 ml, 2.8%) were then added and this was left to boil for 10 min and then cooled on ice. The absorbance was read at 520 nm and the % inhibition calculated (see section 3.2.4.9).

3.2.4.5 Nitric oxide radical scavenging activity (NO)

The nitric oxide radical scavenging activity was determined using the method of Garrat (1964). Into each test tube, 2 ml of sodium nitroprusside, 0.5 ml phosphate buffer and 0.5 ml plant extract were added. The mixture was incubated at 25ºC for 150 minutes where after incubation 0.5 ml of the reaction mixture was added into different test tubes. Sulfanilic acid (1 ml, 0.33%) was also added and the test tubes were mixed and allowed to stand for 5 minutes. Then 1 ml of naphthylethylenediamine dichloride (5%) was added. This was mixed and again allowed to stand for 30 minutes in diffuse light. Absorbance was read at 540 nm using a plate reader. The % scavenging activity was calculated (see section 3.2.4.9).

3.2.4.6 Chelating activity on Fe^{2+}

Each plant extract (1 ml) concentration (0-5 mg/100 ml) was mixed with 3.75 ml deionised water, 0.1 ml of 2 mM $FeCl_2$ and 0.2 ml of 5 mM ferrozine. The mixture was left to stand for 10 minutes. The iron chelating activity was tested using the method described by Decker and Welch (1990). Absorption was read at 562 nm. Citric acid and EDTA were used as standards. % chelating was calculated (see section 3.2.4.9).

3.2.4.7 Reducing Power

The reducing power of the plants extracts was measured following the method indicated by Oyaizu (1986). The plants extracts (1-5 mg/100 ml) were mixed with 2.5 ml of phosphate buffer (0.2 M pH 6.6), and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated for 20 minutes. TCA (2.5 ml, 10 %) was added and left to stand for 10 minutes and then centrifuged (1000 rpm; 10 minutes), whereafter 2.5 ml of the supernatant was obtained and diluted (1:1) with distilled water and mixed with 0.5 ml of 0.1% $FeCl_3$. The absorbance was read at 700 nm. The higher the absorbance, the higher the reducing power of the extract.

3.2.4.8 Total antioxidant capacity

The assay was based on the reduction of Mo (VI) to Mo (V) as described by Dasgupta and De (2004). Plant extracts (0.3 ml) was combined with 3 ml of the reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The mixture was incubated for 90 min at 95^{o}C and this was cooled to room temperature. The absorbance of the solution was measured at 695 nm against blank. The oxidant activity was expressed as the number of equivalents of ascorbic acid and BHT.

3.2.4.9 Calculation of antioxidant activity

Unless otherwise stated, ascorbic acid, BHT, and Trolox were used as standards. All assays were repeated three times and the mean ± S.E. reported. The inhibitory effect of the extracts on each parameter was calculated as:

$$\% \text{ inhibition} = [1-A_t/A_0]*100$$

Where A_t is the value of the test and A_0 is the value of the fully oxidized control.

The inhibitory concentration providing 50% inhibition (IC_{50}) was calculated from the graph of percentage inhibition against extract concentration.

3.2.5. Sulfhydryl content

The SH^- content of each plant was measured by the method described by Cohen and Lyle (1966). Separate extraction (1:5w/v) of the plants material was done using cold distilled water containing 30 µmoles/ml EDTA. Different concentrations (0-10 µg/ml) of reduced glutathione (GSH) were prepared. Into each corresponding test tubes 2.5 ml of 0.1M phosphate buffer (pH 8) and 0.1 ml of 1% o-phthaldehyde were added to 2 ml GSH and plant extract. After 15-20 min at room temperature the fluorescence was read at 420 nm (emission) and 350 nm (excitation). The standard GSH graph was used to estimate the concentrations of SH in the plant extracts.

3.2.6 Antipyretic activity

Approval for experimental procedures was obtained from the Research Animal Ethic Committee, University of Zululand (see Appendix C).

Pyrexia was induced in Sprague-Dawley rats by subcutaneous injection of 12% brewer's yeast (1ml/kg bw). Twenty-four hours later the anal temperatures of the animals were taken, and those that showed a minimum rise of 1.5^0C were selected as pyretic. Pyretic rats were divided into five groups of five animals each (figure 3.1). Group one was the control and received carboxy-methylcellulose (CMC), the drug vehicle. Groups two, three and four received the plant extract dissolved in CMC (100, 500, 1000 mg/kg body weight, respectively). Group five was administered with

paracetamol (panado) as the standard drug (100 mg/kg). The drugs were orally administered with the use of a canulor. The rats had free access to food and water. The anal temperatures were taken (three times each period) at 30 minutes, 1 hour, 2 hours and 4 hours after the administration of the drugs.

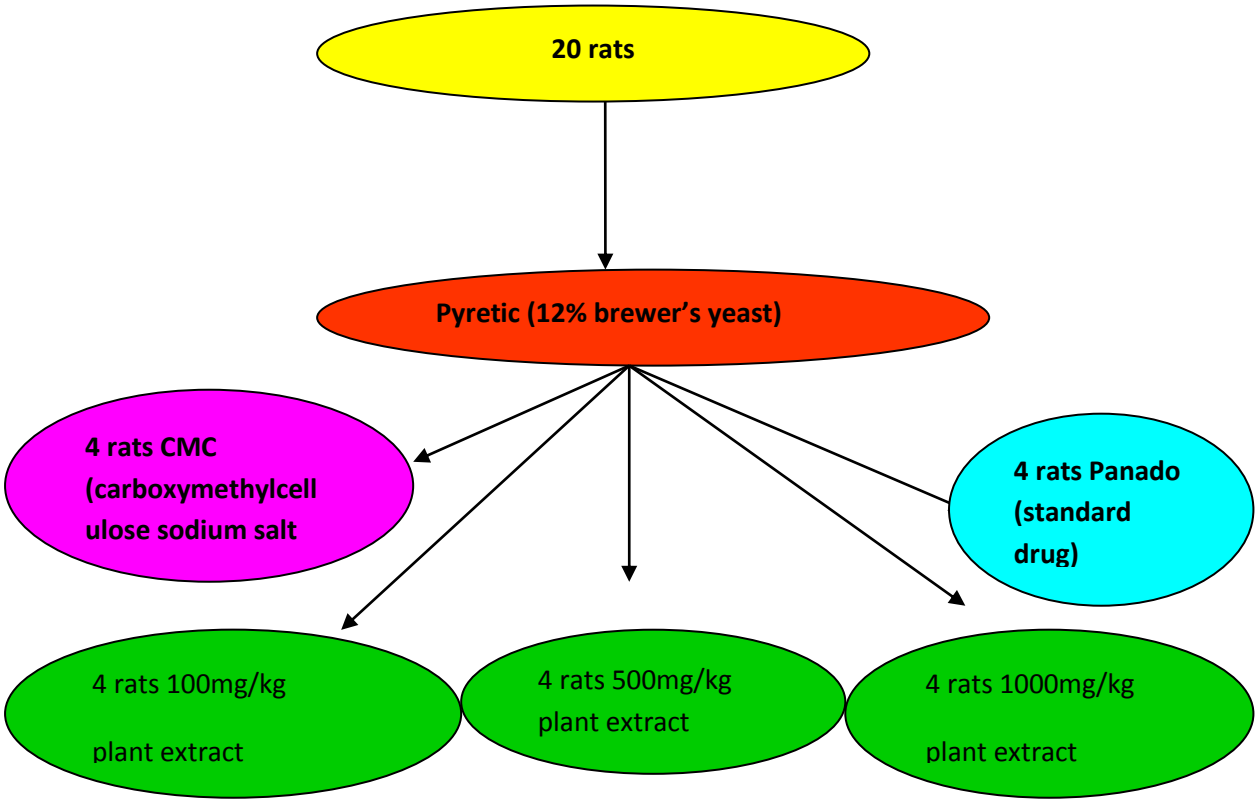


Figure 3.1 Experimental method layout for the *in vivo* antipyretic activity study

3.2.7 *In vitro* Antiplasmodial activity

The plant extracts were tested in triplicate against chloroquine sensitive (CQS) strain of *Plasmodium falciparum* (D10). Continuous *in vitro* cultures of asexual erythrocytes stages of *P. falciparum* were maintained using a modified method of Trager and

Jensen (1976). Quantitative assessment of antiplasmodial activity *in vitro* was determined via the parasite lactate dehydrogenase assay using a modified method described by Makler *et al*., (1993). The samples were prepared to a 2 mg/ml stock solution in 10% DMSO or 10% methanol and sonicated to enhance solubility. Stock solutions were stored at -20^{o}C. Further dilutions were prepared on the day of the experiment. Chloroquine (CQ) was used as the reference drug in all experiments. Test samples were tested at three concentrations which were 20 µg/ml, 10 µg/ml and 5 µg/ml. CQ was tested at three concentrations (30 ng/ml, 14 ng/ml and 7.5 ng/ml). A full dose was performed on active samples to determine the concentration inhibiting 50% of parasite growth (IC_{50}-value). Test samples were tested at a starting concentration of 100 µg/ml, which was then serially diluted 2-fold in complete medium to give 10 concentrations; with the lowest concentration being 0.2 µg/ml. The same dilution technique was used for all samples. CQ was tested at a starting concentration of 100 ng/ml. The highest concentration of solvent to which the parasites were exposed to had no measurable effect on the parasite viability (data not shown). The IC_{50} values were obtained using a non-linear dose-response curve fitting analysis via Graphpad Prism version 4.0 software.

3.2.8 Cytotoxicity

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] cell proliferation assay (Mosman, 1983) was used to determine the cytotoxicity of the extracts against human embryonic kidney (HEK293) and human hepatocellular carcinoma (HepG2) cells. The cells were seeded in a 48-well plate at a density of 2.5 x 10^4 cells per well. Following an overnight incubation at 37^{o}C, the cells were incubated with the compound at different concentrations (50, 100, 150, 200, 250, 300, and 350 µg/200 µl) in medium (MEM + Gutamax + antibiotics + 10% fetal bovine serum) for 48 hours.

Thereafter, the medium was removed from the cells and 200 µl MTT solutions (5 mg/ml phosphate buffer saline) as well as 200 µl of cell culture medium was added to the corresponding wells. The cells were incubated at 37^{o}C for 4 hours and the reaction was terminated by addition of DMSO (100/200/400 µl). The cells viability was determined spectrophotometrically (Biomate spectrophotometer) at 570nm. The experiment was done in triplicate and the results were expressed as mean ± SD. Lethal concentration of the compound that results in 50% cell death (LC_{50}) was determined by regression analysis using QED statistics programmer.

3.2.9 Larvicidal test

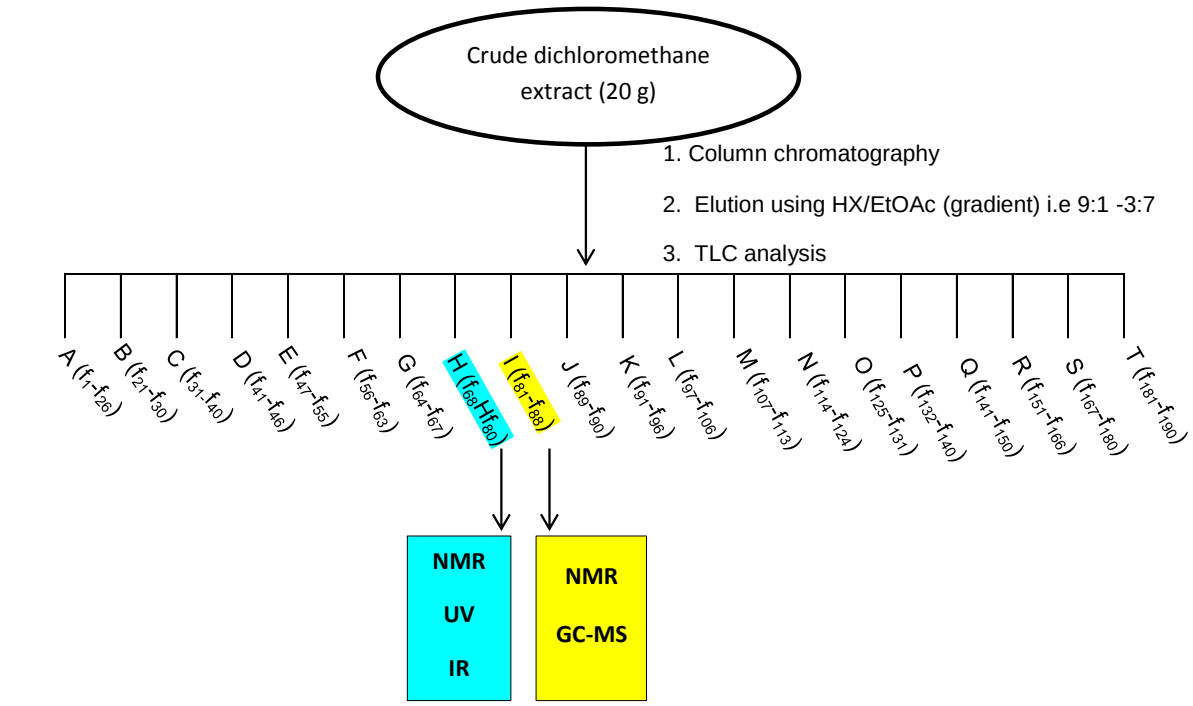
Larvae of *Culex quinquefasciatus* were collected from the Hatchery unit of the Department of Zoology, University of Zululand, KwaDlangezwa. They were maintained at ambient rearing conditions in the environmental room. All bioassays were conducted at 28 ± 1^{o}C, 60.0 ± 5% R H and 12 hours dark photoperiod. A 10% yeast suspension was used as food source. Test for mosquito larval activity was conducted with some modifications using the method of Cheng *et al.,* (2004) and Rafikali and Nair (2001). Ten fourth-instar mosquito larvae were collected with a Pasteur pipette, placed on a filter paper to remove excess water and transferred to the Petri-dishes (100 ml) each containing 29.0 ml of degassed distilled water and 1000 µL of different concentrations of plant extract (10- 250 µg/ml) in 1% DMSO with a tiny brush. Each Petri-dish was shaken lightly to ensure a homogenous test solution and was left at room temperature. Each test was performed in triplicate. The control was prepared with 29.0 ml of degassed distilled water and 1000 µL of DMSO solution without the plant extract to which larvae were added. Observation on larvae

mortality was recorded after 24 hours exposure, during which no food was given to the larvae. Larvae were considered dead, when they did not react to touching with a needle. The percentage of mortality and lethal concentrations (LC_{50}) values were determined using Abbots formula and Probit analysis program, version 1.5 respectively.

3.2.10 Isolation, purification and characterisation of compounds

3.2.10.1 Isolation

The crude dichloromethane extract (20 g) of *Acanthospermum australe* (showed the most *in vitro* antiplasmodial activity) was subjected to silica gel column chromatography (20 mm x 500 mm; Silica gel 60; 0.063 – 0.2 mm; 70-230 mesh ASTM), eluted with a gradient of hexane: ethyl acetate solvent system. A total of 20 combined fractions were obtained (figure 3.2) and analysed using thin layer chromatography (TLC) technique (silica gel 60 TLC aluminium sheets 20 cm x 20 cm, F_{254}, hexane: ethyl acetate solvent system 9:1-7:3). The TLC plates were viewed using ultraviolet (UV) light where after they were developed with 10% H_2SO_4 spray reagent and then exposed to heat. Fractions that exhibited similar profile were combined and concentrated *in vacuo*. Their weights were recorded. The eighth (MF/01/H) and ninth (MF/01/I) fractions were separately recrystallized in hexane and methanol respectively to obtain compounds ($MF/01/H_1$, 0.05 g) and ($MF/01/I_1$ 0.03 g).



1. Concentrated *in vacuo*

2. Recrystallized in methanol or hexane

Figure 3.2: Schematic representation of the isolation and purification of MF/01/H_1 and MF/01/I_1 through column chromatography.

3.2.10.2 Structure elucidation

MF/01/H_1 and MF/01/I_1 were analysed using 1D and 2D NMR techniques (^{1}H-^{1}H, ^{13}C-^{13}C, DEPT, COSY, HMQC, HMBC and NOESY) (In $CDCL_3$, Bruker 600 MHz), infrared (IR) (Perkin-Elmer 100 FTIR), UV (in $CHCL_3$, Varian- Cary 50 UV-visible spectrophotometer) and gas chromatography-mass spectrometry (GC-MS). The melting point (Reichert Thermovar) was also determined. Compounds were identified using the resulting spectra analysis (Appendix E) by comparing to the standards and library materials.

3.2.11 *in vitro* antiplasmodial activity of the pure compound (MF/01/H_1)

The *in vitro* antiplasmodial activity of the isolated pure compound was determined as previously reported (see section 3.2.9).

3.2.12 Statistical analysis

Data were analysed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test. The results were presented as mean ± S.E. Statistical difference was accepted at $p<0.05$. Student's t-test was used to analyse statistical difference between control and treated groups.

CHAPTER 4

RESULTS

Nine medicinal plants (*Gardenia thunbergia, Siphonochilus aethiopicus, Schotia brachypetala, Acorus calamus, Withania somnifera, Elaeodendron transvalense, Hypoxis hemerocallidea, Vernonia adoensis* and *Acanthospermum australe*) were separately extracted with methanol, dichloromethane, and distilled water. The extracts were tested for antioxidant, antipyretic, larvicidal, *in vitro* antiplasmodial, and cytotoxicity activity. An attempt was made to isolate, purify and characterise the active constituents in the most active *in vitro* antiplasmodial extract (dichloromethane extract of *Acanthospermum australe*). The isolated compound was also screened for *in vitro* antiplasmodial activity.

The results of these experiments are presented in this chapter.

4.1 Phytochemical screening

The results of qualitative screening of the plants for their chemicals composition are presented in Table 4.1. The phytochemical screening showed the presence of tannins, terpenoids, cardiac glycosides, saponins, flavonoids, alkaloids and anthraquinones in the different plants.

Table 4.1: The Phytochemicals of the plants. Present phytochemicals are denoted by the "+" sign and absent phytochemicals by the "-" sign.

Plants	Phytochemicals						
	Saponins	Flavonoids	Tannins	Terpenoids	Cardiac glycoside	Alkaloids	Anthroquinones
H. heme	-	-	+	+	+	-	+
A. Aust	+	+	+	-	+	+	-
V. Adoe (r)	+	-	+	+	+	+	+
V. Adoe (l)	-	+	+	+	+	-	+
A. cala	-	+	-	+	+	+	-
E. tran	+	+	+	+	+	-	+
W. somn	+	+	+	+	+	+	-
G. thun	+	-	+	-	-	+	+
S. aeth	+	+	-	+	+	+	-
S. brac	+	-	+	+	-	-	-

4.2 Total phenol, Proanthocyanidin, and flavonoids content of the plants.

Quantitative analysis of some of the phytochemicals present in the plants (Table 4.2) indicates that total phenol, proanthocyanidin and flavonoids were present in appreciable amounts in all the plants.

Table 4.2: Total phenol, Proanthocyanidin and flavonoids (mg/g) content of the plants.

Plant	Total phenol (mg/g)	Proanthocyanidin (mg/g)	Flavonoid (mg/g)
H. hemerocallidea	0.12	0.32	0.07
A. australe	0.13	0.17	0.09
V. adoensis (roots)	0.16	0.05	0.07
V. adoensis (leaves)	0.05	0.21	0.02
A. calamus	0.051	0.11	0.107
E. transvalense	0.035	0.25	0.098
W. somnifera	0.051	0.21	0.050
G. thunbergia	0.17	0.16	0.07
S. aethiopicus	0.22	0.11	0 .15
S. brachypetala	0.36	2.74	0.12

4.3 Extraction

Table 4.3 shows the percentage yield of the crude extracts.

Table 4.3: Percentage yield (w/w; dry weight) of the crude extracts

PLANTS	**Yield (%)**		
	Methanol	**Dichloromethane**	**Water**
H. hemerocallidea	21	2.4	6
A. australe	5	2	2
V. adoensis (roots)	4.2	1	4
V. adoensis (leaves)	20	4	7
A. calamus	15.1	9.8	3.3
E. transvalense	6.6	1.7	5.2
W. somnifera	2.1	1.1	1.8
G. thunbergia	3.45	6.8	1
S. aethiopicus	4.68	0.27	1.46
S. brachypetala	19.5	3.42	1.25

It is apparent that methanol extracted more components from the plants than dichloromethane and water.

4.4 *In vitro* antioxidant activity of the methanol extracts.

The antioxidant properties (table 4.4 and figure 4) indicate that the extracts exhibited a concentration dependent antioxidant activity (See appendix D).

Table 4.4: *In vitro* Antioxidant activity (IC_{50}) of the methanol plants extracts. ND=Not determined.

	Activity IC_{50} µg/ml					
Plant Name	**DPPH**	**ABTS**	**Fe^{2+} Chelating**	**SO**	**OH**	**N O**
H. hemerocallidea	1.13	5	5	2.6	0.8	2.1
A. australe	3.2	5	5	0.84	0.82	2.05
V. adoensis (roots)	2.3	5.2	5	0.70	0.78	3.4
V. adoensis (leaves)	4	1.04	4	2.68	0.88	1.57
A. calamus	2.8	>5	>5	1.8	3.0	4.2
E. transvalense	0.7	4.1	3.9	1.6	3.6	3.6
W. somnifera	2.2	4.6	4.3	0.8	1.2	4.7
G. thunbergia	6.39	5.58	7.48	16.47	4.35	20.2
S. aethiopicus	3.62	13.72	>5	8.58	>5	15.26
S. brachypetala	8.12	11.00	>5	0.82	7.21	4.45
BHT	0.7	3	ND	2.46	1.37	2.74
Ascorbic acid	0.5	5	ND	0.84	3.7	3.76
EDTA	ND	ND	0.6	ND	ND	ND
Citric Acid	ND	ND	2.9	ND	ND	ND

However, except the *E. transvalense* and the *V. adoensis* (leaves) extracts (IC_{50} of 0. 7 µg/ml and 1.04 µg/ml, respectively) all the other extracts were poorer scavengers of DPPH and ABTS than the standards (AA and BHT). On the other hand, most of the extracts showed relatively better ability to scavenge the biological free radicals

(SO, OH, and NO) than the standards. Most of the plant extracts did not show any potential to inhibit Fe^{2+} chelating.

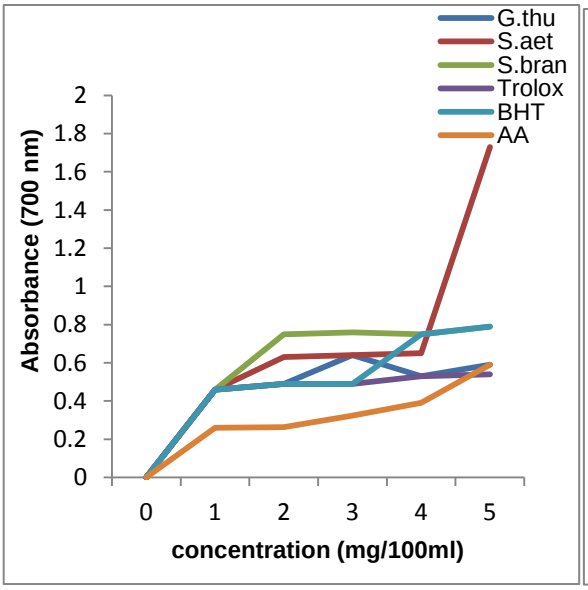


Figure 4.1 a: Reducing power of *G. thunbergia, S. aethiopicus* and *S. brachypetala*

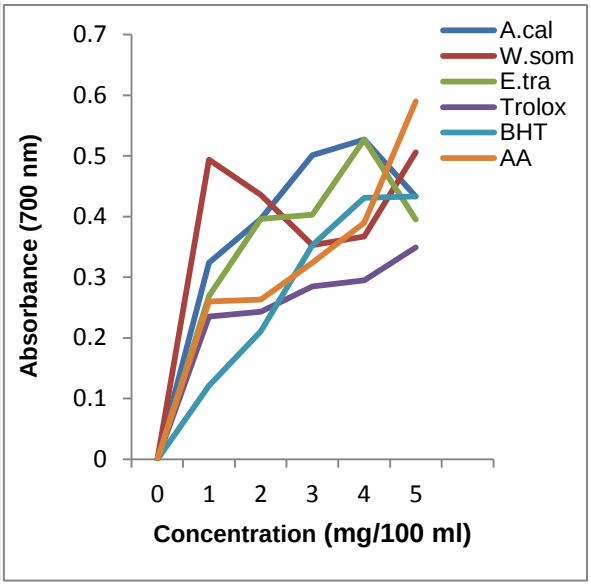


Figure 4.1 b: Reducing power of *A.calamus W.somnifera* and *E.transvalense*

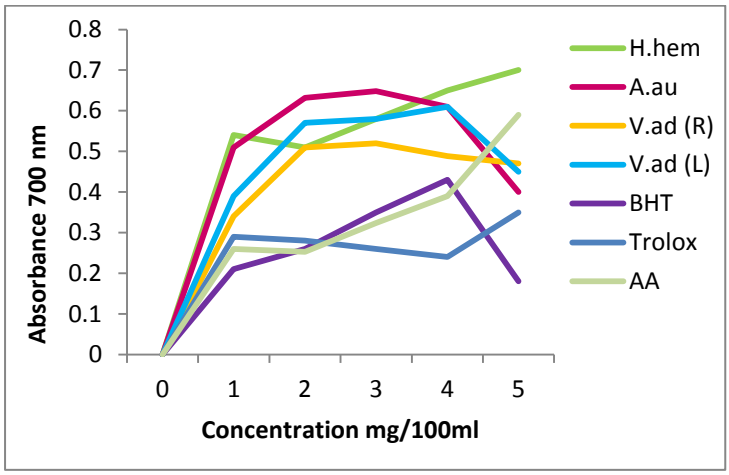


Figure 4.1 c: Reducing power of *H. hemerocallidea, A.australe* and *V. adoensis* (roots) and *V. adoensis* (leaves).

Figure 4.1: Reduction potential of the extracts of the plants and the standard drugs, BHT, trolox and ascorbic acid.

The extracts exhibited a concentration dependent reducing power (figure 4.1) with *H. hemerocallidea* and *S. aethiopicus* exhibiting high reducing power compared to the standards BHT, trolox and ascorbic acid.

4.5 SH content and total antioxidant capacity of the plants.

SH content and antioxidant capacity of the plants are presented in table 4.5. Most plants showed appreciable amount of SH and relatively high total antioxidant capacity. *S. branchypetala* had the highest SH content (7.25 µg/g), however, it did not exhibit any high total antioxidant capacity. *S. aethiopicus* showed traces of SH content ($1.25x10^{-03}$ µg/g).

Table 4.5: SH content and total antioxidant capacity of the plants.

Plants	**SH content (µg/g)**	**Total antioxidant capacity measured in equivalence to BHT and AA**	
		BHT	**AA**
H. hemerocallidea	1.2	41	79
A. australe	0.8	47	84
V. adoensis (leaves)	0.43	55	100
V. adoensis (roots)	1.58	35	63
A. calamus	2.57	82.4	32
E. transvalense	0.36	172.8	67.8
W. somnifera	2.74	58.7	23
G. thunbergia	1.35	52.7	38.8
S. aethiopicus	$1.25x10^{-03}$	78.1	31.8
S. brachypetala	7.25	40.3	11.13

4.6 The bioassay: The larvicidal, *in vivo* antipyretic, *in vitro* antiplasmodial activities and the cytotoxicity of the plants extracts.

The bioassay results are shown in Table 4.6.1 to 4.6.4.

4.6.1 Larvicidal activity of the plants extracts.

The effectiveness of the plants extracts as a larvicide against the larvae of *Culex quinquefasciatus* was observed to be dose dependent. The plant extracts either killed or reduced spontaneous movement in *C. quinquefasciatus* larvae after 24 hours following treatment (table 4.6.1). The results of the percentage mortality of the fourth-instar larvae of *C. quinquefasciatus* indicate that the aqueous extracts of the plants had little or no larvicidal activity. The dichloromethane extracts and the methanol extracts were the most efficient (≥ 50% mortality).

Table 4.6.1: Larvicidal activity of the plants extracts against Larvae of *Culex quinquefasciatus.* ND= Not determined.

Plants name	Plant Extracts larvicidal activity					
	% larvae mortality			IC_{50} (µg/ml)		
	Dichloromethane	Methanol	Water	Dichloromethane	Methanol	Water
H. hemerocallidea	40	57	0	ND	ND	ND
A. australe	65	60	0	ND	ND	ND
V. adoensis (leaves)	80	73	0	ND	ND	ND
V. adoensis (roots)	40	43	0	ND	ND	ND
A. calamus	64	58	37	10.98	11.6	23.67
E. transvalense	60	47	35	18.18	9.78	ND
W. somnifera	77	80	33	14.78	22.88	47.66
G. thunbergia	52	77	0	0	ND	0
S. aethiopicus	66	0	35	ND	ND	ND
S. brachypetala	74	60	30	ND	ND	84.56

4.6.2 The *in vivo* antipyretic activity of the plants extracts

The *in vivo* antipyretic activity of the plants extracts are presented in table 4.6.2.

Most of the plant extracts exhibited the potential to reduce pyrexia in the induced rats. The activity was time and concentration dependent with most of the extracts showing activity as early as from 30 minutes even at the lowest concentration (100 mg/kg). The dichloromethane and aqueous extracts of *W. somnifera* (500 mg/kg) exhibited significant ($P<0.05$) activity. A similar activity was also observed for the *E. transvalense* dichloromethane extracts.

E. transvalense methanol extract showed significant ($P<0.001$) activity that was comparable to that of the reference drug (Panado). *A calamus* methanol extract also exhibited significant ($P<0.0001$) activity at the highest concentration (1000 mg/kg) and this was also concentration dependent.

Table 4.6.2 a: The antipyretic activity of plants extracts. Data represents the mean ± S.E. of the experiment at 100 mg/kg. *P<0.05, **P<0.001, ***p<0.0001.

Name	**Plant extract**	**Treatment**	**Induced Temp**	**Minutes after plant extract feeding**			
				30	**60**	**120**	**240**
CMC	Control	100 mg/kg	37.5±0.06	37.8±0.1	37.6±0.1	37.4±0.1	37.5±0.1
Panado	Standard	100 mg/kg	37.6±0.2	36.5±0.1***	36.0±0.1***	35.6±0.1***	36.0±0.1***
A. australe	DCM	100 mg/kg	37.9±0.1	36..4±0.2*	37.6±0.0*	37.3±0.5*	37.8±0.0
A. australe	Methanol	100 mg/kg	37.8±0.4	37.8±0.1	37.7±0.3	37.6±0.5	37.6±0.2
A. australe	Aqueous	100 mg/kg	37.9±0.2	37.8±0.0*	37.8±0.1*	37.5±0.1*	37.4±0.2*
V. adoensis (leaves)	DCM	100 mg/kg	38±0.6	37.5±0.5	37.5±0.3*	37.2±0.7*	37.0±0.5
V. adoensis (leaves)	Methanol	100 mg/kg	38.5±0.2	38.2±0.2*	37.8±0.8*	37.5±0.2*	37.0±0.4*
V. adoensis (leaves)	Aqueous	100 mg/kg	39±0.1	38.0±0.2*	37.9±048*	37.7±0.2*	37.0±0.3*
V. adoensis (roots)	DCM	100 mg/kg	37.4±10.79	37.8±10.9	37.6±10.87	35.7±10.32	35.6±10.29
V. adoensis (roots)	Methanol	100 mg/kg	38.5±11.12	37.9±10.96	37.6±10.5	36.9±10.66	36.9±10.66
V. adoensis (roots)	Aqueous	100 mg/kg	38.0±0.0	37.8±0.3	37.8±0.6	37.7±0.8	37.8±0.1
A. calamus	DCM	100 mg/kg	38.7±0.3	37.5±0.4	37.8±07	37.6±0.6	37.8±0.1
A. calamus	Methanol	100 mg/kg	37.4±0.06	37.1±0.13*	37.2±0.2	37.1±0.3	37.0 ±0.2
A. calamus	Aqueous	100 mg/kg	36.3±10.5	36.6±10.6	36.9±10.7	35.7±10.3	36.2±10.5
H. hemerocalllidea	DCM	100 mg/kg	38.1±11.0	38.0±9.8	37.7±10.9	36.4±10.5	37.2±10.7

Table 4.6.2 a continued

H. hemerocalllidea	Methanol	100 mg/kg	38±0.2	37.8±0.2*	37.6±0.5*	37.5±0.3*	37.6±0.7
E. transvalense	DCM	100 mg/kg	37.7±0.1	37.9±0.2*	37.6±02*	37.8±0.1*	37.5±0.3*
E. transvalense	Methanol	100 mg/kg	38.3±0.2	38.4±0.3*	38.3±03*	38.1±0.19**	38.0±0.15**
E. transvalense	Aqueous	100 mg/kg	38.3±11.03	37.5±10.82	37.7±10.88	37.6±10.84	37.1±10.73
W. somnifera	DCM	100 mg/kg	37.3±0.3	36.2±0.2*	35.7±07*	35.5±0.2*	36.0±0.4*
W. somnifera	Methanol	100 mg/kg	37.6±0.12	37.5±0.13	36.8±0.2*	36.6±0.2**	36.9±0.4*
W. somnifera	Aqueous	100 mg/kg	37.6±0.1	37.7±0.3*	36.7±04*	36.3±0.5*	37.0±0.2*
W. somnifera	DCM	100 mg/kg	37.3±0.3	36.2±0.2*	35.7±07*	35.5±0.2*	36.0±0.4*
W. somnifera	Methanol	100 mg/kg	37.6±0.12	37.5±0.13	36.8±0.2*	36.6±0.2**	36.9±0.4*
W. somnifera	Aqueous	100 mg/kg	37.6±0.1	37.7±0.3*	36.7±04*	36.3±0.5*	37.0±0.2*
G. thunbergia	DCM	100 mg/kg	8.4±0.2	37.5±0.4	37.8±07	37.6±0.6	37.8±0.6
G. thunbergia	Methanol	100 mg/kg	36.4±10.55	35.7±10.3	34.9±10.09	34.7±10.05	34.1±9.84
G. thunbergia	Aqueous	100 mg/kg	36.9±10.68	35.6±10.29	36.7±10.59	35.8±10.33	35.7±10.30
S. aethiopicus	DCM	100 mg/kg	37.3±10.76	38.1±10.98	38.1±10.99	37.7±10.87	36.7±10.51
S. aethiopicus	Methanol	100 mg/kg	36.9±10.64	37.7±10.87	37.4±10.79	37.4±10.79	36.5±10.53
S. brachypetala	DCM	100 mg/kg	35.3±10.19	35.7±10.29	35.9±10.39	33.2±9.58	35.7±10.32
S. brachypetala	Methanol	100 mg/kg	36.7±10.59	37.9±10.97	36.7±10.59	36.2±10.46	35.9±10.39
S. brachypetala	Aqueous	100 mg/kg	38.4±0.08	37.1±0.13*	37.2±0.2	37.1±0.3	37.0 ±0.3

Table 4.6.2 b: The antipyretic activity of plants extracts. Data represents the mean ± SE of the experiment at 500 mg/kg. *P< 0.05, **P< 0.001, ***p<0.0001.

Name	**Plant extract**	**Treatment**	**Induced Temp**	**Minutes after plant extract feeding**			
				30	**60**	**120**	**240**
CMC	Control	500 mg/kg	37.5±0.06	37.8±0.1	37.6±0.1	37.4±0.1	37.5±0.1
Panado	Standard	500 mg/kg	37.6±0.2	36.5±0.1***	36.0±0.1***	35.6±0.1***	36.0±0.1***
A. australe	DCM	500 mg/kg	38.7±0.3	36.8±0.2*	37.7±0.2*	37.3±0.3*	38.3±0.1*
A. australe	Methanol	500 mg/kg	38±0.4	37.9±0.2	37.7±0.5	37.8±0.3	37.7±0.3
A. australe	Aqueous	500 mg/kg	38.2±0.3	38±0.2*	37.9±0.6*	37.6±0.5*	37.6±0.3*
V. adoensis (leaves)	DCM	500 mg/kg	38±0.6	37.9±0.3*	37.5±0.3	37.4±0.4*	37±0.2
V. adoensis (leaves)	Methanol	500 mg/kg	38.6±0.5	38.5±0.3*	37.6±0.6 *	37±0.1*	36±0.2*
V. adoensis (leaves)	Aqueous	500 mg/kg	38.9±0.1	38.7±0.5*	38±0.6 *	37.5±0.1*	37.2±0.7*
V. adoensis (roots)	DCM	500 mg/kg	38.5±11.1	37.0±10.9	37.7±10.9	36.8±10.6	36.7±10.6
V. adoensis (roots)	Methanol	500 mg/kg	38.3±11.04	38.8±11.2	37.9±10.9	38.0±10.9	38.0±10.9
V. adoensis (roots)	Aqueous	500 mg/kg	38±0.1	38±0.1*	37.9±0.3*	37.8±0.8	37.9±0.3*
A. calamus	DCM	500 mg/kg	38.6±0.1	37.9±0.02*	37.5±0.3*	37.5±0.5	37.7±0.2*
A. calamus	Methanol	500 mg/kg	37.6±0.1	36.9±0.16**	37.0±0.2	37.2±0.3	37.0±0.2

Table 4.6.2 b continued

A. calamus	Aqueous	500 mg/kg	36.7±10.6	37.3±10.8	37.8±10.9	37.5±10.8	36.9±10.7
H. hemerocallidea	DCM	500 mg/kg	36.9±10.7	37.7±10.9	37.2±10.7	36.7±10.6	36.6±10.6
H. hemerocallidea	Methanol	500 mg/kg	38.9±0.1	38.7±0.5*	38±0.6*	37.5 ±0.1*	37.2±0.7*
E. transvalense	DCM	500 mg/kg	38.0±0.2	37.9±0.2*	37.5±0.4*	37. ±0.6**	37.1±0.3*
E. transvalense	Methanol	500 mg/kg	38.6±0.16	38.3±0.2**	38.2±0.18**	37.9±0.14**	38.1±0.2**
E. transvalense	Aqueous	500 mg/kg	37.6±10.86	37.6±10.84	37.7±10.90	38.2±11.02	37.5±10.85
W. somnifera	DCM	500 mg/kg	37.4±0.3	36.3±0.3*	36.6±0.5*	36.5±0.3*	35.3±0.3*
W. somnifera	Methanol	500 mg/kg	37.7±0.13	36.6±0.4	36.2±0.4*	36.3±0.7**	34.1±0.9**
W. somnifera	Aqueous	500 mg/kg	38.7±0.3	36.6±0.2*	36.3±0.2*	38.4±0.3*	38.3±0.3*
G. thunbergia	DCM	500 mg/kg	35.7±0.4	35±0.4*	34.4±0.1*	35.6±0.2	35±0.3*
G. thunbergia	Methanol	500 mg/kg	35.9±10.38	34.9±10.13	35.2±10.23	34.3±9.94	34.9±10.13
G. thunbergia	Aqueous	500 mg/kg	37.4±10.71	35.2±10.21	35.3±10.21	35.8±10.35	25.4±11.23
S. aethiopicus	DCM	500 mg/kg	36.7±10.6	36.9±10.68	36.9±10.68	36.4±10.54	35.1±10.15
S. aethiopicus	Methanol	500 mg/kg	40.5±12.10	37.3±10.76	37.0±10.68	35.7±10.32	36.8±10.63
S. brachypetala	DCM	500 mg/kg	35.4±10.21	35.9±10.39	35.6±10.28	35.1±10.14	36.2±10.46
S. brachypetala	Methanol	500 mg/kg	36.9±10.69	37.0±10.69	36.7±10.61	35.2±10.18	35.8±10.34
S. brachypetala	Aqueous	500 mg/kg	38±0.2	37±0.1*	34.9±0.3*	38.8±0.3	35.9±0.3*

Table 4.6.2 c: The antipyretic activity of plants extracts. Data represents the mean ± SE of the experiment at 1000 mg/kg. *P< 0.05, **P< 0.001, ***p<0.0001.

Name	**Plant extract**	**Treatment**	**Induced Temp**	**Minutes after plant extract feeding**			
				30	**60**	**120**	**240**
CMC	Control	1000 mg/kg	37.5±0.06	37.8±0.1	37.6±0.1	37.4±0.1	37.5±0.1
Panado	Standard	1000 mg/kg	37.6±0.2	36.5±0.1***	36.0±0.1***	35.6±0.1***	36.0±0.1***
A. australe	DCM	1000 mg/kg	38.2±0.1	37.9±0.5*	37.7±0.3	38±0.5*	38±0.4*
A. australe	Methanol	1000 mg/kg	38.2±0.6	38±0.2	37.9±0.2	38±0.3	37.8±0.2
A. australe	Aqueous	1000 mg/kg	38.3±0.3	38±0.2	37.6±1.0	37.5±0.2	36±0.1*
V. adoensis (leaves)	DCM	1000 mg/kg	38±0.4	37.9±0.4*	37.6±0.2*	37.5±0.5*	37.2±0.3
V. adoensis (leaves)	Methanol	1000 mg/kg	38.7±0.7	38.3±0.1*	37.2±0.5*	35±0.0	35±0.6*
V. adoensis (leaves)	Aqueous	1000 mg/kg	38.2±0.5	37±0.3*	37.2±0.6*	36.6±0.6*	36.3±0.1*
V. adoensis (roots)	DCM	1000 mg/kg	37.4±10.8	37.9±10.9	37.5±10.83	36.3±10.47	36.8±10.62
V. adoensis (roots)	Methanol	1000 mg/kg	38.3±11.06	37.4±10.78	37.6±10.86	36.5±10.54	36.5±10.54
V. adoensis (roots)	Aqueous	1000 mg/kg	37.9±0.3	37.7±0.1	37.5±0.4*	37.6±0.4	37.5±0.2*
A. calamus	DCM	1000 mg/kg	36.9±0.4	37.6±0.1*	37.5±0.04*	37.6±0.3	37.8±0.3**
A. calamus	Methanol	1000 mg/kg	37.7±0.1	37.1±0.13*	37.1±0.06*	36.8±0.1**	36.7±0.1 ***
A. calamus	Aqueous	1000 mg/kg	37.0±10.7	36.5±10.5	36.5±10.6	37.3±10.8	36.2±10.5
H. hemerocallidea	DCM	1000 mg/kg	37.3±10.8	37.0±10.7	37.2±10.8	35.9±10.4	36.5±10.6
H. hemerocallidea	Methanol	100 mg/kg	38.2±0.5	37±0.3*	37.2±0.6*	36.6±0.6*	36.3±0.1*
E. transvalense	DCM	1000 mg/kg	38.3±0.3	37.8±0.2*	37.6±0.2*	36.5±0.2*	36.4±0.1*

Table 4.6.2 c continued							
E. transvalense	Methanol	1000 mg/kg	38.7±0.12	37.8±0.3**	37.6±0.04**	37.2±0.3**	37.6±0.3**
E. transvalense	Aqueous	1000 mg/kg	38.4±11.07	37.2±10.76	38.2±11.02	37.6±10.85	37.1±10.71
W. somnifera	DCM	1000 mg/kg	36.5±0.4	36.1±0.1*	37.2±0.04*	36.1±0.2*	35.8±0.5*
W. somnifera	Methanol	1000 mg/kg	37.5±0.03	37.5±0.3	36.8±0.4	35.5±0.3	36.8±0.5
W. somnifera	Aqueous	1000 mg/kg	37.2±0.1	38.8±0.5*	37.8±0.3	38.5±0.5*	38.4±0.4*
H. hemerocalledea	DCM	1000 mg/kg	37.3±10.8	37.0±10.7	37.18±10.8	37.18±10.8	35.9±10.4
G. thunbergia	DCM	1000 mg/kg	37.9±0.5	37.6±0.1*	37.5±0.04*	37.6±0.3	37.8±0.3**
G. thunbergia	Methanol	1000 mg/kg	37.5±10.8	33.7±9.7	34.3±9.9	34.3±9.9	31.9±9.6
G. thunbergia	Aqueous	1000 mg/kg	37.7±10.9	36.2±10.5	27.8±12.3	27.5±12.1	27.0±11.9
S. aethiopicus	DCM	1000 mg/kg	37.1±10.7	37.6±10.9	37.5±10.5	36.9±10.7	36.7±10.6
S. aethiopicus	Methanol	1000 mg/kg	37.23±10.77	36.9±10.60	36.9±10.68	37.6±10.86	37.6±10.7
S. brachypetala	DCM	1000 mg/kg	35.8±10.35	35.8±10.34	36.2±10.45	38.3±11.6	35.9±10.4
S. brachypetala	Methanol	1000 mg/kg	35.9±10.4	36.7±10.6	36.3±10.5	35.9±10.4	35.9±10.4
S. brachypetala	Aqueous	1000 mg/kg	38.7±0.1	37.1±0.13*	37.1±0.06*	36.8±0.1**	36.7±0.1 ***

4.6.3 *In vitro* antiplasmodial activity of the plants extracts

Plants extracts activity against *Plasmodium falciparum* (chloroquine sensitive (CQS)) D10 strain is presented in table 4.6.3. The antiplasmodial results revealed that dichloromethane extract of *A. australe* was the most active at 5 µg/ml (Table 4.6.3 a). The dichloromethane extracts of *E. transvalense* and *W. somnifera* (Table 4.6.3 a), and methanolic extracts of *V. adoensis* (leaves) and *G. thunbergia* (Table 4.6.3 b) were also active with less than 50% survival at the lowest concentration. The dichloromethane extracts of *V. adoensis*, *S. aethiopicus, S. brachypetala,* and *G. thunbergia* (Table 4.6.3 a), and the methanol extracts of *A. calamus* showed activity between 10 to 20 µg/ml (Table 4.6.3 b). The dichloromethane extract of *E. transvalense*, *W. somnifera* and methanol extract of *G. thunbergia* showed good activity with IC_{50} values ≤ 5 µg/ml (Table 4.6.3 a). The aqueous extracts of the plants did not exhibit any activity at the concentration tested (Table 4.6.3 c). IC_{50} values were determined for extracts showing activity at ≤ 5 µg/ml.

Table 4.6.3 a: *In vitro* antiplasmodial activity of the dichloromethane plants extracts against *Plasmodium falciparum* D10 strain.

Plant	**Plant Extract**	**% Parasite Survival**			**IC_{50} µg/ml**	**Activity µg/ml**
		20 µg/ml	**10 µg/ml**	**5 µg/ml**		
H. hemerocallidea	Dichloromethane	105.84	109.86	75.72	ND	NA
A. *australe*	Dichloromethane	0.00	0.00	11.47	1.04	5
V. adoensis (leaves)	Dichloromethane	56.12	73.93	89.46	ND	10-20
V. adoensis (roots)	Dichloromethane	35.98	77.73	93.02	ND	NA
A. calamus	Dichloromethane	67.94	101.33	100.60	NA	NA
E. transvalense	Dichloromethane	10.67	4.14	36.53	5.07	≤5
W. somnifera	Dichloromethane	22.06	35.38	47.34	4.94	≤5
G. thunbergia	Dichloromethane	46.71	75.99	77.34	ND	10-20
S. aethiopicus	Dichloromethane	44.89	90.01	92.63	ND	10-20
S. brachypetala	Dichloromethane	38.51	67.30	82.98	ND	10-20
		30 ng/ml	**15 ng/ml**	**7.5 ng/ml**		
Chloroquine (CQ) (n=8)		4.14	23.26	32.55	27.73 ng/ml (n=1)	

NA=Not active at concentration tested, ND=Not determined, n=number of data sets averaged

Table 4.6.3 b: *In vitro* antiplasmodial activity of the methanol plants extracts against *Plasmodium falciparum* (CQS) D10 strain

Plant	Plant Extract	% Parasite Survival			IC_{50} µg/ml	Activity µg/ml
		20 µg/ml	**10 µg/ml**	**5 µg/ml**		
H. hemerocallidea	Methanol	98.95	96.34	110.86	ND	NA
A. australe	Methanol	41.13	46.78	76.86	ND	5-10
V. adoensis (leaves)	Methanol	0.00	44.23	41.68	2.90	5
V. adoensis (roots)	Methanol	92.25	109.49	105.52	ND	NA
A. calamus	Methanol	47.52	70.40	91.64	ND	10-20
E. transvalense	Methanol	57.03	86.23	94.70	ND	NA
W. somnifera	Methanol	70.17	97.39	104.62	ND	NA
G. thunbergia	Methanol	12.11	33.88	39.72	4.36	≤5
S. aethiopicus	Methanol	106.95	99.32	102.53	ND	NA
S. brachypetala	Methanol	69.24	95.30	92.62	ND	NA
		30 ng/ml	**15 ng/ml**	**7.5 ng/ml**		
Chloroquine (CQ) (n=8)		4.14	23.26	32.55	27.73 ng/ml (n=1)	

NA=Not active at concentration tested, n=number of data sets averaged

Table 4.6.3 c: *In vitro* antiplasmodial activity of the aqueous plants extracts against *Plasmodium falciparum* (CQS) D10 strain

Plant	Plant Extract	% Parasite Survival			IC_{50} µg/ml	Activity
		20 µg/ml	**10 µg/ml**	**5 µg/ml**		
H. hemerocallidea	Aqueous	82.82	112.14	110.77	ND	NA
A. australe	Aqueous	57.33	83.23	100.27	ND	NA
V. adoensis (leaves)	Aqueous	61.38	86.53	89.70	ND	NA
V. adoensis (roots)	Aqueous	95.09	90.58	98.77	ND	NA
A. calamus	Aqueous	103.09	97.69	104.63	ND	NA
E. transvalense	Aqueous	96.92	107.68	93.80	ND	NA
W. somnifera	Aqueous	93.22	105.79	96.04	ND	NA
G. thunbergia	Aqueous	95.07	97.38	92.62	ND	NA
S. aethiopicus	Aqueous	100.14	107.31	89.25	ND	NA
S. brachypetala	Aqueous	81.17	101.86	89.39	ND	NA
		30 ng/ml	**15 ng/ml**	**7.5 ng/ml**		
Chloroquine (CQ) (n=8)		4.14	23.26	32.55	27.73 ng/ml (n=1)	

NA=Not active at concentration tested, n=number of data sets averaged

4.6.4 Cytotoxicity of the plants active for *in vitro* antiplasmodial activity

Cytotoxicity levels of the samples showing encouraging (IC_{50}-values) *in vitro* antiplasmodial activity were determined against both human kidney (HEK293) and human hepatocellular carcinoma (HepG2) cell lines. It is apparent that the plant extracts exhibit weak toxicity (Table 4.6.4).

Table 4.6.4: Cytotoxicity of the plants active for *in vitro* antiplasmodial activity against HEK293 (Human embryonic kidney) cells and HepG2 (Human hepatocellular carcinoma) cells.

Plant Name	**Extract**	**MTT Assay LC_{50} (µg/ml)**	
		Human embryonic Kidney cells	Human hepatocellular carcinoma cells
A. australe	Dichloromethane	534	512
V. adoensis (leaves)	Methanol	361	421
E. transvalense	Dichloromethane	512	394
W. somnifera	Dichloromethane	635	911
G. thunbergia	Methanol	363	338

4.7 Isolation and characterisation

The isolation and purification of the crude dichloromethane extract of *A. australe* (which exhibited the highest antiplasmodial activity) yielded two major compounds (MF/01/H_1 and MF/01/I_1) that were further identified through IR, UV, and GC-MS techniques. The 2D NMR (HSQC, HMBC, NOESY and COSY) were used to assign carbons and protons (see appendix E for NMR and other spectra for the compounds). Comprehensive assignment of the ^{13}C-NMR and significant ^{1}H-NMR of MF/01/H_1 in comparison with literature values is presented in Table 4.7a and 4.7b.

Table 4.7a: ^{1}H-NMR and ^{13}C-NMR spectral data of compound MF/01/H_1a.

Position	δ_H (ppm)	δ_H (ppm) [RC]	δ_c (ppm)	δ_c (ppm) [RC]
1			37.3	37.2
2			31.7	31.7
3	3.51 (1H, *m*)	3.20 (1H, *m*)	71.8	71.8
4			42.2	41.8
5			140.8	140.8
6	5.32 (1H, *m*)	5.36 (1H, *m*)	121.7	121.8
7			31.9	32.0
8			31.8	31.8
9			50.1	50.8
10			37.3	37.7
11			21.1	21.1
12			39.8	39.8
13			42.3	42.7
14			56.8	56.5
15			26.1	26.3
16			25.4	25.9
17			56.1	56.2
18			36.2	36.1
19			19.4	19.4
20			33.9	33.9
21			26.1	26.4
22			45.9	46.1
23			23.1	23.2
24			12.3	12.2
25			31.7	30.4
26			21.1	21.0
27			21.2	21.0
28			19.1	19.3
29			12.1	12.0

RC= Reference compound

Table 4.7 b: ^{1}H-NMR and ^{13}C-NMR spectral data of compound MF/01/H_1b.

Position	δ_H (ppm)	δ_H (ppm) [RC]	δ_c (ppm)	δ_c (ppm) [RC]
1			37.3	37.2
2			31.7	31.7
3	3.51 (1H, *m*)	3.20 (1H, *m*)	71.8	71.8
4			42.2	41.8
5			140.8	140.8
6	5.32 (1H, *m*)	5.26 (1H, *m*)	121.7	121.8
7			31.9	32.0
8			31.8	31.8
9			50.1	50.8
10			37.3	37.7
11			21.1	21.1
12			39.8	39.9
13			42.3	43.0
14			56.8	56.6
15			26.1	26.4
16			28.9	28.6
17			56.1	56.4
18			40.5	40.1
19			19.8	20.2
20	5.13 (1H, *dd*, *J* = 16Hz, 8Hz	5.15 (1H, *d*, *J* = 8Hz)	138.3	138.3
21	5.08 (1H, *dd*, *J* = 8 Hz, 8Hz)	5.04 (1H, *d*, *J* = 8Hz)	129.3	129.5
22			51.2	52.2
23			25.4	25.4
24			12.3	12.3
25			31.9	31.9
26			21.1	21.1
27			21.2	21.1
28			19.4	19.3
29			12.3	12.3

RC= Reference compound

Compound MF/01/H_1 was obtained as white flakes with melting point 110-112^{o}C. The ^{1}H-NMR spectra (appendix E) of compound MF/01/H_1 showed a sterol proton pattern. The proton NMR showed the proton of H-3 and H-6 appeared as a multiplet

at δ 3.51 and δ 3.52 respectively (table 4.7a and 4.7b). The proton NMR also showed proton of H-20 and H-21 as a duplex of a duplex at δ 5.13 (1H, *dd*, *J* = 16Hz, 8Hz and 5.08 (1H, *dd*, *J* = 8 Hz, 8Hz) respectively. The ^{13}C-NMR has shown recognisable signals 140.8 and 121.7 ppm allocated to C_5 and C_6 double bonds respectively as in Δ^5 spirostene (Kamboj *et al.*, 2011). The spectra showed 29 carbon signal and 3 cyclohexane rings and a cyclopentane ring. The alkene carbons appeared at δ 140.8, δ 121.7 and δ 129.3 (Kamboj *et al.*, 2011).

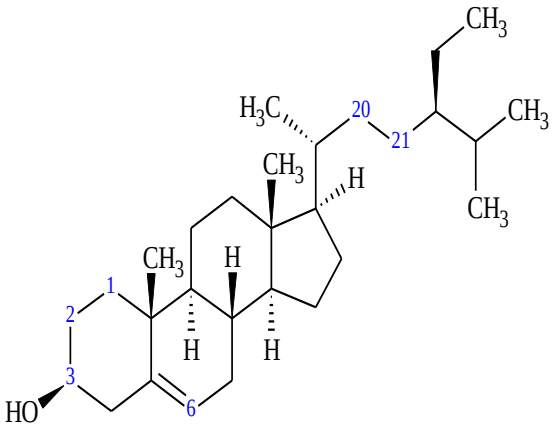


Figure 4.2a: Chemical structure of β-sitosterol (MF/01/H_1a)

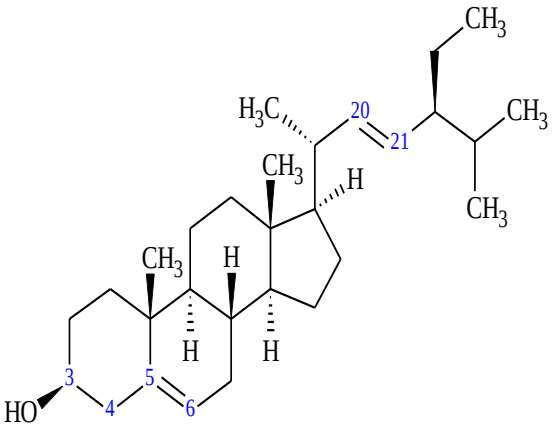


Figure 4.2b: Chemical structure of stigmasterol (MF/01/H_1b)

Figure 4.2: Chemical structure of MF/01/H_1

The signal corresponding proton and carbon NMR spectrum data of the sample suggests that sample MF/01/H_1 contains two similar compounds MF/01/H_1a and MF/01/H_1b. The NMR spectra data (appendix E) in comparison with those described in literatures indicated that the compounds presenting MF/01/H_1 are β-sitosterol and stigmasterol (figures 4.2 a. and b). The double bonds present at C_5 and C_6, and C_{20} and C_{21} suggest that MF/01/H_1b is a stigmasterol. And the presence of a double

bond in C_5 and C_6, and the single bond in C_{20} and C_{21} suggest that MF/01/H_1a is a β-sitosterol.

Sample MF/01/I_1 was obtained as a white powder and even though it appeared as a single spot on TLC, it was found to have some impurities when analysed through NMR and therefore could not be adequately identified. However, it was further analysed through GC-MS and the spectra (appendix E) revealed a mixture of compounds**:** Camphene, 1,2,5-oxadiazole, gamma sitosterol, beta sitosterol, 2-ethylacridine, decane and traces of compound MF/01/H_1.

4.8 *In vitro* antiplasmodial activity of MF/01/H1.

In vitro antiplasmodial activity of the pure compound MF/01/H1 against *Plasmodium falciparum* (CQS) D10 strain show no antiplasmodial activity (table 4.8).

Table 4.8: *In vitro* antiplasmodial activity of MF/01/H1 from *A.australe* dichloromethane extract against *Plasmodium falciparum* (CQS) D10 strain.

Plant	**% Parasite Survival**			**IC_{50} µg/ml**
	20 µg/ml	**10 µg/ml**	**5 µg/ml**	
Sitosterol	92.5	94.1	95.4	NA
	30 ng/ml	**15 ng/ml**	**7.5 ng/ml**	
Chloroquine (CQ) (n=4)	21.7	35.9	75.0	16.7 ng/ml

NA=Not active at concentration tested, n=number of triplicates

CHAPTER 5

DISCUSSION

Despite the many and varying efforts to control the disease, malaria still remains a threat to the human race. People in under-developed countries cannot afford the means of protecting themselves against the disease and, on the other hand, the parasite has developed an increasing resistance to available antimalarial drugs. This resistance is also favoured by the population movement introducing resistant parasite to areas previously free of drug resistance. Since the discovery of quinine (isolated from *Chinchona officinalis* L., Rubiaceae), the potential of investigating plants for new anti-malarial compounds has been on the increase. It is apparent that some of the plants (A. *australe*, *E. transvalense, G. thunbergia, V. adoensis* (leaves) and *W. somnifera)* investigated in this study possess antimalarial properties.

Antioxidant activity

Free radicals have been implicated in a variety of diseases ranging from cancer to neurological diseases (Rafikali and Nair, 2001). In severe malaria, the red blood cell membrane is exposed to a local increase in oxidative stress (Griffiths *et al*., 2001). It is thus suggested (Griffiths *et al*., 2001; Ngouela *et al*., 2006) that compounds exhibiting both anti-plasmodial and antioxidant activities could be leads for new antimalarial drugs. Although there are medications to manage free radical damage and to protect the body against oxidative stress the drugs available and in use are known to have severe side effects. The need for natural antioxidants to replace the synthesized ones (BHT, BTA) cannot be overemphasised. Most of the extracts showed relatively better ability to scavenge the biological free radicals (SO, OH, and NO) than the standards. Secondly, the relatively high content of SH, phenol,

proanthocyanidin, and flavonoids (table 4.4) suggests that the plants in this study have the potential of combating oxidative stress (Moure *et al*., 2001). For example, a dual role of NO has been implicated in many neurological disorders of the body. Its roles in the pathogenesis of major depression and modulatory activity of various antidepressants have been indicated by recent researches (Stermler *et al.,* 1992; Galigniana *et al.,* 1999; Lee *et al.,* 2004). If production of nitric oxide is unremitting this could be a threat to the biological system. The results in this study suggest that most of the plant extracts were inhibitors of nitric oxide. Two inhibitors of NO production have been isolated from plants that are used for their insecticidal activity (Hossay *et al*., 2011).

The reducing power capacity of many plant extracts have been correlated with their antioxidant activity (Pin-Der-Duh, 1998). In addition, reducing properties have been associated with the presence of reductones, which exert antioxidant action by breaking the free radical chains through hydrogen atom donation (Gordon, 1990; Pin-Der-Duh, 1998). The results suggest the plants in this study to be good reductones. The plant extracts ability to scavenge hydroxyl radical can help prevent lipid peroxidation as hydroxyls are the major active oxygen species causing lipid peroxidation and biological damages (Pin-Der-Duh, 1998).

Larvicidal, cytotoxicity and *in vitro* antiplasmodial activity.

Mosquitoes have achievably attained the utmost public notoriety than any other arthropod. Over 2 million people die annually from mosquito-borne diseases such as malaria, filarial disease and viruses such as dengue and yellow fever (Abdullahi *et al.*, 2011).The control of mosquitoes, and thus malaria and other related diseases, is a major concern of both governmental and non-governmental organisations. It is

envisioned that the only successful method of reducing mosquito densities to an appreciable level for which a malaria epidemic can be controlled is by attacking the larval breeding places through the use of larvicides (WHO, 1996). Plants generally offer an alternative source of insect control agents, because they are available locally, cheap and contain a wide range of bioactive chemicals, which have little or no harmful effect on non-target organisms and the environment.

The results for larvicidal activity of the extracts (Table 4.6.1) demonstrated the larvicidal potency of the dichloromethane extracts. The larvicidal activity of some plants has been reported (Howard *et al*., 2011) and linked to the presence of flavonoids and saponins in the plants (Abdullahi *et al.*, 2011); however, such plants were not reported to exhibit any antiplasmodial activity. Nonetheless, additional study on specific potency of these phytochemicals (and others) needs to be undertaken to establish which of these chemical could be liable for larvicidal effectiveness.

The results from this study indicate that the dichloromethane extracts of *A. australe, E. transvalense* and *W. somnifera* and methanolic extracts of *V. adoensis* (leaves) and *G. thunbergia* contain antimalarial substances which also showed the most activity. This investigation also shows that the parasite-killing ability of the extracts is concentration dependent. This was evident in the observation, whereby the cells treated with a higher concentration showed higher percentage mortality compared to the lower concentration. It is worth noting that *A. australe* extracts exhibited the most active antioxidant and antiplasmodial properties at the lowest concentration tested.

Previous studies have shown that alkaloids are the dominating active constituents present in most antimalarial drugs (Ahmed *et al*, 2010). The antiplasmodial activity of

alkaloids (Warhurst, 2007) and flavonoids derivatives (De Monbrison *et al.*, 2006) has been demonstrated. The results observed therefore may account, at least in part, to the presence of these phytochemicals in the extracts (table 4.1). However, further study would have to be conducted to elucidate the active ingredient in these plants. The present study supports the earlier claims on the antimalarial activity of these plants by traditional healers. It is apparent, however that traditional healers may not be optimizing the effectiveness of the plants since their concoctions are often prepared with water and this study indicates that the organic extracts were most active.

A good drug has to be active; then again it should be non toxic. Nevertheless, toxicity of a drug will depend on the anticipated use and activity. So activity alone of a drug is not enough without the knowledge of its toxicity levels. Svensson *et al.*, (2005) illustrated that plant extract toxicity depends on the concentration of specific compounds. The cell lines are considered to be a good replacement of animal based methods (Betrabet *et al.*, 2004). The samples showing encouraging *in vitro* antiplasmodial activity were therefore selected to determine their cytotoxicity levels against both human kidney (HEK293) and human hepatocellular carcinoma (HepG2) cell lines. The results revealed moderate cytotoxicity activity of the plant extracts. According to the American National Cancer Institute guidelines, a compound is considered significantly active with IC_{50} value less than 30µg/ml (Suffness and Pezzuto, 1990). The results show that these plants can be used as future possible malaria drugs with no threat of toxicity.

The phytochemicals screening of the plants confirmed the presence of many constituents such as tannins, flavonoids, terpinoids and alkaloids which have been associated with antimalarial properties (Ahmed *et al*., 2010).

However, the isolated and purified compounds (β-sitosterol--MF/01/H_1a; and stigmasterol-- MF/01/H_1 b) obtained from *A australe* did not reveal any *in vitro* antiplasmodial activity. Literature review has revealed that sitosterol is difficult to be obtained in pure form (Kamboj and Saluja, 2011). It is apparent that the active component in *A. australe* is not a phytosterol. Each plant contains different phytochemicals which may be acting singly or in synergy with one another to exert the observed activity. The loss of activity could also be due to decomposition during fractionation or the removal of protective matrix. The active compound could also be any one of the minor components not identified. Phytosterols are not known to possess antimalarial properties. However, previous studies have revealed that beta-sitosterol is one of those remarkable plant nutrients which are important, non-toxic nutrients, important for the maintenance of health against several disorders. Phytosterols are very stable and, therefore, cannot be affected by intense processes such as boiling, neutralization, bleaching and deodorisation (Normen *et al.*, 1999; Bortolomeazzi *et al.*, 2000). Beta sitosterol display scientifically recognised health benefits such as anti-tumour activities (Romero and Lichtenberger, 1990; Janezic and Rao, 1992), antidiabetic (Ivorra, 1988) and antibacterial (Hess *et a.l,* 1995; Padmaja *et al.*, 1993) properties, lowering cholesterol level (Lees *et al.,* 1977) and antifungal abilities (Smania *et al.,* 2003).

Antipyretic Activity

Most of the plant extracts exhibited the potential to reduce pyrexia in the induced rats (Table 4.6.2). The activity was time and concentration dependent with most of the extracts showing activity as early as from 30 minutes even at the lowest concentration (100 mg/kg). In folk medicine, fever is the early symptom of malaria. Even though fever may be caused by other infections, the onset of fever is easily attributed to malaria infection. Most of the extracts in this study did not exhibit antiplasmodial activity but showed antipyretic properties. Plants with such activities would offer substantial relief to malaria patients. It is likely that traditional healers use these plants to treat the symptoms rather than the *plasmodium* infection.

CHAPTER 6

CONCLUSION

Traditional healers rely on plants to treat their patients. The results of this study scientifically validate the usage of the plants (*A. australe, G. thunbergia, W. somnifera*, *V. adoensis* (leaves) and *E. transvalense*) as treatment for malaria by traditional healers. With the relatively high antipyretic, antioxidant and larvicidal activity, coupled to the low levels of cytotoxicity, the plants could be candidates for the isolation and characterisation of the active compounds.

However, the results showed that the major isolates, β-sitosterol and stigmasterol did not exhibit any antiplasmodial activity *in vitro*. The active compound could be any one of the minor components not identified in this study. The antioxidant activity of the plants may be a mechanism that contributes to their antimalarial activity.

6.1 Suggestion for further studies

Further studies of these plants are suggested along these lines:

(i) Isolation and characterisation of the active compounds.

(ii) *In vitro* antiplasmodial activity on other *plasmodium* strains.

(iii) *In vivo* antiplasmodial activity.

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Appendix A

Details of Reagents preparations

A1 Dragendorff's reagent:

Solution A: 0.85 g of subnitrate bismuth and 20ml of glacial acetic acid were dissolved in 40 ml of distilled water.

Solution B: 0.8 g of potassium iodide was dissolved in 20ml of distilled water. Both solutions A and B were stored in dark bottles separately. 5ml of each solution (A and B) And glacial acetic acid were mixed and made up to 100ml with distilled water.

A2 Mayer's reagent:

Solution A: 1.36 g mercury (II) chloride was dissolved in 60 ml of distilled water.

Solution B: 5.0 g potassium iodide was dissolved in 20 ml of distilled water.

Both solutions were mixed and made up to 100ml with distilled water.

A3 1% TBA

1 g of TBA was dissolved in 50% glacial acetic acid solution.

A4 DPPH

2 mg % of DPPH was prepared in methanol.

A5 ABTS*

10 mg (1 tablet) of ABTS was dissolved in 3 ml of distilled water and 2 mg of potassium persulfate was added to the solution. The mixture was incubated in the

dark for 16 hours at room temperature. ABTS* was then diluted with methanol (1ml ABTS*: 60 ml methanol) and used immediately.

A6 Phosphate buffer (pH6.6)

18 ml of 0.2 M potassium hydroxide was mixed with 50 ml of 0.2 M potassium dihydrogenphosphate and made up to 100 ml with distilled water. The pH was then adjusted to the required value.

A7 Sodium carbonate buffer (pH 10.5)

18 ml of 0.1 mol/litre sodium bicarbonate was made up to 100 ml with 0.1 mol/litre sodium carbonate.

A8 Sulphanilic acid reagent

20 ml of glacial acetic acid was made up to 100 ml with distilled water. This was used to dissolve 33 grams of sulphanilic acid.

A9 Tris buffer (pH 7.4) containing EDTA and NaCl

10.227 g of 175 mM NaCl, 7.88 g of 50 mM Tiris-HCl and 2.79 g of 7.5 mM were dissolved and made up to 100 ml using distilled water.

A10 2% $AlCl_3$

2 g of $AlCl_3$ was prepared and made up to 100ml in 80% methanol.

A11 Vanillin

4% vanillin in 80% methanol was prepared.

A12 GSH

Distilled water (5 ml) containing 30 mmol/ml of EDTA was used to dissolve 1 mg of GSH. The solution was kept in ice.

Appendix B

Method details

B1 Extraction:

The plants materials were washed and dried in the oven at 50±2^{o}C for 24hr. The dried materials were ground into powder and passed through 2mm meshscreen (2 mm mesh) and stored in brown bottles at 4^{0}C until used for extraction. 40 g of each powder was separately incubated (1:5w/v) with methanol, distilled water, and dichloromethane for 24 hours on orbital shaker, 150 rpm at room temperature. This was then filtered using Whatman No. 1 filter paper. The extracts obtained after filtrations were concentrated under reduced pressure; the aqueous extracts were freeze-dried. The extracts were then dissolved in 10 ml of their respective solvents and stored at 4^{0}C.

B2 Phytochemical screening:

B2.1 Test for tannins

0.5g of methanolic extract was boiled with 10ml of water for 15 minutes, filtered and made up to 10 ml. Into 2 ml of the filtrate few drops of 0.1% $FeCl_3$ solution were added. A blue-green or bluish-black green precipitate was taken as preliminary evidence of the presence of tannins.

B2.2 Test for saponins

2.5 g of the plant material was extracted with boiling water. The extract was shaken vigorously to froth after cooling. The extract was then allowed to stand for 15-20

minutes and was classified for saponin content as follows: No froth = Negative, froth less than 1 cm = Weakly positive, froth 1.2 cm high = Positive, froth greater than 2 cm high = Strongly positive.

B2.3 Test for alkaloids

25 mg of extract was dissolved in water and filtered. The filtrate was acidified with 1M HCl. 1 ml of the filtrate was treated with two drops of Mayer's reagent. A precipitate was taken as preliminary evidence of the presence of alkaloids. Another 1ml of the filtrate was treated with Dragendroff's reagent. Turbidity or precipitate was also taken as preliminary evidence for the presence of alkaloids.

B2.4 Test for terpenoids (Salkowski test)

5 ml of methanolic extract was mixed in 2 ml of chloroform. 3 ml of concentrated H_2SO_4 was carefully added to form a layer. A reddish brown colouration of the interface was formed to show positive results for the presence of terpenoids.

B2.5 Test for anthraquinones

5 g of the extract was mixed with 10 ml of benzene. After shaking the mixture was filtered. 5 ml of 10% ammonium solution was added to the filtrate and shaken to form a mixture. The presence of pink, red or violet colour in ammonia solution (low phase) shows positive results.

B2.6 Test for flavonoids

Two tests were used for the screening of flavonoids:

B2.6.1 Ferric chloride test

1 ml of the extract was mixed with 1 ml of 10% $FeCl_3$. Formation of a dark brown (dirty brown) colour precipitate is evidence for positive results.

B2.6.2 Sodium hydroxide test

1 ml of extract was mixed with 1ml of diluted NaOH. A golden yellow precipitate shows positive results.

B2.7 Test for cardiac glycosides

Three tests were performed for the screening of cardiac glycosides:

B2.7.1 Liebermann's test:

0.5 ml of the extracts was mixed with 2 ml of acetic anhydride. The mixture was cooled well in ice. Carefully 1 ml of concentrated sulphuric acid was added. A colour change from violet to blue and green was observed as an indication of the presence of steroidal nucleus i.e. the aglycone portion of the cardiac glycosides.

B2.7.2 Salkowski test

0.5 g of the extract was mixed with 2 ml of concentrated sulphuric acid (which was carefully added to form a layer) and 2 ml of chloroform. A reddish brown colour at interphase was taken as an indication of the presence of a steroidal ring (aglycone portion of the cardiac glycoside).

B2.7.3 Keller-Kilian test:

0.5 g of the extract was dissolved in 2 ml glacial acetic acid containing 1 drop of 10% $FeCL_3$ solution. This was underlayered with sulphuric acid (1 ml). A brown, violet or greenish ring at the interphase indicated the presence of deoxysugar characteristic of cardenolides.

B3 Flavonoid content (quercetin equivalent)

Various concentrations (0.01, 0.02, 0.04, 0.06, 0.08 and 0.1 mg/ml) of quercetin were prepared in diethyl ether. The plant powder was dissolved in diethyl ether and then the diethyl ether was allowed to evaporate. The plant residue and the various concentrations of quercetin were dissolved in 0.5 ml of 2% $AlCl_3$ solution. It was allowed to stand at room temperature for an hour; a yellow colour indicated the presence of flavonoid. The absorbance was read at 420 nm. The standard graph of quercetin was plotted (figure B1). Flavonoid was estimated as quercetin equivalent from the plotted graph.

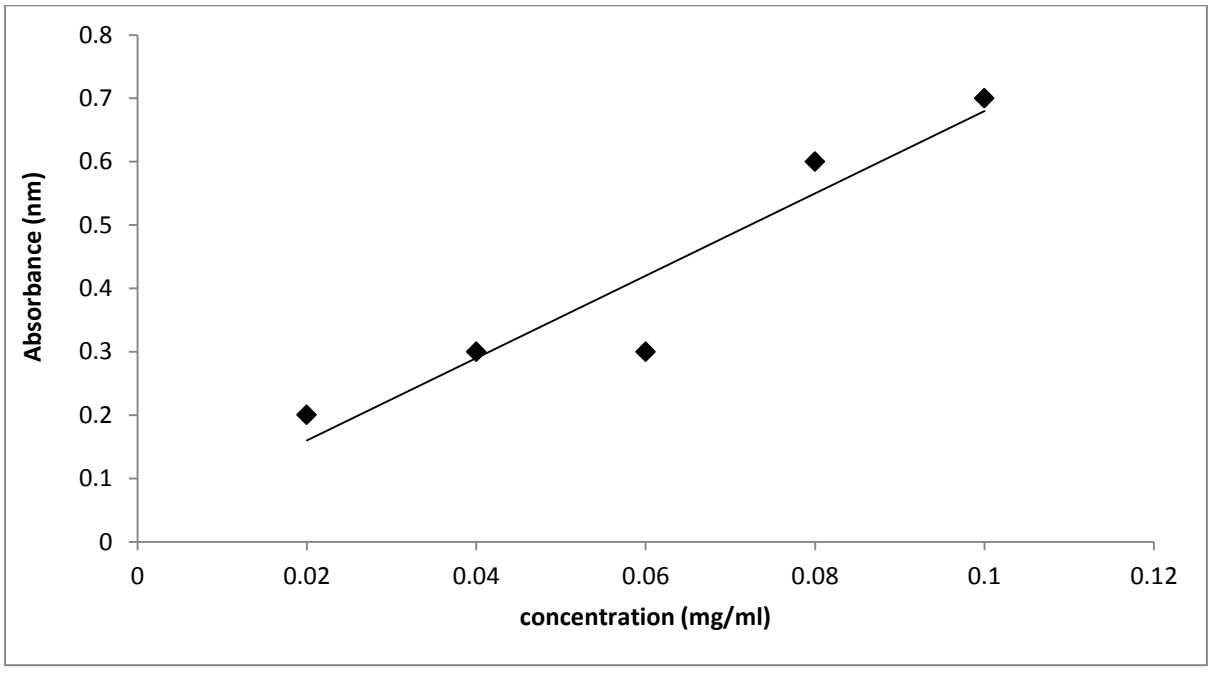

Figure B1: Calibration curve of quercetin.

The curve was used to determine the flavonoid content of the plants as quercitin equivalent.

B4 Total phenolic content (Gallic acid equivalent- GAE)

Different concentrations of gallic acid were prepared as in flavonoid content. Plant powder was mixed with diethyl ether (1g/5ml). Diethyl ether was allowed to evaporate off in both gallic acid and plant powder to leave the residue. Sodium carbonate (7.5 g/100 ml) and Folinciocalteu reagent (1:10 dilution) were prepared.

A set of test tubes (in duplicate) was set up and into each test tube containing the residues 1.5 ml Folinciocalteu reagent, and 1.2 ml sodium carbonate were also added into the solution. It was allowed to stand in the dark for 30 minutes, and absorbance was read at 765 nm. The standard graph of gallic acid was plotted, see figure B1.2. Total phenolic content was estimated as gallic acid equivalent from the plotted graph.

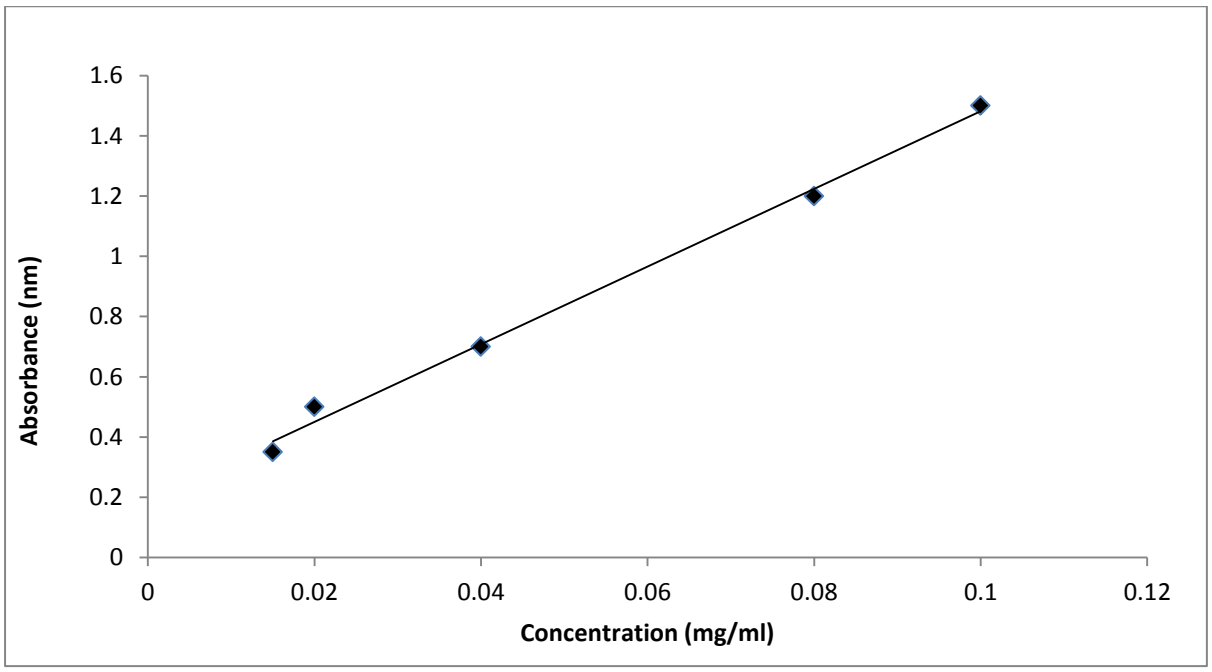

Figure B2: Calibration curve of gallic acid.

The curve was used to determine the total phenolic content of the plants as gallic acid equivalent.

B5 Proanthocyanidin content (Catechin equivalent)

Different concentrations of catechin in diethyl ether were prepared as in flavonoid content. The plant powder was extracted with diethyl ether (1 g: 5 ml). Diethyl ether was allowed to evaporate off. Vanillin (4%) and 1% HCL were also prepared. Test tubes were set in duplicates. Into each test tube containing the residue 3 ml of vanillin, and 1.5 ml HCL were added. The mixture was allowed to stand for 15 minutes at room temperature and absorbance was read at 500. The standard graph was plotted, see figure B3. Proanthocyanidin was estimated as catechin equivalent from the graph.

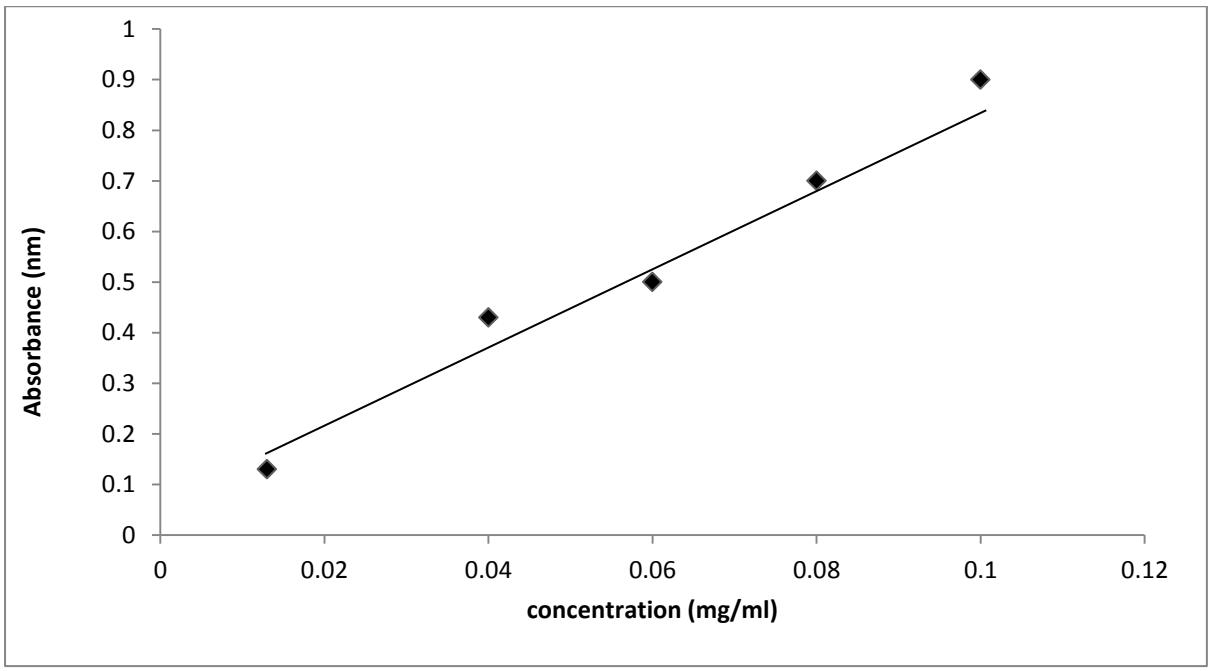

Figure B3: Calibration curve of catechin.

The curve was used to determine the proanthocyanidin content of the plants as catechin equivalent.

B6 Antioxidant activity *in vitro*

B6.1 Free radical scavenging

B6.1.1 DPPH

DPPH (2 mg/100 ml) was prepared in methanol. Different plant extract concentrations (1-5 mg/100 ml) were also prepared in methanol. Six test tubes in duplicate (12 test tubes) were set up for each extract concentration. 2 ml of DPPH solution was poured into each test tube. 2 ml of each extract was added into each corresponding test tube. The mixture was mixed and left to stand for 30-60 minutes. The absorption was read at A_{517}. The percentage scavenger was calculated:

$\%\ Scav = [1-A_t/A_0]\cdot 100.$

Methanol was used as the blank.

Graphs of the percentage scavenging activity versus concentration of extract (mg/100 ml) were plotted.

B6.1.2 ABTS:

Different concentrations of the extracts (1-5 mg/100 ml) were prepared in methanol. Test tubes were set as in the DPPH experiment. 1ml of ABTS* was put in each test tube and 1 ml of the extract was added into each corresponding test tube. This was mixed and left to stand for 6 minutes. The absorbance was read at 734 nm. Methanol was used as the blank. Percentage scavenging activity was calculated and

graphs of percentage scavenging activity versus concentration (mg/100 ml) were constructed.

B6.1.3 Reducing Power:

Different concentrations of the extracts (1-5 mg/100 ml) were prepared in methanol. The following were also prepared: Phosphate buffer (PB), 1% potassium ferricyanide (PF), 10% Trichloroacetic acid (TCA) and 0.1% Ferric Chloride ($FeCl_3$). Test tubes were set up as in DPPH experiment. 1 ml of extract was mixed with 2.5 ml PB (pH 6.6, 0.2 M) and 2.5 ml PF (1%). The mixture was incubated at 50^{o}C for 20 minutes. After incubation 2.5 ml of 10% TCA was added and left to stand for 5-10 minutes. The mixture was centrifuged at 1000 rpm for 10 minutes. 2.5 ml of the supernatant was diluted with 2.5 ml of distilled water. This was mixed with 0.5 ml of 0.1% $FeCl_3$. The absorbance was read at 700 nm and distilled water was used as blank. The graph of absorbance (nm) versus concentration of extract (mg/100 ml) was constructed.

B6.1.4 Chelating activity on Fe^{2+}:

Test tubes were set as in DPPH experiment. 1 ml of extract was diluted with 3.75 ml of distilled water and then mixed with 0.1 ml 2 mM $FeCl_2$ and 0.2 ml 5 mM ferrozine. The mixture was left to stand for 10 minutes (into mix). The absorbance was read at 562 nm. The percentage chelating was calculated as follows:

% chelating = $(1-A_t/A_0) \times 100$

Percentage chelating versus concentration of extract (mg/100 ml) graphs were constructed.

B6.1.5 Superoxide anion scavenging

Test tubes were set as in DPPH experiment. Into each test tube, 0.48 ml sodium carbonate buffer (0.05 M), 0.02 ml xanthine (3 mM), 0.02 ml EDTA (3 mM), 0.02 ml BSA (0.15%), 0.02 ml NBT (0.75 mM), and 0.02 ml of plant extract were added. The mixture was incubated for 20 minutes at 25ºC. After incubation, 0.02 ml XOD (6 mM) was added. The mixture was incubated again for 20 minutes at 25ºC. After incubation 0.02 ml of CuCl (6 mM) was added and the absorbance was read at A_{560}. The percentage scavenging activity was calculated using the formula: % scavenging activity [1-At-A0]*100. Percentage scavenging activity versus concentration of extract (mg/100 ml) graphs was constructed.

B6.1.6 Hydroxyl radical scavenging assay

Test tubes were set as in DPPH experiment. Into each test tube, 0.2 ml $FeSO_4.7H_2O$ was added. Then 0.2 ml of plant extract or 0.2 ml of phosphate buffer instead of plant extract was added, and also 0.2 ml deoxy ribose, 0.1 ml phosphate buffer and 0.2 ml H_2O_2 was also added to all test tubes. The mixture was incubated at 37ºC for four hours. After incubation, 1 ml TCA and 1 ml TBA were added. The mixture was boiled in boiling water for 10 minutes. The tubes were cooled on ice after boiling and the absorbance was read at A_{520}. OH scavenging activity was determined using the formula: % Scav = $[1-(A_t/A_0)]*100$ % scavenging activity versus concentration (mg/100 ml) graph was plotted.

B6.1.7 Sulfhydryl content

Plant powder was extracted with ice cold distilled water containing 30 mM/ml EDTA solution, in 1:5 ratios, centrifuged at 1000 rpm for 10 minutes, and filtered. 3 test

tubes in duplicate (1 GSH, 1 extract, 1 GSH) were set up. 0.1 M phosphate buffer and 1% o-phthalaldehyde (OPT) were also prepared. Into each test tube, 2 ml of GSH/Extract, 0.5 ml of PB (2.5 ml PB for blank) and 0.1 ml of OPT were added and mixed. The mixture was allowed to stand for 15-20 minutes at room temperature. Fluorescence was read at 420 UV.

The standard graph (figure B4) of GSH was plotted and the SH content of the plant was estimated from it.

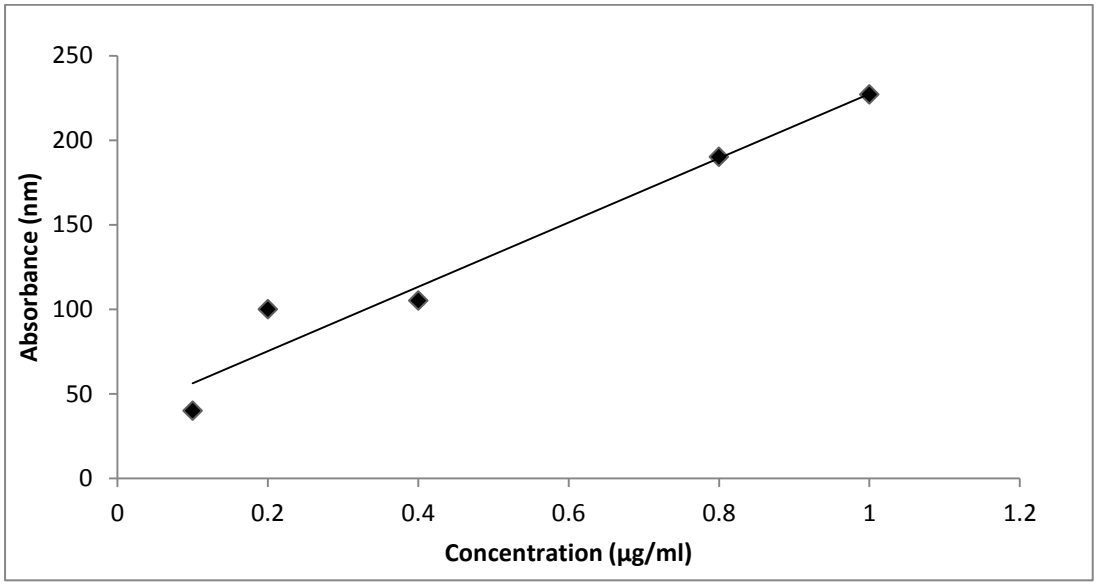

Figure B.4 Calibration curve of sulfhydryl content (µg/ml) against absorbance (nm). This was used for the determination of sulfhydryl content.

B6.1.8 Nitric oxide radical scavenging

Test tubes were set as in DPPH experiment. Into each test tube, 2 ml sodium nitroprosside, 0.5 ml PBS, and 0.5 ml plant extract. The mixture was incubated at 25ºC for 150 minutes. After incubation, 0.5 ml of mixture was transferred into different test tubes. Sulfanilic acid (1 ml) was added and mixed. The mixture was allowed to stand for 5 minutes and 1 ml of naphthylamine was added. It was mixed

and allowed to stand for 30 minutes. The absorbance was read at 540 nm. % scavenging activity was calculated using the following the formula: % Scav = [1-(A_t/A_0)]*100 % scavenging activity versus concentration (mg/100 ml) graph was plotted.

B6.1.9 Total antioxidant capacity

The plant powder was extracted with distilled water (1:5 w/v) and filtered. Reagent solution (0.6 M H_2SO_4, 28 mM sodium phosphate, 4mM ammonium molybdate) was also prepared. 3 test tubes in duplicate were set up. Into each test tube, 0.3 ml of filtered plant extract and 3 ml of reagent solution was poured. It was all mixed and incubated at 95ºC for 90 minutes. After incubation, it was cooled at room temperature and absorbance was read at 695 nm. Antioxidant activity was expressed as the number of equivalents of ascorbic acid and BHT.

B7 Antipyretic activity

For the animal experiment 20 rats were used. They were divided into five groups of four animals each. Group one contained rats that were not treated with the plant extract (control) instead they were given carboxy-methylcellulose (CMC). Group two; three and four were treated with the plant extract, 100, 500, 1000 mg/kg body weight respectively. Group five was treated with Panado (paracetamol) as the standard drug (100 mg/kg). The rats were acclimatised for four days and had free access to food and water ad lib. Before induction, the rats were weighed and their temperature was taken. Pyretic conditions were induced with 12% brewer's yeast injection (subcutaneously), according to body weight and left for 24hrs. Only those whose temperature rose by 1.5ºC were noted as pyretic and, therefore, were used for the studies. Pyretic rats then received their respective drugs using a canulor and their

temperatures (anal) taken at 30 min, 1hr, 2hrs and 4 hrs. All treatments were dissolved in CMC.

B8 *In vitro* antiplasmodial activity

In vitro antiplasmodial activity was screened. The compounds were tested in triplicate against chloroquine sensitive (CQS) strain of *Plasmodium falciparum* (D10). Continuous *in vitro* cultures of asexual erythrocytes stages of *P. falciparum* were maintained. Quantitative assessment of antiplasmodial activity *in vitro* was determined via the parasite lactate dehydrogenase assay. The samples were prepared to a 2 mg/ml stock solution in 10 DMSO or 10% methanol and sonicated to enhance solubility. Samples were tested as a suspension if not completely dissolved. Stock solutions were stored at -20^{o}C. Further dilutions were prepared on the day of the experiment. Chloroquine (CQ) was used as the reference drug in all experiments. Test samples were tested at three concentrations which were 20 µg/ml. 10 µg/ml and 5 µg/ml. CQ was tested at three concentrations (30 ng/ml, 14 ng/ml and 7.5 ng/ml). A full dose was performed on active samples to determine the concentration inhibiting 50% of parasite growth (IC_{50}-value). Test samples were tested at a starting concentration of 100 µg/ml, which was then serially diluted 2-fold in complete medium to give 10 concentrations; with the lowest concentration being 0.2 µg/ml. The same dilution technique was used for all samples. CQ was tested at a starting concentration of 100 ng/ml. The highest concentration of solvent to which the parasites were exposed to had no measurable effect on the parasite viability (data not shown). The IC_{50} values were obtained using a non-linear dose-response curve fitting analysis via Graph Pad Prism v.4.0 software.

B9 larvicidal activity

Larvae of *Culex quinquefascitus* were collected from the Hatchery Unit of the Department of Zoology, University of Zululand, KwaDlangezwa. They were maintained at ambient rearing conditions in the environmental room. All bioassays were conducted at 28 ± 1^{o}C, 60.0 ± 5 R H and 12 h dark photoperiod. A 10% yeast suspension was used as food source. Test for mosquito larval activity was conducted. Ten fourth-instar mosquito larvae were collected with a Pasteur pipette, placed on a filter paper to remove excess water and transferred to the Petri-dishes (100ml) each containing 29.0 ml of degassed distilled water and 1000 µL of different concentrations of plant extract (10-250 µg/ml) in 1% DMSO with a tiny brush. Each Petri-dish was shaken lightly to ensure a homogenous test solution and was left at room temperature. Each test was performed in triplicate. The control was prepared with 29.0 ml of degassed distilled water and 1000 µL of DMSO solution without the plant extract to which larvae were added. Observation on larvae mortality was recorded after 24hr exposure, during which no food was given to the larvae. Larvae were considered dead, when they did not react to touching with a needle. The percentage of mortality and lethal concentrations (LC_{50}) values were determined using Abbots formula and probit analysis program, version 1.5 respectively.

B10 Cytotoxicity

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] cell proliferation assay was used to determine the cytotoxicity of the extracts against human embryonic kidney (HEK293) and human hepatocellular carcinoma (HepG2) cells. The cells were seeded in a 48-well plate at a density of 2.5 x 104 cells per well. Following an overnight incubation at 37^{o}C, the cells were incubated with the

compound at different concentrations (50, 100, 150, 200, 250, 300, and 350 µg/200 µl) in medium (MEM + Gutamax + antibiotics + 10% fetal bovine serum) for 48h. Thereafter, the medium was removed from the cells and 200 µl MTT solution (5 mg/ml phosphate buffer saline) as well as 200 µl of cell culture medium was added to the corresponding wells. The cells were incubated at 37^{o}C for 4 h and the reaction was terminated by addition of DMSO (100/200/400 µl). The cells viability was determined spectrophotometrically (Biomate spectrophotometer) at 570 nm. The experiment was replicated thrice and the results were expressed as mean ± SD. Lethal concentration of the compound that results in 50% cell death (LC_{50}) was determined by regression analysis using QED statistics programme.

B11 Isolation, purification and characterisation

B11.1 Isolation and purification

Methanol, dichloromethane, ethyl acetate and hexane were distilled using simple distillation method for the use in running column chromatography and TLC. Packing of the column chromatography of the dichloromethane extract of *Acanthospermum australe* was done and slurry was prepared by mixing silica gel 60 0.063-0.200 mm (70-230 mesh ASTM) with hexane: ethyl acetate (the initial solvent). This was used for elution. The column was packed and allowed to reach a constant level before it could be sealed with a small amount of sand (about 0.1-0.3 mm; mesh) just enough to cover the top. The crude extract (20 g) was loaded into the column and allowed to settle. This was then sealed with more sand just enough to cover the top. The initial solvent (hexane: ethyl acetate, 9:1) was also added to allow the column to run. This was run several times to equilibrate the column and to ensure tight packing of the

materials as well as to get rid of any bubbles present. Series of 20 ml fractions collection were collected into beakers. Elution was done with the use of hexane:ethyl acetate solvent system beginning from 9:1-4:6. TLC analysis of the collected fractions was performed and plates were viewed under UV light to identify the fractions with a common outline where after they were developed using 10% sulphuric acid spray reagent and then exposed to heat. A total of 190 of 20 ml fractions were collected. Fractions of a similar profile were separated and combined to give a total of 20 combined fractions to give MF/01/A (Af_1-f_{20}) MF/01/B (Bf_{21}-f_{30});...; MF/01/S (Sf_{167}-f_{180}), MF/01/T (Tf_{181}-f_{190}) (See figure 3.2). The combined fractions were separately concentrated *in vacuo* and their weights were determined.

Based on the nature of (MF/01/H) and (MF/01/I), the two compounds were separately dissolved in hexane (MF/01/H) and methanol (MF/01/I) to recrystallise. The mixtures where then filtered separately to obtain residues (MF/01/H_1 and (MF/01/I_1). The samples were analysed using TLC and the residues were found to show purity and these were subjected to NMR techniques for characterisation and structural elucidation.

B11.2 Structure elucidation

MF/01/H_1 and MF/01/I_1 were further analysed using 1D and 2D NMR techniques (^{1}H-^{1}H, ^{13}C-^{13}C, DEPT, COSY, HMQC, HMBC and NOESY) (In $CDCL_3$, Bruker 600 MHz), infrared (IR) (Perkin-Elmer 100 FTIR with ATR sampling accessory), UV (in $CHCL_3$, Varian-Cary 50 UV-visible spectrophotometer and GC-MS (in DCM, 7890A GC system coupled with Agilent technologies 5975C VLMSD with triple-axis detector). The resulting spectra where analysed and the compound identified by comparing them to similar compounds in literature library.

APPENDIX C

C1. Ethic clearance

Ethics Committee
Faculty of Science and Agriculture
University of Zululand
C/O Mr L Vivier
Department of Zoology
University of Zululand
Private Bag 1001
KwaDlangezwa
3886
Tel: 035 – 902 6741
Email: lvivier@pan.uzulu.ac.za

18 October 2010

To whom it may concern

ETHICS EVALUATION OF RESEARCH PROJECT PROPOSAL

This letter serves to confirm that **Nethengwe MF** (Student No **20054033**), registered for a MSc Degree in the Department of Biochemistry and Microbiology at the University of Zululand, in accordance with appropriate rules submitted a research project proposal to the Ethics Committee of the Faculty of Science and Agriculture at the University of Zululand. The research project will investigate: **Antiplasmodial/Antipyretic activity of some Zulu medicinal plants**.

Based on the research protocol stipulated, this committee could find no reason from an ethical standpoint to reject the proposed research. The administration of Scheduled Medicinal Substances (Medicines Control Act) should be done with the approval of Dr GF Fourie, the Veterinary Surgeon on the Ethics Committee.

Yours sincerely

Mr L Vivier
Chairperson
Ethics Committee
Faculty of Science and Agriculture
University of Zululand

C2 Interview of Traditional Healers

RESEARCH QUESTIONNAIRES

Date: Questionnaire No.

Name of the Interviewer:

Particulars of the area
GPS reading:
Name of the area:
Name of the Sub-local/Sub-Area:
Name of the Village (Precise place):

Sociodemographic data
Gender: **Age:**

Male	
Female	

15-24	
25-34	
35-44	
45-54	
55-64	

Plant Species Particulars

Zulu names:

Plant 1: _____

Plant 2: _____

Plant 3: _____

Plant 4: _____

Scientific names:
Plant
1: _____

Plant 2: _____

Plant 3:

Plant 4:

English names:

Plant 1:

Plant 2:

Plant 3:

Plant 4:

Source of plant material:

Collected from the wild	
Cultivated (home-garden)	

What are the other uses of the plant?

Plant 1:

Plant 2:

Plant 3:

Plant 4:

Plant usage and collection

Question	**Usage**

Which part(s) used?	
Are the plants sold?	
In which state are the plants sold? (fresh or dry)	
If collected from the wild, when? (season)	
Any specific time for collection during the day?	
What places does the plant prefer to grow in? (wetland, dry land, grassland, forests, old fields, as weeds among the plants.	

Preparation Method:

a) How is the medicine taken (e.g. by mouth or as enema)?

_____

b) How is the medicine prepared?

_____

Storage Method:

_____

Dosage:

a) What is the dosage (e.g. one cup three times a day?)

_____

b) For how many days is the medicine taken?

_____

c) Are there any known side effects?

d) Where did the knowledge come from (e.g. grandmother, relative)?

Age Group:

Infants	
Children	
Adults	

APPENDIX D

GRAPHS

D1 Antioxidant Activity- free radical scavenging activity

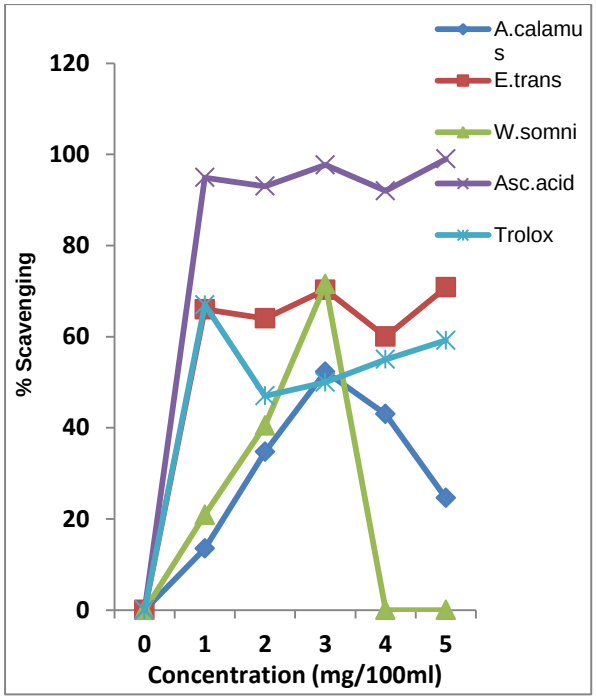

Figure D1.1 a: Activity of *A. calamus*, *E .transvalense* and *W. somnifera* on DPPH radicals

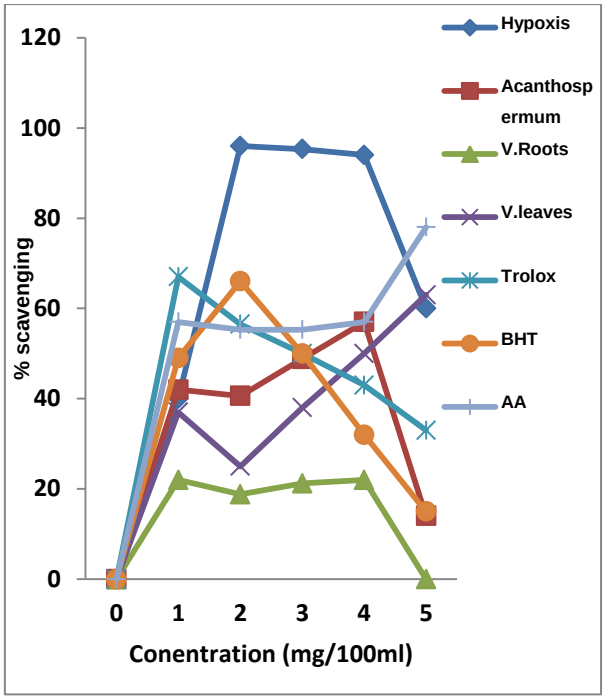

Figure D1.1 b: Activity of *H. hemerocallidea, A. australe, V. adoensis* (roots) and *V.adoensis* (leaves) on DPPH radicals

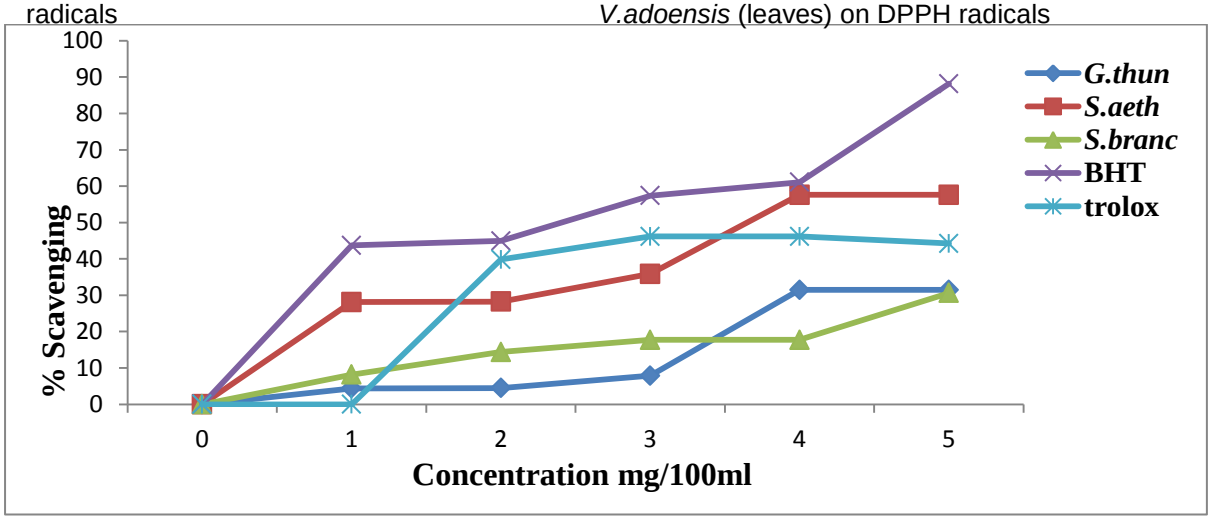

Figure D1.1 c: Activity of *G. thunbergia, S aethiopicus* and *S. brachypetala* on DPPH radicals

Figure D1.1 Percentage scavenging activity of the methanolic plant extracts on DPPH radicals. Standards BHT, ascorbic acid and Trolox.

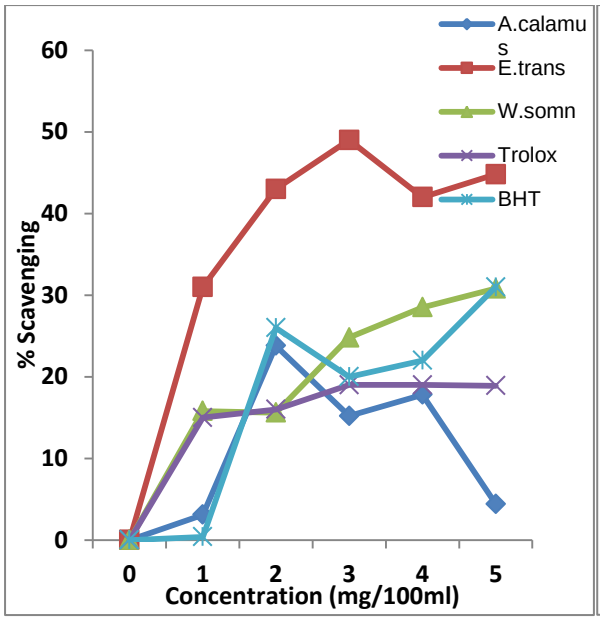

Figure D1.2 a: Activity of *A. calamus, E .transvalense* and *W. somnifera* on ABTS radicals

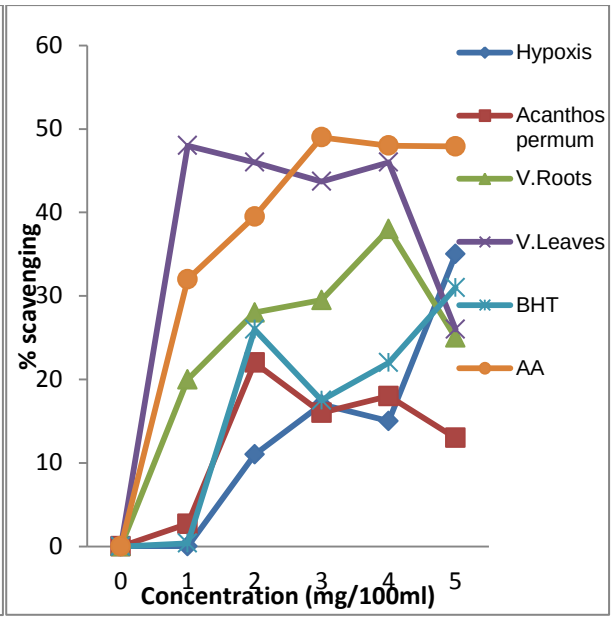

Figure D1.2 b: Activity of *H. hemerocallidea, A. australe, V. adoensis* (roots) and *V.adoensis* (leaves) on ABTS radicals

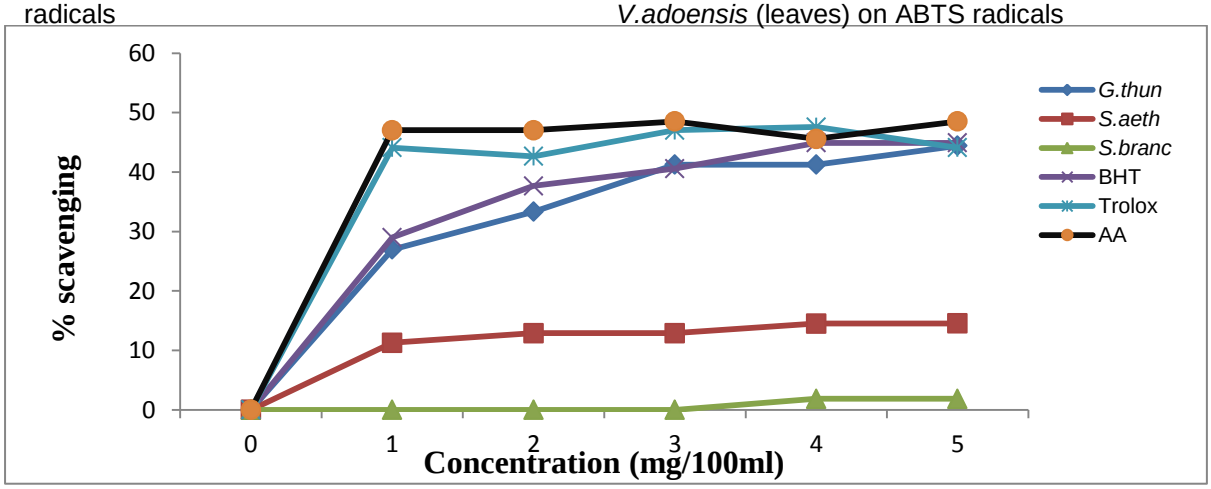

Figure D1.2 c: Activity of *G. thunbergia, S aethiopicus* and *S. brachypetala* on ABTS radical

Figure D1.2: Percentage scavenging activity of the methanolic plants extracts on ABTS radicals. Standards BHT, ascorbic acid and Trolox.

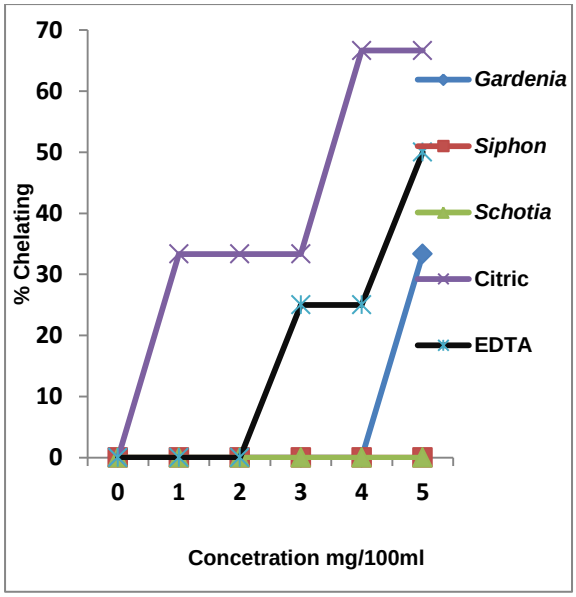

Figure D1.3 a: Chelating activity of *G. thunbergia, S. aethiopicus* and *S. brachypetala* on Fe^{2+}

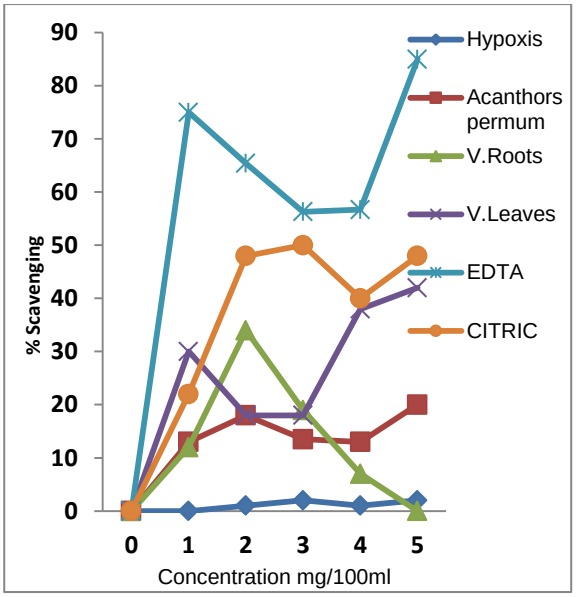

Figure D1.3 b: Chelating activity of *H. hemerocallidea, A. australe, V. adoensis* (roots) and *V.adoensis* (leaves) on Fe^{2+}

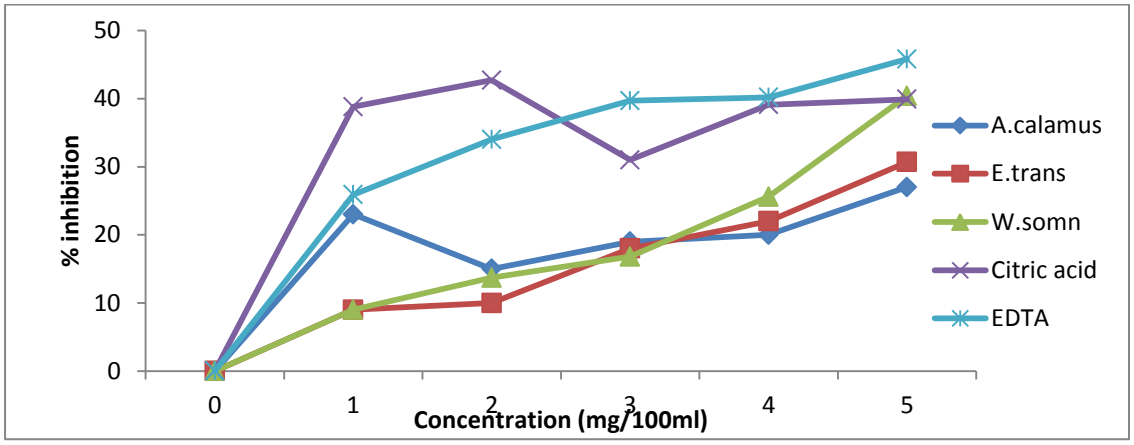

Figure D1.3 c: Chelating activity of *A.calamus, E. transvalense and W. somnifera* on Fe^{2+}

Figure D1.3: Percentage chelating activity of the methanolic plants extracts on Fe^{2+}. Satndard drugs EDTA and citric acid.

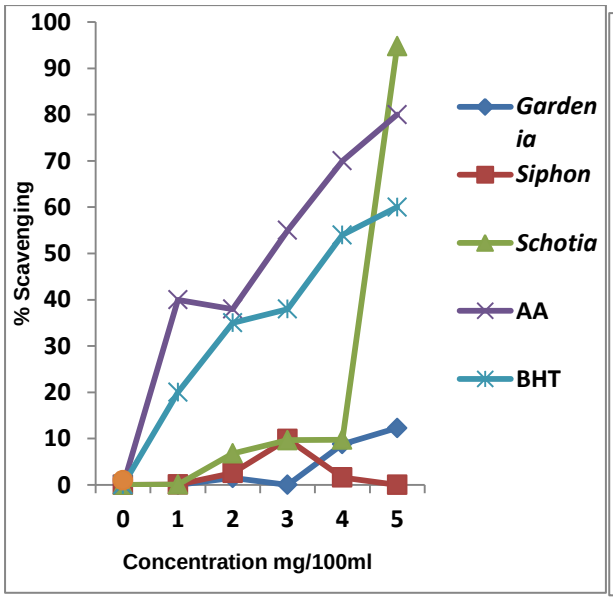

Figure D1.4 a: Nitric oxide radical scavenging activity of *G. thunbergia, S. aethiopicus* and *S. brachypetala*

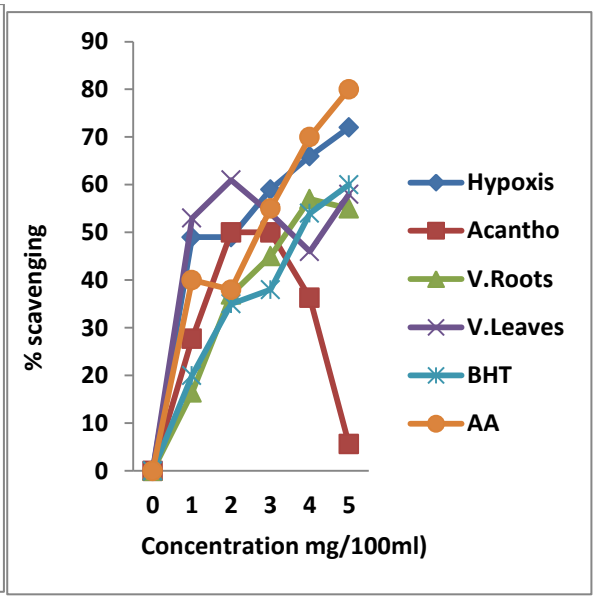

Figure D1.4 b: Nitric oxide radical scavenging activity of *H. hemerocallidea, A. australe, V. adoensis* (roots) and *V.adoensis* (leaves)

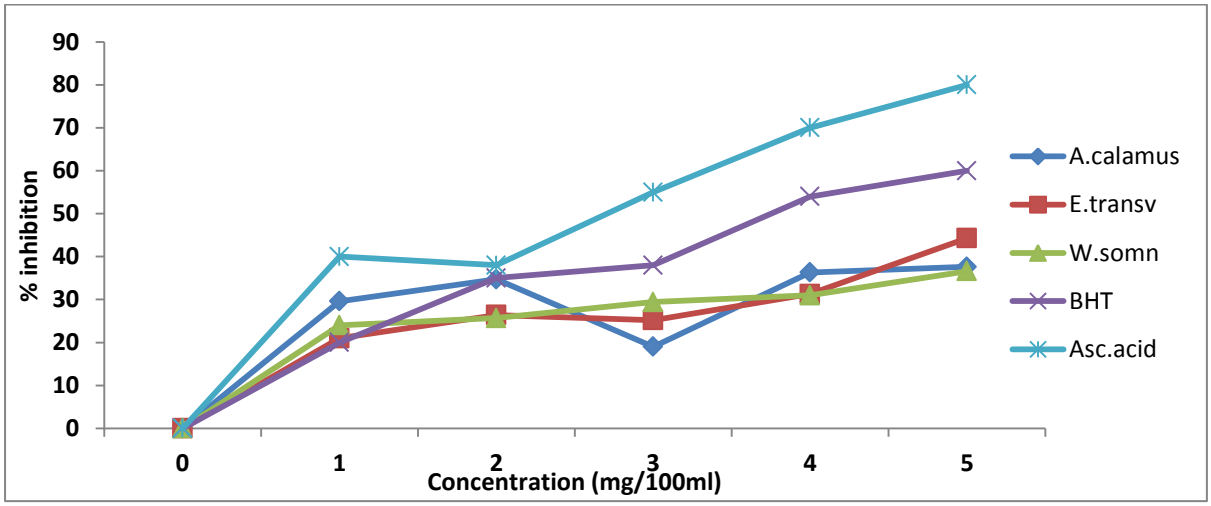

Figure D1.4 c: Nitric oxide radical scavenging activity of *A. calamus, E .transvalense* and *W. somnifera*

Figure D1.4: Nitric oxide radical scavenging activity of the methanolic plants extracts

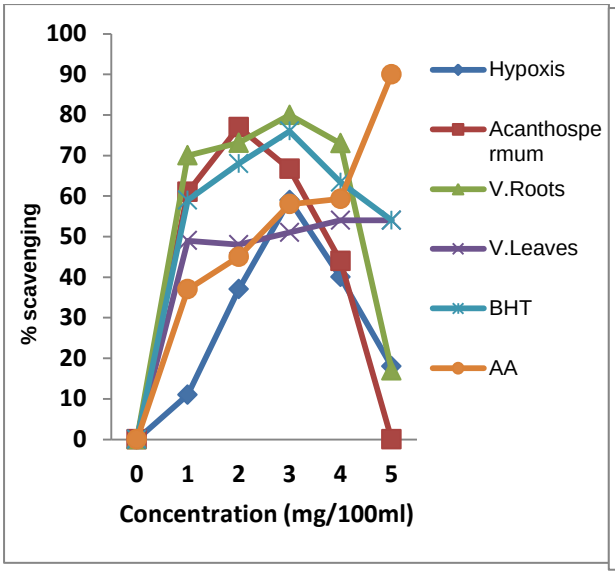

Figure D1.5 a: Super oxide scavenging activity of *H. hemerocallidea, A.australe, V. adoensis* (roots) and *V.adoensis* (leaves)

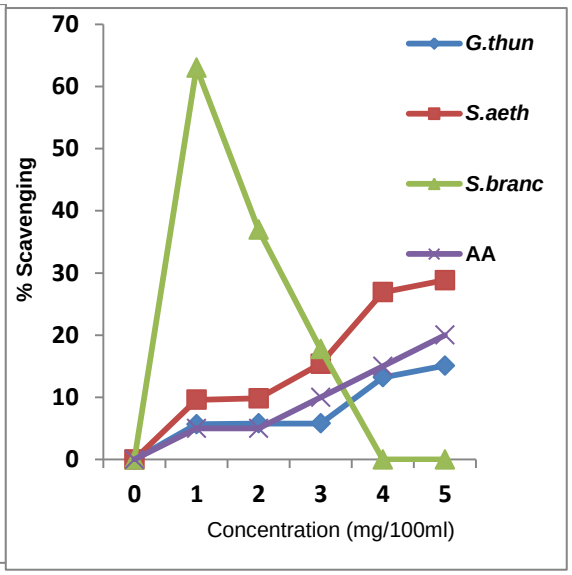

Figure D1.5 b: Super oxide scavenging activity of *G. thunbergia, S aethiopicus* and *S. brachypetala*

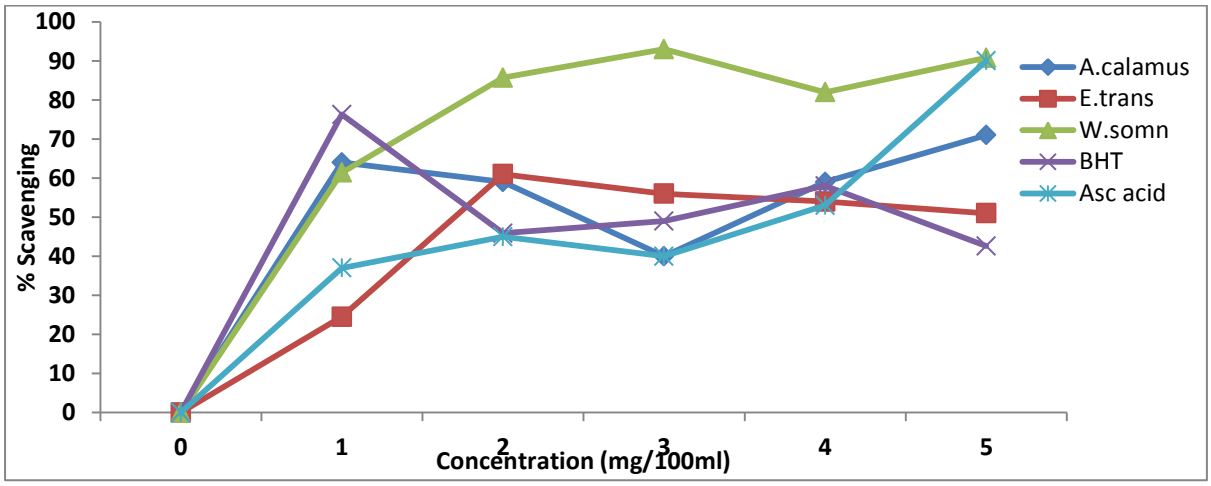

Figure D1.5 c: Super oxide anion scavenging activity of *A. calamus, E .transvalense* and *W. somnifera*

Figure D1.5: Super oxide anion scavenging activity of the methanolic plant extracts.

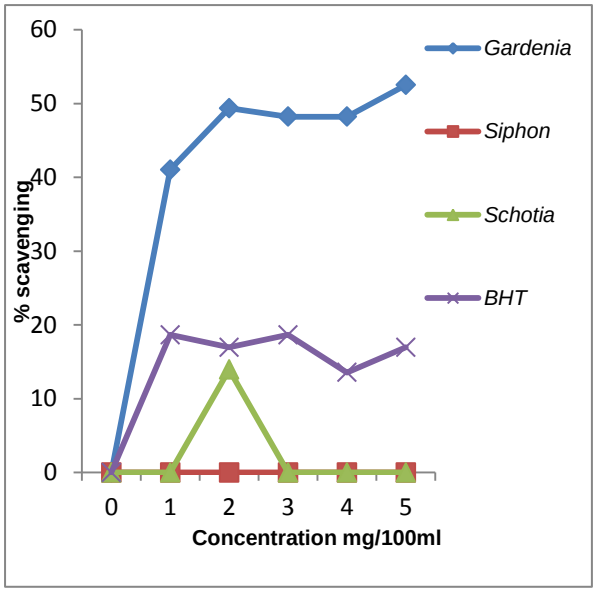

Figure D1.6 a: Hydroxyl radical scavenging activity of *G. thunbergia, S aethiopicus* and *S. brachypetala*

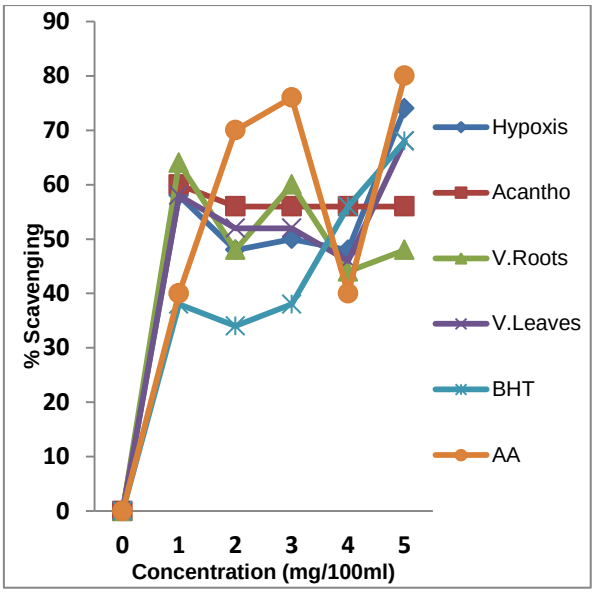

Figure D1.6 b: hydroxyl radical scavenging activity of *H. hemerocallidea, A. acanthospermum, V. adoensis* (roots) and *V.adoensis* (leaves)

Figure D1.6: Hydroxyl radical scavenging activity of the methanolic plants extracts.

APPENDIX E

SPECTRA

E1 Spectral data for compound MF/01/H_1

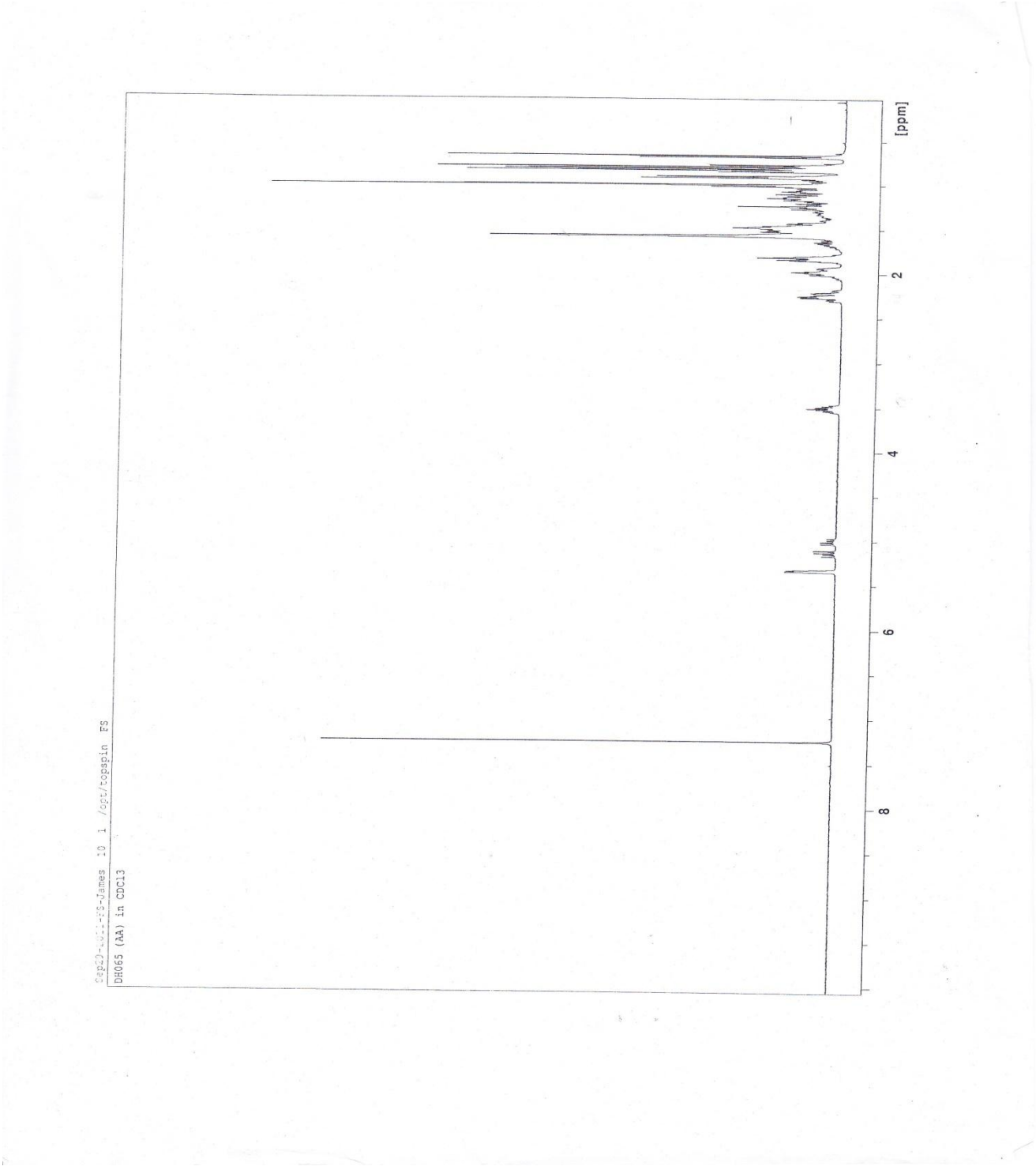

Figure E1.1 H-NMR spectrum of compound MF/01/H_1

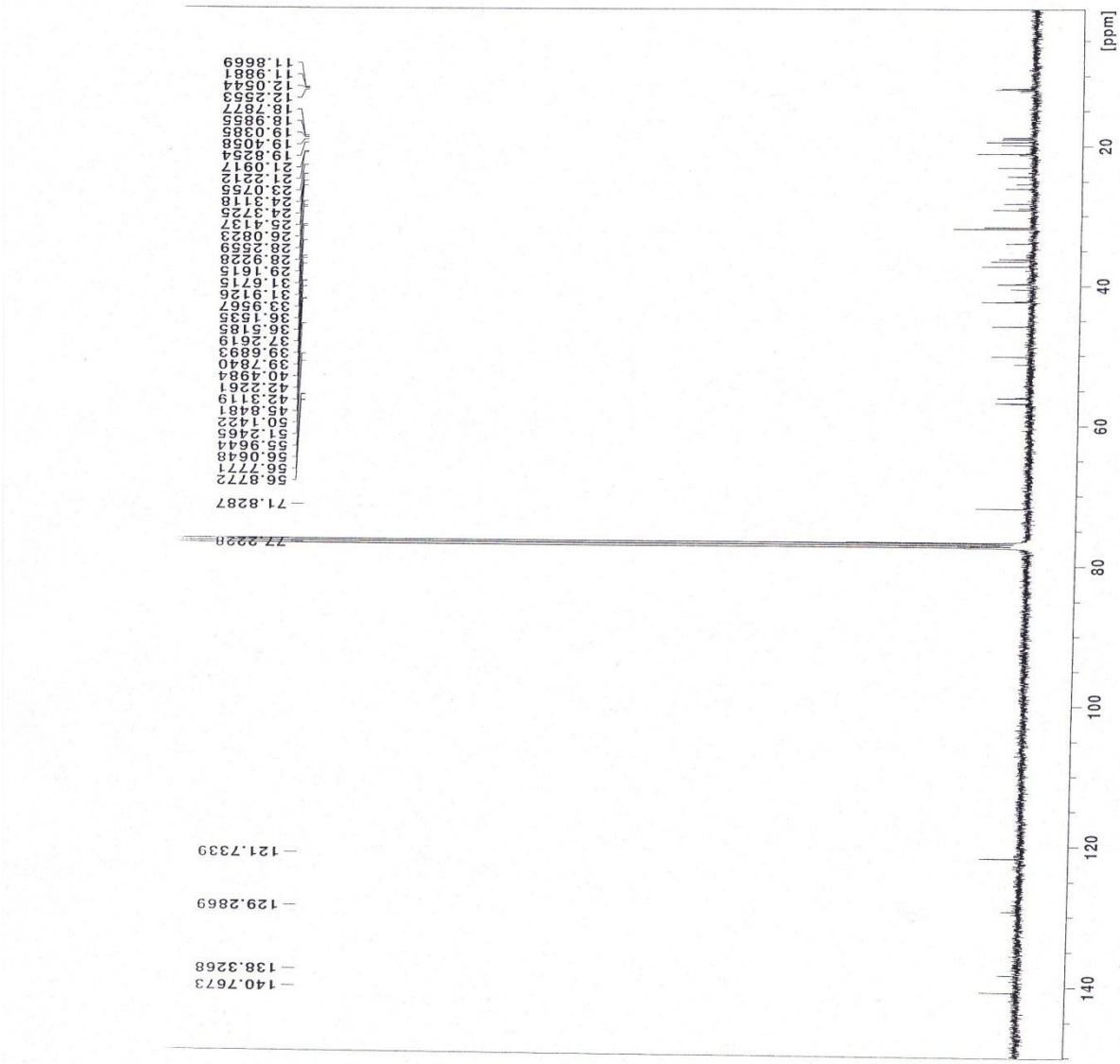

Figure E1.2: ^{13}C-NMR, spectrum of compound MF/01/H_1

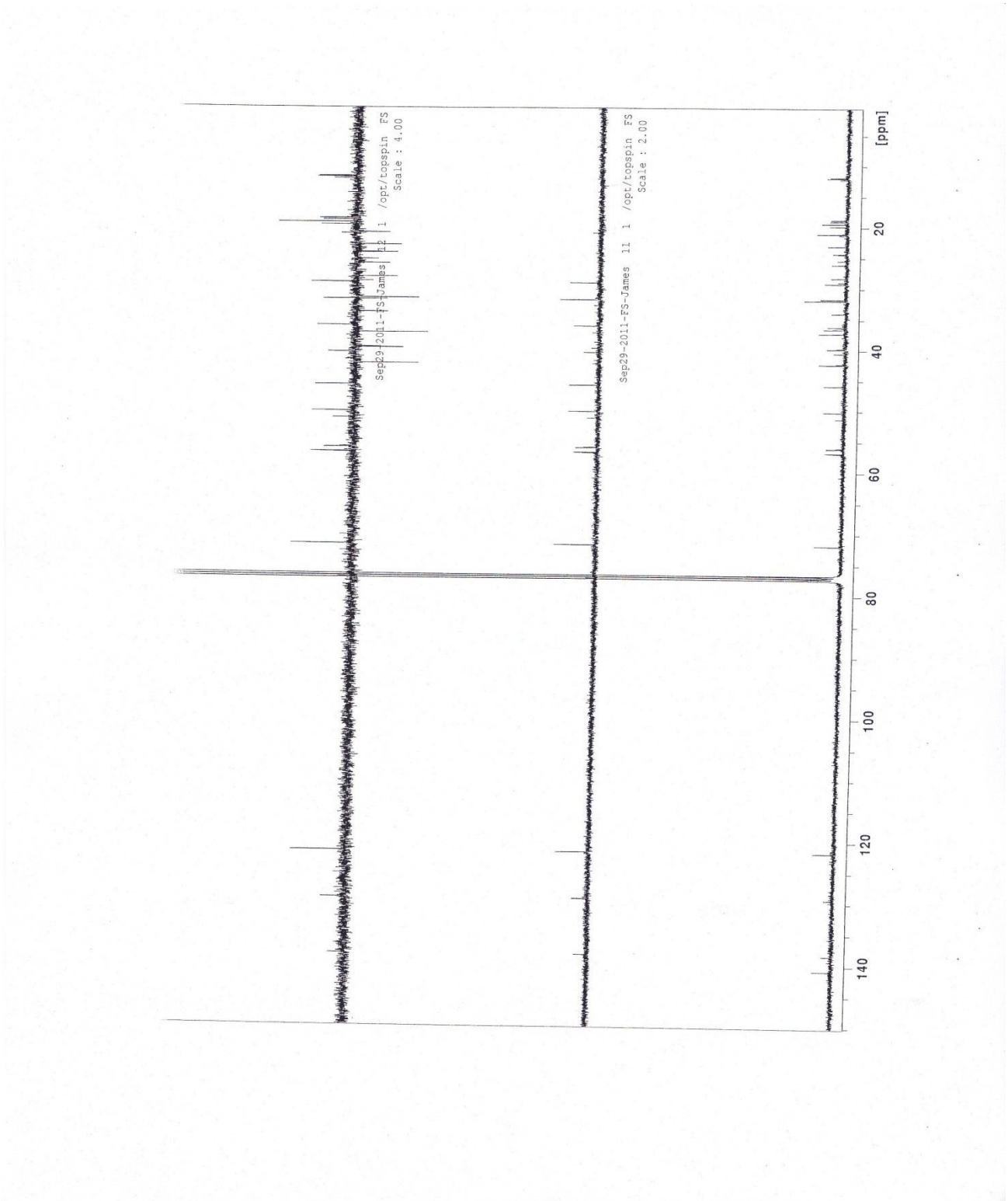

Figure E.1.3: ^{13}C-NMR, DEPT 90 and DEPT 135 spectra of copound MF/01/H_1

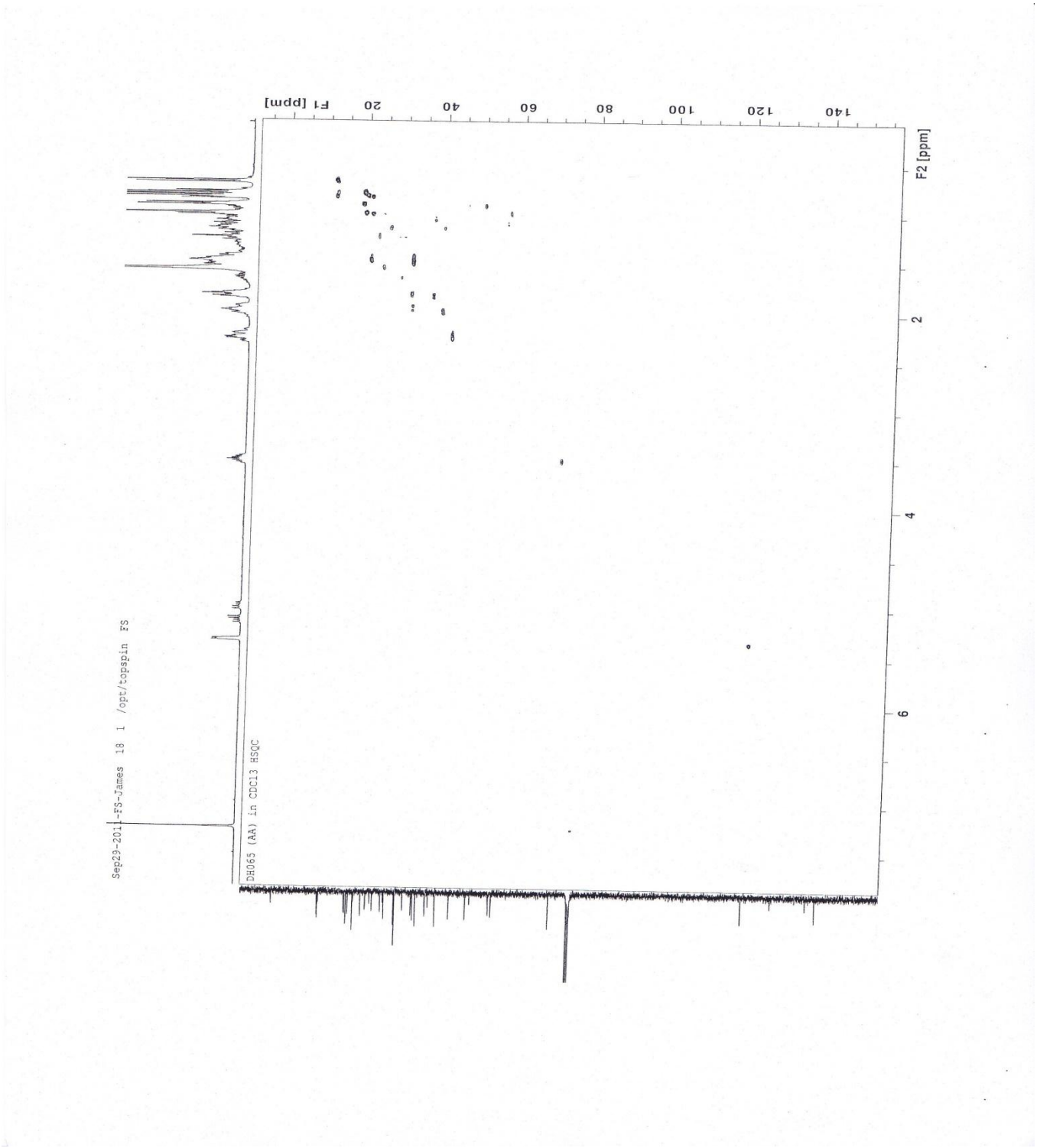

Figure E1.4 a: HSQC NMR spectrum of compound MF/01/H_1

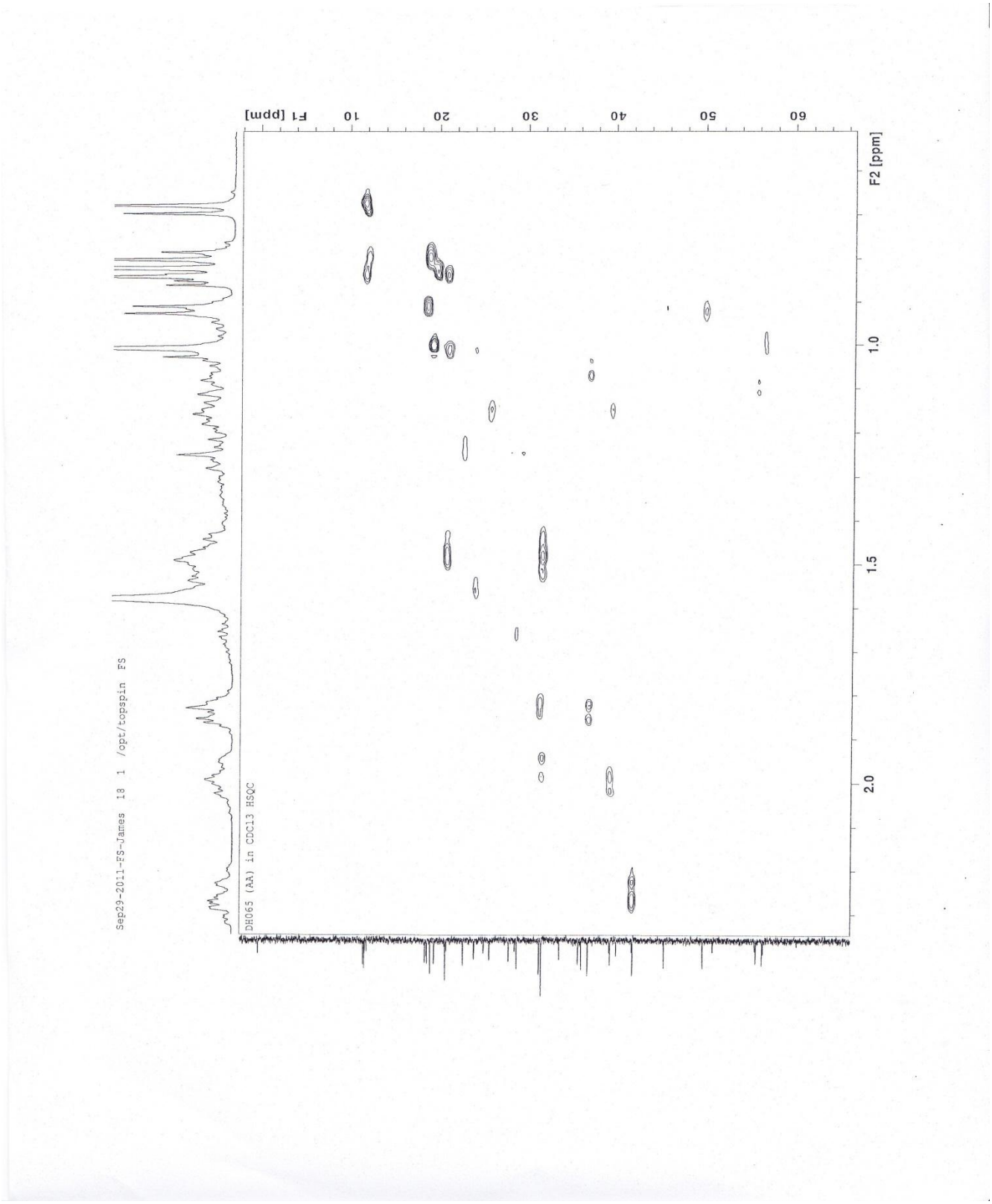

Figure E1.4b: HSQC NMR spectrum of compound MF/01/H_1

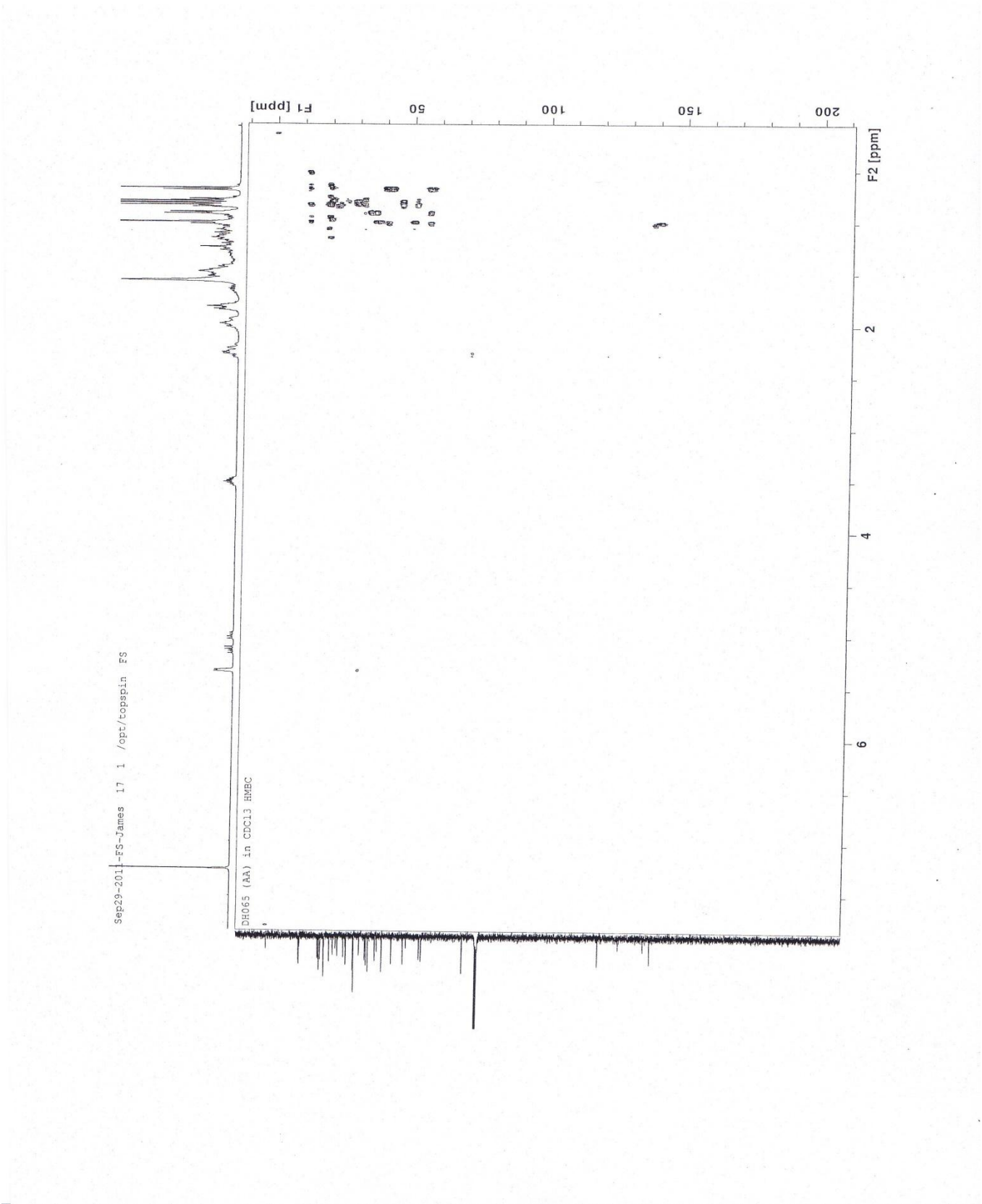

Figure E1.5a: HMBC NMR spectrum of compound MF/01/H_1

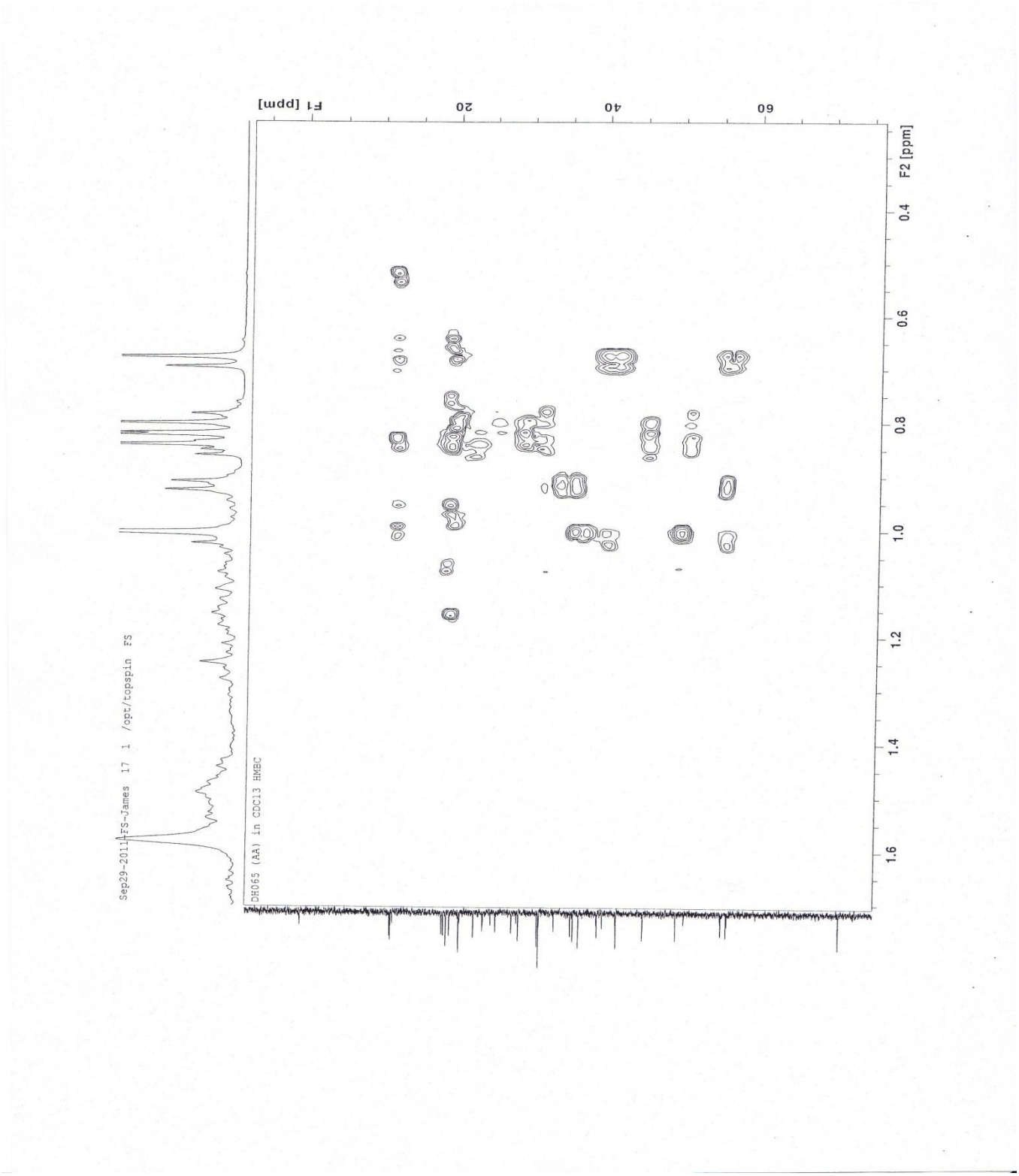

Figure E1.5b: HMBC NMR spectrum of compound MF/01/H_1

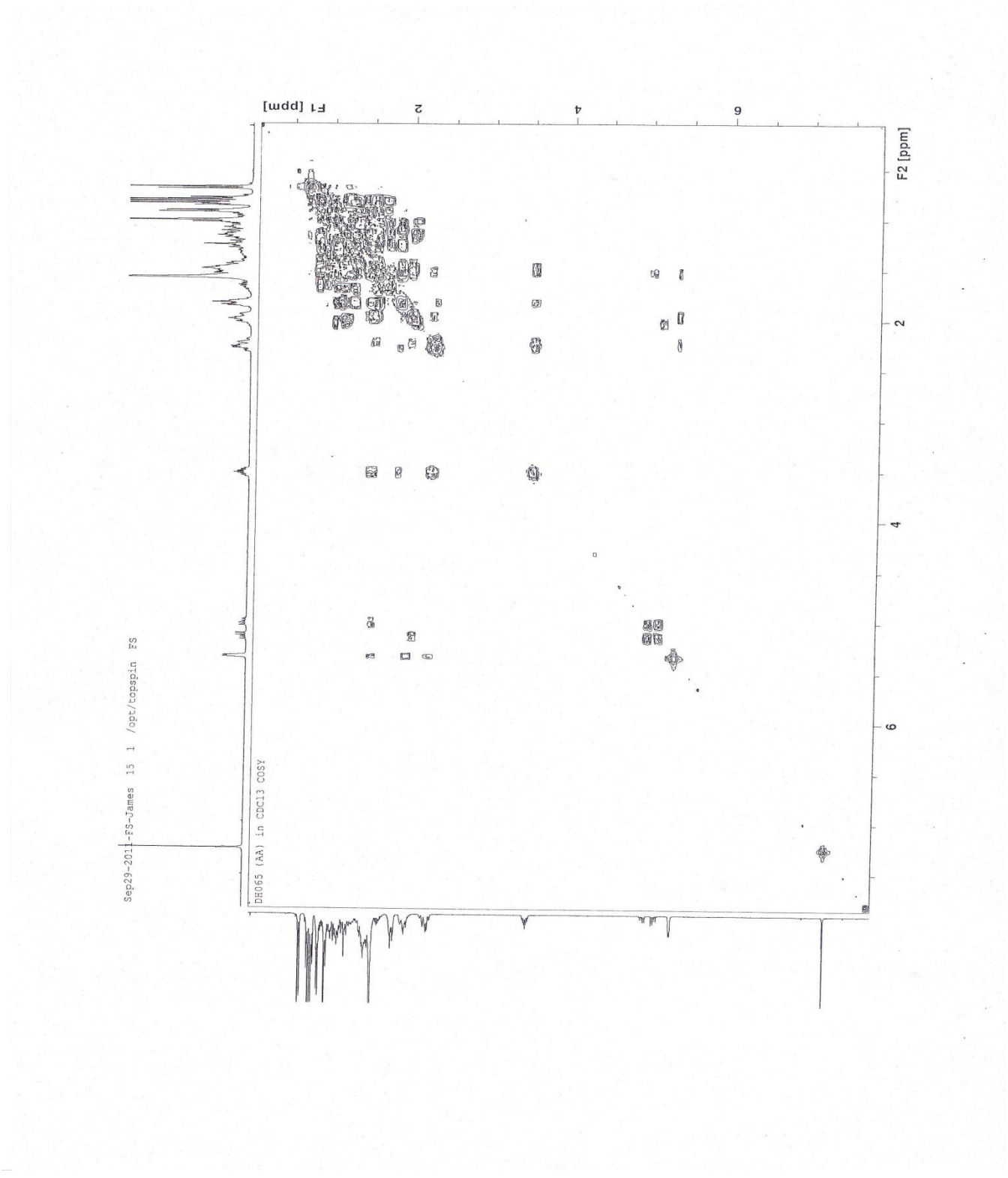

Figure E1.6: COSY NMR spectrum of compound MF/01/H_1

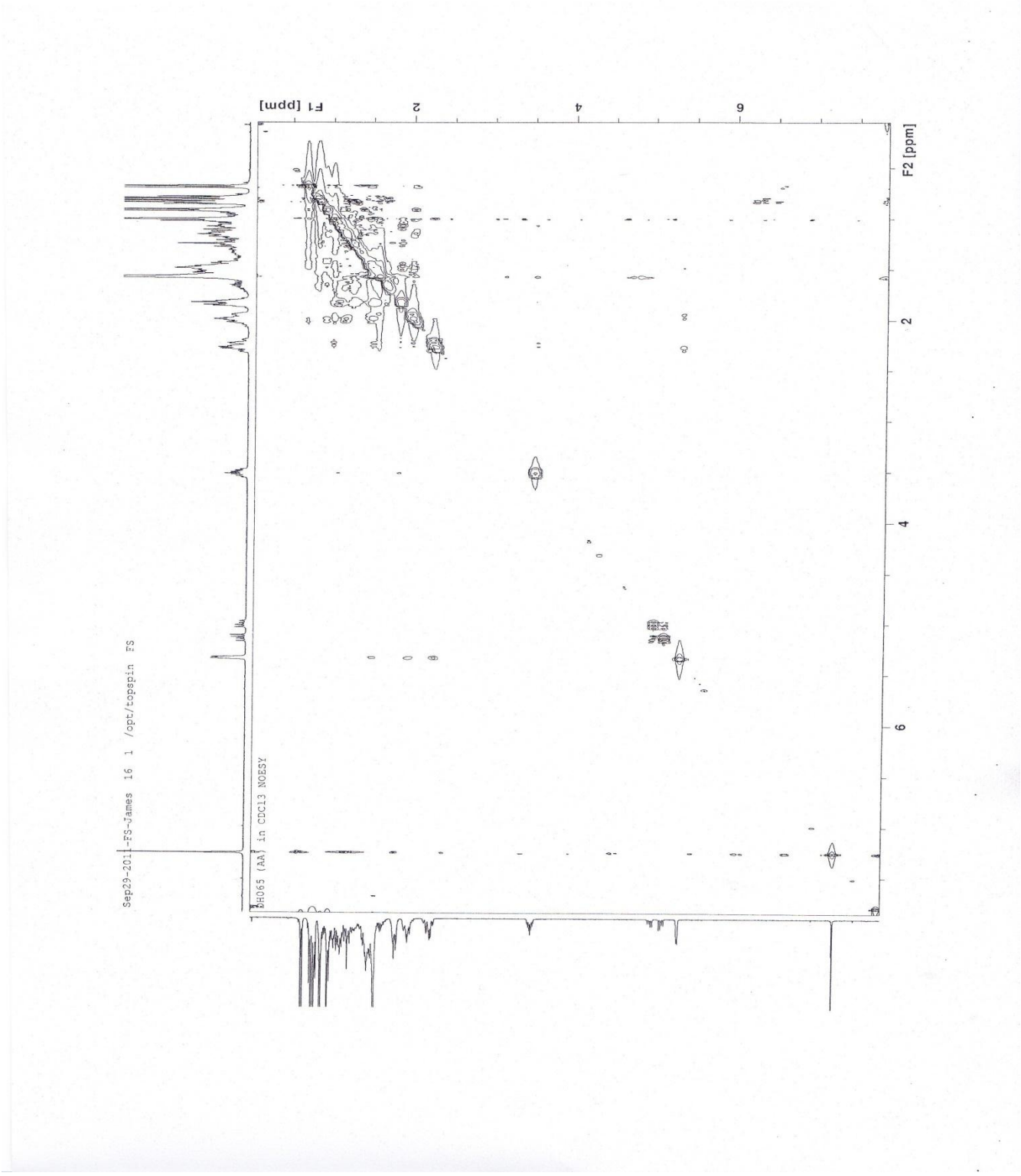

Figure E1.7: NOESY NMR spectrum of compound MF/01/H_1

E2 Spectral data for compound MF/01/I_1

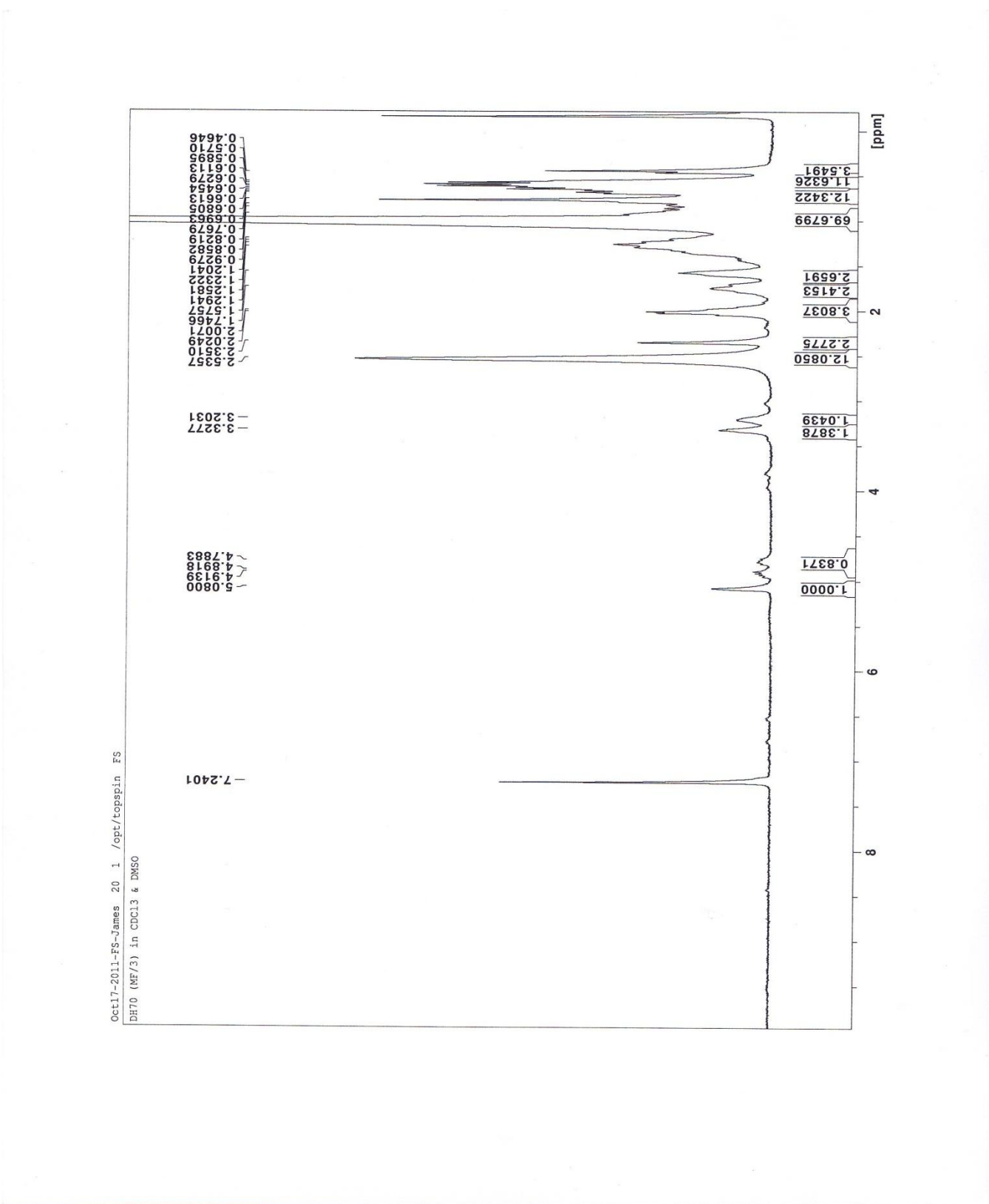

Figure E2.1: ^{1}H-NMR spectrum of compound MF/01/I_1

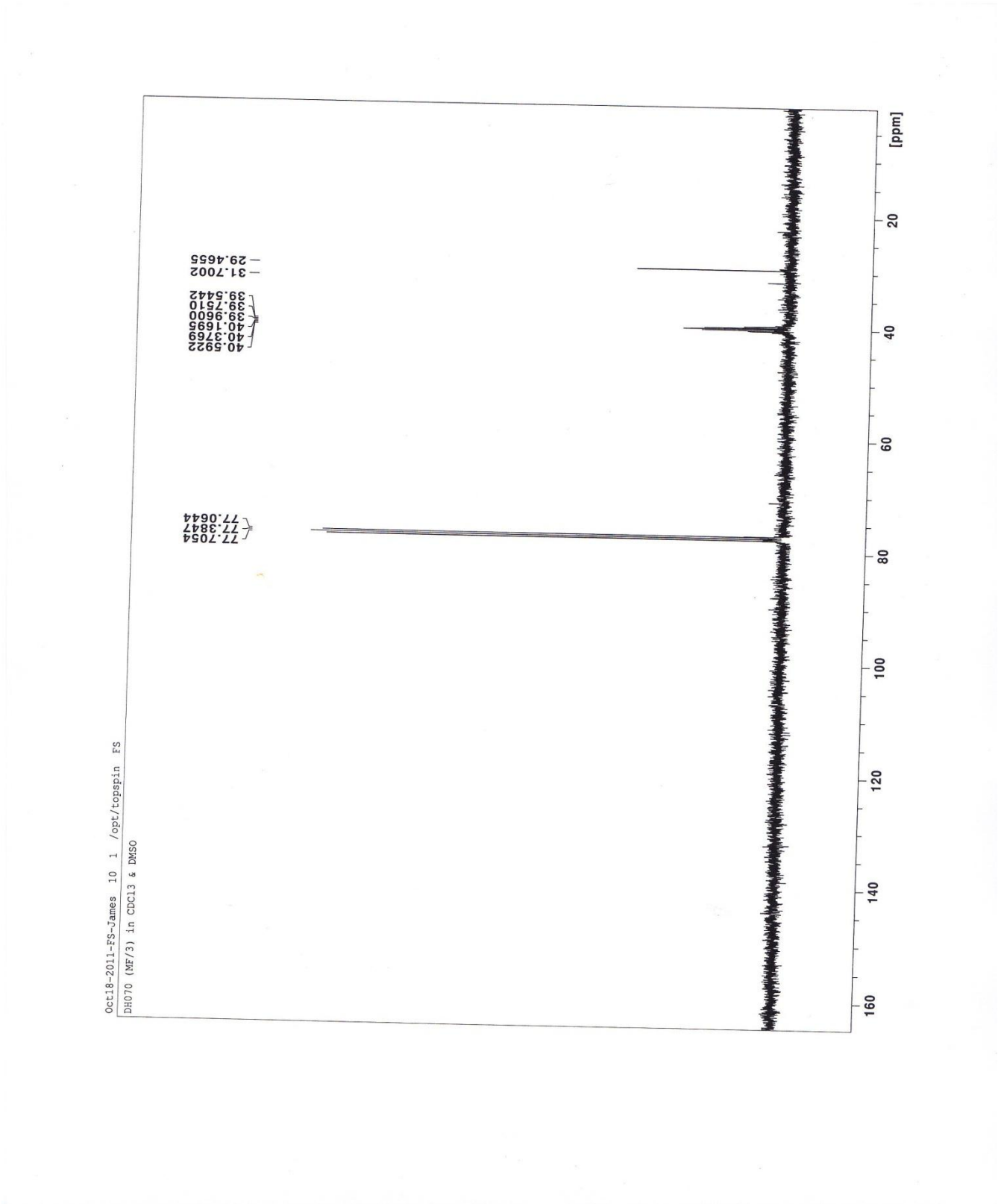

Figure E2.2: ^{13}C-NMR spectrum of compound MF/01/I_1

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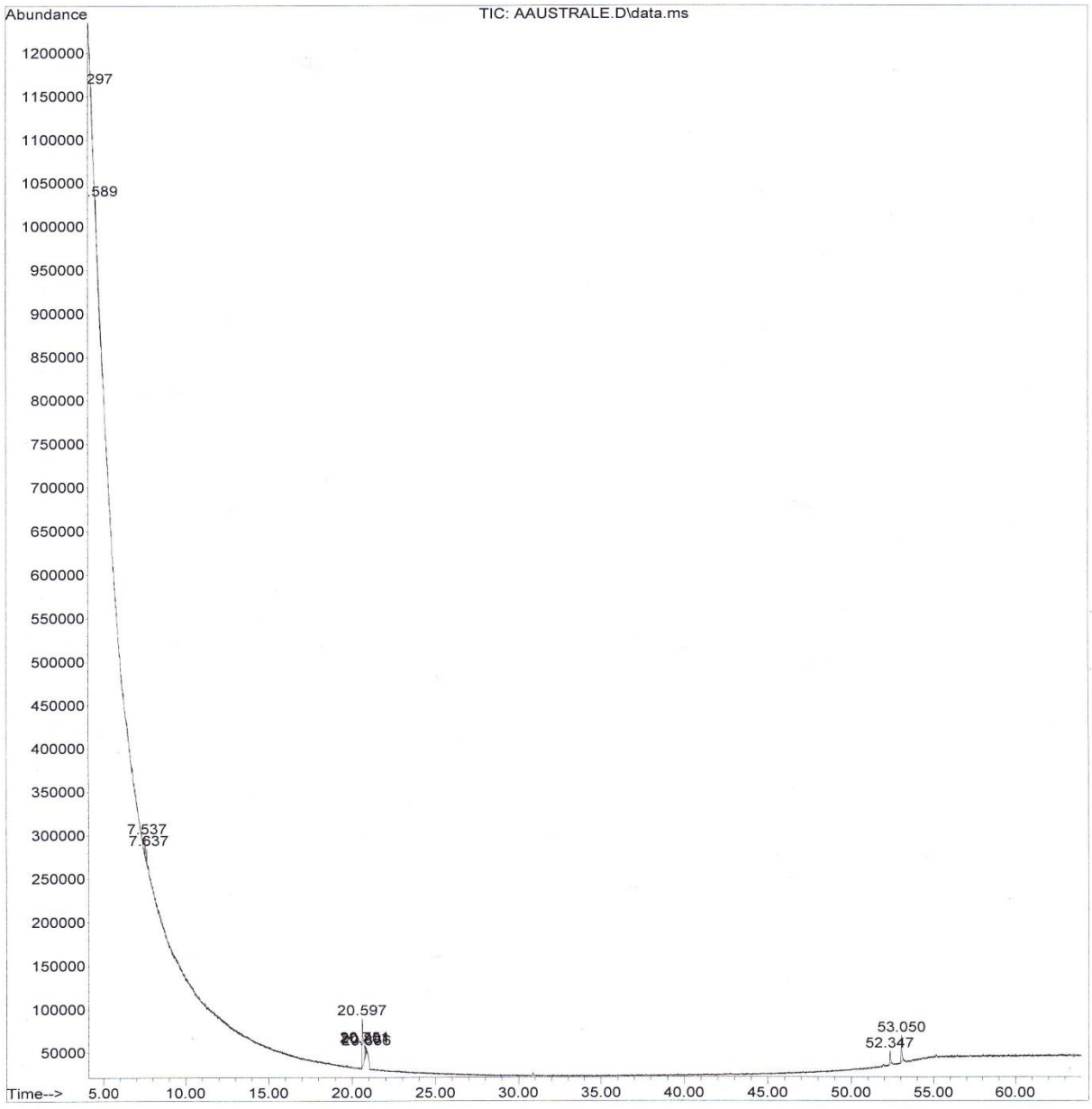

Biochem slow.M Thu Nov 10 12:59:48 2011 Page: 2

Figure E2.3: GC-MS spectrum of compound MF/01/I_1

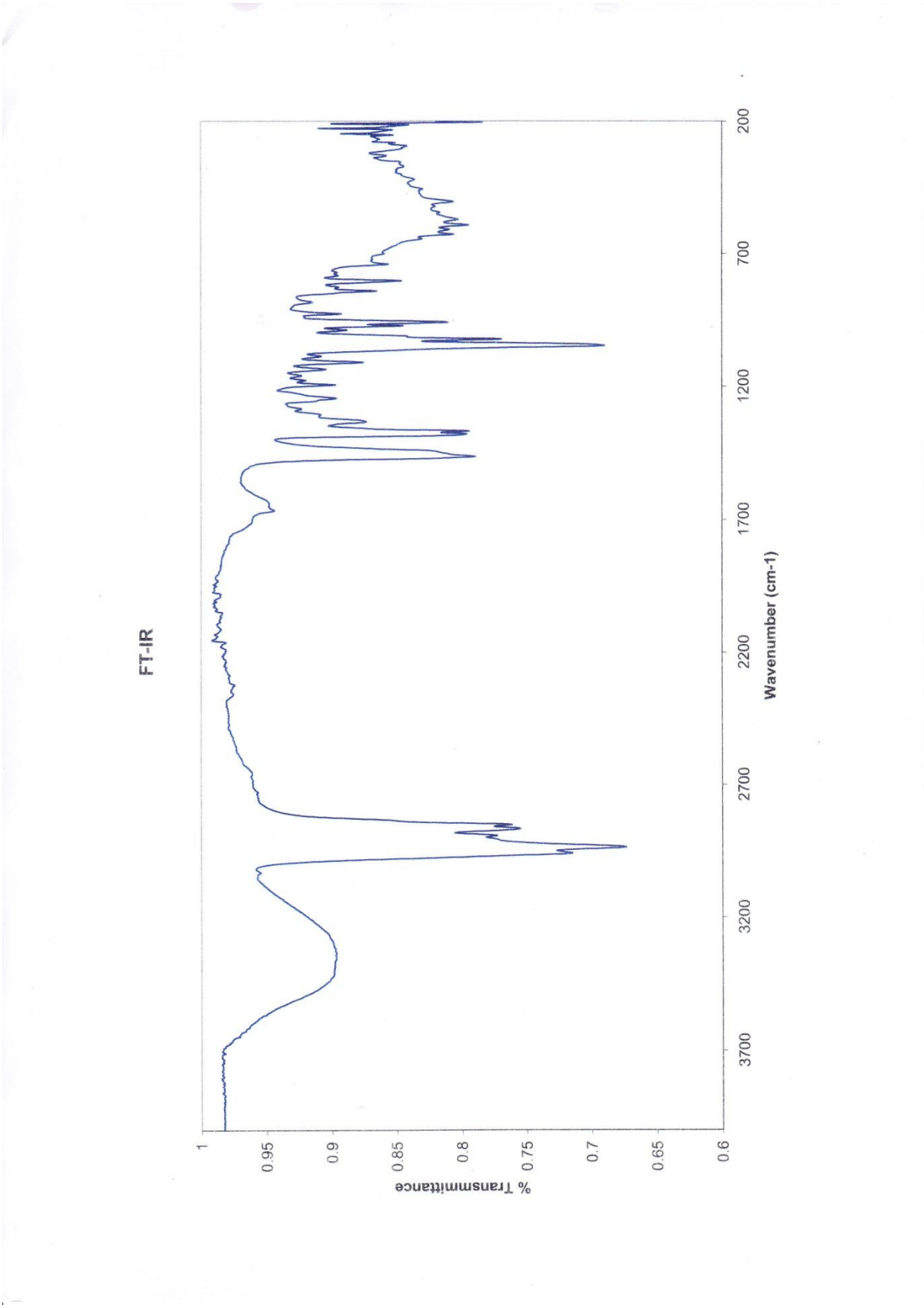

Figure E2.4: IR spectrum of compound MF/01/I_1

E2.5 GC-MS spectrum of mixture of compounds found to be present in compound MF/01/I_1

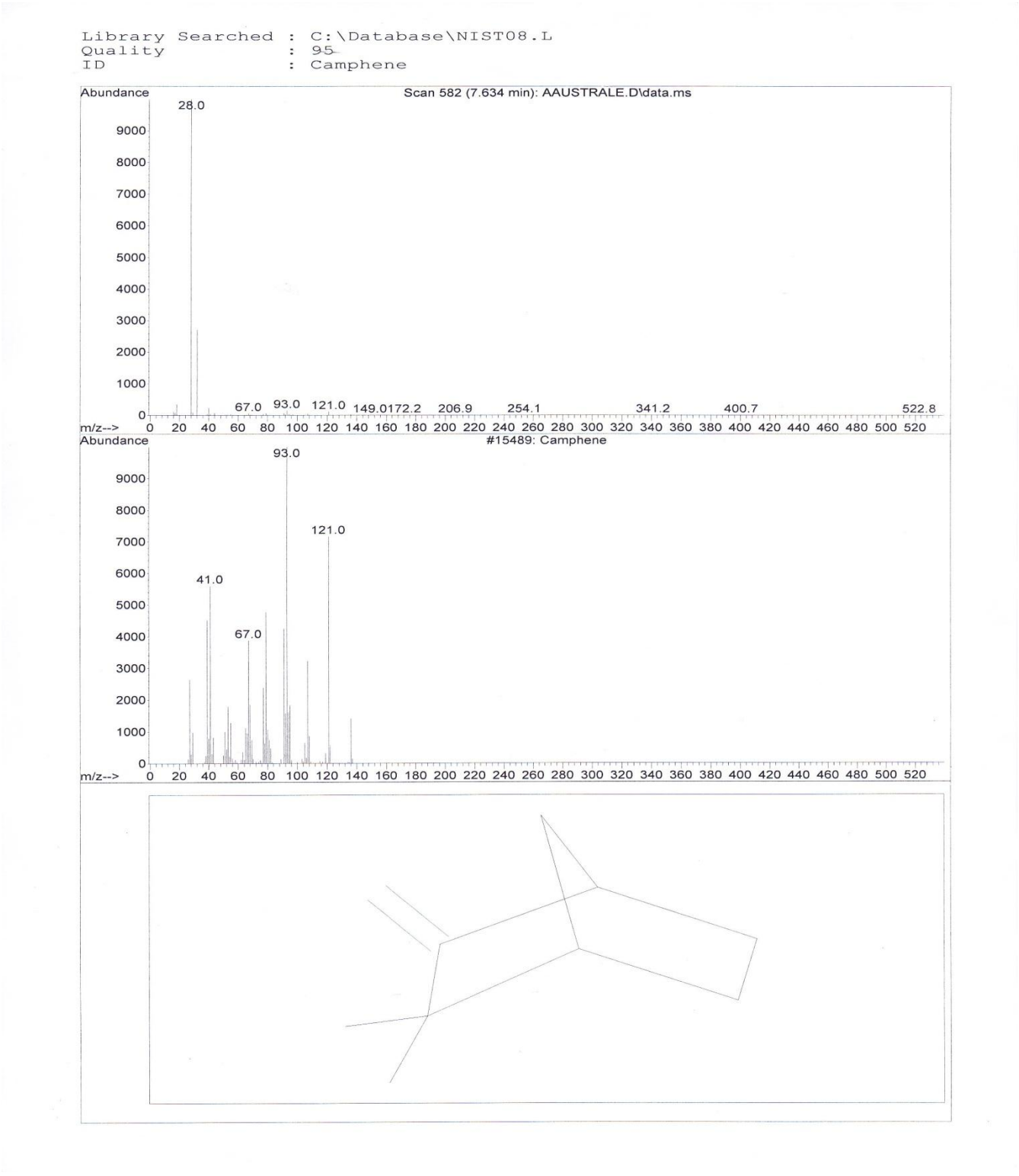

Figure E2.5a: Camphene

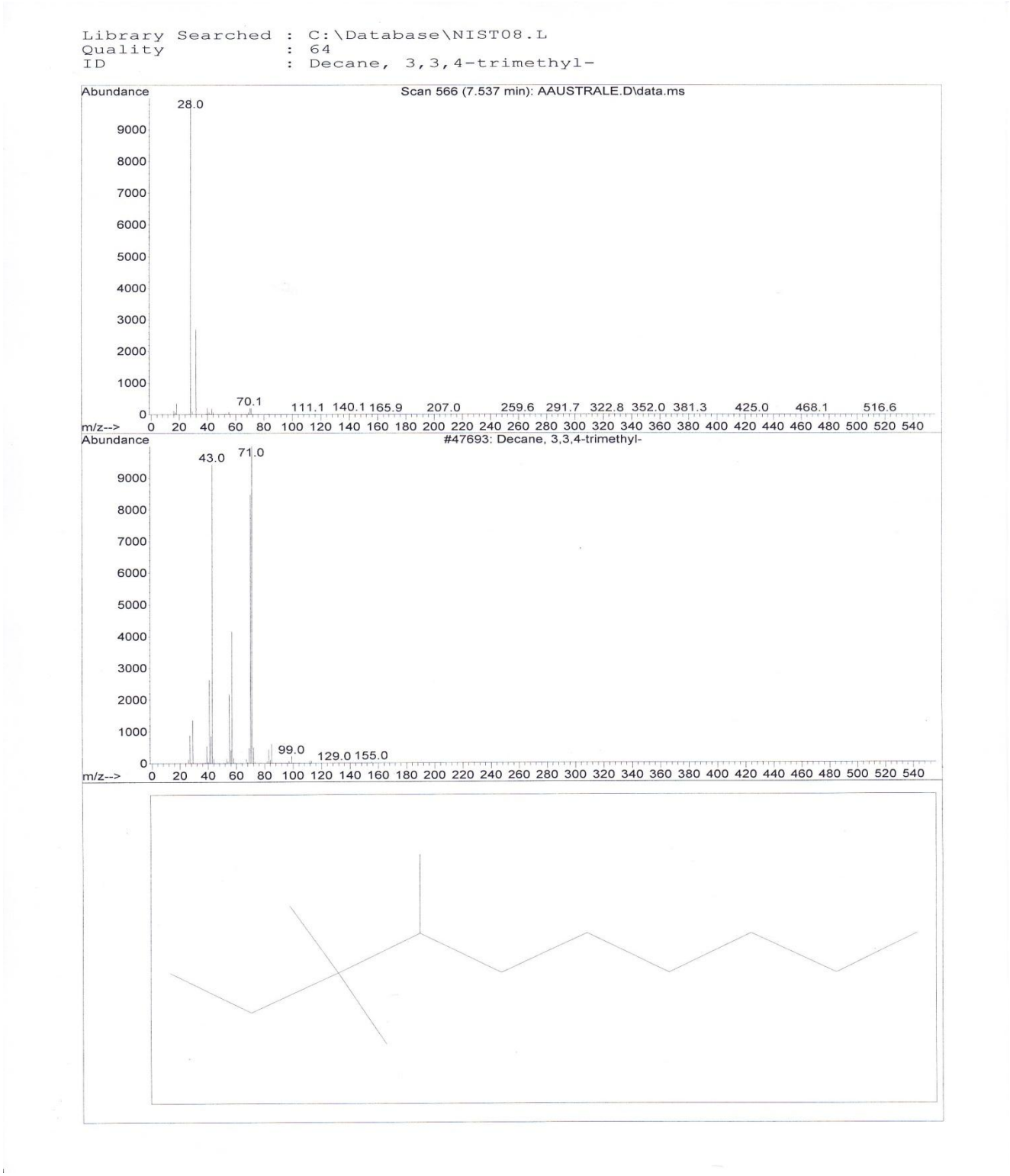

Figure E2.5b: Decane, 3, 3, 4-trimethyl-

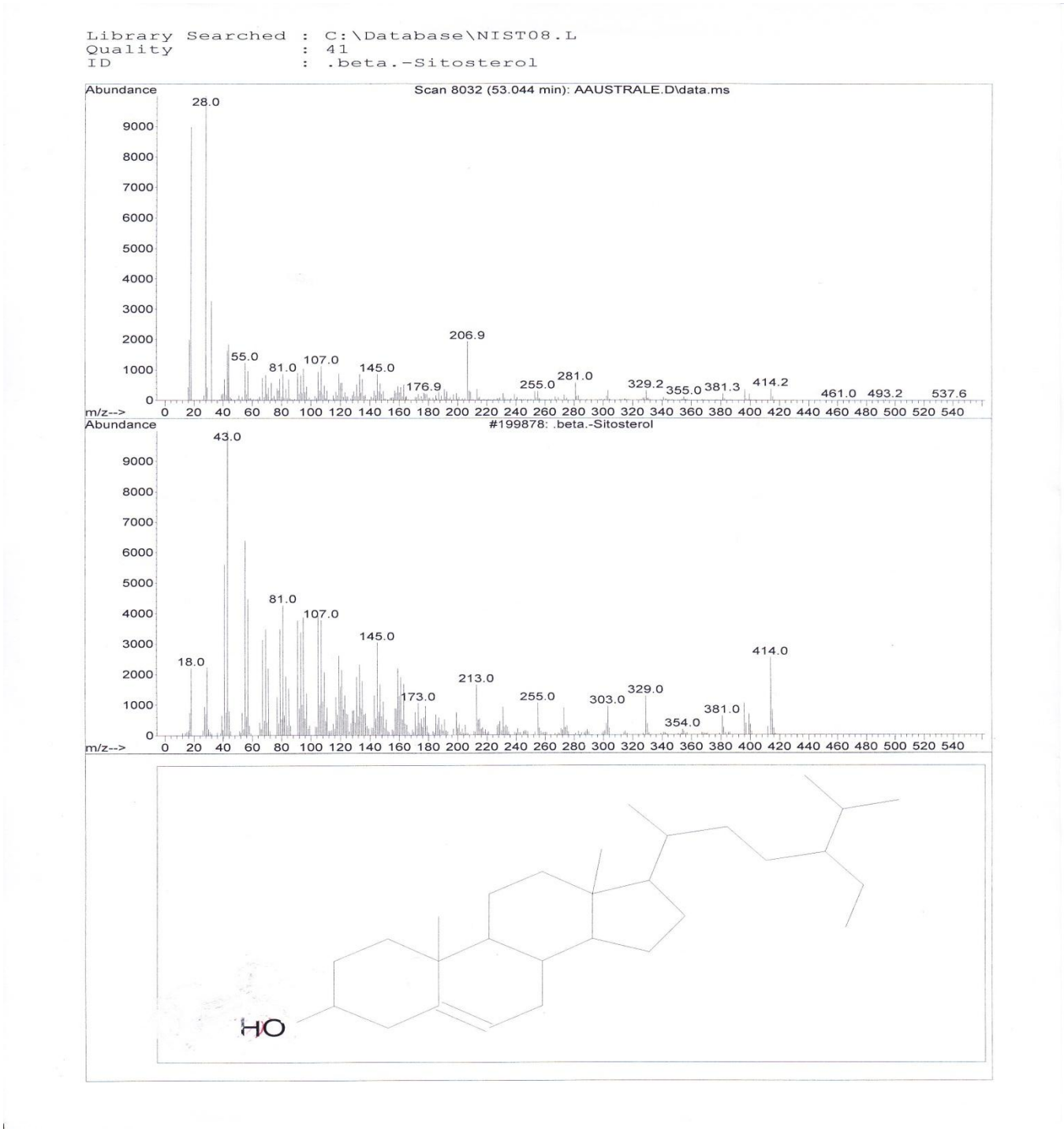

Figure E2.5c: Beta-Sitosterol

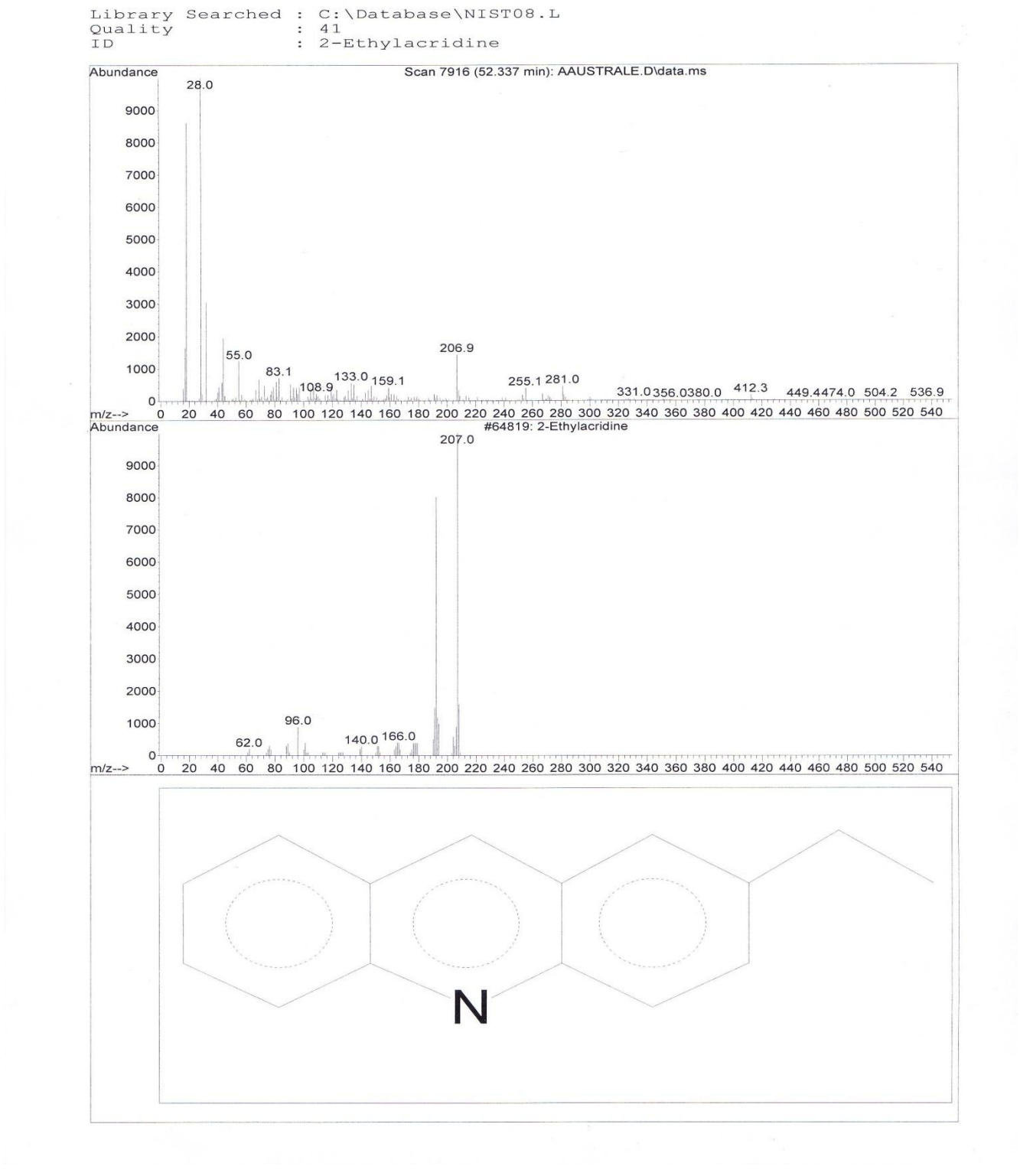

Figure E2.5d: 2-ethylacridine

APPENDIX F

CONTRIBUTION TO KNOWLEDGE

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Full Length Research Paper

Larvicidal, antipyretic and antiplasmodial activity of some Zulu medicinal plants

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Gardenia thunbergia, Siphonochilus aethiopicus, Schotia brachypetala, Acorus calamus, Withania somnifera, Elaeodendron transvalense, Hypoxis hemerocallidea, Vernonia adoensis **and** ***Acanthosperum australe*** **are medicinal plants commonly used by traditional healers in South Africa to treat malaria. Aqueous, dichloromethane and methanol extracts of these plants were screened for larvicidal, antioxidant, *in vivo* antipyretic and *in vitro* antiplasmodial activities. The plant extracts either killed or reduced spontaneous movement in *Culex quinquefascitus* larvae after 24 h following treatment. Methanol extracts exhibited antioxidant (2,2-diphenyl-1-picrylhydrazyl free radical (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) scavenging, Fe^{2+} chelating) activity, albeit to varying degree of efficiency. The dichloromethane and methanol extracts significantly ($p \leq 0.05$) reduced pyrexia with activity increasing in a concentration dependent manner. The antiplasmodial activity against chloroquine sensitive strain of *Plasmodium falciparum* (D10) showed that the methanol extracts of *G. thunbergia, V. adoensis* and the dichloromethane extracts of *E. transvalense, A. australe* and *W. somnifera* were active (IC_{50} of 1.04 to 5.07 µg/ml). The results support the use of these plants in folk medicine and suggest that these plants contained constituents that could be developed as potent antimalarial drugs (mosquito larvicide, anti-fever and anti-plasmodial).**

Key words: Antiplasmodial, larvicidal, antipyretic activity, medicinal plants.

INTRODUCTION

Malaria is one of the most live threatening and widespread infectious diseases of our time. It has been estimated that about 40% of the world's population live in countries where the disease is prevalent and about 250 million people suffer from the disease every year (WHO, 2008). Malaria is caused by parasites of the genus *Plasmodium* (*Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale* and *Plasmodium malariae*). The currently used methods of prevention and treatment of malaria include the use of insecticides and larvicide spraying and the use of bed nets and antimalarial drugs. Drugs are also available for *falciparum* malaria which is the most dangerous of the human malaria parasite. However, the malaria parasite has over the years developed resistance to available drugs, including artermisinin-based combination therapies (Afonso et al., 2006) which are otherwise the most effective antimalarial agents in current use. This is the reason why the search for alternative antimalarial therapies is an on-going exercise. In addition, the disease is well associated with poverty and underdeveloped communities. Poor people cannot afford mosquito nets and insecticides as a method of prevention of mosquito bites.

Historically, the majority of antimalarial drugs have been derived from plants (Newman et al., 2003). Phytochemicals are known to be used by plants to prevent attack from phytophagous (plant eating) insects.

Curtis et al. (1986) showed that this repellent property of plants to pest insects and to mosquitoes has been of

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Larvicidal and Antimalarial Activity of Some Zulu Medicinal Plants

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Plasmodium falciparum malaria is one of the most significant obstacles to world health. The parasite's increasing resistance to current drugs and the alarming side effects of such drugs to humans encourage the search for alternate, safer and effective medications[1]. This study investigated the bioactivity of some Zulu medicinal plants.

METHODS: *G. thunbergia*, *S. aethiopicus*, *S. brachypetala*, *A. calamus*, *W. somnifera*, *E. transvalense*, *H. hemerocallidea*, *V. adoensis* and *A. australe* were separately extracted with methanol, dichloromethane and water. Extracts were screened for antioxidant activity[2,3]

Larvicidal activity of the extracts was tested against *C. quinquefascitus* larvae[4]. *In vitro* antiplasmodial activity was tested against chloroquine sensitive (CQS) strain of *Plasmodium falciparum* (D10)[5]. The MTT cytotoxicity test of the extracts against human embryonic kidney (HEK293) and human hepatocellular carcinoma (HepG2) cells was investigated [6]. Pyretic conditions were induced in rats by means of yeast (1ml/kg bw 12%) injection. Pyretic rats then received the extracts and their temperatures taken (anal) over 4 hours.

RESULTS AND DISCUSSION:

Table 1: Antioxidant activity (IC_{50}) of plants

Plant Name	DPPH	ABTS	Fe^{2+} Chelating	Activity IC_{50} µg/ml SO	OH	N O
H. hemerocallidea	1.13	5	5	2.6	0.8	2.1
A. australe	3.2	5	5	0.84	0.82	2.05
V. adoensis (roots)	2.3	5.2	5	0.70	0.78	3.4
V. adoensis (leaves)	4	1.04	4	2.68	0.88	1.5
A. calamus	2.8	>5	>5	1.8	3.0	4.2
E. transvalense	0.7	4.1	3.9	1.6	3.6	3.6
W. somnifera	2.2	4.6	4.3	0.8	1.2	4.7
G. thunbergia	6.39	5.58	7.48	16.47	4.35	20.2
S. aethiopicus	3.62	13.72	>5	8.58	>5	15.26
S. brachypetala	8.12	11.00	>5	0.82	7.21	4.45
BHT	**0.7**	**3**	**NA**	**2.46**	**1.37**	**2.74**
Ascorbic acid	**0.5**	**5**	**NA**	**0.84**	**3.7**	**3.76**
EDTA	**NA**	**NA**	**0.6**	**NA**	**NA**	**NA**
Citric Acid	**NA**	**NA**	**2.9**	**NA**	**NA**	**NA**

The extracts showed a concentration dependent antioxidant activity (Table 1). They demonstrated great potential to scavenge biological free radicals.

The plant extracts either killed or reduced spontaneous movement in *Culex quinquefascitus* larvae after 24 hours following treatment (Table 2).

Most of the extracts showed antiplasmodial activity, but those listed in table 3 exhibited activity at concentrations <10µg/ml with low IC_{50} values. The DCM extracts of *A. australe* was the most active.

Table 2: Larvicidal activity of plants (Extracts with < 50% activity not shown)

Plant	extract	Lavicidal Activity(µg/ml) %Mortality	IC_{50}
W. somnifera:	Dichloromethane	77	14.78
	Methanol	80	22.88
E. transvalense:	Dichloromethane	60	18.18
A. calamus:	Dichloromethane	64	10.98

Table 3: I*n vitro* antiplasmodial activity and cytotoxicity of plants

Plant extract		*In vitro* Antiplasmodial (µg/ml) Activity	IC_{50}	MTT Assay LC_{50}µg/ml HEK293	HepG2
E.transvalence	DCM	5µg/ml	5.07	512	394
V. adoensis (leaves)	Meth	5µg/ml	2.90	361	421
A. australe	DCM	5µg/ml	1.04	534	512
G. thunbergia	Meth	<10µg/ml	4.36	363	338
W. Somnifera	DCM	5µg/ml	4 .94	635	911

Table 4: The antipyretic activity of plant extracts at 500 mg/kg. Data represents the mean ± SE of the experiment. $^{*}P < 0.05$, $^{**}P < 0.01$ $^{***}P < 0.001$.

Plant	extract	treatment	Induced temp	Temperature after feeding 30	60	120	240 min
CMC	Control	100 mg/kg	37.5±0.06	37.8±0.1	37.6±0.1	37.4±0.1	37.5±0.1
Panado	Stand	100 mg/kg	37.6±0.2	36.5±0.1***	36.0±0.1***	35.6±0.1***	36.0±0.1***
A. cala	Meth	500 mg/kg	37.6±0.1	36.9±0.16**	37.0±0.2	37.2±0.3	37.0±0.2
E. trans	Meth	500 mg/kg	38.6±0.16	38.3±0.2**	38.2±0.18**	37.9±0.14**	38.1±0.2**
E. trans	DCM	500 mg/kg	38.0±0.2	37.9±0.2*	37.5±0.4*	37. ±0.6**	37.1±0.3*
W. somn	DCM	500 mg/kg	37.4±0.3	36.3±0.3*	36.6±0.5*	36.5±0.3*	35.3±0.3*
W. somn	Aque	500 mg/kg	38.7±0.3	36.6±0.2*	36.3±0.2*	38.4±0.3*	38.3±0.3*
A. aust	DCM	500 mg/kg	38.7±0.3	36.8±0.2*	37.7±0.2*	37.3±0.3*	38.3±0.1*
V. ado(L)	Meth	500 mg/kg	38.6±0.5	38.5±0.3*	37.6±0.6*	37±0.1*	36±0.2*
V. ado(L)	Aque	500 mg/kg	38.9±0.1	38.7±0.5*	38±0.6*	37.5±0.1*	37.2±0.7*

The extracts significantly lowered the body temperature of the rats; the activity was comparable to that of panado (table 4)

CONCLUSION: It is apparent that the traditional healers use these plants in managing fever (a symptom of malaria). We found that *A. australe*, *G. thunbergia*, *W. somnifera and E. transvalense* possessed components that showed anti-plasmodial activity. With the relatively high anti pyretic, anti oxidant and larvicidal activity, coupled to the low levels of cytotoxicity, the plants could be candidates for the isolation and characterisation of compound that could be used in the management of malaria.

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Antiplasmodial, Antipyretic and Antioxidant activity of some Zulu Medicinal Plants

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INTRODUCTION: Malaria is one of the most life threatening diseases; it is caused by parasites of the genus *Plasmodium* with over 300 million cases and one million deaths per year[1]. The parasite's increasing resistance to commonly used antimalarial drugs enthused search for alternative drugs[2]. Many plants are used by traditional healers in South Africa to treat malaria [3]. The objective of this study is to screen some Zulu medicinal plants for antiplasmodial, antipyretic, and antioxidant activity.

METHODS: Nine plants (*G. thunbergia, S. aethiopicus, S. branchypetala, A. calamus, W. somnifera, E. transvalense, H. hemerocallidea, V. adoensis* and *A. australe)* were separately extracted with methanol, dichloromethane and water. Extracts were screened for antioxidant activity: DPPH, ABTS, super oxide, nitric oxide, hydroxyl radical scavenging and chelating activity[4,5,6,7,8,9].
The SH content and phenolic contents of the plants were determined[10].
Pyretic conditions were induced in rats by means of yeast (1ml/kg bw 12%) injection (subcutaneous). Confirmed pyretic rats then received their respective drugs and their temperatures taken (anal) at 4hours.
In vitro antiplasmodial activity was tested against chloroquine sensitive (CQS) strain of *Plasmodium falciparum* (D10)[11].

RESULTS AND DISCUSION

		Activity (IC_{50}µg/ml)		
	Plant		BHT	AA
DPPH	*E. transvalense*	0.7	0.7	0.5
ABTS*	*V. adoensis* (leaves)	1.04	3	5
SO	*V. adoensis* (roots)	0.70	2.46	0.84
OH	*V. adoensis* (roots)	0.78	1.37	3.7
NO	*V. adoensis* (leaves)	1.57	2.74	3.76
A.A / Fe^{2+}	*A. australe*	1.0	N/A	N/A
			EDTA	CitricA
Chelating	*E. transvalense*	3.9	0.6	2.9

Table1: Antioxidant activity (IC_{50}) of the plants (N/A=Not applicable)

The extracts showed a concentration dependent antioxidant activity. They demonstrated great potential to scavenge readily existing free radicals at a very low concentration (Table 1). Phenolic compounds are known to contribute to the antioxidant properties of plants. Table 2 shows the quantitative values of such compounds.

Plant	SH	Total phenol	Proanthocyanidin	Flavonoid
H. hemerocallidea	1.28	0.12	0.32	0.07
A. calamus	2.57	0.05	0.11	0.11
V. adoensis (leaves)	0.43	0.16	0.05	0.07
S. branchypetala	7.25	0.36	2.74	0.12
E.transvalense	0.36	0.035	0.25	0.098
W.somnifera	2.74	0.051	0.21	0.050

Table 2: Total phenol, Proanthocyanidin, flavonoids (mg/g) and SH (µg/g) content the plants

Most of the extracts showed antiplasmodial activity; those listed in table 3 exhibited activity at concentrations <10µg/ml with low IC_{50} values. The DCM extracts of *A. australe* was the most active.

References will be provided on request.

Plant	Extract	Activity	IC_{50} (µg/ml)
E.transvalence	DCM	5µg/ml	5.07
V. adoensis (leaves)	Meth	5µg/ml	2.90
A. australe	DCM	5µg/ml	1.04
G. thunbergia	Meth	<10µg/ml	4.36
W. Somnifera	DCM	5µg/ml	4 .94

Table 3: *In vitro* antiplasmodial activity of the plants

Plant	extract	100mg/kg	500mg/kg	1000mg/kg	Panado	CMC
E. transvalense	DCM	36.6 ± 0.4*** 36.6 ± 0.4***	36.2 ± 0.4*** 36.2 ± 0.4***	36.3 ± 0.70*** 36.3 ± 0.70**	34.1± 0.9***	37.7±0.13***
A. australe	DCM	33.1±0.8** 37.5±0.2	33.1±0.8** 38.1±0.3*	33.1±0.8** 38.0±0.1*	37.5±0.8**	33.1±0.8**
G. thunbergia	Met	35.17±0.4 35.17±0.4	35.05±0.4* 34.36±0.4	34.36±0.9* 34.36±0.9	35.15±0.7	35.6±0.26
W. somnifera	DCM	37.9 ± 0.19*** 37.9 ± 0.19	37.6 ± 0.15*** 37.6 ± 0.15*	36.7 ± 0.16*** 36.7 ± 0.16***	35.2 ± 0.8***	37.8±0.08***
V. adoensis (leaves)	Met	36.3±0.2** 37.4±0.2	36.3±0.2** 37.5±0.2*	36.3±0.2** 37.6±0.1*	37.3±0.1**	36.3±0.2**
V. adoensis (leaves)	DCM	37.4±0.1** 37.6±0.0*	37.4±0.1** 37.9±0.1**	37.4±0.1 37.5±0.4	37.1±0.1*	37.4±0.1*

Table 4: The antipyretic activity of the plant extracts. ***Data represents the mean ± SE of the experiment. *P < 0.05, **P < 0.01 ***P < 0.001. Red represents the plant extract compared to the control and the black colour when plant extract is compared to panado.***

Table 4 represents the antipyretic activity of the extracts. The extracts significantly lowered the body temperature of the rats; the activity was comparable to that of panado.

CONCLUSION:
Free radicals have been implicated as the causative agents of various diseases. The plants showed potential to scavenge free radicals. The plants extracts exhibited antipyretic activity; high levels of antiplasmodial activity were observed in some of the extracts.
These results apparently support the use of these plants in managing malaria and fever (a symptom of malaria) in folk medicine.

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